Faculty of Pharmacy, Nursing and Health Professions

Master's Program in Clinical Laboratory Science

The frequency of JAK2V617F and JAK2 exon 12 mutations among patients suspected to have Polycythemia Vera in West Bank- Palestine

By

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Advisors

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Dedication

I dedicate this thesis to My Dear Friend, Family and My Parents.

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NAL

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List of Abbreviations

RBC	Red Blood Cells
WBC	White Blood Cells
PLTs	Platelets
HSC	Hematopoietic Stem Cells
YS	Yolk Sac
EMP	Erythroid-Myeloid Progenitors
FL	Fetal Liver
BM	Bone Marrow
TPO	Thrombopoietin
GM-CSF	Granulocyte-Macrophage Colony Stimulating Factor
IL	Interleukin
SCF	Stem Cell Factor
PI3K	Phosphotidal-inositol-3-Kinases
PLC -g	Phospholipase C- gamma
EPO	Erythropoietin
BMP	Bone Morphogenetic Protein
SMAD	S-Mothers Against Decapentaplegic
STAT	Signal Transducer and Activator of Transcription
GR	Glucocorticoid Receptor
PMDs	Myeloproliferative Disorders
CMPDs	Chronic Myeloproliferative Disorders
WHO	World Health Organization
MPNs	Myeloproliferative Neoplasms
CML	Chronic Myelogenous Leukemia
PN-MPNs	Philadelphia Negative – Myeloproliferative Neoplasms
PV	Polycythemia Vera
ET	Essential Thrombocythemia

PMF	**Primary Myelofibrosis**
JAK2	**Janus Kinase 2**
CALR	**Calreticulin**
MPL	**Myeloproliferative Leukemia**
AML	**Acute Myeloid Leukemia**
PVSG	**Polycythemia Vera Study Group**
Hb	**Hemoglobin**
Hct	**Hematocrit**
EEC	**Endogenous Erythroid Colony**
mPV	**masked Polycythemia Vera**
TYK2	**Tyrosine Kinase 2**
JH	**JAK Homology**
EPO-R	**Erythropoietin Receptor**
TPO-R	**Thrombopoietin Receptor**
G-CSF-R	**Granulocyte Colony- Stimulating Factor Receptor**
SOCS	**Suppressor Of Cytokine Signaling**
HP-1α	**Heterochromatin Protein-1 α**
LMO2	**LIM domain Only 2**
Tp53	**Tumor Protein 53**
CBB	**Central Blood Bank**
MOH	**Ministry Of Health**
CBC	**Complete Blood Count**
PCR	**Polymerase Chain Reaction**
ARMS	**Amplification Refractory Mutation System**
RFLP	**Restriction Fragment Length Polymorphism**
HRM	**High Resolution Melting**
FO	**Forward Outer**
RO	**Reverse Outer**
FWT	**Forward Wild Type**

RMT **Reverse Mutant**

NTC **No Template Control**

NGS **Next Generation Sequencing**

Abstract

INTRODUCTION: Polycythemia Vera (PV) is a myeloproliferative disorder, characterized by excessive overproduction of normal erythrocytes. It could be transformed into aggressive leukemia. Thus, understanding the molecular genetics and cellular biology of this disorder should improve the diagnosis and management of PV.

Several genetic studies have shown that *JAK2V617F* is the most common mutation associated with PV features (95%), other mutations of JAK2 exon 12 are present in JAK2V617F-negative PV (4%). Both affect the tyrosine kinase activity and are associated with the erythropoietin-hypersensitive expansion of erythroid progenitors. The most common mutations detected in JAK2 exon 12 are *F537-K539delinsL,* and *K539L.*

OBJECTIVE: to determine the frequencies of *JAK2V617*, JAK2 exon 12 mutations (*F537-K539delinsL,* and *K539L)* among Palestinian patients suspected to have polycythemia Vera, using different molecular techniques.

MATERIALS AND METHODS: This case-control study was designed to determine the presence of mutations among Palestinian patients with PV features and control group. A total of 129 individuals were enrolled in this study; PV (patients group 1) who have Hb level >18.5g/dL for men and >16.5g/dL for women, 52 patients (40.3%); 4 (7.7%) females and 48 (92.3%) males. The control group consisted of 46 (35.7%) healthy individuals who have Hb level <16.5 g/dL for men and Hb < 16.0 g/dL for women; of them 4 (8.7%) were females and 42 (91.3%) were males. The (patients group 2), consisted of 31 (24%) males with Hb level between 16.6 g/dL and 18.5 g/dL but doesn't meet the 2016 WHO criteria. The average age of study

subjects ranged between 18 - 83 years, with a median of 32 years. Samples were tested for CBC and EPO level. The presence of *JAK2V617F* mutation was tested using amplification-refractory mutation system (ARMS) PCR, and restriction fragment length polymorphism (RFLP). While the presence of JAK2 exon 12 mutations were tested using ARMS-PCR and high resolution melting curve (HRM).

Results: the rate of *JAK2V617F* in the sample tested was 69.2% (36/52), 21.7% (10/46), and 51.6% (16/31) in patients group 1, controls group, and patients group 2 respectively. These results are approximately similar to those published in neighboring and foreign countries. While JAK2 exon 12 mutations were detected as wild-type in all specimens 100%. All Statistically significant association was found between the presence of *JAK2V617F* and developing the PV disorder among study groups, X^2 = 22.2, with p-value <0.001 .

CONCLUSION: This study is the first in West-Bank, Palestine intended for the assessment of PV patients. According to our results regarding the presence of *JAK2V617F* mutation among Palestinian population. The following may need to be taken into consideration:

- presence of *JAK2V617F* in majority but not all of patients groups; probably suggest the presence of another gain-of-function mutation contributing to the disease other than those identified in this study. Thus, more investigations and research are required.
- Identifying the *JAK2V617F* mutation in healthy people increased the possibility that the mutation is harbored before the onset of the disease, and this must be confirmed by adequate evaluation of healthy people carrying the mutation. However, the burden of mutated alleles should be identified to

determine a cut-off value that can be helpful in PV diagnosis. Furthermore, genetic stability based on DNA repair is effective in normal hematopoietic stem cells, but decreases in differentiating cells, making them susceptible to mutations, including *JAK2V617F* . However, epigenetic modification may repress the effects of JAK2V617F in healthy individuals due to express or silence important gene, thus preventing PV features.

Our understanding of the molecular pathogenesis of PV is very important for the classification, diagnosis, and choice of the best treatment methods that have been proven to alter the natural history of this myeloid neoplasm.

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ملخص الدراسة

مقدمة: تعرف ال Polycythemia vera (PV) بسرطان الدم الذي يتميز بالإفراط في إنتاج خلايا الدم الحمراء مع إمكانية تحوله إلى سرطان دم خبيث. وبالتالي، فإن ازدياد فهم الوراثة الجزيئية والبيولوجيا الخلوية لهذا الاضطراب يحسن من التشخيص والتحكم في سير هذا المرض.

أظهرت العديد من الدراسات الوراثية أن *JAK2V617F* هو الطفرة الأكثر انتشارا لهذا المرض وتقدر بمعدل 95٪ ،وطفرة JAK2 exon 12 موجودة بنسبة 3٪ لدى الذين لا يحملون طفرة *JAK2V617F* . التي تؤثرعلى نشاط انزيم التيروسين كاينيز والمرتبط بزيادة حساسية الإريثروبويتين لإنتاج كريات الدم الحمراء.

الأهداف: الهدف الأساسي من هذه الدراسة هو تحديد نسبة طفرات *JAK2V617* و JAK2 exon 12 بين المرضى الفلسطينيين, التي تشير الدلائل أنهم قد يعانون من ارتفاع كريات الدم الحمراء الحقيقي باستخدام عدة تقنيات جزيئية مختلفة.

المواد و الطرق: تم تصميم هذه الدراسة للمقارنة بين من يحملون الطفرات المذكورة من المرضى الفلسطينيين الذين لديهم ميزات ارتفاع كريات الدم الحمراء الحقيقي ومجموعة تحكم لا يوجد لديها ميزات ارتفاع كريات الدم الحمراء الحقيقي. تم اشتراك 129 فردًا في هذه الدراسة, وتم توزيعهم على ثلاث مجموعات على النحو التالي ، مرضى ارتفاع كريات الدم الحمراء الذين لديهم مستوى هيموغلوبين أكثر من 18.5 g/dL للرجال وأكثر من 16.5 g/dL للنساء ، ويتألف من 52 (40.3٪) من الأفراد ؛ منهم 4 (7.7٪) من الإناث و 48 (92.3٪) من الذكور. المجموعة الضابطة التي تتكون من 46 (35.7٪) من الأفراد الأصحاء الذين لديهم مستوى هيموغلوبين أقل من 16.5 g/dL للرجال وأقل من 16.0 g/dL للنساء ؛ منهم 4 (8.7٪) من الإناث و 42 (91.3٪) من الذكور. وتتكون المجموعة المرضى

الثانية 31 (24٪) من الذكور الذين لديهم مستوى هيموغلوبين بين 16.6 g/dL وg/dL18.5. تراوحت أعمار الأفراد من جميع الفئات ما بين 18- 83 سنة ، وكان متوسط العمر 32 سنة.

تم جمع عينات الدم من المشاركين واختبارها لمستوى تعداد الدم الكامل وهرمون الإرثروبويتين, كما تم عزل الحمض النووي لإجراء المزيد من التحليل الجزيئي للكشف عن وجود الطفرات.

الطرق الجزيئية التي استخدمت لهذا الغرض تشمل : نظام طفرة التضخيم الحراري - تفاعل البلمرة المتسلسل, و تفاعل البلمرة المتسلسل - تقييد طول القطعة تعدد الأشكال, و منحنى الذوبان عالي الدقة.

تم فصل منتجات تفاعل البلمرة المتسلسل على 2٪ أجاروز جل وصبغها باستخدام إيثيديوم برومايد و تقييمها.

النتائج: باستخدام عدة طرق جزيئية نظام طفرة التضخيم الحراري - تفاعل البلمرة المتسلسل,و تفاعل البلمرة المتسلسل -تقييد طول القطعة تعدد الأشكال؛ تم العثور على JAK2V617F بنسبة 69.2 ٪ (52/36) و 21.7٪ (46/10) و 51.6٪ (31/16) في مجموعة المرضى 1 ومجموعة التحكم ومجموعة المرضى 2 على التوالي ، وهي قريبة تقريبًا من أبحاث الدول المجاورة والأجنبية. بينما لم يتم الكشف عن طفرات JAK2 exon 12 في أي فرد.

تم العثور على ارتباط ذي دلالة إحصائية بين وجود JAK2V617F وتطوير اضطراب ارتفاع كريات الدم الحمراء الحقيقي بين مجموعات الدراسة ، X2 = 22.2 ، مع قيمة p <0.001

الخلاصة: هذه الدراسة هي الأولى في الضفة الغربية ، فلسطين وتهدف إلى تقييم مرضى ارتفاع كريات الدم الحمراء الحقيقي. وفقًا لملاحظتنا بشأن وجود طفرات JAK2V617F بين السكان الفلسطينيين. قد يلزم أخذ ما يلي في الاعتبار:

- وجود JAK2V617F في غالبية وليس كل مجموعات المرضى ؛ ربما يقترحون وجود طفرة أخرى في اكتساب الوظيفة تساهم في المرض بخلاف تلك المحددة في هذه الدراسة. وبالتالي ، هناك حاجة إلى مزيد من التحقيقات والأبحاث.
- أدى التعرف على طفرة JAK2V617F في الأشخاص الأصحاء إلى زيادة احتمالية وجود الطفرة قبل ظهور المرض ، ويجب تأكيد ذلك من خلال الملاحظة الكافية للأشخاص الأصحاء الذين يحملون الطفرة. علاوة على ذلك ، فإن الاستقرار الجيني القائم على إصلاح الحمض النووي فعال في الخلايا الجذعية المكونة للدم الطبيعي ، ولكنه ينخفض في الخلايا المتمايزة ، مما يجعلها عرضة للطفرات ، بما في ذلك *JAK2V617F*.

قد تسهم النتائج في زيادة كبيرة لفهم هذا المرض وإمكانية تشخيصه بدقه وينعكس ذلك في تعزيز النتائج القدرة العلاجية وتقليل نسبة خطر تحوله إلى سرطان الدم - اللوكيميا.

Chapter 1: Introduction

During the third week of embryonic life, the formation of the body begins and occurs through the process of gastrulation (1), which consists of establishing the three germ layers of the embryonic disc; that includes ectoderm, mesoderm, and endoderm (2,3).

Each of them transforms into specific tissues of many organs and body systems; in order to form full-grown human body.

The ectoderm gives rise to the epidermis, skin, hair, mammary glands, lens and inner ear (4), neural tube and crest to form the nervous system, and other structures, as seen in Figure 1 . The endoderm leads to the formation of respiratory passages and organs, digestive tract, liver and pancreas (5).

The third mesoderm layer; generates the shape of the head mesenchyme, axial skeleton, trunk skeletal muscles, and connective tissues, excretory and reproductive organs (6). The mesoderm is also the source of bone marrow, heart, blood vessels and blood cells formation (4,5).

1.1. Hematopoiesis:

It is the dynamic and continuous processes of production and development of all blood cells (7). This process includes erythropoiesis (production of red blood cells (RBC)), leukopoiesis (production of white blood cells (WBC)), and thrombopoiesis (production of platelets (PLTs)) (3,4).These different blood cell lines are formed from multipotent and self-renewing hematopoietic stem cells; this leads to the fact that intermediate progenitor cells differentiate into all blood cells under a complex network of tissues, organs, and regulatory factors to produce white blood cells that are essential for the immune system and responsible for defense against infection;

red blood cells carry and transport oxygen and carbon dioxide between the lungs and tissues in the body; and platelets are responsible for the coagulation system and to prevent bleeding (3,4).

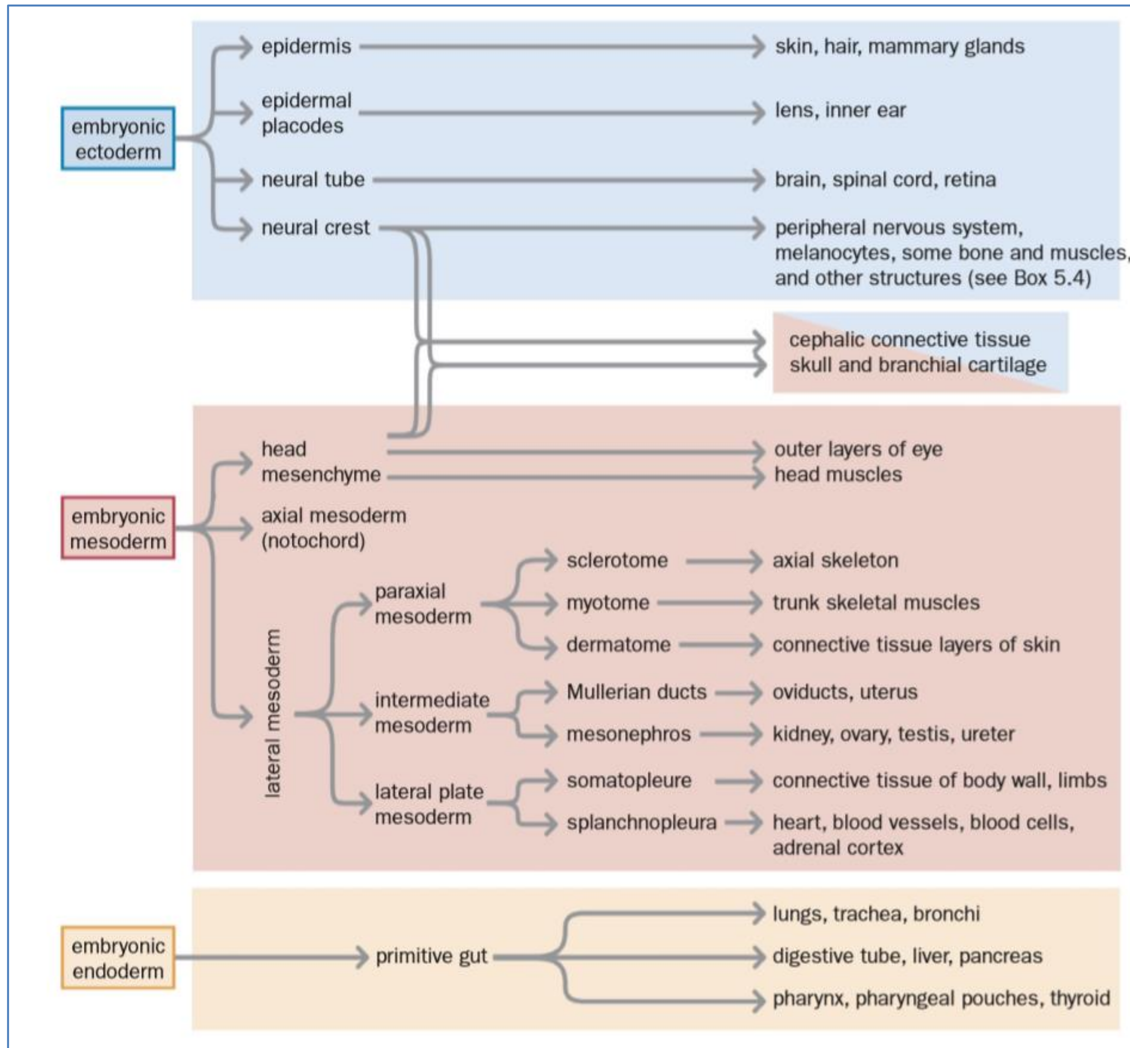

Figure 1: Principal derivatives of the three germ layers.

(T. Strachan. Human molecular genetics, 4th ed, Garland Science, Taylor & Francis Group, LLC,2011; chapter5;133-135).

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1.1.1. Origin of hematopoiesis:

During early embryonic life, the development of the hematopoietic system occurs in specific and special different waves (5). The primitive wave in which blood cells are generated without an intermediate progenitor(2), unlike the definitive wave that involves the production of Hematopoietic stem cells (HSC), the most powerful and self-renewing cells of hematopoietic system, which is able to produce all blood cell types via intermediate ancestor's cells (6).

In the early stage of embryonic life; specially at neural tube stage, mesodermal cells migrate to the yolk sac (YS) to form blood islands called hemangioblast, which is a common precursor for generation of both the first blood cells, and endothelial cells to form the first vascular structures in the embryonic life (1,2,3).

In these blood islands primitive blood cells are produced, includes large nucleated erythrocytes which are three to six times larger than that of adult erythrocytes, lymphocyte and rare precursors of megakaryocytes (1,2). After decline of the YS which begins at 6th week and end in about the second months of embryonic life (2,6); as in figure 2, the primitive wave switches over into the definitive wave, in which produces functionally more complex erythroid - myeloid progenitors (EMPs) (1).

1.1.2. Multipotent-progenitors are formed during a definitive wave

The second wave of hemogenesis temporarly produce EMPs with more complex function, these enucleated cells express high levels of the tyrosine receptor kinase and several markers: CD117, CD41, and CD16/32 (1,3), and express of adult (βmajor) globin. However, EMP is differing from HSC by lacking lymphoid cell potential, which is generated separately in the same wave (1). Taken together, the

second wave cells with several hematopoietic transcription factors play important role in the generation of the third wave that promote generation of HSC in the embryo (5,6).

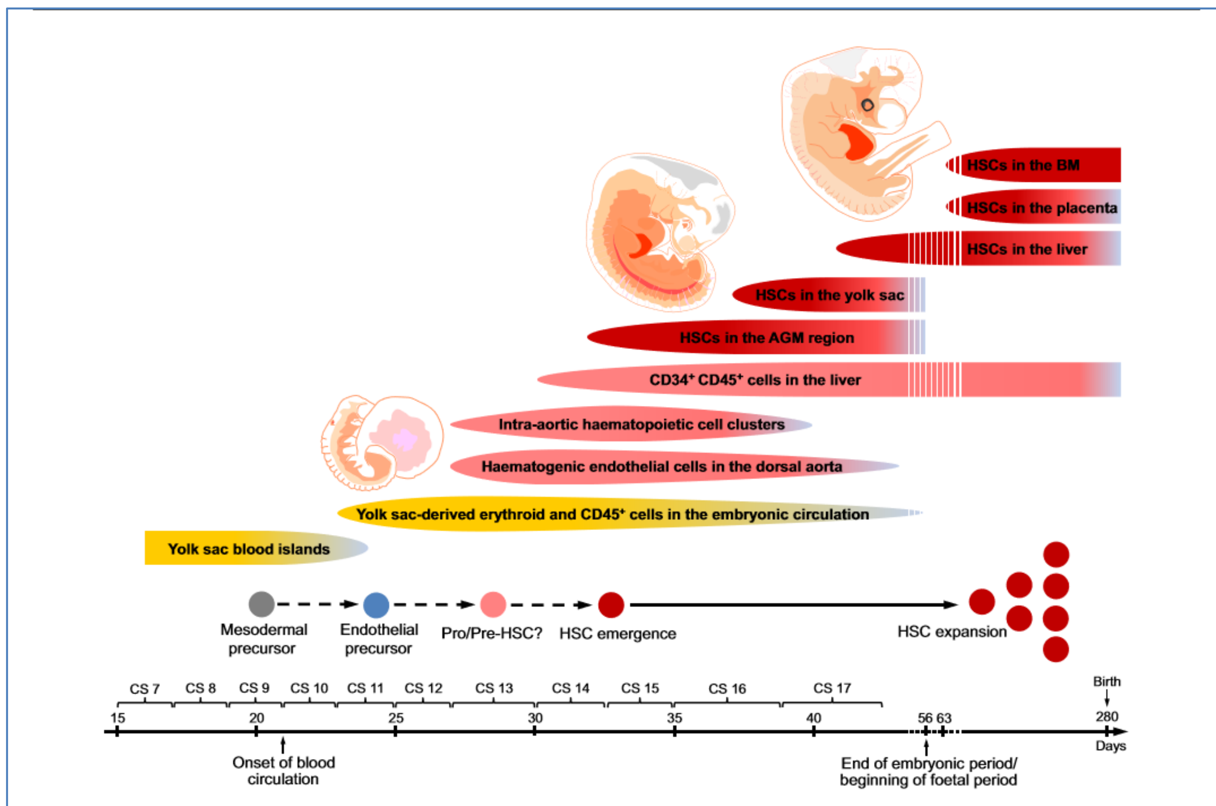

Figure 2: Chronology of human hematopoietic development.

(**Andrejs Ivanovs and Alexander Medvinsky. Human haematopoietic stem cell development: from the embryo to the dish .Published by The Company of Biologists Ltd. Development (2017) 144, 2323-2337).**

1.1.3. Hematopoietic stem cells (HSCs) generation

hematopoietic stem cells the most potent and self renewing cells, that differentiate to produce all functional blood cells types (7). The site of blood cells production moves into the fetal liver (FL) followed by the spleen. Until the seventh month of fetal life

and after birth; bone marrow becomes the major site of blood cells production and continues throughout adult life (2,4). But with age, hemogenesis becomes limited to specific sites such as the skull, sternum, ribs, vertebrae, and pelvis (3), see Figure 3. While yellow marrow consists of fat cells, and with age red bone marrow becomes replaced with yellow marrow (fat cells) and restricts the powerful blood cells formation. However, the yellow marrow is able to reproduce blood cells under certain stressful conditions (4).

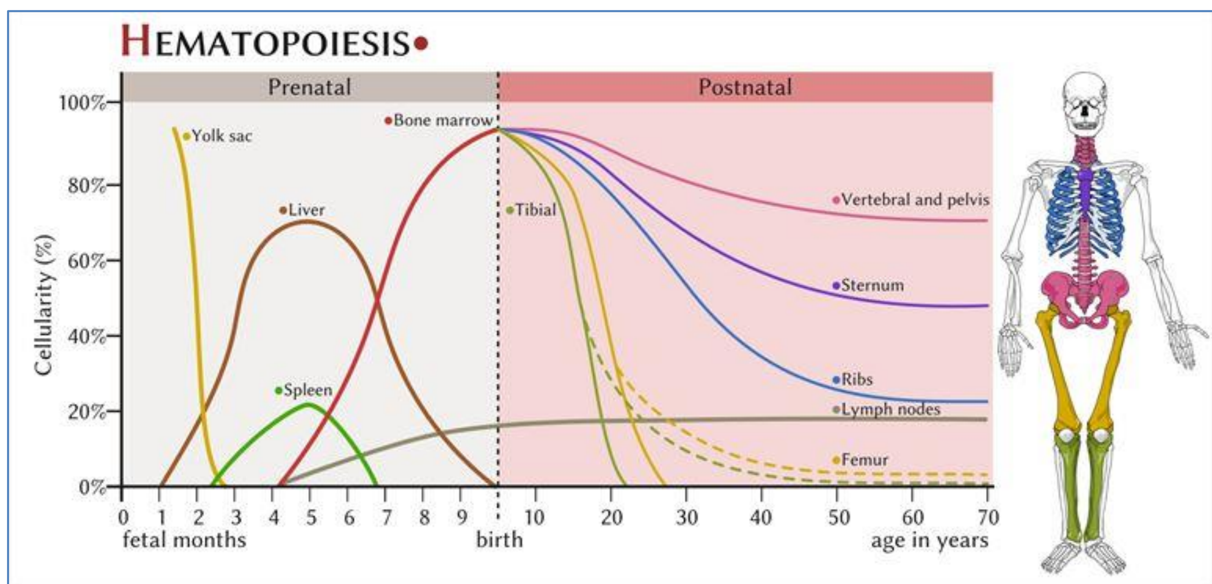

Figure 3: Chronology of hematopoiesis during human life.

(Marie L. Kotter and Susan G. Trevithick. Alteration in oxygen transport. Chapter 13, page 261. Basicmedical Key. 2017).

1.1.4. Hematopoietic Stem Cell Niche

Controlling and promoting the function of HSC such as self-renewing, differentiation, homing, and indolence is achieved through interaction between HSCs and the microenvironment of the marrow that is called hematopoietic stem cell niche (2). This interaction involves direct contact cell-to-cell, secretion of cytokines, and different growth factors that are essential for regulating the function of HSCs in hematopoiesis

(7), in order to maintains the differentiation of mature blood cells from HSCs, and need continuous process of controlling to change signals and transcriptional factors triggered by the HSC niche, which either stimulate production or prevent overproduction of blood cells, therefore providing balance numbers of different blood cell types needed for life. Furthermore, the poor function of the niche may result in critical diseases such as hematopoietic malignancies (2).

1.1.5. Hematopoietic Stem Cell Markers

In the bone marrow (BM), hematopoietic stem cells constitute approximately 1/10000 of marrow cells . Thus, the identification of human HSCs in the bone marrow depends on presence of specific markers such as CD34+, CD38-, CD90+, CD45R-, and Lineage negative cells (Lin-) (2,7).

1.2. Erythropoiesis: Regulation and Niche

The most common cell population in whole blood is the red blood cells, RBCs are produced in the BM and have a life span of 120 days. Each microliter of human blood contains about ~5 X 10^6 red blood cell, the reference range of RBCs per microliter for males is 4.7 to 6.1 X 10^6 , and for females is 4.2 to 5.4 X 10^6 (3).

In the BM, the complex niche includes endothelial cells of the vascular system, hematopoietic stem cells, osteoblasts, stromal cells and the extracellular matrix, all together supports the interaction between developing hematopoietic cells and cell adhesion molecules, cytokines, and growth factors to produce mature red blood cells (erythrocytes). The cytokines triggere the initial erythroid progenitors include

thrombopoietin (TPO), granulocyte macrophage- colony stimulating factor (GM-CSF), interleukin-3 (IL-3), IL-11, and particularly stem cell factor (SCF) which stimulates different pathways such as phosphotidalinositol-3 (PI-3) kinase, Src kinases, and phospholipase C- gamma (PLC-g) (3,8). This stimulation occurs by binding a tyrosine kinase receptor called KIT. Sometimes in a certain phase, SCF and erythropoietin (EPO) cooperate to stimulate the proliferation and differentiation of the developing erythroid ancestors and play an essential role in phosphorylating the EPO receptor itself.

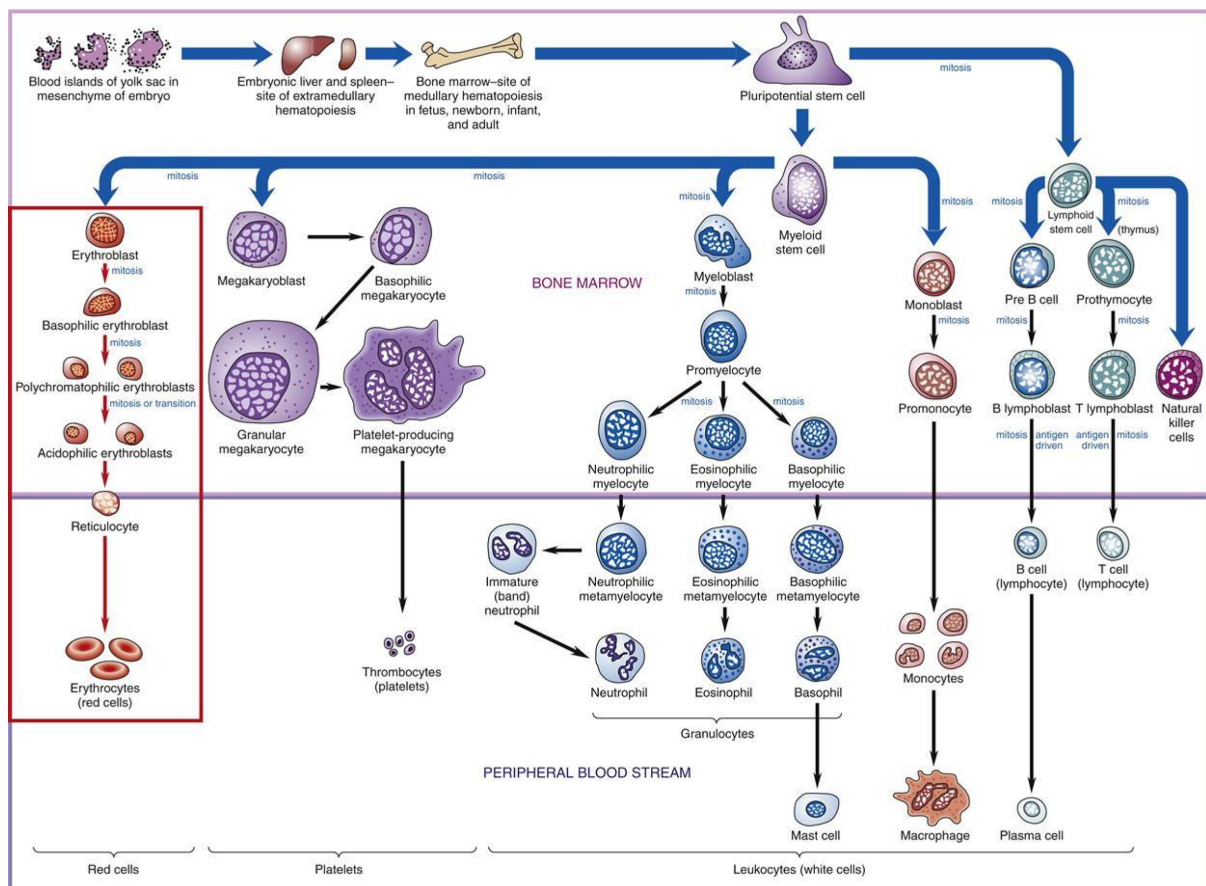

Figure 4: Hematopoiesis flowchart.

(Marie L. Kotter and Susan G. Trevithick. Alteration in oxygen transport. Chapter 13, page 259. Basicmedical Key. 2017).

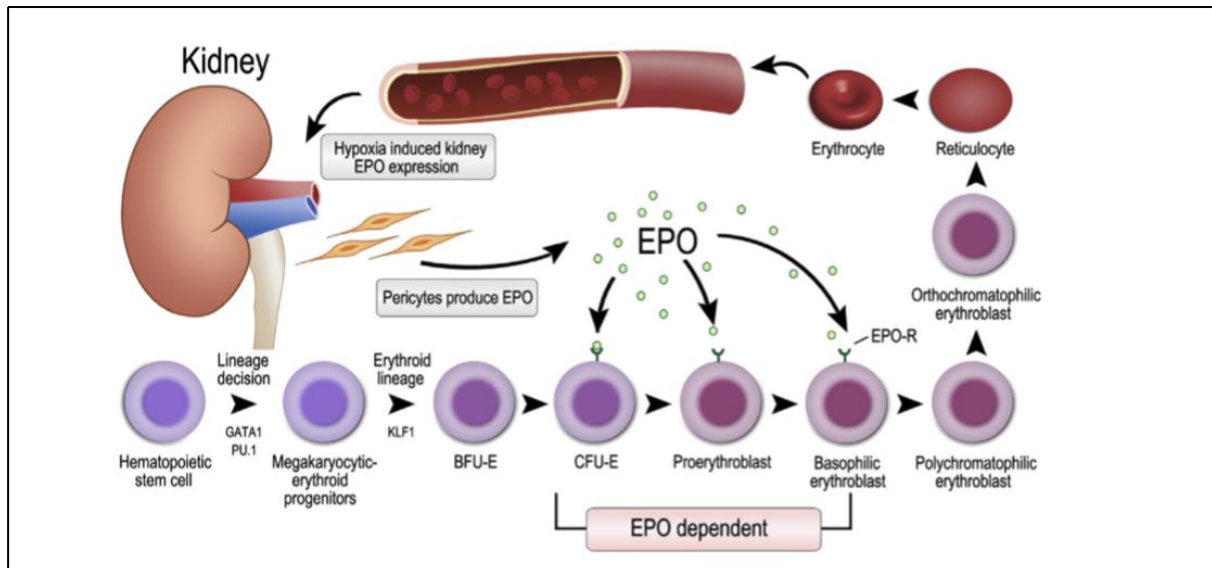

Figure 5: Stages of erythroid differentiation and oxygen dependent feedback loop regulated by kidney EPO.

EPO, erythropoietin; EPOR, erythropoietin receptor; KLF1, Kru ̈ppel-like factor 1; BFU-E, burst-forming unit-erythroid; CFU-E, colony-forming unit-erythroid. (Hong-Mou Shih and Shuei-Liong Lin. Physiology and pathophysiology of renal erythropoietin-producing cells. Journal of the Formosan Medical Association (2018) 117, 955e963).

At the final phases of differentiation, the erythroid ancestors, erythroid cells are no longer capable to proliferate because they have lost their nucleus, mitochondria, and endoplasmic reticulum. Approximately ~2.4 x 10^6 new erythrocytes have to be produced in each second, in order to save the red blood cell number in the ~5 L of blood volume of an adult person (3). Mature RBCs have a small size with a diameter of 6-8 mm and biconcave shape that increase surface area of erythrocytes. This is an important feature for the function of RBCs during gas exchange which facilitates easy entering of the cells into the small capillaries in different tissues in the body (8).

In normal conditions, about ~1% of the erythrocytes are cleared every day and replaced by new cells (3). However, this rate can increase in response to hypoxic stress when insufficient oxygen supply to tissues or insufficient numbers of functional

erythrocytes. In these cases, EPO production in kidney is increased to stimulate production of more erythrocytes in the BM to compensate for the hypoxic conditions. Furthermore, in the bone marrow there are other factors involved to support and play a role in expansion of the erythroid progenitor self-renewal, such as bone morphogenetic protein4 (BMP4)/SMAD5), signal tranceducer and activator of transcription 5 (STAT5) , SCF/ KIT, and the glucocorticoid receptor (GR) (3,8).

However, increasing erythrocytes production under erythropoietic stress may decrease the rate of apoptosis of erythroid progenitors. While increasing rate of apoptosis may occur due to failure to up-regulate expression of anti-apoptotic protein. Furthermore, under stress conditions the FAS/FASL system which contributes to the apoptotic process, it may reduce the expression of FAS receptors and FASL ligands in erythroid cells in order to increase their survival (3).

Chapter 2: Literature review

2.1. Myeloprolifertaive Neoplasms - Historical perspective

The term myeloproliferative disorders (PMDs) was first described by Damashek in 1951 (9,10,11), as a group of acquired malignant disorder characterized by excessive production and proliferation of hematopoietic cells and fibroblasts in the bone marrow (12,13).

2.1.1. Definition and classification

The chronic myeloproliferative disorders (CMPDs) was described as a group of heterogeneous diseases. CMPDs originate from the clonal expansion of the hematopoietic pluripotent stem cells, and leads to excessive and over production of one or more of the blood formed elements in the peripheral blood, which are red blood cells, white blood cells and platelets (14,15).

According to the World Health Organization (WHO) 2008 classification and the 2016 revision (13,15), the term CMPD was replaced with classical myeloprolifertaive neoplasms (MPNs), that encompass both chronic myelogenous leukemia (CML) and BCR-ABL negative MPNs (Philadelphia-negative MPNs; PN-MPNs) (16,17); characterized by excessive production of terminally differentiated fully functional blood cells. PN-MPNs are classified into three clinical entities: Polycythemia Vera (PV) that is overproduction of erythrocytes (18), Essential Thrombocythemia (ET) which is overproduction of platelets, and Primary Myelofibrosis (PMF) characterized by the prominence of marrow fibrosis and extramedullary hematopoiesis in the liver and spleen (17). These disorders occur due to presence of different somatic point

mutations; that include *JAK2 V617F* gain- of- function mutation in the Janus kinase 2 (JAK2) gene, calreticulin (*CALR*) mutation (19,20), and myeloproliferative leukemia (MPL) virus oncogene termed as (*W515L/K*) (21,22). The frequencies of these mutations are ~95%, 0%, and 0% in PV; 60%, 20%, and 3% in ET; and 60%, 25%, and 7% in PMF, respectively (23,24). However, these mutations can't completely explain the phenotypic heterogeneity of PN-PMNs and the ability of development to acute myeloid leukemia (AML) (25,26).

In European countries and based on several European studies, the annual incidence rate of PN-MPNs ranges from 0.4 to 2.8 for PV, 0.38 - 1.7 for ET, and 0.1-1.0 for PMF, per 100,000 per year (24).

Furthermore, and according to the WHO classification system for hematopoietic tumors, MPNs constitute one of the five categories of myeloid malignancies (27,28), and this research project will study the molecular characteristics of PV among Palestinian population, in order to increase the understanding of molecular and cellular biology aspects of PV, that could lead to improve both diagnosis and management of PV patients.

2.2. Polycythemia Vera (PV)

PV was first described by Vaques in 1892 then Osler in 1903, as a chronic disease characterized by cyanosis, polycythemia and splenomegaly (29). Increasing in both granulocytes and megakaryocytic precursors was added as important tools to characterize PV in 1904 by Turk (30,31). Whereas not until 1935, Hirsch added the presence of marrow fibrosis to the hematological findings of PV. In 1967 Wasserman had founded formally the Polycythemia Vera Study Group (PVSG) and data continue

to be used in the management of PV (11,21). The PVSG conducted a number of clinical trials in PV including its seminal contribution of drug leukemogenicity associated with chlorambucil and p32. The PVSG is also credited for the first consensus diagnostic criteria in PV, which have since undergone substantial revisions by the WHO subcommittee for classification of myeloid malignancies (11,21,32).

2.2.1. PV definition

PV is a chronic abnormality of the hematopoietic stem cell, characterized by uncontrolled proliferation of erythroid, granulocytic and megakaryocytic cells, and complicated by extramedullary hematopoiesis, myelofibrosis, and acute leukemia (33,34). PV should be distinguished from secondary polycythemia in which the erythrocytes are only increased from an increased erythropoietin hormone to compensate the hypoxic conditions, and relative erythrocytosis which is due to decrease in plasma volume (35,36). The median age at diagnosis is 65 years (21) and PV may be found in all age ranges and a male predominance was noted (11,21).

The 2008 WHO criteria for diagnosis of PV requires meeting both major criteria and one minor criterion, or the first major criterion and two minor criteria (18,19).

The major criteria: Hemoglobin (Hb) > 18.5 g/dL (men), Hb > 16.5 g/dL (women), or Hb or Hematocrit (Hct) > 99th percentile of reference range for each of age, sex, or altitude of residence, or Hb >17 g/dL (men), Hb >15 g/dL (women) if associated with a sustained increase of ≥ 2 g/dL from base line that can't be attributed to correction of iron deficiency, or elevated red cell mass >25% above normal mean predicted value, And presence of *JAK2 V617* or similar mutation.

The minor criteria: Bone marrow trilineage myeloproliferation, subnormal serum erythropoietin (EPO) level, and endogenous erythroid colony (EEC) growth. However, recently the 2016 WHO criteria for diagnosis of PV has changed as in shown Table 1.

Table 1: 2016 WHO criteria for diagnosis polycythemia vera, (Barbui et al. Blood Cancer Journal (2018) 8:15)

Table 1 2016 World Health Organization diagnostic criteria for polycythemia vera and essential thrombocythemia

	Polycythemia vera (PV)[a]	*Essential thrombocythemia (ET)*[b]
Major criteria		
1	Hemoglobin > 16.5 g/dL(men)Hemoglobin > 16.0 g/dL (women)or Hematocrit > 49% (men) Hematocrit > 48% (women) orincreased red cell mass (RCM)[c]	Platelet count ≥ 450 × 10^9/L
2	BM biopsy showing hypercellularity for age with trilineage growth (panmyelosis) including prominent erythroid, granulocytic and megakaryocytic proliferation with pleomorphic, mature megakaryocytes (differences in size)	BM biopsy showing proliferation mainly of the megakaryocyte lineage with increased numbers of enlarged, mature megakaryocytes with hyperlobulated nuclei. No significant left-shift of neutrophil granulopoiesis or erythropoiesis and very rarely minor (grade 1) increase in reticulin fibers[d]
3	Presence of *JAK2* or *JAK2* exon 12 mutation	Not meeting WHO criteria for *BCR-ABL1* + CML, PV, PMF, MDS, or other myeloid neoplasms
4		Presence of *JAK2*, *CALR* or *MPL* mutation
Minor criteria		
1	Subnormal serum erythropoietin level	Presence of a clonal marker (e.g., abnormal karyotype) or absence of evidence for reactive thrombocytosis

Table adapted from Barbui T et al. *Blood Cancer J* 2015; 5:e337[103] and Arber et al. *Blood* 2016;127:2391–2405[2]
BM, bone marrow; *CML*, chronic myeloid leukemia; *MDS*, myelodysplastic syndrome
[a]PV diagnosis requires meeting either all three major criteria or the first two major criteria and one minor criterion
[b]ET diagnosis requires meeting all four major criteria or first three major criteria and one minor criterion
[c]More than 25% above mean normal predicted value
[d]Grading of BM fibers[87]
Criterion number 2 (BM biopsy) may not be required in cases with sustained absolute erythrocytosis: hemoglobin levels. 18.5 g/dL in men (hematocrit, 55.5%) or 16.5 g/dL in women (hematocrit, 49.5%) if major criterion 3 and the minor criterion are present. However, initial myelofibrosis (present in up to 20% of patients) can only be detected by performing a BM biopsy; this finding may predict a more rapid progression to overt myelofibrosis (post-PV MF)

These guidelines differed from those of WHO 2008 because of :

- The discovery of new molecular findings that provide deeper knowledge to understand the pathology of MPNs, which enhance the good influence on diagnosis and outcome.

- Reducing the diagnostic thresholds of both hemoglobin (Hb) and hematocrit (Hct) with the introduction of masked PV (mPV), which markedly changed the diagnostic landscape of this MPN subtype and, therefore, treatment options and outcomes, revealing that PV has not been diagnosed in the past.
- In this context, the histology of BM was increased from insignificant to the main diagnostic criterion by recognizing its reproducible characteristics of morphological features (20,21,22).

Thus, depending on the accuracy of the initial diagnosis and the discovery of the genetic abnormality in almost all of the patients with PV, leads to enhance their treatment, management and outcomes.

2.2.2. Janus Kinase 2 (JAK2) and PV pathogenesis:

JAK2 is a cytoplasmic tyrosine kinase encoded by a gene on the short arm of chromosome 9 (37), that interact with several receptors for cytokines which are important in regulating hematopoietic differentiation (38,39), and function including those for EPO and TPO (40).

In 2002, the JAK2 locus was found affected in 30% of PV patients that include a 9p loss of heterozygosity (41). The discovery of JAK2V617F mutation in 2005 has significantly contributed toward knowledge and understanding of the MPNs pathogenesis (42,43).

The JAK family contains 4 kinases: JAK1, JAK2, JAK3 and tyrosine kinase 2 (TYK2) that attach to cytokine receptor cytosolic domains and lacks intrinsic tyrosine kinase activity (44,45). JAK2 kinase structure has four distinct domains (46), the two highly

similar domains present at the carboxyl terminus: the first one is an active kinase domain called JAK homology-1 (JH1), and the second is an “inactive” catalytically pseudokinase domain (JH2) which functions as a negative regulator of the JH1 kinase activity (47,48). At the N-terminus of the JAK kinases, the JH5-JH7 domains called a FERM contains Band-4.1, ezrin, radixin, and moesin, that take place in the connecting to the cytosolic domain of cognate cytokine receptors (49,50), see Figure 6. The last domain is called SH2 that mediates interactions with negative or positive regulators of JAK kinase activity (48).

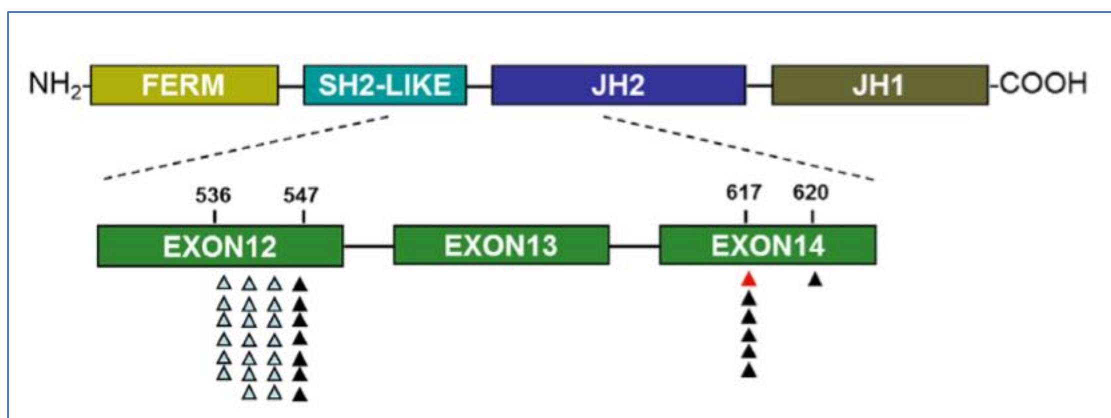

Figure 6: Schematic representation of JAK2 gene and mutation hotspots.

Locations of JAK2 mutations are indicated by colored triangles: red for V617F, black for other SNP mutations, and blue for insertions/deletions. Representative codon numbers are marked above exon boxes. (Jerald Z. Gong and et al. Laboratory Practice Guidelines for Detecting and Reporting JAK2 and MPL Mutations in Myeloproliferative Neoplasms. Jmd. 2013; Vol. 15. No 6.).

JAK2 plays an important role in the signaling of “myeloid” cytokine receptors includes erythropoietin receptor (EPO-R), myeloproliferative leukemia (MPL); thrombopoitin receptor (TPO-R), and granulocyte colony- stimulating factor receptor (G-CSF-R), and it is the only JAK can mediate the signaling of (EPO-R) and (MPL)(48). JAK2 also maintains trafficking of these 2 receptors to the cell surface

and provides their stability (51). Therefore, presence of certain mutation may affect the work of these functional units, specially the function required for the promotion of *JAK2V617F* signaling.

2.2.3. Activation of kinase JAK2

In case of low level of cytokine, JAK2 is normally phosphorylated on serine-523 and tyrosine-570 of JH2 domain (48): This will inhibit the interactions with the JH1 domain, and resulting in suppressing the activity of JAK2. While in case of cytokine binding with its receptor, this makes conformational changes of the receptor and JAK2 that activates kinase JAK2 by auto phosphorylation on both tyrosine 1007 and 1008, and decrease the level of phosphorylated serine-523 and tyrosine-570 (13,51).

In erythroid precursor cells; activation of JAK2 includes two signaling pathways; canonical and non-canonical (52). Both are induced by binding EPO with EPOR, which makes structural changes within EPOR and JAK2, and leads to start auto-phosphorylation of tyrosine residues in the cytoplasmic domain that provides docking sites to initiate signaling pathways of cytoplasmic proteins. The canonical signaling pathway activates proteins such as STAT5 which is one of signal- transducer- and-activator- of- transcription (STAT) family, and also activates the RAS-MAPK and PI3-AKT signaling pathways that prevent apoptosis of hematopoietic progenitor cells by promoting survival and proliferation (52,53). Accumulation of STAT5 protein dimmers then translocation into the nucleus and binding to its target sites in the regulatory regions, that results in transcription of target genes such as suppressor of cytokine signaling-1 (SOCS1) and serine/ threonine kinase (PIM1); SOCS1 responses for proteosomal degradation, and PIM1 supports SOCS function by providing phosphorylation and stabilization to these proteins, in order to enhance a negative

feedback loop to suppress JAK/STAT signaling pathway. Although the non-canonical signaling pathway is unclear, but it has an important function in the relaxation of heterochromatic region of the genome, and allow transcription of functionally necessary genes. JAK2 kinase makes epigenomic alterations that leads to rendering oncogenic effects. This occurs by phosphorylation of tyrosine-41 of histone H3 and prevent engagement of the transcriptional repressor heterochromatin protein-1α (HP-1α) to this site, leading to increase expression of the oncogene lmo2, and the levels of phosphorylated H3Y41 increased by increasing the activity of JAK2, therefore the affinity of HP-1α for histone H3 decreased by increasing the levels of phosphorylated H3Y41. Therefore, JAK2 inhibitors suppress H3Y41 phosphorylation and enhance chromatin-bound HP-1α in cells, leading to repressing HP-1α-regulated genes (52), see Figure 7.

2.2.4. The founding mutations

The *JAK2V617F* or JAK2 exon 12 mutations inconveniently activate the intracellular signaling molecule Janus kinase 2 (JAK2) as shown in Figure 8, and these are the most important acquired genetic mutations that have been accounted in 96% and 3% of PV cases respectively (46).

JAK2V617F mutation

In exon 14 of the gene JAK2, changing of guanine to thymine at nucleotide 1849 leads to the *JAK2V617F* mutation that convert a valine to a phenylalanine at codon 617, which is located between JH1 and JH2 domains, resulting in preventing the inhibition of the JH2 domain on the kinase domain, by changes in JH1-JH2 conformation and adenosine triphosphate (ATP) binding (53). In the absence of cytokine receptor ligation the expression of JAK2V617F has been shown to allow for

JAK2 signaling, but the expression of type I cytokine receptors and a functional FERM domain are still required for JAK2 signaling and cytokine-independent growth. Furthermore, the *JAK2V617F* mutation may allow for the escape from negative regulation by the suppressor of cytokine signaling 3 (SOCS3). Thus, constitutive activation of the JAK2 kinase which activate the downstream pathways STAT5, PI3K, AKT (or protein kinase B) and mitogen activated protein kinase (ERK)(54), However, there is evidence that *JAK2V617F* can increase the production of reactive oxygen species and reduce the apoptotic response to DNA damage by inhibiting the Bcl-xl deamidation pathway (55). All of which are implicated in EPO signaling and regulate cell proliferation, differentiation and apoptosis.

JAK2 exon 12 mutations

In the *JAK2V617F*-negative PV (5%), various somatic gain-of function mutations in exon 12 of JAK2 were detected (56). A literature review published four years ago; has reported a 37 different variants in the distal half of JAK2 exon 12; from 172 individual cases , these variants included 20 deletions variants, six duplications variants, and eleven non-synonymous base (13). These mutations are located in the region between the SH2 and JH2 domains, a region between amino acids 536 and 547, around Lys 539. The most frequent mutations being the *N542-E543del* (23%), *E543-D544del* (11%), *F537-K539delinsL* and *K539L* (10%) (15), and the *R541-E543 delinsK* (8%) (26). Furthermore, these mutations are not localized in the pseudokinase domain, but they can modify the structure of the JH2 domain in a very similar way to *V617F*, which also induce constitutive activation but to a greater degree than *JAK2V617F*, with greater JAK2 phosphorylation and MAPK pathway activation (55), and also result in cytokine-independent growth. Along these lines, it was shown that the F595 residue, located in helix C of the pseudokinase domain, is

necessary for both the *V617F* mutants and *K539L*, but not important for cytokine-induced JAK2 activation (13,26).

Unlike *JAK2V617F*, exon 12 mutations are associated with only erythrocytosis phenotype (7,26), and not associated with ET and PMF, thus it could be diagnosed as PV or idiopathic erythrocytosis (IE). Despite these differences, JAK2 exon 12 PV have the same incidence rates with *JAK2 V617F* PV for thrombosis and bleeding, and can progress to a secondary myelofibrosis (55).

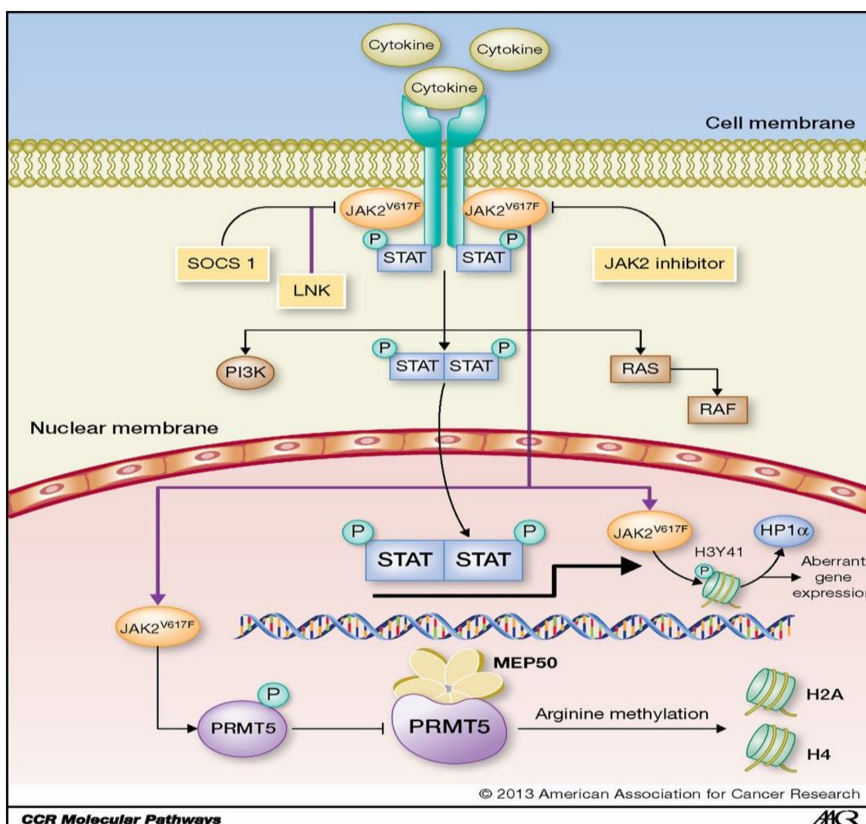

Figure 7: JAK/STAT pathway in MPNs.

(Alfonso Q. and Srdan V. Molecular Pathways: JAK/STAT Pathway: Mutations, Inhibitors, and Resistance. Clin Cancer Res; 2013; 19(8); 1933–40).

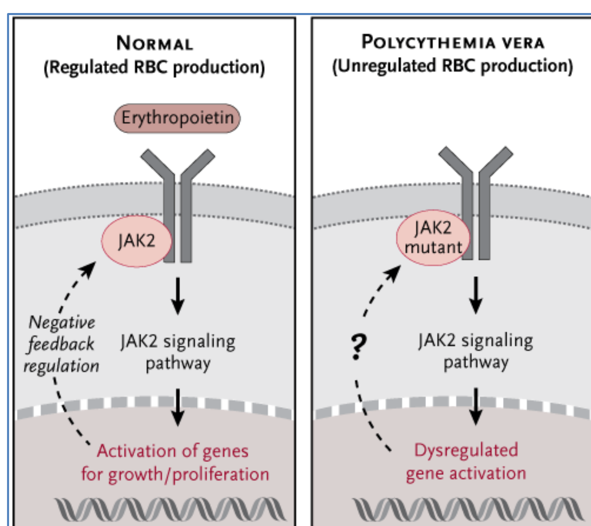

Figure 8: Normal vs. JAK2 mutant signaling in PV.

(Yvonna Reekie. Small-molecule Kinase Inhibitor Shows Promise for Treating Family of Blood Disorders. Cancer Drug Takes Fast Track to Clinical Trials | Harvard Medical School. April 18, 2008).

2.2.5. Cellular consequences of JAK2 mutations

Mutations in JAK2 have been shown to occur in cells near the top of the hematopoietic hierarchy and can be found in the hematopoietic stem cell (HSC) compartment (56,57). These observations are consistent with the long-term persistence of JAK2-mutated MPNs, and the mutation can be found in cells of the lymphoid as well as myeloid lineages in some cases (58). Several lines of evidence indicate that *JAK2 V617F* does not confer an advantage at the HSC level. Xenografts of JAK2-mutant patient cells into immunodeficient animals suggest that JAK2 mutations do not result in a strong self-renewal advantage (21); a finding that is recapitulated in several knock-in mouse models (59,60). Instead, JAK2 mutant HSCs are skewed towards symmetrical differentiation with a subsequent expansion of the progenitor pool, rather than self-renewal, and not demonstrate an advantage in competitive transplantation experiments. These observations suggest that *JAK2V617F* alone is not enough for initiation the disease and required additional

mutations. This is consistent with the detection of the *JAK2 V617F* mutation on its own in patients without overt myeloid malignancy (61,62). Another explanation for these findings is that the expansion of the progenitor (rather than the stem cell) pool is sufficient to mediate disease development (63), an idea that is reinforced by recent studies which demonstrate that a pool of long-term multipotent progenitors are the main drivers of adult hematopoiesis (64). Finally, it is likely that there is functional heterogeneity within the JAK2-mutated HSC pool and across disease subtypes (65). For instance, there is evidence that the ability to self-renew, and therefore stably engraft, may decrease with increasing levels of JAK2 expression, similar to the differences in stem cell behavior seen at different expression levels of STAT5 and oncogenic NRAS (66).

2.3. Clinical features:

PV usually has an insidious onset as a chronic disease, and many patients are asymptomatic and are diagnosed incidentally on routine blood work. But some patients with PV have a symptomatic burden that impacts quality of life (21). Patients may present with thrombosis and or bleeding secondary to erythroid expansion, hyperviscosity, and elevated platelets. Symptoms that negatively impacted quality of life, with their associated prevalence based on survey data of 402 patients, included fatigue (97%), insomnia (58%), pruritus (40%), sexual dysfunction (51%), abdominal discomfort (45%), early satiety (62%), difficulty with concentration (58%), and sad mood (57%) (67). Also symptoms may include headache, epistaxis, ischemic or hemorrhagic, stroke, angina, myocardial infarction, and claudication.

Other complication of PV include gout in 5-10% (21) and increased risk for peptic ulcer disease due to increased histamine release from basophiles. Furthermore, splenomegaly is the most common physical findings in PV (50%-80%).

2.3.1. Prognosis and Risk Factors for PV Complications

The most complications associated with PV include its thrombotic tendency, a long-term possibility of evolution to MF or AML, and compromised longevity (Figure 9) (68). According to the European Collaboration on Low-Dose Aspirin in Polycythemia Vera (ECLAP), the risk of thrombosis based on data from 2004 was 4.4% of patients per year (69). Recently, the Cytoreductive Therapy in Polycythemia Vera (CYTO-PV) study found the risk of thrombosis at 2.7% of patients per year (70); this lower rate may be reflective of more aggressive treatment. Traditionally, thrombosis risk assessment has been based on age and thrombosis history. Furthermore, some analysis of the CYTO-PV data identified leukocytosis as an additional risk factor for thrombosis (71). Other contributing mechanisms for thrombosis may include inflammatory stress, activation of the endothelium and platelets, and activated protein C resistance (72) and increased *JAK2V617* allele burden (73) considered as critical risk factor.

Additionally, a long-term consequence of PV is evolution to post PV MF, which has a prevalence of approximately 5% at 10 years and 6% to 14% at 15 years (74). The risk factors for progression to MF include older age, leukocytosis, splenomegaly, marrow fibrosis at diagnosis, JAK2 allele burden greater than 50% (75), and use of agents such as radioactive phosphorus (32P), chlorambucil, or pipobroman. Furthermore, using agent such as hydroxyurea (HU) or busulfan has a controversial association with leukemic transformation (11). In a study of 826 patients with PV at

Mayo Clinic, survival was 14 years for those older than 60 years and 24 years for those under 60 years (76). Risk factors for mortality and leukemic transformation in another recent study of 1545 patients with PV included older age, leukocytosis, thrombosis, and abnormal karyotype (11).

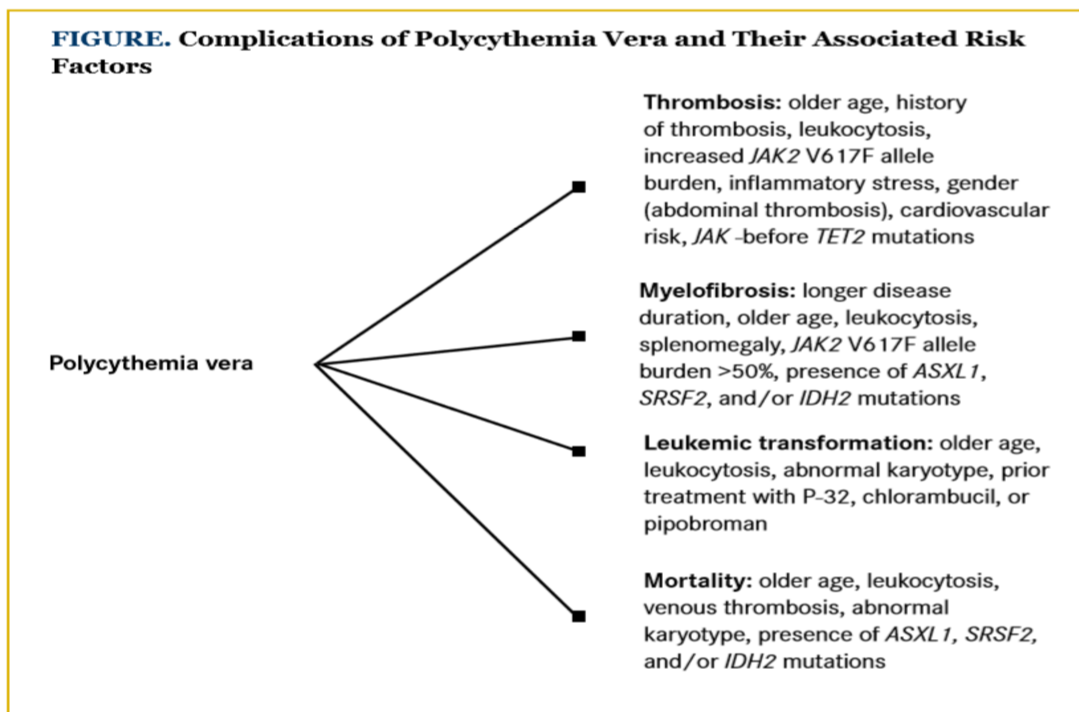

Figure 9: Prognosis and Risk Factors for Complications of PV.

(Saba S. Shaikh and Brady L. Stein. Polycythemia Vera: Contemporary Updates in Diagnosis, Prognosis, and Treatment. AJHO. 2017;13(9):23-31).

2.4. Transformation into acute myeloid leukemia

As mention previously; the MPNs include group of diseases associated with a high prevalence of *JAK2V617F*, uncontrolled production of mature blood cells, and

variable risk rates of transformation into acute myeloid leukemia (AML) (77), that increased specially after treatment with genotoxic therapy.

The risk of leukemic transformation in PV over 10 years period was found to be approximately 4%, in ET 1%, and in PMF 20% (13,27). MPN patients with secondary AML have bad outcomes, median survival of less than 6 months, and not improves by using standard AML therapies (27). Several articles have shown that the presence of two or more somatic mutation in an MPN patients significantly increased the risk of leukemic transformation in 25% of patients with ET, and in 36% in patients with PV or MF(13). There are several mutations that are associated with secondary leukemia transformation such as, mutations in *ASXL1, SRSF2, IDH1/2, TET2, CBL,* and *LNK*. Also mutation in *RUNX1* (30% of the cases), *FLT3*- internal tandom duplication (15%), *N-RAS*, *NFI*, and deletion in *1KZF1* and *CUX1* (9,15).

Tumor protein 53 (*TP53*) a major tumor suppressor gene, encodes p53 protein, which is involved in controlling cell-cycle checkpoints and apoptosis. TP53 mutation was found in 40% to 50% of secondary leukemia of MPNs (15). And this usually includes deletions or missense mutations in the TP53 gene, or amplification of chromosome 1q targeting MDM4; a TP53 transcriptional inhibitor (15). In early MPN heterozygous TP53-mutated clones can be found, but remain low until complete loss of tp53 by losing the second allele, in other words; until transition from heterozygosity to homozygosity for the mutation (78), that followed by rapid clonal expansion. The presence of *TP53* mutation associated with poor prognosis, poor overall survival, and reduced response to chemotherapy (79,80). Presently, the only mouse model which leads to leukemia is the JAK2V617-positive in a *TP53*-null, and they died within 100 days of transplantation (13,15). The important factors for developing secondary AML are old aging, chronic inflammation, abnormal karyotype,

and predisposing factors like presence of more than one mutation which increase the development of the disease (80).

2.5. General Objective

In this study, finding the genetic abnormality such as *JAK2V617*, JAK2 exon 12 such as *F537-K539delinsL* and *K539L* mutations, in Palestinian patients with PV will lead to improve our understanding and the ability to manage its treatment, diagnosis and management.

2.6. Specific Objectives

- To determine the frequency of the presence *JAK2 V617F*, JAK2 exon 12 mutations include *F537-K539delinsL* and *K539L* in blood cells of patients suspected to have Polycythemia Vera.

Chapter 3: Materials and Methods

3.1. Study design:

A case-control study design was used in this research project.

Samples were collected from Palestinian Ministry of Health (MOH) hospitals in the West Bank, and included; Central Blood Bank (CBB)-Ramallah, Blood Bank of Alia Governmental Hospital-Hebron and Jenin Governmental Hospital.

3.2. Sampling

Patients group 1; included 52 patients suspected to have Polycythemia Vera according to the guidelines of the 2016 WHO diagnostic criteria, they meet the following criteria: Hb > 18.5 g/dL for men and Hb > 16.5 g/dL for women, so criterion number 2 (BM biopsy) not required, and subnormal serum EPO level (either normal or low).

The subjects of control group were 46 donors, selected to be free of erythrocytosis and other PV features, but match the patients group in number, gender and age. The third group is (patients group 2); of 31 subjects that collected according to 2016 WHO criteria; with Hb level above 16.5 g/dL and below 18.5 g/dL for men, but without making BM biopsy.

All subjects with pulmonary disease, high EPO level, high-altitude living and athletes were excluded.

Standardized questionnaire (shown in Appendix B), was prepared and completed by each individual of all groups, in order to obtain relevant details of current health status, age, gender, health history and life style.

3.3. Specimens collections:

Peripheral blood samples (volume 5 ml) were collected by venipuncture into EDTA and plain tubes, following aseptic technique from PV patients and control individuals. The collected sample was used to perform the following tests: Complete Blood Count (CBC) using automated cell counter, EPO serum level, and DNA extraction.

3.4. Methodology:

3.4.1. **CBC test**: was done for each individual patients and control groups, using automatic blood cell counter machine (sysmex-XP-300).

3.4.2. **Serum EPO level**: EPO is a hormone, produced primarily by the kidneys, and act as a hematopoietic growth factor, that is responsible of differentiation and maturation of erythroid progenitors. Physiologically Increased production of EPO occurs in case of anemia and hypoxia that leads to secondary polycythemia, while in primary PV; normal or reduced EPO level must be found. Therefore, serum EPO level was done for all study samples, in order to distinguish between the different types of polycythemia.

Procedure principle:

The test was done using IMMULITE/1000 EPO, which is a solid-phase, enzyme-labeled chemiluminescent immunometric assay, and based on manufacture protocol. The reference range for the kit used is 5.4 to 31 mIU/ml. Quality control samples

were used with each run. Any sample has EPO level above the 31 mIU/ml was excluded.

3.4.3. Molecular methodology:

3.4.3.1. DNA extraction

The QIAamp DNA Blood, mini kit 250 (lot number of 154050024), was used for DNA extraction according to insert in the kit provided by the manufacturer.

DNA concentration was determined using Thermo nanodrop lite spectrophotometer, by measuring the absorbance at wavelength of 260 nm. And the purity by determining the absorbance ratio at wavelength 260/280 nm. In addition, purity of the extracted DNA was checked on 2% agarose gel electrophoresis as shown in Figure 10.

3.4.3.2. Amplification-refractory mutation system (ARMS) PCR / Allele-specific PCR for detection JAK2V617F

ARMS-PCR, is considered one of the most sensitive (0.01% - 5%) methods to detect changes in a single base conversion mutation from G to T mutation in the JAK2 gene (39,93).

For analysis of allelic variants, 4 primers ARMS-PCR technique were used to detect *JAK2V617F* mutation (Table 2) as follow: a primer pairs of Forward Outer (FO) and Reverse Outer (RO) were used first to amplify a product of 463 bp. Then, this product was used as template for the next two separate reactions; to detect wild type allele a primer Forward Wild-type specific (FWT) with a primer RO were used to generate a band of 229 bp. To detect mutant allele; a primer Reverse Mutant specific

(RMT) with a primer FO were used to generate a band of 279 bp. All PCR products were electrophoresed on 2% standard agarose gel at 5V/ 1cm of gel for 45 minutes, and visualized after staining of ethidium bromide (stock 10mg/ml) under UV transilluminator (BIO Rad, Gel Doc^{TM} XR), and photographed (41,44).

Table 2: Primers used in the ARMS-PCR for JAK2V617F (41,44):

Primer	Sequences (5' – 3')
FO	5'-TCCTCAGAACGTTGATGGCAG-3'
RO	5'-ATTGCTTCCTTTTTCACAAGAT-3'
FWT	5'-GCATTTGGTTTTAAATTATGGAGTATATG-3'
RMT	5'-GTTTTACTTACTCTCGTCTCCACAAAA-3'

PCR amplification conditions

The PCR amplification was performed in a total volume of 25 µl, containing about 25 ng DNA, 12.5 µl (1X) of PCR Go Tag Green master mix 2X (Promega, USA), 0.5 µl (0.2 µmol) of each FO, RO and 1µl (0.8µmol) of each FWT and RMT primer. The PCR conditions on the thermal cycler (Bio RAD-T100^{TM}) were as follows:

Initial denaturation at 95^{0}C for 3 minutes, followed by 35 cycles of denaturation at 95^{0}C for 30 sec, annealing at 58°C for 35 sec, extension at 72°C for 40 sec, and the final extension step at 72°c for 7 minutes.

All amplification reactions included a no template control (NTC) as blank, and positive control (sample previously detected with heterozygosity of JAK2V617F mutation). A 100 bp DNA ladder RTU (Cat.No. DM001-R500) was used to identify the approximate size of PCR products.

3.4.3.3. Restriction fragment length polymorphism (RFLP) for JAK2V617F

The PCR-RFLP allow rapid detection of *JAK2V617F* mutation after the amplification of target genomic sequences by PCR. The restriction enzyme *BsaXI* will distinguish the *JAK2V617F* mutation (360bp); whereas *JAK2* wild type generated a fragment of (180 bp) and identified directly in stained agarose gel, thus; zygosity determination was based on *BsaXI* restriction digest patterns (38).

PCR amplification conditions

The PCR amplification was performed in a total volume of 25 µl, containing about 25 ng DNA, 12.5 µl (1X) of PCR Go Tag Green master mix 2X (Promega,USA), 0.5 µl (0.2 µmol) of each Forward and Reverse primers for RFLP (Table 3). The PCR conditions on the thermal cycler (Bio RAD-T100^{TM}) were as follows:

Initial denaturation at 95°C for 3 minutes, followed by 25 cycles of denaturation at 95°C for 30 sec, annealing at 58°C for 35 sec, extension at 72°C for 40 sec, and the final extension step at 72°C for 7 minutes.

All sample runs were included a no template control (NTC) as blank to ensure no cross-contamination occurred, and positive control (sample previously detected with heterozygosity of *JAK2V617F* mutation), and 50bp DNA ladder RTU (Cat.No. DM012-R500) was used to identify the approximate size of PCR products.

After amplified-PCR, the specific amplified product subjected to enzymatic digestion using the restriction enzyme *BsaXI* following the manufacture's protocol. The reaction mixture was prepared in a final volume of 25mcl using 1 µl of enzyme *BsaXI* (2U), 4 µl from 10X enzyme buffer(1X) supplied with the kit, 13µl of free nuclease D.W and 7µl of specific PCR-product. Then incubated at 37°C for 3 hours.

Then, by using staind agarose gel; the 360 bp amplicon remained undigested in mutant *JAK2V617F*, while *JAK2* wild type generated a fragment of 180 bp as previously described.

Table 3: Primers used in the RFLP-PCR for JAK2V617F (38):

Primer	Sequences
RFLP-F	5'- TGCTGAAAGTAGGAGAAAGTGCAT-3'
RFLP-R	5'- TCCTACAGTGTTTTCAGTTTCAA-3'

3.4.3.4. Amplification-refractory mutation system (ARMS) PCR / Allele-specific PCR for detection JAK2-Exon 12 mutations

We performed ARMS-PCR to detect JAK2 exon 12 mutations, include : the F537-K539delinsL mutation (leading to the replacement of phenylalanine at position 537 through lysine at position 539 by a single leucine), and the K539L mutation (result in replacement of lysine at position 539 with a leucine)(81). The outer primers pair used to generate a wild-type 496bp in the first amplification, this PCR product used as a template for the next PCR, and the specific forward primer with the reverse outer (separate tube for each mutation) used to generate two bands including 496bp and 342bp in case of mutant allele presence.

PCR amplification conditions

The PCR amplification was performed in a total volume of 25 µl, containing about 25ng DNA, 12.5 µl (1X) of PCR Go Tag Green master mix 2X (Promega, USA), 0.5 µl (0.2µmol) of each FO and RO . And in second PCR; 0.25 µl (0.1µmol) of forward specific primer and 0.25 µl (0.1µmol) of RO (common) primer were used (Table 4) to detect each of *F537-K539delinsL,* and *K539L* mutations in separate tubes. The PCR conditions on the thermal cycler (Bio RAD-T100^{TM}) were as follows:

Initial denaturation at 95°C for 3 minutes, followed by 30 cycles of denaturation at 95°C for 30 sec, annealing at 58°C for 35 sec, extension at 72°C for 40 sec, and the final extension step at 72°C for 7 minutes.

All sample runs were included a no template control (NTC) as blank, and 100bp DNA ladder RTU (Cat.No. DM001-R500) was used to identify the approximate size of PCR products as seen in Table 4.

Table 4: Primers used in the ARMS-PCR for JAK2-Exon 12 mutations (33,43):

Primer	Sequences	Size (bp)
FO	5'- CTCCTCTTTGGAGCAATTCA-3'	
RO(common)	5'- GAGAACTTGGGAGTTGCGATA-3'	496 bp
Forward:F537-K539delinsL	5'- CATATGAACCAAATGGTGTTAATC-3'	342 bp
Forward:K539L	5'- CATATGAACCAAATGGTGTTTTCACTT -3'	342 bp

3.4.3.5. High resolution melting curve for JAK2-Exon 12 mutations

High resolution melting curve analysis represents a new mutation scanning technology , it is simple, time saving, sensitive and specific tool for detection DNA variations including 1-bp substitution (31). The melting curve analysis is a post-PCR technique; used in the presence of a DNA-binding dye, to distinguish the variance in nucleic acid sequences, based on their different melting points of DNA fragments; by increasing the temperature; they are denatured from a double-strand to a single-strand form. The melting temperature of a PCR product depends on several factors; such as amplicon length, sequence, and GC content (33,46).

The PCR amplification was performed in a total volume of 25 µl, containing about 25ng DNA, 12.5 µl (1X) of PCR Go Taq colorless master mix 2X (Promega, USA, 0000378487), 0.25 µl (0.2µmol) of each primer (Table 4), and 1 µL of a 1000-fold dilution of SYBR Green I dye from Roche, and adjusted with free nuclease water to a final volume of 25µL, separate reaction for each mutation was done. The PCR conditions on the thermal cycler (Bio RAD-T100^{TM}) were as follows: Initial denaturation at 95°C for 3 minutes, followed by 30 cycles of denaturation at 95°C for 30 sec, annealing at 58°C for 35 sec, extension at 72°C for 40 sec, and the final extension step at 72°C for 7 minutes. All sample runs were performed with no template control (NTC) as blank.

After PCR amplification, the amplicon examined for detection melting temperature, using glass capillaries and Light Cycler instrument (Roche, Germany, Ser. No. 1404061). The PCR products were heated at 95℃ for 2 min, then cooled at 40℃ for 1 min in preparation of a melting curve analysis. The melting curve analysis was

performed afterward with an increased of 0.2ºC/s from 40°C to 95°C. Then, the melt curve data were subjected to gene scanning analysis by Light Cycler software, version 3.5.28 (Roche) to identify the melting temperature peak in melting curves, which indicate the presence of variation in target sequence. The specificity of PCR products were also checked by 2% agarose gel electrophoresis for 30min/70V. And 100bp DNA ladder RTU (Cat.No. DM001-R500) was used to identify the approximate size of PCR products.

Optimization of a Homemade SYBR Green I Reaction Mixture:

SYBR Green I homemade Real-time PCR master mix was prepared according to Karsai et al descriptions (82), and was used in this study to evaluate the effect of melting behavior of JAK2 Exon-12 amplicons. The amplification efficiency and reproducibility of the real-time PCR was compared with the SYBR Green I master mixture from Roche Molecular Biochemicals. The BSA was excluded according to Karsai et al due to better amplification. In this study, also excluding Triton X-100 gave better amplification sensitivity. The SYBR Green I was diluted in TE 1:1000 (pH 8.0) and stored at −20°C. After thawing, the SYBR Green I aliquot was kept at 4°C and protected from light.

Freshly suspended aliquots were made every 3 weeks; since the dye remain stable as long as 18 days according to Karsai et al (94), afterward the SYBR Green I degradation products become potent inhibitors of PCR. The influence of SYBR Green I concentration was assessed by using different volumes of the 1000-fold dilution for the 25 μL reaction volume: 0.4 μL, 1 μL, or 1.4 μL. Good results for all primer pairs were obtained with the 1 μL volume per reaction. A concentration of 2 mM $MgCl_2$ was first used and good results were obtained with this concentration.

3.5. Statistical Analysis

Statistical analysis for all data was performed using IPM Statistical Package for Social Sciences (SPSS) statistics version 20 . The statistical significant differences for the variables among study groups analyzed by using independent samples t-test and one way ANOVA. A statistically significant difference was defined as a p-value less than < 0.05.

3.6. Ethical consideration:

Birzeit University regulation and scientific research ethics standards were strictly adhered to during this study. All data collected maintained with privacy. And acknowledge will be for all members and participants who participated in this study.

Chapter 4: Results

A total of 129 individuals were enrolled in this study, and distributed into three groups. The first group is PV the patients who have Hb level of >18.5 g/dL for men and >16.5 g/dL for women, and consist of 52 (40.3%) individuals; of them 4 (7.7%) females and 48 (92.3%) were males. The second group is the control group that consist of 46 (35.7%) healthy individuals who have Hb level <16.5 g/dL for men and Hb < 16.0 g/dL for women; of them 4 (8.7%) were females and 42 (91.3%) were males. The third group is the impaired group and consist of 31 (24%) males who have Hb level between 16.6g/dL - 18.5g/dL, as shown in (Table 5). The participants ages among all groups ranged between 18-83 years old, with the median of 32 years old.

The raw data of hematological parameters for CBC test, DNA concentration and serum EPO results as shown in (see Appendix A). The frequencies of these results are shown in Table 6. By using independent t-test, to determine whether there is a statistically significant difference between the parameters means of each two unrelated groups; there was a statistically significant difference between groups in Hct, Hb level and RBC count (Tables 7,8,9). While there was no statistically significant difference between groups in PLTs count, WBCs count and EPO level. Also, one way ANOVA statistically test was used to determine if statistically significant difference between the three groups (Table 10). However, in this study, out of 29 individuals (22.5%) were non smoker, while about 100 individuals (77.5%) were smokers, distribution of smoking habits among study groups shown in (Table 11). Even though; the difference between level of smoking among the study groups was statistically insignificant with p-value of 0.069 (Table 12).

Furthermore; statistically significant differences between groups were found in the blood donation frequency, presence of familial high Hb (in family members) , face redness, headache and night sweating. While there was no statistically significant differences between groups in frequencies of doing exercise, suffering from splenomegaly, gout, itching, stomach ulcer and dizziness as shown in (Table12).

By using two molecular methods ARMS-PCR and PCR-RFLP; as shown in Table 13; the presence of *JAK2V617F* mutation was identified at 69.2% (36/52) in patients group 1, of them 33 (91.7%) were males with median age of 35 years (Hb and RBCs mean ± SD is 18.8 ± 1.3 g/dL, 6.4 ± 0.7 10^6/µl respectively), and 3 (8.3%) were females with median age of 64 years (Hb mean ± SD is 18.2 ± 1.3 g/dL), see Table 14. The mutation also detected at 51.6% (16/31) in males of patients group 2 who have Hb mean ± SD of 17.5 ± 0.4 g/dL, and median age of 35 years. However; it is also identified at 21.7 (10/46) in males of healthy controls group who have Hb mean ± SD of 15.3 ± 0.6 g/dL. All *JAK2V617F* positive samples have a mixed pattern of wild-type and mutant alleles, as shown in Figure 11 and Figure 12.

The analysis for detection JAK2 Exon 12 mutations was proceeded to determine the most-common mutations of JAK2 Exon 12, which are: *F537-K539delinsL,* and *K539L.* By using ARMS-PCR; the two mutations of *F537-K539delinsL* and *K539L* were not detected in all study population; producing a band 496bp of wild-type pattern as in Figure 13. The results of JAK2 exon 12 mutations; *F537-K539delinsL* and *K539L* were confirmed by using HRM, that show a melting curve peak at 76.3^{o}C, and 76.0^{o}C, respectively as shown in (Figure 14,15).

Table 5: Frequency table of Gender and age among the study groups

Group	Number	Male (%)	Female (%)	Median (years)	Minimum (years)	Maximum (years)
Patients 1	52	48 (92.3%)	4 (7.7%)	37	18	83
Control	46	42 (91.3%)	4 (8.7%)	30	19	70
Patients 2	31	31 (100%)	None	31	19	65
Total	129	121(93.8%)	8 (6.2%)	32	18	83

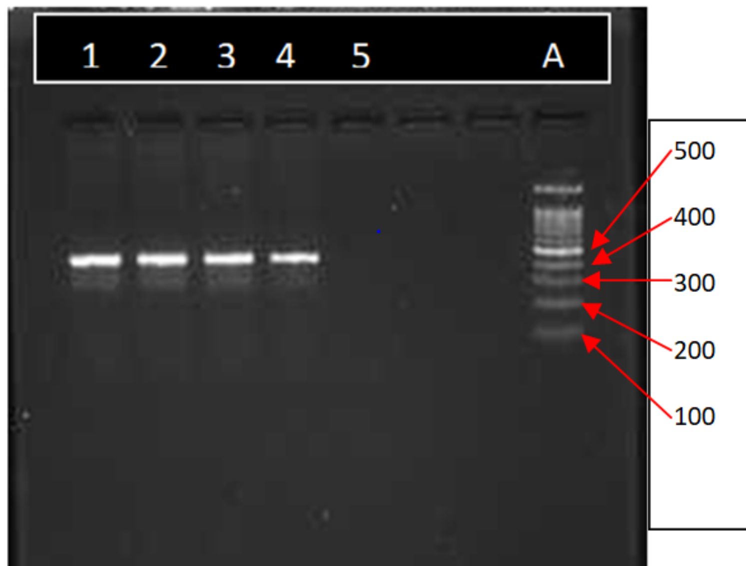

Figure 10: Agarose gel electrophoresis (2%) of extracted DNA to check purity and integrity.

Lane 1-4: DNA of different samples with 463bp, Lane 5: No template control (NTC), Lane A: 100 bp DNA ladder.

Table 6: Hematological parameters and EPO level distribution among study groups.

Group	Number	Hb (g/dL) Mean ± SD	Hct (%) Mean ± SD	RBC (10^6/µl) Mean ± SD	WBC (10^6/µl) Mean ± SD	PLTs (10^6/µl) Mean ± SD	EPO (mIU/ml) Mean ± SD
Patients1	M 48	18.8 ± 1.15	54.6 ± 2.68	6.4 ± 0.65	8.6 ± 2.97	225 ± 116	7.61 ± 5.75
	F 4	17.9 ± 1.25	53.8 ± 5.0	6.2 ± 0.56	11.1 ± 2.3	319 ± 167	7.36 ± 10.5
Control	M 42	15.4 ± 0.96	44.5 ± 2.34	5.3 ± 0.32	7.76 ± 2.07	224 ± 49.5	8.0 ± 3.97
	F 4	13.5 ± 1.56	40.6 ± 3.63	5.0 ± 0.39	7.7 ± 1.63	241 ± 39.9	8.39 ± 1.02
Patients 2	M 31	17.4 ± 0.39	50.5 ± 1.3	5.87 ± 0.33	8.45 ± 2.06	207.9 ± 51	8.56 ± 4.30

Table 7: Differences between hematological parameters and EPO level among the patients group 1 and control group.

Parameters	GROUP	Total N	Mean	Std. Deviation	p- value*
HB	Patients group1	52	18.767	1.1764	.001
	Controls group	46	15.322	1.1531	
HCT	Patients group1	52	54.552	2.8563	.001
	Controls group	46	44.189	2.6692	
RBC	Patients group1	52	6.4185	.64334	.001
	Controls group	46	5.3122	.34232	
PLTs	Patients group1	52	232.29	121.555	0.724
	Controls group	46	225.50	48.628	
WBC	Patients group1	52	8.8173	2.99187	0.045
	Controls group	46	7.7565	2.02832	
EPO	Patients group1	52	7.5964	6.09003	0.668
	Controls group	46	8.0448	3.80138	

*Statistics analysis was done using independent t-test.

Table 8: Differences between hematological parameters and EPO level among the patients group 1 and patients group 2.

Parameters	GROUP	Total N	Mean	Std. Deviation	p-value*
HB	Patients group 1	52	18.767	1.1764	
	Patients group 2	31	17.461	.3955	.001
HCT	Patients group 1	52	54.552	2.8563	.001
	Patients group 2	31	50.548	1.3339	
RBC	Patients group 1	52	6.4185	.64334	.001
	Patients group 2	31	5.8742	.33190	
PLTs	Patients group 1	52	232.29	121.555	0.293
	Patients group 2	31	207.97	51.105	
WBC	Patients group 1	52	8.8173	2.99187	0.550
	Patients group 2	31	8.4516	2.06492	
EPO	Patients group 1	52	7.5964	6.09003	0.438
	Patients group 2	31	8.5694	4.30679	

*Statistics analysis was done using independent t-test.

Table 9: Differences between hematological parameters and EPO level among the control group and Patients group 2.

	GROUP	N	Mean	Std. Deviation	p-value*
HB	Controls group	46	15.322	1.1531	.001
	Patients group 2	31	17.461	.3955	
HCT	Controls group	46	44.189	2.6692	.001
	Patients group 2	31	50.548	1.3339	
RBC	Controls group	46	5.3122	.34232	.001
	Patients group 2	31	5.8742	.33190	
PLTs	Controls group	46	225.50	48.628	0.133
	Patients group 2	31	207.97	51.105	
WBC	Controls group	46	7.7565	2.02832	0.147
	Patients group 2	31	8.4516	2.06492	
EPO	Controls group	46	8.0448	3.80138	0.575
	Patients group 2	31	8.5694	4.30679	

*Statistics analysis was done using independent t-test.

Table 10: Differences between hematological parameters and EPO level among the study population.

Parameters / Groups		Total N	Mean	Std. Deviation	P-value* Between groups
HB	Patients group 1	52	18.767	1.1764	.001
	Controls group	46	15.322	1.1531	
	Patients group 2	31	17.461	.3955	
HCT	Patients group 1	52	54.552	2.8563	.001
	Controls group	46	44.189	2.6692	
	Patients group 2	31	50.548	1.3339	
RBC	Patients group 1	52	6.4185	.64334	.001
	Controls group	46	5.3122	.34232	
	Patients group 2	31	5.8742	.33190	
PLTs	Patients group 1	52	232.29	121.555	0.460
	Controls group	46	225.50	48.628	
	Patients group 2	31	207.97	51.105	
WBC	Patients group 1	52	8.8173	2.99187	0.106
	Controls group	46	7.7565	2.02832	
	Patients group 2	31	8.4516	2.06492	
EPO	Patients group 1	52	7.5964	6.09003	0.686
	Controls group	46	8.0448	3.80138	
	Patients group 2	31	8.5694	4.30679	

*Statistics was done by using one way ANOVA.

Table 11: Distribution of Smoking habit among study groups

		SMOKING				Total
		NO SMOKING	LIGHT SMOKING	MEDIUM SMOKING	HEAVY SMOKING	
Patients group 1	Count	12	4	17	19	52
	% within GROUP	23.1%	7.7%	32.7%	36.5%	100.0%
Controls group	Count	14	6	19	7	46
	% within GROUP	30.4%	13.0%	41.3%	15.2%	100.0%
Patients group 2	Count	3	4	16	8	31
	% within GROUP	9.7%	12.9%	51.6%	25.8%	100.0%
Total	Count	29	14	52	34	129
	% within GROUP	22.5%	10.9%	40.3%	26.4%	100.0%

Table 12: Other most common variables of lifestyle and symptoms significance among study groups

		Sum of Squares	df	Mean Square	F	p-value*
Smoking	Between Groups	6.341	2	3.170	2.727	.069
	Within Groups	146.465	126	1.162		
	Total	152.806	128			
Blood Donation	Between Groups	9.104	2	4.552	25.034	.001
	Within Groups	22.911	126	.182		
	Total	32.016	128			
Familial High Hb	Between Groups	1.683	2	.841	5.391	.006
	Within Groups	19.666	126	.156		
	Total	21.349	128			
Exercises	Between Groups	5.221	2	2.610	2.662	.074
	Within Groups	123.539	126	.980		
	Total	128.760	128			
Splenomegaly	Between Groups	.020	2	.010	.649	.524
	Within Groups	1.949	125	.016		
	Total	1.969	127			
Gout	Between Groups	.039	2	.020	.859	.426
	Within Groups	2.891	126	.023		
	Total	2.930	128			
Itching	Between Groups	3.163	2	1.581	1.900	.154
	Within Groups	104.899	126	.833		
	Total	108.062	128			
Face Redness	Between Groups	23.683	2	11.842	11.229	.001
	Within Groups	132.875	126	1.055		
	Total	156.558	128			
Headache	Between Groups	15.862	2	7.931	8.848	.001
	Within Groups	112.944	126	.896		
	Total	128.806	128			
Sweating	Between Groups	22.884	2	11.442	12.930	.001
	Within Groups	111.504	126	.885		
	Total	134.388	128			
Stomach Ulcer	Between Groups	2.995	2	1.497	2.813	.064
	Within Groups	67.068	126	.532		
	Total	70.062	128			
Dizziness	Between Groups	1.121	2	.560	.710	.494
	Within Groups	99.500	126	.790		
	Total	100.620	128			

*Statistical analysis was done using one way ANOVA.

Table 13: Frequencies of JAK2V617F mutation detected by ARMS assay among study population

Group	Total Number	JAK2V617 Positive(%)	Male (%)	Female (%)	JAK2V617 Negative	Male	Female
Patients1	52	36 (69.2%)	33(91.7%)	3(8.3%)	16	15	1
Patients 2	31	16 (51.6%)	16 (100%)	0(0%)	15	15	0
Controls	46	10 (21.7%)	10(100%)	0(0%)	36	32	4

Table 14: Study population details and Summary of results distributed according to presence of JAK2V617F mutation

GROUP	JAK2V617F	Gender	N	AGE years Median (min-max)	HB g/dL Mean ± SD	RBC 10^6/µL Mean ± SD	WBC 10^3/µL Mean ± SD	PLTs 10^3/µL Mean ± SD	EPO mIU/ml Mean ± SD
Patients group 1	Mutated	Male	33	35 (18-78)	18.8 ± 1.3	6.4 ± 0.7	8.1 ± 2.3	214 ± 90	8.1 ± 5.6
		Female	3	64 (60-83)	18.2 ± 1.3	6.4 ± 0.6	10.0 ± 0.7	358 ± 181	2.1 ± 1.0
	Wild -Type	Male	15	36 (28-68)	18.8 ± 0.7	6.4 ± 0.45	9.6 ± 3.9	247 ± 162	6.4 ± 6.1
		Female	1	64 (64-64)	16.9 ± 0	5.8 ± 0	14.5 ± 0	203 ± 0	23.2 ± 0
Patients group 2	Mutated	Male	16	35 (19-65)	17.5 ± 0.4	5.8 ± 0.32	8.3 ± 2.0	205 ± 41	9.3 ± 5.4
	Wild -Type	Male	15	29 (22-44)	17.4 ± 0.3	5.9 ± 0.34	8.5 ± 2.14	210 ± 61	7.7 ± 2.7
Controls group	Mutated	Male	10	31 (21-65)	15.3 ± 0.6	5.1 ± 0.32	8.1 ± 1.76	212 ± 57	6.7 ± 3.78
	Wild -Type	Male	32	27 (19-70)	15.5 ± 1.0	5.4 ± 0.30	7.6 ± 2.18	227 ± 47	8.42 ± 4.0
		Female	4	54 (32-67)	13.5 ± 1.5	5.0 ± 0.39	7.7 ± 1.6	241 ± 40	8.4 ± 1.0

Table 15: Study groups according to JAK2V617F mutation status

Group	Total	JAK2V617		p-value*
		Mutated	Wild-type	
Patients 1	52	36 (69.2%)	16(30.8%)	
Patients 2	31	16 (51.6%)	15(48.4%)	0.001
Controls	46	10 (21.7%)	36(78.3%)	
Total	129	62(48.1)	67(51.9%)	

*Statistics was done by using Chi square test

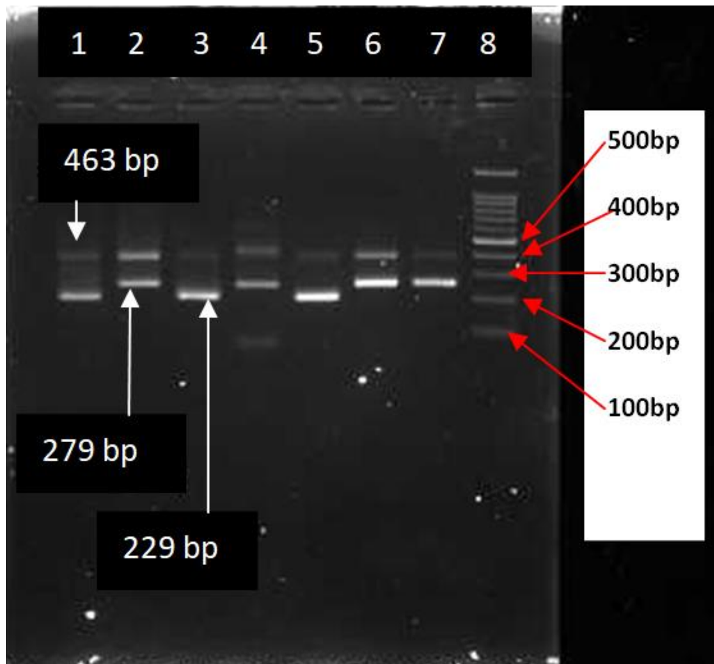

Figure 11: Agarose 2% gel electrophoresis for identifying JAK2V617F using ARMS-PCR.

Lane 1, 3, and 5 shows wild type band 229bp, Lane 2, 4, and 6 shows mutated band 279bp, a band of 463bp indicates outer template. Lane 7 positive mutated control 279bp, Lane L 100bp DNA ladder.

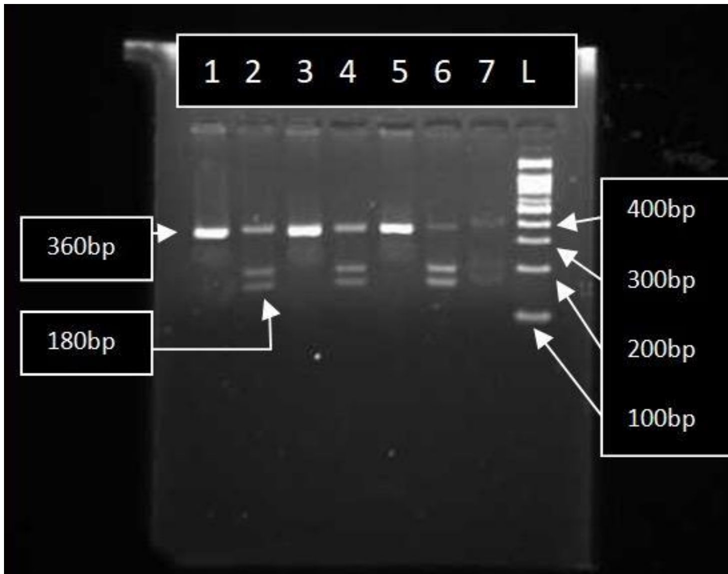

Figure 12: Agarose gel electrophoresis 2% for identifying JAK2V617F using PCR-RFLP.

Lane 1,3 and 5 different amplicons before *BsaXI* digestion with band of 360bp, Lane 2,4 and 6 for same amplicons after *BsaXI* digestion with mixed pattern of both mutant (360bp) and wild-type (180bp) alleles. Lane 7 for positive control digested by *BsaXI*, Lane L 100bp DNA ladder.

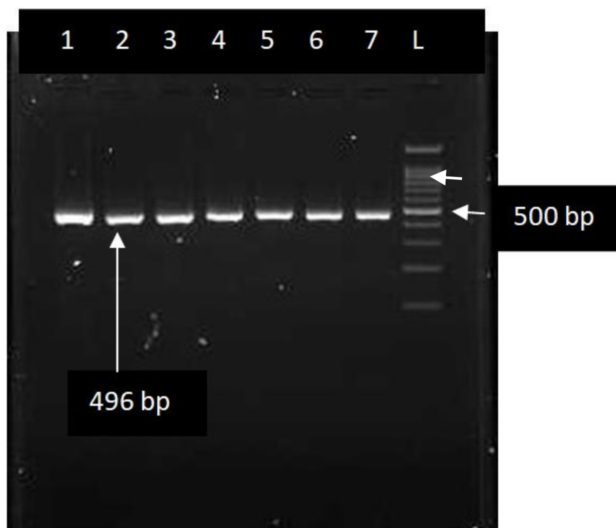

Figure 13: Identifying ARMS assay for JAK2 Exon -12 mutations; using 8% polyaccrylamide gel electrophoresis.

Lanes L :100bp DNA ladder, Lanes from 1 to 4 show wild-type alleles for (F537-K539delinsLf) with band of 496bp, Lanes 5 to 7 show wild-type alleles for (K539L) with band 496bp.

Although HRM curve analysis was done using specimens of controls group as a wild type control, all specimens for all groups approximately have the same melting temperature, wild-type allele has melting peaks at 76.3°C for *F537-K539delinsL* (Figure 14), and 76.0°C for*K539L* mutation (Figure 15).

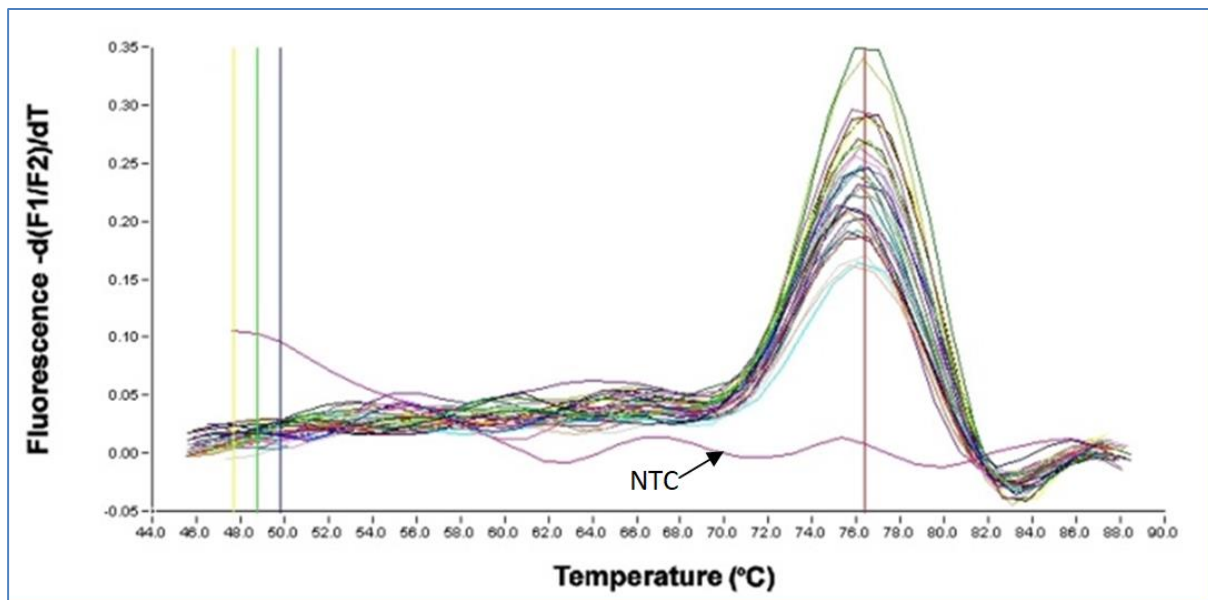

Figure 14: High resolution melting curve for JAK2 exon-12 (F357-K539delinsLf).
Melting curve of different samples selected randomly from all groups, to identify JAK2 Exon-12 mutation (F357-K539delinsLf); all DNA samples show wild-type alleles with melting temperature of 76.3°C. NTC: No template control.

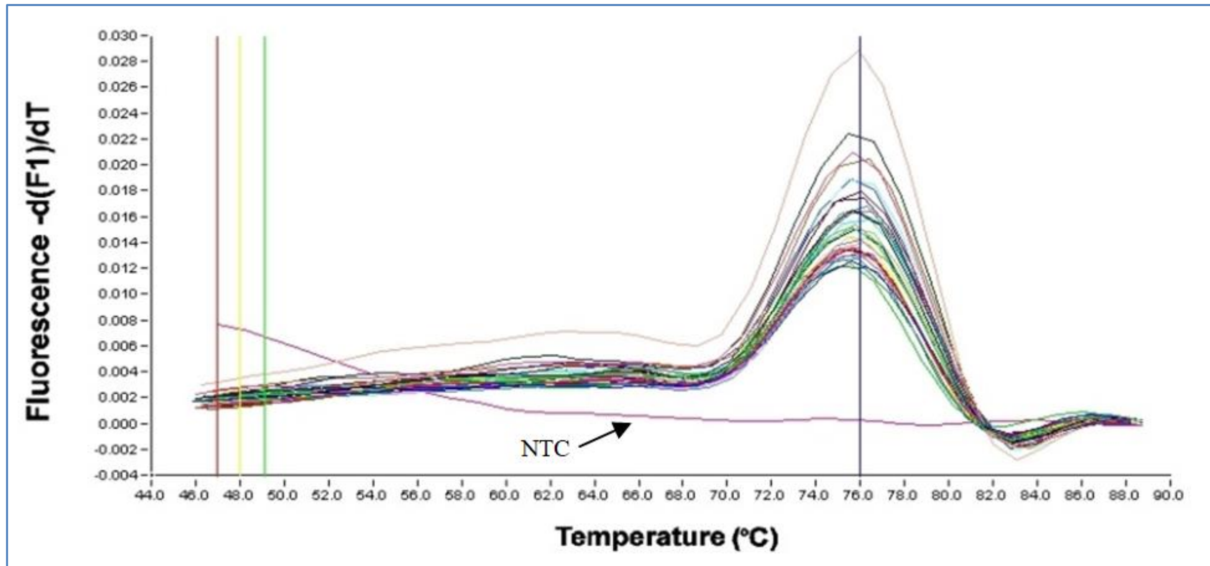

Figure 15: High resolution melting curve for JAK2 exon-12 (K539L).

Melting curve of different samples selected randomly from study groups, to identify JAK2 Exon-12 mutation (K539L); the DNA samples show wild-type alleles with melting temperature of 76.0°C. NTC: No template control.

Chapter 5: Discussion

This study is the first molecular study to determine the frequency of presence the JAK2 gene mutations in the West Bank - Palestine, among patients suspected to have PV. The most common JAK2 gene mutations include *JAK2V617F* that is present in 95% of PV cases, and JAK2 exon 12 mutations which are present in about 2-3% of PV cases, and there is rare of PV cases lacking these mutations (JAK2 negative-PV) (81). These two somatic mutations which are gain - of - function mutations induce spontaneous activation of tyrosine kinase signaling pathways independently of EPO, through the disrupting auto inhibitory function of the pseudokinase domain. This leads to increase proliferation and differentiation of hematopoietic myeloid cellular lineages, especially in erythroid pool, and increase production of RBCs, WBCs and PLTs, which are the most common features of PV.

According to 2016 WHO criteria the patients should meet the three major criteria for PV diagnosis, including Hb > 16.5 g/dL (men) and Hb > 16g/dL (women), BM biopsy showing hypercellularity, and presence of *JAK2V617F* or JAK2 exon 12 mutations. With an exceptional of no need for making BM biopsy if Hb > 18.5 g/dL (men) and Hb > 16.5 g/dL (women). Thus, the study population consistent of (patients group 1) who have Hb > 18.5 g/dL (men) and Hb >16.5 g/dL (women), controls group who have Hb < 16.5 g/dL (men) and Hb <16 g/dL (women), and the third group called (patients group 2) who have Hb between 16.5 g/dL and 18.5 g/dL for men (but doesn't meet the criterion of making BM biopsy).

In this study, the current data showed a statistically significant differences in Hb, Hct, and RBCs values among the three study groups, and WBCs count has higher count value with statistically difference in the patients group comparing with controls group

with p-value <0.001 , while the mean of other hematological parameters has no significantly differ among study populations. Also the variables of lifestyle and symptoms has no statistical significant differences among populations, except of suffering from face redness, headache, night sweating ,blood donation, and presence of relative familial of high Hb value, with p-value of < 0.05 for them.

By using both of ARMS-PCR and PCR-RFLP; we found the *JAK2V617F* mutation in 69.2% (36/52) of patients group 1, of them 33 (91.7%) were males with median age of 35 years (Hb and RBCs mean ± SD is 18.8 ± 1.3 g/dL, 6.4 ± 0.7 10^6/µl respectively), and 3 (8.3%) were females with median age of 64 years (Hb mean ± SD is 18.2 ± 1.3 g/dL), see Table 14. The mutation also detected at 51.6% (16/31) in males of patients group 2 who have Hb mean ± SD of 17.5 ± 0.4 g/dL, and median age of 35 years. However; it is also identified at 21.7 (10/46) in males of healthy controls group who have Hb mean ± SD of 15.3 ± 0.6 g/dL. All *JAK2V617F* positive samples have a mixed pattern of wild-type and mutant alleles, as shown in Figure 11 and Figure 12. And there is an enough evidence to suggest an association between the presence of JAK2V617F mutation and developing the PV, X^2 = 22.2 with p-value <0.001, as shown in Table 15.
Furthermore; none of JAK2 exon 12 mutations was identified in all study population. The results of JAK2 exon 12 mutations were detected by ARMS-PCR and HRM methods.

In particularly; the frequency of *JAK2V617F* mutation among PV patients group-1 in this study was 69.2%, that variable to those reported by other studies worldwide, and the mutation rate in patients was lower than other neighboring population such as Lebanese 100% (83), Egyptian 81.4% (84), Iranian studies78.3% (41), while our rate is close to Iraqi 65% (44), and Jordanian 70% (85), but none of them has controls

group (free of PV features) in their studies except Iranian study (but even JAK2V617F not detected in healthy controls) , none of them has used more than one molecular method, and three of them had been used ARMS-PCR method.

Also; we found the *JAK2V617F* mutation in 21.7% (10/46) in healthy controls group, which is higher than what reported in previous articles worldwide. The frequency of detection *JAK2V617F* mutation in blood of healthy individuals with normal hematological parameters ranging from 1 to 10% (86), but it might be higher than expected in the early detection of PV disorder. Four of case control studies have found few frequencies of *JAK2V617F* mutation among healthy individuals as follow: *JAK2V617F* mutation identified in 10% (5/52) (87), in 37 of 3935 (88), in 21.3% (20/94) (89), and in 1.6% (2/127) of healthy individuals (43). However; in our country there was another study in Hadassah-medical center in Jerusalem 2017 (90), they had been detected the mutation 17.3% (17/98) of normal donors and the mutation burden was very low (<0.01%), which somewhat close to our findings in this study. While some case control studies were not detected the *JAK2V617F* mutation in any of healthy controls group (91,92,93).

Furthermore, a recent article analyzed 175 blood donors with high Hb value and erythrocytosis, and they didn't have *JAK2V617F* mutation (86). Another study in Kashmir-India has shown *JAK2V617F* mutation rate 100% among PV patients in their case-control study, using Allele specific-PCR, while the mutation was not detected in healthy controls (91). Another study in Spain has used case control study, by using HRM and validating methods, the *JAK2V617F* was detected in 29 (93.5%) of 31 PV patients and in 2 (1.58%) of 126 controls group (43). Alireza Moradabadi group (46) compared the different molecular methods to detect *JAK2V617F*, include RFLP-PCR, ARMS-PCR, and HRM with the gold standard

methods of Real-Time PCR and sequencing, overall results were similar and comparable to each others. Although the gold standard method to identified mutations is the sequencing, but it is limited by high cost and low sensitivity (10-20%) (16). Thus; ARMS-PCR is a widely used method, cost effective, and considered one of the most sensitive (0.01% - 5%) molecular methods used to detect *JAK2V617F* (94,95). The RFLP-PCR was the first method used to identify the *JAK2V617F* mutation, therefore; it is a reference method (46) and depends on the enzyme efficiency and the control sequence, but it is time-consuming and challenged by incomplete digestion with the restriction enzyme. The HRM was introduced as an easy, sensitive and reliable method to detect JAK2 gene mutations (43,86). Several articles have made comparison between several molecular techniques to identify JAK2 gene mutations, and all have revealed 100% comparable between the techniques (46,94).

In addition to; a recent study has found a novel mutation in exon 14 (*c.1849_1853 GTCTG>TTTCT;p.V617F/C618L*) in one patient, and another compound mutation of (*c.1849G>T;1852T>C; p.V617F/C618R*) in another patient, by using next generation sequencing (NGS) that targeted the entire coding region of JAK2 gene(81). Another article has identified another compound mutation in PV, *JAK2 L611V/V617F* (96). There are several compound mutations in exon 14 have been reported such as: *L611V/V617F*, *L611S/V617F*, *V615F/V617F*, *V617F/C618F*, and *V617F/D620E* (81). Regardless the association between the presence of JAK2V617F mutation and developing the PV among our study groups; absence of this mutation in other patients who had PV features; indicate that another JAK2 gene mutations could be found and induce the activity of JAK2 tyrosine kinase; resulting in erythroid proliferations other than *JAK2V617F*, or presence any of JAK2 exon 12 mutations, or

any other gene (40). And if it's not! Why there are cases of MPN and PV with strong features and didn't have the JAK2V617F mutation! This issue simply has an evidence of presence another mutations affect the JAK/STAT pathway and result in progression the disease.

Presence of *JAK2V617F* mutation in the majority -but not in all- of our patients groups (62.6%) indicate some suggestions, firstly: probability of presence another mutation that associated with the progression of the PV. Secondly: identifying the *JAK2V617F* mutation in healthy people (21.7%), increased the possibility that the mutation harbors before the onset of the disease (86,89), and *JAK2V617F* presence may indicate that "a very early molecular event has occurred prior to the manifestation of PV disorder" (86,89), therefore; this must be confirmed by adequate observation of healthy people carrying the mutation. Thirdly: JAK2V617F in healthy people is might be due to the fact that the first precursor carrying JAK2V617F may be more differentiated in healthy people than in patients with PV. At this stage, the potential for self-renewal and the ability to differentiate the mutated cell are reduced, and the created clone can be subjected to death. In this way; the mutated clone does not create a clinical phenotype and may disappear upon serial evaluation. Fourthly, genetic stability based on DNA repair is effective in normal hematopoietic stem cells, but decreases in differentiating cells, making them susceptible to mutations, including JAK2V617F (89). Finally, presence of JAK2V617F mutation in healthy individuals without PV phenotypes or features; it might be due to an epigenetic alteration and modification that change or repress the way in which important gene may be expressed or silenced.

Therefore, advanced research should be done to investigate findings of this study, may be using NGS for JAK2 gene, covering at least JAK2 exon 12, exon 13, and

exon 14, in which is highly sensitive and can identify rare mutations. Or using targeted gene panels assessed by NGS that cover several genes in a single assay, thereby examines all of target genes involved in the PV pathogenesis. Moreover, performing a BM examination to detect the hypercellularity; is very important as an essential criterion in the 2016 WHO for diagnosis of PV disorder.

Finally; which is the most important; for using quantitative real time-PCR in further research, which is the best method for detection of very low burden mutations, and it's sensitivity is 0.01-5% (90,97), in order to determine the *JAK2V617F* allele burden in our healthy individuals to determine the cut-off value between negative and positive JAK2V617F, and to evaluate the effect of the JAK2V617F allelic burden on clinical and laboratory parameters in Palestinian patients with PV (95,98).

Chapter 6: Conclusion:

According to our observation regarding the presence of *JAK2V617F* mutation in majority –but not in all- of patients groups 62.6% (52/83), and in minority of healthy controls group 21.7% (10/46), raising the possibility for presence of different gain of function mutations other than JAK2 gene mutations between different ethnic groups; that associated with the PV features and phenotypes; in which somatic mutation could affect the morphological pattern based on inter-individual differences in genetic factors. Also; presence of *JAK2V617F* in blood of healthy people might be due to genetic stability based on DNA repair is effective in normal hematopoietic stem cells, but decreases in differentiating cells, making them susceptible to mutations. Furthermore; the mutation may be dose-dependent, and the progression of PV depends on the number of mutated alleles occupied the majority of blood cells, thus it's important to determine the cut-off value of *JAK2V617F* burden allele in our population, and to find out the genetic changes or the target mutation in which has the synergism effect with *JAK2V617F* mutation; to contribute in the progressing and pathogenesis of PV. However; as mentioned before, *JAK2V617F* associated with three different diseases phenotypes PV, ET, and MPF but the reason why it remains unclear, which approve suggestion of another mutation may be found alone or in multiple for combination with JAK2 mutations; that induce continuous activation of the JAK/STAT pathway and triggered PV phenotypes as a multifactorial disease. Recently, an evidence that the MPNs resulted due to combination of genetic dysregulation with several mutated genes outside of JAK2 locus; involved in the regulation of epigenetic mechanisms and splicing machinery, that play important role in these malignancies. These mutations appear to provide a selective in vivo

advantages to cells, and current research is aimed at more accurately determining their functional effects.

The exact role of target mutations, and their effect on PV phenotype and features that might be due to epigenetic alteration; will require further future studies, and more focusing on the relationship of external factors and acquired genetic abnormalities in the onset and progression of the disease.

Hopefully, future research in our country should be done using more advanced molecular technique, such as quantitative real time-PCR, NGS, and targeted gene panels assessed by NGS, that cover several genes in a single assay, thereby examines all of target genes involved in the PV pathogenesis. NGS provides a wide range of gene analysis without significantly increasing the amount of work required to complete the test. In addition to perform BM examination for accurate diagnosis of PV disorder.

Finally, our understanding of the molecular pathogenesis of PV is very important for the classification, diagnosis, and choice the best treatment methods that have been proven to alter the natural history of this myeloid neoplasm.

Recommendations:

Further research should be done in order to investigate findings of this study. Also, further research must be done using specimens of Palestinian population and other foreign population to confirm our findings. And to determine the cut-off value of JAK2V617F burden allele in Palestinian population which is helpful in correct diagnosis of PV.

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Appendix

Appendix A: Hematological parameters of CBC, EPO levels, and DNA concentration and ratio for specimens.

PG1: patients group 1, CG: controls group, PG2: patients group 2.

Sample number	HB g/dl	HCT %	RBC $10^6/\mu L$	WBC $10^3/\mu L$	PLTS $10^3/\mu L$	EPO (5.4 to 31 mIU/ml)	DNA Concentration (μg/ml)	DNA Ratio (A_{260}/A_{280})nm
1PG1	19.6	59.3	7.83	6.6	218	11.8	50.6	1.88
2PG1	18.8	54.7	6.2	11.1	129	2.87	53.8	1.72
3PG1	20.2	57.7	6.58	7.1	122	6.86	61.6	1.71
4PG1	18.1	54	5.95	7.3	103	3.33	52.3	1.62
5PG1	17.7	50.1	5.89	6.4	141	7.13	53.9	1.68
6PG1	17.6	51	5.64	7.9	324	7.51	50.7	1.8
7PG1	18.3	52.7	6.04	11.6	302	6.41	60.8	1.81
8PG1	18.2	52	6.25	5.2	270	9.04	42.5	1.87
9PG1	18.5	54.8	5.91	6.2	112	5.19	45.3	1.77
10PG1	19.1	55.5	6.68	6.6	213	15.6	60.2	1.78
11PG1	18.1	52.1	5.91	6.1	195	3.5	59.1	1.87
12PG1	18.2	54.6	7.6	19.7	819	1	98.4	1.88
13PG1	20	57.5	6.67	11.1	200	6.23	72.7	1.84
14PG1	19.8	55.9	6.61	5.2	129	7.11	66.4	1.75
15PG1	17.7	51.7	5.84	8.1	220	9.8	65.7	1.72
16PG1	18.9	54.9	6.55	7.7	188	6.35	42.1	1.86
17PG1	17.9	51.9	6.28	9.6	237	11	81	1.9
18PG1	19.8	53.3	6.21	11	301	4.67	54.1	1.89
19PG1	15.2	56.4	9.3	10.2	509	1.73	64	1.83
20PG1	19.7	61.2	7.06	9.4	566	<1	50.2	1.85
21PG1	18.6	53.4	6.26	9.3	134	4.92	43.5	1.87
22PG1	18.8	56.3	6.64	10.7	185	2.44	50.4	1.86
23PG1	18.6	53	6.12	13.7	221	5.05	40.4	1.83
24PG1	16.9	50.2	5.8	14.5	203	23.2	66.9	1.83
25PG1	19.4	54.1	5.84	7.7	190	3.02	29.2	1.85
26PG1	19.2	56.3	6.26	12.7	228	2.98	54.3	1.86
27PG1	18.7	53.3	6.33	7.8	213	3.05	42.3	1.84
28PG1	20.9	57.5	6.6	7	184	6.53	60.1	1.9
29PG1	20.5	62	7.18	8.6	187	25.3	25.1	1.9
30PG1	18.9	53.3	6.07	6.6	174	4.88	35.5	1.9
31PG1	21.3	57.7	6.88	6.2	236	10.9	42.2	1.9
32PG1	18.8	52.9	5.93	8.4	179	4.27	46.7	1.88

Appendix A: Hematological parameters of CBC, EPO levels, and DNA concentration and ratio for specimens.
PG1: patients group 1, CG: controls group, PG2: patients group 2.

Sample number	HB g/dl	HCT %	RBC 10^6/µL	WBC 10^3/µL	PLTS 10^3/µL	EPO (5.4 to 31 mIU/ml)	DNA Concentration (µg/ml)	DNA Ratio (A_{260}/A_{280})nm
33PG1	19.6	52.9	6.21	7.4	140	15	35.9	1.8
34PG1	19.1	53.5	5.64	6	234	9.58	40	1.88
35PG1	20.1	55.8	6.45	6.7	228	8.16	37.4	1.89
36PG1	19.1	58.6	6.7	10.7	100	16.5	44.8	1.87
37PG1	20.1	56.4	6.75	8.1	105	8.12	53.3	1.84
38PG1	19.2	54.8	6.54	9.2	214	7.84	37.1	1.73
39PG1	17.2	51	6.2	10.9	275	2.21	35	1.75
40PG1	18.6	58.2	6.91	11.6	288	8.28	44	1.8
41PG1	17.9	53	5.93	9.9	233	3.03	36.2	1.79
42PG1	18.6	55.4	6.43	3.2	175	20.8	36.2	1.75
43PG1	19	57.6	6.86	8.3	242	27	41.9	1.81
44PG1	17.7	52.6	6.13	8.1	211	4.83	29.3	1.74
45PG1	18.5	51.2	6.12	7.8	168	12.7	56.1	1.82
46PG1	19.1	53.5	6.42	5.9	168	4.4	41.4	1.83
47PG1	18.5	55.1	6.42	13.4	289	5.31	59.6	1.74
48PG1	18.9	53.5	6.3	7.4	298	4.72	41.1	1.78
49PG1	21	57	6.41	5.9	196	7.47	26.5	1.61
50PG1	18.4	51.8	6.02	6.4	233	3.51	34.3	1.72
51PG1	15.5	47.3	5.1	8.8	246	0.899	72.6	1.83
52PG1	17.8	56.2	7.31	15.5	404	0.284	92.2	1.8
1CG	14.3	42.2	5.03	8.3	293	6.9	64.6	1.79
2CG	16.2	45.9	5.31	8.2	243	5.96	43.8	1.73
3CG	15.6	42.5	5.24	5.9	275	3.45	62.1	1.77
4CG	16.4	45.1	5.36	9.1	269	4.65	57.8	1.75
5CG	16.4	44.9	5.39	9.3	252	8.46	75.9	1.77
6CG	16	44.9	5.39	10.5	266	5.74	65.4	1.78
7CG	14.1	43.6	5.82	8.3	257	7.86	29.3	1.73
8CG	14.6	42.5	4.74	8.1	158	3.95	28.4	1.72
9CG	14.3	39.6	4.63	6.4	234	11.4	25.3	1.7
10CG	15.8	45	5.08	9.9	223	5.25	31.3	1.69
11CG	16.1	45.4	5.33	7.5	242	8	26.3	1.57

Appendix A: Hematological parameters of CBC, EPO levels, and DNA concentration and ratio for specimens.
PG1: patients group 1, CG: controls group, PG2: patients group 2.

Sample number	HB g/dl	HCT %	RBC $10^6/\mu L$	WBC $10^3/\mu L$	PLTS $10^3/\mu L$	EPO (5.4 to 31 mIU/ml)	DNA Concentration (µg/ml)	DNA Ratio (A_{260}/A_{280})nm
12CG	16.4	47.4	5.68	11.6	182	6.94	40.3	1.73
13CG	15.9	45.2	5.54	6.7	233	8.03	50.6	1.84
14CG	13.1	38.4	4.75	6.3	278	16.8	42.3	1.85
15CG	16.6	45.9	5.19	6.3	229	6.02	40.9	1.82
16CG	15.8	45.8	5.34	8.4	279	7.22	62.7	1.79
17CG	13.1	40.4	5.37	7.8	259	9.42	49.3	1.79
18CG	15.4	43.3	5.31	12.6	237	5.08	54.6	1.81
19CG	12.3	37.6	4.8	9.2	240	7.87	70.2	1.8
20CG	14.3	42.2	5.01	7.6	259	6.34	73.1	1.72
21CG	16.5	46	5.48	5.4	219	6.88	66	1.55
22CG	16.9	48.4	5.61	7.6	222	6	57.8	1.83
23CG	12.8	38.7	4.58	5.4	186	9.05	36.6	1.79
24CG	15.8	44.5	5.19	9.5	299	2.91	54.8	1.83
25CG	16.9	46.3	5.5	5.8	289	8.47	50.3	1.85
26CG	16.2	44	5.4	10.8	175	7.12	55.3	1.83
27CG	15.8	44.1	5.69	5.9	211	4.65	37.9	1.73
28CG	14.5	42.8	5.09	4.1	270	8.4	26.5	1.75
29CG	15.4	43.3	4.94	10.8	254	6.47	40.5	1.74
30CG	13.3	41.4	5.54	7.7	256	20.9	52.3	1.76
31CG	15	43.8	5.29	6.8	192	15.2	38.8	1.81
32CG	14.5	41.6	4.89	9.8	83	7.49	45.5	1.85
33CG	15.6	44.8	4.98	9.5	242	3.88	55.9	1.82
34CG	15.1	43.3	4.89	4	174	5.18	28.4	1.76
35CG	14.7	43.3	5.38	5.6	267	9.91	35.6	1.8
36CG	15.5	45.9	5.84	8.6	179	11	41	1.8
37CG	16.4	47.5	5.43	8	199	14.8	53.7	1.79
38CG	15.1	44.7	5.76	8.4	208	9.97	37.9	1.79
39CG	15.9	46.7	5.55	4.6	195	14.95	38.8	1.76
40CG	15.3	46.4	6.12	5.8	270	5.23	60.7	1.84
41CG	16.6	48.2	5.82	6	190	10.23	29.7	1.74
42CG	16.6	50.6	5.55	10.6	82	7.48	25.1	1.73

Appendix A: Hematological parameters of CBC, EPO levels, and DNA concentration and ratio for specimens.
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Sample number	HB g/dl	HCT %	RBC $10^6/\mu L$	WBC $10^3/\mu L$	PLTS $10^3/\mu L$	EPO (5.4 to 31 mIU/ml)	DNA Concentration (µg/ml)	DNA Ratio (A_{260}/A_{280})nm
43CG	16.5	47.2	5.37	5.8	151	12.8	43.6	1.78
44CG	15.5	44.2	5.2	9.4	201	3.87	63.1	1.8
45CG	14.1	42.5	5.62	6.7	249	3.41	41.3	1.84
46CG	15.6	44.7	5.34	6.2	202	8.47	59.2	1.8
1 PG2	17.1	49.1	5.59	8.4	66	9.44	51.9	1.84
2 PG2	17.6	50.5	5.75	7.1	231	10.3	50.9	1.82
3 PG2	17.5	49.6	5.62	8.5	283	8.08	56.1	1.86
4 PG2	17.2	48.5	5.6	10	232	9.24	63.8	1.75
5 PG2	17.7	51.6	6.11	9.6	193	7.69	64.5	1.75
6 PG2	17.4	52.2	6.32	7.2	229	5.63	65.1	1.74
7 PG2	17.1	51.4	6.21	4.4	242	7.29	30.1	1.65
8 PG2	17.7	50.8	6.15	11	220	5.17	62.9	1.65
9 PG2	18.1	52.9	5.82	10.2	216	8.46	80.5	1.84
10 PG2	18.2	51.8	6.23	8.1	177	12.4	58.7	1.83
11 PG2	17.1	49.1	5.42	7.5	208	1.97	62.7	1.81
12 PG2	17.9	50	5.86	5.5	159	6	47	1.86
13 PG2	17.4	48.5	5.51	6.1	234	6.47	47.5	1.8
14 PG2	18	51.8	6.23	3.3	241	4.93	36.3	1.62
15 PG2	18.1	53.2	6.53	12.2	336	3.71	63.6	1.69
16 PG2	17	48.9	5.39	8.6	160	9.97	49.8	1.81
17 PG2	17.6	51.1	6.03	10.3	182	3.93	49.4	1.77
18 PG2	17.5	49.8	5.7	10.4	161	7.11	67.9	1.81
19 PG2	17.2	50.7	5.88	10.1	193	2.1	50.1	1.81
20 PG2	17.2	49.9	5.75	10.3	210	8.93	55.5	1.81
21 PG2	17.4	50	5.74	6.3	187	6.11	39.1	1.8
22 PG2	18	50.2	5.67	9	150	10.5	41.3	1.81
23 IPG2	18.2	52.3	5.77	7.5	227	5.75	43.1	1.8
24 PG2	17.3	52	6.36	9.9	175	13	52.9	1.8
25 PG2	17.4	51.2	5.4	8.8	150	14.4	62.2	1.87
26 PG2	17.1	50.9	5.81	9.3	249	20.2	65.6	1.87

Appendix A: Hematological parameters of CBC, EPO levels, and DNA concentration and ratio for specimens.
PG1: patients group 1, CG: controls group, PG2: patients group 2

Sample number	HB g/dl	HCT %	RBC 10^6/µL	WBC 10^3/µL	PLTS 10^3/µL	EPO (5.4 to 31 mIU/ml)	DNA Concentration (µg/ml)	DNA Ratio (A_{260}/A_{280})nm
27 PG2	**17.3**	**50.4**	**6.2**	**5.8**	**241**	**18.35**	**44.5**	**1.85**
28 PG2	**17**	**51.2**	**6.32**	**8.8**	**251**	**13.9**	**67.5**	**1.85**
29 PG2	**17**	**49.1**	**5.59**	**11.1**	**289**	**4.92**	**70.3**	**1.81**
30 PG2	**17**	**48**	**5.35**	**9.5**	**157**	**7.8**	**32.4**	**1.73**
31 PG2	**17**	**50.3**	**6.19**	**7.2**	**198**	**11.9**	**37.9**	**1.75**

Appendix B : Form of approval of the participant in scientific research:

Dear participant

Before you agree, you should read the following data:

- The aims of this scientific research are:

1. To know the reasons of why high numbers of blood cells occur in Palestinian population.
2. To search of the genetic mutations like JAK2V617F, JAK2 exon 12; that enhance increasing the blood cells and cause the disease which may develop with time to aggressive acute myeloid leukemia.
3. The results of this scientific research may help to enhance patients life and improve their following and treatment.

- How will be the participation take place:

1. A blood sample (5ml) will be drawn from each participant to do the tests of : CBC, Erythropoietin level, and DNA extraction.
2. No any complication occur from drawing blood.
3. The participant should fill the form of participation by complete some information about his self such as name, gender, age, life style and health status. These information helpful in this research.
4. All of data and information will be very confidential and only for the scientific research.

5. This study will contains two groups of participants; group of patients who have high hemoglobin levels, and the second group will be for participants of normal hemoglobin levels; to control the work.

- **Obligation**

The results of this scientific research and all of data is the right of Birzeit University and no legal entity has the right to obtain it. Each participant is entitled to know its own results later on.

Your participation in this scientific study and answering the questions precisely; helps ensure quality results and will help achieve the goals of this study.

Thank you for your cooperation and participation.

Signature of participant___________ Date____________

Questionnaire form:

Name:_____________ Gender: Female/Male

Age:_______________ Occupation:_________________

Address:____________ Phone:______________________

1. Health status

- Please answer the following questions:

  Have you suffering from:

No.	Health status	Never	Rarely	Sometimes	Always
1	Headache				
2	Weakness				
3	Sweating				
4	Stomach ulcer				
5	Facial redness				
6	Dizziness				
7	Body itching				

2. Diseases

- Have you suffering of : (choose Yes or No)

No.	Disease	Yes	No
1	Diabetes		
2	Hypertension		
3	Breathing problems		
4	Kidney problems		
5	Splenomegaly		
6	Gout		
7	Under medication		

If you take medicine, what type of medication?__________________.

3. Life style

- Please choose the suitable choice:

  Are you:

No.	Life style	Never	Light	Medium	Heavy
1	Smoking				
2	Playing exercise				
3	Eating vegetables				
4	Eating red meat				
5	Eating chicken				
6	Eating fish				
7	Eating cooked liver				
8	Eating cooked spleen				

4. Health history questions:

- Are there people in your family who suffer from high hemoglobin level?

  Yes_____ No_______

  If yes, what is the relative relation with him?___________

- Do you donate blood ?

  Yes _______ No_______

  If yes:

  1. How many times per year? ____________
  2. What is the reason for donation?

     Therapeutic_________ Volunteer________

Thank Your participation

نموذج موافقة المشترك في البحث العلمي::

عزيزي المشارك / ة:- قبل الموافقة يجب قراءة البيانات التالية:

- إن الهدف من هذا البحث العلمي هو:

1. معرفة سبب حدوث ارتفاع عدد خلايا الدم الحقيقي لدى المرضى في المجتمع الفلسطيني.
2. البحث عن وجود الطفرات الجينية التالية :

JAK2V617F and JAK2 exon 12 mutations والتي قد تسبب هذا المرض وتساهم في تطوره إلى مراحل أصعب.

3. نتائج هذا البحث سوف تساهم في تحسين الوضع الصحي لهؤلاء المرضى وتوفير العناية اللازمة والملائمة لهم.

  - سوف يتم أخذ عينة دم (5 مل) من كل مشارك حتى يتم عمل الفحوصات اللازمة, وهي
  - , Erythropoietin level ,DNA extraction, and CBC

- لا يوجد أي خطورة تترتب على المشارك جراء سحب الدم.
- سوف يطلب من المشارك كتابة اسمه وبعض البيانات الخاصة اللازمة لتوضيح مجريات البحث العلمي , لكن جميع البيانات سيتم التعامل معها برموز وسرية تامة.
- هذه الدراسة ستضمن مجموعتان من المشاركين: مجموعة اللذين لديهم أعراض ارتفاع عدد خلايا الدم, ومجموعة ضابطة للمقارنة وضمان جودة النتائج.
- **تعهد**: أن هذه الدراسة وجميع البيانات والنتائج هي ملك لجامعة بيرزيت ولا يحق لأي جهة غير قانونية بالحصول عليها. ويحق لأي مشترك معرفة النتائج لاحقا.

إن اشتراكك في هذه الدراسة العلمية الطبية, وإجاباتك للأسئلة المطروحة بدقة وشفافية يساعد في ضمان جودة نتائج هذه الدراسة العلمية ، والتي تهدف إلى الكشف عن مسببات ارتفاع عدد خلايا الدم الحمراء لدى المرضى في المجتمع الفلسطيني ، للبحث في إمكانية تطوير الخدمات الطبية المقدمة لهم ، وتحسين طرق العلاج لحمايتهم من تفاقم الوضع الصحي .

نشكر تعاونك واشتراكك في هذا البحث العلمي الطبي. توقيع المشارك _________ التاريخ____________.

نموذج الاستبيان :

الاسم :- ____________________ الجنس :- ذكر/ أنثى

العمر :- __________________ المهنة :- _______________________

مكان :- ___________________ رقم الهاتف :- ____________________

- أرجو الإجابة عن الأسئلة التالية بوضع إشارة (√) في المكان المناسب

الرقم	الحالة الصحية	لا	نادراً	أحياناً	دائماً
1	هل تعاني من وجع في الرأس؟				
2	هل تعاني من ضعف عام؟				
3	هل تعاني من التعرق؟				
4	هل تعاني من قرحة في المعدة؟				
5	هل تعاني من احمرار الوجه؟				
6	هل تعاني من دوخة؟				
7	هل تشعر في رغبة ملحة لحك الجسم؟				

- أرجو الإجابة عن الأسئلة التالية باختيار نعم أو لا

الرقم	الحالة الصحية	نعم	لا
1	هل تعاني من مرض السكري؟ إذا نعم, منذ متى؟------------------		
2	هل تعاني من ارتفاع ضغط الدم؟ إذا نعم, منذ متى؟------------------		
3	هل لديك مشاكل في التنفس؟		
4	هل لديك مشاكل في الكلى؟		
5	هل لديك تضخم في الطحال؟		
6	هل تعاني من مرض النقرس؟		
7	هل تتناول أدوية بشكل مستمر؟ ما هي؟-----------------------------------		

- أرجو الإجابة عن الأسئلة التالية بوضع إشارة (√) في المكان المناسب

الرقم	أساليب حياتية	لا	خفيف	متوسط	بكثرة
1	هل أنت مدخن				
	هل تمارس الرياضة				
	هل تتناول الخضراوات				
	هل تتناول اللحوم الحمراء				
	هل تتناول الدجاج				
	هل تتناول الأسماك				
	هل تتناول الكبد				
	هل تتناول الطحال (طحال الخروف أو العجل مثلا)				

- هل يوجد أشخاص في العائلة لديهم ارتفاع في نسبة الدم ؟

نعم لا

ما هي صلة القرابة؟________________________________

- هل تقوم بالتبرع بالدم ؟

نعم لا

إذا كانت الإجابة نعم كم مرة في السنة ؟ ____________________

إذا كانت الإجابة نعم ما هو سبب التبرع؟ ....طوعي ......علاجي.

شكرا لمشاركتك معنا