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Value of Nuclear Factor- Erythroid2 Related Factor in Diagnosis of Myeloproliferative Neoplasms Subtypes

A thesis

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بِسْمِ اللَّـهِ الرَّحْمَـٰنِ الرَّحِيمِ

﴿ يَرْفَعِ اللَّهُ الَّذِينَ آمَنُوا مِنْكُمْ وَالَّذِينَ أُوتُوا الْعِلْمَ دَرَجَاتٍ ۚ وَاللَّهُ بِمَا تَعْمَلُونَ خَبِير﴾

صدق الله العليّ العظيم

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Certification of Supervision

We certify that this thesis entitled " **Value of NF-E2 in Diagnosis of Myeloproliferative Neoplasms Subtypes** " was prepared under our supervision at the ***College of Medicine / University of Babylon*** as a partial requirement for the master degree in Pathology.

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Dedication

This study is wholeheartedly dedicated to my beloved parents, who have been my source of inspiration and gave me strength when I thought of giving up, who continually provide their moral, spiritual, emotional, and financial support

To my brother, sisters, who shared their words of advice and encouragement to finish this study.

To my small family my husband, thanks for your support and patience and your love and believing in me and encouraging me ,my children thank you for giving me the energy to continue every time I look at you

To my friends and colleagues who became my supporter and help me in any problem I faced

And lastly, we dedicated this thesis to Allah , thank you for the guidance, strength, power of mind, protection and skills and for giving us a healthy life. All of these, I offer to you.

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Abstract

Background: The myeloproliferative neoplasms (MPNs) are clonal hematological disorders that are defined by an excess of differentiating hematopoietic cells in the chronic phase. The three main disorders that make up the Philadelphia-negative MPNs are polycythemia vera (PV), essential thrombocythemia (ET), and myelofibrosis (Mf). The precise differential diagnosis of certain MPN entities can be difficult, especially in the early stages of the diseases. Because useful additional tests are not yet available, pathologists have had to rely solely on histomorphological interpretation of bone marrow biopsies in conjunction with laboratory data. Even molecular testing, like as JAK2 mutation analyses, are ineffective in differentiating ET and PMF because both are associated with the V617F mutation in 50% of cases. Overexpression of the transcription factor NF-E2 in MPN was recently identified.

Objectives: this study was conducted to assess the value of NF-E2 in diagnosis of myeloproliferative neoplasms subtypes

Patients and methods: A retrospective cross sectional study was done in Pathology and Forensic Medicine department, collage of Medicine in Babylon University, the study sample consist of tissue blocks fixed in formalin, embedded in paraffin were collected from archived material from Baghdad medical city from 16 October 2022 to 20 February 2023. The paraffin blocks represent (70) cases including: (60) Myeloproliferative Neoplasms and (10) control

group.The myeloproliferative neoplasms include: (20) Myelofibrosis and (20)Polycythemia Vera ,(20) Essential Thrombocythemia with patients age ranging from 22 to 80 years old. which had been referred to and evaluated in department of pathology in Baghdad medical city between 2019 to 2022 was stained immunohistochemically for NF-E2 and analyzed regarding the subcellular localization of NF-E2 in erythroid progenitor cells.

Results: the percentage of NF-E2 is significantly higher in patients with myelofibrosis than in those with polycythemia vera an essential thrombocythemia an with very low levels in control group

Conclusion:. This study showed that NF-E2 immunohistochemistry can help to distinguish between myeloproliferative neoplasms subtypes even in early stages of the diseases. An MPN unclassified showing a proportion of more than 20% nuclear positive erythroblast can be classified as a primary myelofibrosis with 88.33% accuracy.

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List of Abbreviation

Abbreviation	Key
AML	Acute myeloid leukemia
ASXL1	Additional sex combs like1
ATP	Adenosine Triphosphate
AVWD	Acquired Von willbrand disease
BFU-E	Blood forming unit – erythroblast
BCR-ABL1	Brea kpoint cluster region_Abelson proto oncogene
b-TRCP	Beta transducing repeat containing protein
bZIP	Basic leucin zipper
CALR	Calreticulin
CNC	Cap n collar
CML	Chronic myeloid leukemia
CMPDs	Chronic myeloproliferative diseases
CXCL1	Chemokine ligand1
CXCR	CXC chemokine receptor
DAB	Diaminobenzidine
DI	Deionized water
DNMT3	DNA methyltransferase 3
ECM	Extracellular matrix
EECs	Endogenous erythrocyte colonies
EMH	Extramedullary hematopoiesis
EPO	Erythropoietin
EPOR	Erythropoietin receptors

ERK/MAPK	Extracellular signal-regulated kinase /mitogen – activated protein kinase
ERK	Extracellular signal regulated protein kinase
ET	Essential thrombocythemia
EZH2	Enhancer of zeste homolog2
FERM	Band 4.1 protein ezrin radixin moesin
FGF	Fibroblast Growth Factor
FGFR1	Fibroblast Growth Factor Receptor 1
FRA	Fos related antigen
GsK3b	Glycogen synthase –kinase 3b
HS-CRP	Highly sensitive C reactive protein
HCT	Hematocrit
Hgh	Hemoglobin
HIF	Hypoxia Inducible Factor
HSCs	Hematopoietic stem cell
ICD-O-3	International classification of diseases for oncology
IDH1	Isocitrate dehydrogenase_1
IDH2	Isocitrate dehydrogenase_2
JAK2	Janus kinase2
JAK-STAT	Janus kinase-signal transducer and activator of transcription
JH1	Jersey haplotype1
JNK	c-jun n-terminal kinase
Keap-1	Klech like ECH associated protein
LDH	Lactic dehydrogenase

MPNs	Myeloproliferative neoplasms
MPDs	Myeloproliferative diseases
MPN-U	Myeloproliferative neoplasms- unclassified
MPL	Myeloprolpiferative leukemia protein
Mpv	Masked Polycythemia Vera
M-TOR	Mammalian target of rapamycin
MEG	Megakaryocytes
MDS-F	Myelodysplastic syndrome with myelofibrosis
NF-E2	Nuclear Factor- Erythroid 2
Neh domain	Nuclear factor –ECH homology
PBS	Phosphate buffered saline
PV	Polycythemia Vera
PMF	Primary Myelofibrosis
PDGFRA	Platelet derived growth factor receptor alpha
PDGFRB	Platelet derived growth factor receptor beta
PI3K	Phosphotidylinositol3- Kinase
PI3K/AKT	Phosphotidylinositol3- Kinase /Protein Kinase (AKT)
PTXP3	Pentraxin 3 related protein
PARS-T	Protease activated receptor
RCM	Red cell mass
ROC	Reactive oxygen species
ROC curve	Receiver operating characteristics
RT	Room temperature
SRSF2	Serine/arginine-rich splicing factor
SF3B1	Splicing factor 3B subunit

SH2	Src homology 2
SP	Super plus
SCF	Skip 1-cul1 box protein
TBS	Tris buffered saline
TSPI	Thrombospondin 1
TET2	Ten _eleven translocation 2
TP53	Tumor protein 53
TGF	Transforming growth factor
V617F	Valine 617 phenylalanine
VHL	Von –hippel –landau
VWF	Von will brand factor
WHO	World health organization
ZRS2	Zinc finger RNA binding motif and serine /arginine rich 2

Chapter one

Introduction

Chapter one

Introduction

1.1. Introduction

Classical (Philadelphia-ve) MPNs are clonal hematopoietic disorders of stem cells defined by an excess in myeloid progenitor cells production, culminating in an excess of mature blood cells. The WHO categorizes various disorders as MPNs; nevertheless, (PV), essential (ET), myelofibrosis (PMF) are regarded as the main subgroup of Philadelphia –ve MPNs.(1).

These are presented with high red cell mass, a high platelet count, and bone marrow fibrosis in PV,ET.PMF respectively. These three diseases have a lot in common, such as hyper cellular BM, a a propensity to thrombosis and hemorrhage, and a chance of developing leukemia in long term. (2)

Furthermore, a prefibrotic form of myelofibrosis (pre-PMF) is becoming more recognized as a distinct MPN. Furthermore, there are patients have many characteristics of an MPN but they didn’t meet diagnostic criteria for PMF, ET, or PV, and they are classified as MPNs-unclassifiable (MPN-U). MPNs are frequently discussed together because they share pathobiologic and clinical characteristics. Furthermore, patients with ET and PV can develop myelofibrosis (MF), which is known as post-ET MF and post-PV MF, respectively.(3)

William Damashek, established first categorization of these conditions. which he initially referred to as myeloproliferative disorders, in 1951. (MPDs). . The MPDs, according to Damashek's proposals, were

erythroleukemia (Di Guglelmo syndrom), CML, PV, ET (megakaryocytic leukemia), PMF (idiopathic/ or agnogenic myeloid metaplasia).(4)

Chronic myeloproliferative disorders (CMPDs), which remained undefined as a separate diagnostic group, were important inclusions in Damashk's classification, which was adopted by the WHO Classification of Hematological Tumor in 1976.(5) MPNs were first identified as myeloproliferative disorders in 1951.(4) It wasn't until the third edition of the International Classification of Diseases for Oncology (ICD-O-3) upgraded MPNs' status from uncertain / borderline to malignant in 2000 that the malignant character of MPNs was routinely acknowledged..(6)

Since 2005, clonal abnormalities in classic MPNs have been discovered, defining the natures of the acquired clone. Which found in ninety five percent of PV patients and approximately fifty percent of ET and PMF patients.(7)

In many of those who did not have a JAK2 mutation, (MPL) mutations and then calreticulin (CALR) mutations were discovered(8)(9).There are still a small number of people who have no mutation and are referred to as triple negative.(10) .These disorders primarily affect adults and the elderly, having a yearly incidence of 1-5 cases/100,000 (11).

Males are afflicted more frequently than females., although each entity's epidemiology is unique. The primary lineage of differentiation, the molecular environment, and illness stage all influence clinical and laboratory features of MPNs...Those who are not treated have a lower life expectancy than the normal population due to the increased risk of hemorrhage and

thrombosis, failure of the bone marrow, organ damages, and blast transformation as the disease progresses.(12). The following are some of the clinical features of MPN-Unclassified at presentation: Early stage MPN-Unclassified patients are more likely to have high number of blood cell (thrombocytosis, leukocytosis, and/or increse Hb concentrations), but advanced stages are typically associated with cytopenia with splenomegaly and hepatomegaly. It can be challenging to make a diagnosis because it calls for screening other illnesses that are similar, like infections and toxins or drug exposure .Identifying MPN driver gene mutation, like (MPL or JAK2) or myeloid neoplasms that had gene relation (EZH2 , IDH1 , IDH2 , SRSF2 , and SF3B1) in this context provides evidence of hematopoiesis clonal nature and enables the separation of reactive situations.(13) (14). Although discovery of a JAK2V617F activating point mutation in majority of MPN patients(15)(16), Differentiating ET from PMF can be difficult(17).)ET and PMF can be similar clinically and by histopathological appearance , especially in the early stages, a problem can cause difficulty in recognizing the criteria for diagnosis, that including WHO classification (18) (19). Additionally, the WHO categorization has come under fire for relying on histology, which may be highly variable between observers (18)(20). However, as ET and PMF have markedly different clinical outcomes, it is crucial to correctly classify and diagnose these entities (19).

Goerttler et al(21) established that the overexpression of (NF-E2), that regulates maturationof both erythroid and megakaryocytic cells , is common in PV. A recent study from the above group outlined the possible diagnostic value of immunostaining for NF-E2 in distinguishing MPN subtypes using BM biopsy (22)

1.2.Aim of Study

1.Differentiation between subtypes of MPNs by nuclear factor erythroid2(NF-E2) immunohistochemistry staining of erythroblasts

2.Diagnosis of primary myelofibrosis in pre fibrotic stage.

Chapter two

Review of literature

Chapter Two

Literature Review

2.1.Myeloproliferative Neoplasms(MPNs) Definition:

Philadelphia-negative myeloproliferative neoplasms (MPNs) are clonal hematopoietic neoplasms that are distinguished by a hyper activation of signal transduction pathways including JAK-STAT.(23) (24), This causes a pro-inflammatory condition and an excess of myeloid blood cells to be produced.(23) (24)

2.2.MPNs History:

MPNs have a long history, related to 19th century, when severe leukocytosis and/or erythrocytosis had been reported that drew attention to a group of blood disorders that presented with markedly high hematopoiesis(25). CML was the first MPN to be described in detail by John Bennett.(26) It wasn't until 1879 when another one Gustav, who described 2 patients with enlarge spleen, leukoerythroblastosis, and bone marrow fibrosis, distinguishing it from Chronic myeloid leukemia.(27)PV was described in 1882 by Vaquez, who described fourty year-old male with vascular congestion and erythrocytosis. Heart abnormalities didn’t presented during the autopsy, but he did notice enlarged liver and spleen. Vaquez concluded this abnormality caused by hyperactivity hematopoietic.(28).ET was the last formally described MPN. Two Austrian pathologists, described sever thrombocytosis in patient ("more than three times normal values") with hyperplasia megakaryocytic. He experienced recurring muco cutaneous bleeding(29) . Damashek proposed the first classification of

conditions, that initially referred to as myeloproliferative disorders, in 1951. (MPDs). Previous studies had revealed that there was differences in the prognosis and biology of MPDs over the years, leading to the suggestion that these entities be divided into 2 groups: myeloleukemia syndromes presented with single lineage hematopoiesis and a high blast transformation proclivity; and myeloproliferative syndromes, which have slower clinical course, , high rates of fibrotic evolution and non-destructive hematopoiesis (30). Depend on questionable assumptions, this distinction was latterly classified as an acute leukemia/myelodysplastic syndrome.(31), and chronic myeloid leukemia revealed molecular and clinical characteristics that distinguished them from all other MPNs(32).

These MPDs were classified as chronic myeloproliferative disease under the more general heading of myeloid malignancies in the 2001 World Health Organization (WHO) classification of myeloid malignancies (CMPD). The name "CMPD" was changed to "myeloproliferative neoplasm" in the new 2008 WHO classification system (MPN)(33) Mastocytoses were included in the MPN spectrum, on other hand myeloid or lymphoid neoplasms with rearrangements of PDGFRA, PDGFRB, and FGFR1 separately considered (34), WHO Classification at 2016 preserved this matter, with the exclusion of Mastocytoses, which considered separated part due to their distinct features(23).

2.3.MPNs Subtypes:

Classical MPNs have been subdivided into three entities: **Polycythemia Vera(PV), Essential Thrombocythemia (ET),and Primary Myelofibrosis(PMF),** which frequently have disease-related complications like venous and arterial thrombosis, hemorrhages, and transformation to acute myeloid leukemia (AML).

2.3.1.Polycythemia Vera:

Polycythemia vera is a stem cell disorder characterized by myeloid lineage hyperproliferation and the presence of the activating JAK2 mutation(35)**.** Pathogenic changes in MPNs are thought to originate in MPN stem cells, which give rise to diseased clonal progeny(35). The most common feature of Polycythemia Vera is the total red blood cell mass increase, though increases the neutrophil number and /or platelet number (36). Polycythemia Vera was described for the first time in 1892. by French physician Louis Henri Vaquez (1860-1936)(37) . He described a patient who had polycythemia (elevated hemoglobin) but no cardiac or pulmonary diseases. Louis Henry Vasquez was the first who suggested categorizing increased red blood cell counts in to two categories : (38):

- Absolute erythrocytosis (increased red blood cell mass).
- Relative erythrocytosis [a condition characterized by low plasma volume without increasing red cell mass].

Olser (39) supported idea about Polycythemia Vera. Osler discovered individuals with red blood cell increase had a specific clinical condition in his clinical practice.(38). Another physician, observed Polycythemia

Vera caused an increase in all hematopoietic lineages, that include WBC and platelets count. According to that finding, Polycythemia Vera not limited to erythroid lineage. The initial suggestion , Polycythemia Vera was a hematopoietic stem cell disease, due to that cell gives rise to all lineages.(38). Myeloproliferative neoplasms were defined by William Dameshek in 1953 considered disorder that included Polycythemia Vera, Essential Thrombocythemia, and Myelofibrosis, and considered the Polycythemia Vera as bone marrow proliferative activity produced by no known trigger. (4). Polycythemia Vera Study Group created official criteria for diagnosis, that emphasized importance of therapeutic phlebotomy, and advocated for using of hydroxyurea as a therapeutic choice from 1967 to 1997. It was hypothesized that hydroxyurea inhibited ribonucleotide reductase, that essential for the proliferation and cellular division.(39). During 2005, a study that showed JAK2 V617F mutation in Polycythemia Vera. A gene that encodes the Janus kinase 2 protein, and a point mutation that converts valine to phenylalanine directly. (40) (41). In 2008, (WHO) had classify system for myeloproliferative neoplasms, which includes polycythemia vera. In this classification, the JAK2 V617F mutation (or JAK2 exon 12 mutations) was included as a significant criterion required for diagnosis. The hemoglobin threshold for Polycythemia Vera in men was 18.5 g/dl and 16.5 g/dl in women. Because hemoglobin is the primary protein in red blood cells, these hemoglobin levels were thought to be good surrogates for an increase in red blood cell mass.(40). In 2016, the World Health Organization changed the Polycythemia Vera classification scheme and diagnostic criteria. A bone marrow biopsy demonstrating

hypercellularity in all three cell lines (red blood cells, white blood cells, and platelets) as well as the presence of a JAK2 mutation (either V617F in exon 14 or a mutation in exon 12) is now required, as is hemoglobin levels greater than 16.5 g/dl in men and 16 g/dl in women. These are the most important criteria. The minor requirement is a low erythropoietin level.(23).

2.3.1.1.Epidmiology:

PV has an estimated incidence of 2.3-2.8 per 100,000 people per year, with a median age at diagnosis of around 60 years and a male/female ratio of 1.2:1(42),and prevalence is 1/3300 people, considering the long life expectancy(43). According to some publications, the risk of myeloid diseases is higher in young females, whereas it is overexpressed in males at older ages. (45) (46)

The incidence rate increased significantly in patients over the age of 50, and even more so in those over the age of 75. (47) PV risk was lower among women in all age groups.(44)The median survival time is 14.1 years, with thrombotic complications or progression to leukemia or myelofibrosis being the leading causes of death.(45)Older age, male sex, White race, and European descent are non-modifiable risk factors for increased morbidity in PV(46) (47) (48),while Smoking, obesity, hypertension, diabetes, and hyperlipidemia are all modifiable risk factors for morbidity in PV(49). In Japan, the reported incidence of PV ranges from 0.2 cases per million 53 , to approximately 20 cases per million in Minnesota.(54) Disparity in incidence of illness ,explained by distinct proclivity for PV between different racial and ethnic groups, with Ashkenazi Jews in the United States and Israel

having a fourfold rise (50). African Americans less affected than rest of US population. (51) PV has been linked to ionizing radiation exposure, with survivors of the Hiroshima atomic bomb detonation having a 20-fold higher incidence than general population, and US military personnel involved in an atomic device detonation having a 20-fold higher incidence than the general population. A group of PV cases was recently identified at Tamaqua, Pennsylvania, on the site of a prior toxic waste dump, suggesting that environmental exposure may possibly play a role in PV propensity. Although the exact nature of the chemical exposure was unknown, the incidence of PV among local inhabitants was 4.5 times higher than in neighboring country.(52)

2.3.1.2 Pathogenesis of Polycythemia Vera:

Clonality

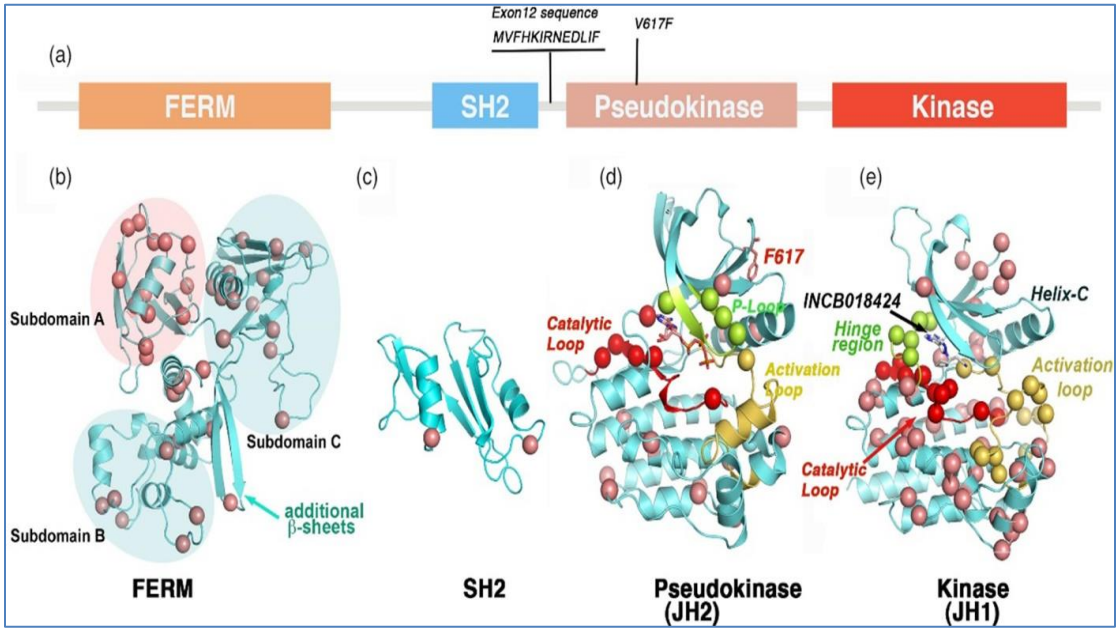
Hematopoiesis in polycythemia vera is abnormal at the multipotent progenitor or stem cell level due to increased production and turnover of erythrocytes, neutrophils, and platelets, together with hypercellular bone marrow. A previous study of 2 female PV patients who were heterozygous for X-linked G6PD provided evidence to support this hypothesis .The skin fibroblasts and lymphocytes in these patients had A-type and B-type glucose-6-phosphate dehydrogenase isozymes, as expected. However, red blood cells, granulocytes, and platelets contained only one isozyme (type A), indicating possible clonal origin of that disorder at the pluripotent hematopoietic progenitor level. Another patient, the majority of B lymphocytes belonged to same disease clone, indicating involvement of an earlier hematopoietic progenitor with lymphoid, myeloid, erythroid, and

megakaryocytic differentiation potential(53).

Molecular Genetic Features

Myeloproliferative disorders patients more often have a gain-of-function mutation on chromosome 9, where valine at position 617 of the Janus kinase 2 gene is replaced by phenylalanine (JAK2 V617F),(54) which had a substantial effect on the approach to PV diagnosis and investigations in it's pathogenesis, This mutation causes tyrosine kinase signaling via the JAK/STAT pathways to be constitutively activated. JAK2 V617F is found that about 95% of PV patients at diagnosis time.(55) However, because a significant proportion of patients with essential thrombocytosis and myelofibrosis also have the JAK2 V617F mutation, this mutation is not pathognomonic for PV. The significant proportion of PV patients who do not have the JAK2 V617F mutation have other mutations in JAK2 exon 12.(56). Mutations of Exon 14 V617F have been linked to an increase in erythrocyte, platelet, and granulocyte cell counts, whereas exon 12 mutations are linked to an increase in solitary erythrocytosis. (57). Myeloid cell proliferation is controlled by an orderly mechanism involving a JAK2/STAT signaling pathway. JAK2 gene codes for tyrosine kinase JAK2, that phosphorylates STAT proteins, causing these to homodimerize and translocate into cells' nucleus These homodimers' translocation upregulates or repress the transcription of target genes that control cellular proliferation.(58) Exon 14 or 12 mutations and JAK2 mutation, resulting in activated dysregulation of the JAK2/STAT pathway and uncontrolled cellular proliferation. JAK2 kinase has several structural (fig.2.1) domains, with special emphasis on JH2 pseudokinase domain and nearby JH1 active catalytic site for phosphorylation of STAT transcription factors that promote

cellular proliferation.(59) Mutation of JAK2 V617F exon 14 point and the various exon 12 mutations in or near the pseudokinase JH2 domain cause abnormal domain to domain protein expression. Mutation of JAK2 V617F, molecular modeling predicts that replacing amino acid valine with phenylalanine at codon 617 will destabilize the pseudokinase domain JH2 (60) The structural destabilization of the pseudokinase domain is thought to favor gain-up (constitutive activation) of the neighboring JH1 catalytic domain.(41) As reported by several investigators, gain in function linked to high disease activity characterized by pruritus, erythrocytosis, need for treatment by cytoreductive therapy, and the propensity to fibrosis.(61)



(Fig. 2.1.) Dual Pseudo kinase JH2 and JH1 domains wherein an errant 617F phenylalanine substitution shown in JH2 causes a gain-up of activation of the contiguous ATP/catalytic site. (59)

Erythropoiesis in PV is autonomous and does not require erythropoietin (EPO). PV patients have lower plasma levels of this hormone, and unlike normal ones, PV progenitor cells can survive in vitro and give rise to erythroid colonies (BFU-E) in the absence of added erythropoietin (endogenous erythroid colonies, EECs). PV erythroid progenitors exhibit increased sensitivity to EPO, as well as insulin-like growth factor-1, thrombopoietin, interleukin-3, and granulocyte/monocyte colony-stimulating factor. erythropoietin receptor (EPOR) mutation are well-known to present in inherited polycythaemia, but they are not found in patients with PV .(36)

The thrombopoietin receptor (Mpl) has been found to be reduced or not present on the platelets of patients with PV and myelofibrosis people. The abnormality in Mpl expression and function is both a marker for PV and a potential pathophysiologic contributor by suppressing apoptosis. The utility of Mpl as a PV diagnostic marker has been called into question because its expression in PV may overlap with the level of expression in healthy people.(53)

2.3.1.3.Clinical features:

Patients with PV may be diagnosed by chance if they exhibit nonspecific symptoms such as mild hypertension, fatigue, intense itching, and headache. Symptoms of hyper viscosity, with increased red cell mass (RCM) as well as thrombocytosis, may lead a clinician to suspect PV. Other symptoms include arterial and venous thrombosis with cardiovascular events, Budd-Chiari syndrome or mesenteric ischemia, bleeding complications ranging from nonspecific ecchymosis to major hemorrhage, hypertension, headache and dizziness, visual disturbances, vertigo, tinnitus,

claudication, and Erythromelalgia .(62) Some patients may apply as a result of incidentally discovered elevated blood cell counts during medical evaluations for other reasons. Splenomegaly, hepatomegaly, plethora, are the most common physical findings.(63)

Pruritus, particularly after hot baths ,are reported in about 70% patients and be related to the degranulation of increased mast cells in the skin of PV patients, releasing the histamine and the inflammatory mediators. Aqua genic pruritus has been identified as a pathognomonic sign of Polycythemia Vera (PV). PV-associated pruritus was first reported in 1985, and its estimated prevalence ranges from 31% to 69% of patients. Particularly, in nearly half of cases, the onset of aqua genic pruritus precedes the diagnosis of PV. Aqua genic pruritus has substantial impact on patients' quality of life .Aqua genic pruritus cause sleep disturbances and can result in the development of a psychological phobia and the cessation of bathing. Furthermore, the majority of patients think that pruritus is the most difficult aspect of PV, which can lead to a reduced physical and social life in more severe cases(64).

Thrombotic events occur in approximately 25% of patients before or at the time of diagnosis, with roughly two-thirds being of arterial origin and the remaining third being of venous origin. There is still a thrombotic risk after diagnosis, with approximately 20% of patients who are experiencing an event during the course of their disease. Aside from major thrombotic events, patients can experience micro vascular circulatory disturbances, which are seen in about one-third of patients at presentation.(65) Venous thrombosis manifest themselves as deep vein thrombosis in the extremities, pulmonary emboli, and venous thrombosis in unusual locations such as the

splanchnic or sinus sagittalis superior vein thrombosis. Acute coronary syndrome, stroke, cerebrovascular arterial thrombosis, and acute myocardial infarction were among the most common arterial events in patients with PV, while deep vein thrombosis, splanchnic vein thrombosis, pulmonary embolism, and superficial venous thrombosis were some of the most common venous events, according to recent observational studies. (66)(67) The mechanisms underlying thrombosis pathogenesis are complex, involving numerous cell types, prothrombotic factors, and inflammatory markers. Bar-Natan and Hoffman's January 2019 paper describes a pro-inflammatory MPN milieu - a delicate effect on vessel endothelium, cell adhesion molecules (including integrins and selectins), platelet/red blood cell interactions, and the presence of mutations JAK2 V617F.(68) Old age, more duration of disease, history of thrombosis, erythrocytosis, leukocytosis, and JAK2 V617F allele burden are considered as PV-associated thrombosis risk factors .(Table 2.1)(69)

Table 2.1 Unique Risk Factors For Thrombotic Complications(69)

Risk Factors	Comments
Gender Differences	Women: ● ↓ age at diagnosis ● ↑ rates of AVT*
Mutational Status	● ↑ JAK2 V617F allele burden
Microparticles	MPs associated with: ● ↓ thrombin inhibition ● ↑ CD41 ● ↑ splenomegaly
Inflammatory Markers	Role of Pentraxins: ● ↑ hs-CRP ● ↓ PTX3
Neutrophil Extracellular Trap	Result in NETosis and apoptosis

Patients with PV develop progressive splenomegaly over time. Splenomegaly in MPN is frequently caused by extramedullary hematopoiesis caused by dysfunction of bone marrow and occur in fibrotic disease patients.(70)

15% to 20% of PV patients progress to a spent phase of the disease, similar to primary myelofibrosis. Furthermore, approximately 5% to 10% of patients develop PV at a faster rate. This accelerated phase is clinically similar to acute myeloid leukemia (AML) and may or may not be preceded by an MF phase. Transformation is thought to occur as a result of genetic instability, which leads to the acquisition of new mutations. The JAK2 V617F or JAK2 exon 12 mutations are not more common in transformed

MPN—in fact, AML clones frequently lack the JAK2 mutation. Mutations in other genes, such as TET2, ASXL1, EZH2, and DNMT3, are found in PV patients at 5% to 15% frequency, but none of these have been shown to have transforming potential in PV patients.(71)

2.3.1.4 Diagnosis:

Stage 1 Investigations

Full Blood Count

The full blood count analysis will not only confirm a raised Hct but will also detect neutrophilia and thrombocytosis, that are common in JAK2 V617F-positive PV and part of the JAK2-negative PV criteria. Because smokers have a significantly higher neutrophil count than nonsmokers, neutrophilia is defined as a neutrophil count of more than 12.5x 10^9/l. All patients should have a blood film reviewed to look for any unusual features. Abnormalities such as circulating blasts and leucoerythroblastic features and monocytosis would be indicators for bone marrow evaluation. have been observed in those with confirmed PV.(72)

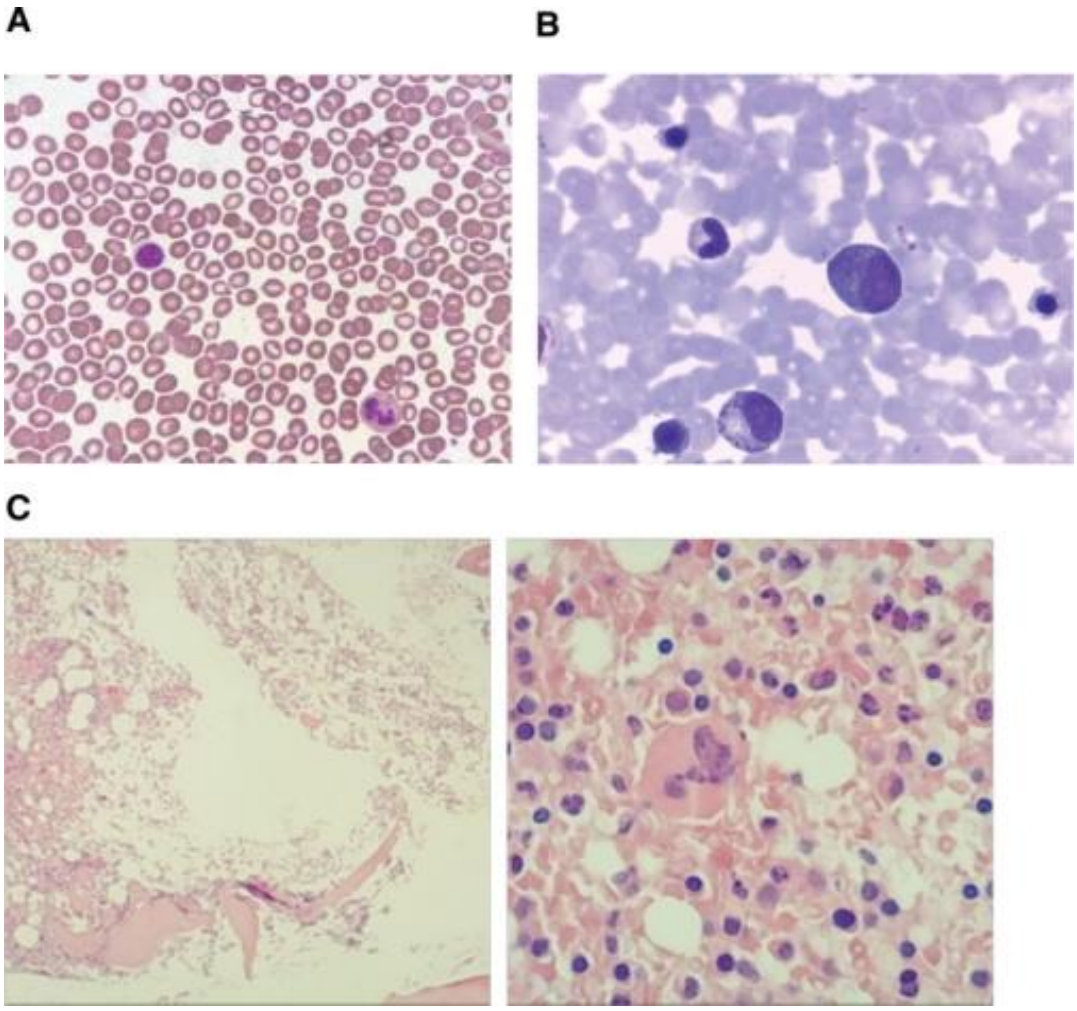
Blood film:

The results of peripheral blood tests vary depending on the stage of disease at the time of diagnosis. Peripheral blood usually has an excess of normochromic, normocytic red blood cells during the prepolycythemic and overtpolycythemic stages. If there is an iron deficiency, hypochromic, microcytic red cells may be present. Thrombocytosis is common, and approximately 15% of cases may be mistaken for essential thrombocytosis. Leukocytosis can occur even when there is no fever or infection. However,

because neutrophils, not lymphocytes or monocytes, are increased in PV, the total WBC count may not accurately reflect disease activity. While immature cells can be seen, blasts are not a common occurrence. In contrast, in the postpolycythemic myelofibrosis stage, a leukoerythroblastic picture develops with teardrop-shaped red blood cells (dacryocytes), poikilocytosis, and neutrophilia.(73)

Bone marrow examination:

A bone marrow examination is not commonly used. Its usefulness is largely limited to cases where the clinical suspicion of polycythemia vera is high, despite the absence of a JAK2 (V617F) mutation, or when testing facilities for the mutation are unavailable. When combined with other suggestive hematologic parameters, classic findings can help support a diagnosis of polycythemia vera. A hypercellular marrow with erythroid hyperplasia and subtle megakaryocytic atypia are both highly suggestive findings.Tri-lineage hyperproliferation is also anticipated.(73)



(Fig 2.2) Morphologic features in peripheral blood, bone marrow aspirate and bone marrow biopsy at the time of diagnosis with PV. (A) Morphologic review of the peripheral blood smear (B) The bone marrow aspirate at the time of diagnosis with PV showed hypercellular bone marrow with a G/E ratio of 1.22:1. Erythroid hyperplasia was prominent, mainly in polychromatophilic and late erythroblasts. (C) Bone marrow biopsy at the time of diagnosis with PV revealed prominent hyperplasia (approximately 70%) with grade 1 reticular fibers(74)

Stage2 Dignostic criteria:

Recently, some researchers have proposed revising the World Health Organization (WHO) criteria, particularly in light of the discovery of masked PV (mPV) in a subset of PV patients. Patients with mPV have normal or borderline haemoglobin (Hgb) and haematocrit (Hct) values, but they are usually positive for JAK2 mutations, have bone marrow features consistent with PV, and have low serum erythropoietin levels. According to Barbui, a revision to the current WHO diagnostic criteria emphasizing a lower Hgb threshold and/or the use of Hct threshold values could aid in accurately diagnosing those with mPV and allow for appropriate and prompt treatment of these patients. The WHO criteria from 2008 were modified based on their proposals.(75)

(Table 2 .2)world health organization (WHO) diagnostic criteria for polycythemia Vera(76)

	2008 WHO diagnostic criteria[a]	**2016 WHO Diagnostic criteria[b]**
Major Criteria	1) Hgb >18.5 gm/dL (male) and>16.5 gm/dL (women) 2)Presence of JAK2V617F or JAK2 exon 12 mutation	1) Hgb >16.5 gm/dL (male)and >16.0gm/dL (female) OR HCT>49% (male) and HCT >48% (female)OR increased red cell mass(RCM)* 2) B0ne marrow hypercellular for age with trilineage myeloproliferation 3) Presence of JAK2V617F or JAK2 exon 12 mutation
Minor Criteria	1) Bone marrow hypercellular for age and trilineage myelo-proliferation 2) Endogenous erythroid colony formation in vitro 3) Subnormal serum EPO Level.	1) Subnormal serum erythropoietin level

a. PV diagnosis requires meeting BOTH major criteria and 1 minor criterion OR first major criterion and 2 minor criteria
b. PV diagnosis requires meeting ALL 3 major criteria, OR first 2 major criteria and the minor criteria

***-More than 25% above mean predicted value.**

According to the 5th edition of WHO diagnostic criteria of haematolymphoid tumours there is a minor change in diagnostic criteria of PV :

PV diagnostic criteria is defined by increase the haemoglobin concentration and/or haematocrit, trilineage hyperplasia ,pleomorphic mature megakaryocytes in the bone marrow, and NM 004972: JAK2 p.V617F or JAK2 exon 12 mutations. Because determining high red cell mass with 51Cr-labeled red cells has become not common in routine practice, it has been excluded from diagnostic criteria.(77)

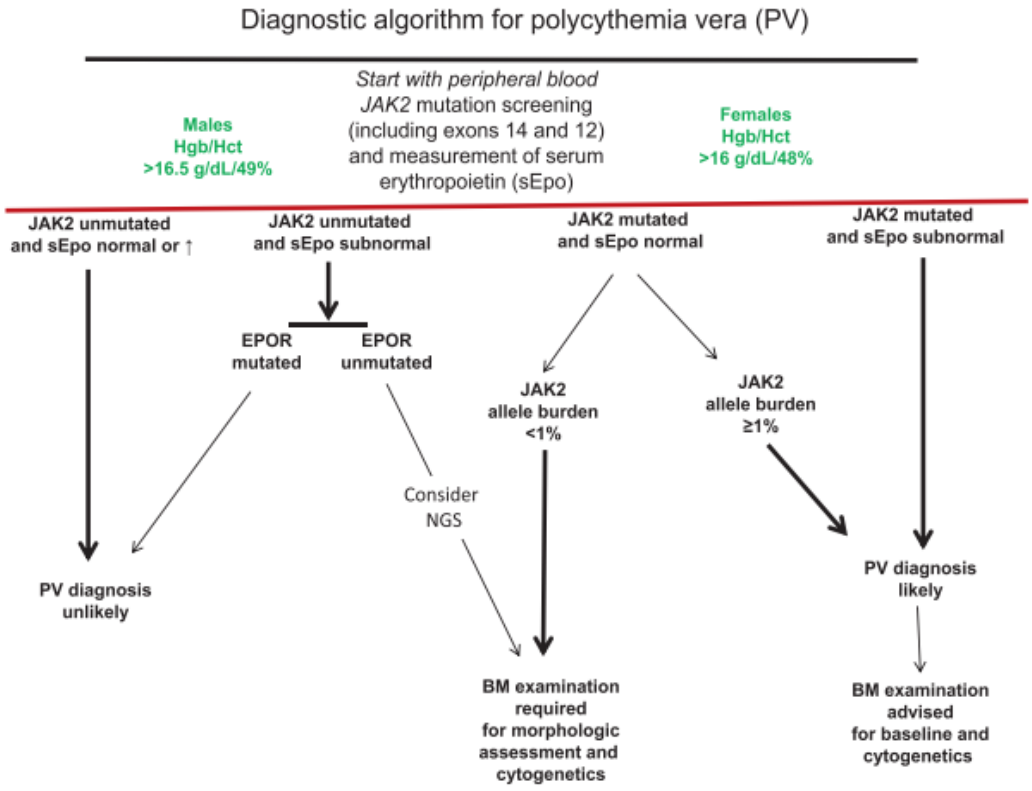


Fig. 2.3. : Current diagnostic algorithm for polycythemia vera(78)

Table (2.3.) The BCSH guidelines recommend the following diagnostic criteria for Polycythemia Vera.(79)

JAK2-positive PV
A1- High PCV (>0.52 in men, >0.48 in women) OR
increased red cell mass (>25% above predicted)∗
A2- JAK2 Mutation
Diagnosis requires both A1 AND A2 to be present
JAK2-negative PV
A1- increased red cell mass (>25% above predicted) OR
PCV >0.60 in men, >0.56 in women.
A2- Absence of JAK2 mutation
A3- No cause of secondary erythrocytosis*
A4- Palpable splenomegaly
A5- Presence of an acquired genetic abnormality (excluding
BCR–ABL) in the hematopoietic cells
B1 -Thrombocytosis (platelet count >450 × 10^9/L)
B2 -Neutrophil leukocytosis (neutrophil count >10 × 10^9/L
in non-smokers and >12.5 × 10^9/L in smokers)
B3- Splenomegaly by abdominal ultrasound
B4 -Endogenous erythroid colonies or low serum
erythropoietin
Diagnosis requires A1 + A2 + A3 + either another A or two
B criteria

2.3.1.5 Causes of Secondary Erythrocytosis: (36)

- **Congenital**
  - Abnormal Hb with increased oxygen affinity
  - Reduced 2,3-bisphosphoglycerate
  - von Hippel–Lindau (VHL) gene Mutation
  - proline dehydroxylase genes Mutations
  - hypoxia inducible factor (HIF) genes Mutations
- **Acquired (increased erythropoietin level):**
  - Conditions that cause low oxygen levels (high altitude, chronic lung disease, some congenital heart diseases)
  - Renal diseases – tumours; (hyper-nephroma), cysts (mostly benign), hydronephrosis, after kidney transplantation
  - Liver diseases (hepatoma, cirrhosis, hepatitis)
  - Tumours (bronchial cancer, uterine fibroid, cerebellar haemangiomata)
  - Disease of endocrine (Cushing's syndrome, Phaeochromocytoma)
  - Medications (erythropoietin and androgens)
- **Idiopathic (undefined primary or secondary)**
- Resolve or pathology may be masked initially.

2.3.2. Essential Thrombocythemia

2.3.2.1.Overview

ET is an uncommon but dangerous myeloproliferative neoplasm with thrombocytosis, bone marrow megakaryocytic hyperplasia, and predisposition to thrombotic or hemorrhagic consequences. Essential thrombocytosis (primary thrombocythemia) is a chronic, non-reactive myeloproliferative neoplasm that primarily affects men and women aged 50 to 60. Megakaryocyte proliferation causes a persistent rise in the quantity of circulating platelets. Approximately 90% of patients with essential thrombocytosis have Janus kinase 2 (JAK2), calreticulin (CALR), or myeloproliferative leukemia (MPL) mutations.(80) Most ET patients have a normal life expectancy, and progression of disease into fibrotic or leukemic transformation is uncommon.(81)Recently, researchers collaborated to develop an integrated clinical and genetic survival risk model for ET.(82) The novel mutation-enhanced international prognostic model for ET (MIPSS-ET) identified independent risk factors for overall survival as being age >60 years, male sex, leukocytosis (11x 10^9/L), and SF3B1/SRSF2 mutations (occurring in 10% of patients). Drug therapy is aimed at reducing the risk of thrombosis rather than improving survival in ET, as of yet. 4 risk factors are recognized in this regard.(83).Table 2.4

Table (2.4)thrombotic risk factors in Essential Thrombocythemia(83).

Risk categories	Criteria
very low risk	age 60 years, absent history of thrombosis, JAK2 wild type
low risk	age 60 years, absent history of thrombosis, JAK2 mutated
intermediate risk	age > 60 years, absent history of thrombosis, JAK2 wild type
greater risk	age > 60 years or thrombosis with JAK2 mutation

2.3.2.2.Epidemiology

Annual incidence of ET in the United States is 2.5 cases per 100 000 with a prevalence of 24 cases per 100 000. Another studies estimated annual ET incidence in Western countries to be between 0.2 and 2.5 cases per 100,000 people, with a prevalence of 38 to 57 cases per 100,000 people. Furthermore,the incidence is higher in female patients than in male patients , with a roughly 2:1 ratio. The median age at diagnosis is 60 years, with 20% of patients being under the age of 41. According to some studies conducted in recent years, the average age of ET diagnosis has decreased: 56 years as opposed to the previous data of 60 years(83) ET has an overall favorable prognosis when compared to the other MPNs (though ET has a shorter life expectancy than the general population), with an expected survival of 18 to 19.8 years (compared to 13.5 years in PV and 5.9 years in PMF). Survival appears to be significantly better in patients at low risk of thrombosis (26.7

years)(84)

2.3.2.3. Pathogenesis

Over the last 15 years, advances in molecular biology have created a wealth of knowledge that has improved the ability to diagnose MPNs. JAK2 (janus activated kinase 2) is a gene on chromosome 9, position p24. The V617F JAK2 mutation was first reported in 2005 and is the most common mutation in ET, with an estimated frequency of 50% to 60%.(85) Furthermore, additional driver mutations in the MPL and CALR (calreticulin)genes were identified in 2006 and 2013, respectively The MPL gene, also known as the TPO receptor, is found on chromosome 1p34. About 5% of ET patients have mutations in the MPL gene.(86) Finally, the CALR gene is found on chromosome 19p13.2. CALR mutations, according to some authors, are linked to production platelet and found in patients with thrombocytosis other than ET, such as PMF or refractory anemia with ring sideroblasts and marked thrombocytosis.(86) The most common CALR mutations are a 52 bp deletion (type 1) ,and a 5 bp insertion (type 2)(87) Absence of them does not rule out ET diagnosis because 20% of ET patients are triple negative. Germ line JAK2 mutation (JAK2V617I) has also been linked to hereditary thrombocytosis with vascular events but not fibrotic/leukemic progression. JAK2 mutations patients are typically older, high hemoglobin level or platelet count, and have aberrant serum erythropoietin levels. Thrombosis more likely to occur .(88)

2.3.2.4.Clinical features

Patients present with persistent and progressive thrombocytosis, which is frequently associated with no symptoms. Mild splenomegaly and

leukocytosis affect a variable proportion of patients.(89) Symptoms and signs of disease are generally classified as life-threatening or not at the time of presentation as well as during the clinical course of the disease. Non-life-threatening events occur in 30%-50% of patients and include vasomotor symp- toms (headache, , lightheadedness and visual symptoms, atypical chest pain, erythromelalgia, acral dysesthesia, and increased risk of first trimester miscarriages while bleeding ,thrombosis, , and disease transformation into AML or post-ET myelofibrosis are all life threatening complications of ET.(90)

Thrombosis are frequent complication of ET and can occur in both arterial and venous vascular beds, lead to transient ischemic attack, deep vein thrombosis/pulmonary embolism, hepatic, portal, splenic, and coronary artery ischemia. AML and/or post-ET myelofibrosis transformation are uncommon but serious reasons of death in ET.(91)On other hand, bleeding events risks in ET is less common, ranging between 5% to 30% . The commonest sites are GIT and urogenital and intracranial bleeding can occur as well.(92) Extreme thrombocytosis (platelet count > 1000-1500x10^9/L) is the highest factor for bleeding as it can be associated with acquired von Willebrand disease (AVWD). This occur due to adsorption of large VW factor multimers by the platelet membrane in the setting of high platelet counts . Measuring the ristocetin activity and VW factor antigen level in cases of severe thrombocytosis, especially if bleeding has occurred. Antiplatelet therapy should be stopped when VWF activity levels are 30% or more.(93)

2.3.2.5.Diagnosis

WHO 2016 criteria for diagnosis of ET used, which include combination of laboratory data and morphological features along with finding of molecular-genetic(23). Recent WHO guidelines outline 4 diagnosing criteria for ET, as did the previous two editions (in 2001 and 2008): high platelet count (>450x10^9/L), characteristic bone marrow (BM) histology finding, no defining features of any other MPNs or myeloid neoplasms, and presence of characteristic clonal genetic information. Aside from CALR, the 2016 WHO criteria place a greater emphasis on BM histology/morphology, particularly in distinguishing 'true' ET from prefibrotic/early PMF (prePMF). These entities are distinguished by histologic features, with prePMF exhibiting atypical megakaryocytic morphology and grade 1 reticulin fibrosis (or less), whereas true ET lacks these characteristics.(23)

To be diagnosed with ET, you must meet all four major criteria, or at least the first three, which include A positive JAK2, CALR, or MPL mutation, thrombocytosis, big megakaryocytes in bone marrow, Philadelphia negative MPN, or JAK2 positive, CALR, or MPL mutation. The minor criterion shows the presence of a clonal marker or the absence of reactive thrombocytosis.**(Table2.5):**(88)

Table2. 5: Essential throbocythemia(2016) WHO diagnostic criteria:

Major criteria
1. Platelet count ≥450 × 109/L **2.** Bone marrow biopsy showing proliferation mainly of the megakaryocyte lineage with increased numbers of enlarged, mature megakaryocytes with hyper-lobulated nuclei. No significant increase or left shift in neutrophil granulopoiesis or erythropoiesis and very rarely minor (grade 1) increase in reticulin fibers. **3.** Not meeting WHO criteria for BCR-ABL1+ CML, PV, PMF, myelodysplastic syndromes, or other myeloid neoplasms. **4.** Presence of JAK2, CALR, or MPL mutation
Minor criteria
Presence of a clonal marker or absence of evidence for reactive thrombocytosis*.

Diagnosis requires all major criteria or the first three major criteria plus a minor criterion

Criteria for ET diagnosis are wellestablished and have not changed

causes of reactive thrombocytosis:(94)

- Bacterial infections and tuberculosis
- Inflammatory diseases
- Hemolytic anemias and acute blood loss

- Advanced malignancies
- Asplenia (congenital or functional) and post splenectomy
- Rebound after chemotherapy-induced thrombocytopenia
- Iron deficiency
- Rebound after vitamin B12 repletion in pernicious anemia

Blood and Marrow Findings:

Platelet counts can range from slightly higher than normal to several million per microliter. Platelets can be large, pale blue in appearance, and hypogranular. Nucleated megakaryocyte fragments with a lymphoblastoid appearance may be seen in the blood film on occasion. Mild leukocytosis may occur in some patients, with Hb concentrations ranging from normal to mild anemia. Normally, the leukocyte differential count is normal, with no nucleated red cells.(95) Marrow cellularity is increased, with megakaryocytic hyperplasia and masses of platelet debris ("platelet drifts"). Megakaryocytes are frequently massive, ploidy-increased, and found in clusters. It is uncommon to have severe megakaryocytic dysplasia. One-fourth of ET patients may have no splenomegaly or marrow fibrosis, as well as no blood teardrops or leukoerythroblastic morphological features. The majority of patients progress to myelofibrosis..(95)

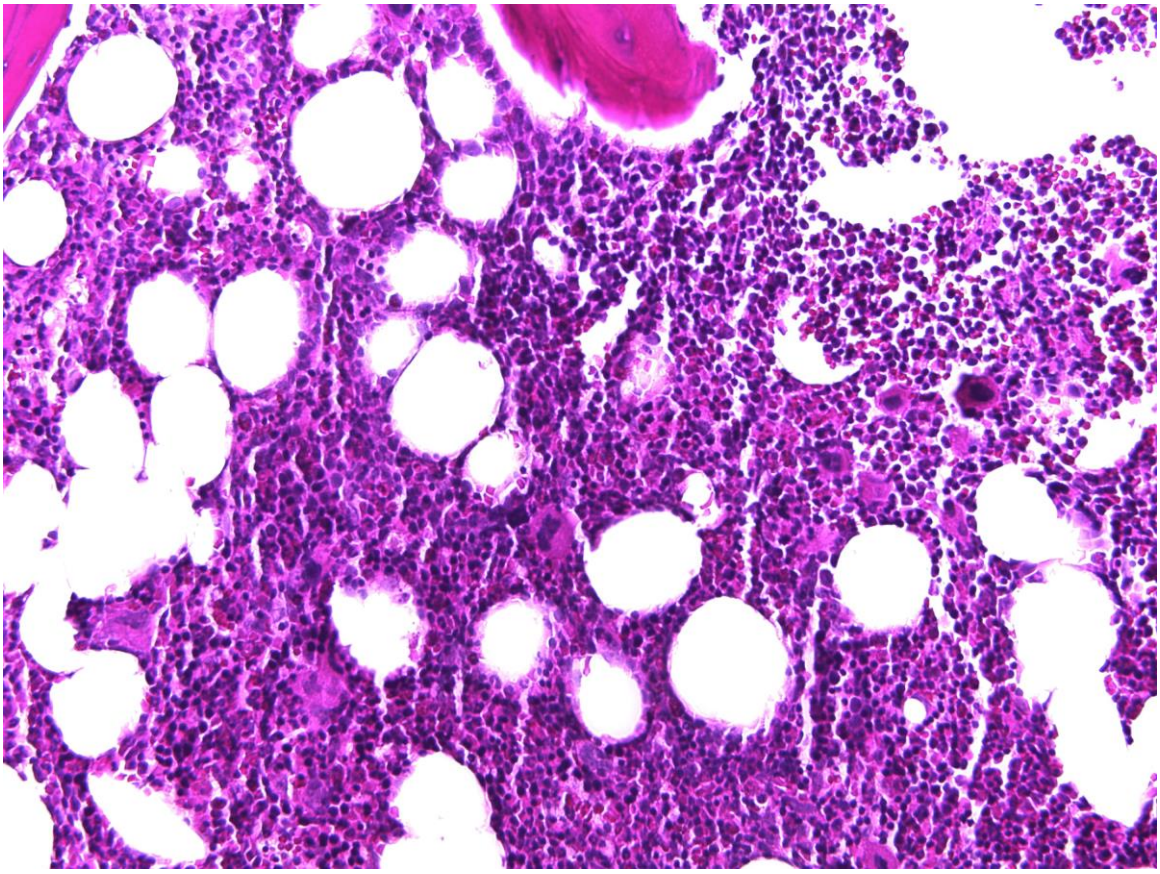


Fig 2.4.Bone marrow biopsy for ET showing megakaryocytic hyperplasia (96)

2.3.3.Primary Myelofibrosis

PMF is a type of myeloproliferative neoplasm (MPN) that is distinguished by clonal hematopoietic cell expansion and bone marrow fibrosis. PMF has the worst prognosis of the classic Philadelphia chromosome-negative MPNs, with a 6-year median overall survival (OS). Although haematologic transformation and disease progression are the most common causes of death, patients with PMF are at high risk for other competing causes of mortality and morbidity.(97) Primary myelofibrosis is the least frequent type of Philadelphia -ve

myeloproliferative neoplasm.(98).

William Dameshek, a hematologist, defined PMF in 1951 as one of a category of diseases known as myeloproliferative disorders.(99) PMF was previously known as agnogenic myeloid metaplasia, chronic idiopathic myelofibrosis, and myelofibrosis with myeloid metaplasia, with the most recent designation being primary myelofibrosis.(99)

WHO Classification of Tumor of Hematopoietic and Lymphoid Tissues,4th edition, changed name myeloproliferative disorders to myeloproliferative neoplasms in 2008. PMF has changed the disease name chronic idiopathic myelofibrosis. MPNs were also classified depended on clinical features ,bone marrow morphology, and genetic data.(100) WHO in 2016 amended the fourth edition, resulting in adjustments to MPN categorization. The classification of MPNs remains depend on bone marrow morphology, clinical characteristics, and genetics, as previously indicated, but the revised edition contains more molecular genetic data as a result of the identification of new somatic mutations.(101) Prefibrotic/early primary myelofibrosis (pre-PMF) and overt PMF are the two kinds of PMF.(101) This classification distinguishes between "true" essential thrombocythemia (ET) and pre-PMF.(102)

In a self-perpetuating vicious cycle, MF represents the paradigm of onco-inflammatory disorders: chronic inflammation, fueled by the malignant hematopoietic clone by itself, contributes to systemic manifestations and elicits clonal evolution.(103)(104) In fact, while the disease's onset is clearly caused by acquired somatic mutations in target myeloid genes, its progression is clearly driven, at least in part, by inflammation.(105)

Dysregulation of the JAK/STAT pathway caused by one of three so-called "driver mutations," namely JAK2 exon 14 and exon 12 mutations, thrombopoietin receptor gene (Myeloproliferative Leukemia Protein, MPL) mutations, or calreticulin (CALR) mutations, has traditionally been identified as the correlation between the neoplastic clone and cytokine overproduction in MF (and MPN in general)(106)

2.3.3.1.Etiology and pathogenesis

The MPNs are clonal hematologic diseases that are believed to be caused by a hematopoietic stem cell transformation. In erythroid and myeloid cells, Ph-negative MPNs have increased signaling through the Janus kinase (JAK) signal transducer and activator of transcription (STAT) pathway as well as the phosphatidylinositol 3-kinase (PI3K)-AKT (also known as protein kinase B) pathway.(107)

Driver mutations

Majority of PMF patients have one of three disease-causing driver mutations that activate the Janus kinase 2 and signal transducer and activator of transcription (JAK2-STAT) pathways, leading uncontrolled myeloproliferation. About 50-65% of PMF patients, a mutation in the JAK2 gene, specifically the JAK2V617F exon 14 mutation, is discovered.(108)(109) These patients are older, have greater hemoglobin levels and WBC counts, but lower platelet counts. A calreticulin (CALR) gene mutation has been detected about 20-30% of patients with PMFis associated with smaller aged patients, lower WBC counts and hemoglobin, and a higher platelet count. .(108)(109) The least common driver mutation, detected in roughly 10% of PMF patients, is a mutation in the

myeloproliferative leukemia (MPL) gene. A tiny number of PMF patients do not have any of the three driver mutations and are classified "triple negative" cases..(108)

Patients with triple negative PMF have the worst prognosis and are difficult to identify from other myeloid disorders. Furthermore, some non-driver mutations have been linked to PMF patients and are likely to play a role in disease development and progression to acute leukemia. (Table2.6)(110) differentiate from other myeloid disorders and has the worst prognosis.

Table (2.6.) Frequent Non-driver Somatic Mutations in PMF

Mutation	Mutation Mutational Frequency
TET2 (TET oncogene family member 2)	~17%
SRSF2 (Serine/arginine-rich splicing factor 2)	~17%
ASXL1 (Additional Sex Combs-Like 1)	~13%
U2AF1 (U2 Small Nuclear RNA Auxiliary Factor 1)	~16%
DNMT3A (DNA cytosine methyltransferase 3a)	~7%
EZH2 (Enhancer of zeste homolog 2)	~7%
IDH1/IDH2 (Isocitrate dehydrogenase 1 and 2)	~4%
SF3B1 (Splicing factor 3B subunit 1)	~7%

TP53 (Tumor protein p53)	~4%

DNA methylation (TET2, DNMT3A, and IDH1/IDH2), RNA splicing (SF3B1), chromatin modifications (ASXL1, EZH2), and DNA repair (TP53) are all affected by non-driver mutations. Appearance of one or more of these non-driver mutations in triple negative individuals may aid in diagnosis, but it frequently implies a poor prognosis..(111)

Mechanism of fibrosis

TPO binding to MPL stimulates multiple tyrosine kinase pathways, most notably the JAK-STAT pathway, in normal signaling.(112) JAK-STAT activation can be increased by mutations in both MPL and the ER chaperone calreticulin, the latter through boosting protein recruitment to MPL promoter region, which activates JAK-STAT. (113) Activation of JAK-STAT alters gene expression, which leads to alterations in the cytokine and growth factor milieu, which causes fibrosis..(114) The cause of bone marrow fibrosis is uncertain, aberrant megakaryocytes with multilobulated nuclei could be a beginning component. Unlike normal megakaryocytes, these aberrant cells, according to one hypothesized explanation, express the cell adhesion molecule P-selectin on their intracytoplasmic vacuoles and demarcation membrane system rather than the -granule membrane, which is normal position..(114) This altered expression allows P- selectin to coordinate neutrophils and eosinophils engulfment to megakaryocytes cytoplasm and to stimulate cytokines release like transforming growth factor beta (TGF-), platelet-derived growth factor (PDGF), and fibroblast growth factor (FGF) from the granules of engulfed cells. The activated cytokine pathways are

responsible for the fibrosis, neoangiogenesis, and osteosclerosis. (114)

The fibrosis induced by a rise in reticulin fibers, laminin, collagen types I, III, IV, and V, and glycoproteins (vitronectin, fibronectin, and tenascin) (several of which are triggered by TGF-).)(115) As a result, collagen and other ECM components undergo less proteolysis. Furthermore, TSP-1, a known TGF-1 activator, is overexpressed in all stages of PMF, regardless of fibrosis. TSP-1 also suppresses MMP activity, which adds to the procedure described above..(116) Hypoxic circumstances are caused by insufficient hematopoiesis in the bone marrow, which can disrupt the CXCL12/CXCR4 axis and cause HSC migration from the bone marrow to the spleen, contributing to extramedullary hematopoiesis..(70) Endothelial cells that express CXCL12 in the splenic sinusoids are likely to capture circulating HSCs and induce the establishment of a bone marrow-like niche. TGF- signaling may also help extramedullary hematopoiesis develop. Increased TGF- signaling in the bone marrow of PMF patients promotes osteoblast differentiation while lowering ubiquitin-mediated proteolysis, a mechanism required for erythroid maturation..(117) In contrast, TGF- has no effect on ubiquitin-mediated proteolysis in the spleen. This, along with enhanced splenic mTOR signaling, could explain splenic extramedullary hematopoiesis in clinically advanced illness..(117) The mTOR signaling pathway is essential for both normal and pathologic erythropoiesis; chronic stimulation can lead to MPNs and acute leukemias..(118) In PMF patients, however, hematopoietic cells express fewer TGF- receptors. (115) This may explain why a considerable number of PMF patients show an increase in circulating myeloid cells. PMF pathophysiology has recently been related to chronic inflammation. According to Hasselbach (2012), MPNs are

characterized by a persistent inflammatory state that initiates clonal evolution and catalyzes the transition from the early disease phases to the myelofibrotic burn-out phase..(119) Chronic inflammation may cause significant stem cells mutations through generating oxidative damage to DNA in hematopoietic cells..(119)(120) ROS activate proinflammatory pathways like NF-B, causing more ROS to be produced and causing genetic damage. These pathways stimulate the synthesis of PMF-related cytokines such as interleukin (IL)-1, `IL-2, IL-6, IL-8, tumor necrosis factor- (TNF-), vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), basic fibroblast growth factor (bFGF), and -interferon..(121) The higher levels of IL-8, IL-2 receptor (IL-2R), IL-12, IL-15, and CXCL10 in PMF patients, that independently predict poor mortality, give clinical evidence of the relevance of inflammation..(121) Elevated levels of IL-8 and IL-2R, in particular, have been associated to the development of acute myeloid leukemia (AML). (121)

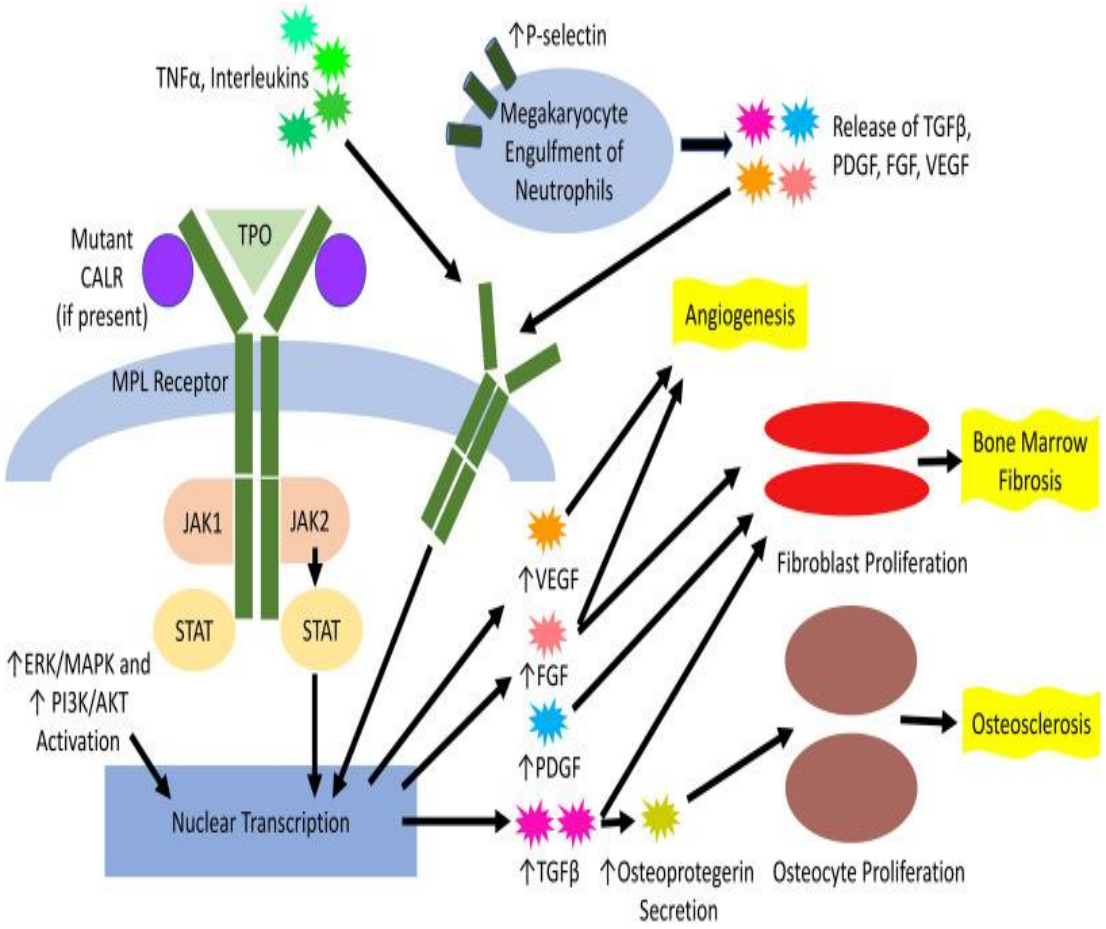


Fig.2.5. Bone Marrow Fibrosis Pathophysiology..(114)

2.3.3.2.Clinical features

2.3.3.2.1.Epidemiology

The annual incidence of primary myelofibrosis (PMF) is 0.4-1.4 per 100,000.People, with an older male predilection, though younger patients can be affected. PMF is the least common of the MPNs, but it is associated with a lower survival rate of 2 to 5 years after diagnosis and symptom onset.(122) PMF prevalence rates range from 1.76 to 4.05 per 100,000 people.(111) PMF and MF that arise from a pre-existing PV tend to have a male predominance. However, studies show that many females are

diagnosed with post-ET MF..(123) Previous study discovered young girls below the age of 34 were more likely than males to develop PMF, while blacks aged 35-49 had a greater frequency of PMF than whites..(44) PMF more common prevalent in white and non-Hispanic patients. Clinically, these changes appear to be minor. The incidence of PMF increases with age, with individuals over 75 having a greater incidence, but the majority of cases occurring between the ages of 50 and 74; the median age of diagnosis is 70. Many case reports link MF to autoimmune diseases; however, without additional research, it is unclear whether this is a risk factor..(114)

2.3.3.2.2.Clinical manifestations

Anemia, constitutional symptoms like lethargy, increase body temperature , and night sweating), with enlarged spleen are the most common clinical signs of PMF. Spleno-megaly is a prevalent feature in patients with PMF, accounting for over 90% of cases..(98) Splenomegaly. is caused by extramedullary hematopoiesis, which causes splenic architecture abnormalities and an increase in the the existence of megakaryocytes.(111) It is often a distressing symptom that contributes to complication. Other results include platelet counts changes, hemorrhage, bone pain, headache, enlarged liver (40-70% of individuals), thrombosis, and low body weight.. (124)

Other complications of the disease include symptomatic portal hypertension, which can cause variceal bleeding or ascites, and non-hepatosplenic EMH, which can cause cord compression, ascites, pleural effusion, pulmonary hypertension, or diffuse extremity pain. It is currently assumed that PMF-associated bone marrow stromal changes, ineffective

erythropoiesis, EMH, cachexia, and constitutional symptoms are caused by abnormal cytokine production by clonal cells and a host immune response.(124) Many patients die from comorbid conditions such as cardio-vascular events and the consequences of cytopenias, such as infection or bleeding, in addition to leukemic progression, which occurs in approximately 20% of patients.(125)

2.3.3.3.Diagnosis

Prefibrotic/Early Primary Myelofibrosis and overt myelofibrosis. A prefibrotic PMF and overt myelofibrosis is diagnosed using a set of clinical, laboratory, morphological, and molecular criteria.**Table2. 7.**(126)

Table(2.7)Prefibrotic/Early PMF and overt myelofibrosis.(126)

Prefibrotic Myelofibrosi	**Overt Myelofibrosis**
Clinical and Laboratory Features	
Splenomegaly Anemia Leukocytosis ≥ 11 × 109 /L Elevated LDH Leucoerythroblastosis	Splenomegaly Anemia Leukocytosis ≥ 11 × 109 /L Elevated LDH
Morphology	
Hypercellular marrow with atypical MEG proliferation (cloud-like nuclei) and dense Clusters peritrabecular fat, Increased GRAN and Frequently reduced ERY Fibrosis ≤ 1	**Atypical proliferation of MEG Variable cellularity (including subtotal aplasia)** intrasinusoidal hematopoiesis osteosclerosis Fibrosis ≥ 2
Molecular Features §	
JAK2, CALR or MPL mutation or other clonal marker	**JAK2, CALR or MPL mutation or other clonal marker**
Differential Diagnosis	
ET RARS-T MDS-F	Post-PV or post-ET MF

The current PMF diagnosis is based on WHO criteria from revised 2016and includes a composite assessment of clinical and laboratory features **(Table2.8).**

(Table 2.8.) 2016 revised world health organization (WHO) diagnostic criteria for primary myelofibrosis(124)

Primary myelofibrosis (pre-fibrotic)	**Primary myelofibrosis (overtly fibrotic)**
Major criteria:	Major criteria:
1. Typical megakaryocyte changes, accompanied by ≤ grade 1 reticulin/collagen fibrosis	1. Typical megakaryocyte changes, accompanied by ≥ grade 2 reticulin/collagen fibrosis
2. Presence of JAK2, CALR or MPL mutations, or presence of other clonal markers, or absence of evidence for reactive bone marrow fibrosis	2. Presence of JAK2, CALR or MPL mutations, or presence of other clonal markers, or absence of evidence for reactive bone marrow fibrosis
3. Not meeting WHO criteria for other myeloid neoplasms	3. Not meeting WHO criteria for other myeloid neoplasms
Minor criteria:	Minor criteria:
1.Anemia not otherwise explained	1.Anemia not otherwise explained
2.Leukocytosis ≥ 11 × 109/L	2.Leukocytosis ≥ 11 × 109/L
3.Increased serum lactate dehydrogenase	3.increased serum lactate dehydrogenase
4.Palpable splenomegaly	4.Palpable splenomegaly
	5.A leukoerythroblastic blood smear

Diagnosis requires meeting all three major criteria and one minor criterion. And according to 5th edition diagnostic criteria Primary myelofibrosis (PMF) is presented by abnormal megakaryocytes and granulocytes proliferating in bone marrow, which is associated in fibrotic stages with a polyclonal increase in fibroblasts driving secondary reticulin and/or collagen marrow fibrosis, osteosclerosis, and extramedullary haematopoiesis.

Prefibrotic PMF must still be distinguished from ET and PV, as well as from fibrotic PMF . The significance of serial monitoring of bone marrow fibrosis and spleen size using reproducible and standardized criteria remains relevant, particularly in JAK1/2 inhibitors receiving patients. PV and ET progress to AP (10-19% blasts) and BP (20% blasts) in a minority of cases, but leukaemia is more common in PMF, and leukaemia-free survival is shorter in fibrotic PMF compared to prefibrotic PMF.(77) While mutations in JAK2, CALR, and MPL are considered driver events, mutations in other genes, particularly TET2, ASXL1, and DNMT3A, are found in more than half of MPN patients. Splicing regulators (SRSF2, SF3B1, U2AF1, ZRSR2) and other chromatin structure, epigenetic functions, and cellular signaling regulators (e.g., EZH2, IDH1, IDH2, CBL, KRAS, NRAS, STAG2, TP53) mutations are less common. These additional mutations are more common in PMF and advanced disease than in PV and ET, and some have been linked to a poorer prognosis (e.g., EZH2, IDH1, IDH2, SRSF2, U2AF1, and ASXL1 mutations in PMF).(77)

2.3.3.4.Post ET and post PV myelofibrosis

The World Health Organization's (WHO) diagnostic criteria for PMF combine laboratory data with molecular and genetic findings, as well as morphologic features of the bone marrow. According to revised WHO criteria published in 2016, bone marrow biopsy has become critical for the diagnosis of MPNs, particularly to distinguish ET from early prefibrotic MF (pre-MF; **Table(2.9)** and to reflect the recent recognition of pre-MF by several groups.

Given that pre-MF behaves more aggressively than ET, this consider significant advancement in efforts to diagnose and predict patients prognosis .(127)

Table (2.9.) Criteria for Post–Polycythemia Vera Myelofibrosis and Post–Essential Thrombocythemia Myelofibrosis(128).

Post polycythemia vera Myelofibrosis		Post essential thrombocythemia myelofibrosis
Major criteria (all required)		
1 Documentation of a previous diagnosis of PV or ET as defined by WHO criteria		
2 Bone marrow fibrosis grade 2-3 (on scale of 0-3)24 c Or grade 3-4 (on scale of 0-4)		
Minor criteria (≥2 required)		
1	**Anemia or sustained loss of requirement of either phlebotomy (in absence of cytoreductive therapy) or cytoreductive treat- ment for erythrocytosis**	**Anemia and decrease in hemoglobin level of>2 g/dL from baseline**
2	**Increased LDH (above reference level)**	**Increased LDH (above reference level)**
3	**Leukoerythroblastosis**	
4	**Increasing splenomegaly, defined as either an increase in palpable splenomegaly of >5 cm (below left costal margin) or the appearance of newly palpable splenomegaly**	
5	**Development of >1 of 3 constitutional symptoms: >10% weight loss in 6 months, night sweats, and unexplained fever (>37.5°C)**	

2.4.Nuclear Factor –Erythroid2(NF-E2)

2.4.1.NFE2 Definition

The nuclear factor erythroid-2 gene (NF-E2), a hematopoietic transcription factor, is required for proper erythroblast and megakaryocyte differentiation.(128)

2.4.2.NFE2 History and Strucrure

The NFE2 transcription factor was found as one of two DNA binding activities in erythroid cell extracts in the late 1980s. The first protein, previously known as NF-E1 (nuclear factor-erythroid 1), is now known as the GATA1 transcription factor. The second protein, NF-E2, was found as a protein that recognized a sequence containing an activator protein 1 (AP-1) core motif, but it differed from other AP-1 factors in that it required residues outside of the core region for optimal binding..(129)

NF-E2 is a basic leucine zipper transcription factor that belongs to the cap "n" collar (CNC) subfamily. Nrf2-erythroid-derived CNC homology protein homology (Neh) domains are highly conserved domains of NRF2. Nrf2 is a modular protein structure of the Nrf2 protein consists of 605 amino acids (aa)with 7 Nrf2-ECH homology domains (Neh1-7) that serve distinct functions (130) Neh1 domain is a CNC and basic leucine zipper domain that interacts with partner proteins to form heterodimers.(131)

The Neh3 domain, which is located at the extremely end of NRF2's carboxyl terminus, is involved in NRF2 transactivation. Neh4 and Neh5 interact with the cyclic adenosine monophosphate response element binding protein-binding protein in a cooperative manner.(131)

Under normal conditions, Kelch-like ECH-associated protein 1 (Keap1), a cytoplasmic, cysteine-rich, actin-bound protein, sequesters Nrf2 in the cytoplasm Keap1's Kelch repeat domains (Kelch1-Kelch6) directly bind to Nrf2's NH2-terminal Neh2 domain and actin filaments, resulting in Nrf2 cytoplasmic sequestration.(132)

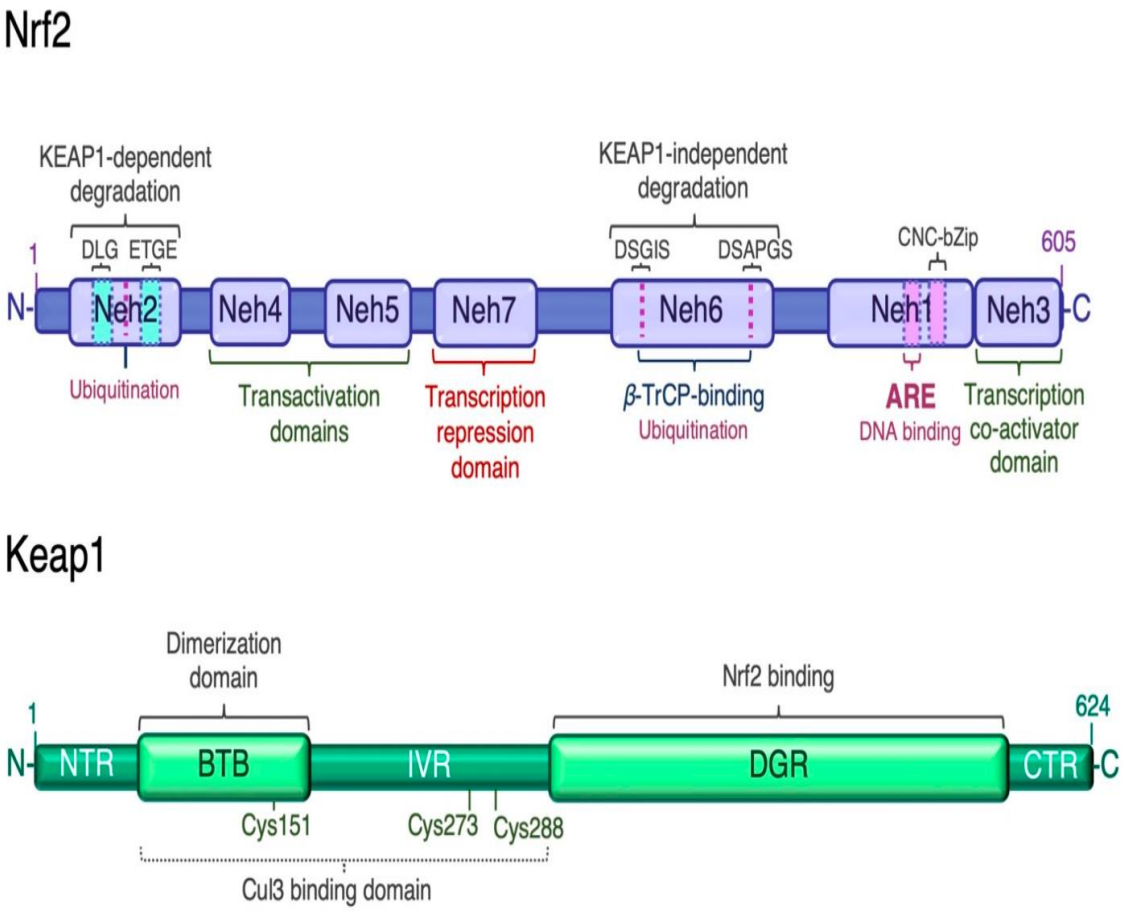
The Neh6 domain is a serine-rich region that is involved in the independent negative regulation of Nrf2 stability. It contains two conserved peptide motifs, DSGIS and DSAPGS, that are recognized by b-TrCP (b-transducing repeat-containing protein) (24). After glycogen synthase kinase-3b (Gsk-3b)-mediated phosphorylation of the DSGIS motif, b-TrCP binds more efficiently to the Neh6 domain, promoting the recruitment of the Skp1-Cul1-F-box protein (SCF) ubiquitin ligase complex and subsequent proteasomal degradation of Nrf2.(133)

By a physical association between the two proteins, the Neh7 domain is involved in the repression of Nrf2 transcriptional activity by the retinoid X receptor.(134)

Furthermore, oxidative stress can promote the dissociation of Keap1 and Nrf2, which speeds up Nrf2 phosphorylation and nuclear translocation. Nrf2 can form a heterodimer with other bZIP transcription factors to express its downstream target genes. Phosphorylation of Nrf2 by extracellular signal-regulated protein kinase (ERK), glycogen synthase kinase 3 (GSK3), phosphatidylinositol 3-kinase (PI3K), and c-JUN N-terminal kinase (JNK) is positively regulated, whereas cellular oncogene fos (c-Fos), fos related antigen-1 (Fra-1)(135)

The gene encoding the transcription factor NFE2 is found on human

chromosome 12 and mouse chromosome 15, and it was cloned more than 20 years ago. There are two transcripts, each with a different non-coding region, one of which is more abundant in adult hematopoietic locations and the other in fetal liver..(129)



figure(2.6.). Human Nrf2 and Keap1 structure. The Kelch domain of Keap1 binds the high-affinity ETGE and low-affinity DLG motifs in Nrf2's Neh2 domain for Nrf2 ubiquitination and degradation. ARE stands for antioxidant response element; BTB stands for broad-complex, tramtrack, and bric-a-brac domain; CTR stands for C-terminal region; DGR or Kelch stands for double glycine repeat domain; IVR stands for intervening region; Neh stands for Nrf2-erythroid-derived CNC homology (ECH) domain; NTR stands for N-terminal region.(136)

NF-E2-related factor 2) is a master regulator of cellular responses against environmental stresses. NF-E2 induces the expression of detoxification and antioxidant enzymes and Keap1. Under the stress condition, Keap1 induces NF-E2 translocation from the cytoplasm to the nucleus and thus activates the expression of multiple target genes .The target genes of Nrf2 include the genes encoding antioxidant enzymes, drug-metabolizing enzymes and transporters, and heme and iron metabolic enzymes .(137).The expression level of NF-E2 is particularly high in the detoxification organs or tissues which directly counter the environment, such as the intestine, lung, and choroid plexus of the brain in a mouse embryo .(137)

2.4.3.Role of NFE2 in Myeloproliferative Neoplasms*:*

Although discovery of an activating point mutation in the JAK2 kinase (JAK2V617F) in the majority of MPN patients, distinguishing ET from PMF can be difficult. Both clinical characteristic and the histopathological finding of ET and PMF can be the same, especially in the early stages, a problem that has sparked lively debate about distinguishing for diagnosis criteria, including those of the WHO classification. Further more, WHO classification has been chastised for depend on histopathology, which can be highly variable between observers. However, due to the significant clinical course and outcome differences between ET and PMF, accurate classification and diagnosis of these entities is critical.(22)

Nuclear factor erythroid-2 (NFE2), a hematopoietic transcription factor, plays a role in the pathophysiology of myeloproliferative neoplasms (MPNs), as the majority of MPN patients have elevated NFE2 levels, and elevated NFE2 expression recapitulates MPN hallmarks in vitro and in

vivo**.**(138)

The molecular mechanism causing elevated NFE2 levels in MPN patients is unknown at this time, and little is known about NFE2 target genes. We recently reported that epigenetic mechanisms, including JAK2-mediated histone phosphorylation at the NFE2 locus, regulate NFE2 expression.(138) It has previously been reported that the transcription factor nuclear factor erythroid 2 (NF-E2) is abnormally elevated in MPN patients. The presence or absence of the JAK2V617F mutation has no effect on NF-E2 overexpression in ET and PMF. Furthermore, elevated NF-E2 levels cause an MPN phenotype in a murine model.(22) .

The correct differential diagnosis of certain MPN entities can be difficult, especially in the early stages of the diseases. Because relevant ancillary tests are not yet available, pathologists have had to rely primarily on histomorphological interpretation of bone marrow biopsies in conjunction with laboratory data. Even molecular testing, like as JAK2 mutation analyses, are unsuccessful in discriminating between ET and PMF because both are associated with the V617F mutation in 50% of instances. Recently, it was discovered that the transcription factor NF-E2 is overexpressed in MPN..(139)

Chapter Three

Materials and Methods

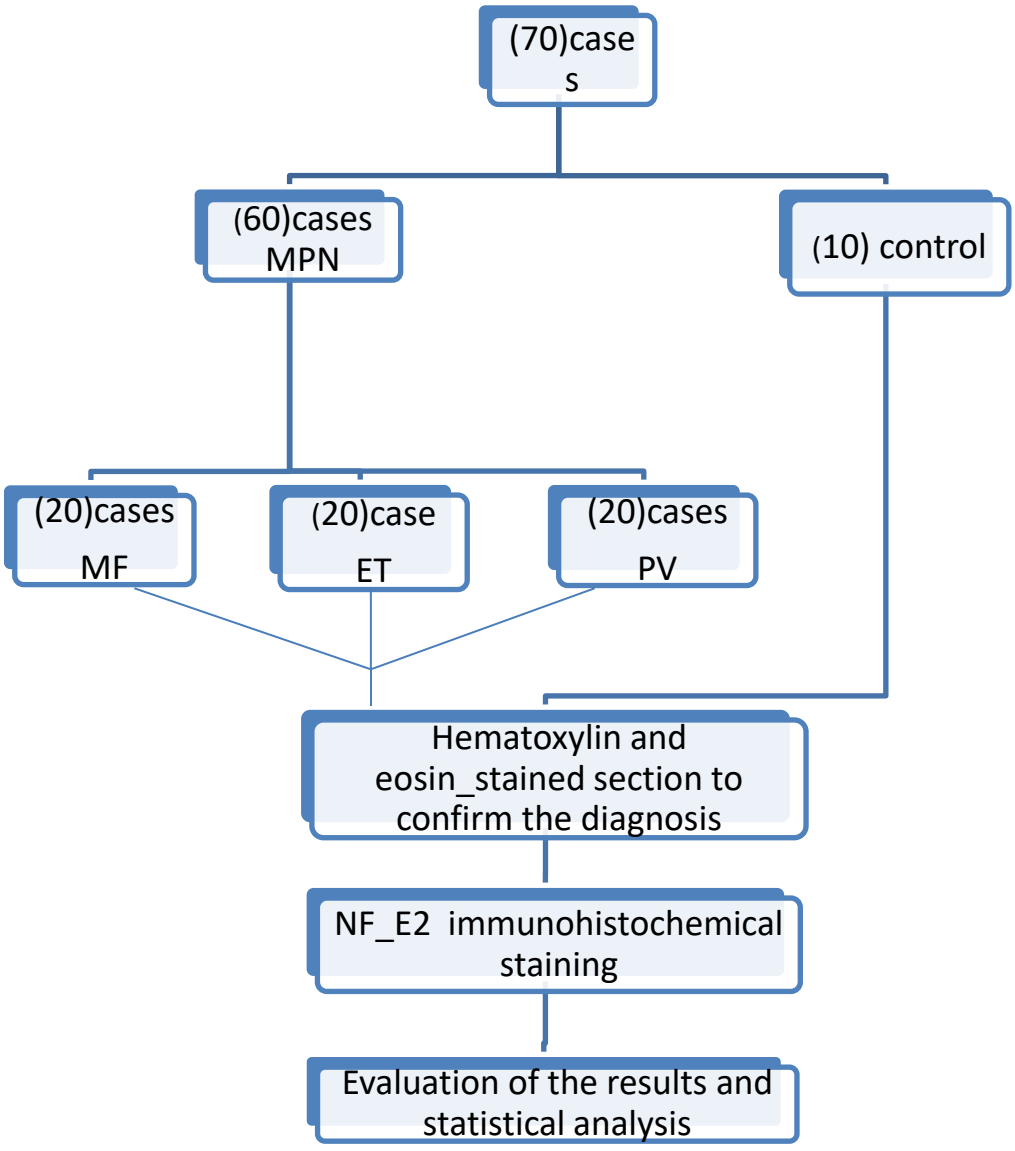
Chapter Three

Materials and methods

3.1. Tissue Samples:

A retrospective cross sectional study was done in Pathology and Forensic Medicine department, collage of Medicine in Babylon University, the study sample consist of tissue blocks fixed in formalin, embedded in paraffin were collected from archived material from Baghdad medical city from 16 October 2022 to 20 February 2023.The paraffin blocks represent (70) cases including: (60) Myeloproliferative Neoplasms and (10) control group.The myeloproliferative neoplasms include: (20) Myelofibrosis and(20)Polycythemia Vera ,(20) Essential Thrombocythemia with patients age ranging from 22 to 80 years old. From each block, two sections of 4μm thickness were taken, one stained with (H&E) for histopathological revision, the other section was stained immunohistochemically for NF-E2 marker. Nrf2 (Acetyl Lys599) Polyclonal Antibody (BT LAB(bioassay technology laboratory) was used in this study with retrieving kit (Elabscience) was used in this study

3.2.Study design diagram



3.3 Staining method

3.3.1Equipment and material

The following equipment and materials were used throughout this

Table 3.1:

	Material	Company	Origin
1	Nrf2 (Acetyl Lys599) Polyclonal Antibody	BT LAB(bioassay technology laboratory)	China
2	Retrieving kit:	Elabscience	USA
3	Electrical microtome	Histo lab	Italy
4	Positive charge slides	AFCO	Lebanon
5	Microwave oven	Memmert	Germany
6	Coplin jars		Germany
7	Water bath	Flat plate	England
8	Micropipette and tips	Slamed	Germany
9	Timer	Digital timer	China
10	Disposable syringe	Changzhou kangfulai	China
11	Microscopic slide	AFCO	Lebanon
12	Cover slide	AFCO	Lebanon
13	Light microscope	Olympus	Japan
14	Others: gloves ,tissue paper, humidity chamber, wax pen, cotton swabs, hot plate, staining gars ,xylene, deionized water, ethanol of different concentrations.		

3.3.2 Hematoxylin and eosin staining method:

1. Deparaffinization of sections has been performed by incubating the sections in an oven at 65 degree for two hours, then two changes in xylene 5 minutes for each change.
2. Then tissue was rehydrated in decreasing grades of ethanol including 3 changes of 100 % ethanol each for 5 minutes; followed by 95 % ethanol for 5 minutes; 70% ethanol for 5 minutes and washing in distilled water.
3. Subsequently sections were stained with hematoxylin for 3-10 minutes.
4. After that slide were washed well in running tap water.
5. Followed by removing excess stain by differentiating the sections in 1% acid alcohol solution (1% hydrochloric acid + 70% ethanol) for 5-10 seconds.
6. Then slides were washed well in tap water until sections regain their blue color.
7. Later sections were stained in eosin for 5 seconds.
8. Dehydration slowly through increasing grades of alcohols (i.e., 70%, 90%, 100% each for 5 minutes.
9. Followed by clearing in 2 changes of xylene, each for 5 minutes.
10. Finally slides were mounted with DPX (Distyrene, plasticizer and xylene) and covered with coverslip.

3.3.3 Immunohistochemical Testing:

3.3.3.A. Immunohistochemical Staining Procedure:

NFE2L2 (primary antibody) encodes a transcription factor (nuclear factor, erythroid 2 like 2). (140)

That is a member of a family of basic leucine zipper (bZIP) proteins. Encoded transcription factor regulates genes that contain antioxidant response elements (ARE) in their promoters; many of these genes encode proteins that participate in response to the injury and inflammation that involve production of free radicals. Multiple transcript variants encoding different isoforms have been characterized for NFE2L.

3.3.3.B. Immunohistochemical Staining Protocol:

The immunostaining method used in the current study was the Elabscience(141) is a highly sensitive and rapid immunohistochemical broad spectrum detection reagent applied for NF_E2 antibody

1. Four microns sections of formalin-fixed and paraffin-embedded tissue were mounted on positive charged slides.
2. Deparaffinization was done by incubating the sections in an oven at 60 C℃for 2 hours, followed by 3 changes in xylene each for 5 minutes, then rehydration in decreasing grades of ethanol (100%, 90% each for 10 minutes then 70% for 5 minutes)
3. Dewaxing/Antigen Retrieval working solution had put in the repair box, and heat until it boils.
4. The slides had put into the boiling Dewaxing/Antigen Retrieval working solution for making tissue immersed (To ensure the pH of the solution, the metal slide rack should not be used).
5. Repair the antigen with medium-low power for 15~30 min, and avoid drying the tissue through the process. Hold the repair box to a RT environment, and it cool down naturally.
6. Hold out slides when the solution cooled down to RT, then wash the

slides with DI water, be certain that there is no residue of Dewaxing/Antigen Retrieval working solution.

7. By using absorbent paper, dry the liquid around the tissue, incubate with SP Reagent B (Peroxidase Blocking Buffer) at RT for 15 min to eliminate endogenous peroxidase activity. Wash with PBS or TBS, 2 min × 3 times.
8. By absorbent paper, dry the PBS then with an oily pen draw a circle around the tissue. Add primary antibody with proper dilution ratio using SP Reagent G (Antibody Dilution Buffer), incubate at RT or 37°C for 30 min~1h or at 4°C overnight (then rewarm at 37°C for 30 min). Wash with PBS or TBS, 2 min × 3 times.
9. By absorbent paper, dry the PBS, add a drop of SP Reagent C (Polyperoxidase-anti-Rabbit/Mouse IgG), incubat at RT or 37 degree for half an hour. Wash with PBS or TBS, 2 min × 3 times.
10. Put 1 drop (approximately 50 μL) of SP Reagent D (High Sensitive DAB Concentrate) into each 1 mL of SP Reagent E (High Sensitive DAB Substrate), mix fully and the mixed reagent is the DAB Working Solution. Prepare fresh solution before use and the prepared solution should be stored in the dark. Fresh prepared DAB Working Solution is valid within 4 hours and the unused solution must be abandoned.
11. Dry the PBS with absorbent paper, Take control of the DAB coloration period, the color of tan or brownish yellow is the positive signal. Avoid of excessive reaction. Wash the section with DI water to terminate the chromogenic reaction.
12. By using absorbent paper, dry the liquid around the tissue, incubate with SP Reagent F (Hematoxylin Staining Buffer) at RT for about 5 to

10 minutes, and wash the slides with DI water.

13. Transfer slides into alkaline water for 1 minute, then wash thoroughly with water for about 5 to10 minutes.
14. Dehydrate by using gradient alcohol, transparent with transparent agent, drop Neutral Balsam near tissue, and cover it with the cover glass and dry the slide.
15. Slides were examined under light microscope.

3.3.4 Evaluation of Immunostaining:

The evaluation of positive immunohistochemical reaction for NF_E2 antibody is by the diffuse brown staining of the nucleus and/or cytoplasm and scoring the results according to intensity of the stain and percentage of stained cells

Score 0 : no evidence of stain

Score 1+ : weak stain

Score 2 + : moderate stain

Score 3+ : strong stain

Data analysis

The statistical analysis was carried out using the statistical package for social sciences SPSS version 27. Categorical variables presented as frequencies and percentages, continuous variables presented as mean, standard deviation and range. Chi-square and Fisher's exact tests were used to find the association between variables. ANOVA test was used to compare means among four groups. P value of ≤ 0.05 was considered as significant.

Chapter Four

Results

Chapter four

Results

4.1 Socio-demographic distribution of study group

Table 4.1. show patients distribution with myeloproliferative neoplasms regarding their socio-demographic characteristics including (age and gender). Mean age of them was (58.13 ± 11.53), the median was(60.5) with maximum age was 80 years and minimum age was 22 years. About one third of them presented with age group (60-70) years (N=20, 33.3%). More than half of patients were males (N=34, 56.7%) of patients.

Table 4.1: The Distribution of Patients with Myeloproliferative Neoplasms Regarding Their Socio-demographic Characteristics (N=60)

Socio-demographic characteristics	Number	%
Age (years)		
< 40 years	4	6.7%
40-50 years	6	10.0%
50-60 years	19	31.7%
60-70 years	20	33.3%
≥ 70 years	11	18.3%
Total	60	100.0%
Gender		
Male	34	56.7%
Female	26	43.3%
Total	60	100.0%

4.2 Distribution of Patients of MPN According to Subtypes

Figure4.1: show distribution of patients with myeloproliferative neoplasms according to subtypes including (Myelofibrosis, Polycythemia Vera and Essential Thrombocythemia). All three subtypes represent 20 patients (33.3%) of total patients.

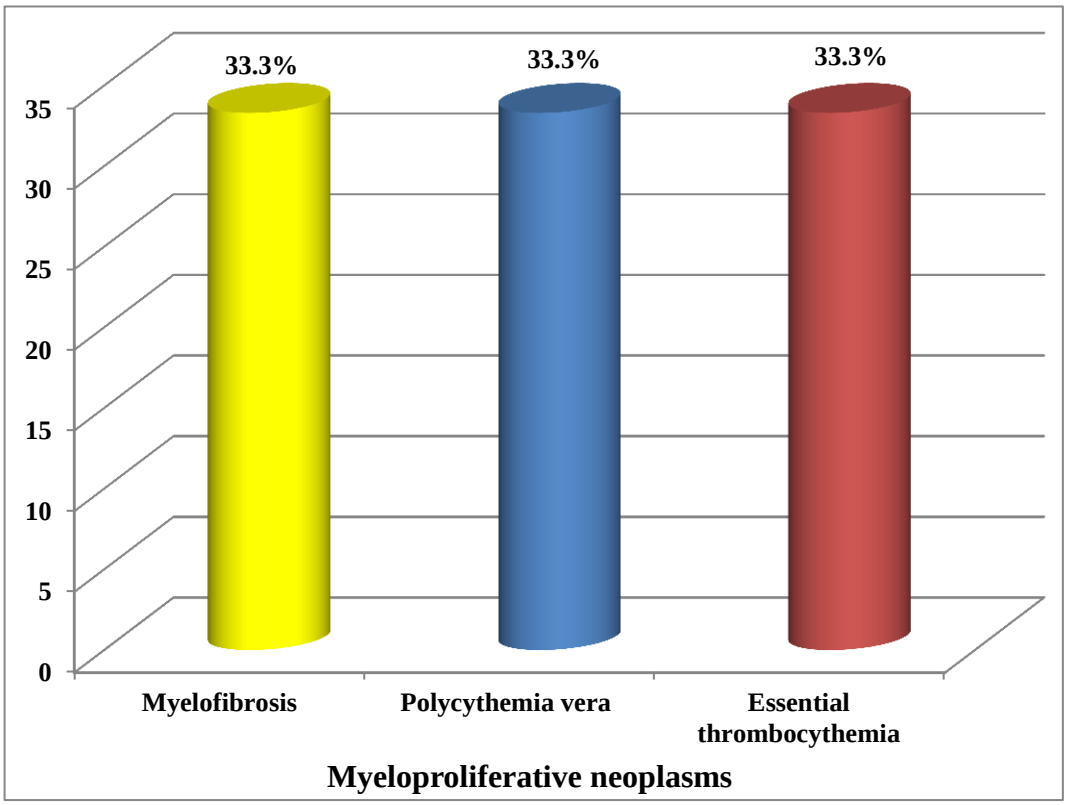


Figure4.1: Distribution of patients with myeloproliferative neoplasms according to subtypes (N =60)

4.3. Mean Differences of Age (years) Related to The Diagnosis :

Table 4.2: Mean differences of age (years) according to diagnosis including (Myelofibrosis, Polycythaemia Vera, Essential Thrombocythemia and Control group). No significant differences was found among means of age (years) related to the diagnosis.

Table 4.2: Mean differences of age (years) related to the diagnosis (N=70)

Variable	DX	N	Mean ± SD	F test	P value
Age (years)	Myelofibrosis	20	61.50 ± 6.85	1.323	0.274
	Polycythemia vera	20	58.20 ± 9.15		
	Essential thrombocythemia	20	54.70 ± 16.05		
	Control group	10	58.50 ± 6.11		

4.4 The Association Between Diagnosis and The Study Variables (age,splenomegally and score)

Table 4.3: There was significant association between diagnosis and study variables including (splenomegaly and score).

Study variables	Diagnosis				P-value
	Myelofibrosis	Polycythemia vera	Essential thrombocythemia	Control group	
Gender					0.437
Male	14 (70.0)	11 (55.0)	9 (45.0)	5 (50.0)	
Female	6 (30.0)	9 (45.0)	11 (55.0)	5 (50.0)	
Total	20 (100.0)	20 (100.0)	20 (100.0)	10 (100.0)	
Splenomegaly					**<0.001***
Present	18 (90.0)	4 (20.0)	8 (40.0)	0 (0.0)	
Absent	2 (10.0)	16 (80.0)	12 (60.0)	10 (100.0)	
Total	20 (100.0)	20 (100.0)	20 (100.0)	10 (100.0)	
score					**<0.001***
+1	0 (0.0)	20 (100.0)	7 (35.0)	10 (100.0)	
+2	7 (35.0)	0 (0.0)	12 (60.0)	0 (0.0)	
+3	13 (65.0)	0 (0.0)	1 (5.0)	0 (0.0)	
Total	20 (100.0)	20 (100.0)	20 (100.0)	10 (100.0)	

4.5 Mean Differences of Haemoglobin (mg/dl) According to Diagnosis

Table4.4: The mean differences of Haemoglobin (g/dL) according to diagnosis including (Myelofibrosis, Polycythemia vera, Essential Thrombocythemia and Control group). A significant differences was found among means of Haemoglobin (g/dL) regarding to the diagnosis.

Table 4.4: Mean differences of Haemoglobin (g/dL) according to diagnosis (N=70)

Study variable	Diagnosis	N	Mean ± SD	F-test	P-value
Haemoglobin (g/dL)	Myelofibrosis	20	10.97 ± 2.42	27.405	<0.001*
	Polycythemia vera	20	17.37 ± 3.26		
	Essential thrombocythemia	20	12.92 ± 1.76		
	Control group	10	11.35 ± 1.20		

4.6 The Mean Differences of PCV (%) Related to Diagnosis

Table 4.5: The mean differences of PCV (%) according to diagnosis including (Myelofibrosis, Polycythemia vera, Essential thrombocythemia and Control group). There were significant differences between means of PCV (%) according to diagnosis.

Table 4.5: Mean Differences of PCV (%) According to Diagnosis (N=70)

Variable	DX	N	Mean ± SD	F-test	P-value
PCV (%)	Myelofibrosis	20	33.31 ± 7.14	33.037	<0.001*
	Polycythaemia Vera	20	53.70 ± 8.58		
	Essential thrombocythemia	20	40.35 ± 6.33		
	Control group	10	34.61 ± 2.98		

4.7 Mean Differences of WBC ($X10^9/L$) According to Diagnosis:

Table4.6: The mean differences of WBC ($X10^9/L$) according to diagnosis including (Myelofibrosis, Polycythemia vera, Essential thrombocythemia and Control group). A significant differences was found between means of WBC ($X10^9/L$) according to the diagnosis.

Table4.6: Mean Differences of WBC ($X10^9/L$) According to Diagnosis (N=70)

Study variable	Diagnosis	N	Mean ± SD	F-test	P-value
WBC ($X10^9/L$)	Myelofibrosis	20	8.59 ± 2.75	**13.528**	**<0.001***
	Polycythemia vera	20	16.01 ± 4.44		
	Essential thrombocythemia	20	15.93 ± 3.90		
	Control group	10	11.56 ± 6.88		

4.8 Mean Differences of Platelet Count (X10/L) According to Diagnosis

Table 4.7: The mean differences of Platelet count ($X10^9$/L) according to diagnosis including (Myelofibrosis, Polycythemia vera, Essential thrombocythemia and Control group). A significant differences was found among means of Platelet count ($X10^9$/L) related to the diagnosis.

Table 4.7: Mean Differences of Platelet (X109/L) Related to the Diagnosis (N=70) (N=70)

Variable	Diagnosis	N	Mean ± SD	F-test	P-value
Platelet count ($X10^9$/L)	Myelofibrosis	20	264.60 ± 168.51	**35.353**	**<0.001***
	Polycythemia vera	20	626.65 ± 238.18		
	Essential thrombocythemia	20	1349.15 ± 600.49		
	Control group	10	316.10 ± 110.63		

4.9 Mean Differences of NF-E2 (%) Related to The Diagnosis

Table 4.8: Mean differences of NF-E2 (%) regarding the diagnosis including (Myelofibrosis, Polycythaemia Vera , Essential Thrombocythemia and Control group). A significant differences was found between means of NF-E2 (%)regarding the diagnosis.

Table4.8: Mean differences of NF-E2 (%) related to the diagnosis (N=70)

Variable	DX	N	Mean ± SD (Percentage)	F-test	P-value
NF-E2 (%)	Myelofibrosis	20	29.80 ± 3.58	47.166	<0.001*
	Polycythemia vera	20	8.85 ± 8.61		
	Essential thrombocythemia	20	16.45 ± 8.50		
	Control group	10	3.00 ± 1.41		

immunohistochemistry staining for NF-E2 results:

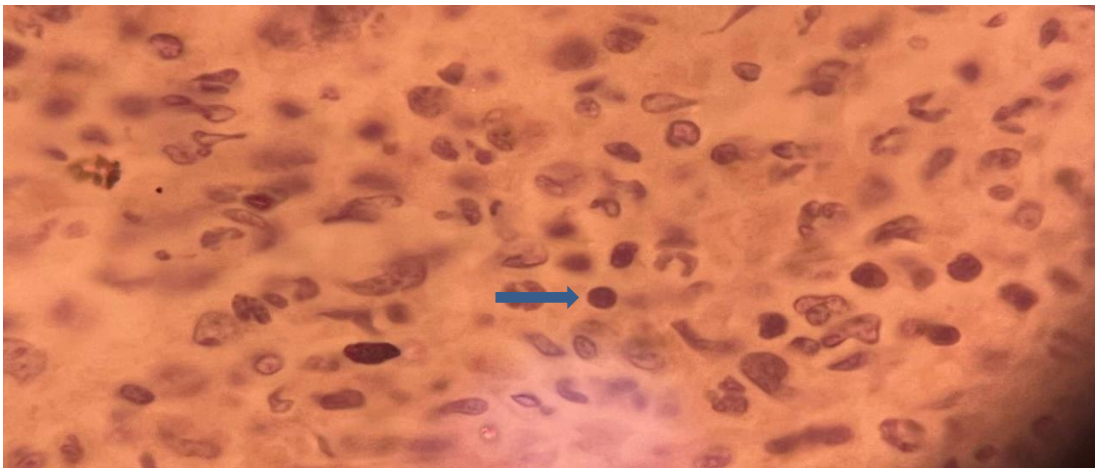


Fig.4.2a negative results for NF-E2(small dark stained nucleus erythroblast)

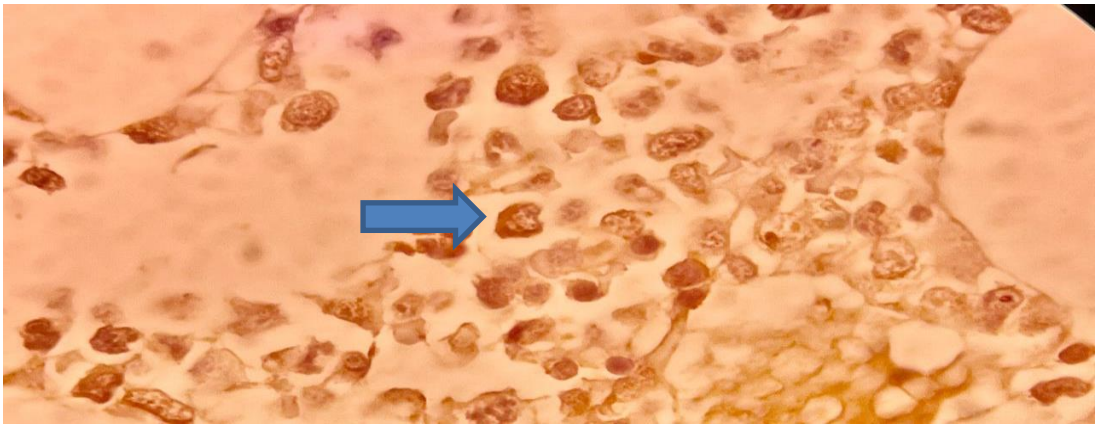


Fig4.2b positive results for NF-E2(cytoplasmic staining of erythroblast)

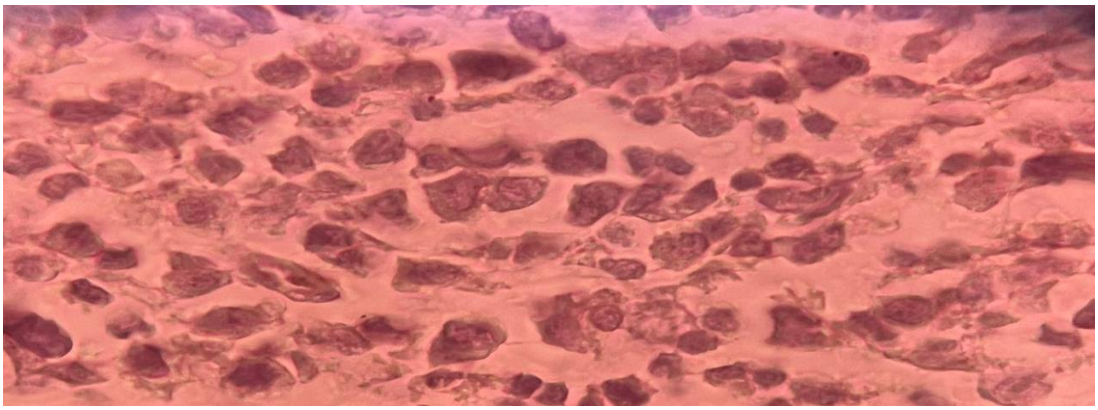


Fig 4.2c score 1(weak staining)

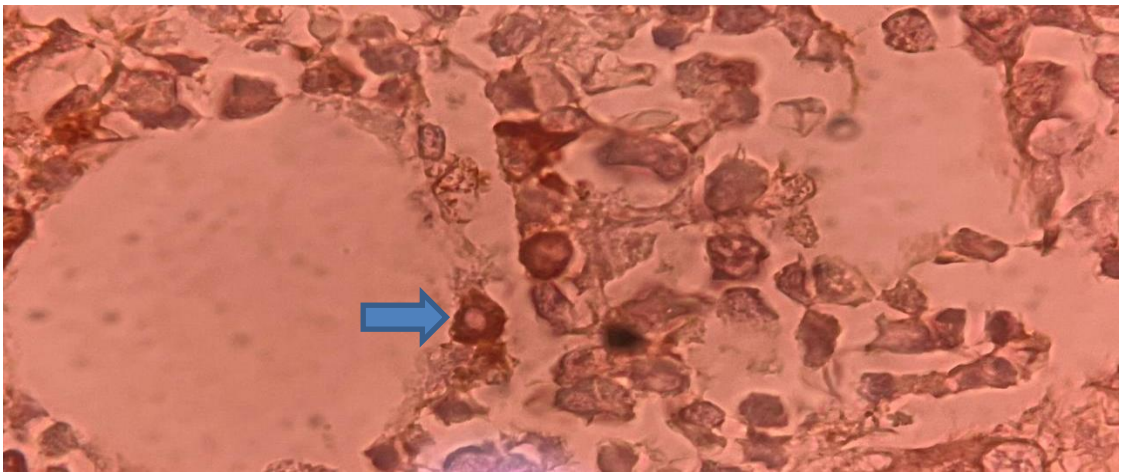


Fig4.2d positive results for NF-E2 (score2)

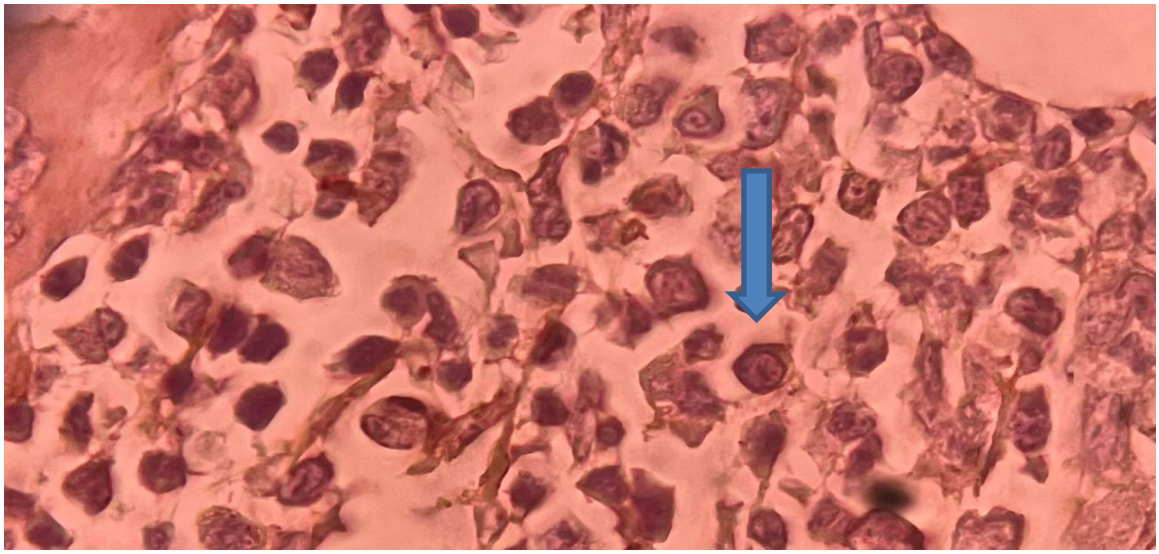


Fig 4.2e positive results for NF-E2 score 3(strong cytoplasmic and neuclear staining of erythroblast)

Figure 4.3 show the ROC curve for sensitivity and specificity of NF-E2 in diagnosis of myeloproliferative neoplasms patients. (**AUC=0.949, P<0.001*) ,** 95% CI (0.90-0.998) and the optimal cut off value to predict myeloproliferative neoplasms patients was (≥ 5.5) (sensitivity=86.7% ,specificity=100.0% and overall accuracy =88.57%).

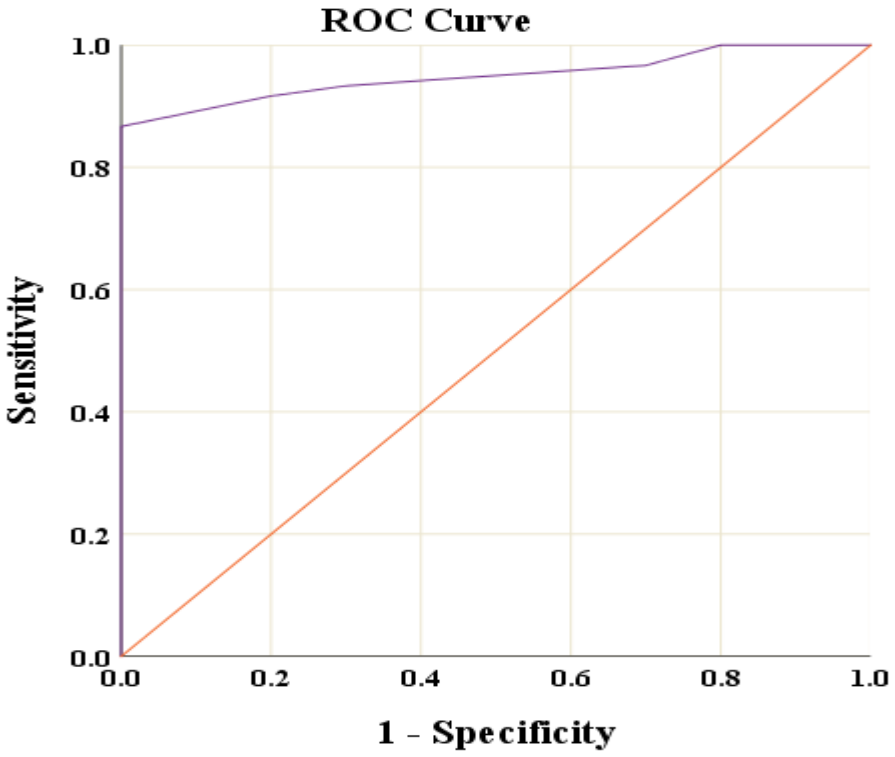


Figure4.3: ROC curve for sensitivity and specificity of NF-E2 in diagnosis of myeloproliferative neoplasms patients (N=70)

Figure 4.4 show the ROC curve for sensitivity and specificity of NF-E2 in diagnosis of Myelofibrosis and differentiated it from other myeloproliferative neoplasms subtypes. (**AUC=0.904, P<0.001*) ,** 95% CI (0.826-0.981) and the optimal cut off value to predict Myelofibrosis was (≥ 20.5) (sensitivity=100.0% ,specificity=82.5% and overall accuracy =88.33%).

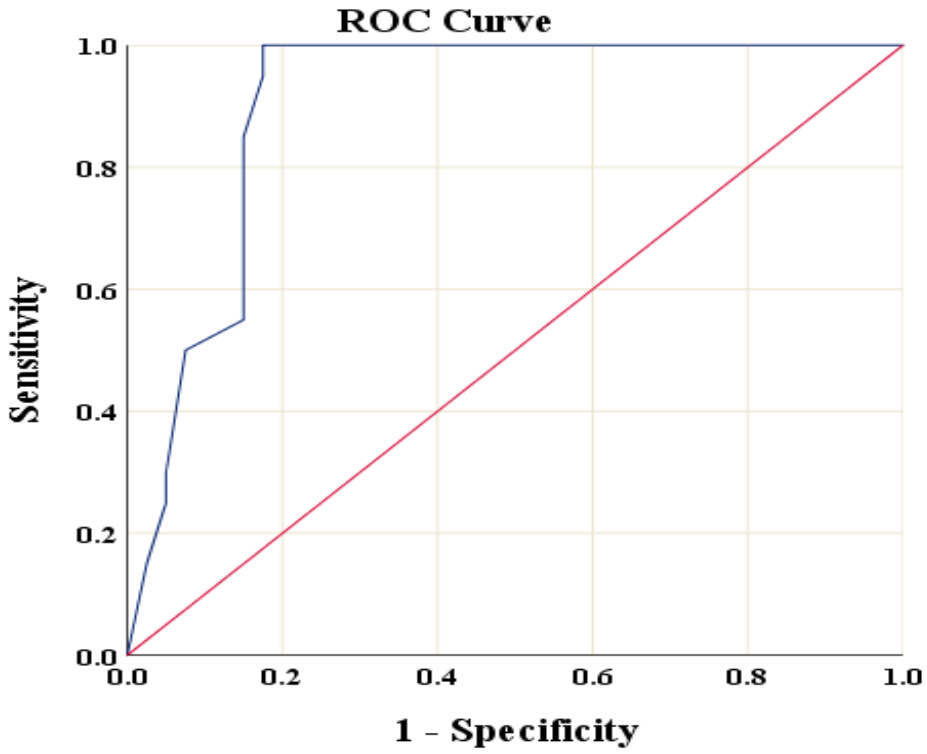


Figure 4.4 ROC curve for sensitivity and specificity of NF-E2 in diagnosis of Myelofibrosis (N=60)

Chapter Five

Discussion

Chapter five

Discussion

5. Discussion

The aim of the current study was to study the value of NF-E2 in myeloproliferative neoplasms subtypes diagnosis. In spite of the high number of molecular abnormalities that detected in MPN persons, distinguishing between these related entities, particularly early, prefibrotic phase of proliferative mylofibrosis and essential thrombocythemia, still difficult and depend primarily on the histology and clinical diagnosis. And that because none of mutation detected in patients of myeloproliferative neoplasms are specific to one entity.

Histology is still very helpful in distinguish between thrombocythemia and pre fibrotic PMF in patients with isolated thrombocytosis. However, it has been argued that histology alone lacks discriminatory power, and inter observer variability still a concern. A simple, reproducible immunehistochemical stain with low inter observer variability and a high level of accuracy. The proportion of nuclearNF-E2 staining in patients with PMF was already significantly higher at diagnosis and remains unchange over time

5.1 Age and gender distribution

In our study patients distribution with myeloproliferative neoplasms regarding their socio-demographic characteristics that include the age and gender (table 4.1). Median age of patients was (58.13 ± 11.53) with the maximum age was 80 years and the minimum age was 22 years. One third

of them presented with age group (60-70) years (N=20, 33.3%). More than half of patients were males (N=34, 56.7%) of patients which approximately agree with study of **Mohammed SK et al(2020):** from Iraq over time.(142).

The mean age distribution of patient with myelofibrosis in current study (table 4.2. A)was 62 year,the result were comparable to other studies by O'Sullivan JM et al(2018):, Tefferi A et al (2018):, Mughal TI et al(2014): .(142)(143)(144) in which the mean age of myelofibrosis patients is 64 ,64 ,65 respectively with male to female ratio 2:1

The mean age of distribution of pat7ients with polycythemia vera in current study 55.5 years this result agree with the study of Malignancies, Lymphoid(145) in which the mean age was 55_60 years with male to female ratio 1.5:1

In current study age distribution in patients of essential thrombocythemia was 60.5 with female predominance which agree with previous studies by Accurso V et al(2019):, Accurso V et al(2020):, Lawrence NH (2021):.(87)(84)(89) in which the mean age was 60 years old and female to male ratio 2:1.however no significant differences was found between mean of age (years) according to diagnosis.

5.2 distribution of patients with myeloproliferative neoplasms according to splenomegaly

In current study the distribution of patients with myeloproliferative neoplasms regarding to splenomegaly that include (present and absent) (Fig 4.1). Splenomegaly was positive in half of patients with myeloproliferative neoplasms which represent 30 patients (50.0%) of total patients including

control, and according to subtypes of myeloproliferative neoplasms 90% of pateints of myelofibrosis having splenomegaly which agree with previous study done by ***Mughal TI et al (2014)*:**, ***Oon SF et al (2019): , Butina M et al (2021):***.(143)(122)(111) in which splenomegaly palpable in more than 80%, 90%,90% respectively.

while only 20% of polycythemia vera have splenomegaly in our study and was disagree with previous study done by ***Lee MW et al (2022):*** (144) in which estimate splenomegaly in polycythemia vera (0.8%) these may indicate that cases with splenomegaly in our study have progressive disease or due to sample selection.

In current study 40% of essential thrombocythemia have splenomegaly and was approximately agree with study in which was 35.3% by ***Sah SK et al(2022):*** .(145),there was significant association between diagnosis and splenomegaly in current study.

5.3 Distribution according to score of intensity and percentage

In regard to intensity of NF-E2 staining (table 4.3)in current study scored as(+1 ,+2,+3) in myelofibrosis there were 7 cases scored +2 and 13 cases scored +3 apart from 20 cases ,in polycythemia vera the all 20 cases scored as +1 while in essential thrombocythemia 7 cases scored+1 and 12 cases scored as+2 and only 1 case scored+3 apart of 20 cases and therefore there was significant association between diagnosis and this score is unique to our study.

5.4 mean differences of Hemoglobin level(g/dL)according to the diagnosis

In current study the mean differences of Hemoglobin level(mg/dl)according to the diagnosis (table 4. 4.A) in myelofibrosis the mean hemoglobin level was (10.97±2.42 g/dL) and in patients of polycythemia vera was (17.37±3.26 g/dL)and in those with essential thrombocythemia was(12.92±1.76g/dL)which aproximately agreed with previous study done by ***Al-Saadi EAKD et al(2021):*** in which the mean hemoglobin levels were (10.5±3.7g/dL)(146) in which the mean hemoglobin levels were (10.5 ± 3.7g/dL) , (16.0 ± 3.2 g/dL),(11.0 ± 2.7 g/dL) respectively.

There were significant differences between means of hemoglobin level according to diagnosis with p value (< 0.001).

5.5 the mean differences of PCV(%) according to diagnosis

In regard to mean differences of PCV(%) in current study(table 4.5 A); the mean ±SD of PCV in myelofibrosis was(33.31%±7.14%) which approximately agreed with study done by **Mohammed SK et al (2020):** (142) in which the mean of pcv was(35.2%±0.4%).

And the mean of PCV in patients with polycythemia vera in current study was (53.70%±8.58%) and in Essential thrombocythemia was (40.35%±6.33%) approximately agreed with previous study by ***Silver RT et al(2019):***(147) in which the mean PCV percentage was(50.9%±4.4% in male and 51.2%±5.8 %) for PV and and (43.5%±4.4%)in male and(42.8%±2.2%)in female) for ET patients. There were significant differences

between means of PCV according to diagnosis with P-value<0.001.

5.6 Mean differences of WBC count (x 10^9/L)according to diagnosis

The mean differences of WBC count (x 10^9/L) in current study (table4.6) the mean WBC count of patients with myelofibrosis was (8.59±2.75x10^9/L) which approximately agreed with study done by ***Birgegard G. et al (2019):*** which the mean WBC count was (9.8±7.2x10^9/L).(148)

In regard to mean WBC count in polycythemia vera in current study was (16.01±4.44x10^9/L) which approximately agreed with previous study done by ***Lee MW et al (2022):*** in Korea (144)

In current study the mean WBC count in patients with essential thrombocythemia was(15.93±3.90 x10^9/L) which approximately agreed with previous study done by ***Shen CL et al(2021):***in Taiwan (149) in which mean WBC count was (16.5±12.2 x10^9/L).

There were significant differences regarding mean WBC count according to diagnosis.

5.7 The mean platelet count according to diagnosis

Regarding mean platelet count in current study in myelofibrosis was (264.60±168.51)x 10 ^{9}/L which approximately agreed with previous study done by ***Al-Saadi EAKD et al (2021):*** in Iraq(146) in which the mean platelet count was (218.9 ±170.4x10^9/L). and in essential thrombocythemia was (1349±110 x10^9) which nearly agree with study done by ***Elbager S et al (2020):*** (150).

While regarding mean platelate count in patients with polycythemia vera in current study was (626.65±238.18 x 10^9/L) that nearly come in agreement with study by ***Lee MW et al(2022):*** (144) .

There was significant differences between means of platelet count according to diagnosis.

5.8 The mean differences in percentage of NF-E2 according to diagnosis

It has been suggested that immunohistochemistry staining for transcription factor NF-E2 can be used for distinguishing between unclassified MPN subtypes (MPN-U) for MPN patients who do not clearly fulfill the diagnostic criteria for PV,ET ,PMF; especially between ET and PMF even in early stages of the diseases and that what we found in current study by obtaining the percentage and intensity of erythroblast nuclear and cytoplasmic staining of NF-E2 percentage were the mean percentage of NF-E2 in myelofibrosis was (29.80%±3.58 %) which considered statistically highly significantly elevated compared with essential thrombocythemia in which the mean percentage of NF-E2 was (16.45%± 8.50%) that considered slightly elevated and that come in agreement with study done by ***Aumann K et al (2013):*** in Germany in university of Freiburg (22) while the mean percentage of NF-E2 in current study in patients with polycythemia vera was(8.85%±8.61%) which disagree with the above previous study in which the percentage of NF-E2 was (23.9%±10%) and this might attributable to insufficient samples of polycythemia vera in our study. there were significant differences between means of percentage of NF-E2 according to diagnosis, with cut off value to predict myelofibrosis ≥20 .

In this study we propose that quantitative NF-E2 immunohistochemistry of bone marrow biobsies presented with thrombocytosis is important method that can support the distinction between MPN subtypes especially between ET and pre_fibrotic PMF and this considered to be very important for both prognosis and treatment decision.

Chapter six

Conclusion and Recommendations

Chapter six

Conclusion and recommendations

6.1Conclusion

It concluded that:

1. NF-E2 expressed in all patients with myeloproliferative neoplasms an can aid in diagnosis with optimal cut off value to predict MPN patients ≥ 5.5 with sensitivity =86.7% ,specificity =100% and overall accuracy =88.57%
2. NF-E2 highly significantly positive in myelofibrosis patients so can aid distinguishing unclassified MPN subtypes especially between ET and prefibrotic PMF with cut off value to predict myelofibrosis ≥20 (sensitivity =100% ,specificity =82.5% and overall accuracy =88.33%)
3. The proportion of nuclear positive erythroblasts of all erythroid precursor cells and NF-E2 immunohistochemistry can be helpful for distinguishing between ET and PMF .

6.2 Recommendations

It recommended that:

1. Further studies with follow up of patients, larger sample size and wider age range are recommended to substantiate our results and evaluate the potential role of NF-E2 in diagnosis MPN subtypes .
2. Further studies needed to certain the differences in NF-E2 staining with a second alternative antibody against different NF-E2 peptide.

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الخلاصة

الخلفيه:الاورام التكاثرية النقوية هي امراض دموية نسيلية يتم تعريفها على انها زيادة في تمايز الخلايا المكونة للدم في المرحلة المزمنة.الانواع الثلاثة الرئيسية التي تشكل الامراض التكاثرية النقوية(سلبية فيلاديلفيا) تتضمن هي كثرة خلايا الدم الحمراء وكثرة الصفيحات الاساسية والتليف النقوي .قد يكون التشخيص التفريقي الدقيق لبعض كيانات الاورام التكاثرية النقوية امرا صعبا ,خاصة في المراحل المبكرة من الامراض.

نظرا لعدم توفر اختبارات اضافية مفيدة بعد, كان على اخصائي علم الامراض الاعتماد بشكل اساسي على البيانات المختبرية والتحليل النسيجي والمورفولوجي لخزعات نخاع العظم. حتى الاختبارات الجزيئية مثل تحليل طفرة (JAK2) , غير فعالة في التمييز بين الخثار الاساسي والتليف النقوي الاولي لأن كلاهما مرتبط بطفرة v617 f في حوالي خمسين بالمائة من الحالات.تم مؤخرا تحديد الافراط في التعبير عن العامل النووي المشتق من خلايا الدم الحمراء في انواع الامراض التكاثرية النقوية.

الهدف من الدراسة: اجريت هذه الدراسة لتقييم قيمة العامل النووي المشتق من خلايا الدم الحمراء في تشخيص الانواع الفرعية للأورام التكاثرية النقوية

المرضى وطريقة العمل:مجموعة من 70 خزعة نخاع عظم تتضمن (60) عينة مشخصة كامراض نقوية تكاثرية(وتتضمن (20) عينات من نوع كثرة خلايا الدم الحمراء و(20)عينات من نوع زيادة الاقراص الدموية الاساسي و(20) عينات من نوع التليف النقوي)), و10 عينات تتضمن المجموعة الضابطة التي تم تقييمها في قسم علم الامراض في المدينة الطبية في بغداد م بين عاميي (2019) و (2022) تم تلطيخها كيميائيا مناعيا بالعامل النويي المشتق من خلايا الدم الحمراء نوع 2 وتحليلها من قبل خبراء علم الامراض.

النتائج: أظهرت هذه الدراسة أن الكيمياء الهيستولوجية المناعية للعامل النووي – المشتق من كريات الدم الحمراء 2 يمكن أن تساعد في تحديد الأنواع الفرعية للأورام التكاثرية النقوية حتى في المراحل المبكرة من المرض. بدقة 88.33٪ ، يمكن إجراء تشخيص أولي للتليف النقوي للأورام التكاثرية النقوية التي لم يتم توصيفها بعد وتظهر نسبة تزيد عن 20٪ من الأورمات الحمراء النووية الإيجابية.

الخلاصة: النسبة المئوية للعامل النووي – كريات الدم الحمراء 2 أعلى بكثير في المرضى الذين يعانون من التليف النقوي مقارنة بأولئك الذين يعانون من كثرة الحمر الحقيقية ، وهي كثرة الصفيحات الأساسية مع مستويات منخفضة جدا في المجموعة الضابطة.

جمهورية العراق
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جامعة بابل
كلية الطب / فرع الامراض

قيمة العامل النووي الثاني المشتق من كريات الدم الحمراء في تشخيص الانواع الفرعية للاورام التكاثرية النقوية

رسالة مقدمة إلى مجلس كلية الطب في جامعة بابل

كجزء من متطلبات نيل درجة الماجستير في الطب\ علم الامراض

من قبل

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