Histopathological study of myelofibrosis (PMF) and essential thrombocythaemia (ET) and correlation with clinical and laboratory feature

A Dissertation Submitted in part fulfillment of the rules and regulations for the M.D. Degree Branch III (Pathology) Examinations of The Tamil Nadu Dr. M.G.R. Medical University, Chennai to be held in April 2015

CERTIFICATE

This is to certify that this dissertation titled **"Histopathological study of myelofibrosis (PMF) and essential thrombocythaemia(ET) and correlation with clinical and laboratory feature"** is a bonafide work done by Dr H.Pradeep in part fulfillment of rules and regulations for the M.D. Branch III (Pathology) Degree Examination of the Tamil Nadu Dr. M.G.R. Medical University, to be held in April 2015.

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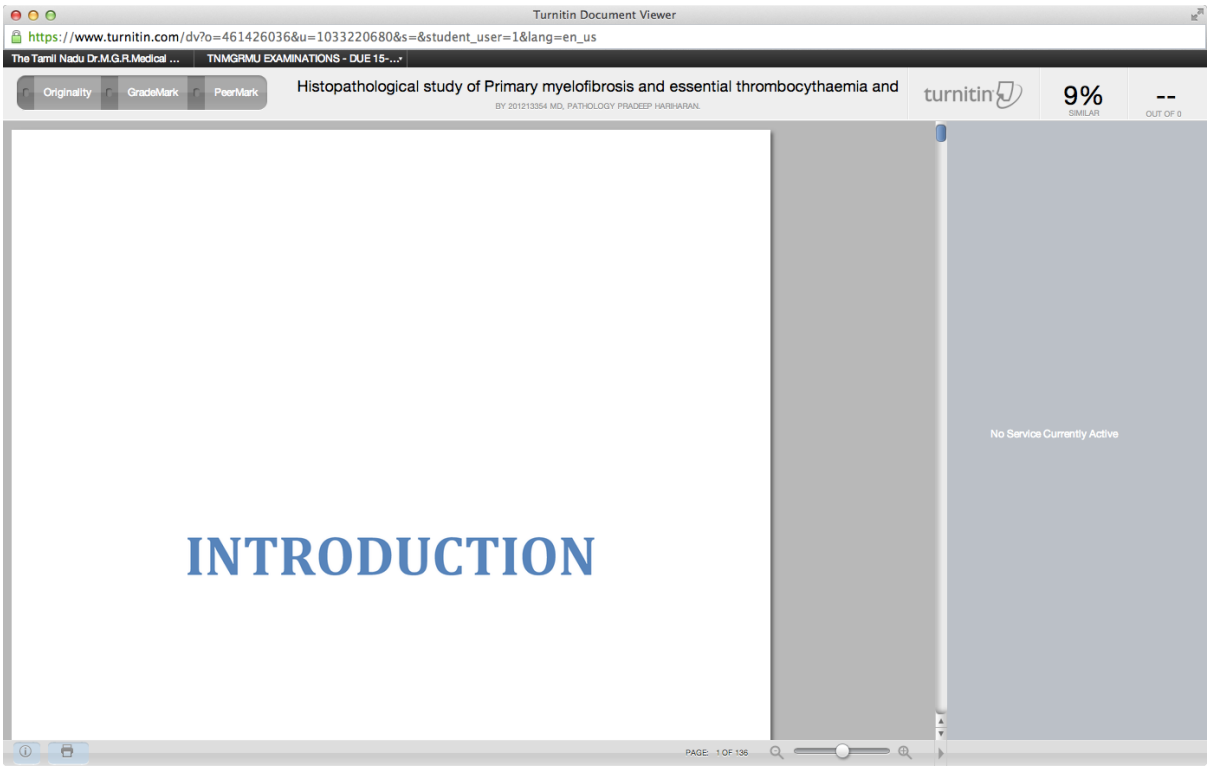
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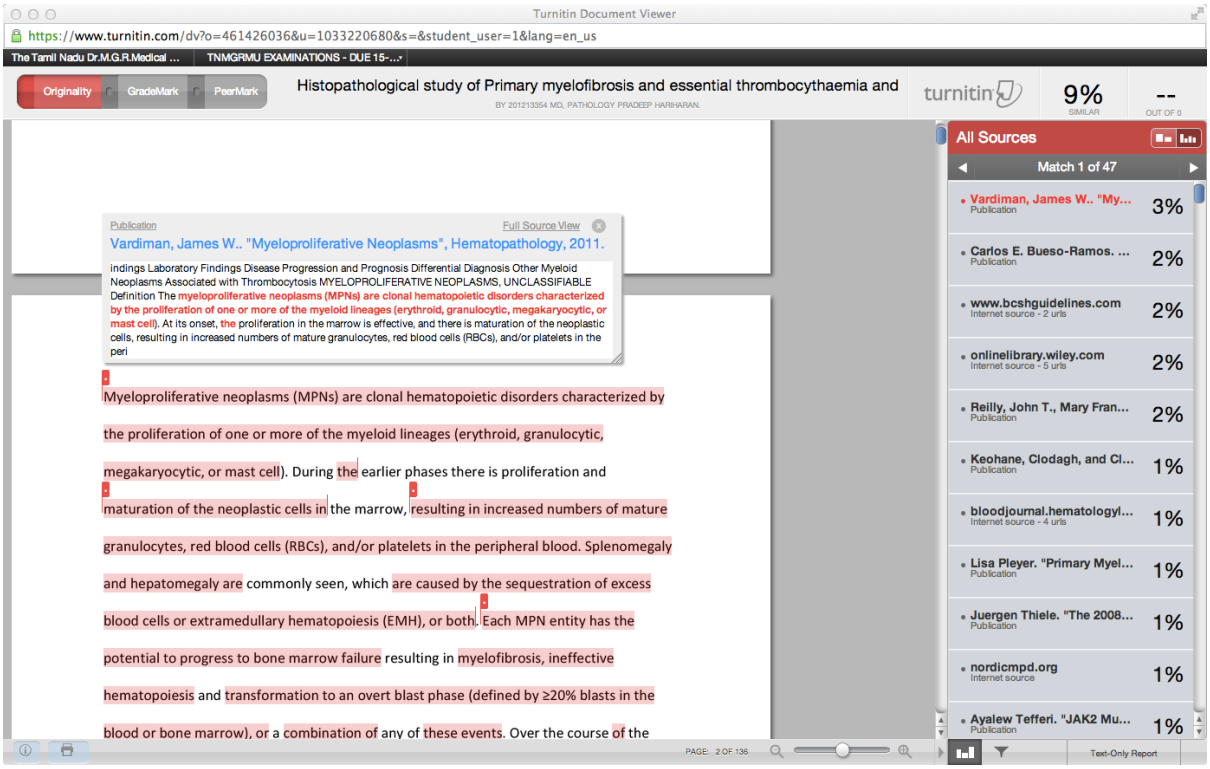
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indings Laboratory Findings Disease Progression and Prognosis Differential Diagnosis Other Myeloid Neoplasms Associated with Thrombocytosis MYELOPROLIFERATIVE NEOPLASMS, UNCLASSIFIABLE Definition The myeloproliferative neoplasms (MPNs) are clonal hematopoietic disorders characterized by the proliferation of one or more of the myeloid lineages (erythroid, granulocytic, megakaryocytic, or mast cell). At its onset, the proliferation in the marrow is effective, and there is maturation of the neoplastic cells, resulting in increased numbers of mature granulocytes, red blood cells (RBCs), and/or platelets in the peri
Myeloproliferative neoplasms (MPNs) are clonal hematopoietic disorders characterized by the proliferation of one or more of the myeloid lineages (erythroid, granulocytic, megakaryocytic, or mast cell). During the earlier phases there is proliferation and maturation of the neoplastic cells in the marrow, resulting in increased numbers of mature granulocytes, red blood cells (RBCs), and/or platelets in the peripheral blood. Splenomegaly and hepatomegaly are commonly seen, which are caused by the sequestration of excess blood cells or extramedullary hematopoiesis (EMH), or both. Each MPN entity has the potential to progress to bone marrow failure resulting in myelofibrosis, ineffective hematopoiesis and transformation to an overt blast phase (defined by ≥20% blasts in the blood or bone marrow), or a combination of any of these events. Over the course of the
PAGE: 2 OF 136
Text-Only Report

Index

INDEX OF TABLES

INDEX OF FIGURES

Abbreviations

1) AML -Acute myeloid leukaemia

2) ALL –Acute lymphoblastic leukaemia

3) BTB –Bone marrow trephine biopsies

4) CML-Chronic myeloid leukaemia

5) DC-Differential count

6) DIPSS-Dynamic International Prognostic Scoring system

7) ET-Essential Thrombocythaemia

8) IPSS-International Prognostic scoring system

9) JAK-Janus Kinase

10) MDS-Myelodysplastic syndrome

11) MPN-Myeloproliferative Neoplasm

11) PNH- Paroxysmal nocturnal Haemoglobinuria

12) PMF-Primary Myelofibrosis

13) PV- Polycythemia Vera

14) PVSG- Polycythemia Vera Sub Group

15)USG: Ultrasonogram

15) WBC- White Blood cell

Title of abstract: Histopathological study of myelofibrosis (PMF) and essential thrombocythaemia(ET) and correlation with clinical and laboratory features .

Department: General Pathology

Name of the candidate: H. Pradeep

Degree and subject: MD (PATHOLOGY)

Name of the guide: Vivi Srivastava

AIM: 1) To describe the bone marrow morphology in primary myelofibrosis and essential thrombocythaemia.

2) To develop a scoring system for the diagnosis of these conditions based the various histological parameters seen in bone marrow trephine biopsies in these groups of patients.

3) To determine whether such a scoring system reliably distinguishes between ET and prefibrotic phase of PMF.

4) To compare the blood and bone marrow findings in patients with and without JAK2V617 mutations.

Methods: This was a study done on retrieved bone marrow trephine biopsies in Department of General Pathology, Christian Medical College, Vellore . 134 cases were included in the study, which included 27 cases of ET , 107 cases of PMF , including 13 cases of prefibrotic phase of PMF and correlated with relevant laboratory and clinical parameters .

Parameters evaluated

The histomorphological features of all cases was studied in detail with a score applied for each histomorphological parameter with a maximum score of 21.

Relevant statistical analysis for Categorical variables and continuous variables were done using chi square test and fisher t test.

Results

All cases of ET had a total score of <10 and scores of prefibrotic MF ranged from 12-17 in our 21 point scoring system thus allowing a distinction between them. JAK2 positivity did have a influence on certain laboratory and histomorphological parameters such JAK2V617 positive cases of PMF had a higher haemoglobin and total leukocyte count at presentation. On histopathology hyperchromatic dysplastic nuclei and small clusters were found more commonly in JAK2V617 positive MF. JAK2WT ET had a higher platelet count at diagnosis and also large clusters being more frequently present in JAK2V617 positive ET.

INTRODUCTION

Myeloproliferative neoplasms (MPNs) are clonal hematopoietic disorders characterized by the proliferation of one or more of the myeloid lineages (erythroid, granulocytic, megakaryocytic, or mast cell). During the earlier phases there is proliferation and maturation of the neoplastic cells in the marrow, resulting in increased numbers of mature granulocytes, red blood cells (RBCs), and/or platelets in the peripheral blood. Splenomegaly and hepatomegaly are commonly seen, which are caused by the sequestration of excess blood cells or extramedullary hematopoiesis (EMH), or both. Each MPN entity has the potential to progress to bone marrow failure resulting in myelofibrosis, ineffective hematopoiesis and transformation to an overt blast phase (defined by ≥20% blasts in the blood or bone marrow), or a combination of any of these events. Over the course of the disease accumulating genetic defects usually accompany morphologic evidence of disease progression .There are eight neoplasms classified under myeloproliferative neoplasms according to World Health Organisation (WHO). (2)

WHO CLASSIFICATION OF MYELOPROLIFERATIVE NEOPLASMS

- Chronic myelogenous leukaemia (CML), *BCR-ABL1* positive
- Chronic neutrophilic leukaemia (CNL)
- Polycythaemia vera (PV)
- Primary myelofibrosis (PMF)
- Essential thrombocythaemia (ET)
- Chronic eosinophilic leukaemia, not otherwise specified (CEL, NOS)
- Mast cell disease (MCD)
- Myeloproliferative neoplasm, unclassifiable (MPN, U)

The present study will look into the various histopathological features of PMF (prefibrotic phase and overt fibrotic phase) and ET in detail on the bone marrow biopsies along with

relevant laboratory and clinical findings. Bone marrow biopsy is an essential part in the diagnostic workup of PMF and ET. Both these myeloproliferative neoplasms have definitive changes in the erythroid , myeloid and especially megakaryocytic lineages. Pre-fibrotic phase of PMF and ET often present with thrombocytosis and hence need to differentiated as both these entities have different modalities of treatment. The histopathological features of these two myeloproliferative neoplasms will be dealt in detail and comparison of findings will be done in the present study.

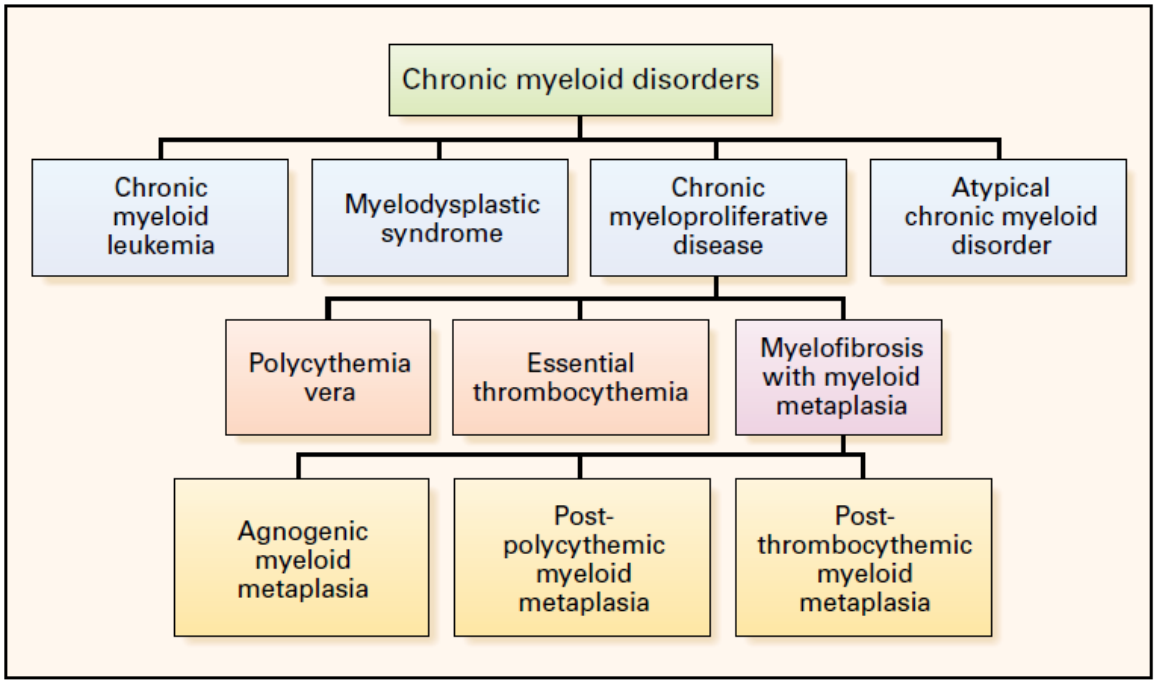


Figure 1 Working classification of chronic myeloproliferative disorder

AIMS

1) To describe the bone marrow morphology in primary myelofibrosis and essential thrombocythaemia.

2) To develop a scoring system for the diagnosis of these conditions based the various histological parameters seen in bone marrow trephine biopsies in these groups of patients.

3) To determine whether such a scoring system reliably distinguishes between ET and prefibrotic phase of PMF.

4) To compare the blood and bone marrow findings in patients with and without JAK2V617 mutations.

REVIEW OF LITERATURE

Historical Perspective:

Surgical trephine biopsy is an older procedure than needle aspiration of the marrow. Pianese in 1903 was the first to obtain marrow from the epiphysis of the femur.

Sternum, iliac crest and tibia were subsequently used by workers for trephine biopsy.

Thus bone marrow aspiration continued to remain an important diagnostic tool for the diagnosis of haematological disorders. But in cases of fibrosis of marrow aspirates usually results in dry tap and hence trephine biopsy is vital is arriving at a correct diagnosis .(1) Jamshidi and Swaim in 1971 devised a new biopsy needle which is now used for bone marrow trephine biopsies.

BONE MARROW STRUCTURE

Bone marrow is a soft, semisolid, red gelatinous substance which occupies the medullary cavities of the axial skeleton and in adults, bone marrow is the 5 primary site of haematopoiesis and is composed of bone, blood vessels , lymphatics and haematopoietic tissue.(1,2) The medullary cavity is encased by trabecular bone consisting of periosteum, cortical and the subcortical bone. The medullary cavity contains hematopoietic cells, stromal cells and extra-cellular matrix. Arterioles, sinusoids and peripheral nerves traverse the interstitial space. (1)

The age of the patient is taken into consideration while assessing bone marrow cellularity which refers to relative amount of haematopoietic and fat cells with .The cellularity is highest in the first decade and gradually decreasing to around 29% by 8^{th} decade (1).

NORMAL MARROW STRUCTURE:

ERYTHROID CELLS: Erythroid series of cells are in inter-trabecular location forming islands, nodules or clumps. The immature cells are in the centre of the island, with

mature cells at the periphery of the islands. Reticuloendothelial cells are seen in the vicinity of the erythroid islands. All the stages of maturation can be seen in the population. Normal myeloid-erythroid ratio in a trephine biopsy is 1.5-3:1.

GRANULOCYTES: All the series of granulocytes and their precursors are identifiable in the bone marrow biopsy. They are located near the bone trabeculae (paratrabecular).The more mature cells are towards the marrow space with immature forms near the bone trabeculae. Neutrophils and eosinophilis are readily identified (larger and yellowish red granules). Basophils are infrequently seen. (1,3)

MEGAKARYOCYTES: They are perisinusoidal in location with platelets directly shed into the sinusoids. These are the largest cells seen in the bone marrow ranging in size from 12-150 microns . Mature megakaryocytes show eosinophilic cytoplasm with variable granularity, the nucleus being coarsely cerebriform and multilobated. Emperipoliesis may be seen in the megakaryocytes. (1)

MONONUCLEAR MACROPHAGE SYSTEM:

MONOCYTE SERIES: Monoblasts are morphologically similar to myeloblasts except that their nuclear shape may be slightly clefted or lobulated. Monocytes (15-18 microns) have abundant cytoplasm with intracytoplasmic vacuoles with eccentrically placed kidney shaped nucleus with fine and lacy chromatin. Macrophages are derivatives of monocytes and function as phagocytic cells in the bone marrow and other tissue sites. Macrophages are larger than monocytes measuring 20-30 microns in diameter with nucleus being large, round to oval with lace-like chromatin and have abundant pale blue vacuolated cytoplasm which contain azurophilic granules . They are present usually in the centre of the erythroid islands , plasma cell islands, and adjacent to the reticuloendothelial cells.

LYMPHOCYTES: The earliest morphologically identifiable cells are lymphoblasts

which are usually present in the intertrabecular region. They have a high N/C ratio with a narrow rim of deep blue cytoplasm and an oval hyperchromatic nucleus with one or two nucleoli ,measuring 7-10 microns in diameter and characterized by a round nucleus with coarse, condensed chromatin, inconspicuous nucleoli and scanty blue cytoplasm.

PLASMA CELLS: The characteristic location of plasma cells is along the adventitia of small blood vessels but they can be found singly and in groups with intertrabecular location and measuring 10-18 microns and with an eccentrically placed nucleus , coarse chromatin and deeply basophilic cytoplasm containing a perinuclear clear zone.

MARROW STROMA:

Bone marrow stromal cells consist of adipocytes, osteoclasts osteoblasts, endothelial cells and fibroblast-like reticular cells. Adipocytes (fat cells) are the largest cells in the bone marrow stroma. They lie in close contact with haematopoietic cells and other stromal cells.

Osteoblasts are large, ovoid or cuboidal cells measuring 20-50 microns in diameter having a small eccentric nucleus and an abundant basophilic cytoplasm with a clear Golgi zone located away from nucleus.

Osteoclasts are large, multinucleated cells with abundant cytoplasm which contains numerous azurophilic granules measuring about 100 microns or greater in diameter. The individual nuclei are separate, uniform and round. Endothelial cells are elongated cells containing a flat nucleus with condensed chromatin and a moderate amount of cytoplasm.

Reticular cells are group of cells that form a reticulum or syncytium. These cells are associated with reticular fibers which they produce and which form a three dimensional supporting network that holds the vascular sinuses and haematopoietic elements. The fibers can be visualized by light microscopy and after silver staining.For proper assessment of

marrow cellularity, a trephine biopsy specimen needs to be at least 1.6cm long before processing and contain five to six intertrabaecular spaces.

Bone marrow trephine biopsies are used in diagnosis of a variety of haematological disorders such as leukaemias, staging marrows for lymphomas and other solid tumours. There use is utmost vital importance in cases of marked fibrosis as shown in table 1 and table 2.

Table I. Conditions with increased fibrosis (reticulin fibrosis), but not associated with collagen fibrosis.(1,4)

Conditions

- Hairy cell leukaemia
- HIV infection
- Visceral leishmaniasis
- Pulmonary arterial hypertension
- Treatment with hematopoietic growth factors.

Table 2. Causes of bone marrow fibrosis, GRADE 4 (diffuse, often associated with coarse reticulin fibre network with areas of collagenisation) (1,4)

- Primary myelofibrosis(PMF) and secondary myelofibrosis associated with essential thrombocythaemia (ET) and Polycythaemia rubra vera(PRV)
- Malignant disease such as Acute megakaryoblastic leukaemia , chronic myeloid leukaemia(CML), leukaemia such as (Acute myeloid leukaemia (AML), Acute lymphoblastic leukaemia(ALL)), systemic mastocytosis , lymphomas (Hodgkin and Non-Hodgkin lymphoma Adult T-cell leukaemia/lymphoma and Myelodysplastic syndromes (particularly secondary MDS), Multiple myeloma and tumours metastasising to the bone.
- Paroxysmal nocturnal haemoglobinuria (PNH)
- Bone and connective tissue diseases such as Osteopetrosis, Hyperparathyroidism (Primary and secondary) , Rickets associated with vitamin D deficiency and renal rickets, Osteomalacia and Primary hypertrophic osteoarthropathy.
- Chronic infections such as Tuberculosis , other granulomatous diseases such as Histoplasmosis and Osteomyelitis (Focal or localized)
- Grey platelet syndrome
- Autoimmune conditions such as Systemic lupus erythematoses (SLE), Systemic sclerosis ,Sjogren syndrome and Autoimmune myelofibrosis
- Antiphospholipid antibodies
- Paget's disease of the bone
- Miscellaneous conditions such as bone marrow necrosis, radiation to the bone marrow , sites of healing fracture and sites of previous trephine biopsies

Primary Myelofibrosis (PMF) is a non specific response to various injuries and diseases that involve the bone marrow. It is mediated by a numerous cytokines released from

- megakaryocytes
- cells of the monocyte-macrophage lineage,
- Bone marrow stromal cells.

Marrow fibrosis can be associated with infections or inflammatory conditions that involve the bone marrow .It usually accompanies neoplastic diseases such as carcinoma or lymphoma when they have involvement of the bone marrow. Majority of the myelofibrosis cases are associated with myeloproliferative neoplasms (MPN). PMF is not associated with other secondary conditions and stands out because of a prominent role that bone marrow fibrosis plays in pathogenesis of the disease process. (5,6)

Primary myelofibrosis

Primary myelofibrosis (PMF) is a clonal disorder of the bone marrow which forms a part of the spectrum of myeloproliferative neoplasms (MPN). PMF (which was earlier known as agnogenic myeloid metaplasia) is characterised by the proliferation of megakaryocytes and granulocytes, extramedullary haemopoiesis and progressive fibrosis. In the early stages of myelofibrosis, the marrow is hypercellular. There is progressive splenomegaly due to extra medullary haemopoiesis (EMH). The liver may also be involved in EMH. As the disease progresses, the marrow shows a progressive increase in the number of the reticulin fibres that form its structural framework. In the end stage, the marrow is largely replaced by markedly thickened reticulin fibres with areas of collagen fibrosis. The development of osteosclerosis reduces the marrow space further. (5,6)

Two phases of the disease are recognized:

- The early stage of PMF is also known as the cellular or prefibrotic phase. At this stage, the bone marrow is hypercellular, and the reticulin fibres are normal or only slightly increased. EMH is minimal and may be accompanied by thrombocytosis.
- In the end (fibrotic) stage, the marrow is usually hypocellular due to extensive reticulin and collagen fibrosis. The development of osteosclerosis reduces the marrow space further. The peripheral blood shows a leukoerythroblastic blood picture (LEBP).The spleen is greatly enlarged due to EMH. Hepatomegaly may also be seen (6).

The overt fibrotic phase of PMF has an estimated occurrence of 0.5-1.5 per 100000 persons per year. PMF commonly occurs in the sixth to seventh decade of life, but can also be seen in the fifth decade and both sexes are equally affected. Children are rarely affected and only isolated cases have been reported. Progression to the fibrotic stage is gradual , usually occurring over several years, and is due to the release of cytokines from megakaryocytes, platelets, macrophages and lymphocytes resulting in fibrosis and vascular proliferation in the bone marrow.(6)

Clinical findings

PMF usually develops in the fifth and sixth decade, and affects males and females almost equally. PMF has an insidious onset. Almost a third of patients are asymptomatic and are diagnosed when routine testing shows anaemia or thrombocytosis.

In the early stage before significant fibrosis sets in, patients usually present with constitutional symptoms such as fatigue, weight loss and fever. Other symptoms may night sweats, diffuse bone pains and episodes of bleeding or thrombosis due to elevated platelet counts. Weight loss is defined as a loss of >10% over a period of six months (6)

As the disease progresses, and fibrosis sets in, symptoms may be related to anaemia which is also the most common cytopenia reported with PMF. (5, 6)

Splenomegaly and hepatomegaly become increasingly prominent as the disease progresses. Splenomegaly may be associated with abdominal discomfort or acute abdominal pain due to splenic infarction which have been commonly reported in PMF. Hepatomegaly results from EMH in the fibrotic stage of PMF and is usually accompanied by portal hypertension, ascites and variceal bleeding. Portal hypertension can result from massive splenomegaly (Banti's syndrome) or contribute to the development of splenomegaly. Gout and renal stones related to hyperurcemia may also be seen. (5,6)

Clinical findings

The usual onset of myelofibrosis is in the fifth and sixth decade. There is no sex predilection

The onset of PMF is usually insidious with almost 30% of patients being asymptomatic at presentation, when their illness is discovered by a routine blood count that reveals anaemia or raised platelet counts (thrombocytosis). (5, 6)

In the prefibrotic stage, symptoms are fatigue, easy bruisability and loss of weight which have commonly been reported. Splenomegaly and hepatomegaly are usually absent or only of mild to moderate degree. Patients in this phase may have episodes of bleeding or thrombosis due to elevated platelet counts. Often the clinical picture overlaps with another MPN such as ET. (6)

The latter stages of PMF, i.e. after fibrosis sets in (fibrotic stage) symptoms are usually related to anaemia which is also the most common cytopenia reported with PMF.

Portal hypertension is common in patients with PMF and it can result from a massive splenomegaly (Banti's syndrome) or it contributes to development of splenomegaly. Gouty arthritis and renal stones related to hyperurcemia may be seen. (6)

JAK2V617F mutations may be found in around 50% of patients in fibrotic phase and predicts the progression to a large splenomegaly and leukemic transformation in case of primary myelofibrosis (PMF) .(7) JAK2V617 mutation status in prefibrotic phase of PMF has been reported of late in few literatures.

Pathogenesis of PMF

PMF is accompanied by changes in bone marrow stroma and elevated levels of circulating cytokines, especially in PMF. These cytokines are released from the abnormal megakaryocytes as well as monocytes and nonclonal stromal cells such as fibroblasts. (8)

Elevated cytokine expression in PMF represents an inflammatory response and contributes to the bone marrow fibrosis and constitutional symptoms which is associated with elevated levels of IL-6 and IL-8.Splenomegaly is associated with HGF .Transfusion-dependent anaemia is associated with elevated levels of interleukins (IL-2R, IL-8, IL-10, MIP-1and MCP-1). Leukocytosis in PMF occurs due to IL-2R, HGF and IP-10 and thrombocytopenia with elevated levels of IP-10. (8)

Cytokines that promote increased vascularity and connective tissue deposition include

- macrophage inflammatory protein 1-β(MIP-1β)
- tissue inhibitor of metalloproteinase,
- insulin-like growth binding factor-2,
- Tumor necrosis factor-α (TNF), the levels of which are also markedly increased in PMF.

Plasma levels of many cytokines are elevated in myelofibrosis.(5)

Marrow fibrosis is due to synthesis and release of Fibrogenic factors such as platelet-derived growth factor (PDGF) and transforming growth factor-β(TGF-β) by the neoplastic megakaryocytes and platelets seen in PMF. (5) (7)

Increased serum levels of vascular endothelial growth factor (VEGF) results in prominent neoangiogenesis particularly in the bone marrow and the spleen. An increase in the micro vascular density in the bone marrow and spleen correlates with the degree of fibrosis and EMH .(8)

Nuclear factor- kβ (NF-kβ) also has a role in the development of BM fibrosis in PMF myelofibrosis. Activation of NF-kβ induces the production of IL-1 and TGF-β in monocytes. (9)

β- FGF is a multifunctional growth factor which has a potent mitogenic effect on stromal cells. Extremely high levels of circulating β- FGF are seen in MF. β FGF is released by abnormal and dysplastic megakaryocytes into the surrounding stroma and stimulates stromal cells. In PMF, also βFGF binds to the vascular endothelial cells and contributes to angiogenesis in idiopathic myelofibrosis. (10)

All these cytokines cause excessive deposition of collagen in the bone marrow, leading to pancytopenia and extramedullary haematopoiesis. The commonest site of extramedullary hematopoiesis is the spleen, followed by the liver. The splenic red pulp is expanded by the presence of granulocytic and megakaryocytic cells. Megakaryocytes are often the most conspicuous component of EMH. The debilitating symptoms seen in cases of myelofibrosis (PMF) are driven by the combined effects of elevated levels of proinflammatory cytokines and massive splenomegaly. (6)

Genetic changes play a major role in the development of PMF. The most important is the being JAK2V617F mutation found in about 50% of PMF. (6) This will be discussed in a subsequent section.

Elevated levels of IL-8 and IL-2R are seen in most cases and are associated with disease prognostication and phenotypic correlations. Increased plasma levels of these two cytokines has showed significant association with

a) constitutional symptoms

b) transfusion need

c) leukocytosis

d) overall and leukaemia-free survival in patients with PMF.(8)

Complex pathobiologic genetic mutations contribute to the development of PMF. The most important being JAK2V617F mutation resulting in gain of function point mutation and will be dealt in detail in the genetics subheading.

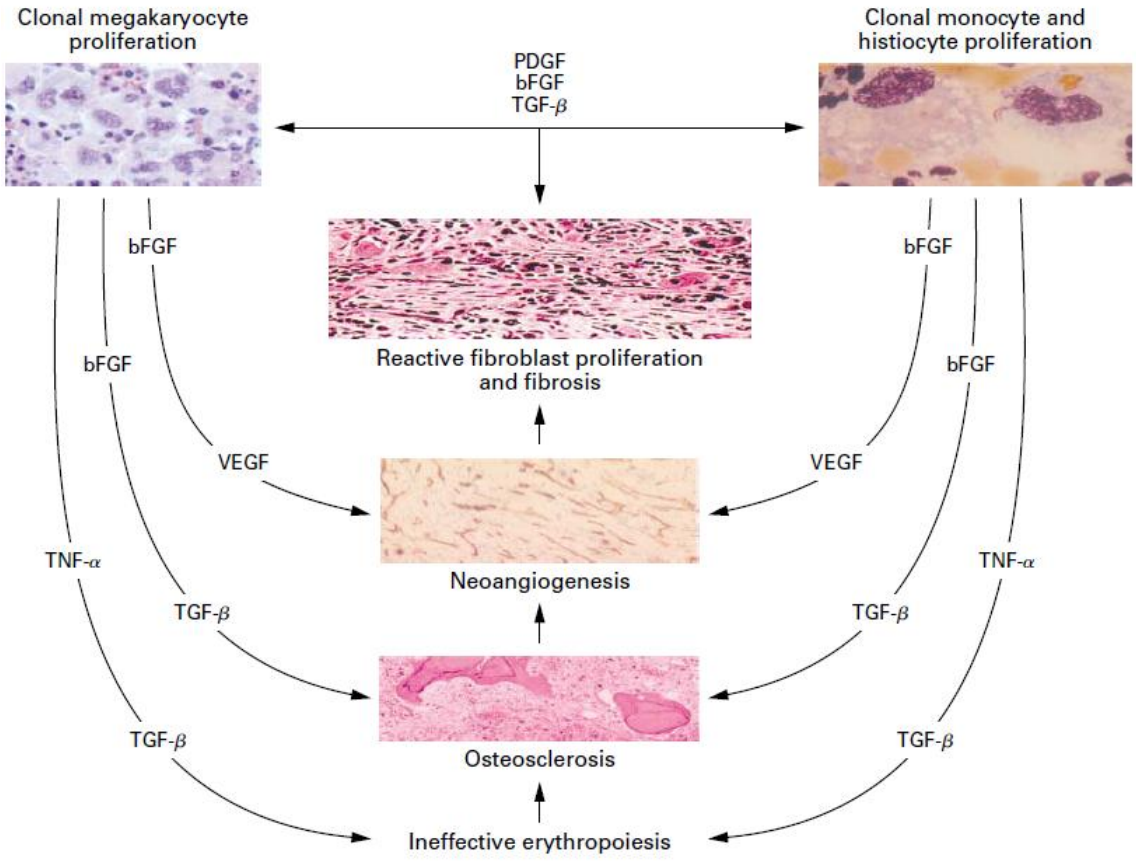


Figure 2- showing the potential cell cytokine interactions in primary myelofibrosis (PMF)

Table3. Definition of morphologic features (11)

Morphologic features	Definition
Staghorn nuclei	Large cells with deeply lobulated nuclei surrounded by mature cytoplasm
Cloud-like nuclei	Enlarged, bulbous, plump nuclei with decreased amount of cytoplasm
Naked nuclei	Compact hyperchromatic megakaryocyte nuclei without visible cytoplasm
Dysmorphic nuclei	Hyperchromatic nuclei with bizarre shapes
Hyperlobulation	Hyperlobulation >6 nuclear lobules; lobules often completely separated by cytoplasm
Hypolobulation	Hypolobulation <4 nuclear lobules surrounded by ample mature cytoplasm
Dense clustering	Dense clustering At least 4 megakaryocytes lying back-to-back without being separated by other cells
Loose clustering	Dispersed cluster of at least 3 megakaryocytes without close contact
Small megakaryocyte cytoplasm	Megakaryocytes <4 myeloid cells in largest dimension
Large megakaryocyte cytoplasm	Megakaryocytes >8 myeloid cells in largest dimension
Dysmorphic megakaryocyte cytoplasm	Dysmorphic megakaryocyte cytoplasm, small to large megakaryocytes with abnormal N/C ratio and a shape other than round
Myeloid erythroid ratio	Myeloid/erythroid ratio of estimated numbers of myeloid and nucleated erythroid cells.
Dilated sinusoids	Dilated sinusoids Visible sinusoids that may or may not be filled with hematopoietic cells.

The above table lists the commonly used terms in the reporting of myeloproliferative neoplasms.

Diagnostic Criteria

The diagnosis of PMF as defined by the WHO classification (Thiele et al, 2008), is based on a combination of features comprising of clinical, morphological, cytogenetic and molecular genetics and requires 3 major and at least 2 minor criteria to be fulfilled.

WHO criteria for the diagnosis of myelofibrosis

Major criteria

1) Presence of megakaryocyte proliferation and atypical megakaryocytes (small to large megakaryocytes with an aberrant nuclear/cytoplasmic ratio and hyperchromatic, bulbous cloud-like nuclei or irregularly folded nuclei and dense clustering of megakaryocytes) which is usually accompanied by reticulin or collagen fibrosis.

Or

In the absence of significant reticulin fibrosis, megakaryocyte changes must be accompanied by increase in cellularity, which is characterised by granulocytic proliferation and often decreased erythropoiesis (i.e. prefibrotic- cellular phase disease).

2) Not meeting the WHO criteria of

i) Polycythaemia Vera (This requires failure of iron replacement therapy to increase haemoglobin level to the polycythaemia vera (PV) range in the presence of decreased serum ferritin. Exclusion of polycythaemia (PV) is based on haemoglobin and hematocrit levels, and red cell mass measurement levels are not required.)

ii) BCR-ABL1+chronic myelogenous leukemia CML) (*This Requires the absence of BCR-ABL1 fusion*)

iii) Myelodysplastic syndrome (This requires the absence of dyserythropoiesis and dysgranulopoiesis)

iv) Other myeloid neoplasms.

3) Demonstration of molecular markers such as JAK2V617 mutation or other clonal markers such as (MPLW515K/L). In the absence of clonal marker there should be no evidence that the bone marrow fibrosis or other changes are secondary to

- infection
- autoimmune disorder
- other chronic inflammatory conditions
- Hairy cell leukemia or other lymphoid neoplasm, metastatic malignancy or toxic chronic myelopathies.(*Note*: *Patients with conditions associated with reactive myelofibrosis are not)immune to PMF, and the diagnosis should be considered in such cases if other criteria are met*).(5,6)

Minor criteria

1) Leukoerythroblastic blood picture showing (nucleated red blood cells(NRBCs) along with immature precursors of myeloid cells (Blasts<20%,promyelocytes,myelocytes, metamyelocytes and band forms) and tear drop cells) (*Degree of abnormality could be border line or marked.*)

2) Increase in serum lactate dehydrogenase level (LDH) (*Degree of abnormality could be border line or marked.)*

3) Anaemia (*Degree of abnormality could be border line or marked*.)

4) Splenomegaly (*Degree of abnormality could be border line or marked*.)(4,5)

Beer et al has shown that a serum LDH of twice an upper limit of normal (normal range 120-240mg/dl) is a reasonable discriminator between PMF from ET/PV. In contrast to other

minor criteria an unspecified increase in LDH levels lacks specificity as a diagnostic criteria for PMF as raised LDH levels are also seen in ET/PV. (12)

Laboratory findings

Peripheral blood smear:

Patients diagnosed with myelofibrosis show a leukoerythroblastic blood picture along with pancytopenia. The blood film is leukoerythroblastic i.e. both nucleated RBCs and erythroblasts are present along with immature WBC (blasts<20%, promyelocytes, metamyelocytes, myelocytes and band forms) and RBCs shows anisocytosis and poikilocytosis resulting in Teardrop RBCs. The RBCs also show polychromasia. Granulocytes and platelets may show features of dysplasia. In the early stages of the PMF the peripheral blood film may show thrombocytosis and leukocytosis. In the later stages of PMF there is reduction in counts of all three cell lines resulting in pancytopenia, leukoerythroblastic blood picture and tear drop poikilocytes become evident. Often there are some giant platelets and occasional circulating micro megakaryocytes. (13, 14)

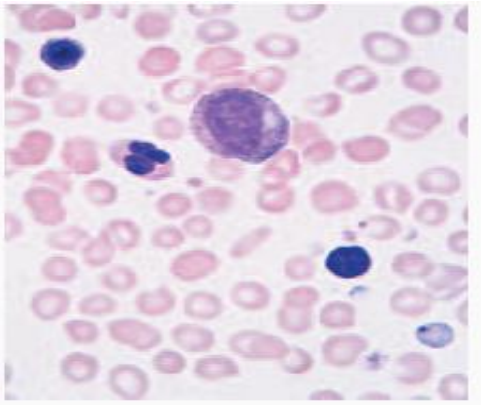


Figure 3: showing a leukoerythroblastic blood picture in a case of myelofibrosis

Bone marrow aspirate: In the Prefibrotic phase of PMF, the aspirate would be hypercellular with increase in all cells (erythroid, myeloid and megakaryocyte) of all lineages. There may

be left shift in myeloid lineage. The maturation of these cells is normal, but there may be some dysplastic megakaryocytes showing hyperchromatic nuclei with clumped chromatin and increased N/C ratio. **I**n the later stages of PMF there is no aspirate obtained resulting in a dry tap, due to increased deposition of reticulin. (5, 6)

Bone marrow trephine biopsy: The examination of bone marrow trephine biopsy is critical for diagnosis of myelofibrosis.

*Prefibrotic phase***:** In the prefibrotic phase of myelofibrosis the bone marrow biopsy(BTB) is hyper cellular with increase in number of neutrophils and megakaryocytes .The myeloid cells show a mild shift to left, band forms, myelocytes , metamyelocytes are seen and the recognition of a significant degree of granulocytic proliferation is important to distinguish prefibrotic phase of PMF from ET.(14) In most cases the erythropoiesis is reduced in quantity, but immature erythroid precursors are prominent.The megakaryocyte morphology is markedly abnormal which is essential to the recognition of the prefibrotic phase of myelofibrosis(PMF).There is clustering of megakaryocytes including small (>3 megakaryocytes) and large (>7 megakaryocytes) clusters along with dense (at least 4 megakaryocytes lying back-to-back without being separated by other cells) and loose clusters (dispersed clusters of at least 3 megakaryocytes without close contact).(11) Loose clusters can be seen in ET and PV but dense clusters are more specific for PMF, indicating its importance in the diagnoses of PMF.(5,11) There is variation in size of the megakaryocytes such as small megakaryocytes are also seen. These megakaryocytes are frequently adjacent to the bone marrow vascular sinuses (Paratrabecular location) and bone trabaeculae. The notable abnormalities of megakaryopoiesis include anisocytosis, abnormal nuclear- cytoplasmic ratios, abnormal chromatin clumping ,dysmorphic nuclei (hyperchromatic nuclei with bizarre shapes) and enlarged, bulbous, plump nuclei with

decreased amount of cytoplasm (cloud like nuclei and hyperlobate (>6 nuclear lobules; lobules often completely separated by cytoplasm) (11). Gianelli et al showed that the recognition of dysmorphic megakaryocytes is important, demonstrating that besides dense clustering, dysmorphic features of the megakaryocytes discriminate prefibrotic phase of PMF from ET.(15) Dysmorphic megakaryocytes were seen more often seen only in prefibrotic phase of PMF and PMF, indicating its specific importance in PMF.(15,16) The megakaryocyte histology is specific for PMF and not for any other myeloproliferative neoplasm. Bone marrow morphological examination is crucial in distinguishing the pre fibrotic phase of PMF from ET. (5)

Brousseau et al (17) evaluated of 102 ET, 18 prefibrotic phase of PMF and seven PMF over a period of 15 years. They devised a scoring system to differentiate between ET and the prefibrotic phase of PMF on the basis of 9 parameters, attaining a global maximum score of 10. They stated that a score of (<=3) would clearly favour a diagnosis of ET (<=3) while a score of (>=6) would clearly favour prefibrotic phase-early PMF. They also suggested that scores of 4–5 were not unequivocally in favour of either condition.

Brosseau et al found that 21 /120 cases had scores of 4-5; thus, they did not assign these "grey zone" cases to either category

Fibrotic phase: The cellularity of bone marrow is markedly reduced with occasional foci of hypercellularity seen in the trephine biopsy. There will be normocellular areas along with hypocellular areas with patches of active hematopoiesis. The hypocellular regions are composed of loose connective tissue and fat. Erythroid cells are markedly reduced .Myeloids may also be reduced with myeloblasts accounting for <10% of the bone marrow cells. Atypical megakaryocytes as described in the prefibrotic phase are the most conspicuous finding .The bone marrow shows clear cut reticulin or collagen fibrosis (MF grade 2 or 3).

Sometimes the bone marrow is completely devoid of haemopoietic cells, showing mainly small islands of haemopoietic precursors situated mostly within vascular sinuses. There is also significant proliferation of vessels showing marked tortuosity and luminal distention (sinusoidal ectasia) often associated with conspicuous intrasinusoidal hematopoiesis with intrasinusoidal megakaryocytes seen. The alteration in the marrow stroma in PMF is responsible for the presence of distended marrow sinusoids with intravascular hematopoiesis.(18) In the end stage of PMF, osteosclerosis will be seen with broad, irregular trabaeculae that can occupies >50%of the bone marrow space. Most of the patients are diagnosed in the fibrotic phase of the disease. (5, 6)

An accelerated phase of the disease shows 10-19% blasts in the peripheral blood and increased number of CD34+ cells by immunohistochemistry with cluster formation and /or a normal endosteal location in the bone marrow .(6)

In cases with > 20% blasts in the peripheral blood or bone marrow at presentation only other findings may suggest myelofibrosis.

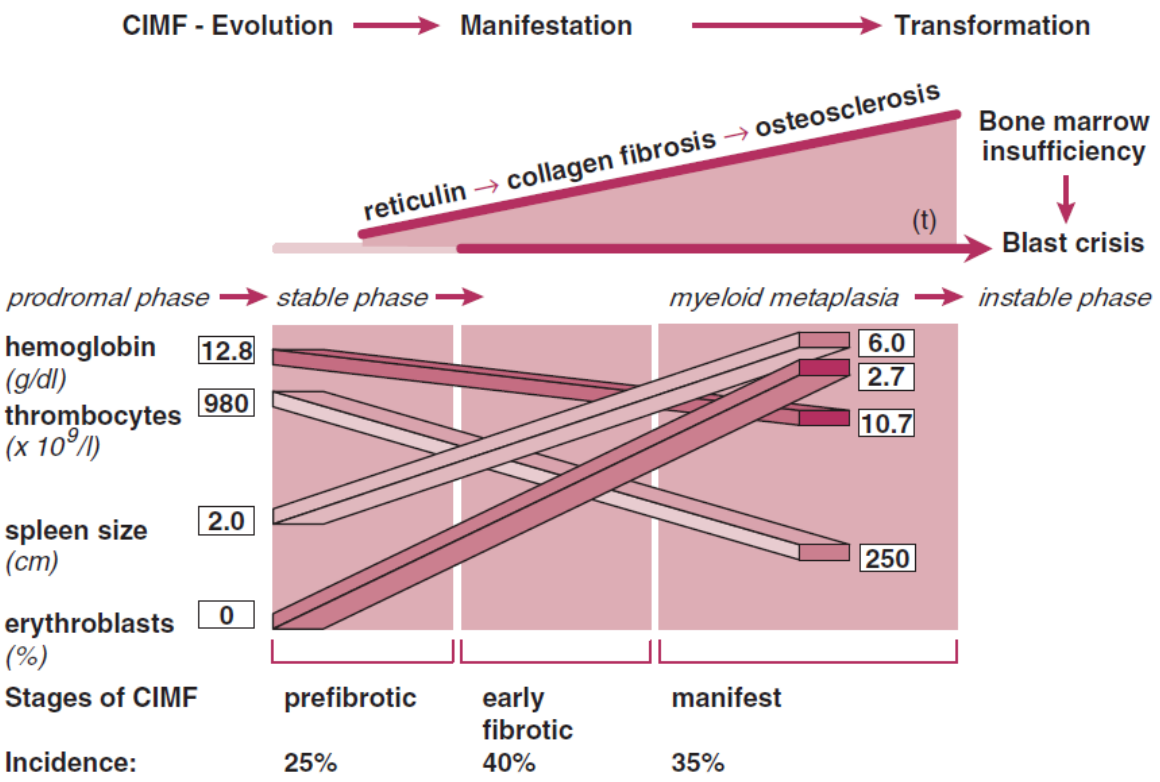


Figure -4 showing the gradual step wise progression from Prefibrotic phase of Myelofibrosis to Fibrotic phase with clinical and laboratory findings.

<u>Blast Phase of Myelofibrosis (BP-Myelofibrosis)</u>

The blast phase (BP) of myelofibrosis (either PMF, post-polycythaemia vera MF or post-ET MF) is synonymous with acute myeloid leukemia (AML). According to the consensus criteria by the International Working Group for Myelofibrosis, BP-MF is defined by either

1) Meeting typical WHO 2008 criteria for AML in the setting of a known prior diagnosis of myelofibrosis (19)

or

2) A persistent increase in peripheral blood blasts to a level greater than 20% for 8 weeks. (20)

The commonest leukaemia arising in a setting of myelofibrosis is AML(M7) acute megakaryocytic leukaemia .(21)

The latter criteria, based on circulating blasts enables a diagnosis to be made in those individuals in whom an aspirate would not possible .The number of blasts in the marrow cannot be determined due to increased replacement of marrow by fibrosis, making an correct estimation of blast percentage very difficult. Several genetic mutations such as IDH1/IDH2, IKZF deletion, NRAS/KRAS, NF1 deletion, TP53 and RUNX1 mutations have been associated with blast phase of myelofibrosis.(22)

A peripheral blood blast percentage >=3% and/or a platelet count <100 $x10^3/\mu L$ at the time of diagnosis are strong and independent predictors of leukemic transformation in patients with primary myelofibrosis.(23)

The majority of individuals with BP-MF usually will survive less than 1 year, despite aggressive treatment. (6)

Semiquantative Grading of bone marrow fibrosis **(WHO 2008 criteria)**

MF0: Scattered linear reticulin with no intersections (cross- over's), corresponding to normal bone marrow.

MF1: Loose network of reticulin with many intersections, especially in perivascular areas.

MF2: Diffuse and dense increase in reticulin with extensive intersections, occasionally with focal bundles of collagen and or focal osteosclerosis.

MF3**:** Diffuse and dense increase in reticulin with extensive intersections and coarse bundles of collagen often associated with osteosclerosis. (5,24)

Genetic Studies

Cytogenetics

Chromosomal abnormalities occur in 32% to 48% of patients with PMF at diagnosis. The karyotype is used to determine prognosis. The commonly seen abnormalities are listed below.

Numerical abnormalities

- Monosomy 5,7 and 17
- Trisomy 8 and 9

Structural abnormalities;

- Deletions of the long (q) arms of chromosomes 5,7,13 and 20.(25)
- Abnormalities of 3q especially the inversion (inv) 9.
- The iso chromosome of 17q and other abnormalities of chromosome 17.

Deletions of 13q and 20q are prominent features of idiopathic myelofibrosis suggesting that gene loss and/or activation is relevant in disease pathogenesis and progression.(25-27)

Karotype and prognosis

Constantine S.T and his colleagues analysed karyotypic abnormalities and its impact seen at the time of diagnosis on survival and stratified patients into the following three categories of cytogenetic abnormalities depending on the median survival of individual. The cytogenetic abnormality testing was done at the time of diagnosis and beyond initial diagnosis.

1) Favourable abnormalities: The patients in this group had a median survival of minimum of 53 months .

2) Unfavourable abnormalities: The median survival for this group of patients was 15 months and 9 months for those evaluated at diagnosis and for those evaluated beyond diagnosis.

3) Very unfavourable abnormalities: The survival of this group of individuals is dismal with all individual dying within 12 months of diagnosis. Regardless of whether the chromosome 17 aberration was discovered at diagnosis or beyond, survival was dismal with all patients projected to have died by 12 months. (28-29)

Table 4 – Cytogenetic abnormalities and its prognostic significance

	Numerical abnormalities	Structural abnormalities
Favourable abnormalities	Trisomy 9,+8	
Unfavourable abnormalities	Deletions in long(q) arm in chromosome 13,20.	Abnormal Chromosome 5or 7
Very unfavourable abnormalities	Monosomy 17, add(17)q25	iso chromosome 17q
Others	Sole trisomy 8, Translocation inv12Q	Deletions in long(q) arm in chromosome 13,or 20 + one other abnormality.

A complex karyotype refers to the presence of more than two abnormalities.(25)

Molecular findings

Screening for somatic Janus Kinase 2 (JAK2V617F) mutation should be carried out routinely in patients with PMF as it is a major diagnostic criterion in the WHO classification .The JAK2 V617F mutation is present in 45-68% of cases of PMF. JAKV617F mutation was detected in 68% of all patients with MPN in an Indian study. In this study, 52% of PMF were positive for the mutation. The presence of JAK2V617F mutation was associated with higher haemoglobin, higher age and higher white blood cell count. (30-40) .The JAK2V617F

mutation correlates with loss of heterozygosity at chromosome 9p. Jak2 plays a major role in haemopoiesis, especially erythropoiesis. JAK2 transmits signals from several cytokine receptors such as EPO, TPO, G-CSF, GM-CSF and IL-3.

When ligands bind to these receptors, the JAK-STAT, MAP-kinase and PI3K pathways are activated. The mutation is seen in the pseudokinase domain (JH2), in exon 14 at position 617 of the JAK2 gene.

Mutations of this region cause constitutive activation of the kinases. This activates the STATs (signal transducers and activators of transcription) even without the action of cytokines such as EPO or TPO. Thus, the JAK2 mutation is a gain of function mutation. STATs act as transcription factors (TFs) regulating the expression of genes that play an important role in cell differentiation, proliferation, and survival. JAK2V617F mutation is commonly found in MPNS in approximately 96%, 50%, and 50% of patients with PV, ET, and MF.

JAK2 has been shown to influence chromatin structure.(41,42) In hematopoietic cells, nuclear JAK2 phosphorylates histone H3Y41, thereby blocking the recruitment of the repressor heterochromatin protein 1α and allowing increased expression of several other genes, such as LMO2 oncogene(43) The discovery of JAK2V617F mutation played an important role in the understanding the pathogenesis of BCR-ABL–negative MPN. A low *JAK2*V617F allele positivity or burden at diagnosis acts as a strong surrogate marker and is associated with shortened survival in cases of PMF. *JAK2* genotyping is mandatory in the diagnostic workup of suspected cases of PMF according to WHO classification as it is a major criteria. A quantitative assay rather than a qualitative assay would be more appropriate to evaluate the JAK2 burden. (44)

Testing for other mutations should be done if atypical features are present on the trephine biopsy, or in cases in the patients lacks a mutation in JAK2 genes.

Other Mutations

1) PDGFRA and PDGFRB rearrangements should be excluded in the presence of significant eosinophilia, as PDGFRA/B-rearranged MPNs are highly sensitive to imatinib therapy.

2) MPLW515L (exon 10) mutations were first described in four cases of JAK2V617F mutation-negative PMF (45), an occurrence that was confirmed by other studies (46-47). MPL mutation-positive patients were older, with a female preponderance and presented with a more severe anaemia (48). Several gain-of-function mutations of MPL have been found in exon 10, resulting in the substitution of a tryptophan 515 to a leucine, lysine, asparagine, or alanine. (49-53).These 5 amino acids play a major role in the cytosolic conformation of MPL and prevent spontaneous activation of the receptor..

3) Mutations in TET oncogene family member 2 (TET2) occur in approximately 15% of cases of PMF and are associated with older age and anaemia. There is no correlation with overall survival or risk of a leukemic transformation. However TET2 testing is not recommended routinely. TET2 (TET oncogene family member 2) is a tumour suppressor gene which is located in the minimal loss-of-heterozygosity (LOH) region of chromosome 4q24. The presence of mutant TET2 does not appear to significantly influence leukocyte count or platelet count in patients with PMF. (54)

4) The clinical significance of mutations in other genes, including – IDH1/2, ASXL1, LNK, IKZF1, CBL and N-RAS is unclear. EZH2 mutations are seen in about 5% of cases and carry a poor prognosis (55).Routine screening of this large gene is still not recommended.

5) Suppressor of cytokine signalling (SOCS) proteins are also important negative regulator. It inhibits JAK signalling which acts by a classic feedback loop. SOCS1-inactivating mutations have been described in B-cell lymphoma. Some mutations in the different SOCS have been found in MPNs, but they are rare in occurrence. SOCS2 may inhibit JAK2V617F signalling,

and its promoter is hypermethylated. At present the role of SOCS proteins in the pathogenesis of MPN remains unclear. (17)

Paediatric myelofibrosis

Primary myelofibrosis in children is rare, with the largest series reporting the findings in 19 children affected with PMF.(56) Children with primary myelofibrosis (PMF) often present with more severe manifestations and have different histopathologic and genetic features from adults. They have an overall megakaryocyte hyperplasia and atypia, proliferation of granulocytes and decrease in erythroid precursors. Micromegakaryocytes were present, but there was absence of hypolobate cloud like megakaryocyte nuclei. Collagen fibrosis was also seen only to a mild degree, when compared with adult cases with no osteosclerosis. Megakaryocytic dysplasia was seen and more than 50% of patients showed eosinophilia. None of the children mutations show JAK2V617F mutations in contrast to adult PMF. Hence it is possible that different mutations than those recognised in adult cases occur in children or it could be that this disorder is not clonal.

There are no reports of malignant transformation in children by either morphology or immunohistochemistry (IHC) for CD34. Only two mutations have been detected, namely changes in the number of copies of RUNX1 and monosomy of chromosome 12p similar to patients with leukaemia, and hence these were considered premalignant changes (57).

Prognosis

A number of scoring systems have been developed for PMF for prognostication and therapy selection, especially to determine the need for allogeneic stem cell transplantation (allo-SCT).

Three main prognostic score systems are in place. (33) One is the Lille score, which includes anaemia and leukopenia or leukocytosis (leukocytes $< 4 \times 10^3/\mu L$ or $> 30 \times 10^3/\mu L$,

respectively) as covariates to identify 3 distinct prognostic groups. The second one, developed at the Mayo Clinic, introduced thrombocytopenia (< 100 × 10^3/µL) and monocytosis (> 10^3/µL).The third one is The IPSS scoring system developed by the International Working Group for myelofibrosis research and treatment.

IPSS — The International Working Group for myelofibrosis research and treatment comprises of haematologists, haematopathologists, and laboratory scientists which has a goal of providing an international platform for scientific dialogue and collaboration in improving the understanding and therapy of the myeloproliferative disorders such as , myelofibrosis. This committee meets annually to discuss and update the various criteria's useful for the diagnosis and management of myeloproliferative disorders. They have come up with a (IPSS prognostic scoring system) based on an evaluation of presenting signs and symptoms in 1054 consecutively-studied patients diagnosed with PMF at seven different centres.(58)The following five adverse prognostic features were noted on multivariate analysis:

i) Presence of constitutional symptoms

- ❖ night sweats,
- ❖ weight loss > 10% over 6 months,
- ❖ unexplained fever (> 37.5°C)
- ❖ diffuse bone pains.

ii) Age greater than >65 years

iii) Haemoglobin less than 10 g/dL

iv) TWBC count >25x10^3/µL

v) Circulating blast cells ≥1 percent

Patients were classified into four risk groups based on the presence of these variables. Each variable was given a score of 1.The total score was used to classify patients into four risk groups

As described below:

a) IPSS zero score: low risk

b) IPSS one score: intermediate risk-1

c) IPSS two score: intermediate risk-2

d) IPSS >= 3 score: high risk

These groups had non-overlapping median overall survivals of 135, 95, 48, and 27 months, respectively. There is also an age-adjusted IPSS for patients less than 65 years of age.(59)

Dynamic IPSS or DIPSS — Gangat et al have modified The IPSS by the addition of a score of 1 for the presence of constitutional symptoms and modifying the score for haemoglobin to 2. (60)

The DIPSS is calculated as follows:

i) Presence of constitutional symptoms: 1 point

ii) Age >65 years: 1 point

iii) Haemoglobin <10 g/ dL: 2 points

iv)Leukocyte count >25,000/ µL: 1 point

v) Circulating blast cells ≥1 percent: 1 point

Subjects with

a) DIPSS zero score: low risk,

b) DIPSS one to two score: intermediate- 1,

c) DIPSS three to four score: intermediate-2,

d) DIPSS 5 to 6 points were considered High risk (60)

In a separate analysis done by Passamonti et al done on 525 patients showed that DIPSS predicted the progression of subjects with PMF to acute myeloid leukaemia (61).The incidence of blast phase development was 0.3, 0.7, 2.6 and 8.6 per 100 patient-years for those in the low-risk, intermediate-1, intermediate-2, and high risk categories, respectively. Worsening of the DIPSS during follow-up also appeared to predict for a significantly higher risk for blast phase development.

DIPSS Plus — IPSS-independent prognostic factors for survival in PMF have been identified. These include

a) Transfusion dependence,

b) Unfavourable karyotype (including +8, -7/7q-, i(17q), inv(3), -5/5q-, 12p-. 11q23 rearrangements and complex karyotype)

c) Platelet count (PC) <10,000/ cu mm.(58-59)

These three additional risk factors were added to the DIPSS score to develop DIPSS-Plus by Gang at et al, with scoring as follows:

Table 5- DIPSS Plus scores

DiPSS Low risk score	0 points
DIPSS intermediate risk-1 score	1 point
DIPSS intermediate risk -2 score	2 points
DIPSS high risk score	3 points
Unfavourable karyotype	1 point
Platelet count <10,000/cumm	1 point
Transfusion need	1 point

When the DIPSS Plus scoring system was applied to 793 consecutive patients with PMF which were seen at the Mayo Clinic, those with zero points (low risk), one point

(intermediate risk-1) two to three points (intermediate risk-2), or four to six points (high risk), had median survivals of 15.4, 6.5, 2.9, and 1.3 years, respectively. When this scoring system was applied to 299 younger (i.e., age <60 years) patients with PMF seen at the Mayo Clinic, median survivals (from the time of their referral) were 20, 14.3, 5.3, and 1.7 years, respectively, confirming the better prognosis of younger patients with PMF .(61)

A "*very high*" risk category, with a median survival of nine months and a two-year mortality rate of 83 percent was obtained from a study of 884 patients with PMF seen at the Mayo Clinic for a period of 34 years (1977 and 2011). This group had either a monsomal karyotype or inv (3) or i(17q) abnormalities or any two of the following

1) circulating blasts >9 percent
2) leukocyte count ≥40,000/ cumm,
3) or other unfavourable karyotype (i.e. complex karyotype or any sole or two abnormalities including +8, -7/7q-, -5/5q-, 12p-, 11q23 rearrangement).

The difference in survival between the "very high" and "high" risk groups was explained, in part, by a difference in the risk of leukemic transformation (two-year incidences of 31 and 7 percent, respectively).(61)

Predicting leukemic transformation — Two of the three additional risk factors (i.e., favourable karyotype, platelet count <1, 00, 000/ cu mm) were used to construct a prognostic model in order to predict leukemic transformation. Those with none of these risk factors or those with one or two of these risk factors had 10-year risk of leukemic transformation of 12 and 31 percent, respectively. (61, 62)

VII) Essential Thrombocythaemia

Essential thrombocythaemia is a rare chronic myeloproliferative neoplasm (MPN) that involves the megakaryocytic lineage. The incidence of essential thrombocythaemia is estimated to be 0.6-2.5-100000 person years according to the Polycythaemia vera study group (PVSG group).(63)

ET is characterized by sustained thrombocytosis >= 4,50,000/cu mm in the peripheral blood while the bone marrow shows increased numbers of large megakaryocytes. Bone marrow biopsy is important at arriving at a diagnosis of ET by excluding

- other myeloid neoplasms associated with excessive platelet counts, such as myelodysplastic syndrome (MDS) associated with deletion (5q)
- myelodysplastic syndrome /myeloproliferative neoplasm (MDS/MPN),
- refractory anaemia with ring sideroblasts (RARS)
- the prefibrotic phase of myelofibrosis.

A few patients may develop BM fibrosis with associated myeloid metaplasia. Transformation to AML or MDS is seen in less than 5% of patients. As with PMF, the presence of the t (9, 22) or its fusion gene, BCR-ABL1 excludes the diagnosis of ET. The JAK2V617 mutation is found in 40-50% of cases of ET and helps exclude reactive causes of thrombocytosis. (63)

Affected individuals usually present with episodes of thrombosis and haemorrhage. ET is an indolent disorder with long symptom-free intervals, interrupted by occasional life threatening, arterial and venous thrombotic events and hemorrhagic episodes.

There are various systems for the diagnosis of essential thrombocythaemia as shown in table 6.

Table- 6 Criteria for diagnosis of essential thrombocythaemia (64)		
WHO 2008 criteria	**Polycythaemia vera study group criteria**	**Committee for British standards in haematology**
1) Sustained platelet count>4,50,000/cumm	1) Sustained platelet count>6,00,000/cumm	1) Sustained platelet count>=4,50,000/cumm
2)Bone marrow biopsy showing megakaryocyte lineage proliferation (without) increase in neutrophil granulopoiesis or erythropoiesis	2) Hematocrit < 40% (or normal red blood cell mass)	2) Presence of an acquired pathogenetic mutation (JAK2 OR MPL)
3)Lack of criteria fulfilment for PV,CML,PMF,MDS or other myeloid neoplasms	3) No myelodysplastic syndrome of Philadelphia chromosome	3) No other myeloid malignancy, (especially CML,MDS,PV,OR PMF)
4)Presence of JAK2V617 or another clonal marker, in the absence there is lack of evidence of reactive thrombocytosis	4) No collagen fibrosis or(less than 1/3 rd of biopsy area)without Leukoerythroblastic blood picture or concomitant splenomegaly	4)No reactive cause of thrombocytosis and normal iron stores
	5)No evidence of reactive thrombocytosis or iron deficiency	5) Bone marrow biopsy showing increased numbers of megakaryocytes with a spectrum of morphology (mostly large with hyperlobate nuclei) and generally no increase in reticulin

The most common secondary (or reactive) causes of thrombocytosis are

- infection, and inflammation
- iron deficiency
- tissue damage
- Haemolysis
- severe exercise,
- malignancy,
- hyposplenism and other causes of an acute phase response.

The platelets are mostly normal sized with a normal mean platelet volume MPV). A peripheral blood film may show features which may indicate an underlying cause, such as acute infective or inflammatory processes. A bone marrow aspirate or trephine biopsy if done due to a uncertainty in diagnosis will show increased number of megakaryocytes (megakaryocytic hyperplasia) with normal mature megakaryocyte morphology and an interstitial distribution without any clustering. The reticulin content is not increased. In cases chronic infective or inflammatory processes there may also be granulocytic hyperplasia and features suggestive of the anaemia of chronic disease.(65)

Laboratory findings

Peripheral blood

There is marked thrombocytosis.The platelets display anisocytosis, ranging from tiny platelets to atypical large, giant platelets ,bizarre shapes; pseudo pods and agranular platelets may be seen. The TWBC count and differential count (DC) are usually within normal limits. Basophilia is usually absent or minimal. The red cells are predominantly normocytic and normochromic unless recurrent haemorrhage has led to iron deficiency. In

such cases the red cells are microcytic and hyphochromic. A leukoerythroblastic blood picture and tear drop cells are absent in ET. (63)

Bone marrow morphology

Bone marrow aspirate: Smears reveal markedly increased numbers of large hyperlobulated staghorn type megakaryocytes dispersed in the background of large sheets of platelets. Perls stain reveals stainable iron in aspirated bone marrow specimens in 40-70% of cases. (63, 66)

Bone marrow biopsy: Normocellular or hypercellular marrow with marked megakaryocytic proliferation with predominance of large and giant forms of megakaryocytes displaying abundant mature cytoplasm and deeply hyperlobulated nuclei (stag-horn like) nuclei. The megakaryocytes are usually dispersed in loose clusters. Bizarre, highly atypical megakaryocytes such as those observed in primary myelofibrosis are not seen. Granulocytic proliferation is usually not seen and erythroid precursors may be found only if patient has a recent episode of haemorrhage.

Reticulin is normal or minimally increased. The presence of increased reticulin or any collagen fibrosis excludes ET. (17,63)

Cytogenetic changes : include +8, abnormalities of 9q,and del (20q).Isolated del 5q has also been reported in ET ,careful morphological examination is required to distinguish such cases from MDS associated abnormalities.(63)

MATERIALS AND METHODS

This study was carried out in the Departments of General Pathology, Haematology and Transfusion Medicine and Immunohaematology, Christian Medical College, Vellore. The period of study was from January 2009 to February 2014.

All patients with a bone marrow trephine biopsy diagnosis of primary myelofibrosis (PMF). and essential thrombocythaemia (ET) were enrolled in this study. Only diagnostic biopsies from patients seen at CMC were included. The bone marrow trephine biopsies were retrieved from archives of the Pathology department and the histological features were reviewed in detail. PMF was further subdivided into the prefibrotic phase and fibrotic phase of PMF.

Cases already on treatment were not included in the study. The other exclusion criteria are listed below.

Exclusion criteria:

A. Myelofibrosis

- Other preexisting myeloproliferative disorders such as chronic myeloid leukemia (CML), polycythaemia Vera (PV) and essential thrombocythaemia (ET).
- Myelodysplastic syndrome with fibrosis or other hematopoietic neoplasms.
- Metastatic malignancy
- Chronic Infections, autoimmune disorders and other chronic inflammatory conditions.

B. Essential thrombocythaemia

Evidence of reactive thrombocytosis such as

- Iron deficiency
- Splenctomy or surgical procedure
- infections and chronic inflammatory disorders including connective tissue disease

- Lymphoid neoplasms and metastatic cancer.

The other clinical and laboratory details including case records were obtained from the archives and electronic databases of the departments of Pathology , Haematology and Transfusion Medicine and Immunohaematology.

Parameters recorded

1**)** Clinical features

- Age, sex, constitutional symptoms and episodes of thrombosis or hemorrhage as appropriate
- Presence of splenomegaly and spleen size

2) Laboratory features

- Haemogram- complete blood count, blood picture to specifically look for the presence of leukoerythroblastosis, tear drop cells in cases of PMF, evidence of thrombocytosis or platelet anisocytosis in cases of ET any other relevant features.
- Serum levels of lactate dehydrogenase (LDH).
- JAK2V617 mutation status when available.
- Results of cytogenetic analysis including FISH for BCR-ABL1 fusion when available.

Histopathological assessment

Bone marrow trephine biopsies from patients with PMF and ET were reviewed in detail by two independent observers with different years of experience (trainee and consultant haematopathologist) without being aware of JAK2 mutation status . The review of each case included at least three slides stained with Haematoxylin and Eosin (H&E) ,Gordon and Sweet Reticulin (GS reticulin) and Periodic acid Schiff (PAS). The following histopathological features of each case were analysed:

- Cellularity - Overall and lineage-wise

- Megakaryocyte location
- Megakaryocyte number and size
- Megakaryocyte nuclei - Hyperlobate staghorn/ Hypolobate cloudlike/ hyperchromatic dysplastic nuclei/ naked nuclei
- Megakaryocyte clusters - size and density*
- Sinusoidal ectasia
- Intrasinusoidal hematopoiesis
- Reticulin grade
- Scoring of biopsies using CMC scoring system for all three subgroups and the Brousseau scoring system for ET and prefibrotic PMF only. The biopsies were reclassified according to the scoring system (see below).

We devised a scoring system in which each of the above parameters was assigned a score from 0-2 . Using our system, the maximum possible score was 21. (Table 7)

Our scoring system used 16 parameters both for classification into ET, prefibrotic phase of PMF and PMF and also for comparison of these entities.

The scoring system devised by Brousseau which listed nine parameters to differentiate between ET and prefibrotic phase of PMF was also used by us to calculate the global score these for two conditions . (Tables 8 & 9) Using the Brousseau scoring system the maximum possible score was 10. We compared our scores and the Brousseau scores in these two conditions.

There were 27 cases of ET, 13 cases of Prefibrotic phase of PMF and 94 cases of PMF after reclassification according to our scoring system. We compared the histological findings of

- ❖ PMF and Prefibrotic phase of PMF
- ❖ ET and the Prefibrotic phase of PMF

❖ Comparison of biopsies with and without JAK2 mutation.

Statistical analysis was done to determine which of the parameters were significant in differentiating the groups.

Table 7

Scoring system devised in CMC

Score / Histological Features	0	1	2
Increased overall cellularity	Absent	Present	-
Granulocyte precursor	Normal / decreased	Hyperplasia	Decreased (02)
Myeloid shift to left	Absent	Present	
Erythroid cellularity	Normal	Increased (01)	Decreased (02)
Megakaryopoiesis	Normal (00)	**Increased (01)**	Decreased(02)
Megakaryocyte location	Perisinusoidal	**Paratrabaecular (01)**	
Type of megakaryocytes			
Size	Normal	Large	Small
Hypolobate cloud like megakaryocyte nuclei	Absent(00)		
Hyperlobate , giant and staghorn megakaryocytic nuclei	Absent (00)	Present(01)	
Hyperchromatic dysplastic megakaryocyte nuclei	Absent(00)	**Present(01)**	
Naked nuclei	Absent(00)	Present(01)	
Megakaryocyte clusters (consists of more than 3 megakaryocytes)	Absent	Present Small - > 4-6 megs	Present Large - > 7 megs
Density of megakaryocyte clusters	Loose – at least 3 megs without close contact	Dense – at least four megs opposed to each other	
Sinusoidal ectasia	Absent(00)	**Present(01)**	
Intra sinusoidal haematopoiesis	Absent(00)	**Present(01)**	
Reticulin grade (MF 0-3)			
	MF 0-Scattered linear reticulin; no intersections	MF 2-Diffuse, dense, extensive intersections; focal collagen bundles / osteosclerosis	
	MF 1-Loose network, many intersections	MF 3- As for 2 with coarse bundles of collagen	

For ET and prefibrotic PMF, we compared our scoring system with that described by Brousseau et al (1). (Table 2) .With this system, the maximum score obtained is 10.

Table 8-Histological parameters evaluated by Brousseau for classification of bone marrow trephine biopsies into PMF, prefibrotic phase of PMF and ET.

Histological parameters		**Score**
Overall cellularity of hematopoiesis	Normal	0
	Increased	1
Reticulin fiber score	MF0	0
	MF1	1
	MF2	2
	MF3	9
Erythroblastic cellularity	Normal	0
	Increased	1
	Decreased	2
Granulocytic cellularity	Normal	0
	Increased	1
Bare megakaryocytic nuclei	Absent	0
	Present	1
Giant and staghorn megakaryocytic nuclei	Absent	0
	Present	1
Cloudlike megakaryocytic nuclei	Absent	0
	Present	1
Hyperchromatic–dysplastic nuclei	Absent	0
	Present	1
Megakaryocytic nucleo–cytoplasmic ratio	Normal	0
	Increased	1
Type of megakaryocytic clusters	Loose	0
	Tight	1
Paratrabecular megakaryocytes	Absent	0
	Present	1

Table 9

Scoring system to calculate the global score described by Brousseau for differentiating between ET and prefibrotic PMF(17).

Score / Histopathological features	0	1	2
Overall cellularity	Normal	Increased	
Granulocytic cellularity	Normal	Increased	
Erythroblastic Cellularity	Normal	Increased	Decreased
Paratrabaecular megakaryocytes	Absent	Present	
Megakaryocyte clustering	Loose	Tight	
Cloud like nuclei	Absent	Present	
Hyperchromatic dysplastic nuclei	Absent	Present	
Bare nuclei	Absent	Present	
Megakaryocyte nuclear cytoplasmic ratio	Normal	Increased	

Patients were also divided into two groups based on their JAK2 mutation status. The laboratory findings in these two groups were compared.

Statistical Analysis:

Data entry was done using the epidata program.

All statistical analysis was done using Statistical package for social sciences(SPSS) data software version 13. Descriptive statistics such as frequency and percentage were used.

A P value of < 0.05 was considered statistically significant.

Categorical variables were analysed using $\chi 2$ test with Yates continued correction and Fischer's exact test.

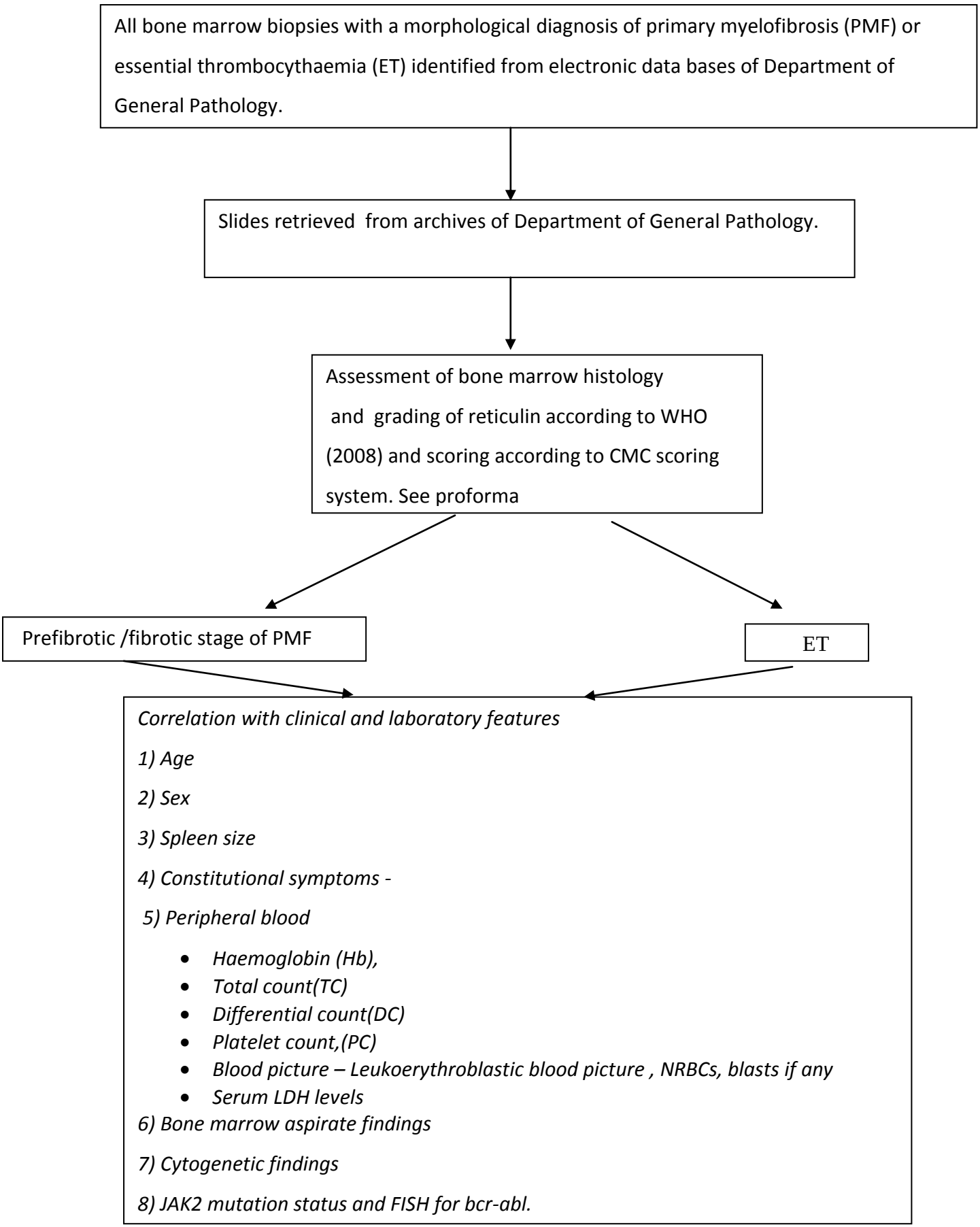
Continuous variables: Shapiro- Wilk W test was done to find out if the variables followed a normal distribution (score >.05).

Continuous variables with *normal distribution* (score >.05): Mean value was taken as a significant score and a paired t-test was applied for comparison of a variable in different groups.

Continuous variables which did not follow a normal distribution (score <.05): Median value was taken as significant and a Mann –Whitney test was done to compare the variable in different groups.

A total of 159 cases were evaluated out of which a total of 27 cases of ET ,107 cases of Primary myelofibrosis (13 cases Prefibrotic phase of PMF and 94 cases of fibrotic phase of PMF) were included in the study .The rest of the cases were excluded based on the exclusion criteria.(Figure 6)

Figure -5 STROVE DIAGRAM OF THE WORK UP PLAN



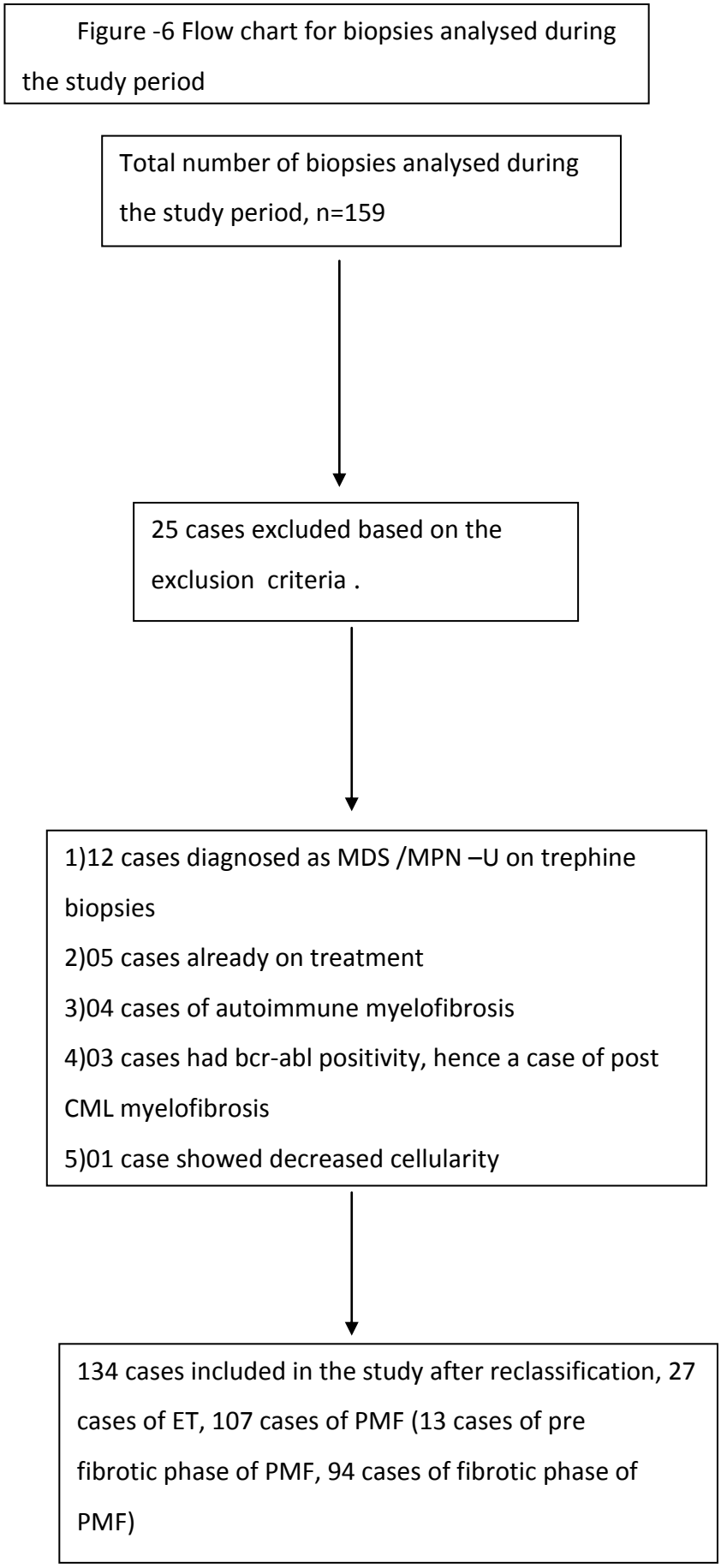
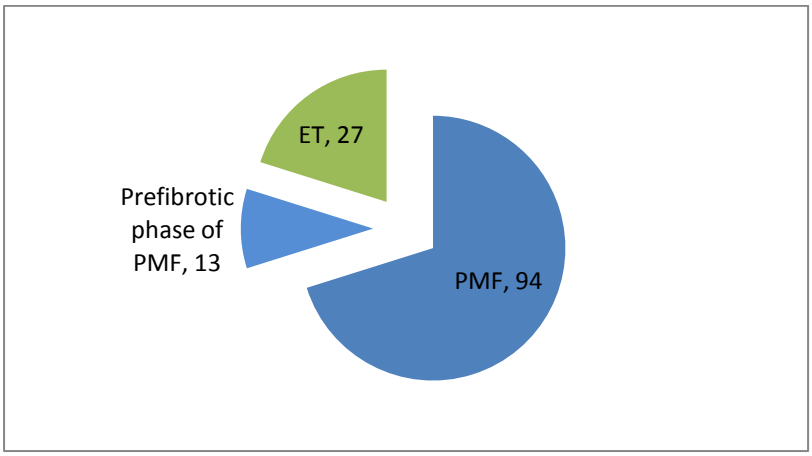


Figure -6 Flow chart for biopsies analysed during the study period
Total number of biopsies analysed during the study period, n=159
25 cases excluded based on the exclusion criteria .
1)12 cases diagnosed as MDS /MPN –U on trephine biopsies
2)05 cases already on treatment
3)04 cases of autoimmune myelofibrosis
4)03 cases had bcr-abl positivity, hence a case of post CML myelofibrosis
5)01 case showed decreased cellularity
134 cases included in the study after reclassification, 27 cases of ET, 107 cases of PMF (13 cases of pre fibrotic phase of PMF, 94 cases of fibrotic phase of PMF)

RESULTS

There were 134 bone marrow trephine biopsies which fulfilled the inclusion criteria for PMF/ET during the study period. These biopsies comprised 107 PMF (including 13 in the pre fibrotic phase) and 27 ET.

Figure 7- Distribution of cases.



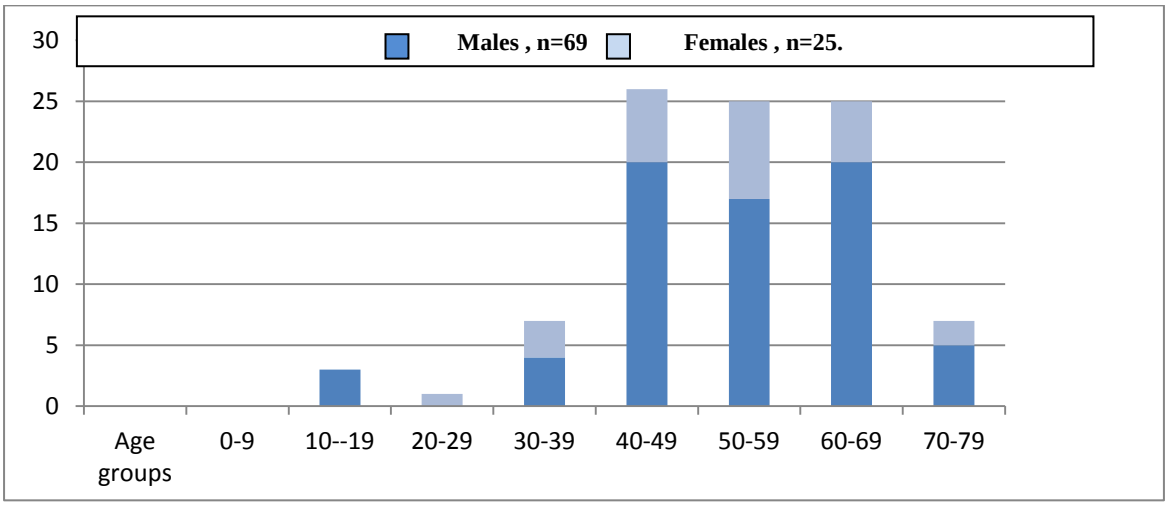
A) Primary myelofibrosis

A .1 Clinical features

A.1. i Age and sex distribution (Figure 8)

The majority of patients (81%) were above 40 years of age .The median age at diagnosis was 53 years, with 76 patients being in the 40-69 age group. There were 69 males and 25 females (sex ratio 2.8:1).

Figure 8- Age and sex distribution



A.1. ii <u>Presenting features</u>

Clinical details were available for 67 patients, 64 (94%) of whom had one or more constitutional symptoms such as drenching night sweats, weight loss (>10% over six months) and unexplained fever (>37.5°C). The common symptoms were easy fatigability (83%), weight loss (52 %), fever (49%) and anaemia (46%). Less common symptoms were a feeling of satiety as well as pruritus in 2.5% of patients. Splenomegaly status at presentation was available for 66 (70%) patients in whom mean spleen size determined by ultrasonography was 20.6cm.

A.2. <u>Laboratory findings at diagnosis for PMF</u> (Table 10)

A.2.i <u>Peripheral blood findings</u>

<u>Haemogram in PMF</u>

There was wide variability in the haemogram**.** The haemoglobin (Hb) ranged from 4.7-17.9 g/dl, total WBC count from 1.2x10³-55.981x10³/µl and platelet count from 2x10³-10,52x10/ µl**.** Anaemia (Hb <10g/dl) was the most common cytopenia and was seen in 40% of patients. Leucopenia (<4 x 10^3/µl) was seen in 8.5% and thrombocytopenia (platelet count (<150 x

10^3/µl) in 2%. Circulating blasts were present in 30% of cases with blast percentages ranging from 1-18 .

Blood picture in PMF

The peripheral blood film reports were available in 77(82%) patients and showed a leukoerythroblastic blood picture in 66 (86%) and tear drop cells in 70 (91%). Other findings included anisocytosis, poikilocytosis with ovalocytes, hypersegmented neutrophils, eosinophilia, basophilia, thrombocytosis and large platelet aggregates.

Serum lactate dehydrogenase (LDH) levels in PMF

LDH values were available in 86 (91%) of patients with a median value of value of 1119 IU/l (range of 329-4043).

Table 10 - Blood findings in PMF

Primary myelofibrosis, n=94			
Parameters	Range	Mean	Median
Haemoglobin(g/dl)	4.70-17.90	10.40	10.48
WBC countx10^3/µl	1.2-55.98	13.18	9.9
Platelet count x10^3/µl	2-1052	242	202
Number of patients with Anaemia(Hb<10g/dl)	38 (40%)		
Leucopenia (<3.5 x 10^3/µl)	08 (8.5%)		
Thrombocytopenia (<150 x 10^3/µl)	20 (21.27%)		
Peripheral blood films available in	77 patients		
Leukoerythroblastic blood picture	66 patients		
Nucleated red blood cells /100 WBC	1-28		
Tear drop cells	70 patients		
Number of patients with circulating blasts	28 (30%)		
Percentage of circulating blasts	1-18%		
Serum LDH values available in	86 patients (91%)		
LDH IU/L	1119 (329-4043)		

A.2.ii <u>Bone marrow trephine biopsy findings in PMF</u> (Table 11)

<u>Cellularity in PMF</u>

The majority of bone marrow trephine biopsies (67, 71%) showed increased cellularity at diagnosis, with normal cellularity in 27 % and decreased cellularity in 2%. Myeloid left shift was present in 43(46%) cases.

Morphology of PMF (Figure 20-32)

The most consistent feature of PMF was increase in reticulin. MF3 reticulin grade was present in 91 (97%) of biopsies with MF2 reticulin grade in the remaining 3%.

Common findings (in more than 80%)

1) Megakaryocytes in paratrabecular location - 92 biopsies (98%)

2) Increased number of megakaryocytes - 89 (95%)

3) Large megakaryocytes - 81 (86%).

4) Hypolobate bulbous cloud-like megakaryocytes - 85(90%),

5) Hyperchromatic dysplastic megakaryocytes - 88(93%).

6) Loose clusters of megakaryocytes - 94%

7) Sinusoidal ectasia - 86 (91%)

8) Intrasinusoidal haematopoiesis - 82 (87%).

Less common findings

1) Dense clusters of megakaryocytes - 70%

2). Small megakaryocytes and naked nuclei - 41% and 53 %.

A .2.iii Scoring of PMF (Table 11 and Figure 9)

CMC Scoring system

The total scores for the 94 PMF were in the range of 11-21 by our 21 point scoring system . The majority (57, 61%) of biopsies had scores of 13-17. Scores at the extreme ends (11 and 21) were seen in approximately five (5%) biopsies each. The low scores (11 and 12) represented end stage PMF with marked fibrosis and low cellularity. Biopsies with high scores (18-21) showed almost all the features evaluated.

Figure 9 - Range of scores in PMF obtained by CMC scoring system.

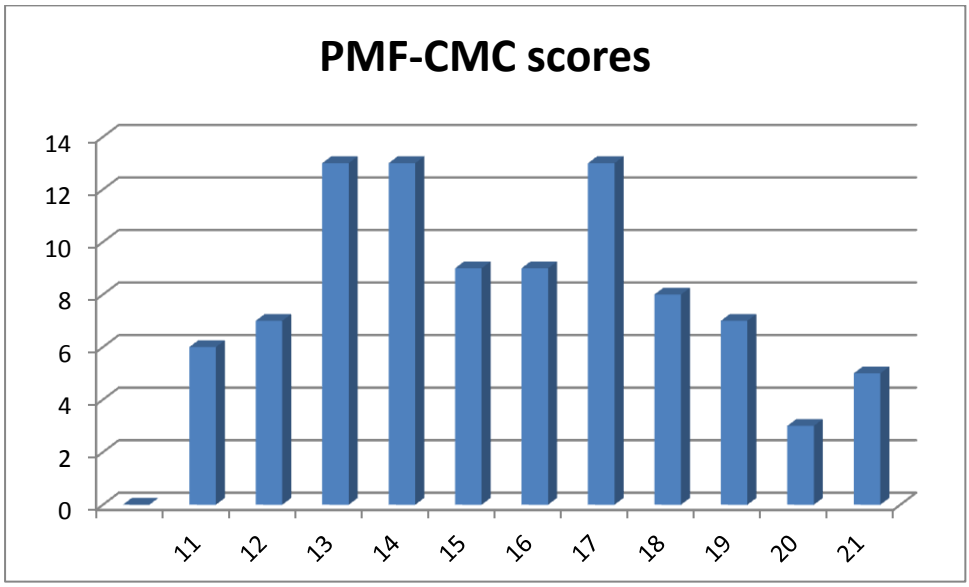


Table 11 - Histological parameters and scores in PMF, CMC scoring system (n=94)

Score / Histological Features	0			1			2		
		N	%		n	%		n	%
Cellularity	Normal	25	27	Increased	67	71			
	Decreased, with fibrosis	2	2						
Granulocyte precursor number	Normal	25	27	Hyperplasia	67	71	Decreased	2	2
Myeloid shift to left	Absent	51	54	Present	43	46			
Erythroid cellularity	Normal	26	28	Increased	03	03	Decreased	65	69
Megakaryopoiesis	Normal	03	03	**Increased**	**89**	**95**	Decreased	2	2
Megakaryocyte morphology and distribution									
Megakaryocyte location	Normal (perisinusoidal)	94	100	**Paratrabecular**	**92**	**98**			
Size	Normal	92	98	Large	81	86	Small	39	41
Megakaryocyte nuclei									
Hypolobate cloud-like megakaryocyte nuclei	Absent	[3]	9	Present	**85**	**91**			
Hyperlobate , giant and staghorn megakaryocytic nuclei	Absent	59	63	Present	35	37			
Hyperchromatic dysplastic megakaryocyte nuclei	Absent	06	06	Present	**88**	**94**			
Naked nuclei	Absent	44	47	Present	50	53			
Megakaryocyte clusters (more than 3 megakaryocytes)***									
Small - 4-6 megs	Absent	02	02	**Present**	**92**	**98**			
Large - > 7 megs	Absent	23	25				Present	71	75
Density	**Loose *– at least 3 megs without close contact**	**88**	**94**	Dense** – at least four megs opposed to each other	66	70			
Sinusoids									
Sinusoidal ectasia	Absent	08	08	**Present**	**86**	**92**			
Intra sinusoidal haematopoiesis	Absent	12	13	Present	82	87			
Reticulin grade									
Reticulin grade (MF 0-3)****	MF 0	0	0	**MF 2**	**3**	**3**			
	MF 1	0	0	**MF 3**	**91**	**97**			

*– at least 3 megs without close contact ** – at least four megs opposed to each other

*** Small clusters - 4-6 megs ; Large clusters - > 7 megs

****MF 0- Scattered linear reticulin, no intersections; MF 1-Loose network, many intersections ; MF 2-Diffuse, dense, extensive intersections, focal collagen bundles / osteosclerosis ; MF 3- As for 2 with coarse bundles of collagen

A.2.iv JAK2 mutation status in PMF (Table 13 , Appendix-2, Figure 49)

The JAK2V617 mutation analysis results were available for 67 cases of which 43 (64%) were positive for the JAK2V617 mutation. Of these 43 cases, 26 (60%) were males.

Comparison of JAK2V617 positive PMF and JAK2 WT PMF (Table 12)

Although the blood counts and the number of small megakaryocytes in JAK2V617 positive PMF were higher than in JAK2WT PMF, only the mean haemoglobin at presentation showed a statistically significant difference between the two groups (10.7 vs 9.3 g / dl in JAK2 WT PMF, P value =.04). There were no other statistically significant differences in the other parameters and histological features did not show any stastically significant differences in patients with and without the mutation. Details of histopathological features of these two groups are given in Appendix 2.

Table 12 - Haematologic and clinical data in JAK2V617 positive and JAK2 WT PMF

Parameters	**JAK2V617POSITIVE PMF n = 43**	**JAK2 WILD TYPE PMF n=24**	**P-value**
Number of cases	43	24	
Age at diagnosis,(median)	53	54	
Sex			
Male	26(61%)	22(92%)	
Female	17(39%)	02(8%)	
Spleen status			
Available in	31/43	16/24	
Mean spleen size by USG*	18 cm (range 13.7-26 cm)	19.9 cm (range 16-28 cm)	.02
Laboratory findings at diagnosis			
Haemoglobin (g/dl)	5.1-17.9 (mean 10.7g/dl)	4.7-16.2 (mean 9.3g/dl)	.04
Leukocyte count x 10^3/µl	1.7-3.3 (median 10)	1.2 -55.98 (median 8.4)	.44
Platelet count x 10^3/µl	600-1052 (median 210)	2-717 (median 184)	.60
Peripheral blood films			
Findings	LEBP** in 33/39 (85%)	LEBP ** in 15/15 (100%)	
Number of cases with blasts	13 (30%)	9 (37%)	
Number of blasts	1-18	1-18	
LDH IU/l	544-4043 (median 1147)	329-2862 (median 1016)	.40

* USG-Ultrasonography**LEBP-Leukoerythroblastic blood picture

A.2.v Cytogenetic findings in PMF (Appendix 9) (Figure 49-55)

A prerequisite for the diagnosis of PMF is the absence of the t (9;22) which may be established either by conventional cytogenetic or fluorescence in situ hybridisation (FISH) analysis. FISH analysis detects BCR/ABL1 fusion which is indicative of the the t(9;22) . Details of cytogenetic studies were available for 71 patients, all of whom were negative for the translocation. Conventional analysis was performed in 56 patients, 24 (43%) of whom showed an abnormal karyotype. Neither form of testing was done in 14 patients.

Of the 94 PMF studied, cytogenetic analysis was done for 56 and FISH for 71 patients. An abnormal karyotype was seen in 24 PMF (42%). Neither form of testing was done in 14 patients.

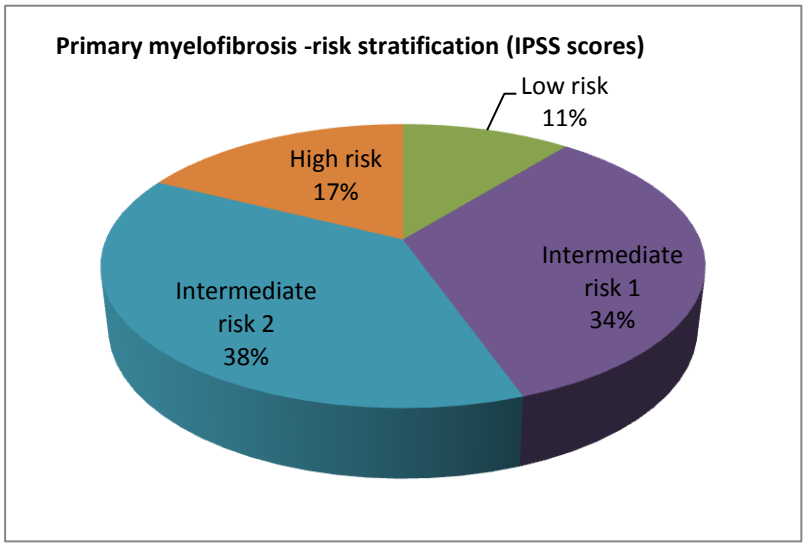
The recurrent MPN-associated abnormalities which were also seen in this series included trisomies 8 and 9, deletions of the long (q) arms of chromosomes 7, 13 and 20 and the short (p) arm of chromosome 12.

The most common abnormality was the deletion 20q seen in six patients followed by the deletion 13q and trisomy 8 in three. Trisomy 9 and loss of chromosome 7q was seen in two patients, either because of a partial deletion or an unbalanced t(1;7) which resulted in monosomy for 7q and trisomy for 1q. Deletion 12p was seen in one patient.

A.2.vi IPSS SCORES IN PMF (Figure 10)

The IPSS score was available for 74 (95%) cases. The majority (72%) of our patients were in intermediate risk 1 and intermediate risk 2 groups. The distribution of characteristics of the 13 high risk cases was as follows : age >65 years (61%), constitutional symptoms and Hb <10g/dl (100%), WBC >25,000/µl (30%), blasts >1% (84%).

Figure 10 - IPSS risk groups in PMF



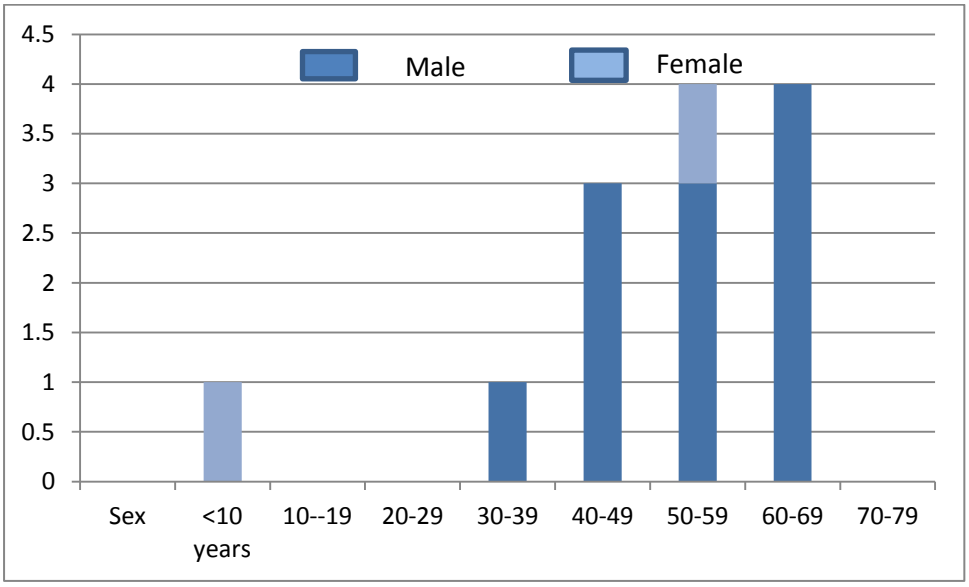
B. Prefibrotic phase of PMF

Thirteen of the 107 PMF were in the early (cellular) prefibrotic phase of PMF.

B .1 Clinical features of Prefibrotic phase of PMF (Table 14)

B.1.i Age and Sex distribution: Eleven of the 13(85%) cases in the prefibrotic phase of PMF were males. All except one were adults (median age 52 years) and eight were above 50 years .One patient was a five-year-old girl. (Figure 11)

Figure 11- Age and sex distribution of prefibrotic phase of PMF



B.1.ii Presenting features

Constitutional symptoms were reported in three patients. Spleen size was recorded in nine patients with median spleen size determined by ultrasonography being 18 cm.

B.2.i Laboratory findings in Prefibrotic phase of PMF (Table 13)

As for PMF, this subgroup of patients also showed marked variation in the haemogram. The peripheral blood films were available in six patients with findings similar to PMF. Serum LDH values were available for nine (69%) patients (median 926 IU/l, range 656-1056 IU/l).

Table 13 - Clinical and laboratory findings in Prefibrotic phase of PMF, n=13

Sex distribution	
Male	11(85%)
Female	2(15%), aged 5 and 51
Age distribution	
Adults	12, median age 52 (range 32-69)
Children	One, aged five years
Spleen status (available in 9/13)	
Splenomegaly	Range 13-20 cm ; median 18 cm
Haemoglobin	mean 12.5 g/dl (range 8-17)
WBC count	median 20.1 x 10^3/µl (range 5.2-67.1)
Platelet count	median 540 x10^3/µl, (range 129 -2021)
Circulating blasts	n=2 , 1-2%
Peripheral blood films (n=9)	
Abnormal	6 (67%)
Leukoerythroblastic blood picture	3
Tear drop cells	4
Anisocytosis,poikilocytosis,ovalocytes	1
Thrombocytosis, basophilia	2
Neutrophilia, eosinophilia	1
Normal	3(33%)
Serum LDH (IU/L)	n= 9; range 656-1231 ; median 926

B.2.ii Bone marrow trephine biopsy findings in Prefibrotic phase of PMF (Table 14, Figure 33-36)

The following findings were seen in all 13 cases:

- Increased cellularity
- Increase in granulocytic precursors
- Increased numbers of megakaryocytes
- Large megakaryocytes
- Paratrabecular location of megakaryocytes.
- Loose clusters of megakaryocytes
- Absence of intrasinusoidal haematopoiesis
- Reticulin grade MF1

Table 14 - Histological features of bone marrow trephine biopsies in Prefibrotic phase of PMF

HISTOLOGICAL FEATURE			n	%
Increased overall cellularity	Present		13	100
Granulocytes	Hyperplasia		13	100
Myeloid left shift	Absent		06	46
	Present		07	54
Erythroid cellularity	Normal		05	39
	Decreased		08	61
Megakaryopoiesis	increased		13	100
Megakaryocytes				
Megakaryocyte location	Paratrabecular		13	100
	Normal perisinusoidal		13	100
Megakaryocyte size	Small		Present in 5/13	38
	Large		13	100
	Normal		13	100
Megakaryocyte nuclei				
Hypolobate bulbous cloud like nuclei	Present		9	69
Hyperlobate , giant and staghorn megakaryocytic nuclei	Absent		04	31
	Present		09	69
Hyperchromatic dysplastic nuclei	Absent		02	15
	Present		11	85
Naked nuclei	Absent		07	54
	Present		06	46
Type of megakaryocyte clusters	Small	Present	13	100
	Large	Present	11	84
	Loose	Present	13	100
	Dense	Present	08	61
Sinusoids				
Sinusoidal ectasia	Absent		09	69
	Present		04	31
Intra sinusoidal haematopoiesis	Absent		13(100)	100
Reticulin				
Reticulin grade	MF1		13(100)	
Reticulin score	0		13(100)	
Scoring				
CMC score	12-17 (median 15)			
Brousseau score	6-10 (median 8)			

0ther significant findings

- Shift to left in myeloid lineage (54%)
- Decreased erythroid cellularity (62%)

- Large clusters of megakaryocytes (85%)
- Dense clusters of megakaryocytes (62%)
- Small megakaryocytes and small clusters of megakaryocytes (38%) each.
- Sinusoidal ectasia (31%)

B.2.iii <u>Prefibrotic phase of PMF - Scores</u> (Table 15 , Figures 12 and 13)

The median score obtained by our 21 point scoring system was 15 with a range of 12-17. With the Brousseau system, our scores ranged from 6-10 with a median score of 7.

Table 15 - Prefibrotic phase of PMF- Comparison of scoring systems

Scoring system		CMC				Brosseau		
S.no	Score / Features	0	1	2		0	1	2
1	Overall cellularity	Normal	Increased n=13, 100%			Normal	Increased n=13,100%	
2	Granulocytic cellularity	Normal	Increased n=13, 100%	Decreased		Normal	Increased n=13, 100%	
3	Erythroid cellularity	Normal n=5, 39%	Increased	Decreased n=8, 61%		Normal n=5, 39%	Increased	Decreased n=8,61%
4	Paratrabecular megakaryocytes	Absent	Present n=13,100%			Absent	Present n=13,100%	
5	Megakaryocyte cluster density	Loose n=13,100%	Tight n=8,61%			Loose n=13,100%	Tight n=8,61%	
6	Cloud-like megakaryocyte nuclei	Absent n=4,31%	Present n=9,69%			Absent N=4,31	Present N=9,69%	
7	Hyperchromatic dysplastic nuclei	Absent n=2,15%	Present n=11,85%			Absent n=2,15%	Present n=11,85%	
8	Bare nuclei	Absent n=7,54%	Present n=6,46%			Absent n=7,54%	Present n=6,46%	
9	Megakaryocyte N/C ratio	Not assessed - requires morphometry for accurate measurement				Absent N=2,15%	Present N=11,85%	
10	Left shift	Absent n=6,46%	Present n=7,54%					
11	Megakaryopoiesis	Normal	Increased n=13,100%	Decreased				
12	Megakaryocyte Size	Normal n=13,100%	Large n=13,100%	Small n=5,38%				
13	Hyperlobate staghorn type nuclei	Absent n=4, 31%	Present n=9,69%					
14	Megakaryocyte clusters		Small n=13,100	Large n=11,84%				
15	Sinusoidal ectasia	Absent n=9,69%	Present n=4,31%					
16	Intrasinusoidal haematopoiesis	Absent n=13,100%	Present					
17.	Reticulin	MF0,MF1-n=13,100%	MF2,MF3					

Figure 12 - CMC scoring system in prefibrotic MF

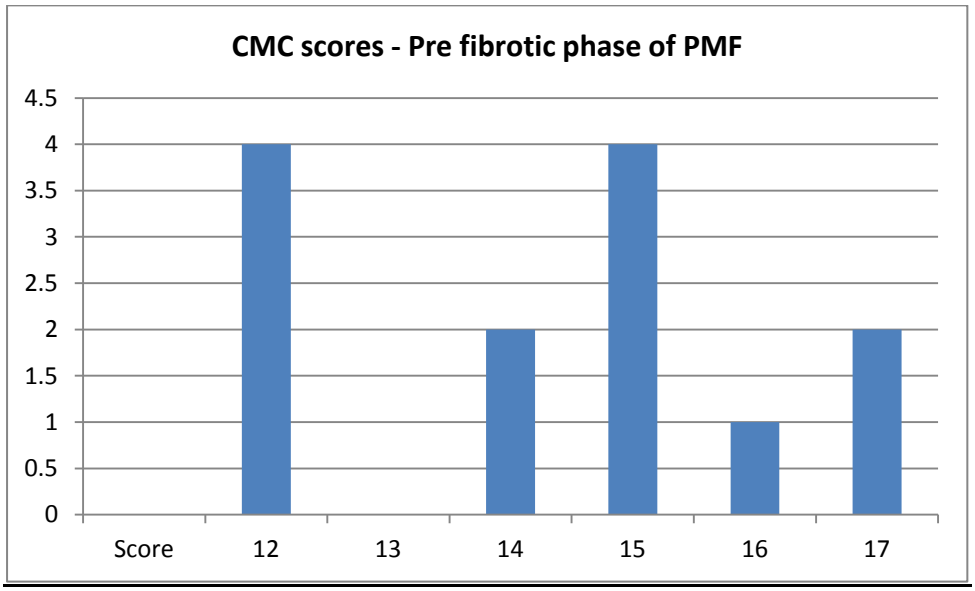
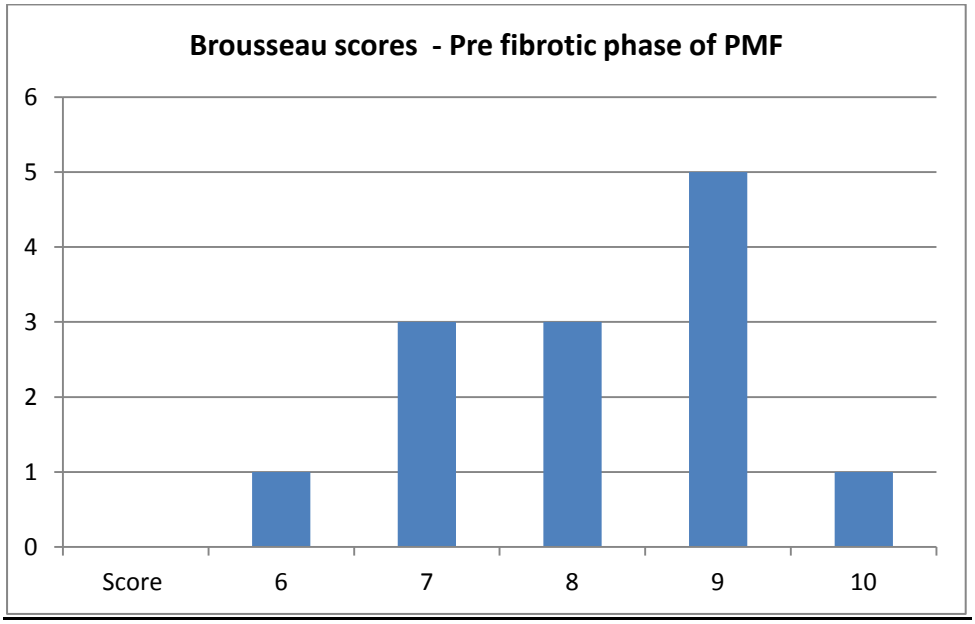


Figure 13 - Brousseau scores in prefibrotic MF



B.2.iv JAK2 mutation in Prefibrotic phase of PMF (Appendix 2 and Appendix 3)

JAK2V617 mutation status was available for six cases of which five (83%) cases were positive for the mutation. The laboratory and histopathological features of these five cases are shown in Appendix 3 and Appendix 4.

B.2.v Cytogenetic findings in Prefibrotic phase of PMF (Appendix 9)

Cytogenetic analysis was done in four patients, two of whom had abnormal karyotypes.

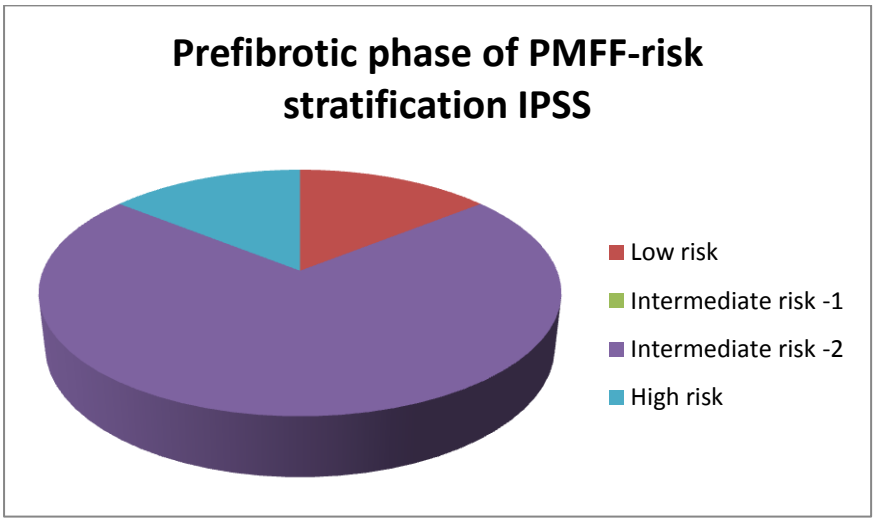
One had 47 chromosomes due to trisomy 9 and a partial deletion of the long (q) arm of chromosome 7 and the other showed an unusual t (4;11)(q25-q27; p13-15).

FISH analysis was done in seven patients, all of whom were negative for BCR/ABL1 fusion.

B.2.vi IPSS scores in Prefibrotic phase of PMF (Figure14)

IPSS scores were available for seven of the 13 prefibrotic phase of PMF, five (71 %) of whom were intermediate risk 1.

Figure 14 - IPSS risk groups in PMF



B.2.vii Comparison of PMF and its Prefibrotic Phase (Tables 16 and 17, and Appendix 4)

The statistically significant differences between PMF and its prefibrotic phase were:

- Larger spleen size in PMF (17.6 vs 20.6 cm , P value =.02).
- Higher blood counts in the prefibrotic phase
    - Haemoglobin (mean 12.48 vs 10.48g/dl, P value =.015).
    - Total WBC count (median 20.1 vs 9 x10^3/µl , P value=.02).
    - Platelet count (median 540 vs 210 x10^3/µl, P value=<.0001)

Findings seen **more commonly in the prefibrotic marrow** than in PMF.

- Increased cellularity and myeloid hyperplasia (P value= .025 each).
- Hyperlobate staghorn-type megakaryocytes (69% vs 37% in PMF, P value=.028) .

Findings seen more **commonly or exclusively in PMF** were

- Sinusoidal ectasia (92% vs 69% in the prefibrotic phase, P value=<.0001)
- Intrasinusoidal haematopoiesis (87% vs nil in the prefibrotic phase, P value=<.0001)

A detailed comparison is shown in Appendix 5.

Table 16 – Comparison of PMF and its Prefibrotic phase – significant clinical and laboratory data

MF, n=67	Myelofibrosis (PMF) , n=94	Prefibrotic phase of PMF, n=13	P-value
Number of cases	94 adults	13 (one child)	
Median age at diagnosis	52	49(excluding one child aged 5)	
Spleen size in cm (mean)	n=66, 13.7- 26 (20.6 cm)	n=9, 13- 20 (17.6cm)	**.02**
Peripheral blood			
Haemoglobin g/dl	4.7-17.9 g (mean 10.5)	8-17 g/dl (mean 12.5)	**.015**
Total WBC count x 10^3/µl	1.2- 55.98 (median 9.9)	5.2-67.1 (median 20.1)	**.02**
Platelet count x10^3/µl	6-1052 (median 210)	129-2021 (median 540)	**<.0001**
LDH IU/L	329-4043 (median 1119)	656-1231(median 921)	**.08**

Table 17 - Comparison of Myelofibrosis and its Prefibrotic phase – significant histological findings

		PMF , n=94		Prefibrotic phase, n=13		P value
		n	%	n	%	
Increased cellularity	Present	67	71	13	100	**.025**
Granulocyte Precursors	Increased	43	46	13	100	**.025**
Hyperlobate , giant and staghorn megakaryocytic nuclei	Absent	59	63	04	31	
	Present	35	37	09	69	**.028**
	Present	88	94	11	85	.247
Sinusoidal ectasia	Absent	8	8	9		
	Present	86	92	4	69	<.0001
Intra sinusoidal haematopoiesis	Absent	12	13	13	100	
	Present	82	87	0	0	<.0001
Reticulin grade	MF0	-	-			
	MF1	-	-	13	100	
	MF2	3	3	-		
	MF3	91	97	-		
CMC score		11-21 (Score of 21 - 5)		12-17		

C) Essential thrombocythaemia (ET)

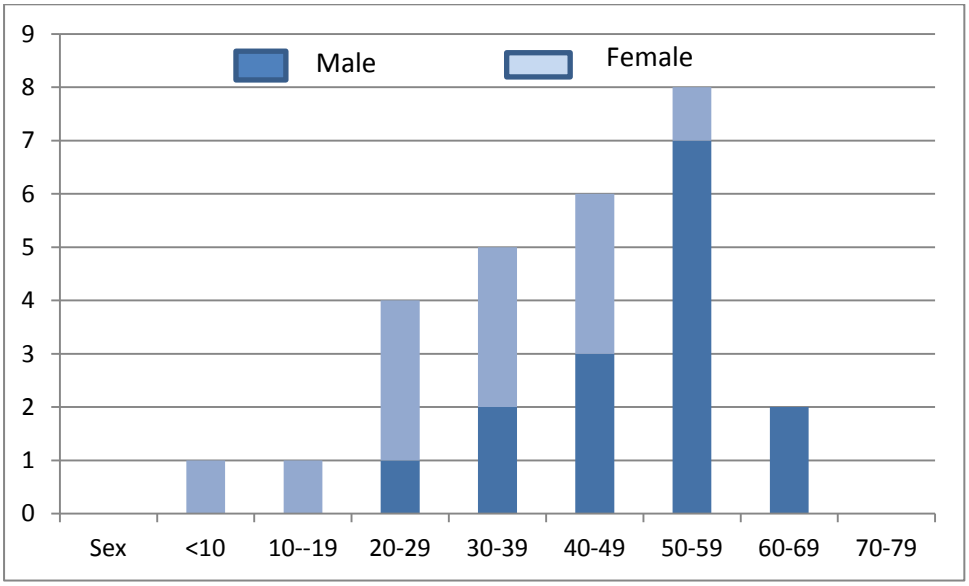
Of the 134 bone marrow trephine biopsies analysed, 27 had histological features of ET.

C .1 Clinical features of ET

C.1. i Age and sex distribution of ET (Figures 15):

Fifteen of the 27 ET (56%) were males (figure-14). There were 25 adults (median age 42 years). There was only one patient below the age of 15, an eight year old girl. There was also a 16 -year-old girl who presented with cortical venous and superior sagittal thrombosis, who was positive for the JAK2 mutation.(figure-15).

Figure 15- Age and Sex distribution in ET



C.1. ii

Presenting features in ET

Details of clinical presentation were available for 18 patients in this group. Five patients presented with thrombotic events , namely, cortical venous thrombosis (two), pulmonary vein thrombosis and Budd Chiari syndrome (one each) .One had non ST elevated myocardial infarction). All five were tested for the JAK2V617 mutation; four of these were JAK2V617 positive with platelet counts in the range of 634 -1484 $x10^3/\mu l$. The patient without the mutation had a platelet count of 594 $x10^3/\mu l$.

There were five patients with splenomegaly, in four of whom spleen size was measured by USG (range 8 -11.9). Seven patients did not have splenomegaly at presentation. Data was not available for the remaining 15.

C.2 Laboratory findings in ET (Table 18)

All 27 patients had thrombocytosis with wide variation in platelet count at diagnosis ranging from 434 - 1484 x10³ /µl (median 949x10³/µl). The mean haemoglobin was 13 g/dl and median WBC count 11.9 x10³/µl (range 7.1 0-48.2). Serum LDH values were available for 18 patients with a median value of 647.50 IU/L (range 319-1402). Peripheral smear showed thrombocytosis on smear in 15 patients with giant platelets in three.

Table 18 - Laboratory and clinical features of ET

ET	n=27
Demographics	
Males	15(56%)
Females	12(45%)
Age	7-65 years
Median age of adults	42 years (range 22-65)
Males	53 (22-65)
Females	32 (7-50) including two <18
Children (<18)	Two , aged 7 and 16
Spleen size	
Normal	24
Splenomegaly	5 patients, range 8 - 11.9 cm
History of thrombosis	5 (19%), see text for details
Laboratory findings	
Haemoglobin (g/dl)	9.4- 15.5, mean 13 g/dl
WBC count (x 10³/µl)	7.1-48.2, median 11.9
Platelet count (x 10³/µl)	434-1384, median 949,mean 1001
Peripheral smear n= 15	
Abnormal	
Thrombocytosis with	15
-Giant platelets	3
-Eosinophilia,basophilia,neutrophilia	2
Circulating blasts	0
Serum LDH (n=18)	
Range (LDH IU/L)	319-1402 (median 647)

C.2.ii Bone marrow trephine biopsy findings in ET (Table 19)

The following findings were **seen in all 27 ET**

- Increased number of megakaryocytes
- Large megakaryocyte with hyperlobate staghorn type nuclei
- Clustering of megakaryocytes with both small and loose clusters
- Perisinusoidal (Normal) location of megakaryocytes
- Absence of myeloid left shift, sinusoidal ectasia and intrasinusoidal haematopoiesis
- Reticulin grade MF0 or MF1.

Other common findings were

- Normal size megakaryocytes (93%)
- Hypolobate bulbous cloud like megakaryocyte (81%)
- Large clusters (78%)

The other parameters were seen in less than a third of our patients **.**

Table 19 - Histological features of bone marrow trephine biopsies in ET, n=27

HISTOLOGICAL DIAGNOSIS	ET n=27		n	%
Overall Cellularity	Normal		7	26
	Increased		20	74
Granulocyte Precursors	Increased		05	18
	Normal		22	82
Left shift	Absent		27	100
Erythroid cellularity	Normal		24	89
	Increased		03	11
Megakaryocyte location	Paratrabecular		08	30
	Normal perisinusoidal		27	100
Megakaryopoiesis	Increased		27	100
Size of megakaryocytes	Small	Absent	27	100
	Normal	Absent	02	7
		Present	25	93
	Large	Present	27(100)	100
Megakaryocyte nuclei				
Hypolobate cloud like megakaryocyte nuclei	Absent		5	19
	Present		22	81
	Present		27	100
Hyperchromatic dysplastic nuclei	Absent		24	89
	Present		3	11
Hyperlobate staghorn type megakaryocytes	Present		27	100
Naked nuclei	Absent		23	85
	Present		4	15
Megakaryocyte clusters				
Size	Small	Present	27	100
	Large	Present	21	78
Type	Loose	Present	27	100
	Dense	Absent	24	89
		Present	3	11
Sinusoids				
Sinusoidal ectasia	Absent		27	100
Intrasinusoidal hematopoiesis	Absent		27	100
Reticulin				
Reticulin	MF0		5	18
	MF1		22	82
	MF2 and MF3		00	
Reticulin score	0		27	100
Scoring				
Total score	5-9, median 8		8,	
Brousseau score , Median, range	0-4, median 2		2,0-4	

C.2.iii <u>Scoring system</u> (Table 20 and Figures 16 and 17)

The median score obtained by our 21 point scoring system was 8 with a range of 5-9. With the Brousseau system, our scores ranged from 0-4 with a median score of 2.

Table 20 –Essential Thrombocythaemia (ET) - Comparison of scoring systems

Scoring system		CMC				Brosseau		
S.no	Feature / Score	0	1	2		0	1	2
1	Overall cellularity	Normal n=7,26%	Increased n=20,74%			Normal n=7,26%	Increased n=20,74%	
2	Granulocytic cellularity	Normal n=22,72%	Increased n=5,18%	Decreased		Normal n=22, 78%	Increased n=5,18%	
3	Erythroid cellularity	Normal n=24,89%	Increased n=3,11%	Decreased		Normal n=24,89%	Increased N=3,11%	Decreased
4	Paratrabecular megakaryocytes	Absent n=19,70%	Present n=8,30%			Absent n=19,70%	Present n=8,30%	
5	Megakaryocyte cluster density	Loose n=27,100%	Tight n=3,11%			Loose n=27,100%	Tight n=3,11%	
6	Cloud-like megakaryocyte nuclei	Absent n=5,19%	Present n=22,81%			Absent n=5,19%	Present n=22,81%	
7	Hyperchromatic dysplastic nuclei	Absent n=24,89%	Present n=3,11%			Absent n=24,89%	Present n=3,11%	
8	Bare nuclei	Absent n=23,85%	Present n=4,15%			Absent n=23,85%	Present n=4,15%	
9	Megakaryocyte N/C ratio (by morphometry)	Not assessed as accurate results are obtained by morphometry				Absent n=24,89%	Present n=9,11%	
10	Left shift	Absent n=27,100%	Present					
11	Megakaryopoiesis	Normal	Increased n=27,100%	Decreased				
12	Megakaryocyte Size	Normal n=25,93%	Large n=27,100%	Small				
13	Hyperlobate staghorn type nuclei		Present n=27,100%					
14	Megakaryocyte clusters		Small n=27,100	Large n=21,78%				
15	Sinusoidal ectasia	Absent n=27,100	Present					
16	Intrasinusoidal haematopoiesis	Absent n=27,100	Present					
17.	Reticulin	MF05(18%) MF1-23,(81%)	MF2,MF3					

Figure 16 - CMC scoring system in ET

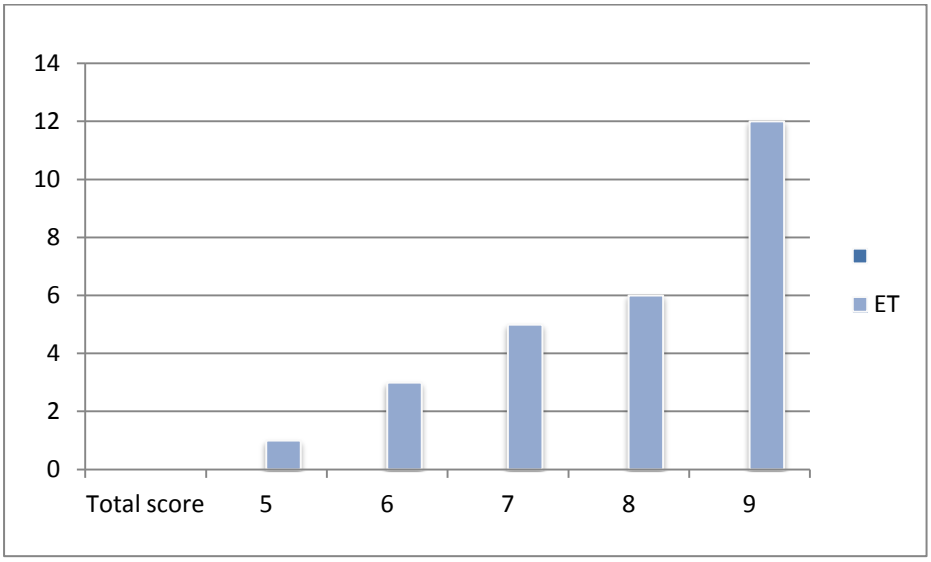
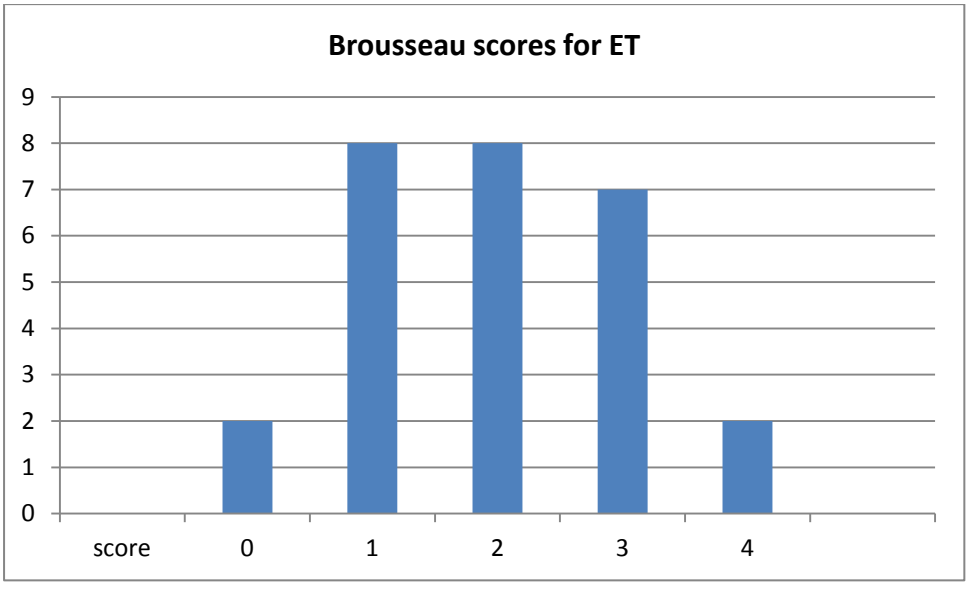


Figure 17- Brousseau scores in ET



C.2.iv JAK2 mutation status in ET

JAK2V617 mutation status was available for 25(92%) of ET, with almost equal numbers of patients with (12, 48%) and without (13, 52%) the mutation. Five (42%) of the 12 JAK2V617 positive cases were males and seven (58 %) were females. (Table 22).

C.2.v Comparison of JAK2V617 positive ET vs JAK2 WT ET (Tables 21 , 22 and Appendix 6)

JAK2V617 positive vs JAK2 WT ET - Laboratory findings

The JAKV617 positive ET was associated with **a lower median platelet count** than JAK2 WT (777 vs 1052 x10^3/ µl , P value =.02). There were no other significant differences between the two groups.

JAK2V617 positive ET vs JAK2 WT ET - Bone marrow trephine biopsy findings

The **presence of large clusters of megakaryocytes in JAK2WT** (92% vs 58% , P value = .048) was the only statistically significant difference between the two groups. Although there were differences in the other parameters none were statistically significant (Appendix 7).

Table 21 - Comparison of JAK2V617 positive and JAK2WT ET – significant differences

Parameter	JAK2V617 POSITIVE, n-12	JAK2 WILD TYPE , n=13	P value
Platelet count x10^3/µl	454 -1484 (median 777)	594-2580 (median 1052)	.02
Large megakaryocyte clusters	7 (58%)	12(92%)	.047

Cytogenetic analysis (Appendix 9)

Karyotypes were available for six patients and results of FISH analysis for 17. All six patients had normal karyotypes .Seven patients did not have either form of testing.

D. Comparison of ET and Prefibrotic phase of PMF (Table 22)

The differences between ET and Prefibrotic phase of PMF were as follows :

D.1 ET versus Prefibrotic phase of PMF - Laboratory findings (Table 22)

The significant differences were

- **A higher platelet count in ET** (949 vs 540 x $10^3/\mu l$, P value = .006) than in the Prefibrotic phase of PMF .
- **Higher LDH levels in the Prefibrotic phase of PMF** (939 vs 704 IU/L , P value = .04)

  The other differences were not statistically significant.
- In addition, circulating blasts and a leukoerythroblastic blood picture were seen only in the Prefibrotic phase of PMF.

Table 22 - ET vs Prefibrotic phase of PMF - Statistically significant laboratory findings

	ET	Prefibrotic phase of PMF	P value
Number	27	13	
Platelet count x $10^3/\mu l$	340-2580 (median 949)	129-202.1 (median 540)	.006
(LDH IU/L)	319-1402 (median 704)	656-1231 (median 939)	.04

D. 2 ET vs Prefibrotic Phase of PMF - bone marrow trephine biopsy findings (Table 23)

The statistically significant histological differences between ET and the Prefibrotic phase of PMF are listed below.

D.2.i ET vs Prefibrotic Phase of PMF - Cellular and vascular changes

Findings in favour of the Prefibrotic phase of PMF, not seen in any of the 27 ET :

- Left shift in myeloid lineage (54% vs nil, P value=<.0001)
- Decreased numbers of erythroid cells (62% vs nil, P value=<.0001)
- Small megakaryocytes (38% vs nil , P value=.002)
- Sinusoidal ectasia (31% vs nil, P value=.008)

Findings **more common in the Prefibrotic phase of PMF than in ET were**:

- Paratrabecular megakaryocytes (100 vs 30%, P value <.0001)
- Increased cellularity (100% vs 74%, P value = .043).
- Myeloid hyperplasia (100% vs 19 %, P value =<.0001).
- Hyperchromatic dysplastic megakaryocyte nuclei (85% vs 11%,P value =.001)
- Naked megakaryocyte nuclei (46% vs 15%, P value =.032)
- Dense clusters of megakaryocytes (62 % vs 11%,P value =.001)

Findings which were **more common in ET than in the Prefibrotic phase of PMF** were:

- Hyperlobate staghorn megakaryocyte nuclei (100% in ET vs 69% , P value= .002)

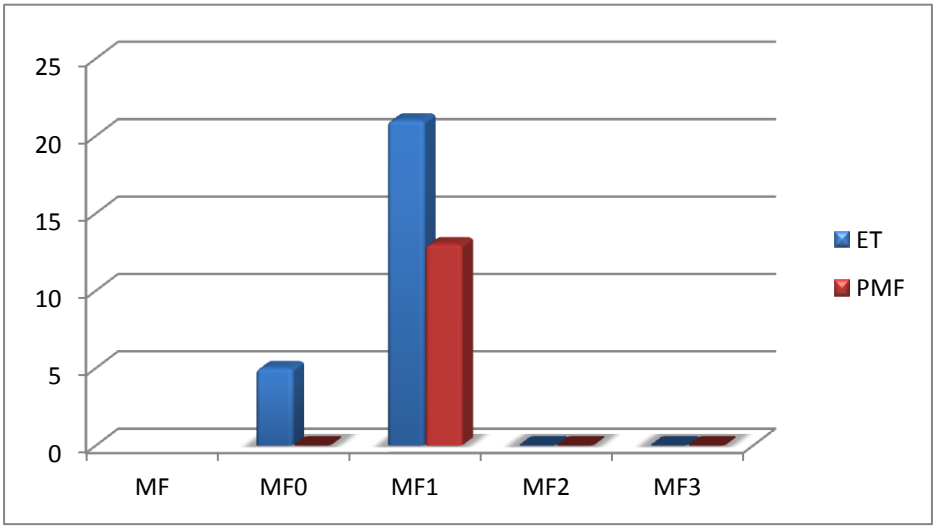
Table 23 – ET vs Prefibrotic phase of PMF - Statistically significant histological differences

HISTOLOGICAL DIAGNOSIS	ET n=27	PMF, n=13	P value
Increased cellularity	20(74%)	13(100%)	.043
Myeloid hyperplasia	5(19%)	13(100%)	<.0001
Myeloid left shift	Nil	7(54%)	<.0001
Decreased erythroid cellularity	Nil	8(62%)	<.0001
Paratrabecular megakaryocytes	8(30%)	13(100%)	<.0001
Small megakaryocytes	Nil	5(38%)	.002
Hyperlobate, giant/ staghorn megakaryocytic nuclei	27(100%)	9(69%)	.002
Hyperchromatic dysplastic nuclei	03(11 %)	11(85%)	<.0001
Naked nuclei	04(15%)	6(46%)	.032
Dense megakaryocyte clusters	03(11%)	8(62%)	.001
Sinusoidal ectasia	Nil	4(31%)	.008
CMC score	5-9 (median 8)	12-17 (median 15)	
Brosseau score	1-4 (median 2)	6-10 (median 8)	

D.2.ii ET vs Prefibrotic Phase of PMF - Reticulin grade (Figure 18)

All 13 Prefibrotic phase of PMF and 81% of ET had MF1 , therefore reticulin grade was not useful to distinguish between these two conditions .

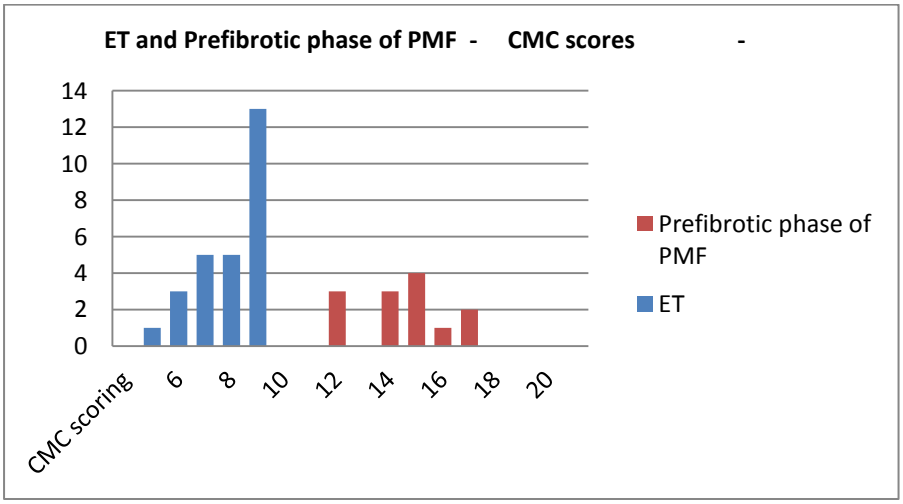
Figure 18 - ET and Prefibrotic phase of PMF - MF grade



D.2.iii ET vs Prefibrotic Phase of PMF - Scores (Table 23 and Figure 19)

Using our scoring system, total score obtained for the Prefibrotic phase of PMF was 12-17 vs 5-9 for ET. The corresponding Brosseau scores were 1-4 for ET

Figure 19 - CMC scores in ET and Prefibrotic phase of PMF



Note : JAK2V617 positive ET and JAK2V617 positive Prefibrotic phase of PMF were compared, statically significance could not calculated the number JAK2V617 positive prefibrotic phase of PMF were less **.(Appendix 8)**

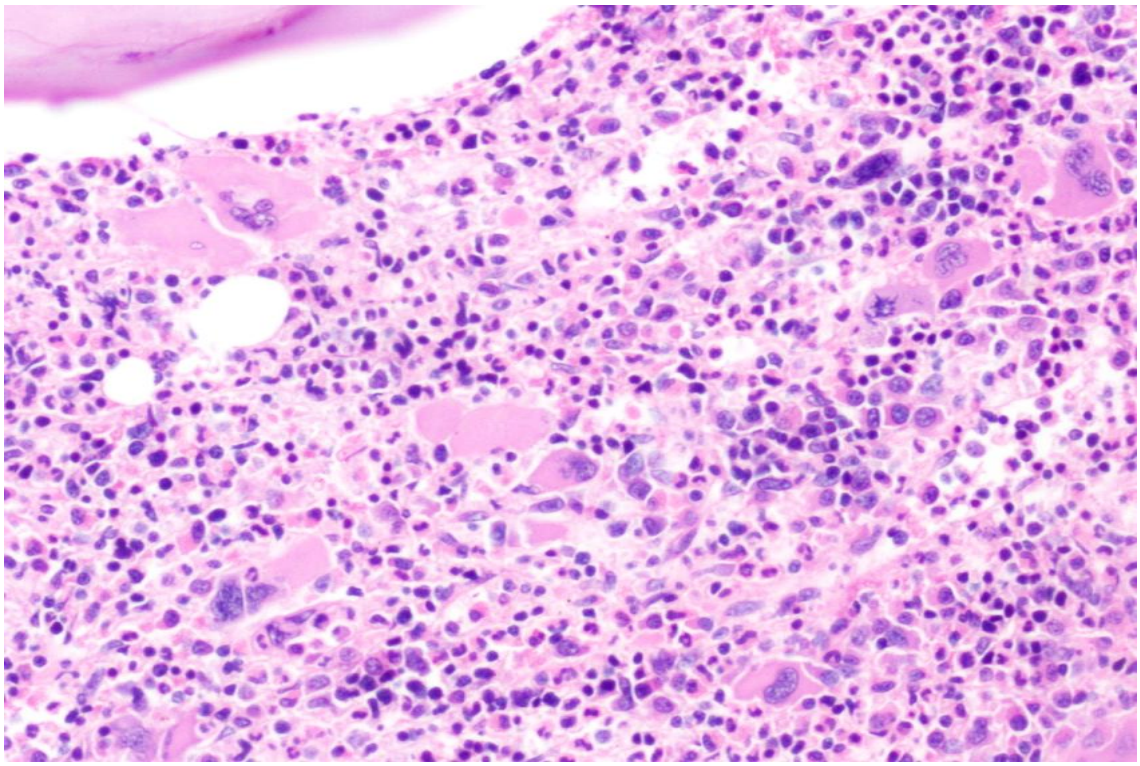


Figure 20-Streaming of haematopoietic cells in PMF. H&E X 200

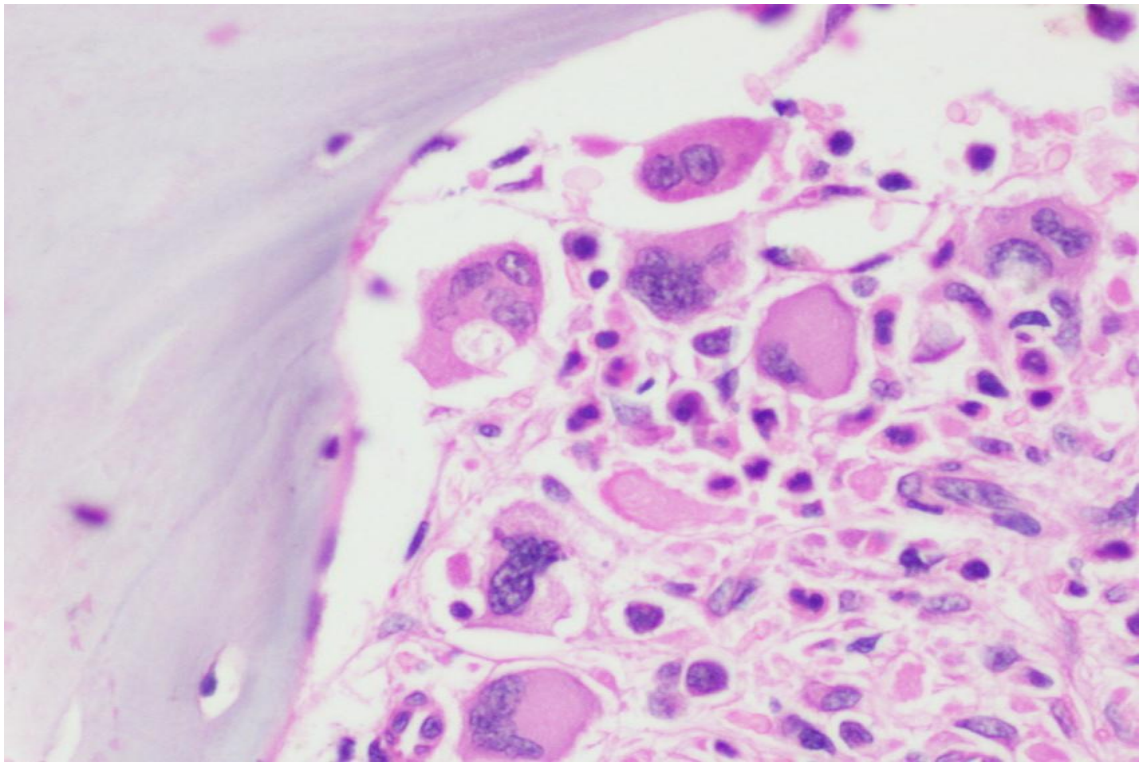


Figure 21-Paratrabecular megakaryocytes in PMF. H&E X 400

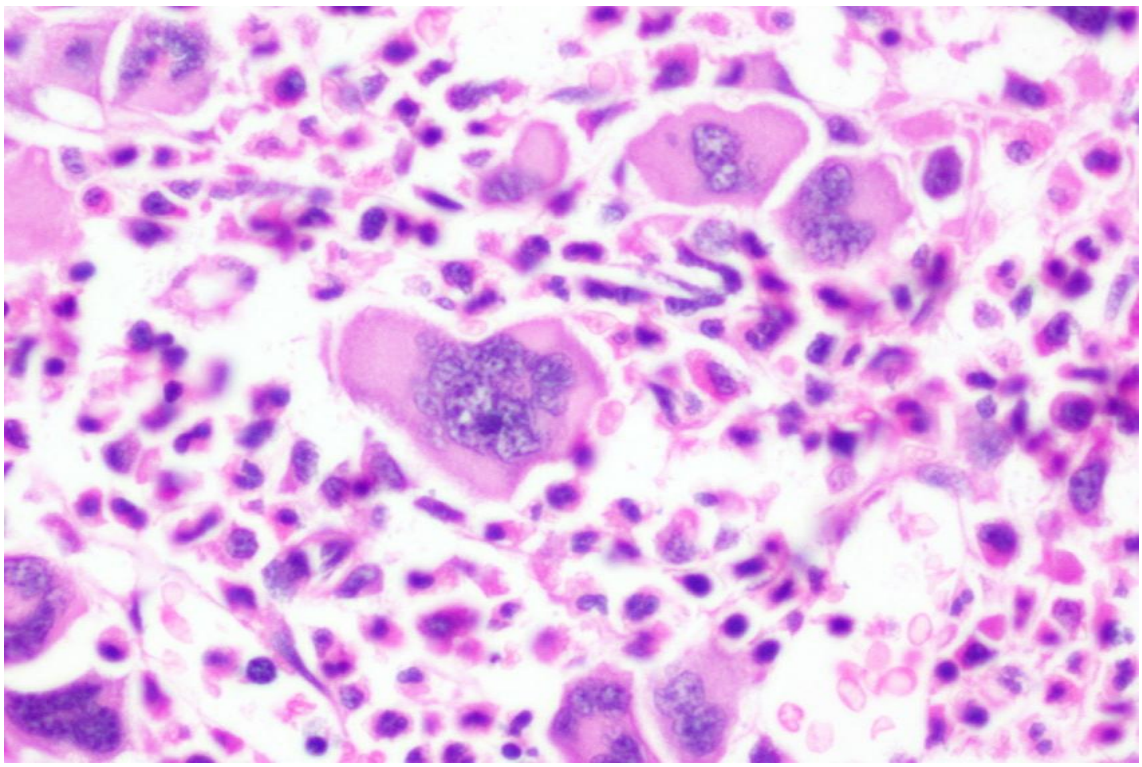


Figure 22-Hypolobate bulbous cloud-like megakaryocytes in PMF. H&E X 400

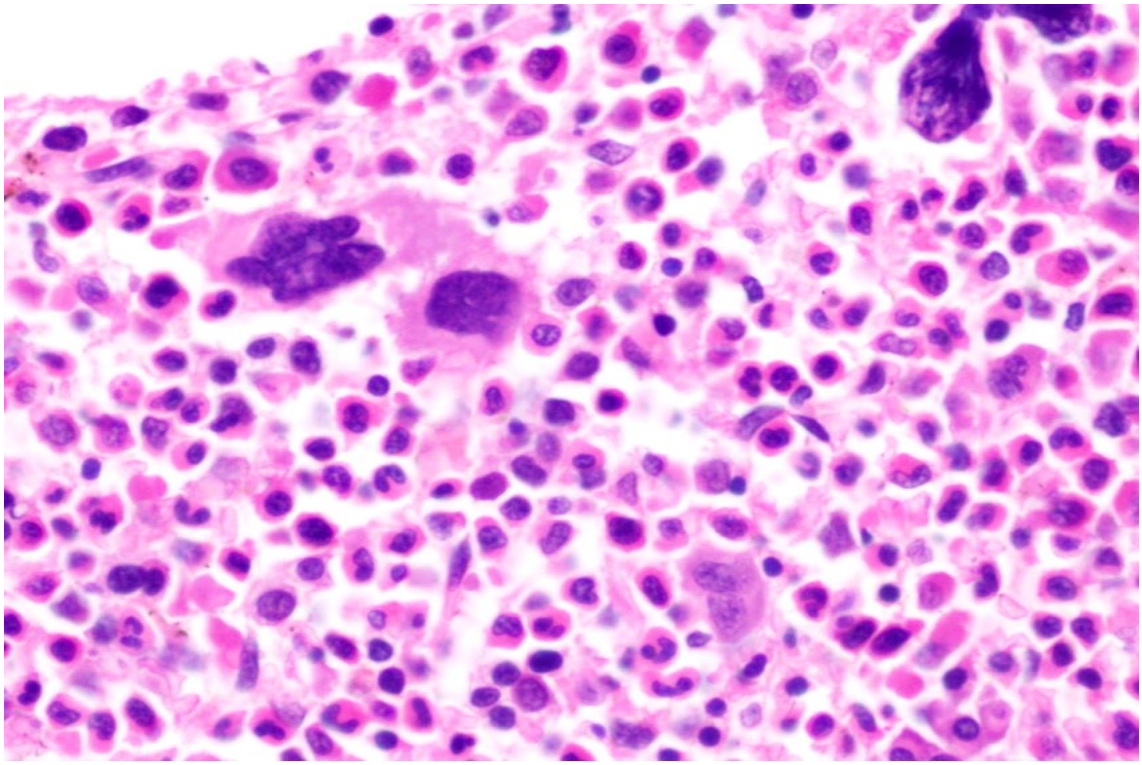


Figure 23-Dysplastic megakaryocyte with hyperchromatic nucleus in PMF. H&E X 400

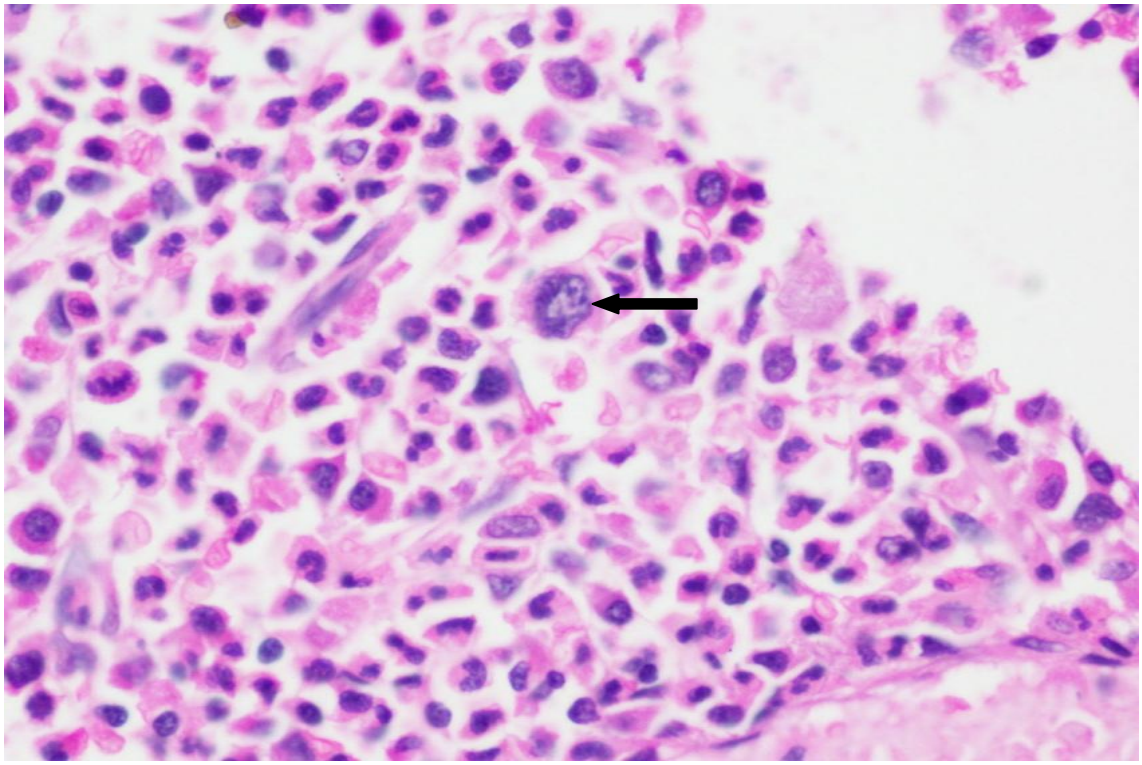


Figure 24-Small megakaryocyte in PMF. H&E X 400

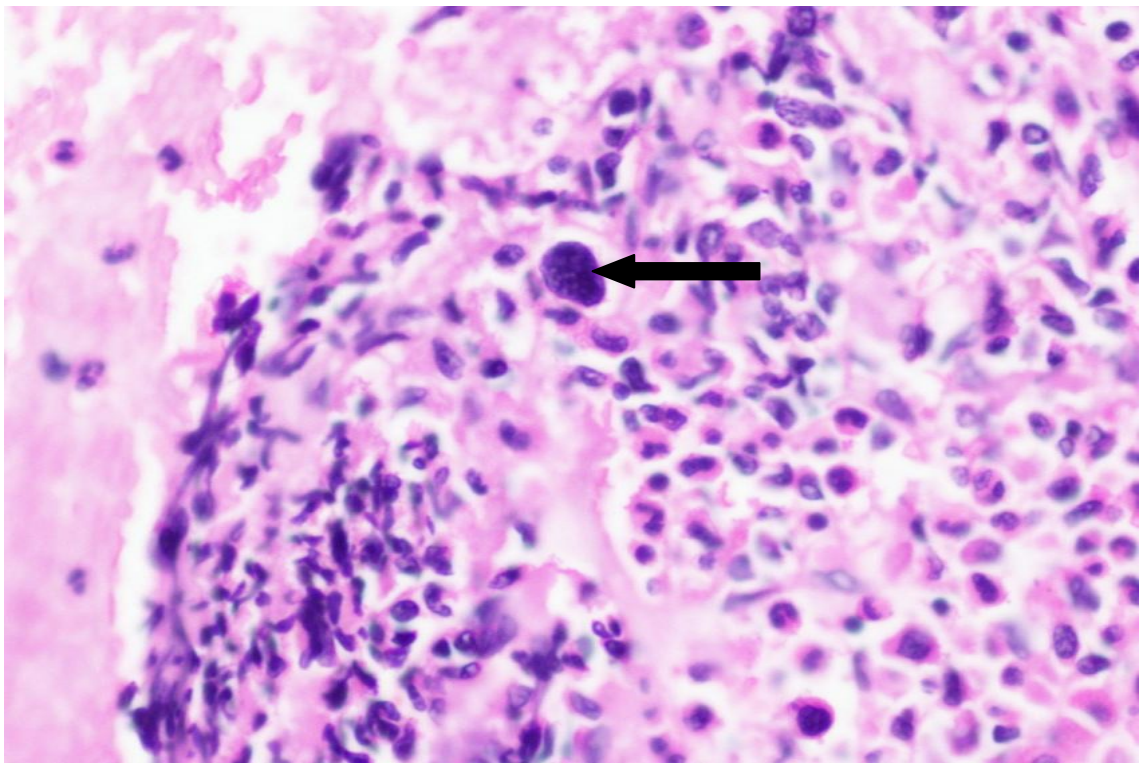


Figure 25-Naked nuclei in PMF. H&E X 400

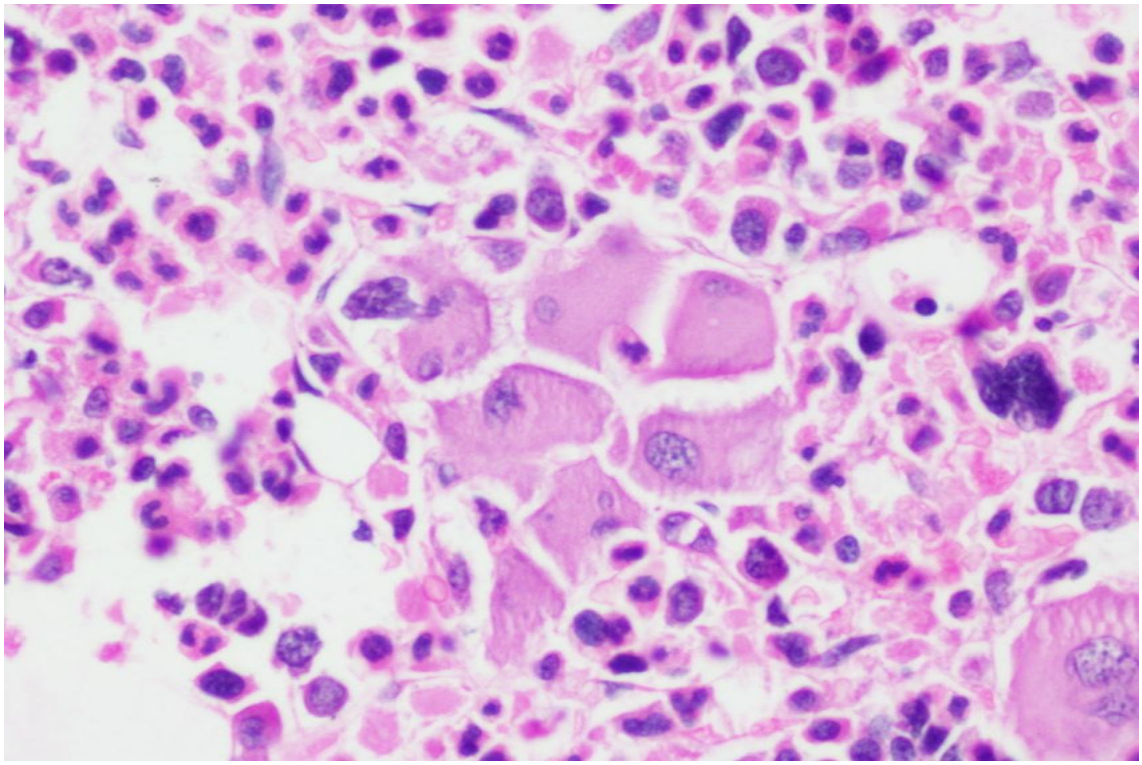


Figure 26-Dense megakaryocyte cluster in PMF, H&E X 400
Note the absence of intervening cells

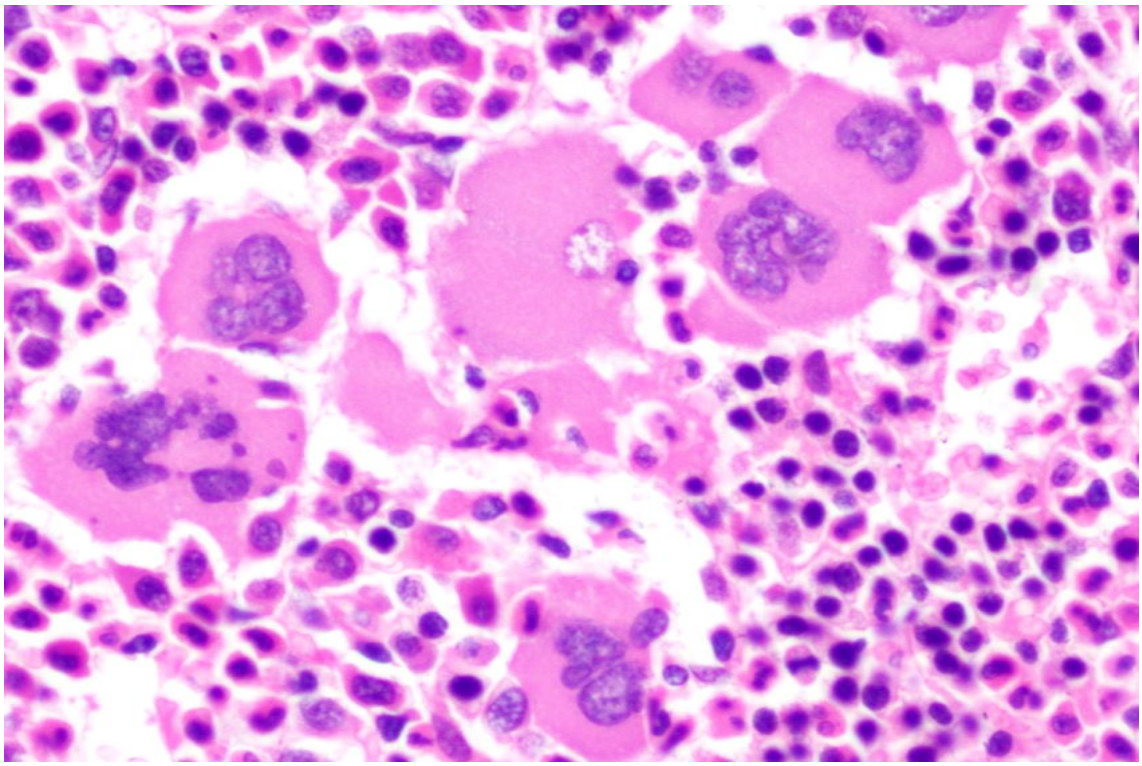


Figure 27-Loose cluster of megakaryocytes Prefibrotic phase of in PMF. H&E X 400
Note The intervening cells in between the megakaryocytes .This feature is also seen in PMF.

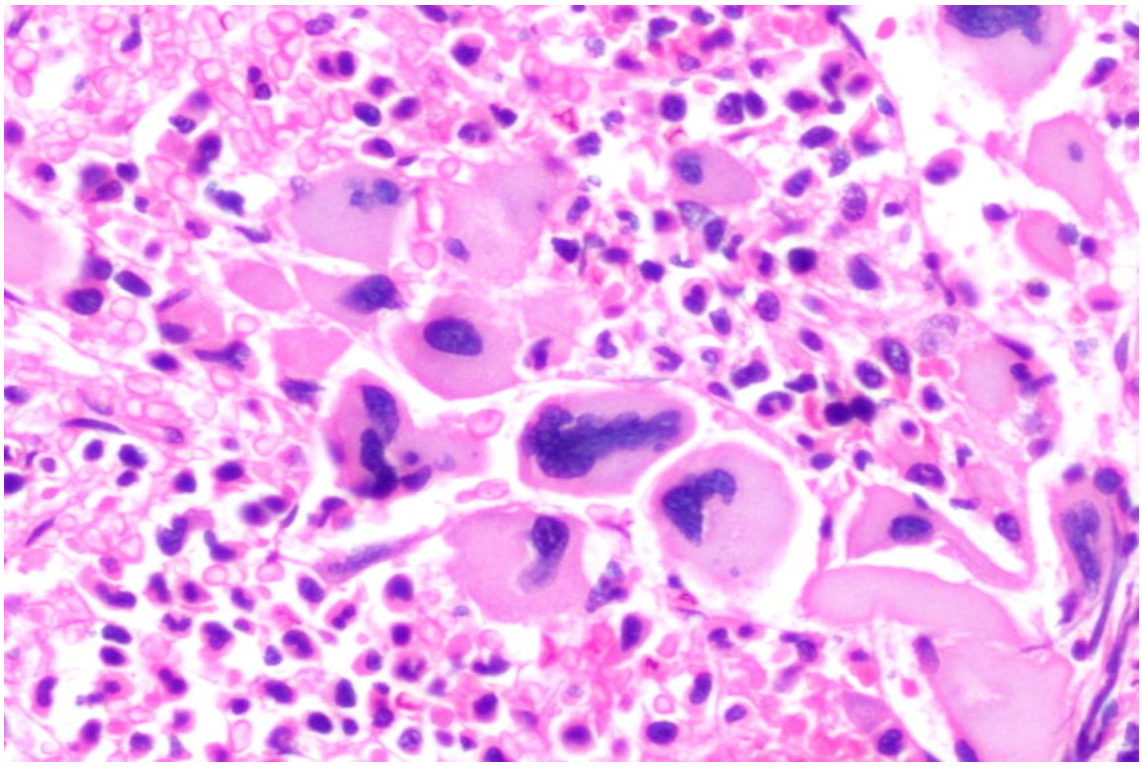


Figure 28-Large cluster of >7 megakaryocytes in PMF. H&E X 400

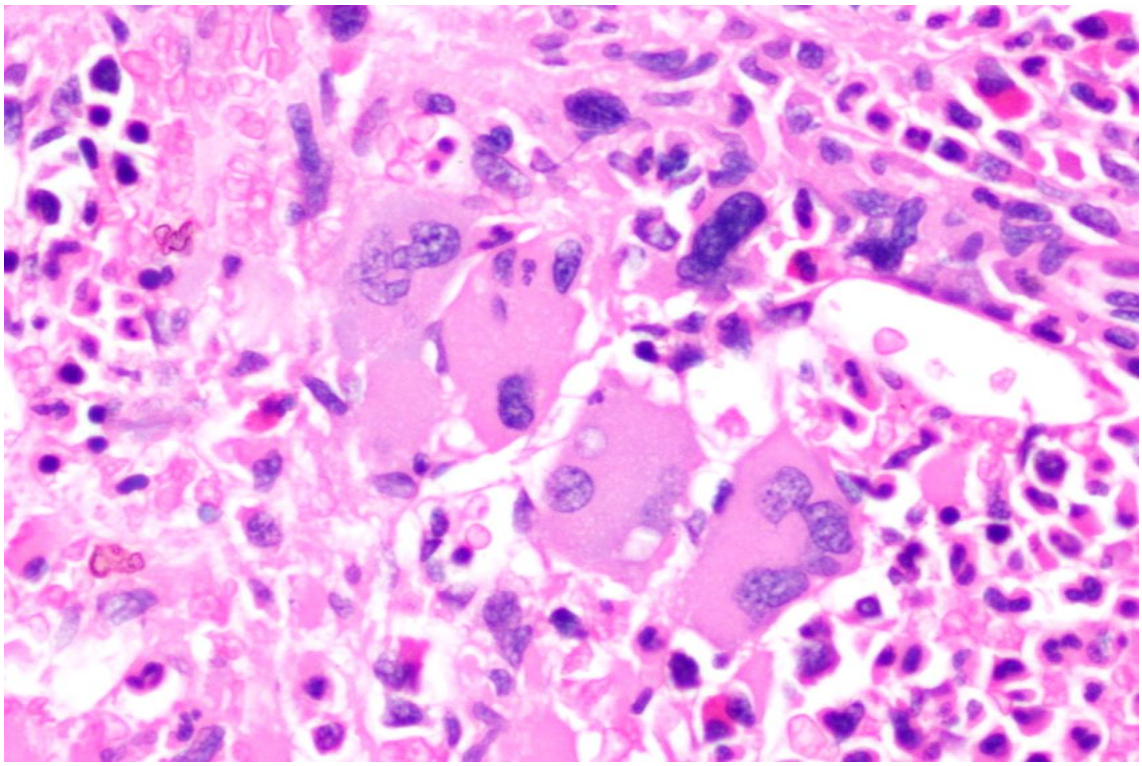


Figure 29-Small cluster of 4-6 megakaryocytes in PMF. H&E X 400

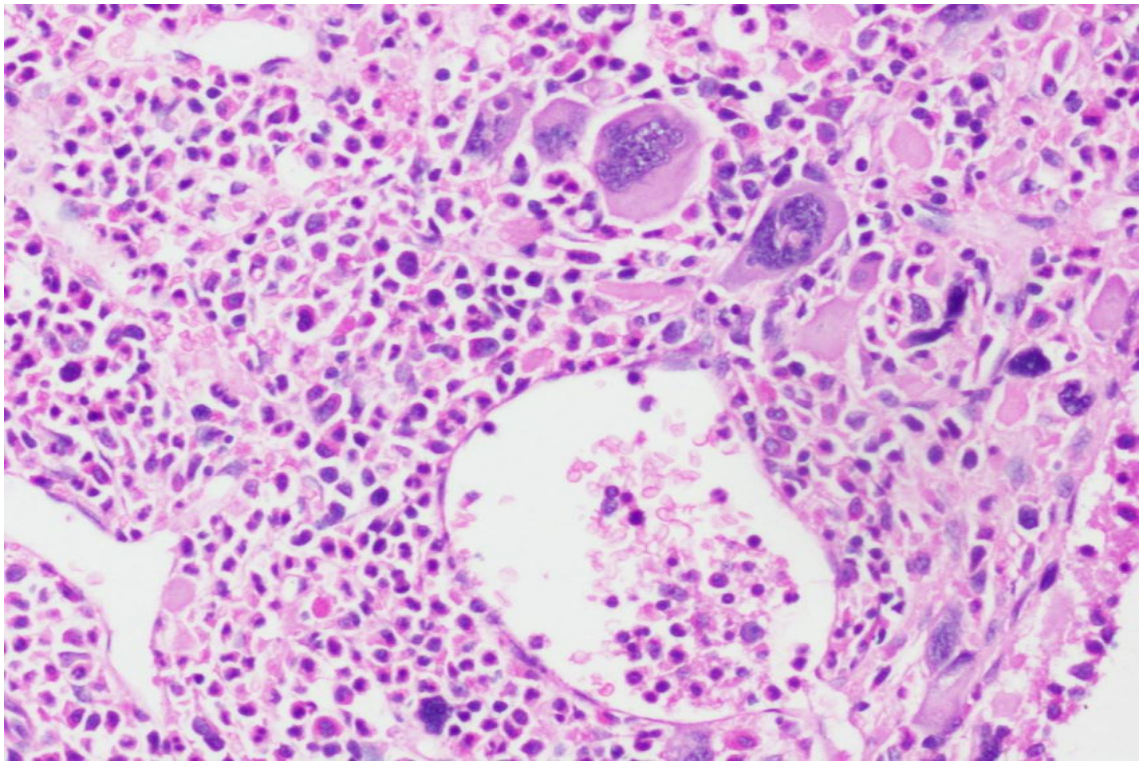


Figure 30- Sinusoidal ectasia with intrasinusoidal haematopoiesis in PMF. H&E X 400

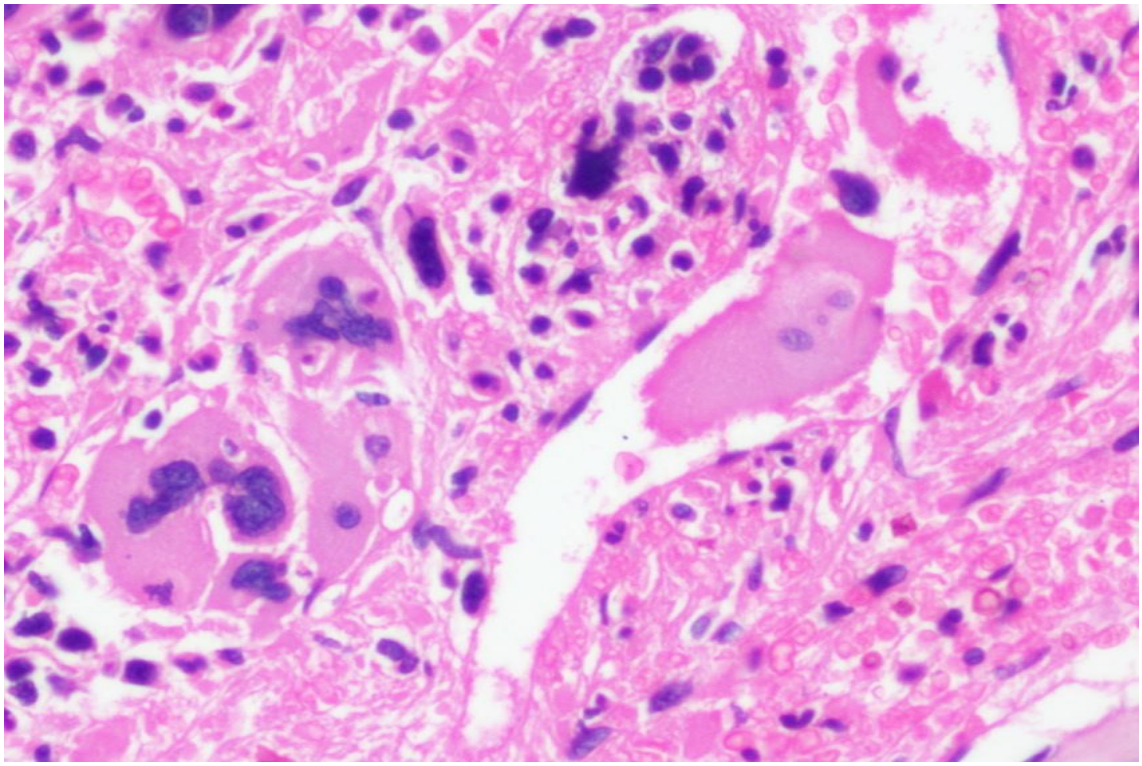


Figure 31-Sinusoidal ectasia with intrasinusoidal megakaryocyte in PMF. H&E X

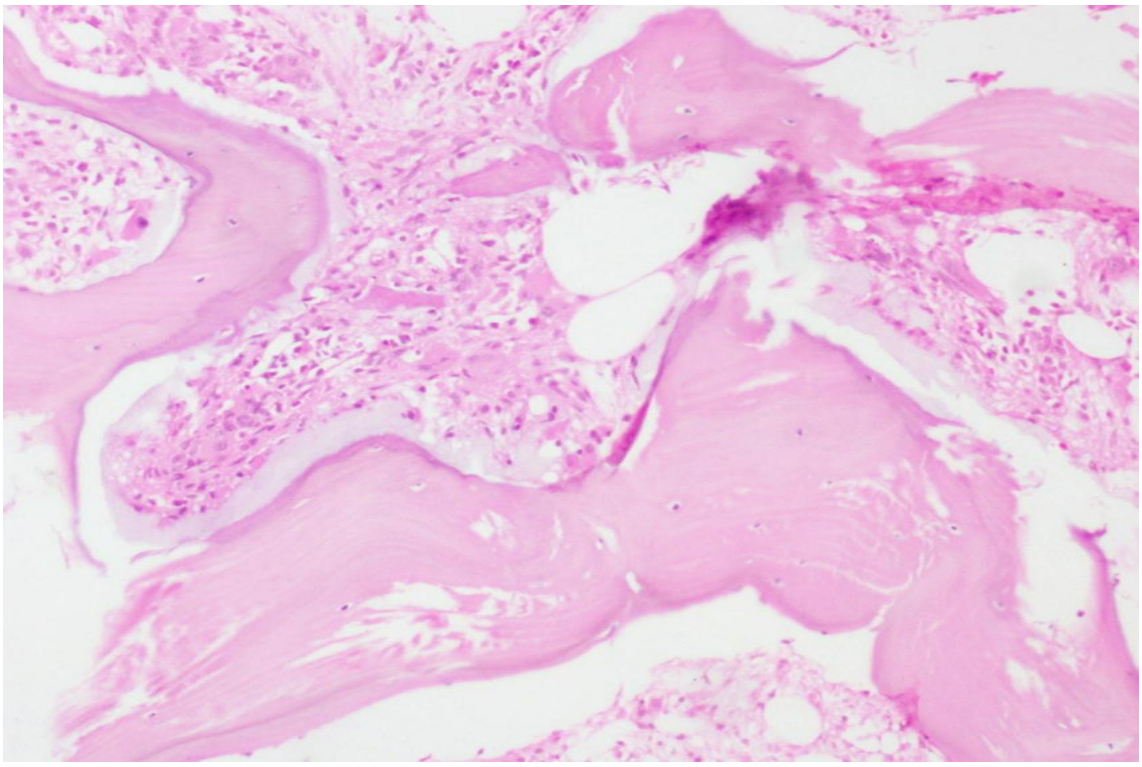


Figure 32-Osteosclerosis in PMF. H&E X 400

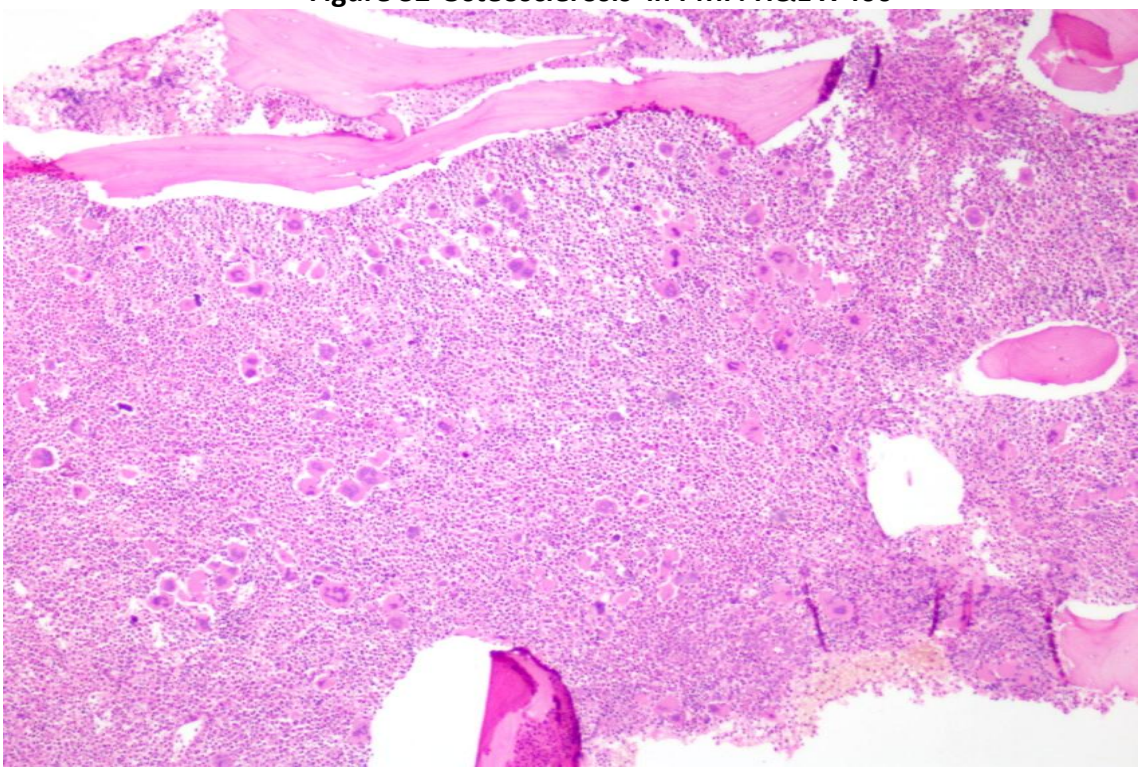


Figure 33-Increased cellularity in Prefibrotic phase of PMF. H&E X 10

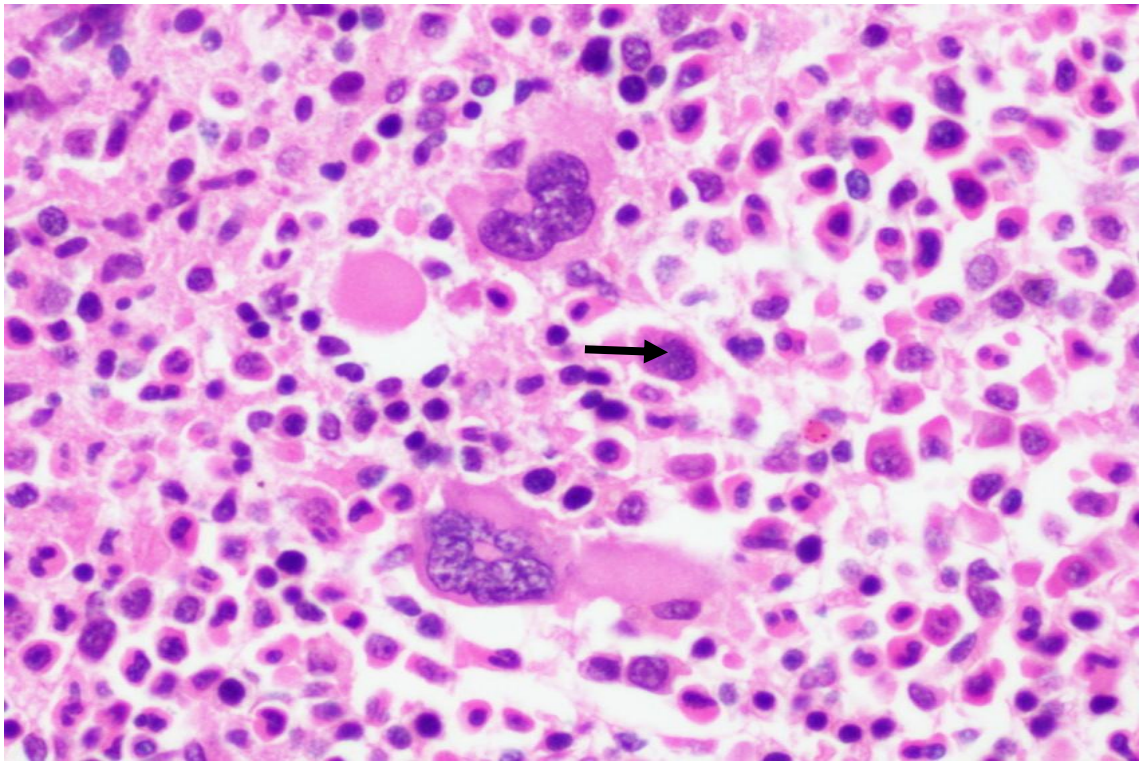


Figure 34-Hypolobate cloud-like megakaryocyte and small megakaryocyte (arrow) in prefibrotic phase of PMF. H&E X 400

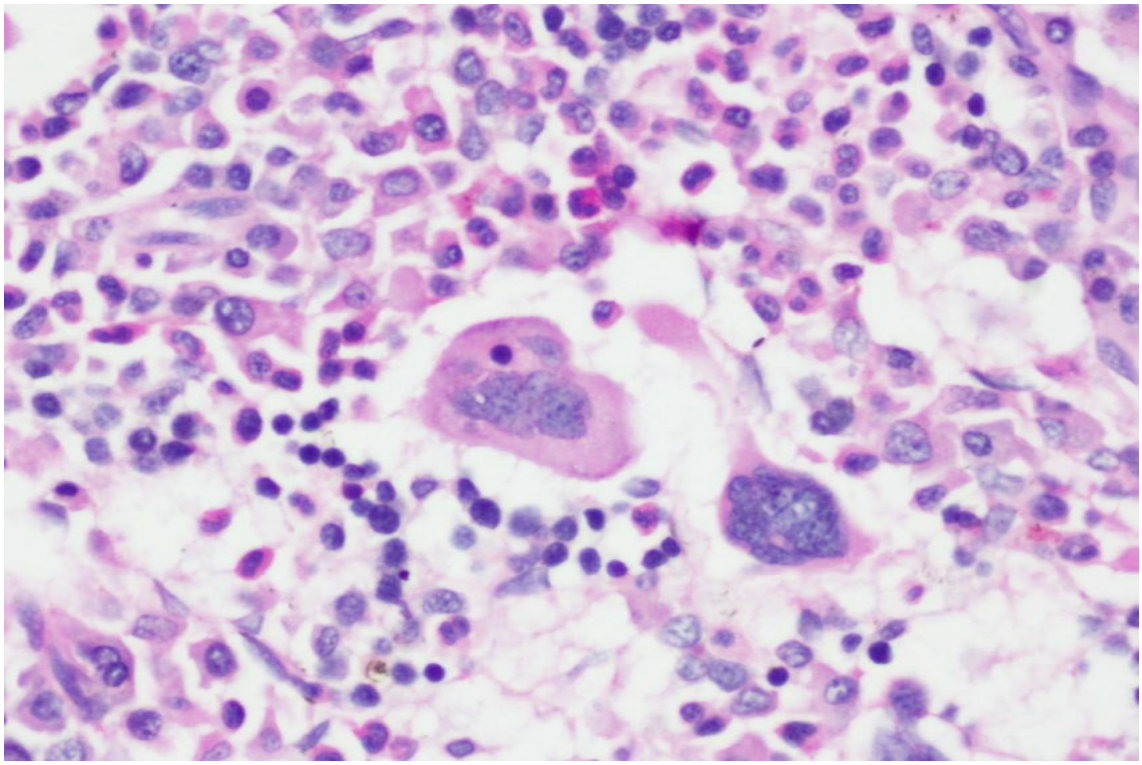


Figure 35-Hypolobate cloud-like megakaryocytes in Prefibrotic phase of PMF. PAS stain X 400

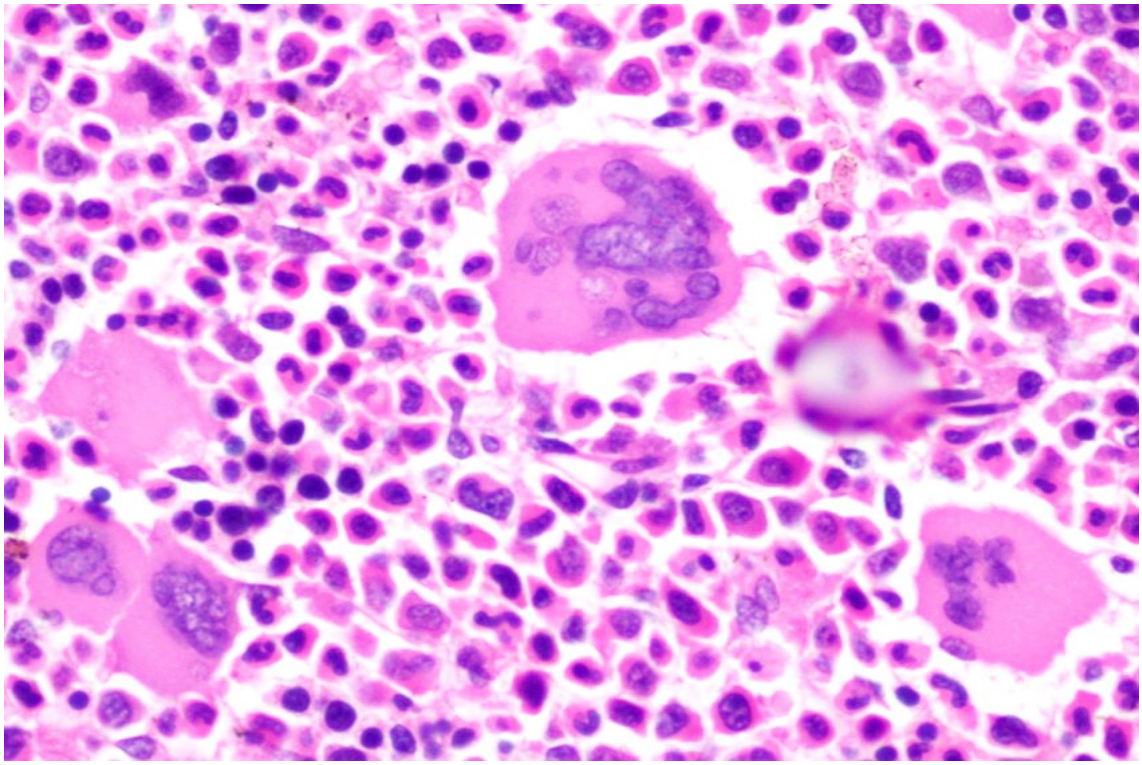


Figure 36-Hyperlobate stag-horn type megakaryocytes in Prefibrotic phase of PMF . H&E X 400

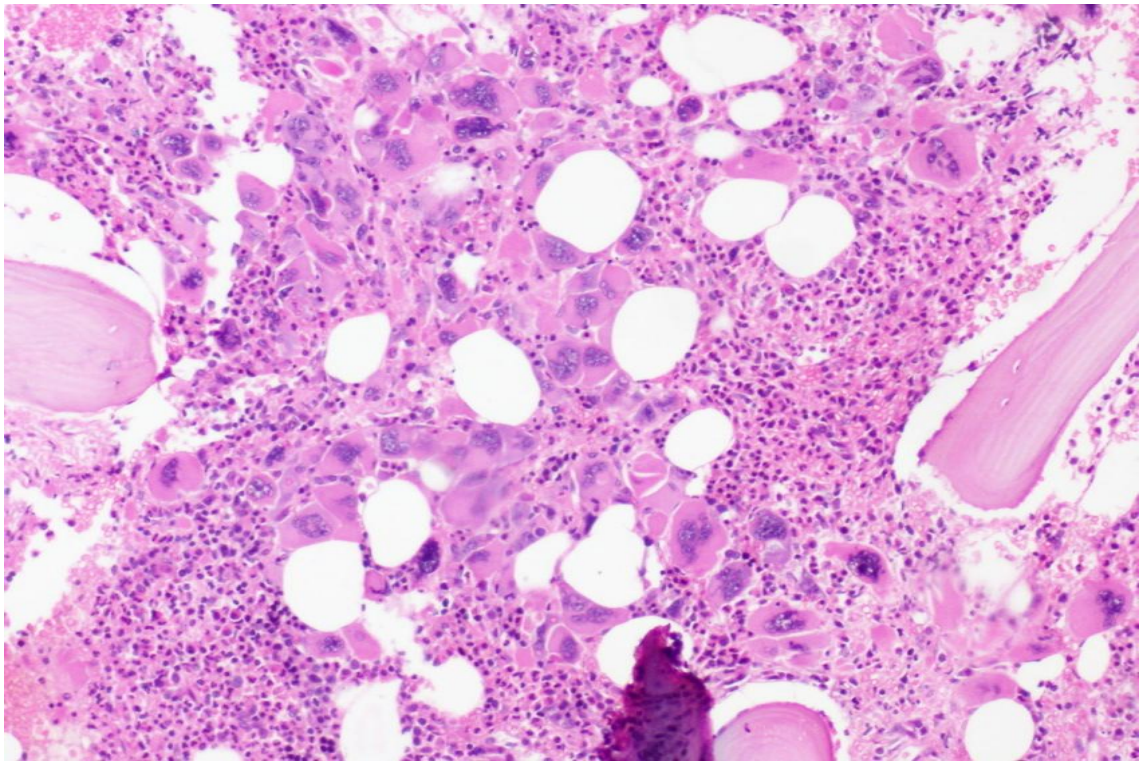


Figure 37 –Clustering of megakaryocytes in ET. H&E X20

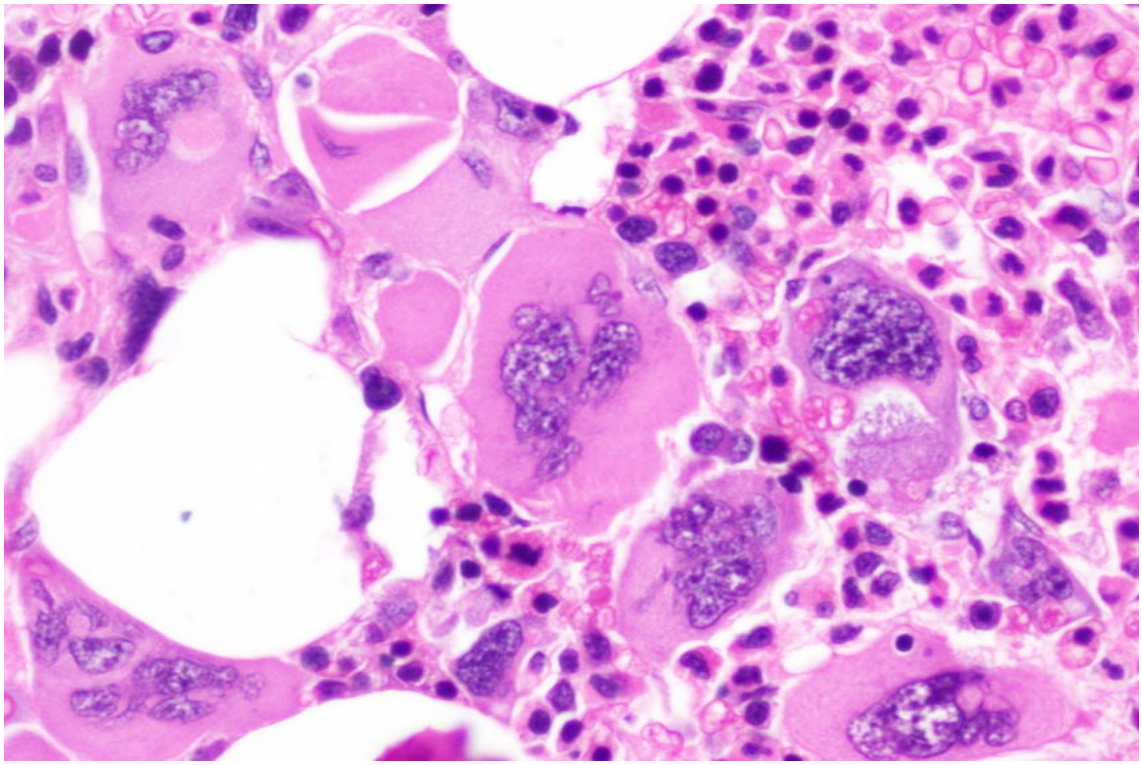


Figure 38-Hyperlobate staghorn type of megakaryocyte in ET. H&E X 400

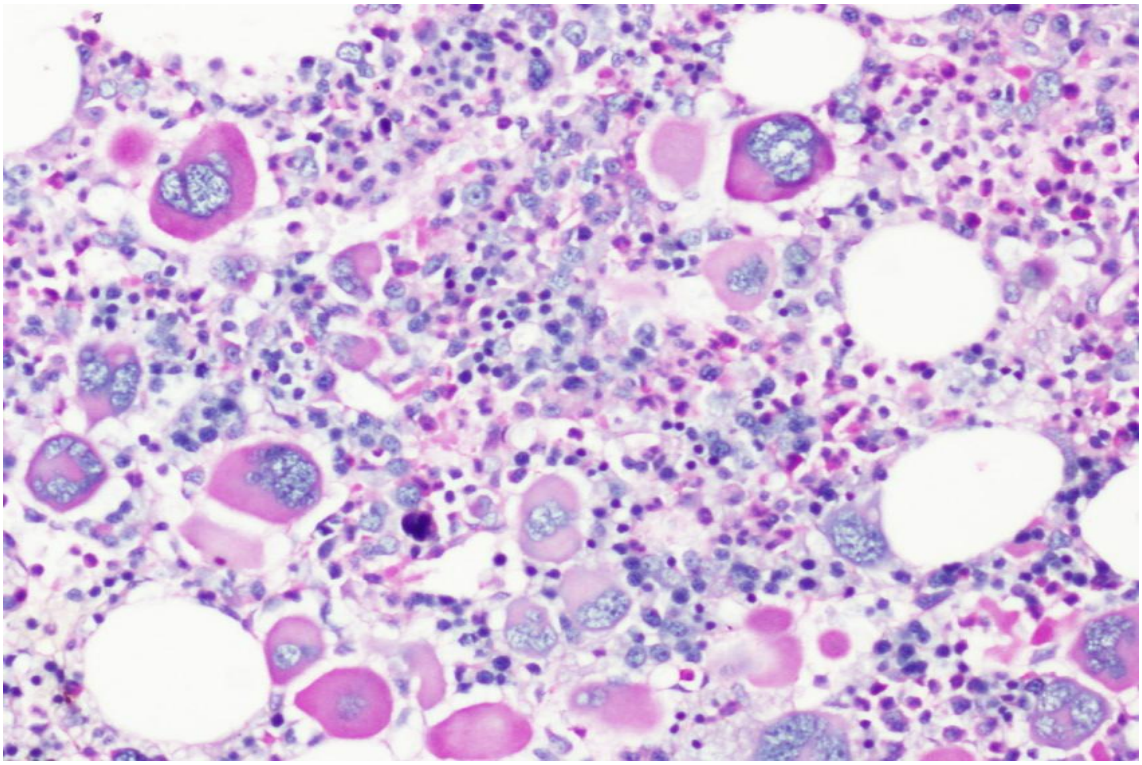


Figure 39-Loose clustering of megakaryocytes in ET , PAS stain. H&E X 400

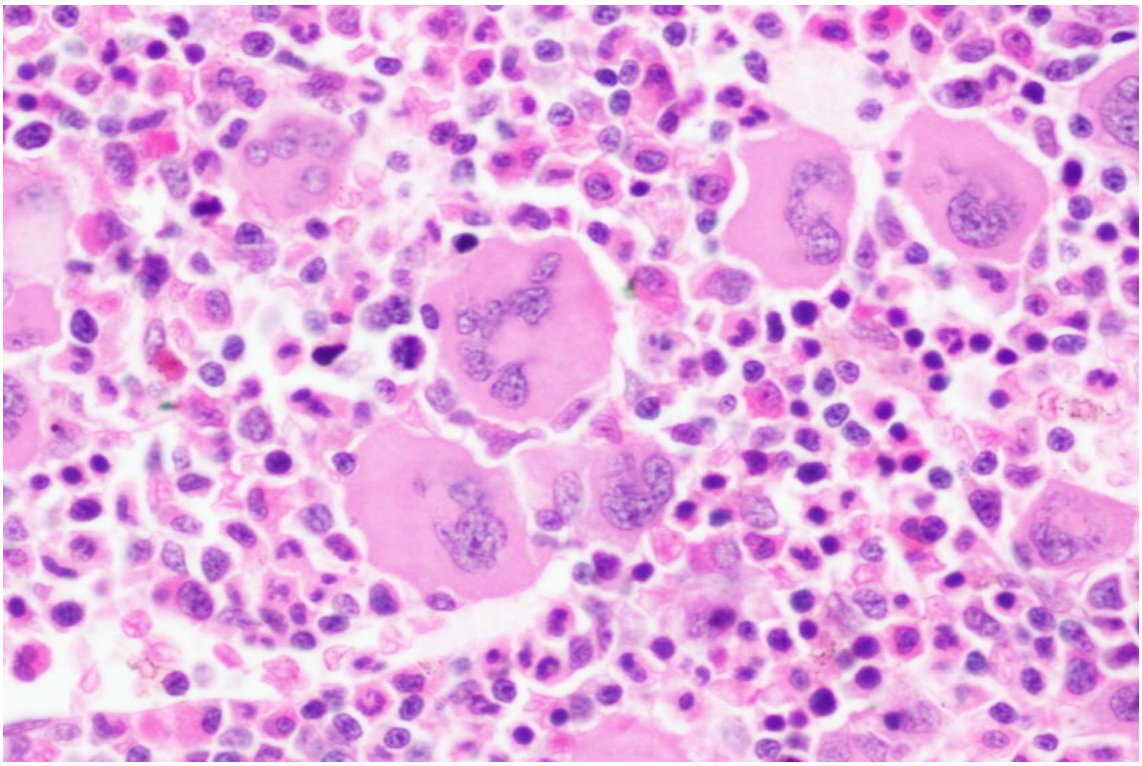


Figure 40-Hyperlobate staghorn type megakaryocytes and small cluster of 4-6 megakaryocytes in ET, H&E X 400x

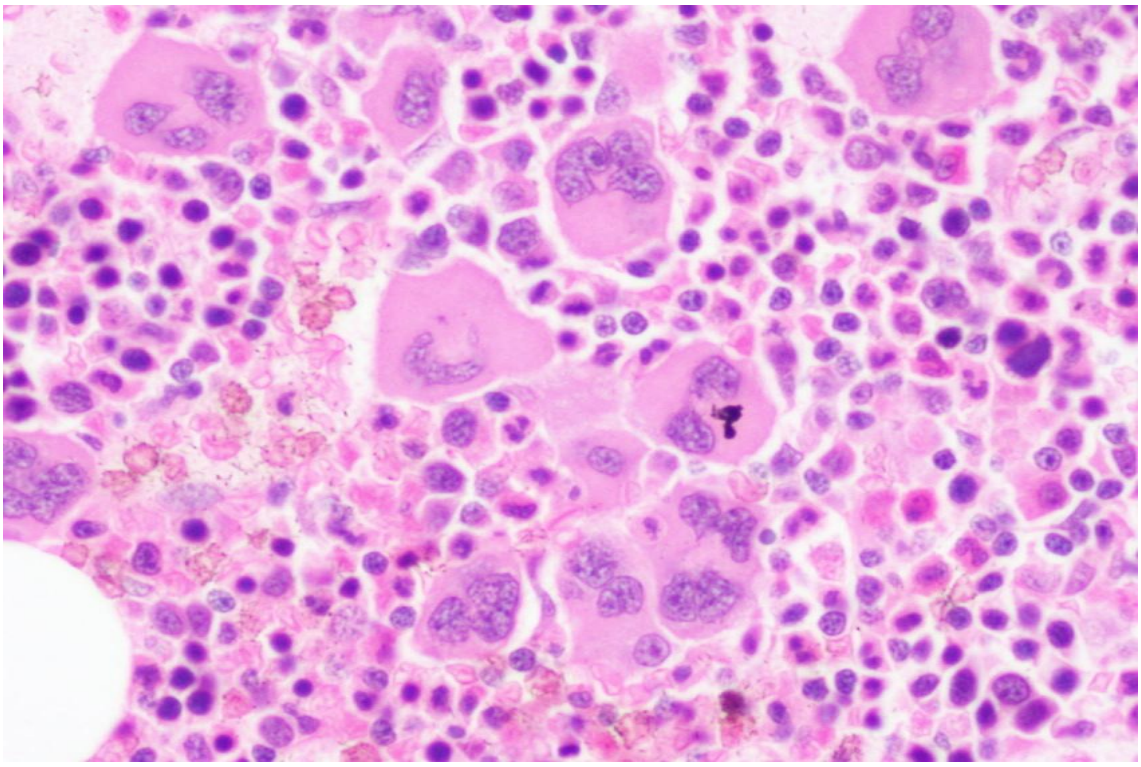


Figure 41 Large cluster of >7 megakaryocytes in ET. H&E X 400

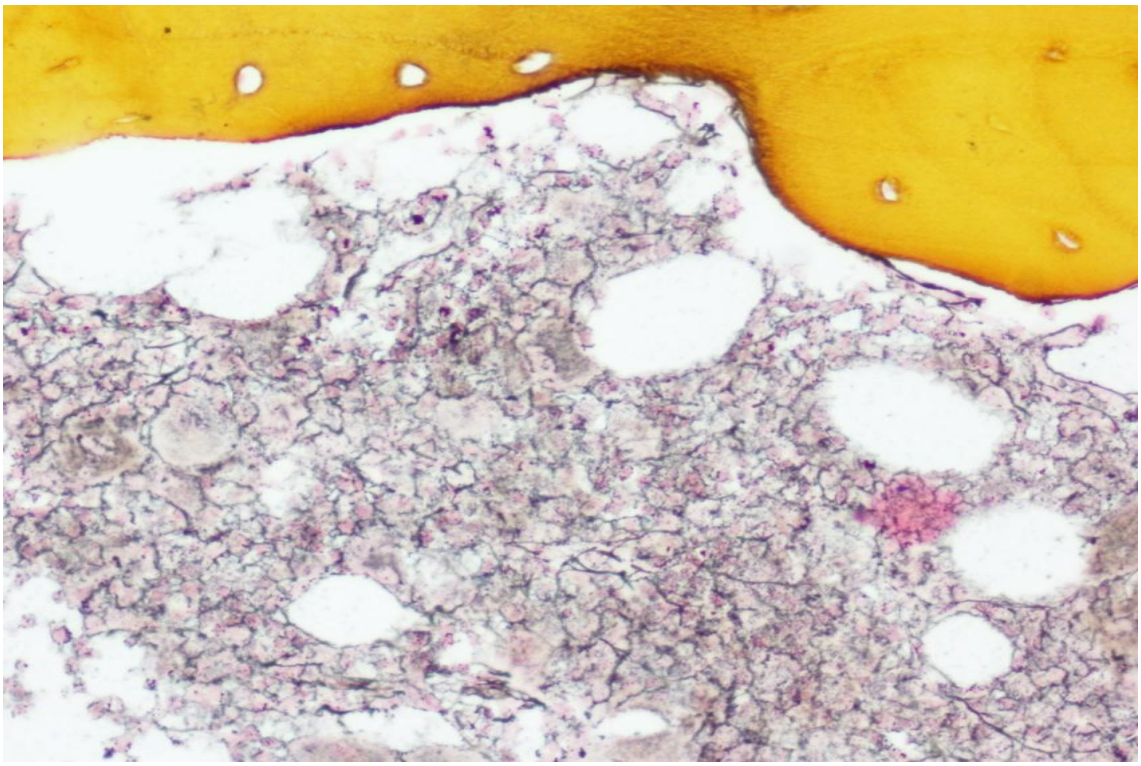


Figure 42 -MF0 reticulin grade in ET.GS reticulin X 400

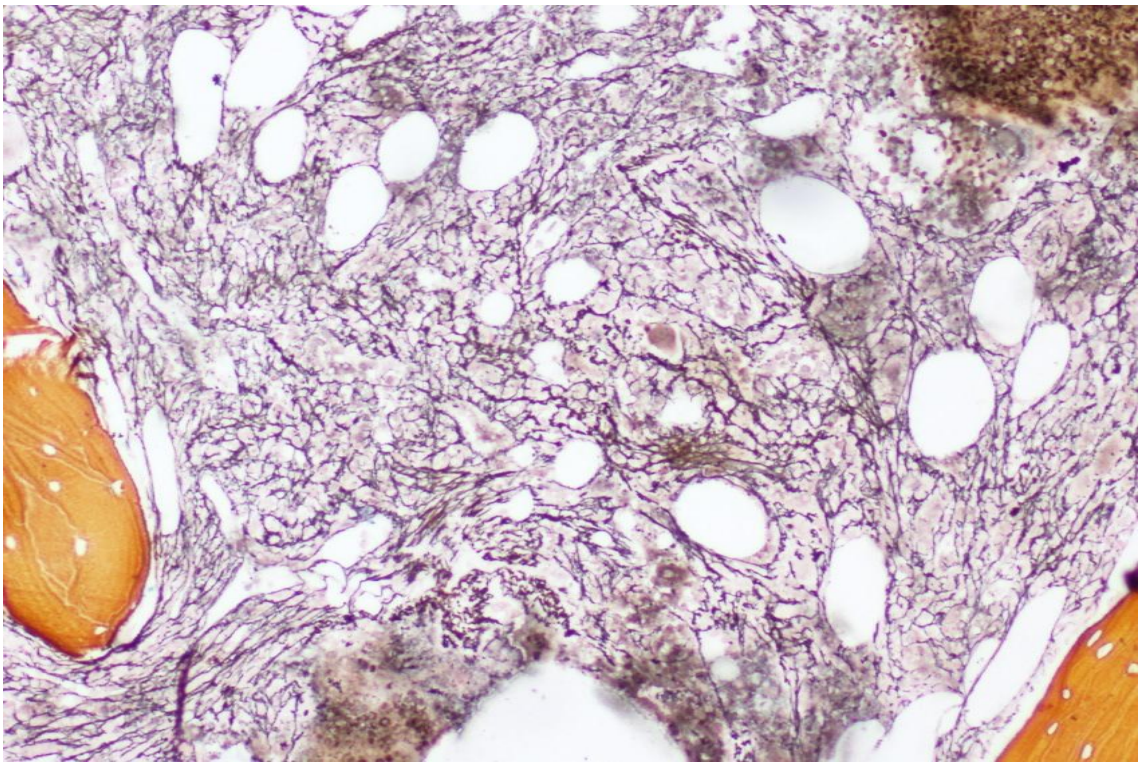


Figure 43-MF1 reticulin grade in ET. GS reticulin X 400

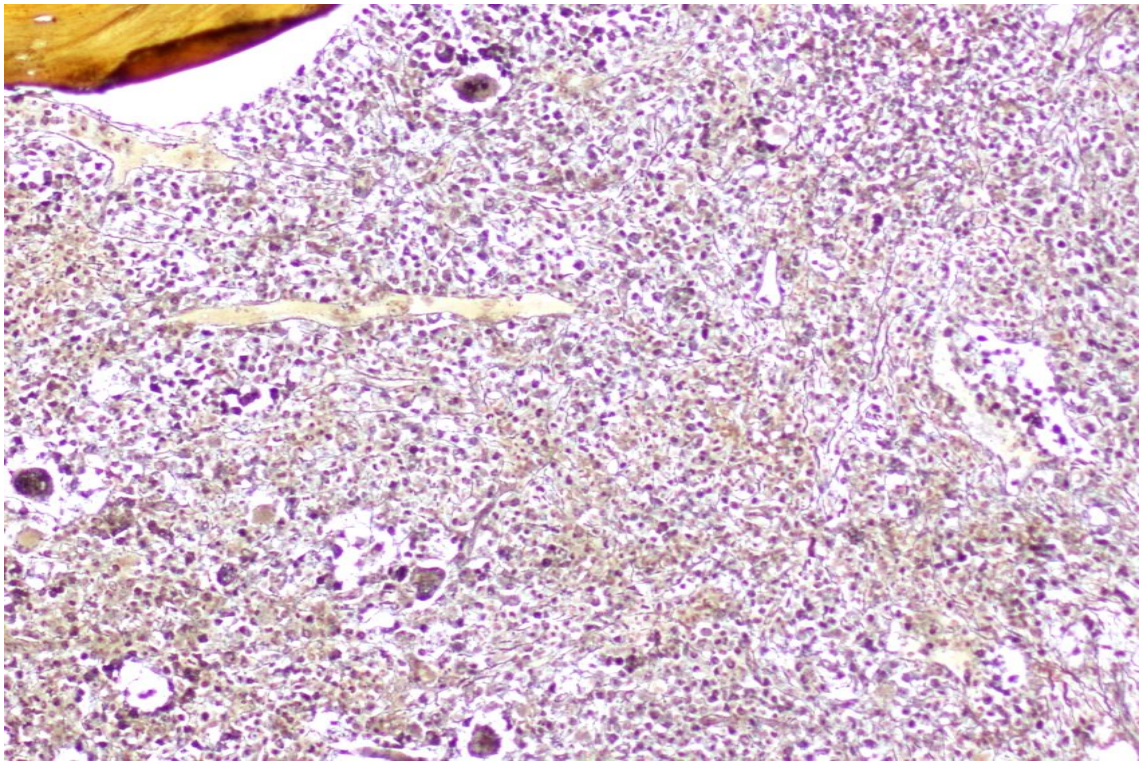


Figure 44- MF1 in Prefibrotic phase of PMF. GS reticulin X 200

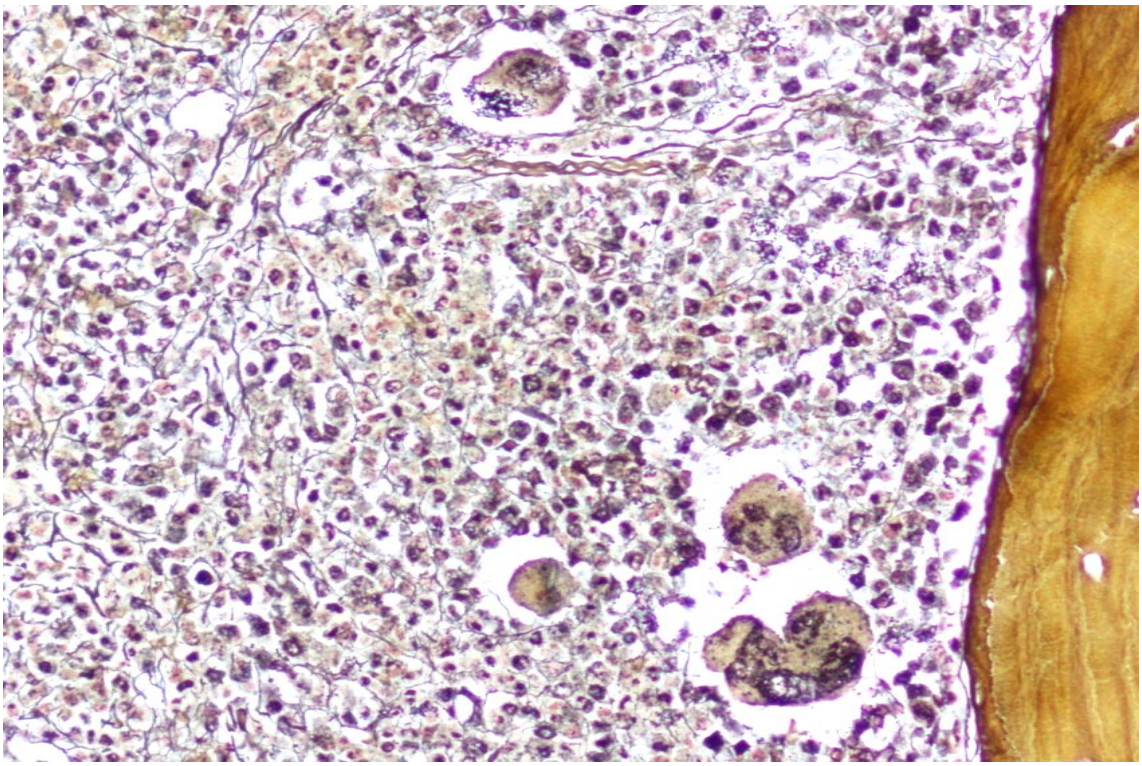


Figure 45- MF1 in prefibrotic phase of PMF with paratrabaecular located megakaryocytes, GS Reticulin x400

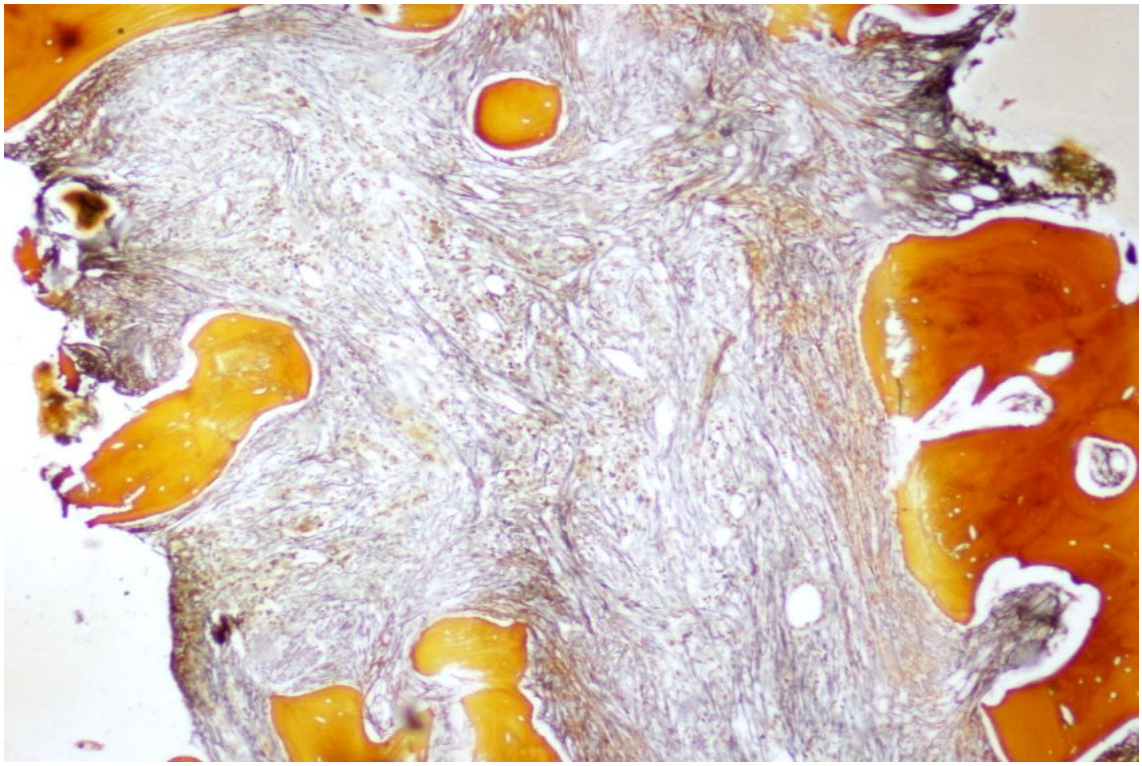


Figure 46- MF2 reticulin grade in PMF. GS Reticulin stain X 200

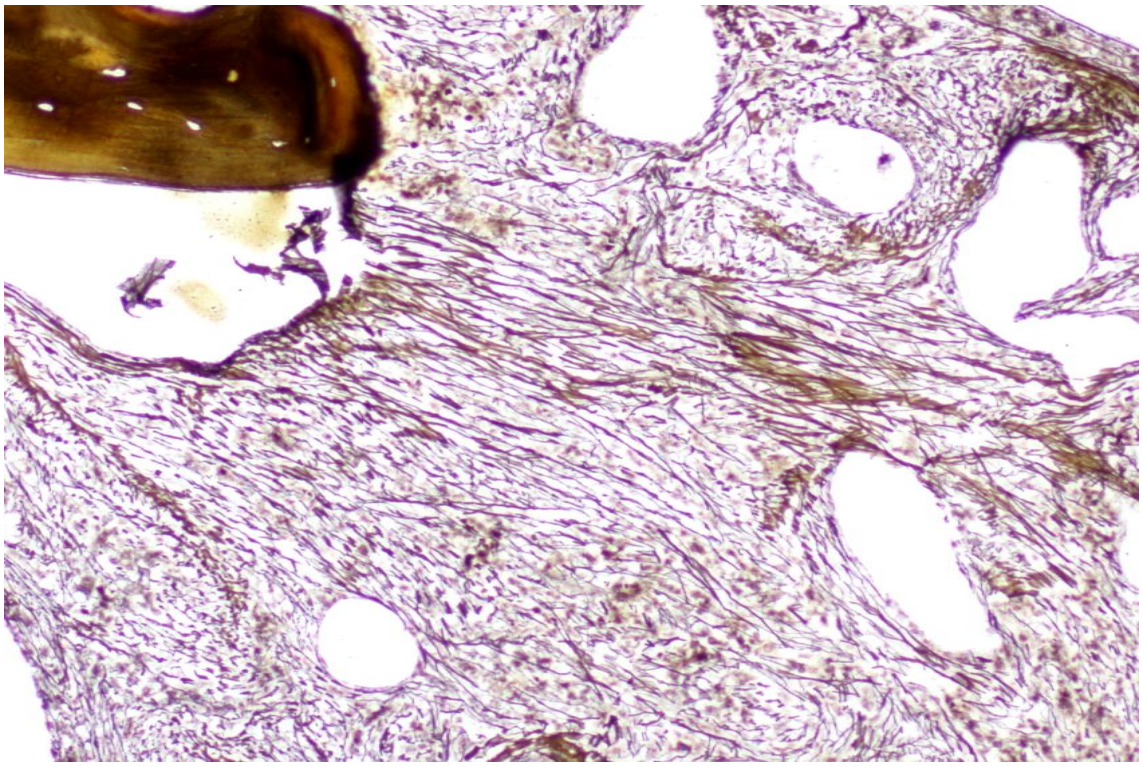


Figure 47- MF3 reticulin grade in PMF. GS Reticulin stain X 200

JAK2 V617F mutation detection by Allele specific PCR followed by agarose gel electrophoresis

Lanes: 1,2 – Jak2 Wild type

3,4 –Positive for V617F

5 – JAK2 V617F positive control

NC- Negative control

100bp MM- molecular marker

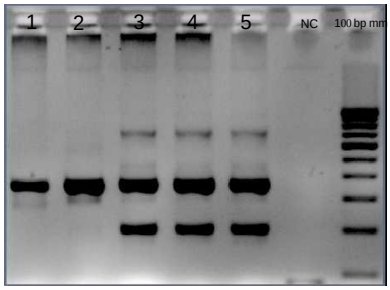


Figure 48 - Allele specific PCR followed by agarose gel electrophoresis

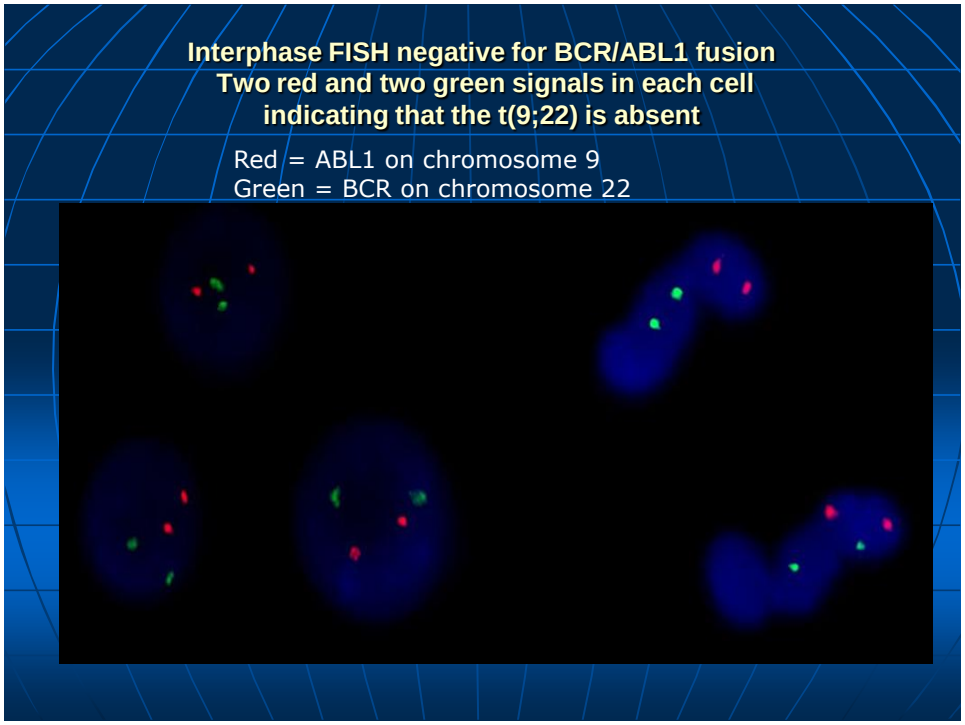


Figure 49 –Interphase FISH negative for BCR/ABL1 with two red and two green signals in each cell indicating that the t(9,22) is absent .

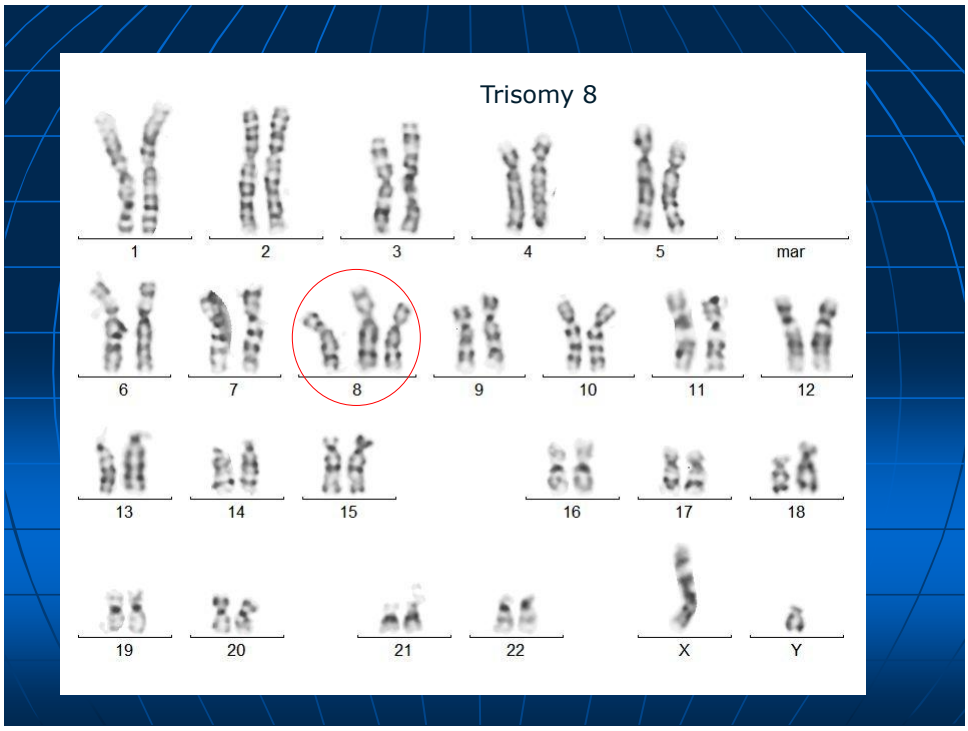


Figure 50- Karyotype in PMF , Trisomy 8

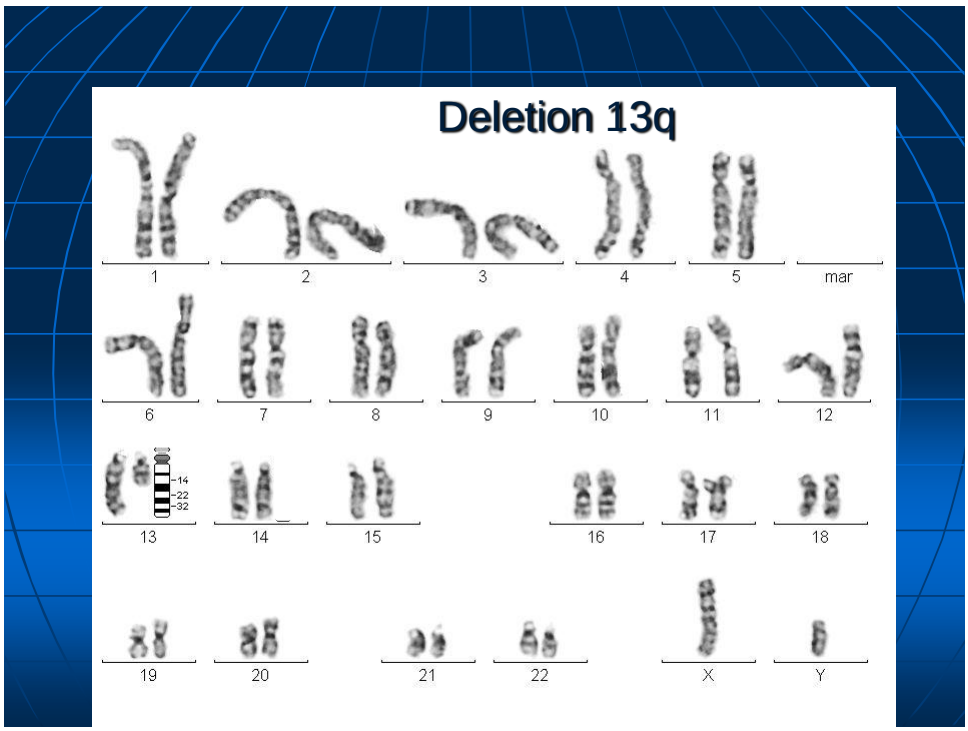


Figure 51 – Karyotype in PMF , Deletion 13q

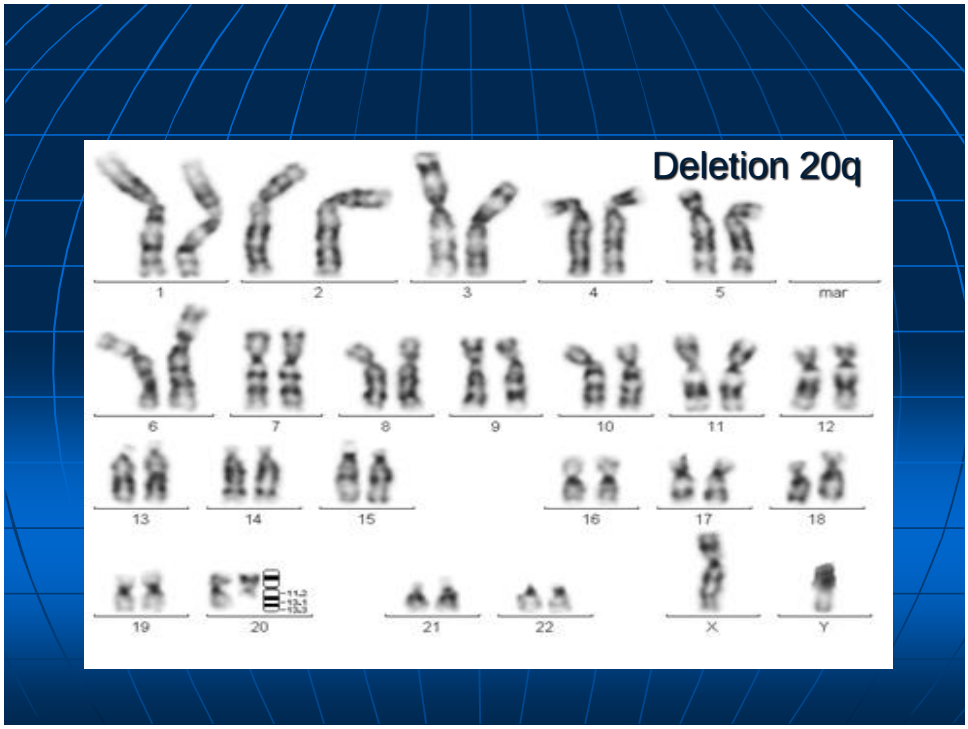


Figure 52 – Karyotype in PMF , Deletion 20q

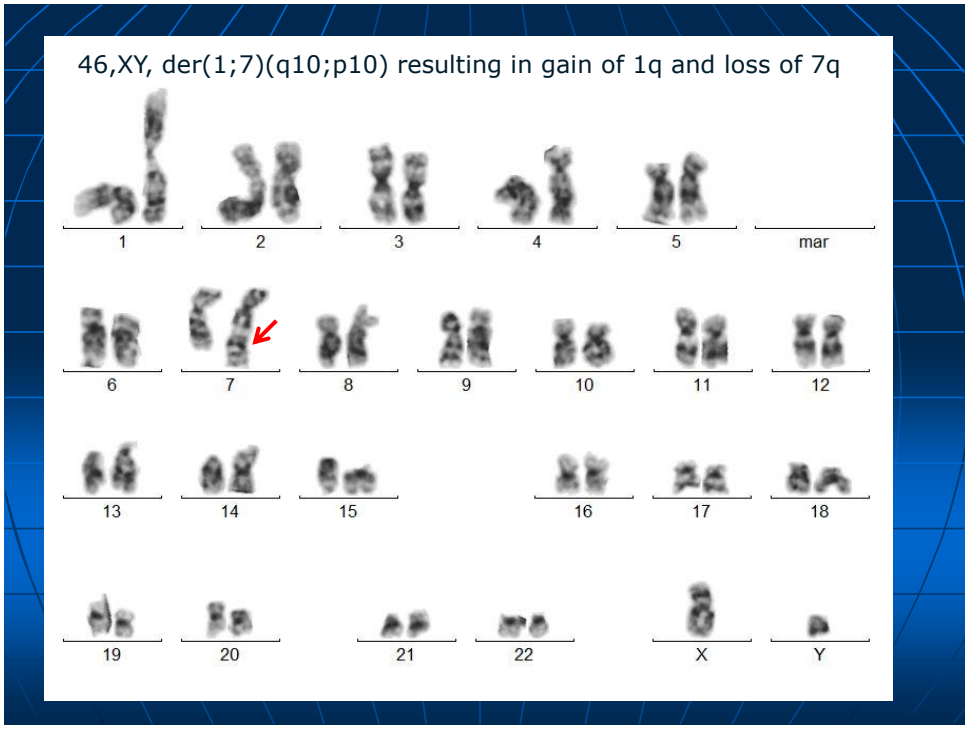


Figure 53 -Karyotype IN PMF, 46XY,der(1;7),(q10,p10) resulting in gain of 1q and loss of 7q.

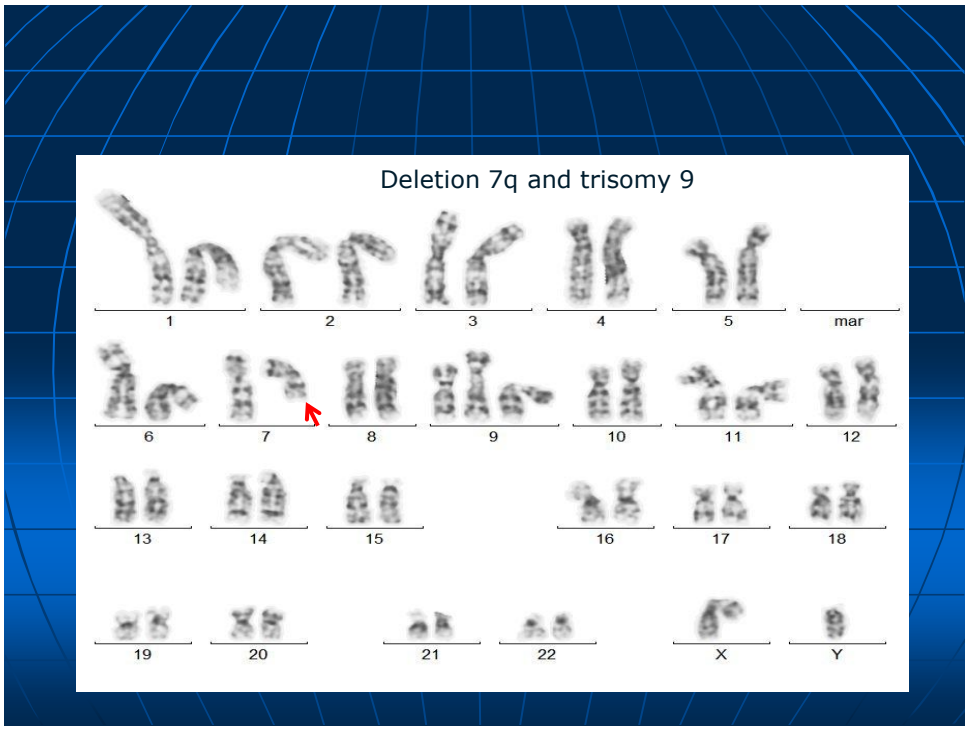


Figure 54 –Karyotype in PMF ,Deletion 7q and trisomy 9

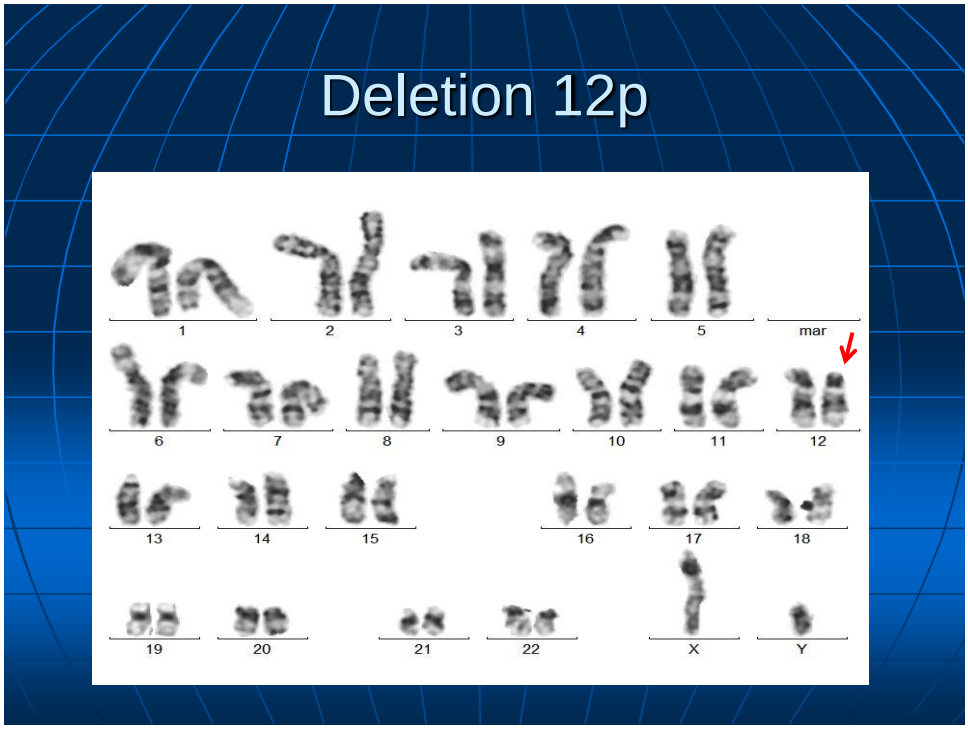


Figure 55– Karyotype in PMF , Deletion 12p

Discussion

Essential Thrombocythemia (ET), polycythaemia vera (PV), and primary myelofibrosis (PMF) form part of the spectrum of myeloproliferative neoplasms (MPN).

Primary myelofibrosis results in bone marrow failure because of replacement of marrow by fibrosis or transformation to acute leukaemia. The characteristic findings of a leukoerythroblastic picture and tear-drop cells in the peripheral smears are suggestive of myelofibrosis. However, these findings may not be present in every patient. In addition, bone marrow aspirates in PMF seldom yield cells (dry tap). Therefore, histological analysis of bone marrow trephine biopsies (BTBs) is critical for the assessment of fibrosis and cellular composition as well as to rule out other pathology that could cause marrow fibrosis (secondary myelofibrosis). In fact, BTBs are considered the gold standard to establish the diagnosis of PMF.

In the initial stage of PMF, known as the prefibrotic phase, the BM is hypercellular with increase in abnormal megakaryocytes and minimal or no fibrosis (reticulin grade MF0-MF1). Thus, the histological features of the prefibrotic phase of PMF show considerable overlap with ET. Therefore, it is important to distinguish between the two conditions because the course of both diseases is very different with respect to survival (76 vs 89% for ET) and the likelihood of progression to overt fibrosis or leukaemia (6-12% vs less than 1% for ET) (66) . As the disease progresses, there is marked fibrosis with decrease in cellular elements, sinusoidal dilatation and marked osteosclerosis resulting in widening of bone trabeculae which replace the marrow space. This leads to peripheral pancytopenia.

Primary myelofibrosis(PMF)

Primary myelofibrosis has been described as a disease of the sixth to seventh decades (5,6). Although the median age of our patients was 53 years, over a third of our patients (39%)

were below 40 years of age with a male predominance. These findings are similar to a report by Xue et al(67) on 642 Chinese patients seen over a period of 24 years, as well as a study from Italy by Barosi et al, of 650 PMF over 15 years (68). There was no difference in the median age of our patients with and without the JAK2V617 mutation. (53vs54)

Other clinical details were available for 67 patients, 64 (94%) of whom had one or more constitutional symptoms. Xue et al (67) described constitutional symptoms in 21% of 642 PMF while Gianelli et al reported constitutional symptoms in only 5% of their 192 PMF (69).

The most common presenting complaint was easy fatigability (83%) while about half of our patients, presented with weight loss or fever or were found to have anaemia. Splenomegaly was present in all 66 patients in whom spleen size was recorded. Xue et al, reported splenomegaly in 45% of their 642 PMF (67).

There was a wide variation in our blood counts, with values ranging from very low to very high. However, our mean values for haemoglobin (10.48 g/dl), total WBC count (131.8 $x10^3$ / µl) and platelet count (242 $x10^3$ / µl) are similar to Xue et al(67). The mean haemoglobin and total WBC counts in the studies by Barosi and Kvasnicka were similar to our study, however, the mean platelet count was higher (432 and 603 x 10^3/µL). (68, 70)

Anaemia (Hb <10g/dl) was the most common cytopenia and was seen in 40% of patients. Circulating blasts were seen in 30 % of PMF, with >1% blasts being seen in 26% .These findings are similar to Xue (68). Only 86 % of our patients showed a leukoerythroblastic blood picture (2,5 ,6, 14).

Serum LDH levels were considerably higher (median value of 1119 IU/L) in our patients as compared the reports by Kvasnicka (70) and Barosi (median 374 and 464).(60)

Almost all (98%) our PMF showed paratrabecular megakaryocytes as well as increased numbers of megakaryocytes (95%). Abnormal megakaryocytes with hyperchromatic dysplastic or hypolobate bulbous cloud-like nuclei, large megakaryocytes, sinusoidal ectasia, and intrasinusoidal haematopoiesis were consistently seen in > 85% of PMF.

The majority (71%) of PMF marrows were hypercellular. A rare (2%) but important finding, was markedly decreased cellularity because of extensive fibrosis and osteosclerosis.

Less common findings were myeloid left shift (46%), naked nuclei (53%) and small megakaryocytes (41%).The presence of small megakaryocytes in PMF has also been reported by Bain and Thiele (2,71), although the WHO classification (2008) has not listed this finding as a characteristic feature of PMF (6). Several other studies have also documented the presence of naked nuclei; however, none of these studies have recorded the actual percentage of this feature in their biopsies. (5, 6, 72,73,74)

Prefibrotic phase of PMF and comparison with PMF

In our study we had 13 patients with a histological diagnosis of Prefibrotic phase of PMF, 11 of whom were males with a median age of 52 years, similar to PMF. The spleen size showed a significant increase with progression of disease, from 17.6 in the Prefibrotic phase to 20.6 cm in PMF similar to Kvasnicka et al (2 cm below the costal margin vs 4 cm in PMF). (71) Barosi used the spleen index (obtained by measuring the longitudinal length x transverse length) to determine splenomegaly. They found a spleen index of 118 cm^2 in the prefibrotic phase as compared to 181 cm^2 in PMF. (68)

There were a few statistically significant differences between PMF and the Prefibrotic phase with regard to blood counts. Overall, as expected, the counts were higher in the Prefibrotic phase of PMF than in PMF, similar to that described by Thiele et al. (71) The mean

haemoglobin (12.5 g/dl) and the median platelet count (540 x 10^3/µl) was similar to other studies of prefibrotic PMF (70,72) .The median total WBC count was higher (22 vs 12 x10^3/µl) than in the study by Kvasnicka but similar to Buhr et al (72) .

These findings again highlight the variability of the peripheral blood findings in these conditions.

A leukoerythroblastic blood picture was seen in 33 % of the Prefibrotic phase as compared to 86 % of PMF in our study. Circulating blasts were seen in 15% of the Prefibrotic phase and 30 % of PMF. Kvasnicka did not report circulating blasts in either group. (70) Serum LDH levels increased as there was progression of disease from the prefibrotic phase to PMF.

Our findings demonstrate the change in spleen size and blood parameters and development of a leukoerythroblastic blood picture with progression from the Prefibrotic phase of PMF to PMF, similar to that described by Thiele et al. (71)

In a study of 136 cases, Barosi et al compared the features of the Prefibrotic phase and PMF. Patients in the Prefibrotic phase presented at a considerably lower age (mean age 38 years versus 54 years in PMF) with a female preponderance in the Prefibrotic phase (68). While the haemoglobin and platelet counts were higher, the total WBC count was reported to be lower in the prefibrotic phase than in PMF. (68) They also reported decrease in serum LDH levels with progression of disease from to PMF. These findings of Barosi are difficult to explain because the prefibrotic phase is characterised by hypercellularity as fibrosis has not yet set in.

These findings again highlight the variability of the peripheral blood findings in these conditions.

Bone marrow trephine biopsy findings - ***Prefibrotic phase vs PMF***

The Prefibrotic phase of PMF was associated with a significantly increase in cellularity and myeloid hyperplasia as compared to PMF (P= <.025). Another significant difference was intrasinusoidal haematopoiesis with intrasinusoidal megakaryocytes which was exclusive to PMF and absent in the prefibrotic phase (p = <.0001), a finding which has also been described by several other studies. (5,6, 73, 74, 75,76) Sinusoidal ectasia was more common in PMF as compared to the prefibrotic phase (P = <.0001). (71)

We also report two other megakaryocytic abnormalities which have not been described in the literature, one of which showed statistical significance.Megakaryocytes with hyperlobate staghorn-type nuclei were present in a significantly higher number of the Prefibrotic phase (69% vs 37%, P =.028). The other hitherto unreported abnormality was the presence of megakaryocytes with hypolobate bulbous cloud-like nuclei (90 % of PMF vs 100% of prefibrotic phase of PMF)

We also noted that hyperchromatic dysplastic megakaryocytes (94% vs 85%) and naked nuclei (53 % vs 46%) were more common in PMF than in its prefibrotic phase. Although the last three differences were not statistically significant, another detailed histopathological study by Thiele et al has also described similar findings. (71)

Scoring system

As described earlier, the histological features of the prefibrotic phase of PMF show considerable overlap with ET, although the prognosis and treatment are very different. Brousseau et al (17) devised a scoring system to differentiate between these two conditions. Brousseau used 12 histological parameters to classify 127 cases into ET, Prefibrotic phase of PMF and PMF. For comparison between the Prefibrotic phase of PMF and ET, they used

nine parameters to achieve a maximum score of 10. They suggested that scores at the ends of the spectrum could be used to distinguish between cases of ET (score <=3) and Prefibrotic phase of PMF (score >=6) , while interpretation might be more difficult with scores in the range of 4–5.

We also attempted to determine whether these two conditions could be better differentiated histologically. Therefore, we devised our own scoring system to ensure a systematic and reproducible analysis of these marrows. Our scoring system which assesses 16 histological parameters, differs in some aspects from the system of Brousseau et al.(17) We have included some features not scored by Brousseau such as left shift in myeloid lineage, megakaryopoiesis ,hyperlobate stag horn type nuclei and sinusoidal ectasia . We did not include the megakaryocyte nuclear-cytoplasmic ratio assessed by Brousseau because this requires morphometric analysis which may not be easily done in routine practice. Each parameter could be awarded a score of 0 to 2 and the maximum score that could be achieved was 21.

We found that the scoring system was largely reproducible by two independent observers who had differing years of experience in reporting BTBs, if the definition of each parameter was kept in mind while scoring. The only parameter where the observers obtained different scores was in the assessment of the number of large and small clusters of megakaryocytes. In occasional cases we had differences in MF grading. However the difference in the final score obtained independently by the two observers was less than 2 in all 134 cases.

CMC Scoring in Prefibrotic phase and PMF

Using our scoring system, we found that the range of scores in PMF and the Prefibrotic phase is similar in most cases. However, as expected, PMF showed a wider range of scores

(11-21) as compared to the prefibrotic phase (12-17). Only PMF reached scores of 18 - 21. Such scores accounted for about 25 % of PMF. However, the score alone cannot be used to categorise the phase of PMF as it is not predictive in all cases. Despite the presence of reticulin fibrosis and a high MF grade, the end stage of PMF can yield scores as low as 11 because of the low cellularity.

Therefore, the most important difference between the prefibrotic phase and PMF was the reticulin grade which is MF0 or MF1 in the prefibrotic phase and MF2 and MF3 in PMF. Regardless of the overall score, the biopsy must be classified as PMF in the presence of reticulin grade MF2 and MF3.

The scoring system showed differences between the Prefibrotic phase of PMF and ET which will be discussed subsequently.

Brousseau scoring system for the Prefibrotic phase

The Brousseau system for the Prefibrotic phase of PMF yielded scores ranging from 6-9 in 12 cases while one had a score of 10. Brousseau et al obtained similar scores of 6-9 in their study of 13 Prefibrotic phase biopsies. (17)

Essential thrombocythaemia (ET)

We had 27 cases of ET with a median age of 42 years and a male: female sex ratio of sex ratio of 1.25:1, similar to Tefferi et al (77). A study of 231 ET by Chim et al reported a median age of 65 years.(78)

In our study, the mean haemoglobin (12.9g /dl) and platelet count (850 -1001 $x10^3/\mu l$.) were similar to other studies (Chinese , Tefferi et al and Cervantes et al) (77,78,79). Our mean total WBC count (14.54 x $10^3/\mu l$) was higher than that reported by a Chinese study(78) and Cervantes et al (79) ($10.4x10^3$ and 9.1 $x10^3/\mu l$) while Tefferi et al (77) reported a WBC count

of (80.5x 10^3/µl) at diagnosis. Splenomegaly though not common was present in (11%); others have noted splenomegaly in 9-24% of ET Chinese (78), Cervantes et al (79) and Tefferi et al (77)studies .

The percentage of patients (19%) with thrombotic events was similar to other studies (13-18%) Chinese study (13%) and Tefferi (17.6%).(77,78)

All our five ET with thrombosis were females with a mean age of 29 years and a mean platelet count of 911x10^3/µl ; four were JAK2V617 positive (80%) . A study by Carobbio et al (80) of 891 ET found that thrombotic events were more common in males and age >60 years with a WBC count of >11x10^3/µl and JAK2V617 positivity also being risk factors for thrombosis. Campbell et al have also described an association between JAK2V617 positivity and thrombosis in 250 patients with ET. (81)

ET is even more rare in children. Girodon et al studied 311 patients over a 20-year- period and found thrombosis in approximately 10-30% of children.(5) We had two children with ET, one of whom presented with cortical and superior sagittal sinus thrombosis and was positive for the JAK2 V617 mutation.(82)

Bone marrow trephine biopsy findings

All 27 ET biopsies showed loose clusters of megakaryocytes and large megakaryocytes with hyperlobate staghorn-type nuclei , both of which have also been described by several others(84,85,86,87,88,89,90,91,92,93). An increase in cellularity and no increase in granulopoiesis was seen in >70% of patients. Dense clusters of megakaryocytes which are typical of the Prefibrotic phase of PMF were seen in only 11 % of ET. The majority (88%) of cases showed MF1 reticulin grade; MF0 reticulin grade was seen in the remaining 18%. These findings were similar to what has been reported in other studies. (83,85,94)

Comparison of ET and Prefibrotic phase of PMF

We also compared ET and the prefibrotic phase of PMF because there is considerable overlap between the histological features of these two conditions. These two conditions have different rates of survival (10-year survival 76 vs 89% for ET ,15 -year survival 59 vs 80% for ET) and leukemic transformation (5.8 vs 0.7 % after 10 years and 11.7 vs 2.1% after 15 years) .(66,95) Moreover, the modalities of treatment are also different.

We found several statistically significant differences between ET and the prefibrotic phase of PMF.

Peripheral blood

Platelet counts were higher in ET (949 vs 540 $x10^3/\mu l$, P value = .006) than in the prefibrotic phase of PMF .

Although ET cases showed lower total WBC count (11.9 vs 20.1 $x10^3/\mu l$) and LDH levels (704 vs 939 IU/L) than the prefibrotic phase of PMF, these differences were not statistically significant.

Bone marrow trephine biopsy

Several histological features were common to both ET as well as the prefibrotic phase of PMF.

All biopsies from both ET as well as Prefibrotic phase of PMF showed increased megakaryopoiesis with large megakaryocytes and all the different types of megakaryocyte clusters; another common feature was absence of intrasinusoidal haematopoiesis which is seen in PMF. All the other parameters studied were present in both conditions, although some features were more common in one or other condition as described earlier.

These overlapping findings illustrate the difficulty in classifying these cases histologically as ET or Prefibrotic phase of PMF. The scoring system was devised by us in an attempt to refine the diagnostic criteria and better classify the cases with diagnostic difficulty.

Features seen only in ET and not in the Prefibrotic phase of PMF were: normal cellularity in 26% , normal numbers of myeloid cells in 81% and reticulin grade MF0 in 18%. Erythroid hypoplasia which was seen in 62 % of Pre fibrotic phase of PMF was not a feature of ET.

However, when ET was compared with the Prefibrotic phase of PMF, the only statistically significant histological finding in favour of ET was the presence of hyperlobate staghorn megakaryocyte nuclei (Pvalue= .002) . Two studies by Thiele et al also noted that hyperlobate staghorn type of megakaryocyte were a common finding in ET; however, neither of these studies gave the percentage of biopsies with this findings. (96,97)

There were several statistically significant findings which favoured the diagnosis of the prefibrotic phase of PMF, namely, granulocytic cellularity, paratrabecular megakaryocytes, dense clusters of megakaryocytes (P value =<.0001 each) and naked nuclei (P value =.032). Gong et al also reported that these findings favoured the diagnosis of prefibrotic phase of PMF over ET (P value = <.05). (98) The other significant findings we found were left shift in myeloid lineage and hyperchromatic dysplastic nuclei (P value =<.0001) and sinusoidal ectasia (P value=.008) which were not reported by Gong et al (98) .

However, we must emphasise that no single feature can be used to differentiate between these two entities. We found that the scoring system was helpful in distinguishing between the two entities.

We compared our findings in these two entities with a similar study by Brousseau et al . (17)

The features found to be in favour of Prefibrotic phase of PMF in both studies were granulocytic cellularity (P value=<.0001 vs P value =.039*), paratrabecular megakaryocytes, hyperchromatic dysplastic nuclei, naked megakaryocyte nuclei (P value=.032 vs P value <.05) dense clusters of megakaryocytes (P value =<.0001 in both studies).

Although we did not find that hypolobate cloud like megakaryocyte nuclei was a significant histological feature, however Brousseau(17) and Ghong et al(98) have reported that this favours Prefibrotic phase of PMF. Brousseau also reported increased megakaryocytic nucleo–cytoplasmic ratio. We did not evaluate this feature separately because this required morphometry for accurately measuring the N/C ratio.We included this feature as part of hyperchromatic dysplastic nuclei.

Other features i**n favour of Prefibrotic phase of PMF found in our study but not in the Brousseau** study were increased cellularity (P value=.043 vs .054*) and decreased erythroid cellularity (Pvalue= <.0001vs.241*). Other significant features found by us but not evaluated by Brousseau were myeloid left shift, sinusoidal ectasia (P value=.008) and small megakaryocytes (P value=.002)

In favour of Prefibrotic phase of PMF in Brousseau study only

- Hypolobate cloud like megakaryocyte nuclei (P value =<.0001 as against P value =.09 in our study).
- Increased megakaryocytic nucleo-cytoplasmic ratio, (P value <.001; not evaluated in our study as accurate results can only be obtained by morphometric analysis which we did not do in our study.

With regard to ET, although we found that the presence of Hyperlobate staghorn megakaryocyte nuclei (P value= .002 vs .563)(14) was **statistically significant,** Brousseau et al did not report this association**.**

Thus we conclude that a scoring system better enables us to differentiate between ET and Prefibrotic phase of PMF. Study of more cases would help us to identify cases that fall in the grey zone and validate our scoring system more robustly.

Six cases initially diagnosed as prefibrotic phase of PMF when the reticulin grade was reported as mild, moderate and severe were later reclassified as PMF when graded MF3 according to the European consensus grading system.

Scoring of Biopsies

ET and PMF were compared using our scoring system as well as the Brousseau scoring system to determine whether the differences between these two entities could be could be identified consistently.

Overall scores

With our scoring system, we noticed a clear difference between the ET and Prefibrotic phase of PMF in contrast to the scores of prefibrotic phase of PMF and PMF. Using our scoring system, four possible cases ET were scored between12-14 and were reclassified as Prefibrotic phase of PMF.

ET had scores of 5-9 while in the Prefibrotic phase of PMF, the scores were 12-17. Nearly half (48%) of our ET had a score of 9. None of our prefibrotic phase of PMF had a score of 11.

Therefore, we suggest that the cut-off value of our scoring system should be 10. If such a score is obtained, the biopsy report should clearly state that an unequivocal categorisation cannot be made.

When we used the Brousseau scoring system, our Prefibrotic phase of PMF had scores of 6-10 while ET ranged from 0-4. We obtained a score of 4 in 2/27 ET. One Prefibrotic phase of PMF was assigned a score of 10 which was not seen in the Brousseau study. (17).

Based upon our findings, we tentatively suggest that the cut-off for a definitive diagnosis for ET be </=5 instead of </=3 as suggested by Brousseau et al.(17) However, we will need to score more ET biopsies to identify cases falling in the grey zone because the number of cases studied by us (27 ET and 13 prefibrotic phase of PMF) are fewer than the Brousseau study (102 cases of ET, 18 cases of prefibrotic phase of PMF).

IPSS Score for PMF and prefibrotic phase of PMF

IPSS scores could be calculated for only five patients with the Prefibrotic phase of PMF, none of whom were in the low risk group. In a study by Gianelli et al, the 58 MF1 prefibrotic phase of PMF fell into all four risk groups, with 19 % in the low risk group although the majority (77%) were in the intermediate risk groups. (99)

Our 91 PMF with MF3 reticulin grade were distributed across all four groups with 11% being in the low risk group.

The 14 PMF with MF3 reticulin grade studied by Gianelli were distributed across all four groups , with low risk in 07%, intermediate risk 1 in 28%, intermediate risk 2 in 43 % and high risk in 22 % in 14 cases of PMF. (99)

JAK2 V617 mutation status

Overall, JAK2V617 mutation analysis results were available for 73 PMF (including six in the prefibrotic phase) and 25 ET.

JAK2 V617 mutation status in PMF

Of the 73 PMF, 48 (66%) were positive for the mutation. This is similar to a study from China by Xu et al (67%) and is higher than what has been reported in the West (43-50 %) as well as another Indian study (52% of 31 patients). (67,100,101,102). Five (83%) of the six in the prefibrotic phase were positive for the JAKV617 mutation. Gong et al (98) found the mutation in 55% of 61 prefibrotic phase of PMF (5). The median age of these patients was 56 which was similar to reports (53-61 years) . (67, 68, 101)

We found that weight loss was the commonest symptom; pruritus was reported by only 2.5% of our patients unlike Tefferi et al who reported that pruritus was the most common symptom. (101)

Most of the patients had palpable splenomegaly while a Chinese study of 642 cases of PMF showed splenomegaly in only 45%(289). (67)

JAK2 V617 Positive vs JAK2 WT PMF

On comparing the JAK2V617 positive and JAK2WT PMF, only the mean haemoglobin at presentation (mean 10.7 vs 9.3 g/ dl) showed a statistically significant difference between the two groups (p value =.04) similar to Vytrva et al.(103) We did not find any other statistically significant differences in the clinical or histological features.

JAK2 mutation status in ET

The JAK2V617 mutation was present in 12 /25 (48%) ET tested. This is lower than reported by other Indian studies (64-70%). (35) (104) but similar to a study by Amy et al (41% of 59 ET)(100). Thrombotic events were seen in four of the 12 patients (33%) with the mutation.

These were cortical venous thrombosis (two), pulmonary venous thrombosis and Budd Chiari syndrome (one each). It has been reported that individuals with the JAK2V617 mutation are prone to thrombotic events (105,106). Primignani et al and Colazzio et al have reported Budd Chiari syndrome in JAK2V617 positive ET.(107,108). None of our patients had bleeding episodes. It is well known that ET is associated with an increased risk of thrombosis. However, thrombocytosis greater 800 x 10^3 /µL is likely to be associated with haemorrhage due to acquired von Willebrand disease. (108)

JAK2 V617 Positive vs JAK2 WT ET

We did not observe an age difference between JAK2V617 positive and JAK2 WT ET (40 vs 39) unlike another Indian study by Ross et al (105) which observed that JAK2V617 patients were a decade older (58 vs 45) (105). Campbell (106) and Kittur et al(110) reported median ages of 60 and 62 years in their ET patients positive for the JAK2 mutation V617 positive ET . Those lacking the mutation had median ages of 52 and 57 .(109) We did not find any difference in spleen size between JAK2V617 positive and JAK2WT ET, similar to other studies.(104,105)

One patient lacking the JAK2 mutation had non ST elevated myocardial infarction.

We found a significant association between the platelet count and the JAK2 mutation status. JAK2WT patients had higher platelet counts (1052 vs 777 x10^3/µl , P value =.02). Kittur et al noticed a similar finding in 176 patients with ET. (110) However, such an association was not reported by Vytrva et al (103) and Campbell et al . (105)

Although our JAK2V617 positive ET patients had a higher haemoglobin and total WBC count similar to other reports, this did not reach statistical significance. ET is associated with

sequestration of platelets in the spleen as there is no extramedullary haemopoiesis except in the late stage.

The bone marrow trephine biopsies showed a statistically significant difference between JAK2V617 positive and JAK2WT ET only with regard to the presence of large clusters of megakaryocytes in JAK2WT (58% versus 92%, P value = .048).This feature has not been described by Vytrva et al. (103), Campbell et al(104) and Nataliya Vytrva et al (103) have reported a statistically significant increase in granulocytic precursors in those with and without the mutation. Although increase in granulocytic precursors was seen in 25% of those with the JAK2 mutation as compared to 15% of those lacking the mutation, this difference was not statistically significant in our study.

Cytogenetics

Cytogenetic aberrations are more common in PMF than in ET. Cytogenetic analysis is difficult in PMF because the marrow often does not yield an aspirate. Karyotyping may then have to be done on peripheral blood. The abnormalities are the same in both conditions. There are no specific abnormalities associated with either disorder reflecting a similar pathogenesis.

The main role of cytogenetics and FISH in these diseases is to demonstrate the presence of a clonal abnormality and to exclude the presence of the t(9;22). The role of cytogenetics in the prognostication of MPN is not as well defined as for the other haematological neoplasms. Tefferi et al suggest that abnormalities such as trisomy 8 and the deletion 12p are associated with a poor prognosis in PMF while the deletions 20q and 13q did not have a prognostic impact. (112). Chromosomal aberrations may be found in 40-50% of PMF. As the disease progresses, structural abnormalities resulting in gain of chromosome 1q and

abnormalities of chromosome 7 may be seen. The karyotypes are usually associated with chromosomal imbalance. The common changes such as trisomies 8 and 9, deletions 13q and 20q and gain of 1q account for 80% of abnormal karyotypes. (113,114)

Summary and conclusions

We have described the histological features of 107 primary myelofibrosis (PMF) and 27 essential thrombocythaemia (ET) over a period of five years.

We have devised a scoring system to classify these condition and to attempt to identify the histological differences between the early (pre-fibrotic) phase of PMF and ET which show considerable overlap.

We have also recorded the clinical and laboratory parameters including JAK2 mutation status and results of FISH for BCR -ABL when available.

The majority of our patients were

- Males (71%)
- Adults (median age 50 years: range 5-72years)

There were 94 cases of PMF excluding the 13 in the prefibrotic phase.

The following histological features were seen in more than (80%) of PMF analysed

- ❖ Megakaryocytes in paratrabecular location
- ❖ Increased number of megakaryocytes
- ❖ Large megakaryocytes
- ❖ Hypolobate bulbous cloud like megakaryocytes
- ❖ Hyperchromatic dysplastic megakaryocytes
- ❖ Sinusoidal ectasia
- ❖ Intrasinusoidal haematopoiesis

All 13 cases in the Prefibrotic phase of PMF showed the following histological features:

- ❖ Increase in granulocytic precursors

- ❖ Increased numbers of megakaryocytes
- ❖ Large megakaryocytes
- ❖ Paratrabecular location of megakaryocytes.
- ❖ Loose clusters of megakaryocytes
- ❖ Absence of intrasinusoidal haematopoiesis
- ❖ Reticulin grade MF1 (scored as 0).

All 27 ET showed

- ❖ Increased numbers of megakaryocytes
- ❖ Large megakaryocyte with hyperlobate staghorn type nuclei
- ❖ Clustering of megakaryocytes with both small and loose clusters
- ❖ Absence of myeloid left shift, sinusoidal ectasia and intrasinusoidal haematopoiesis
- ❖ Reticulin grade MF0 or MF01.

The most important diagnostic criterion of PMF was the reticulin grade.

- ❖ MF3 was seen in 97% of PMF while only 3% had MF2.
- ❖ MF0 - MF1 was seen in ET and prefibrotic phase of PMF.

- There were several statistically significant differences between ET and the Prefibrotic phase of PMF.

Cellular and vascular changes

The statistically significant features in favour of prefibrotic phase of PMF, not seen in any of the 27 cases of ET were

- ❖ Left shift in myeloid lineage
- ❖ Decreased numbers of erythroid cells

- Small megakaryocytes
- Sinusoidal ectasia

Another very significant finding in favour of Pre-fibrotic phase of PMF

- Paratrabecular megakaryocytes

Findings which were seen more commonly in the prefibrotic phase of PMF than in ET were:

- Increased cellularity
- Myeloid hyperplasia
- Hyperchromatic dysplastic megakaryocyte nuclei
- Naked megakaryocyte nuclei
- Dense clusters of megakaryocytes

A finding seen more commonly in ET than in the prefibrotic phase of PMF was:

- Hyperlobate staghorn megakaryocyte nuclei

Reticulin

Reticulin was graded as MF1 in all 13 prefibrotic phase of PMF and in the majority of ET, showing that reticulin pattern does not distinguish between these two conditions.

However, no single feature including reticulin grade can be used to differentiate between these two conditions as there is considerable overlap in the histological features.

We devised a scoring system based on 16 histological features to ensure a systematic assessment.

- Each parameter was scored from 0-2 to obtain scores in the range of 5-21 .
- Scores for ET were 5-9, while the prefibrotic phase of PMF had scores of 12-17.

- We did not have any biopsies with scores of 10-11. It would be difficult to unequivocally distinguish between ET and prefibrotic phase of PMF if the biopsy score is in this range.

JAK2 mutation status

The JAK2 V617 mutation was present in 60 of the 98 patients tested. There did not appear to be statistically significant differences in histological findings between JAK2V617 PMF and JAK2WT PMF.

- With regard to laboratory parameters only the mean haemoglobin at presentation showed a statistically significant difference between the JAK2V617 positive PMF and JAK2WT PMF.
- JAK2WT ET showed a higher platelet count and the presence of large clusters of megakaryocytes in comparison to JAK2 V617positive ET.

Limitation

- This is a purely descriptive study. Although we have adequate laboratory and clinical data , we do not have follow up details**.**

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APPENDIX

APPENDIX- 1

PROFORMA FOR ASSESSMENT OF PMF / ET.

UID number

Biopsy no:

Hospital No **Age**: **Sex**: **Date**

Haemoglobin: g / dL TC: $\times 10^3$ / µ L Platelet count : $/10^3$ / µL

DC: N %, E %, B %, L %, M %, Blasts %, NRBCs: /100

Blood picture

- Tear drop cells
- Poikilocytosis,
- Pseudo Pelger Huet cells
- Platelet morphology

Bone marrow Findings	**Trephine**	**Aspirate**
1) **Length of cores**		
2**) Number of intertrabaecular spaces**		
3) **Increased cellularity** **Absent(00)/Present (01)**		
4) **Granulocyte precursors** Hyperplasia(1)/ hypoplasia(0) /normal(0) /decreased(2)		
5) **Left-shift** (Absent 00/Present01)		

6) **Erythroids** Hyperplasia(1) / normal(0) /decreased(2)		
7)**Megakaryopoiesis** Histotopography/increased number(1) /normal(0)/decreased(2)		
8)Histotopography Location: Paratrabecular(1)		
Perisinusoidal**(**00**)**		
9)Size		
Small(2)		
Increased (Large Size) (1)		
Normal Size(0)		
10)Nuclear features		
Hypolobulation(bulbous/cloud-like) (1)		
Hyperlobulation (staghorn-like) (1)		
Hyperchromatic dysplastic nuclei(1)		
Naked nuclei(1)		
11)Cluster formation		
Small clusters(> 3) megakaryocytes(1)		
Large clusters (> 7)(2)		
12)Cluster density and number		
Dense clusters(1)		
Number of dense clusters		
Loose clusters (0)		
Number of loose clusters		
13)Sinusoidal ectasia (1)		
14)Intrasinusoidal haematopoiesis(1)		
15) Reticulin (WHO 2008 grading)		
MF0: Scattered linear reticulin(0)		
MF1: Loose network, many intersections, perivascular areas.(0)		
MF2: Diffuse, dense, focal bundles of collagen/osteosclerosis.(1)		
MF3: Diffuse, dense, extensive		

intersections, coarse collagen, Osteosclerosis(1)		
16) Cytogenetic analysis Available/ not available List of abnormalities		
17)FISH for BCR/ABL1 fusion Present/Absent/Not available		
18)JAK2 mutation status: Present/Absent/Not available		
19) Serum LDH levels Present (levels)/Not available		

*Trephine biopsy only

**** Prefibrotic and fibrotic phases**

( ) Number in the bracket indicates score.

20) Clinical findings

21) Splenomegaly: Present Size in cm ---------- /absent / Not available

22) Constitutional symptoms: Present / absent / Not available

a) Drenching night sweats

b) Weight loss>10%over six months

c) Unexplained Fever (>37.5°C)

d) Diffuse bone pains

For ET (**Vascular occlusion, haemorrhage such or any thrombotic episode such as splenic or portal vein thrombosis)**

23) IPSS score

APPENDIX 2

Table :1 Histological features of bone marrow trephine biopsies in JAK2V617 positive and JAK2WT myelofibrosis(MF)					
HISTOLOGICAL DIAGNOSIS	**PMF, n=6**		**JAK2V617positive N=43**	**JAK2 Wild type, n=24**	**P value**
1)Increased Overall Cellularity,	Absent		12(27.91)	06(25)	0.797
	Present		31(72.09)	18(75)	
2)Granulocyte Precursors	Increased		11(25.58)	05(20.83)	0.662
	Normal		32(74.42)	19(79.17)	
3)Left shift number	Absent		21(48.84)	10(41.67)	0.572
	Present		22(51.16)	14(58.33)	
4)Erythroid cellularity (number)	Normal		14(32.56)	05(20.83)	
	Increased		01(2.33)	2(8.33)	
	Decreased		28(65.12)	17(70.83)	.35
5)Megakaryopoiesis	Normal		01	01	.693
	Increased		42(97.67)	23(95.83)	
	Decreased		00	00	
6)Megakaryocyte location	Paratrabaecular		43(100)	24(100)	
	Perisinusoidal(Normal)		43(100)	24(100)	
7)Type of megakaryocytes	Small	Absent	24(55.81)	17(70.83)	.328
		Present	19(44.19)	07(29.17)	
	Large	Absent	05(11.63)	02(8.33)	.673
		Present	38(88.37)	22(91.67)	
	Normal	Absent	02(4.65)	00	.283
		Present	41(95.35)	24(100)	
8)Hypolobate cloud like megakaryocyte nuclei	Absent		05(11.63)	02(8.33)	.673
	Present		38(88.37)	22(91.67)	
9)Hyperlobate , giant and staghorn megakaryocytic nuclei	Absent		30(69.77)	15(62.50)	.54
	Present		13(30.23)	09(37.50)	
10)Hyperchromatic dysplastic nuclei	Absent		00	02(8.33)	0.056
	Present		43(100)	22(91.67)	
11)Naked nuclei	Absent		18	10	0.98
	Present		25	14	
12)Type of megakaryocyte clusters	Small	Absent	02	02	.05
		Present	43(100)	22(91.66)	
	Large	Absent	09(20.93)	5(20.83)	.993
		Present	34(79.07)	19(79.17)	
13)Density of Megakarocytes clusters	Loose	Absent	03(6.98)	03(12.50)	.448
		Present	40(93.02)	21(87.50)	
	Dense	Absent	11(25.58)	09(37.50)	.307
		Present	32(74.42)	15(62.50)	
Number of large megakaryocyte clusters/mm(median)			.11	.20	.43
Number of small megakaryocyte clusters/mm(median)			.58	.44	.88
14)Sinusoidal ectasia	Absent		03(6.98)	03(12.50)	.448
	Present		40(93.02)	21(87.50)	
15)Intra sinusoidal haematopoiesis	Absent		4(9.30)	05(20.83)	.184
	Present		39(90.70)	19(79.17)	
	MF2		01(2.33)	00	
	MF3		42(97.67)	24(100)	.452
16)Reticulin score	0		0	0	
	1		43(100)	24(100)	
Total Score (median, range)			15(12-21)	16(13-21)	

Appendix 3

Hematologic and clinical data in JAK2V617 positive prefibrotic phase of PMF

Prefibrotic phase of PMF, n=5		**JAK2V617POSITIVE**		
Number of cases		05		
Age at diagnosis,(median)		Median 52, range -5-69		
Sex				
Male		4(80)		
Female		1(20)		
Laboratory findings		Range	Mean	Median
Haemoglobin (g/dl) at diagnosis		8-17	12	
Leucocyte count x10^3/ µl at diagnosis		6.7-67.1		26
Platelet count x 10^3/µl at diagnosis.		328-712		521
LDH IU/l		850,656-1231	850	656-1231
Splenomegaly	Absent	02		
	No of cases with splenomegaly	03(60%)		
	mean size in cm	15.45 cms		

Appendix -4

Comparison of myelofibrosis and its Prefibrotic phase – histological findings

		PMF , n=94		Prefibrotic phase, n=13		P value
		n	%	n	%	
Increased cellularity	Present	67	71	13	100	**.025**
Granulocyte Precursors	Increased	43	46	13	100	**.025**
Myeloid left shift	Absent	51	54	06	43	
	Present	43	46	07	54	.583
Erythroid cellularity (number)	Normal	26	28	05	38	.615
	Decreased	65	69	08	61	
	Increased	03	03	13	100	
Megakaryopoiesis	Increased	89	95	13	100	.394
Megakaryocyte location	Paratrabecular	92	98	13	100	.595
	Normal perisinusoidal	94	100	13	100	.709
Type of megakaryocytes	Small	39	41	05	38	.835
	Large	81	86	13	100	.153
	Normal	92	98	13	100	.595
Megakaryocyte nuclei						
Hypolobate bulbous cloud like nuclei	Present	85	90	13	100	.496
Hyperlobate , giant and staghorn megakaryocytic nuclei	Absent	59	63	04	31	
	Present	35	37	09	69	**.028**
Hyperchromatic dysplastic nuclei	Absent	6	6	02	15	
	Present	88	94	11	85	.247
Naked nuclei	Absent	44	47	07	54	
	Present	50	53	06	46	.634
Megakaryocyte clusters	Small	92	98	13	100	.595
	Large	71	75	11	84	.468
	Loose	88	94	13	100	.348
	Dense	66	70	08	61	.615
Sinusoids						
Sinusoidal ectasia	Absent	8	8	9		
	Present	86	92	4	69	<.0001
Intra sinusoidal haematopoiesis	Absent	12	13	13	100	
	Present	82	87	0	0	<.0001
Reticulin						
Reticulin grade	MF0	-	-			
	MF1	-	-	13	100	
	MF2	3	3	-		
	MF3	91	97	-		
Reticulin score	0					
	1					
Scores						
CMC score		15,12-21		15,12-17		
Brousseau score (median, range)				8,6-10		

Appendix 5

Histological features of bone marrow trephine biopsies in JAK2V617 positive prefibrotic MF

HISTOLOGICAL DIAGNOSIS	PMF, n=6		JAK2V617positive
Increased cellularity	Present		05(100)
Granulocyte Precursors	Increased		05(100)
Left shift number	Absent		01(20%)
	Present		04(80%)
Erythroid cellularity (number)	Normal		03(60%)
	Decreased		02(40%)
	Increased		05(100)
Megakaryocyte location	Paratrabaecular		05(100)
	Perisinusoidal		05(100)
Type of megakaryocytes	Small	Present	02(40)
	Large	Present	05(100)
	Normal	Present	05(100)
Hyperlobate , giant and staghorn megakaryocytic nuclei	Absent		02(40)
	Present		03(60)
Hyperchromatic dysplastic nuclei	Absent		02(40)
	Present		03(60)
Naked nuclei	Absent		02(40)
	Present		03(60)
Type of megakaryocyte clusters	Small	Present	05(100)
	Large	Present	05(100)
	Loose	Present	05(100)
	Dense	Absent	02(40%)
		Present	03(60%)
Number of large megakaryocyte clusters/mm(median)			0.06
Number of small megakaryocyte clusters/mm(median)			.76
Sinusoidal ectasia	Absent		03(60)
	Present		02(40)
Intra sinusoidal haematopoiesis	Absent		05(100)
	Present		00
Reticulin	MF0		00
	MF1		05(100)
	MF2		00
	MF3		00
Reticulin score	0		05(100)
	1		00
TS (median, range)			15,12-17
Maud Brousseau, et al score (median, range)			07, 6-10

Appendix 6 - Comparison of JAK2V617 positive and JAK2WT ET - clinical and laboratory data

Parameter	**JAK2V617 POSITIVE, n-12**	**JAK2 WILD TYPE , n=13**	**P value**
Number of cases	12	13	
Median age at diagnosis	40	39	
Sex			
Males	5(42%)	9(69%)	
Females	7(58%)	4(31%)	
Spleen size			
No splenomegaly	11	12	
Splenomegaly present in	1 (size 10.6 cm)	1 (size 12.7 cm)	
Thrombotic events			
Present (see text)	4	1	
Laboratory data			
Haemoglobin (g/dl)	10.8-15.5, mean 13 g/dl	9.4-15.3, mean 12.9 g/dl	.91
Leucocyte count $x10^3/\mu l$	7.1-48.2 median 16	5.5-18.9 Median 10	.09
Platelet count $x10^3/\mu l$	454 -1484 Median 777	594-2580 Median 1052	**.02**
Peripheral smear			
Abnormal	4	9	
Thrombocytosis on smear	2	8	
Giant platelets on smear	2	1	
Circulating blasts (%)	0	0	
Serum LDH			
Range IU/L	428.7-1129 (median 705.8)	319-1402 (median 581)	.17

Appendix 7: Comparison of JAK2V617 positive and JAK2WT ET - Histological features of bone marrow trephine

HISTOLOGICAL **FEATURE**			JAK2V617positive , n-12	JAK2 Wild type n-13	P value
Increased cellularity	Absent		03(25)	04(25)	1.0
	Present		09(75)	09(75)	
Granulocyte Precursors	Increased		03(25)	02(15.38)	.645
	Normal		09(75)	11(84.61)	
Myeloid left shift	Absent		12	13	
Erythroid cellularity	Normal		09	12	
	Increased		03(25%)	01(7.6%)	.322
Megakaryocyte location	Paratrabecular		5(41.67)	3(23.08)	0.411
	Perisinusoidal		12(100)	13(100)	
Type of Megakarocytes	Small	Absent	12(100)	13(100)	
		Present	12(100)	13(100)	
	Normal	Absent	1(8.33)	1(7.69)	.95
		Present	11(91.67)	12(92.31)	
Megakaryocyte nuclei					
Hypolobate cloud like megakaryocyte nuclei	Absent		2(16.67)	3(23.08)	.68
	Present		10(83.33)	10(76.92)	
	Present		12(100)	13(100)	
Hyperchromatic dysplastic nuclei	Absent		11(91.67)	12(92.31)	1.00
	Present		1(8.33)	1(7.69)	
Naked nuclei	Absent		10(83.33)	10(76.92)	1.00
	Present		2(16.67)	3(23.08)	
Megakaryocyte clusters					
Size of clusters	Small	Absent	0	0	
		Present	12(100)	13	
	Large	**Absent**	**5(41.67)**	**1(7.69)**	**.047**
		Present	**7(58.33)**	**12(92.31)**	
Density of clusters	Loose	Absent	0	0	
		Present	12(100)	13(100)	
	Dense	Absent	10(83.33)	12(92.30)	
		Present	2(16.67)	1(7.69)	.59
Sinusoids					
Sinusoidal ectasia	Absent		12(100)	13(100)	
Intra sinusoidal haematopoiesis	Absent		12	13	
Reticulin					
Grade	MF0		02(16.67)	03(23.08)	.198
	MF1		10(83.33)	10(76.92)	
	MF2 and MF3		-	-	
Reticulin score	0		12(100)	13(100	
Scoring					
CMC score			5-9 (median 8)	6-9 (median 8)	
Brosseau			1-4 (median 2)	2,1-4(median 2)	

Appendix 8

Comparison of clinical and laboratory data in JAK2 positive cases

		JAK2V617 positive ET, n=12	JAK2V617 positive Prefibrotic phase of PMF, n=13
Number		12	05
Male(number ,%)		05(41.67)	04(80)
Female(number , %)		07(58.33)	01(20)
Age (years, median)		40	56
Haemoglobin(g/l)- (mean , range)		12.85,10.8- 15.5	11.5,8-17
WBC count x10³/µl (median, range)		15.950,7.1-48.2	26,6.7-67.1
Platelet count x10³/µl (median , range)		777, 454-1484	521, 328-712
Circulating blasts, number, %, range		00	00
Peripheral blood films	Abnormal	00	02(40%),LEBP
	Normal	12(100)	03
Splenomegaly	Absent	11	02
	Present)	01(8.33)	03(60%)
	spleen size in cm (mean)	10.6 cms	15.45 cms
No of cases for which LDH values were present (number,%)		07,58.33	05, 100
(LDH IU/l) ((median, range)		705.8,428.7-1129	850,656-1231
Past arterial thrombosis, number (%)		00	00
Past venous thrombosis , number(%)		04(36)	00
Major bleeding /haemorrhage, number(%)		00	00

Appendix -9

Karyotype in PMF, Preffibrotic phase of PMF and ET			
	Karyotype done in	Abnormal Karyotypes in	Karyotypes
PMF	56	23	1)deletion 20q (6 patients) 2)deletion 13q(3 patients) and trisomy 8 (3 Patients). 3)Trisomy 9 and loss of chromosome 7q was seen in (2 patients) because of a partial deletion or an unbalanced t(1;7) which resulted in monosomy for 7q and trisomy for 1q. 4) Deletion 12p was seen in (one patient).
Prefibrotic phase of PMF	04	02	One had 47 chromosomes due to trisomy 9 and a partial deletion of the long (q) arm of chromosome 7 and the other showed an unusual t (4;11)(q25-q27; p13-15).
ET	06	Nil	46XX,46XY