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Toxicology

The Effect of Genetic Polymorphism of STIM1 and ORAI1 Genes on Erythropoietin Resistance in Iraqi Patients with Chronic Renal Failure on Hemodialysis

A Thesis

Submitted to the Council of College of Pharmacy/University of Kerbala as Partial Fulfillment Requirements for the Master's Degree of Science in Pharmacology and Toxicology

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بِسْمِ اللَّهِ الرَّحْمنِ الرَّحِيمِ

{قَالُوا سُبْحَانَكَ لَا عِلْمَ لَنَا إِلَّا مَا عَلَّمْتَنَا إِنَّكَ

أَنتَ الْعَلِيمُ الْحَكِيمُ}

صَدق اللّه العليُ العظيم

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Dedication

To my backbone, my father

To the candle of my life, my mother

To my beloved sisters and friends

To my source of happiness my nieces and nephews

Farah

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List of Abbreviations

Abbreviations	Full text
ACE	Angiotensin-Converting Enzyme
AF	Atrial Fibrillation
ANOVA	Analysis of Variance
ARBs	Angiotensin Receptor Blockers
BFU-E	Burst-Forming Unit-Erythroid
BMI	Body Mass Index
BU	Blood Urea
CFU-E	Colony-Forming Unit-Erythroid
CRF	Chronic Renal Failure
CRP	C Reactive Protein
CVD	Cardiovascular Disease
DD	Dialysis-Dependent
DM	Diabetes Mellitus
DNA	Deoxyribonucleic acid
EDTA	Ethylenediamine Tetra Acetic Acid
EHDx	Expanded Hemodialysis
ELISA	Enzyme-Linked Immunosorbent Assay
EPO	Erythropoietin
EPOR	Erythropoietin receptor
ER	Endoplasmic Reticulum
ERBP	European Renal Best Practice
ESA	Erythropoiesis Stimulating Agent
ESAM	European Survey on Anemia Management
ESRD	End-Stage Renal Disease
GBD	Global Burden of Disease

GFR	Glomerular Filtration Rate
Hb	Hemoglobin
HDF	Hemodiafiltration
HF	Heart Failure
HF dialyzer	High-Flux Dialyzer
HIF	Hypoxia-Inducible Factor
HIF-PHIs	Hypoxia-Inducible Factor Prolyl Hydroxylase Inhibitors
HRP	Horseradish Peroxidase
HTN	Hypertension
IL-6	Interleukin-6
INF-γ	Interferon-Gamma
IP3	Inositol 1,4,5-Trisphosphate
JAK-STAT	Janus Kinase/Signal Transducers and Activators of Transcription
KDIGO	Kidney Disease Improving Global Outcomes
KDOQI	Kidney Disease Outcomes Quality Initiatives
LSD	Least Significant Difference
LVH	Left Ventricular Hypertrophy
MI	Myocardial Infarction
NDD	Non-Dialysis-Dependent
NKF KDOQI	National Kidney Foundation Kidney Disease Outcomes Quality Initiative
OD	Optical Density
ORAI1	calcium Release-Activated Calcium modulator 1
PCR	Polymerase Chain Reaction
PIP2	Phosphatidylinositol 4,5-Bisphosphate
PLC	Phospholipase C
PTH	Parathyroid Hormone
RBC	Red Blood Cell

rHuEPO	Recombinant Human Erythropoietin
RRT	Renal Replacement Therapy
S. Cr	Serum Creatinine
SC	Subcutaneous
SHPT	Secondary Hyperparathyroidism
SLE	Systemic Lupas erythematosus
SNP	Single Nucleotide Polymorphism
SOCs	Store-Operated Calcium Channels
SPSS	Statistical Package for the Social Sciences
STIM1	Stromal Interaction Molecule 1
TBE	Tris/Borate/EDTA
TGF	Transforming Growth Factor
TNF-α	Tumor Necrosis Factor-Alfa
TSAT	Transferrin Saturation
USRDS	US Renal Data System
WHO	World Health Organization

Abstract

Background: Chronic renal failure is a progressive incurable disease defined as damage of kidney structure or abnormalities in kidney function for more than three months; depending on glomerular filtration rate chronic renal failure is classified into five stages. Anemia evolves during the early stages of kidney failure and exacerbates with the progression of renal disease as a result of decreased erythropoietin production. The gold standards for treating anemia in chronic renal failure are both erythropoietin-stimulating agents and iron supplementation, but the resistance to the treatment stands against its goal in many patients. ORAI1 and STIM1 are genes of store-operated calcium channels, it is one of erythropoietin activated pathway and the genetic polymorphism in those genes may contribute to erythropoietin resistance.

Aim of study: to investigate the association of genetic polymorphism in ORAI1 gene rs6486795 T> C, A and STIM1 gene rs1561876 G > A, C, T with erythropoietin resistance.

Methods: A cross-sectional observational study was carried out at Imam Al-Hussain Medical City/ Doctor Adel Al Sabbah Center for Hemodialysis in Karbala. One hundred and twelve patients both male and female, ranging in age from 20 to 79 years old being on hemodialysis and receiving epoetin alfa injection recommended weekly dose for more than 4 months, and sixty-two healthy controls were enrolled in the study as a reference for biochemical tests results. Biochemical and hematological tests were performed on each participant to determine levels of hemoglobin, erythropoietin, serum creatinine, blood urea, and iron levels. Allele-specific polymerase chain reaction technique was used to detect the rs6486795 T> C, A single nucleotide polymorphism in ORAI1 gene and the rs1561876 G > A, C, T single nucleotide polymorphism in STIM1 gene.

Results: The results obtained from the present study showed that regarding the distribution of ORAI1 gene rs6486795 among the patients enrolled the homozygous wild genotype TT represents 39.3 % of the population while the heterozygous mutant TC genotype and the homozygous mutant CC genotype represent 38.4% and 22.3% respectively. Regarding the distribution of STIM1 gene rs1561876 among the enrolled patients the predominant group is the homozygous mutant genotype AA which represents 53.6 % of the population followed by the heterozygous mutant genotype GA 29.5 % while the homozygous wild genotype GG takes the last place between groups with 17% of the population.

Conclusions: according to the results of the current study the findings revealed the genetic polymorphism of store-operated calcium channels genes ORAI1 and STIM1 were stated to be non-significantly associated with erythropoietin resistance but they cannot be excluded from the factors that contribute to erythropoietin resistance because the results showed that CC genotype in ORAI1 rs6486795 gene polymorphism has a statistically significant rise in Hb levels over TT genotype. And CCGG genotype who have the higher hemoglobin level and are considered good responders represent small portion of the patients' population, this might explain the commonness of erythropoietin resistance among these patients.

Chapter One
Introduction

1.1 Chronic Renal Failure

1.1.1 Definition and Classification

Chronic renal failure CRF is defined as damage of kidney structure or abnormalities in kidney function for more than 3 months (Charles & Ferris,2020; Al-Radeef et al., 2018) with glomerular filtration rate [GFR] <60 mL/min/1.73 m2 (Chen et al., 2019) in normal cases the kidneys are responsible for acid-base balance maintenance, wastes removal and controlling fluid and electrolytes balance in the body while in case of CRF electrolyte imbalance, metabolic derangement and waste products accumulation can occur (Al-Hyari et al., 2014).

The most frequent cause of CRF worldwide is Diabetes Mellitus (DM) but other causes like environmental toxins and infections can be more common in developing countries (Jha et al., 2013). In addition to DM, hypertension (HTN) is a common etiologic factor of CRF (Malekmakan et al., 2009). Other main causes include chronic glomerulonephritis, polycystic kidney disease, and chronic pyelonephritis (Ammirati, 2020). The Kidney Disease Outcomes Quality Initiatives (KDOQI) classify CRF into 5 stages depending on the GFR (Foundation, 2006).

Table 1-1 Stages of Chronic Renal Failure GFR Categories (Kakitapalli et al.,2020)

CRF stage	description	GFR (ml/min/1.73 m2)
1	Kidney damage with normal or increased GFR	> 90
2	Kidney damage with mild decrease in GFR	60-89
3	Moderate decrease in GFR	3A 45-59 3B 30-44
4	Severe decrease in GFR	15-29
5	Kidney failure	< 15 or dialysis

In early stages the disease is mainly asymptomatic, kidney function is normal in stage 1 and slightly reduced in stage 2 (Rady &Anwar,2019) while stage 3 is the first to be recognizable by blood test only (Sharma, P. et al.,2010) and the patients in this stage are divided into two subgroups depending on that the risk of kidney outcomes is higher in 3B subgroup than in 3A subgroup (Baek et al.,2012), at stage 4 the patients are peculiarly at risk of cardiovascular events and progression to kidney failure (Chertow et al., 2021), stage 5 which also called end-stage renal disease (ESRD) is known as permanent deterioration in kidney function that needs kidney transplantation or dialysis (Abbasi et al., 2010).

As Albuminuria is considered a highly sensitive marker for evaluation of abnormal kidney function (Raja et al.,2021) CRF is also classified into 3 categories by KDIGO (Kidney Disease Improving Global Outcomes) depending on albuminuria

Table 1-2 Stages of Chronic Renal Failure Albuminuria Categories (Murton et al., 2021)

Category	Albuminuria mg/24hr	Classification
A1	<30	Normal to mildly
A2	30-300	Moderate
A3	>300	Severe

1.1.2 Epidemiology

CRF is a global progressive disease that affects more than ten percent of the population in the world about 800 million individuals. Older people, women, and individuals with DM and HTN are at higher risk (Kovesdy 2022).

Fewer than five percent of patients with early stages CRF report cognizance of their disease (Chen et al., 2019). The global burden of disease (GBD) revealed that CRF is one of the leading causes of death in the world (Correction et al., 2015). As CRF can deteriorate to ESRD and lead to cardiovascular events; this will directly increase the global burden of mortality and morbidity worldwide. The increase in CRF is predominantly resulted from increasing currency of DM, HTN, aging and obesity (Lv & Zhang 2019).

The prevalence of each stage of CRF stages depending on a systematic review and meta-analysis of observational studies was 3.5%, 3.9%, 7.6%, 0.4%, and 0.1% for the five stages respectively (Hill et al.,2016). In 2017 the total number of patients with CRF of all five stages was 843.6 million worldwide (Jager et al., 2019). And by the year 2040 CRF is expected to become the fifth leading cause of death in the world (Foreman et al.,2018). Chronic renal failure prevalence was found to be 6.8% in Basra province in Iraq. (Kamil et al., 2021)

Females have a higher prevalence of CRF than males depending on US Renal Data System (USRDS) (Goldberg &Krause,2016), while a French study showed that males have a higher incidence of CRF than females in all age groups (Jungers et al., 1996) and a Chinese study represent similarity in CRF prevalence in both males and females (Zhang et al.,2008).

1.1.3 Etiology

CRF can be caused by many pathophysiological conditions that affect normal kidney function and result in a decrease in glomerular filtration rate. In the United States, the main causes of CRF are diabetes in the first place followed by hypertension and to a lesser degree glomerulonephritis, cardiovascular disease is also considered one of the important causes, still, there is a portion of the patients

that are free of the previous causes but also have CRF with advance stages especially those older than sixty-five years (Couser et al.,2011) this portion of CRF patients with unknown etiology is higher in developing countries (Sharma, S. K. et al., 2010). CRF can also be caused by autosomal dominant polycystic kidney disease (ADPKD) (Cornec et al.,2019). Some other causes encompass malignancy, polycystic kidney disease, or obstruction as in prostate disease or nephrolithiasis (Al-Radeef et al., 2018) some materials have a large influence on renal function like therapeutic agents that cause kidney damage and environmental heavy metals (cadmium, mercury, and lead) that considered nephrotoxic at high exposure levels (Loh et al.,2009; Kim et al.,2015)

1.1.4 Physiology

Normal kidney functions include waste and toxins clearance, maintenance of fluids, electrolytes, and acid-base balance. Also, regulation of red blood cell RBC production by the hormone erythropoietin EPO which maintains RBC mass by promoting the survival, proliferation, and differentiation of erythrocytic progenitors. EPO is produced mainly by peritubular fibroblasts in the renal cortex and to a lesser degree by hepatocytes and perisinusoidal Ito cells in the liver (Eckardt,2019; Jelkmann,2011; Eckardt,1996)

1.1.5 Pathophysiology

Diabetic nephropathy is the commonest glomerulopathy which is the 1st leading cause of ESRD that about fifty percent of patients with ESRD in the United States are diabetic, hyperglycemia is the first initiator of diabetic nephropathy and without it no development of nephropathy can occur.

Hypertension is the 2nd leading cause of ESRD as it causes nephrosclerotic glomerulopathy characterized by renal vasculopathy, microvascular disease of the glomerular tuft capillaries, diffuse glomerulosclerosis, and interstitial fibrosis. Afterward, GFR will decrease due to a cumulative loss of surface area, increasing glomerular and peritubular fibrosis and mesangial hypertrophy. The nephrons decrease in number during the development of CRF no matter what the etiology is, and the space that is normally taken up by glomeruli and tubules will be replaced by a fibrotic process with an extracellular matrix mainly like scarring tissue. To compensate the execratory needs of the body the leftover nephrons increase their filtration rate, when these nephrons cannot manage the prolonged fluid extra load kidney dysfunction appears. (López-Novoa et al.,2010)

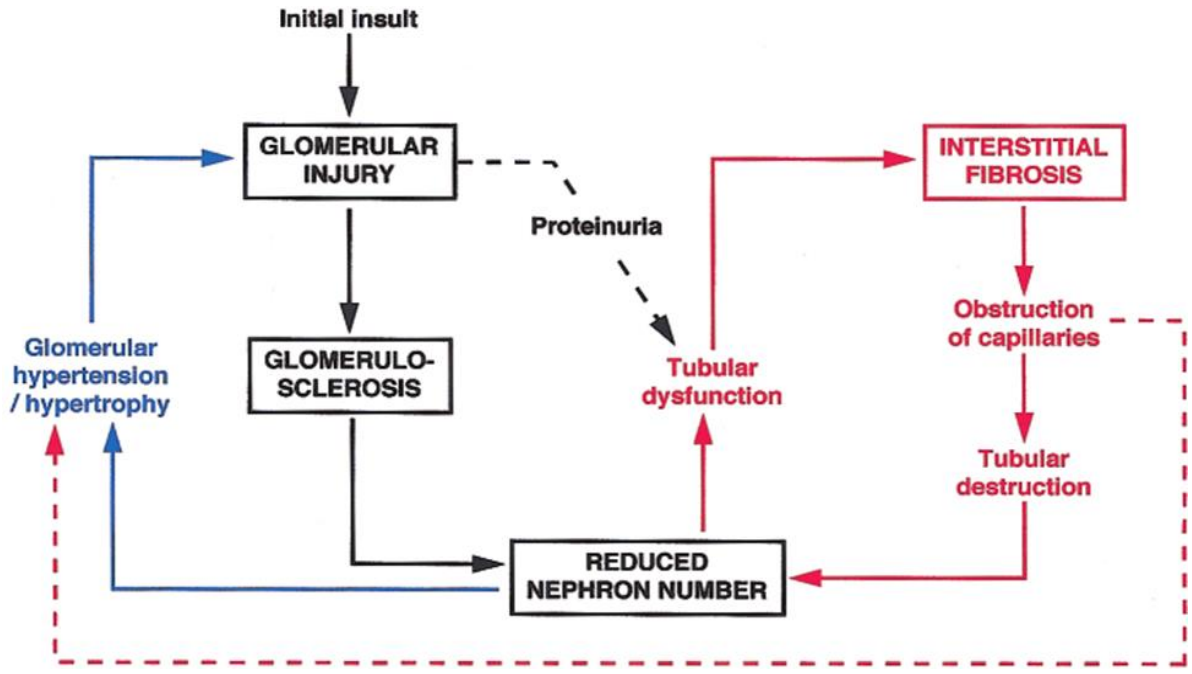

Figure1.1: the mechanisms of progression of glomerular diseases (Rossert et al., 2002)

1.1.6 Clinical presentation

CRF is a perilous widespread health problem that leads to several complications among them ESRD and early mortality (Tuttle et al.,2019) ESRD must be treated by renal replacement therapy RRT which is either kidney transplantation or dialysis, or else it will be fatal (Levin et al.,2017).

In the early stages of the disease only ten percent of patients are aware of having renal disease at the time when therapies can prevent disease progression effectively (Tuttle et al.,2022), CRF presenting acutely is not rare and usually linked with adverse outcomes, in advanced stages of CRF nausea and vomiting occur repeatedly (Wolfe et al.,2010).

Poor clinical outcomes appear as a result of the accretion of nitrogenous and non-nitrogenous toxic compounds like urea which is the dominant nitrogenous toxic compound that gives rise to uremic symptoms. Urates, Hippurates, benzoate, phenol, indoles, and guanidino compounds are other categories of nitrogenous toxic compounds (Al-Radeef et al., 2018).

CRF is commonly asymptomatic in the early course of the disease and symptoms if present are nonspecific in general like weakness, decreased appetite, changes in urination blood in urine or dark-colored urine, foamy or bubbly urine, loin pain, edema, and pale skin. while characteristic symptoms like uremia emerge almost in late stages (Arici, 2014).

Uremia symptoms comprise nausea, vomiting, anorexia, muscle weakness and malaise, asterixis, platelet dysfunction, pericarditis, seizures, changes in mental status, and perhaps coma. Such symptoms are caused by the buildup of various toxins besides urea (Snively & Gutierrez,2004).

1.1.7 Diagnosis

Often in the early stages of CRF, no symptoms appear and the disease become recognizable when a significant decrease in kidney function occurs, if diagnoses happened earlier the development of the disease can be controlled and the complications can be limited (Fink et al.,2012). At advanced stages (4 and 5) considerable organ damage is noticed that will lead to ESRD. Usually, CRF diagnosis is established depending on blood urea BU and serum creatinine S. Cr (Rysz et al.,2017), these biomarkers are not sufficient to distinguish early stages of renal disease. Serum creatinine, GFR, and albuminuria are not very accurate so they recognize renal diseases after time (Wasung et al.,2015). The development of genomics, proteomics, epigenetics, metabolomics, and transcriptomics leads to the introduction of novel techniques, which will permit the identification of novel biomarkers in renal diseases (Gentile & Remuzzi,2016) Criteria of good biomarkers include; specificity for kidney disease, high sensitivity, and ability to identify early stages of renal failure (Mok,2010).

1.1.8 End Stage Renal Disease ESRD

End Stage Renal Disease represents a parlous medical problem that emerges from the progression of chronic kidney disease (Mills et al.,2015), it is a progressive, irrevocable worsening in kidney function making the body unable to remove nitrogenous wastes and urea (uremia) and fails to maintain fluids, electrolyte, and metabolic balance (El-Gohary & Abedl-Karima,2016). In general, ESRD is treated by renal replacement therapy RRT (kidney transplantation or dialysis).

Hemodialysis is the main way of treatment in patients with ESRD and without the treatment fatality will occur within days or weeks because of disastrous complications like pulmonary edema and hyperkalemia (Rodger,2012).

In 2010 about 1.9 million patients with ESRD were on RRT in the world (Anand et al.,2013) and this number was projected to reach 5.439 million in 2030 (Filipska et al., 2021)

Patients with ESRD undergoing hemodialysis are frailer than patients of the same group if they are women, of advanced age, and have DM (Lee & Son,2021).

1.1.9 Complications

CRF is associated with many complications that result in poor clinical outcomes and low quality of life, some of which contribute to death. These complications include cardiovascular disease CVD, HTN, anemia, volume overload, mineral bone disorder, acid-base imbalance, and electrolyte abnormalities, to a lesser degree and in advanced stages nausea, anorexia, cachexia, fatigue, pruritus, and sexual dysfunction can occur (Bello et al.,2017).

A systematic review and meta-analysis showed that hyperuricemia is one of the very significant complications and patients with CRF can develop also malnutrition and inflammation (Rashid et al.,2022).

The biggest cause of morbidity and mortality worldwide nowadays is cardiovascular disease CVD, unfortunately, patients with CRF even in early course of the disease are highly vulnerable to CVD and this risk proportionally increases with the progression of renal disease (Li & Lindholm,2023).

A new study represents one big complication, patients with ESRD are at greater risk of having cancer than other patients with CRF and other healthy individuals. Among all cancerous patients, those with CRF have high prevalence and those who undergo kidney transplantation have 3 times higher chances of getting cancer than the general population (Lees et al., 2023).

It is well known that some chemotherapeutic agents used for the treatment of solid organ tumors are excreted renally and highly nephrotoxic (Perazella,2012), on the other hand some drugs that are used in the treatment of different renal conditions can cause cancer; like cyclophosphamide used to treat glomerulonephritis and severe SLE nephritis may trigger bladder cancer, erythropoiesis stimulating agent ESA prescribed for treatment of renal anemia can decrease survival rate of cancerous patients by its ability to exacerbate cancers that is already exist (Magee,2014).

Also, after a long-term duration use of immunosuppressants that follow kidney transplantation the risk of particular cancer noticeably increases and most of them are viral-associated type such as non-Hodgkin's Lymphoma and Kaposi's sarcoma (Rosales et al.,2020).

A case report study of a 12 yrs. old boy with CRF and bilateral galactorrhea found a very rare endocrinal complication in children (uremic hyperprolactinemia) caused by high peptide hormone serum level that resulted from changes in peripheral metabolism because of CRF (Çamlar et al.,2022).

In addition to the previous CRF has a role in elevating prevalence for the intensity of coronavirus disease and its related complications (Appelman et al.,2022). Other complications involve stroke, hyperkalemia, atrial fibrillation AF, and myocardial infarction MI (Betts et al.,2021).

1.2 Anemia Associated with Chronic Renal Failure

1.2.1 Definition

Anemia evolves during the early stages of kidney failure and exacerbates with the progression of renal disease, it is one of the main consequences of CRF that developed as a result of decreased erythropoietin production; other causes are chronic inflammation, blood loss, decreased erythrocyte life span, and iron insufficiency (Mohammed & Mahmood,2022).

Anemia of CRF is typically hypo-proliferative, normocytic, and normochromic (Hazin,2020) and contributes to increased rates of morbidity and mortality and decreased quality of life (Hussain et al.,2023), in addition to deterioration of renal endurance and maximizing medical costs (Minutolo et al.,2012; Nissenson et al.,2007).

The National Kidney Foundation Kidney Disease Outcomes Quality Initiative (NKF KDOQI) defines anemia as hemoglobin (Hb) level of <13.5g/dL in males and < 12.0g/dL in females (Chatterjee,2014). while the World Health Organization WHO defines it as a hemoglobin level <13 g/dL in male and postmenopausal females, and less than 12 g/dL in premenopausal females. In general anemia is a reduction in one or more RBC measurements, hematocrit, hemoglobin level, or RBC count (Thomas et al.,2008).

A systematic review and meta-analysis found that many risk factors can be associated with anemia of chronic renal failure including; ESRD, body mass index BMI >30 kg /m^2, female gender, hypocalcemia, and albuminuria (Shiferaw et al., 2020).

1.2.2 Epidemiology

Anemia is a pathological complication of CRF that increases in incidence gradually with the permanent deterioration of renal function resulting in repeated hospitalization and negative cardiovascular events (Ribeiro et al.,2013), its prevalence increased from 8.4% at stage 1 to 53.4% at stage 5, and the prevalence also elevated in patients with DM regardless GFR and albuminuria levels, anemia occurred in patients on dialysis more than non-dialysis dependent ones in percent of 93 vs 60 respectively, and dialysis-dependent patients use ESA in about three times as non-dialysis dependent patients (Portolés et al.,2021).

1.2.3 Pathophysiology

Erythropoietin is a glycoprotein hormone produced mainly by the kidney to promote the production of RBCs, and serum EPO levels elevated in case of hypoxia or anemia (Souma et al.,2015).

However, in patients with chronic renal failure EPO levels fail to rise because of the reduced production of EPO hormone due to kidney damage (Zuo et al.,2022) which will interfere with RBCs production (Hedley et al.,2011).

Those patients are usually in a nephrotic inflammatory state that leads to hepcidin production by the liver and its level elevated because of the decrease in renal clearance, this circulatory peptide is considered a potent mediator of anemia in chronic conditions by interfering with iron mobilization and metabolism (Malyszko & Mysliwiec,2007; Van der Weerd et al.,2015).

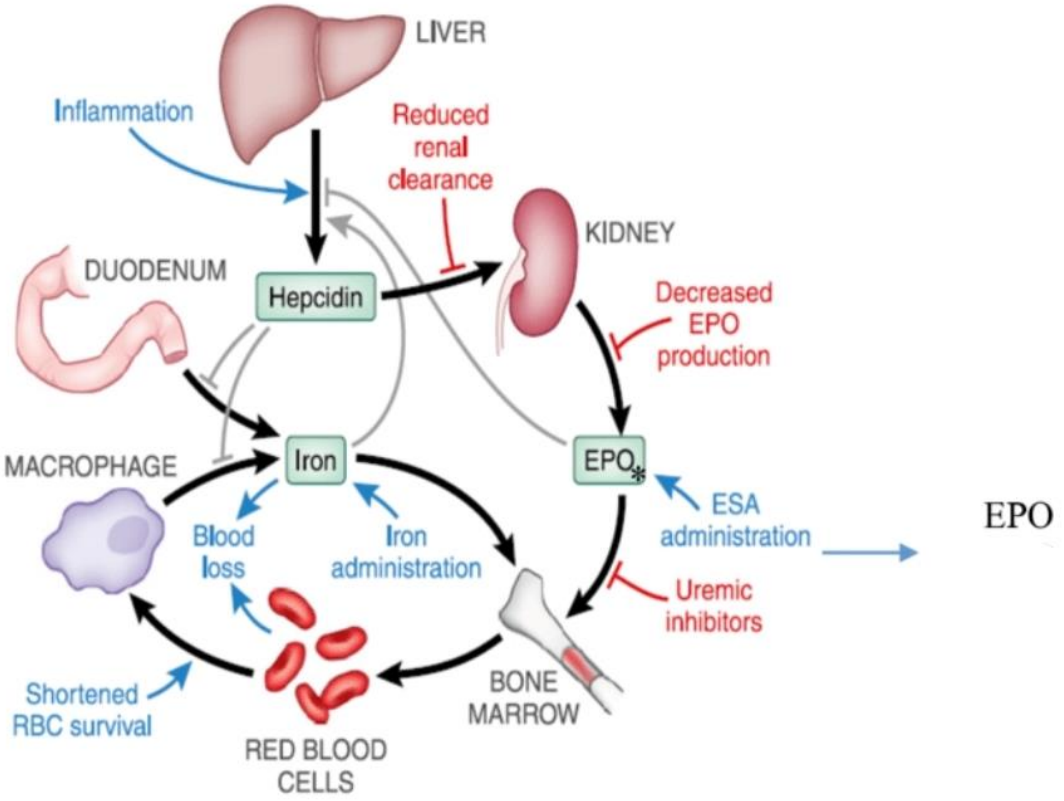

Figure 1.2 Graphical Illustration of the Mechanism of Anemia in CRF. Blue arrow symbolizes activation, red arrow for inhibition, and normal physiological pathways are represented by black and gray arrows. (Binaut et al.,2012)

Other causes include the shortening of erythrocyte lifespan (Ly et al.,2004), extensive loss of blood during dialysis by sampling, clotting in the dialyzer, blood in the dialyzer circuit, and bleeding after dialysis (Sargent &Acchiardo,2004), and uremic toxins which have a role in worsening anemia of CRF by hindering erythropoiesis (Macdougall, 2001). Although a deficiency in folic acid and vitamin b12 does not commonly occur only in about ten percent of dialyzed patients but can aggravate anemia in CRF (Zadrazil &Horak,2015), secondary hyperparathyroidism SHPT contributes to the pathogenesis of anemia in CRF and is related to minimizing the response to ESA in patients with CRF (Limrick &McNichols-Thomas,2009).

Renal anemia can also be related to malnutrition because severe hypoalbuminemia and decreased serum ferritin have an important detriment in the response of patients to ESA (Gaweda et al.,2010). Some drugs can exacerbate the anemic condition like angiotensin-converting enzyme ACE inhibitors and immunosuppressants (Malyszko et al.,2012; Hess et al.,1996).

1.2.4 Clinical manifestations

Patients with anemia of chronic renal failure mainly suffer from being tired, lethargic, exhausted, anorexic, and unable to concentrate or do exercise other symptoms include muscle fatigue, palpitations, decreased libido, and impaired memory (Macdougall,2007). Progression of anemia increases the incidence of negative cardiovascular events such as heart failure HF, angina, and left ventricular hypertrophy LVH which are the main leading causes of death in patients with CRF (Vera-Aviles et al.,2018). generally speaking, symptoms begin to appear when Hb level is below 10 g/dL (Al-Radeef et al., 2018).

1.2.5 Treatment

The gold standards for treating anemia in CRF are both ESA and iron supplementation, depending optimally on diagnosis and severity (Locatelli & Del Vecchio,2023). For dialysis-dependent DD adult patients, ESA therapy must be started when Hb level is between 9.0–10.0 g/dL and for non-dialysis-dependent NDD ones Hb level of <10.0 g/dL leads to the suggestion of initiating ESA with the recommendation of balancing benefits and risks depending on the Kidney Disease Improving Global Outcome (KDIGO) Clinical Practice Guidelines (Locatelli et al., 2013) while according to the European Renal Best Practice (ERBP) ESA therapy

must be considered at Hb level below 11 g/dL in patients with CRF and dosing is flexible conforming to the patients' symptoms, comorbidities, and the coveted Hb target (Locatelli et al.,2010). It is important to know that the Hb target for both DD and NDD patients is no more than 12.0 g/dl to avoid cardiovascular risks and other poor clinical outcomes (Kliger et al.,2013). The crucial role of hemoglobin in tissue oxygenation and its antioxidant function makes it necessary for ESA to be administered to stimulate hematopoiesis (Levin, 2007).

Iron repletion both with ESA administration helps to normalize hemoglobin levels and to evade the risk of blood transfusion plus preventing anemia progression (Parfrey, 2022). Erythropoiesis needs ESA and iron together; while erythropoietin stimulates erythropoiesis, iron deficiency can restrict the process. The importance of iron falls below its active role in hemoglobin synthesis and differentiation of erythroblasts into reticulocytes (Ganz et al.,2023; Batchelor et al.,2020).

A new family of drugs known as hypoxia-inducible factor (HIF) and their revelation led to the invention of HIF prolyl hydroxylase inhibitors (HIF-PHIs) which are oxygen-regulated heterodimeric transcription factors, emerged in the last few years that can stimulate erythropoiesis besides its role in increasing endogenous EPO production by upregulating the expression of genes responsible for the improvement of iron availability, agents in this family can also decrease hepcidin levels (Ku et al.,2023; Rashidi,2023; Portolés et al.,2021).

Erythropoietin stimulating agents (recombinant human erythropoietin rHuEPO) mainly act by increasing the production of RBCs by this they can palliate symptoms of anemia (Yugavathy et al.,2023). Administration of ESA in large doses that are needed to normalize Hb level can raise the incidence of cardiovascular negative outcomes so the clinical practice guidelines of KDIGO for anemia in CRF suggest lowering the target below the normal Hb level (Lee et al,2021).

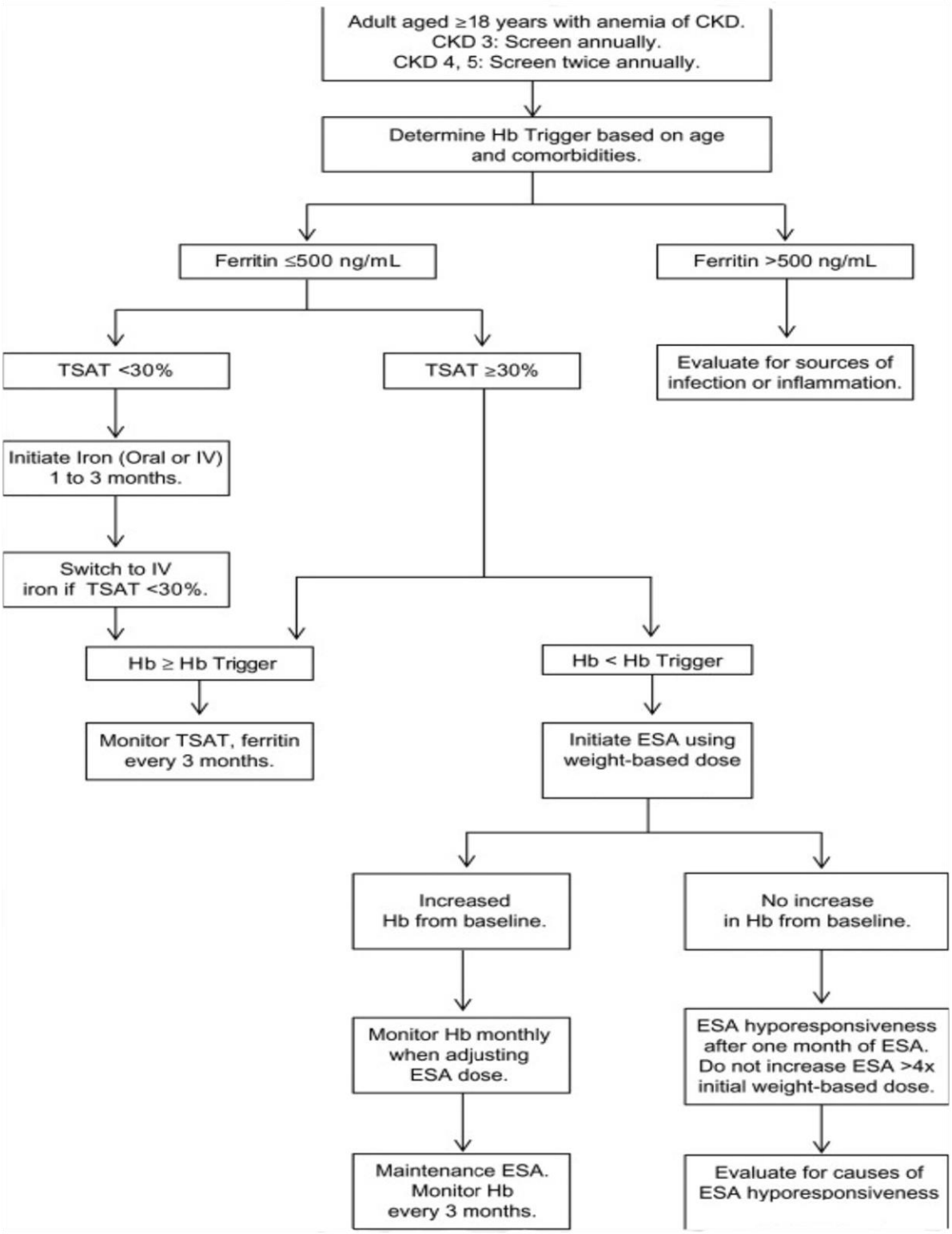

Figure 1.3 Treatment Algorithm of Anemia with CRF in Pre-dialysis Patients (Fernandez & Singh,2015) [Hb: hemoglobin, TSAT: transferrin saturation, ESA: erythropoietic stimulating]

Although a suitable dose of EPO is administered to patients with CRF on regular hemodialysis, about ten percent of the treated patients show resistance to the treatment (Jacovic et al.,2019). while as stated by the European Survey on Anaemia Management (ESAM) about 34% of the treated patients can not reach Hb level of 11 g/dL (Johnson et al.,2007).

1.2.6 Erythropoietin resistance

In addition to erythropoietin deficiency iron deficiency represents an important cause of anemia in CRF, in both absolute(absent iron store) or functional (insufficient iron availability despite adequate stores) (Gafter-Gvili et al.,2019), and the main cause of EPO resistance is iron insufficiency (Macdougall et al.,1991) so iron supplementation in anemia associated with CRF is imperative for maximizing ESAs activity in order to decrease the incidence of adverse effects and the economic load of utilizing ESAs (Batchelor et al.,2020; Lee et al.,2021).

ESAs hyporesponsiveness in both DD and NDD patients correlates with increased mortality rates and consequences of CVD, this hyporesponsiveness can result from inflammation, nutritional disturbance, and iron metabolism (Mase et al.,2023). Inadequate dialysis, infection, hemolysis, blood loss, genetic polymorphisms, hyperparathyroidism, and the use of antihypertensive drugs that interact with ESAs like angiotensin-converting enzyme ACE inhibitors and angiotensin receptor blockers ARB are also associated with resistance (Alves et al., 2015; Samavat et al.,2019; Corredor et al.,2020), other recorded causes of resistance involve uremia, vitamin deficiency, and aluminum toxicity (Gunnell et al., 1999; Wu & Chinnadurai 2022).

EPO resistance is known as tenacious anemia or the need for extremely high doses of ESA (300 IU/kg/week subcutaneously or 450 IU/kg/week intravenously) (Alves et al., 2015).

The NKFKDOQI guidelines define EPO hyporesponsiveness as, at the minimum one of the following states:

- a remarkable increase in EPO dose needed to keep a particular Hb level
- a noticeable decrease in Hb level with constant EPO dose
- inability to elevate Hb level more than 11 g/dL even with the use of EPO dose equivalent to epoetin higher than 500 IU/kg/wk (Al-Radeef et al., 2018)

1.2.6.1 Iron deficiency

CRF patients encounter a noticeable change in iron distribution and balance, some cells and tissues are highly supplied with iron while others have iron insufficiency and this regulatory impairment can mainly interfere with erythropoiesis which makes iron deficiency (in both types absolute or functional) the most common cause of EPO hyperresponsiveness (Wojtaszek et al.,2020; Drüeke, 2001). Iron deficiency occurs when the iron absorbed from diet is not enough to compensate iron losses and in advance stages of CRF iron metabolism can be disturbed by multiple mechanisms (Ganz & Nemeth,2016).

Among patients with anemia associated with CRF, 25-37% of them have iron deficiency and as an optimal treatment IV iron supplementation is recommended for its better effectivity in DD patients (Ribeiro et al.,2013). A lot of risk factors have a hand in iron deficiency like impaired iron absorption, blood loss, and chronic inflammation (Santos et al.,2020).

1.2.6.2 Inflammation

Inflammation can mainly hinder erythropoiesis by the increasing activity of the proinflammatory cytokines (Barany, 2001) about 35% to 65% of DD patients experience inflammation signs which might be the cause of anemia due to bone marrow erythropoiesis suppression by cytokines (Del Vecchio et al., 2005).

Because of bacterial and viral infections, renal disease, and compromised immune system in uremic patients the inflammation prevalence in them is high (Stenvinkel et al., 2000).

DD patients often have elevated levels of inflammatory markers including interleukin-6 (IL-6), c reactive protein (CRP), tumor necrosis factor-alfa (TNF-α), interferon-gamma (INF-γ), transforming growth factor (TGF), and low levels of serum albumin (Ribeiro et al.,2013; Petreski et al., 2021).

As chronic inflammation that is usually present in patients with CRF has a significant role in EPO resistance the future pharmacological treatment strategy in the treatment of EPO resistance associated with inflammation will be the use of anti-cytokine and anti-oxidant (Gluba-Brzózka et al., 2020).

1.2.6.3 Hyperparathyroidism

Among the important factors that can cause EPO resistance are hyperparathyroidism (Benkova-Petrova, 2021), calcitriol deficiency, PTH effect on erythropoietin release (directly or indirectly), RBCs production, survival, and loss are the possible process by which parathyroid hormone causes anemia (Drüeke & Eckardt, 2002).

Elevated PTH levels are negatively associated with decreased Hb levels in patients with CRF on hemodialysis receiving ESAs (Khan, 2017), that a remarkably higher Hb level was observed in patients with PTH levels lower than 300 pg./ml and significantly decreased Hb levels in patients with PTH level exceed 300 pg./ml (Idan & Abdalrahman, 2023).

ESA dose needed for the treatment of renal anemia will decrease with the control of hyperparathyroidism and anemia improvement will be noticed after treating hyperparathyroidism (Madhoun et al.,2023), no specific PTH target is optimal in the treatment of hyperparathyroidism to improve EPO resistance anemia (Ashraf et al.,2022).

1.2.6.4 Inadequate dialysis

Inadequate dialysis is one of the important leading causes of resistance to ESAs in treating anemia of patients with CRF on hemodialysis (Benkova-Petrova et al., 2020), this hypo-responsiveness can result from the accumulation of Uremic toxins by nonselective bone marrow suppression, the adequacy of hemodialysis dose is determined by Kt/V (when K is the dialyzer urea clearance, t is the dialysis time, and V is the patient's urea distribution volume) or urea reduction ratio (Chiang et al.,2022; Manuti, 2021).

The use of expanded hemodialysis EHDx (a novel class of dialysis membranes that are effective in the clearance of middle and large molecules without fluid substitution) has a role in ESA response improvement as compared with the use of a high-flux HF dialyzer and online hemodiafiltration HDF mainly due to the removal of inflammatory cytokines (Yasin & Omran, 2023).

1.2.6.5 Chronic blood loss

Blood loss is recurrent in dialysis-dependent patients due to the use of dialysis machines (Lacquaniti et al.,2020) and it is one of the etiologies that contribute to EPO hypo-responsiveness (Munie & Pintavorn,2021).

1.2.6.6 Aluminum overload

Accumulation of aluminum is a noted consequence in patients undergoing dialysis but the development of hemodialysis technique reduced the occurrence of aluminum overload (Chen et al., 2022), subclinical aluminum toxicity has an inhibitory role on the response to ESAs (Tarng & Huang,1998) aluminum deposition can interfere with bone formation and mineralization resulting in aluminum-related bone disease (Zhong et al., 2020) plasma aluminum level increased due to the use of dialysis fluid, aluminum-containing phosphate binders and antacids (Qunibi, 2020).

1.2.6.7 Nutritional deficiencies

About 23–60% of DD patients have malnutrition which is a remarkably progressing health problem (Feret et al.,2022), malnutrition is one of the risk factors for ESA hypo-responsiveness (Kanbay et al., 2010) and it is largely associated with inflammation and can interrupt response to ESAs by the aid of inflammatory mediators (Yajima et al., 2021).

EPO response and hemoglobin level improvement can be achieved through good nutrition in DD patients (Gityamwi, 2020).

1.2.6.8 Malignancy

Studies showed that some cancers have a role in EPO resistance, and this can be explained by the expression of erythropoietin receptors in several cancer types including breast, lung, prostate, and skin cancers (Lazzari & Silvano, 2020). Usually, cancerous patients experience EPO hypo-responsiveness in the range of 15–75% mainly seen in myelodysplastic syndromes and to a lesser degree in multiple myeloma and chronic lymphocytic leukemia (Johnson et al., 2020).

1.2.6.9 Non-compliance

Compliance for DD patients is divided into three sections including medication, dietary, and dialysis treatment prescription (Kaveh & Kimmel, 2001), either by pharmacy record review or by the questionnaire a less than 90% utilization of prescribed dose is known as non-compliance, noncompliance injections of ESAs is proportionally common and the rate of it about 35% (Wazny et al., 2002) the first common cause of non-compliance is forgetfulness and the second one is injection pain (Johnson et al., 2007).

1.2.6.10 Angiotensin-converting enzyme inhibitors and angiotensin receptor blockers

ACE inhibitors and ARBs both have a role in ESAs hypo-responsiveness in patients with CRF by multiple mechanisms like prevention of angiotensin II-induced erythropoietin release, reduction in the sensitivity to rHuEPO, and an increase in plasma levels of the tetrapeptide N-acetyl-seryl-aspartyl-lysyl-proline (erythropoiesis inhibitor that occurs naturally), which prevents the recruitment of pluripotent hematopoietic stem cells (Kwack &Balakrishnan, 2006).

1.2.6.11 Genetic Polymorphisms

Some genetic polymorphisms can cause EPO response differences in patients with renal anemia like IL-1B and ACE gene polymorphism which has a significant role in regulating EPO response and CRF progression. Studies have shown that ACE DD genotype and IL-1B CC genotype have lower erythropoietin requirement (Nand et al., 2017).

Another study of ACE gene polymorphism showed that Patients with either ACE G2350A (rs4343) II or DD genotype showed better response to rHuEPO than those with ID genotype and Screening for ACE G2350A (rs4343) gene polymorphisms in patients with CRF on hemodialysis before rHuEPO administration may predict patients' response (Hamdan & Mostafa, 2021).

In Kuala Lumpur a study performed showed that EPO gene (rs1617640) polymorphism is associated with low serum EPO in pre-dialysis CRF patients. the recessive HIF-1α gene (rs2057482) model is associated EPO-deficiency. And the recessive gene model of IL-1β (rs1143627) is associated with Hb less than 10 g/dl raising a possible explanation on how all three genes polymorphism can be related to EPO-deficiency anemia in pre-dialysis patients (Yugavathy et al., 2020)

1.3 Erythropoietin

1.3.1 Definition

Erythropoietin (EPO) is a crucial endogenous glycoprotein hormone produced by fibroblast cells in the kidney (90%) and only (10%) by other sources mainly liver cells and principally functions in the regulation of RBCs by controlling the promotion of proliferation, differentiation, and survival of erythroid progenitors (Dahl et al.,2022; Zeisberg & Kalluri, 2015; Lacombe & Mayeux, 1998).

1.3.2 History

In Paris, a pupil of Claude Bernard school Paul Bert described an increased number of RBCs with an increased blood oxygen capacity in high altitudes animals for the first time in 1882 and considered it as genetically derived (Höke, 2006). Viault the French histologist noticed rice in his RBCs after a 2weeks journey from sea level to the mountains in 1890, this furnished the first reveal of increased erythropoiesis after exposure to hypoxia in high altitude by Friedrich Miescher in 1893. Results of an experiment done by Carnot and Deflandre in 1906 showed an increase in RBCs of normal rabbits after infusion of serum from anemic animals they concluded that erythropoiesis is modulated by a humoral factor in the plasma (Bunn, 2013).

This experiment was modified in the middle of the 20th century by Krumdieck (1943) and Erslev (1953) through the addition of precise measurements of reticulocytes and this experiment showed induction of new RBCs production in a group of rabbits in less than a week after injection of serum from anemic animal while a control group of rabbits showed no observable difference in RBCs count after injecting with the same amount of serum from normal rabbits, these experiments led to the conclusion of the presence of a substance capable of stimulating RBCs production (Krumdieck, 1943; Erslev, 1953). This hemopoietic substance was named erythropoietin in 1948 by two Finnish scientists, Bonsdorff and Jalavisto who continued the work on RBC production (Al-Radeef et al., 2018).

The belief of erythropoiesis stimulation by hypoxia was approved in 1950 through the experiment of Reissmann and Ruhenstroth-Bauer by using parabiotic pairs of rats that their circulations were connected and the study showed that both rats the anemic and normal one experienced erythropoiesis and new RBCs production. To determine erythropoietin production site in 1957 Jacobson showed

that only kidney removal prevents erythropoiesis after bleeding (Sytkowski, 2006). Radio-labeled iron uptake in the newly produced RBCs was the most convenient process for studying the impact of anemic plasma on erythropoietin production this was done by W. Fried in 1955 (Fried et al., 1956). By the year 1964 Davin G. Nathan approved that the kidney is the main site for EPO production but not the only one by a study of patients in a renoprival state prepared for kidney transplantation (Nathan et al., 1964).

In 1977 the important work of Goldwasser and his team led to the purification of human erythropoietin from an anemic patient (Miyake et al., 1977), the isolation of human erythropoietin depending on a limited amino acid sequence with the cloning and expression of its gene led to the exploration of physiology and molecular biology of human EPO in 1985 (Lin et al., 1985; Jacobs et al.,1985). In 1989 US Food and Drug Administration approved the use of recombinant human erythropoietin (rHuEPO) in clinical practice for treating anemia in ESRD DD patients (Powe et al., 1992).

1.3.3 Structure

Human erythropoietin is an acidic glycoprotein of 30.4-kDa molecular mass, the composition of the peptide core is 165 amino acids forming two disulfide bridges (Cys7–Cys161, Cys29–Cys33), the carbohydrate portion which represents 40% of the molecule consists of three tetra-antennary N-linked (at Asn 24, Asn 38, and Asn 83) and one small O-linked glycan (at Ser 126) glycans, the N-glycans are crucial for the molecular stability, receptor binding, secretion, and the bioactivity of EPO in vivo (Jelkmann, 2007; Jelkmann, 2016).

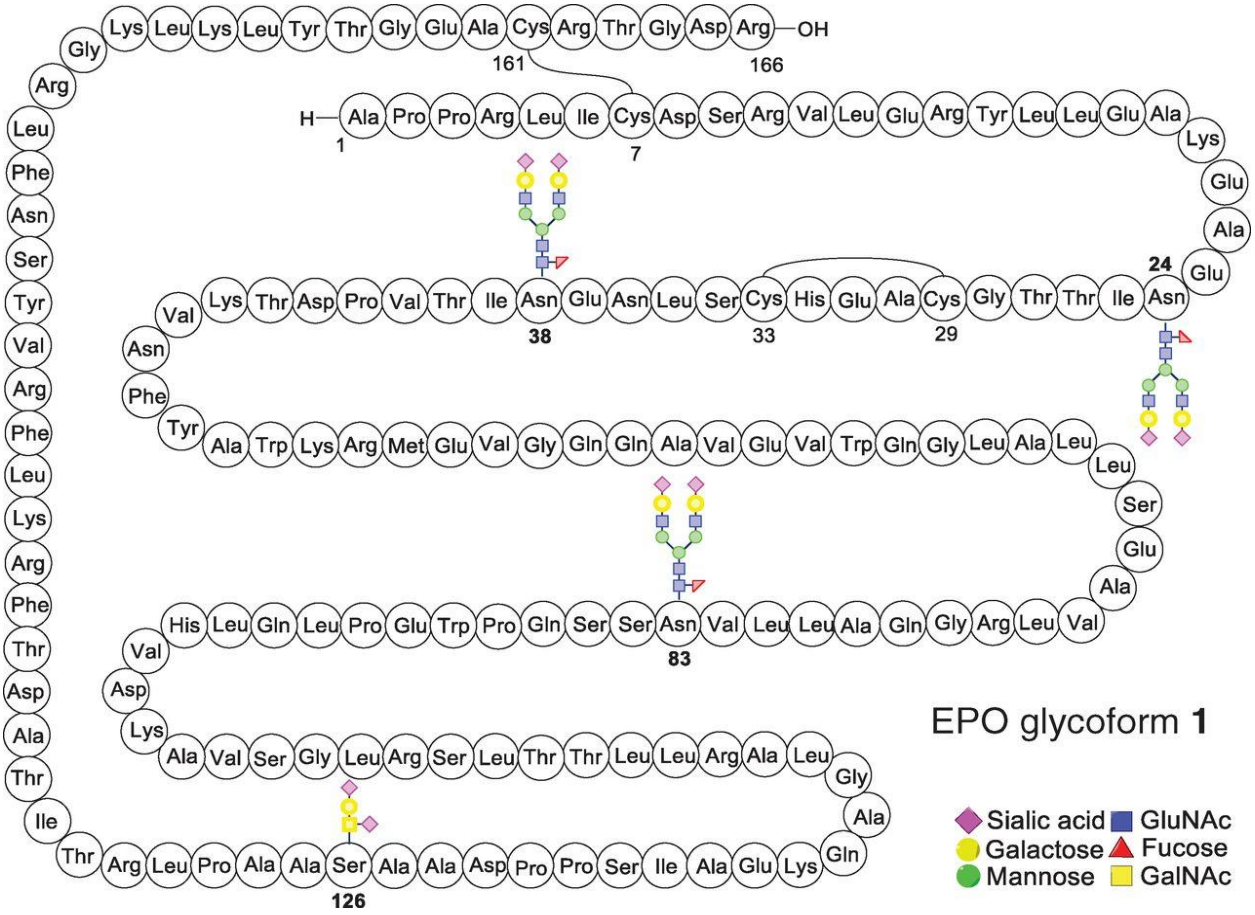

Figure 1.4. Erythropoietin structure shows the 165 circulating amino acids, Two disulfide bonds bind the molecule together between cysteines 29 and 33 and cysteines 7 and 161, Three N-linked sugars are present at asparagines 24, 38, and 83, and one O-linked sugar is present at serine 126 (Wang et al., 2013).

The most important moieties of these glycans are the terminal sialic acid residues and the carbohydrate portions embrace at least 10 molecules of sialic acid which contribute to the low iso-electric pH of erythropoietin (Al-Radeef et al., 2018). EPO is one of the type 1 cytokine superfamily members that is characterized by proportionally rigid globular glycoproteins with four a helices bound together by hydrophobic interactions (Brines & Cerami, 2012). After kidney production of EPO, the plasma half-life is about 5-6 hrs because of the high glycosylation levels (Peng et al., 2020).

1.3.4 Production and Regulation

During fetal life, the main source of erythropoietin production is the liver while in adults the kidney is the major site of EPO production (90% of circulating EPO) and to a very lesser degree the liver (10 % of circulating EPO) (Ohls, 2000; Kietzmann, 2020).

Other organs that participate in EPO production include lung, heart, brain, bone marrow, spleen, reproductive tract, osteoblasts, and hair follicles but the EPO produced by these cells acts locally modulating, for example, cellular viability and regional angiogenesis (Haase, 2013).

Healthy adults produce 200 billion RBCs per day to compensate daily loss of RBCs by senescence in a process called erythropoiesis that is regulated by a mechanism of oxygen-sensing which is responsible for maintaining RBC numbers within the physiological range (Bhoopalan et al.,2020).

Erythropoietin has a key role in erythropoiesis regulation, it is accountable for proliferation, survival, and differentiation of erythroid- progenitors into RBCs, the RBCs carry hemoglobin which is critical for tissue oxygenation (Lanzolla, 2023).

Erythropoietin acts by binding to a specific trans-membrane dimeric receptor which has been found in erythroid and non-erythroid cell types (Foley, 2008).

Hypoxia mainly triggers EPO release to increase oxygen-carrying capacity by stimulating RBCs production and it is an essential characteristic of CRF both with insufficient EPO production. Hypoxia in CRF can be caused by; Loss of peritubular capillaries, decrease in peritubular capillary beds, fibrosis of the tubulointerstitium, oxidative stress, and inflammation (Wojan et al., 2021; Wang et al., 2022).

1.3.5 Erythropoiesis

Erythropoiesis is the process of new RBCs production. To dictate the required RBCs number the bone marrow depends on the kidney in which interstitial fibroblasts in the renal medulla sense hypoxia, leading to the production of the hypoxia-inducible factor 2 (HIF-2) resulting in EPO excretion followed by binding to its receptor on erythroid precursors in the bone marrow to induce their survival, cell division, and differentiation to enucleate, producing reticulocytes that mature to RBCs in the circulation.

It is a complex multi-step process and is divided into three maturational stages, early-stage erythropoiesis, terminal erythroid differentiation, and reticulocyte maturation. Early-stage erythropoiesis consists of two erythroid progenitor stages, burst-forming unit-erythroid (BFU-E) and colony-forming unit-erythroid (CFU-E). The process by which proerythroblasts (Pro) differentiate consecutively to basophilic (Baso), polychromatic (Poly), and orthochromatic (Ortho) erythroblasts that expel their nuclei to become reticulocytes is called terminal erythroid differentiation.

Reticulocyte maturation which is the final step of erythropoiesis includes major changes like; membrane surface area loss via membrane vesiculation, organelle clearance via autophagy, and membrane skeleton reorganization.

The result will be fully functional mature RBCs with maximum hemoglobin-carrying capacity and flexible but stable membranes (Ginzburg et al., 2023).

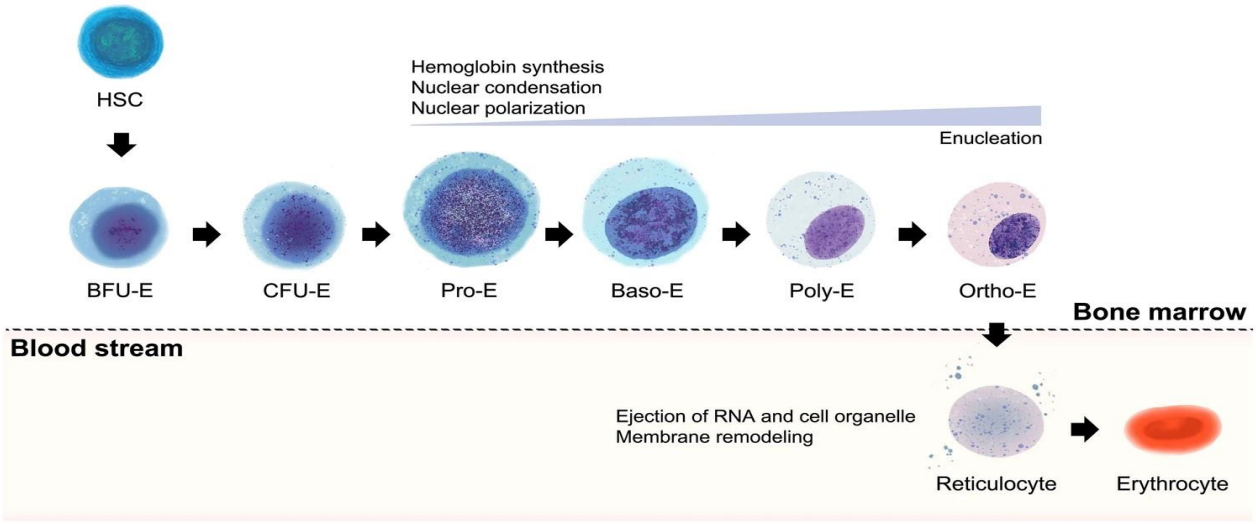

Figure 1.5 Erythropoiesis in vivo with stages and cell types from hematopoietic stem cells. HSC hematopoietic stem cell, BFU-E burst-forming unit erythroid, CFU-E colony-forming unit erythroid, Pro-E proerythroblast, Baso-E basophilic erythroblast, Poly-E polychromatic erythroblast, Ortho-E orthochromatic erythroblast (Han et al., 2023).

1.3.6 Degradation

Erythropoietin is removed from circulation mainly by uptake into erythrocytic cells and other cells that contain EPO receptors and to a lesser degree, EPO is cleared by the kidneys and the liver (Jelkmann, 2004).

1.3.7 Non hematopoietic effects of erythropoietin

EPO has many effects other than the hemopoietic action, during fetal life it is required for embryonic angiogenesis and brain development in addition to liver erythropoiesis. It also has a role in wound healing responses, physiological and pathological angiogenesis, tissue protection, the body's innate response to injury in the brain and heart, and promotion of tumor cell growth or survival (Arcasoy, 2008; Elliott & Sinclair, 2012).

1.4 Epoetin alfa

1.4.1 Definition

Epoetin alpha is chemically synthesized rHuEPO by recombinant DNA technology in Chinese hamster ovary cells and has the identical 165 amino acids sequence of endogenous circulating EPO, it is the first rHuEPO to arrive on the market (Littlewood & Collins, 2005).

1.4.2 Dosing and Administration

Epoetin alfa is available in single-dose or multi-dose, it can be administered via the intravenous or subcutaneous route, shaken or frozen vials must not be used as in such cases the drug is inactive biologically (Patel, S. & Patel, J., 2020). In adult patients with CRF on hemodialysis, the starting dose is 50 IU/kg, thrice weekly, increase or decrease the dose by 25 IU/kg thrice weekly if necessary until the target Hb level is achieved dose adjustment must be done gradually (Compendium, 2016).

1.4.3 Adverse Effects

For patients with renal anemia, a meta-analysis greatly suggests that epoetin alfa effectively increases hemoglobin and hematocrit levels leading to decreases in hospitalizations and transfusions and improvement in quality of life (Jones et al., 2004). On the other hand, epoetin alfa contributes to several unwanted effects including headache, edema, vomiting, tachycardia, nausea, shortness of breath, diarrhea, and iron deficiency. Hb level of more than 11 g/dL or a rapid rise in hemoglobin will increase the risk of negative cardiovascular events; stroke, myocardial infarction, and venous thromboembolism.

A rapid increase in hematocrit results in thrombotic events due to increased blood viscosity and peripheral vascular resistance. Even though epoetin alfa has no direct effect on blood pressure it can cause HTN after administration when a sudden rise in hematocrit happens. Erythema at the site of injection and flu-like symptoms can also occur after administration (Patel, S. & Patel, J., 2020).

1.4.4 Pharmacokinetics

1.4.4.1 Absorption and bioavailability

The half-life t1/2 of erythropoietin administered intravenously ranges from 5-11 hours, the same as t1/2 of endogenous erythropoietin(average t1/2 = 5.2 hours), while Subcutaneous (SC) administered EPO has slower absorption with subsequently low peak plasma levels about 5–10% of IV administered EPO peak plasma levels and extended t1/2 range from 20-25 hours. Peak plasma levels are mostly between 15 and 29 hours.

The bioavailability of SC EPO is about 20% to 40% the loss of material occurred during transport to the blood and lymphatic system from the interstitial space, these pharmacokinetics of rHuEPO approximately are the same in healthy volunteers and patients with CRF (Elliott et al., 2008).

1.4.4.2 Distribution

The volume of distribution is similar to the plasma volume regarding IV administration it is about 40–60 mL/kg signifying restricted extravascular distribution, while it is about 6-fold lower after SC administration (Markham & Bryson, 1995; Elliott et al., 2008).

1.4.4.3 Clearance

At first, it was thought to be established by liver or kidney in the main place, but studies showed that renal clearance is not a significant route other studies demonstrate that EPO exhibits degradation inside the body. Binding of EPO to the EPOR can lead to cellular internalization, at this step the ligand can be degraded(Dinkelaar et al.,1981; Flaharty, 1990; Yoon et al.,1997; Elliott et al., 2008) and the lymphatic system has an important role in decreasing bioavailability after SC administration of proteins (Porter & Charman, 2000).

1.4.5 Pharmacodynamic

Erythropoietin binds to a particular dimeric EPO receptor (EPOR) which is a member of the cytokine receptor superfamily, JAK-STAT-binding receptor (Janus kinase/signal transducers and activators of transcription) on the surface of its target cells. It eventually alters the phosphorylation of intracellular proteins and activates transcription factors to regulate gene expression. EPO induces erythropoiesis leading to stimulation of the proliferation of colony-forming erythroid, Inducing hemoglobin formation and erythroblast maturation, and reticulocytes release in circulation followed by a rise in hematocrit and hemoglobin levels (Patel, S. & Patel, J.,2020; Littlewood &Collins, 2005). Epo induces an increase in intracellular free Ca2+ in human erythroblasts, which is dependent on extracellular Ca2+, by regulating a voltage-independent Ca2+ channel and this Epo-regulated Ca2+ channel belongs to the family of second messenger-operated Ca2+ channels that may have a role in controlling erythroblast differentiation. (Cheung et al., 1997) Among these channel types, store-operated calcium channels (SOCs) are prominent in the non-excitable cells and it is a part of EPO activation signaling pathway (Kao et al., 2021).

1.4.6 Store-operated calcium channel SOCs

Store-operated calcium channels are so named because they are activated by the depletion of Ca2+ from the endoplasmic reticulum (ER), these channels are typically activated by the engagement of cell surface receptors that through G proteins or a tyrosine kinase cascade activate phospholipase C to cleave phosphatidylinositol 4,5-bisphosphate (PIP2) and produce inositol 1,4,5-trisphosphate (IP3), IP3 will induce Ca2+ release through IP3 receptors in the ER membrane.

SOCs are distinctive among ion channels, because of their molecular basis, biophysical properties, and mode of regulation, they have a homeostatic role in furnishing Ca2+ to refill the ER after Ca2+ has been released and pumped out across the plasma membrane.Because of the limited Ca2+ capacity of the ER, Ca2+ release can only generate temporary signals; but protracted store depletion can give rise to Ca2+ entry through SOCs that is sustained for minutes to hours, driving a wide assortment of basic biological processes such as secretion, gene transcription, and modulation of enzymatic activity and motility.

SOCs remained an enigma for two decades after their first proposal, in 2005 STIM1 proteins (Stromal Interaction Molecule 1) were identified as ER Ca2+ sensors, and by the next year ORAI1 (calcium release-activated calcium modulator 1) proteins were identified as SOC subunits. This important discovery helped to explain the molecular mechanisms and functions of SOCs in many cells and tissues (Prakriya & Lewis, 2015).

EPO increases PLC-y1 tyrosine phosphorylation, promotes the formation of membrane complex between PLC-y1 and the EPO receptor itself, and raises the levels of intracellular inositol 1,4,5-trisphosphate and intracellular ca2+ (Ren et al., 1994).

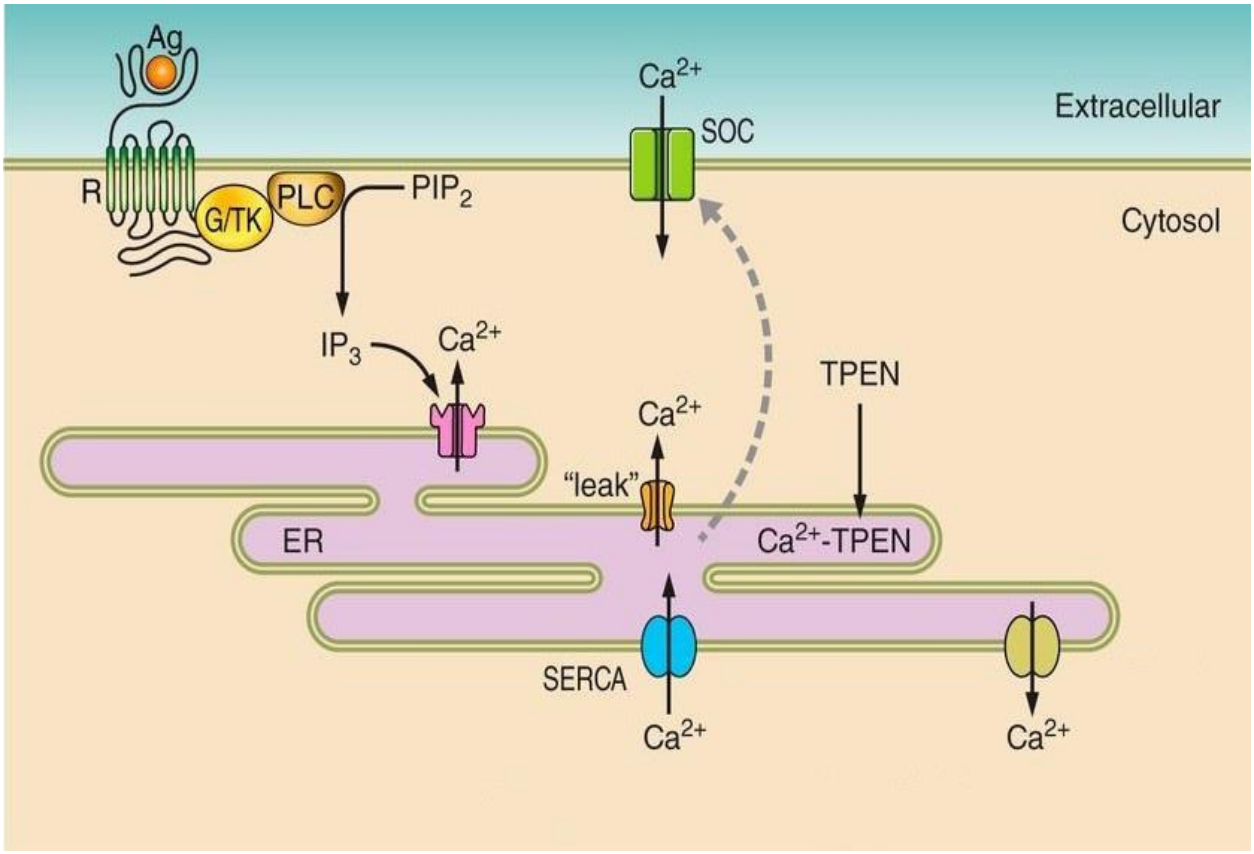

Figure 1.6 diagram represents Store-operated calcium entry mechanism. (Prakriya & Lewis, 2015) Before the discovery of STIM and ORAI1 in normal conditions extracellular agonists (Ag) bind to receptors (R) and activate PLC through a G protein or tyrosine kinase-coupled pathway (G/TK). PLC cleaves PIP2 to produce IP3, which releases Ca2+ from the ER. Store-operated channels (SOCs) are activated by the consequent reduction of ER luminal [Ca2+]. SERCA SarcoEndoplasmic Reticulum Calcium ATPase.

At rest when calcium stores (ER) are filled, ORAI1 (the channel) is found dispersed throughout the plasma membrane and STIM1 (the sensor) throughout endoplasmic reticular membranes. After calcium depletion from its store due to receptor activation ORAI1 and STIM1 become juxtaposed resulting in activation of ORAI1 by binding to STIM1 and thus calcium influx with subsequent stores refilling (Geng et al., 2017).

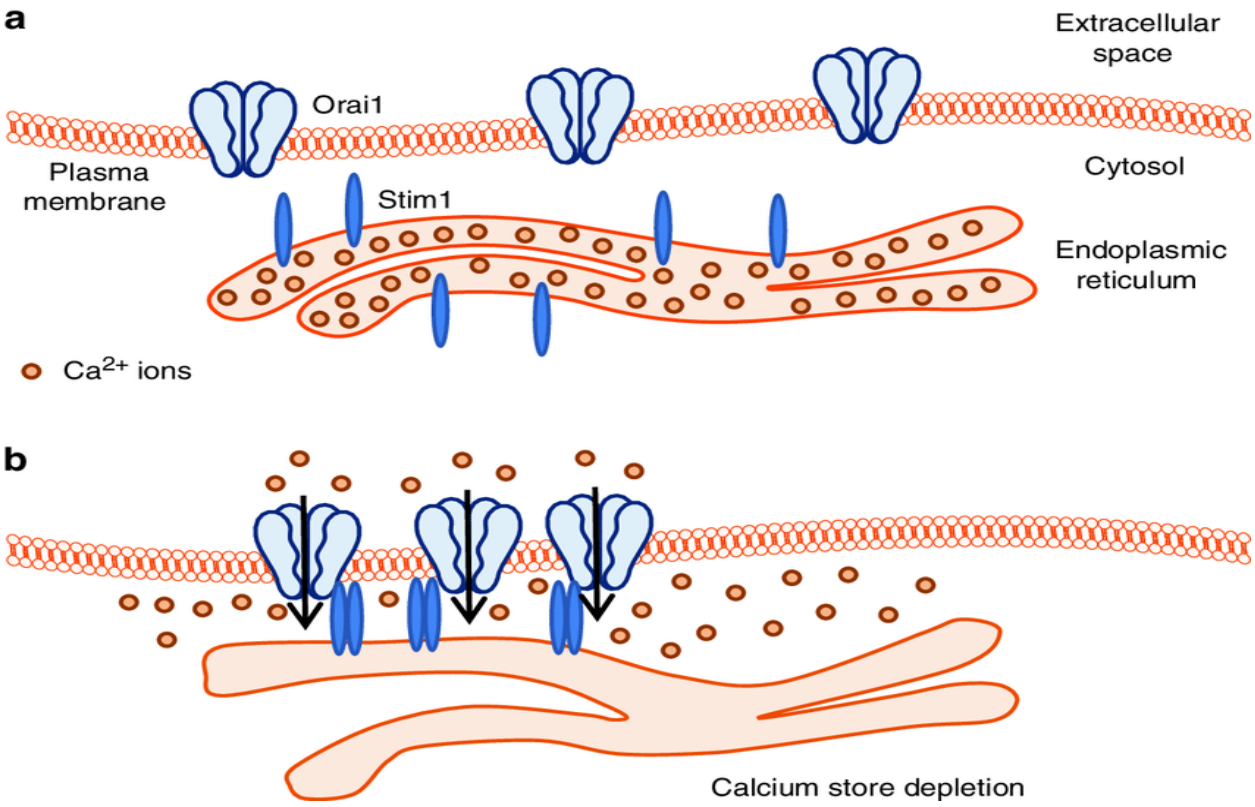

Figure 1.7 Store-operated calcium entry a: at rest. b: after calcium depletion. (Geng et al., 2017)

1.5 Genetic polymorphism in STIM1 and ORAI1 with erythropoietin resistance

When two individuals of the same family inherit the same disease and are treated with the same treatment experience different responses the cause is mainly a genetic factor (Oates & Lopez, 2018).

A single-nucleotide polymorphism (SNP) also known as a genetic variation is the difference in the sequence of nucleotides that may have effects on the pharmacodynamic and pharmacokinetic characteristics of drugs (Yugavathy et al., 2023).

Genetic polymorphisms are naturally occurring variants in the structure of the gene and happen in further than 1% of the population. The study of the changes in drug response due to genetic variations is called pharmacogenomics (in case of studying all genes) or pharmacogenetics (in case of studying a particular gene) (Belle & Singh, 2008).

As store-operated calcium channels play a role in erythropoietin activation pathway, the genetic polymorphism in one or more components of this pathway may lead to disruption of the pathway resulting in EPO resistance.

In 2021 a study performed in Taiwan illustrated that SOC-related genetic polymorphisms have a significant correlation with the risk of EPO resistance in dialysis patients, in genetic polymorphism of STIM1 gene rs1561876 and ORAI1gene rs6486795 patients who carried AA genotype of rs1561876 or CC/CT genotypes of rs6486795 have increased risk of EPO resistance (Kao et al., 2021).

So that the association of genetic polymorphisms in these two genes with EPO resistance is still highly questionable.

Both rs1561876 of STIM1 gene and rs6486795 of ORAI1 gene locate in non-coding regions (3ˈUTR) and (intron) respectively (Lou et al., 2020;Chou et al.,2011).

1.6 Aims of Study

This study is designated to investigate and spotting a light on:

- The distribution of ORAI1 gene polymorphism rs6486795 T > C, A and STIM1 gene polymorphism rs1561876 G > A, C, T in patients population.
- The correlation of STIM1 gene polymorphism rs1561876 G > A, C, T and ORAI1 rs6486795 T > C, A gene polymorphisms with erythropoietin resistance in patients with CRF on hemodialysis in Iraq.
- The impact of the interaction of the two SNPs on Hb blood level and EPO serum level in patients with CRF on hemodialysis in Iraq.

Chapter Two
Materials, Individuals, and Methods

2. Materials, Individuals, and Methods

2.1 Materials

The chemicals, kits, and instruments used in this study with their manufacture and origin are listed in Tables 2-1 and 2-2.

2.1.1 Instruments

Table 2-1: Instruments used in this study with their manufacture and origin.

Instrument	Manufacture/ Origin
Automated hematology analyzer XP series	Sysmex/Japan
Elisa reader PKL	Paramedical /Italy
Cobas c 111 analyzer	Roche /Switzerland
Centrifuge PLC series	Gemmy Industrial/Taiwan
High-speed centrifuge	sigma 3-30K/Germany
Incubator	Binder/Germany
Nanodrop	Thermo Fisher Scientific/USA
Hot plate Stirrer	LabTech / Korea
UV-transilluminator	Major science /Taiwan
Electrophoresis apparatus	Cleaver Scientific Ltd/ UK
PCR -thermal cycler veriti	Thermo Fisher Scientific/USA
Refrigerator	Denka / Japan
Freezer (-20)	Elryan/ China
Electronic scale	G&G /Germany
Digital camera	Canon/UK

2.1.2 Chemicals and Kits

Table 2-2: Kits and chemicals used in this study with their Manufacture and origin.

Kits and chemicals	Manufacture/ Origin
gSYNC DNA extraction kit	Geneaid/ Korea
AccuPower® PCR PreMix	Bioneer/ Korea
100 bp DNA ladder	Bioneer / Korea
10x TBE buffer	MarLiJu /Korea
Human EPO (erythropoietin) ELISA Kit	Elabscience /USA
Agarose powder for gel	MarLiJu /Korea
Primers	Bioneer / Korea
Ethidium bromide	Promega/ USA
Absolute ethanol	Honeywell/ Germany
Distilled water	Pioneer/ Iraq
Erythropoietin vial for injection 4000 U/ EPREX	Cilag AG/ Switzerland

2.2. Individuals

2.2.1. Study Population

This study was a cross-sectional observational study that was carried out at Imam Al-Hussain Medical City/ Doctor Adel Al Sabbah Center for Hemodialysis in Karbala, during the period from November 2022 to April 2023.

2.2.1.1. Ethical Approval

The protocol of the study was approved by the Scientific and Ethical Committee of College of Pharmacy / University of Kerbela, and an informed signed consent form was given by each subject after explaining the nature and purpose of the study.

One hundred and twelve patients (66 male and 46 female) were enrolled in this study with age range from 20 to 79 years, taking erythropoietin vials for injection recommended weekly dose for more than 4 months.

2.2.1.2. Inclusion Criteria

The inclusion criteria involved: patients with chronic renal failure on hemodialysis for at least 4 months taking erythropoietin injection at the recommended weekly dose for a minimum 4 months.

2.2.1.3. Exclusion Criteria

The exclusion criteria involved:

- Patients with viral infections
- Secondary hyperparathyroidism
- Inadequate sessions must be excluded to avoid other causes of EPO resistance.
- Patients undergoing blood transfusion, or taking medication that interferes with ESA.

2.2.1.4. Healthy Controls

Sixty-two healthy subjects were enrolled in the study (30 males and 32 females) as a reference for biochemical tests results.

2.2.2 Clinical Data Collection

During the time of blood sample collection, each subject was questioned about their medical, drug, and family history.

The data were obtained from the medical records of the patients and the patients themselves and these included: age, weight, academic achievement, workplace, marital status, drug history, family history of chronic renal failure, concomitant disease, the dose of erythropoietin injection and duration of the treatment, possible side effects, number of hemodialysis session per week, whether undergone kidney transplant or not, and other drugs used.

2.2.3 Blood Sample Collection

Four ml of blood was collected from each subject that enrolled in the study after taking patient consent, medical and drug history, 1ml was placed in an EDTA tube and 3ml placed in a gel tube.

CBC testing was done by using the blood in the EDTA tube then the tube was saved in a cold place for DNA extraction in the next few days.

The gel tube was centrifuged for 15 minutes at 4000 x g and 2 ml of the resultant serum was transferred to a plain tube and then kept at -20°C till EPO level testing was performed and 1ml used for BU and S. Cr testing.

2.3 Methods

2.3.1 Molecular Analysis

2.3.1.1. DNA Extraction

Genomic DNA was extracted from blood sample as stated by the protocol of gSYNC for blood genomic DNA extraction kit, the following method was adopted for DNA isolation from blood:

1. Blood sample preparation; 200 µl of whole blood was transferred to a 1.5 ml microcentrifuge tube then 20 µl of proteinase k was added and mixed by pipetting after that the tube was incubated at 60°C for 5 minutes.
2. Cell lysis; 200 µl of GSP Buffer was added then mixed by shaking vigorously and incubation for 5 minutes at 60°C was done, every 2 minutes the tube was inverted.
3. DNA Binding; 200 µl of absolute ethanol was added to the sample lysate and mixed immediately by shaking vigorously for 10 seconds
4. A GS column was placed in a 2ml collection tube and all the mixture was transferred to it (including any insoluble precipitate) centrifugation at 14-16000 x g for 1 minute was performed.
5. The 2 ml collection tube containing the flow-through was discarded and the GS column was transferred to a new 2 ml collection tube.
6. Wash; 400 µl of W1 buffer was added to the GS column.
7. Centrifugation at 14-16000 x g for 30 seconds was done and the flow through was discarded
8. The GS column was placed back to the collection tube then 600µl of wash buffer was added and centrifugation for 30 seconds at 14-16000 x g was performed and the flow through was discarded.

9. The GS column was placed back in the 2ml collection tube, again centrifugation for 3 minutes at 14-16000 x g was performed to dry the column matrix.
10. Elution; the dried GS column was transferred to a clean 1.5 ml microcentrifuge tube.
11. Pre-heated elution buffer was added in amount of 100µl to the center of the column matrix, then was let stand for 3 minutes to allow elution buffer to be completely absorbed.
12. Finally, centrifugation at 14-16000 x g for 30 seconds was done to elute purified DNA.
13. The collected DNA was stored at -20 ºC.

2.3.1.1.A Determination of purity and concentration of DNA

DNA concentration and purity were measured by using Nano-spectrophotometer nanodrop. The DNA purity was measured at A260/A280 ratio. 1µl of sample DNA was placed on the micro detector of the device, and then the results were documented.

2.3.1.2. Primers design

The primers were designed by Prof Dr. Hassan Mahmood Musa, Primers of rs6486795 SNP of ORAI1 gene -Snv allele T> C, A and primers of rs1561876 snp of STIM1 genes -Non-coding transcript variant [G/A/C/T]

The primer sequences that were utilized for amplification analysis of ORAI1gene and STIM1gene for SNPs identification are shown in Table 2-3 and Table 2-4 respectively.

Table 2-3 Primers Sequences of rs6486795 SNP of ORAI1 gene T > C, A for allele-specific PCR

Sequence (5'-3')	Template strand	length	Tm	product size	reference
Forward primer	GCTCCAGACGTTTCCAGTGA	20	59.97	462	Prof Dr. Hassan Mahmood Mousa
R-allele T	ATGCCCACAGTGGATGGCA	19	61.92		
R-allele C	ATGCCCACAGTGGATGGCG	19	63.00		
R-allele A	ATGCCCACAGTGGATGGCT	19	61.62		

Table 2-4 Primers Sequences of rs1561876 SNP of STIM1 genes G >A,C,T for allele-specific PCR

Sequence (5'->3')	Template strand	length	Tm	product size	reference
F-allele G	TGTTTCTGTCTCTTGCTTTCG	21	56.78	328	Prof Dr. Hassan Mahmood Mousa
F-allele A	TGTTTCTGTCTCTTGCTTTCA	21	55.67		
F-allele C	TGTTTCTGTCTCTTGCTTTCC	21	56.39		
F-allele T	TGTTTCTGTCTCTTGCTTTCT	21	55.38		
Reverse primer	ATGCCTCTCCCAACCCATTC	20	59.74		

2.3.1.3 Dilution of primers

150µl of distilled water was added to the lyophilized primer in each tube to yield 100 pmoles/µl of stock solution of each according to the instruction of the manufacture

10µl was pipetted from each tube to a new tube with the same label then 90µl of distilled water was added to produce a working solution of each primer.

2.3.1.4 Polymerase Chain Reaction PCR

Allele-specific PCR technique was used to detect the SNP rs1561876 of STIM1 gene and the SNP rs6486795 of ORAI1 gene.

2.3.1.4.A Optimization of the PCR conditions

After several trials of PCR to obtain the best concentration of primers and the best annealing temperature, the optimization of PCR was performed.

2.3.1.4.B Polymerase chain reaction protocol

The PCR mixture was prepared in PCR premix formula as shown in table (2-5)

Table 2-5 Contents of PCR premix tubes

component	Reaction size of 20 µl
Top DNA polymerase	1U
dNTP (dATP, dCTP, dGTP, dTTP)	Each 250 µM
Reaction buffer with 1.5 mM $MgCl_2$	1X
Stabilizer and tracking dye	O

Genotyping for rs6486795 SNP of ORAI1 gene, T>A, C. Bioneer PCR premix was used, 3 tubes were used for each sample DNA, one for detection of the normal allele and the other two tubes for the mutant ones, in each tube 2µl of DNA sample, 0.5µl of primer reverse that was specified to the required allele to be detected, 0.5 µl of primer forward and 19µl of distilled water, the reaction volume became 22µl, all the contents were mixed with the vacuum dried blue pellet by shaking. In the current study, the thermal program for detecting rs6486795 is demonstrated in Table 2-6

Table 2-6 PCR Program for Detecting ORAI1 Gene rs6486795

Step	Temperature	Time	Cycles
Initial denaturation	95°C	5 minutes	1
Denaturation	95°C	20 seconds	30
Annealing	65°C	30 seconds	
Extension	72°C	30 seconds	
Final extension	72°C	5 minutes	1

Genotyping for rs1561876 SNP of STIM1 genes Non-coding transcript variant [G/A/C/T], Bioneer PCR Premix was used, 4 tubes were used for each DNA sample, one for detection of the normal allele and the other three for the mutant ones, in each tube 2 µl of DNA sample, 0.5µl of primer reverse, 0.5 µl of primer forward that was specified to the required allele to be detected and 17µl distilled water, the reaction volume became 20µl, all the contents were mixed with the vacuum dried blue pellet by shaking. In the current study, the thermal program for detecting rs1561876 is demonstrated in Table 2-7

Table 2-7 PCR Program for Detecting STIM1 Gene rs1561876

Step	Temperature	Time	cycles
Initial denaturation	95°C	5 minutes	1
Denaturation	95°C	20 seconds	30
Annealing	60°C	30 seconds	
Extension	72°C	30 seconds	
Final extension	72°C	5 minutes	1

2.3.1.5 Agarose Gel Electrophoresis

1. Agarose gel was prepared at 1.5% g/ml,
   Dimension of gel tray 10cm *15 *0.5 =75cm^2
   1.5 *75= 1.125 gm, 1.125 gm of agarose powder was weighed and transferred to a conical flask
2. The 1x TBE buffer was added in amount of 75 ml, and the conical flask was placed on heater until bubbles formed and the opaque mixture became transparent.
3. After about 2 minutes1.5µl ethidium bromide was added and shaking was performed.
4. The mixture was then poured into the tray after placing the casting dams and the comb and was let to solidify at room temperature.
5. After solidification of the gel the comb was removed lightly away from the tray, the tray was placed in the device tank which was filled with 1x TBE buffer for sample loading to be performed as follows;
   in the first well 5µl of DNA ladder was loaded
   10 µl the PCR product of each sample was loaded in a specific well.
6. The power supply was connected and set at 100 volts, to ensure an electrical field adjusted with (5) v/cm for a 20 cm distance between cathode and anode.
7. After 30 minutes the gel was transferred to the UV transilluminator to visualize the PCR product represented as bands traveling through the gel then the gel was returned to the tray for further electrophoresis, the visualization was repeated after 1 hour, and again after 1.5 hours until the DNA ladder dissociation occurred (Lee et al.,2012).

2.3.2. Biochemical Parameters

2.3.2.1 CBC testing

By using Sysmex xp300TM automated hematology analyzer CBC test was performed.

2.3.2.2 Erythropoietin Blood Level Detection

Erythropoietin blood level was determined by using Human EPO (erythropoietin) ELISA Kit

2.3.2.2.A Dilution method

One step dilution was performed, 5µl sample was added to 495µl sample diluent by this 100-fold dilution was yielded.

2.3.2.2.B Reagent preparation

1. All reagents were brought to room temperature before use.
2. Wash buffer was prepared by dilution of 30 ml of concentrated wash buffer with 720ml of distilled water to get 750 ml of wash buffer
3. Centrifugation of the standard working solution at 10000x g for 1 minute 1ml of reference standard and sample diluent were added then were let to stand for 10 minutes and were inverted gently several times. After it dissolved fully, it was mixed thoroughly with a pipette

   100 mIU/mL working solution was produced from this reconstitution.

   7 EP tubes were taken 500µl of reference standard and sample diluent was added to each tube

500µl of the 100mIU/mL working solution was pipetted to the first tube and was mixed up to produce a 50mIU/mL working solution.

500µL of the solution was pipetted from the former tube to the latter one according to this step.

The illustration below is for reference

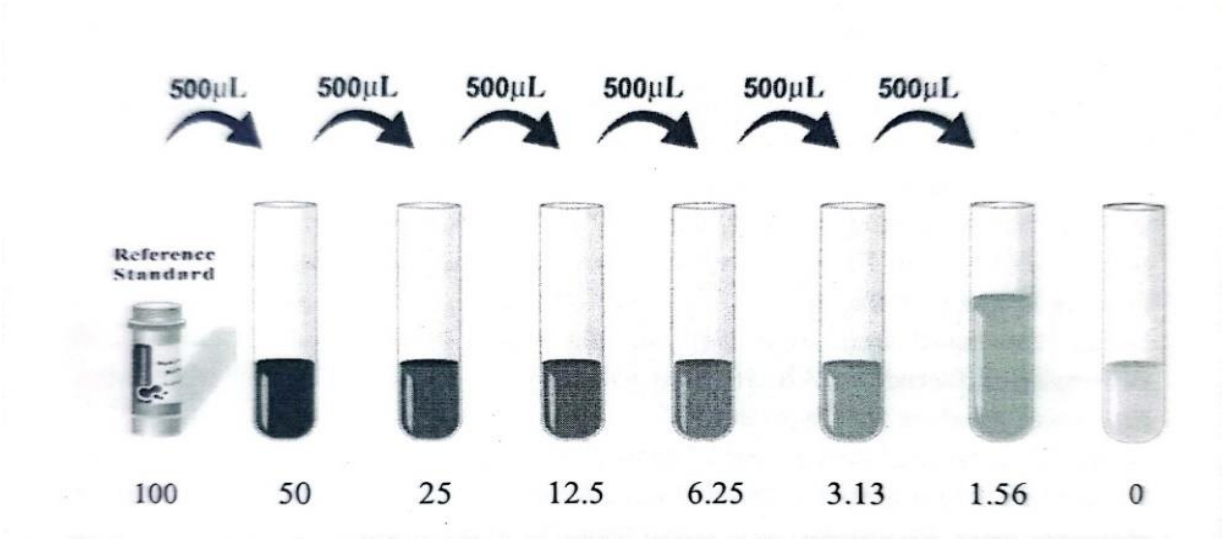

Fig 2.1 Reagent preparation for Erythropoietin blood level detection

The last tube was regarded as blank, no solution was pipetted to it from the former tube.

4. The required amount of biotinylated detection working solution was calculated before the experiment (100µl/well), in preparation slightly more than calculated was prepared as it should have been.

   the concentrated biotinylated detection AB working solution was centrifuged at 800x g for 1 minute

   the 100x concentrated biotinylated detection AB was diluted to 1x working solution with biotinylated detection ab diluent (1:99)

5. The required amount of HRP conjugate working solution was calculated before the experiment (100µL/well).

In preparation slightly more than calculated was prepared as it should be
the concentrated HRP conjugate working solution was centrifuged at 800x g for 1 minute, the 100x concentrated HRP conjugate was diluted to 1x working solution with HRP conjugate diluent (1:99)

2.3.2.2.C Assay procedure

1. Wells were determined for diluted standard, blank, and sample
   100µl added of each standard, blank, and sample into the appropriate wells
   the plate was covered with the sealer provided in the kit.
   Incubation for 90 minutes at 37°C was done
   solutions were added at the bottom of the micro-ELISA plate well
2. The liquid was decanted from each well without wash
   immediately 100µl of biotinylated detection Ab working solution were added to each well, the plate was covered with new sealer
   Incubation for 1 hour at 37°C was performed
3. The solution was decanted from each well, and 350µl of wash buffer was added to each well
   Soaking for 1 minute was done then the solution was decanted from each well and dried against clean absorbent paper, the wash step was repeated 3 times
   The tested strips were used immediately after the wash step; the wells were not allowed to be dried
4. HRP conjugate working solution was added to each well in amount of 100µl
   the plate was covered with new sealer then incubation for 30 minutes at 37°C was performed
5. The solution was decanted from each well, and the wash process was repeated 5 times

6. Substrate reagent were added to each well in amount of 90 µl, the plate was covered with new sealer, incubation for 15 minutes at 37°C was performed, and the plate was protected from light

   The microplate reader was preheated for about 15 minutes before OD measurement.
7. Stop solution was added to each well in amount of 50µl, the addition of stop solution was done in the same order as the substrate solution as it should be
8. Determination of the optical density OD value of each well was performed at once with a microplate reader set to 450nm.

2.3.2.3 Serum Creatinine and Blood Urea Nitrogen testing

By using Cobas c 111 automated analyzer measurement of serum creatinine and blood urea nitrogen was performed.

2.4 Statistical Analysis

The data of the present study was entered and analyzed through the Statistical Package for the Social Sciences (SPSS version 22). The data were presented as frequencies and percentages or mean and standard deviation in appropriate tables and graphs or mean differences in others. Chi-square test, one-way and two-way ANOVA test, and post hoc analysis were used where is appropriate to find out the possible association between the related variables of the current study as LSD was used when equal variances are assumed while Dunnett's T3 were used when equal variances are not assumed depending on Levine's test for homogeneity of variances. Besides, Hardy Weinberg equilibrium was used to detect the prediction of alleles distribution. Statistical association was considered significant when p value equal or less than 0.05 (P value ≤ 0.05).

Chapter Three
Results

3.1 Socio-demographic data and related parameters of patients

The age of the enrolled patients (N=112) ranged from 20-79 years with a mean of 50.94±13.42 years. Male to female ratio was 1.4:1 all of the patients were treated with erythropoietin for at least four months before the study began and more than half of the patients (54.5%) were resistant to erythropoietin. Socio-demographic data illustrated in Table 3-1

Table 3-1 Descriptive statistics of the socio-demographic data of the 112 enrolled patients

Variable		No.	Percentage %
Age (year)	20-39	25	22.3
	40-59	50	44.6
	60-79	37	33.0
Gender	female	46	41.1
	male	66	58.9
Weight (kg)	30-50	14	12.5
	51-70	69	61.6
	71-90	22	19.6
	91-130	7	6.3
Duration of disease (months)	4-60	90	80.4
	61-120	15	13.4
	121-180	7	6.3
Duration of dialysis (months)	4-60	102	91.1
	61-120	8	7.1
	121-180	2	1.8
Duration of treatment (months)	4-50	90	80.4
	51-90	16	14.3
	91-156	6	5.4
Family history	Yes	14	12.5
	No	98	87.5
Response	Responders	51	45.5
	Non-responders	61	54.5

Patients are categorized as responders if their Hb level ≥11g/dl and non-responders if their Hb levels <11 g/dl, for 3 months or more.

Table 3-2 represents descriptive statistics for continuous variables of the 112 enrolled patients, the effect of age on erythropoietin serum level is represented by Table 3-3 in which the patients within the age group (60-79) had a statistically significant rise in EPO levels compared with the patients within the age group (20-39) p-value <0.05

Table 3-2 Descriptive Statistics for Continuous Variables of the 112 Enrolled Patients

Variable	Minimum	Maximum	Mean	Std. Deviation
Age (Year)	22.00	79.00	50.9464	13.42033
Weight (Kg)	33.00	130.00	66.2768	15.74127
Duration of disease (Months)	4.00	180.00	41.8661	41.18525
Duration of dialysis (Months)	4.00	180.00	30.9643	29.17976
Duration of treatment (Months)	4.00	156.00	32.8661	30.10836
Epo mU/ml	2.49	29.80	13.8574	4.46875
Hb g/dl	6.10	13.10	9.6125	1.79835
BU mg/dl	37.00	214.00	115.9865	34.36958
S. Cr mg/dl	3.50	15.00	7.7296	2.09955

[Epo] erythropoietin serum level, [Hb] hemoglobin level, [BU] blood urea, [S. Cr] serum creatinine, [Std] standard.

Table 3-3 Effect of Age on Erythropoietin Level

		EPO levels mU/ml	
Age (year)	No. of patients	Mean ± SD	p-Value
20-39	25	11.87 ± 3.75	
40-59	50	13.88 ± 4.62	
60-79	37	15.16 ± 4.31	0.004 S

[Epo] erythropoietin serum level, [S]= Significant

Table 3-4 represents the association of response with the independent variable (age, duration of dialysis, and duration of the treatment) the results showed that there was no association (p-value <0.05).

Table 3-4 Association between Independent Variables and Response

variable		Responder (No.)	Non-Responders (No.)	P-value
Age (year)	20-39	15	10	0.440 NS
	40-59	29	21	
	60-79	17	20	
Duration of Rx (Months)	4-50	51	39	0.617 NS
	51-90	7	9	
	91-156	3	3	
Duration of dialysis (Months)	4-60	58	44	0.217 NS
	61-120	2	6	
	121-180	1	1	

[NS]= Non significant

Table 3-5 Effect of Gender and Age on The Level of Hb (g/dl)

Age	Gender		Mean difference	SE	P value
20-39	Male	Female	1.972	0.707	0.006 S
40-59	Male	Female	-0.056	0.541	0.905 NS
60-79	Male	Female	0.779	0.578	0.181 NS

[S]= Significant, [NS]= Non significant

3.2 Genotyping of ORAI1 rs6486795 T> C, A genetic polymorphism

3.2.1 Results of Amplification Reaction

The gene polymorphism rs6486795 T> C, A produced a clear band with a molecular size of 462 bps, shown in (Figure 3.1). The size of the amplicon was estimated by comparing it to a 100-2000 bp DNA ladder.

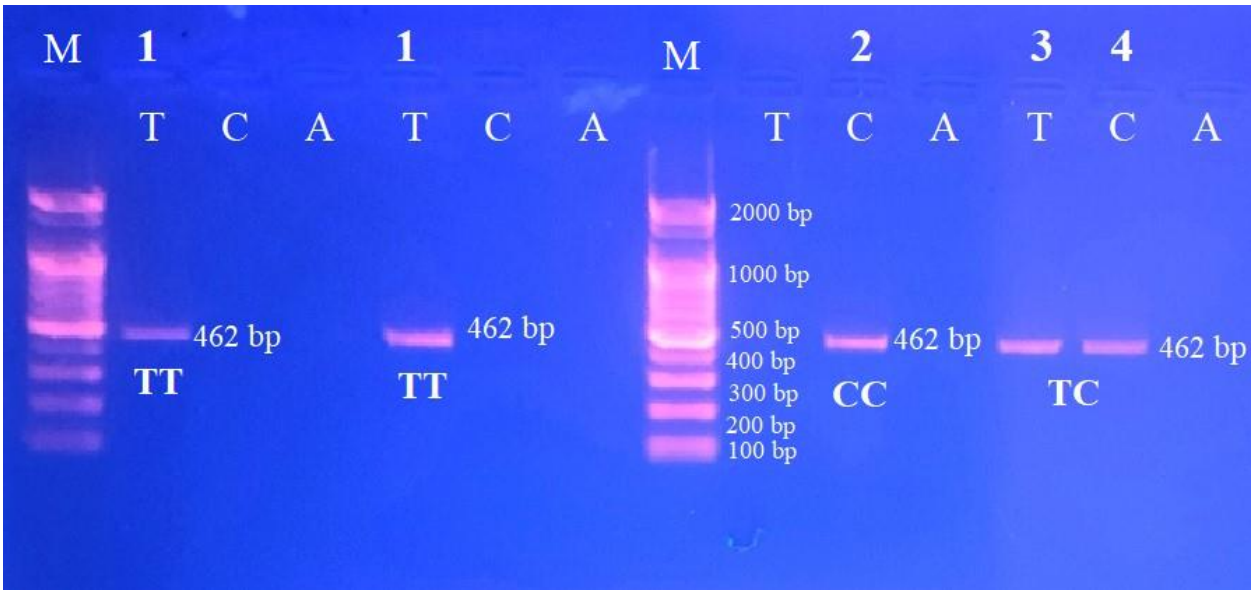

Figure 3.1 Genotyping of ORAI1 rs6486795 genetic polymorphism (T> C, A), Allele-specific PCR technique was used and agarose gel electrophoresis was performed by using 1.5% agarose per TBE buffer with the addition of 1.5µl ethidium bromide. lane M represents the DNA ladder 100-2000 bp, lane 1 represents TT genotype (wild), lane 2 represents CC genotype (homozygous mutant) and 3 and 4 lanes represent TC genotype (heterozygous)..

3.2.2 Distribution of Allele Frequencies of ORAI1 Gene Polymorphism (T> C, A)

According to the aim of this study, the patients were classified according to one of three genotypes for the ORAI1 gene rs6486795 (T> C, A) genetic polymorphism, the wild type homozygous for T allele (TT), heterozygous (TC) and homozygous for the C allele (CC) mutant type. Table 3-6 and Figure 3.2 show the different genotypes among the enrolled 112 patients and no allele frequency was found for A allele in this population.

Table 3-6 Distribution of ORAI1 rs6486795 Gene Polymorphism Different Genotypes in The Enrolled Patients

Variable		Frequency	Percent
ORAI1 rs6486795	TT wild	44	39.3
	TC hetero	43	38.4
	CC homo	25	22.3
	Total	112	100.0

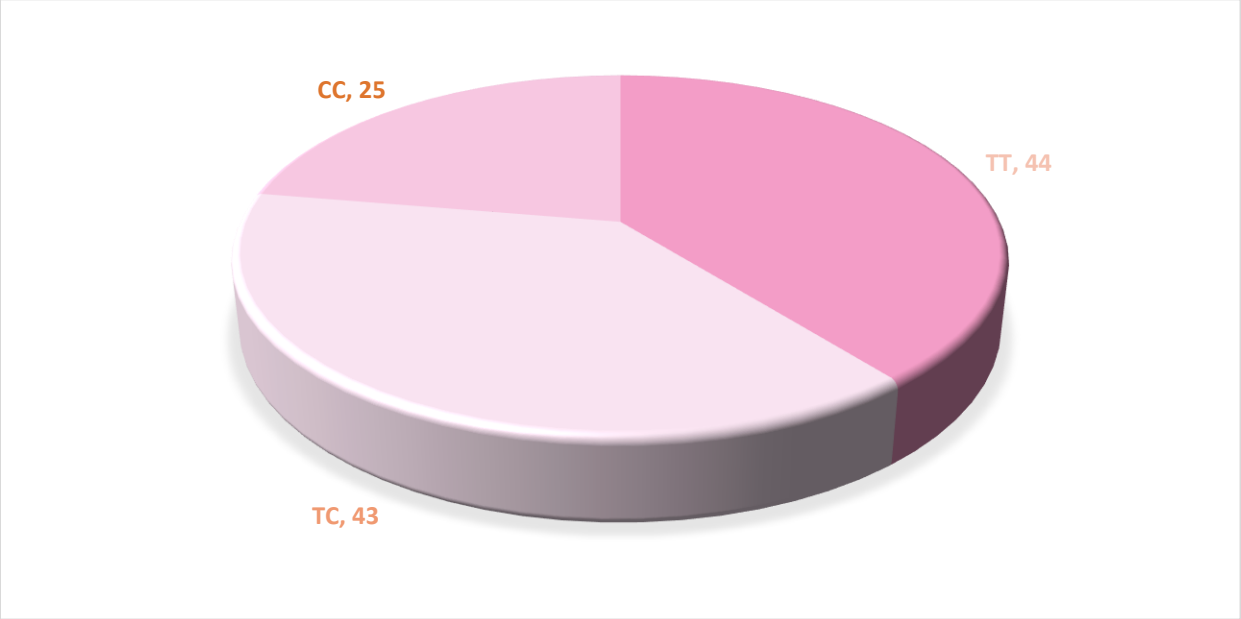

Figure 3.2 Distribution of genetic variants among study patients (ORAI1 rs6486795).

3.2.3 Hardy–Weinberg equilibrium for ORAI1 rs6486795 gene polymorphism

The Hardy-Weinberg equilibrium test was used to show the expected frequency and percent of genotype groups which is statistically significant (p-value <0.05), and the expected predominant group will be the heterozygous TC group based on this study illustrated in Table 3-7 and Figure 3.3.

Table 3-7 Hardy–Weinberg Equilibrium for ORAI1 rs6486795 Gene Polymorphism

Variable			Frequency	Percent	Alleles		Hardy–Weinberg equilibrium X^2 test
Genotype	TT wild	Observed	44	39.3	T	C	P<0.0267 (S)
		expected	38.31	34.2			
	TC hetero	Observed	43	38.4	131 (58.48%)	93 (41.52%)	
		expected	54.39	48.56			
	CC homo	Observed	25	22.3			
		expected	19.31	17.24			

[S]= Significant

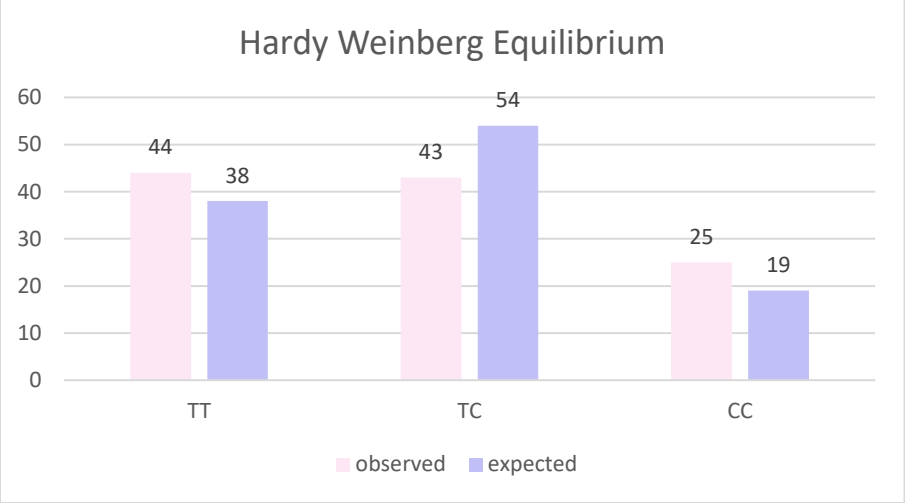

Figure 3.3 Hardy-Weinberg Equilibrium for ORAI1 rs6486795 Gene Polymorphism

3.3 Genotyping of STIM1 rs1561876 G > A, C, T genetic polymorphism

3.3.1 Results of Amplification Reaction

The gene polymorphism rs1561876 G > A, C, T produced a clear band with a molecular size of 328 bps, shown in Figure 3.4. The size of the amplicon was estimated by comparing it to a 100-2000 bp DNA ladder.

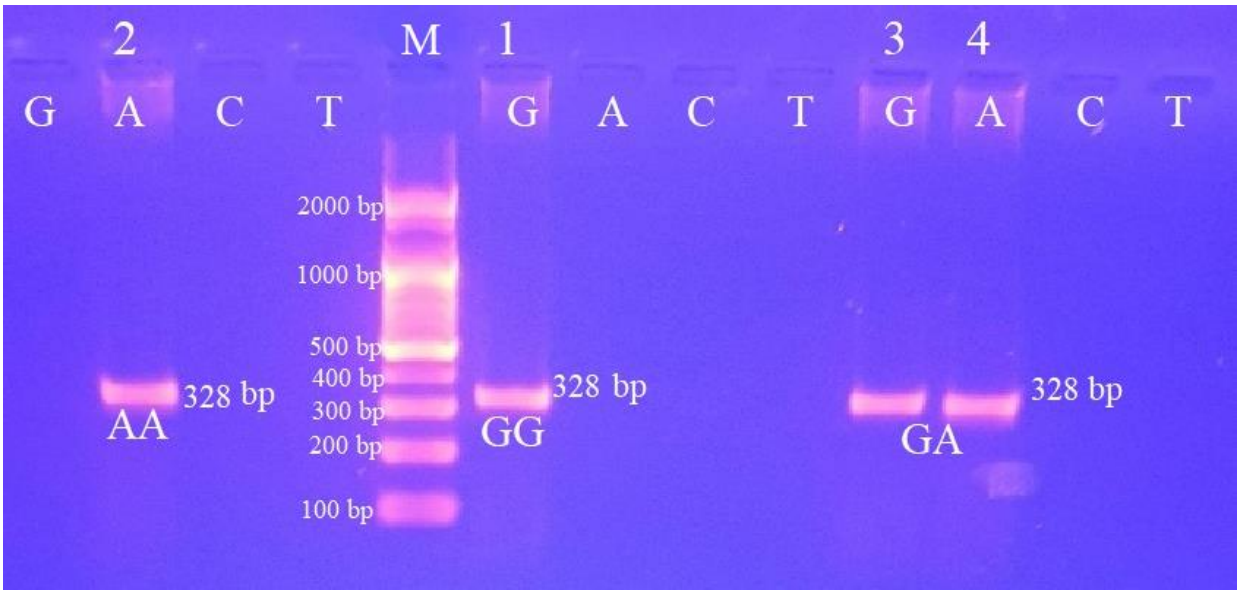

Figure 3.4 Genotyping of STIM1 rs1561876 genetic polymorphism (G>A,C,T), Allele-specific PCR technique was used and agarose gel electrophoresis was performed by using 1.5% agarose per TBE buffer with the addition of 1.5µl ethidium bromide. lane M represents the DNA ladder 100-2000 bp, lane 1 represents GG genotype (wild), lane 2 represents AA genotype (homozygous mutant) and 3 and 4 lanes represent GA genotype (heterozygous).

3.3.2 Distribution of Allele Frequencies of STIM1 Gene Polymorphism G > A, C, T

Consistent with the aim of this study, the patients were classified according to one of three genotypes for the STIM1 gene rs1561876 (G > A, C, T) genetic polymorphism, the wild type homozygous for G allele (GG), heterozygous (GA) and homozygous for the A allele (AA) mutant type. Table 3-8 and Figure 3.5 show the different genotypes among the 112 enrolled patients and no allele frequency was found for T or C alleles in this population.

Table 3-8 Distribution of STIM1 rs1561876 Gene Polymorphism Different Genotypes in The Enrolled Patients

Variable		Frequency	Percent
STIM1 rs1561876	GG wild	19	17
	GA hetero	33	29.5
	AA homo	60	53.6
	Total	112	100.0

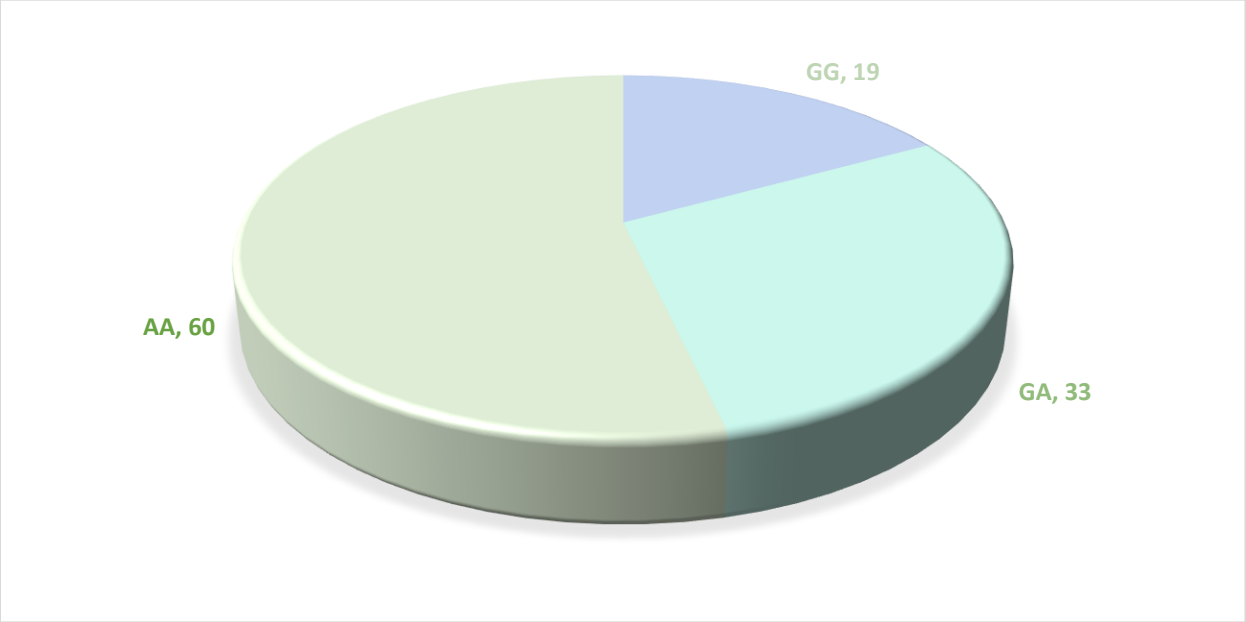

Figure 3.5 Distribution of Genetic Variants among Study Patients (STIM1 rs1561876).

3.3.3 Hardy–Weinberg equilibrium for STIM1 rs1561876 gene polymorphism

The Hardy-Weinberg equilibrium test was used to show the expected frequency and percent of genotype groups, the heterozygous GA group will increase in frequency and percent while both the homozygous wild GG group and the homozygous mutant AA group will decrease in frequency and percent based on this

study these results were statistically significant and summarized in Table 3-9 and Figure 3.6 (p-value <0.05).

Table 3-9 Hardy–Weinberg Equilibrium for STIM1 rs1561876 Gene Polymorphism

Variable			frequency	percent	Alleles		Hardy–Weinberg equilibrium X^2 test
genotype	GG wild	Observed	19	17	G	A	P<0.0007 S
		expected	11.25	10.05			
	GA hetero	Observed	33	29.5	71(31.7)	153(68.3)	
		expected	48.5	43.3			
	AA homo	Observed	60	53.6			
		expected	52.25	46.65			

[S]= Significant

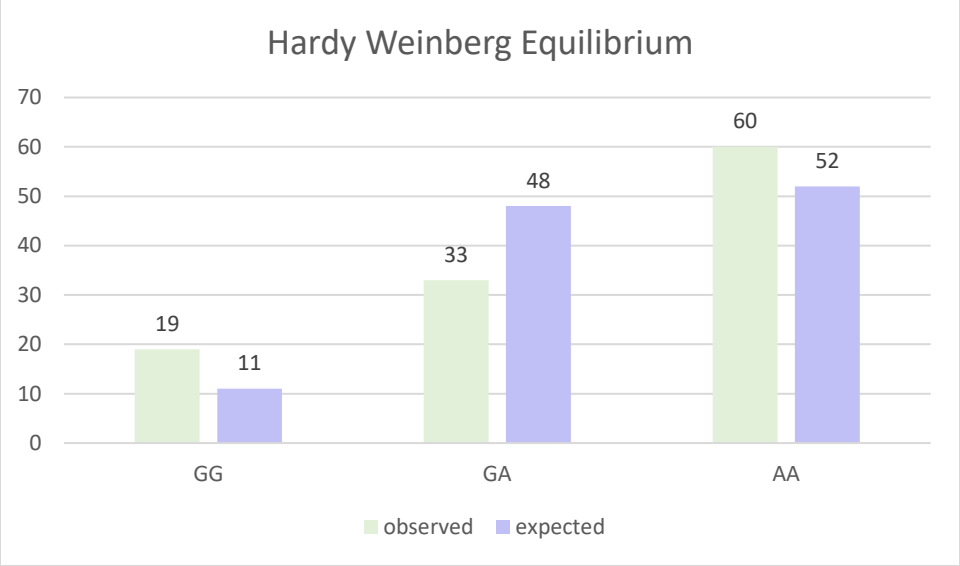

Figure 3.6 Hardy–Weinberg Equilibrium for STIM1 rs1561876 Gene Polymorphism

3.4 Association of Socio-demographic parameters with genetic variation

3.4.1 Socio-demographic parameters with ORAI1 rs6486795 gene polymorphism

The results show that there was an association between genetic variation and response to the treatment represented by Table 3-10 (p-value <0.05).

Table 3-10 Association between Genetic Variants of ORAI1Gene rs6486795 and Response.

variable		Responder (No.)	Non-Responders (No.)	P value
Genotype	TT	14	30	0.031 S
	TC	21	22	
	CC	16	9	

[S]= Significant

There was no association between gender and different genotypes of ORAI1 rs6486795 gene polymorphism this is illustrated in Table 3-11 (p-value <0.05).

Table 3-11 Association between Gender and Genetic Variants of ORAI1Gene rs6486795.

Demographic parameters		Patient genotype (N=112)			P Value
		TT N (44)	TC N (43)	CC N (25)	
Gender	Male	24	28	14	0.572 NS
	Female	20	15	11	

[NS]= Non significant

3.4.2 Socio-demographic data with STIM1 rs1561876 gene polymorphism

The results show that there was an association between gender and genetic variations were represented by Table 3-12(p-value <0.05).

Table 3-12 Association between Gender and Genetic Variants of STIM1 Gene rs1561876

Demographic parameters		Patient genotype (N=112)			P Value
		GG N (19)	GA N (33)	AA N (60)	
Gender	Male	16	16	34	0.036 S
	Female	3	17	26	

[S]= Significant

There was no association between genetic variation and response to the treatment represented by Table 3-13 (p-value <0.05).

Table 3-13 Association between Genetic Variants of STIM1 Gene rs1561876 and Response.

variable		Responder (No.)	Non-Responders (No.)	P value
Genotype	GG	10	9	0.770 NS
	GA	14	19	
	AA	27	33	

[NS]= Non significant

3.5 Association of Socio-demographic and biochemical parameters with genotypes

Regarding ORAI1gene rs6486795 genetic variants the results in Table 3-14 below, show that CC group has a statistically significant rise over TT group in hemoglobin level and also in blood urea, while the control group has a statistically significant difference in hemoglobin level (rise) and serum creatinine and blood urea levels (decrease) compared with the three genetic groups (p-value <0.05).

Table 3-14 Biochemical parameters and their Mean±SD between groups of ORAI1gene rs6486795

parameters	Groups				P-value
	control	TT	TC	CC	
	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	
Epo mU/ml	13.04 ± 3.10	13.52 ± 4.07	14.30 ± 4.70	14.15 ± 4.77	0.362 NS
Hb g/dl	13.48 ± 1.03	9.13 ± 1,69	9.74 ± 1.83	10.23 ± 1.73	0.001 S * 0.005 S **
BU mg/dl	25.48 ± 7.79	112.97 ± 34.15	112.74 ± 33.63	126.84 ± 35.15	0.001 S **
S. Cr mg/dl	0.86 ± 0.16	7.81 ± 2.21	7.78 ± 2.22	7.48 ± 1.69	0.01 S *

[Epo] erythropoietin serum level, [Hb] hemoglobin level, [BU] blood urea, [S. Cr] serum creatinine, [S]= Significant, [NS]= Non-significant, * control group have significant differences with the genetic groups, ** CC group have a significant rise over TT group.

Regarding STIM1 gene rs1561876 genetic variants, the results in Table 3-15 show no statistically significant differences in biochemical parameters appear between genetic groups, only the control group has statistically significant differences in all biochemical parameters in comparison with the genetic groups (p-value <0.05).

Table 3-15Biochemical parameters and their Mean±SD between groups of STIM1 gene rs1561876

parameters	Groups				P-value
	Control	GG	GA	AA	
	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	
Epo mU/ml	13.04 ± 3.10	13.89 ± 4.35	13.48 ± 5.06	14.04 ± 4.21	0.574 NS
Hb g/dl	13.48 ± 1.03	9.78 ± 1.83	9.47 ± 1.85	9.63 ± 1.78	0.001 S *
BU g/dl	25.48 ± 7.79	114.27 ± 25.5	117.56 ± 42.06	115.66 ± 32.56	0.001 S *
S. Cr g/dl	0.86 ± 0.16	7.61 ± 2.19	7.86 ± 2.18	7.69 ± 2.05	0.001 S *

[Epo] erythropoietin serum level, [Hb] hemoglobin level, [BU] blood urea, [S. Cr] serum creatinine, [S]= Significant, [NS]= Non significant, * control group have significant differences with the genetic groups

Table 3-16 and Figure 3.7 represent the study of the effect of two factors genetic variation and type of response on Hb level in ORAI1 rs6486795 gene polymorphism (p-value <0.05).

Table 3-16 Effect of genetic variation of ORAI1 rs6486795 and type of response on Hb levels

ORAI1 rs6486795	Response		Mean difference	SE	P value
TT	responders	Non-responders	3.281	0.233	0.001
TC	responders	Non-responders	3.371	0.210	0.001
CC	responders	Non-responders	3.314	0.287	0.001

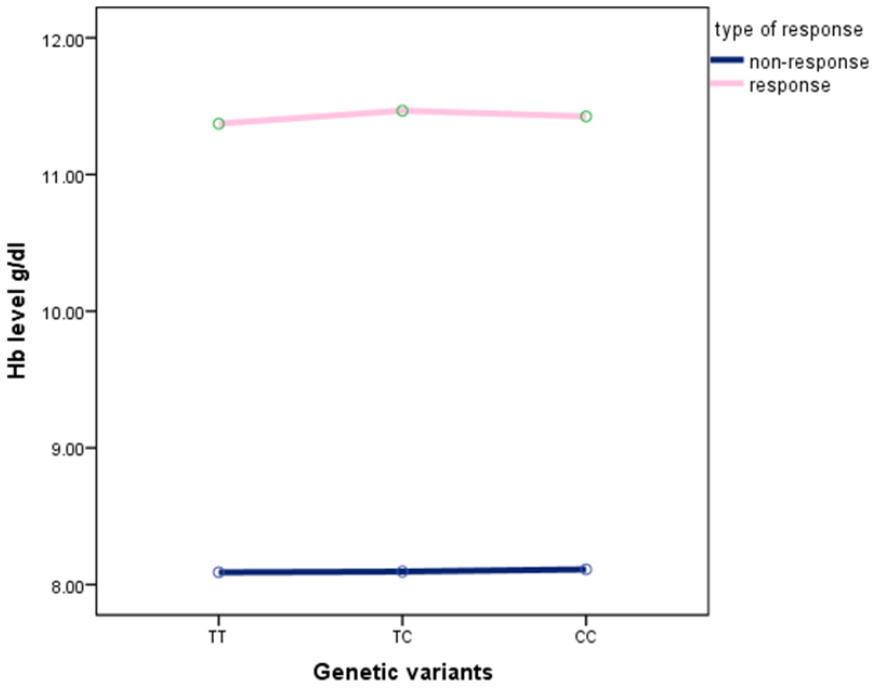

Figure 3.7 The Combined Effect of Genetic Variants and Type of Response on Hb Level

Table 3-17 and Figure 3.8 represent the study of the effect of two factors genetic variation and type of response on Hb level in STIM1 rs1561876 gene polymorphism (p-value <0.05).

Table 3-17 Effect of genetic variation of STIM1 rs1561876 and type of response on Hb levels

STIM1 rs1561876	Response		Mean difference	SE	P value
GG	responders	Non-responders	3.411	0.316	0.001
GA	responders	Non-responders	3.317	0.243	0.001
AA	responders	Non-responders	3.320	0.179	0.001

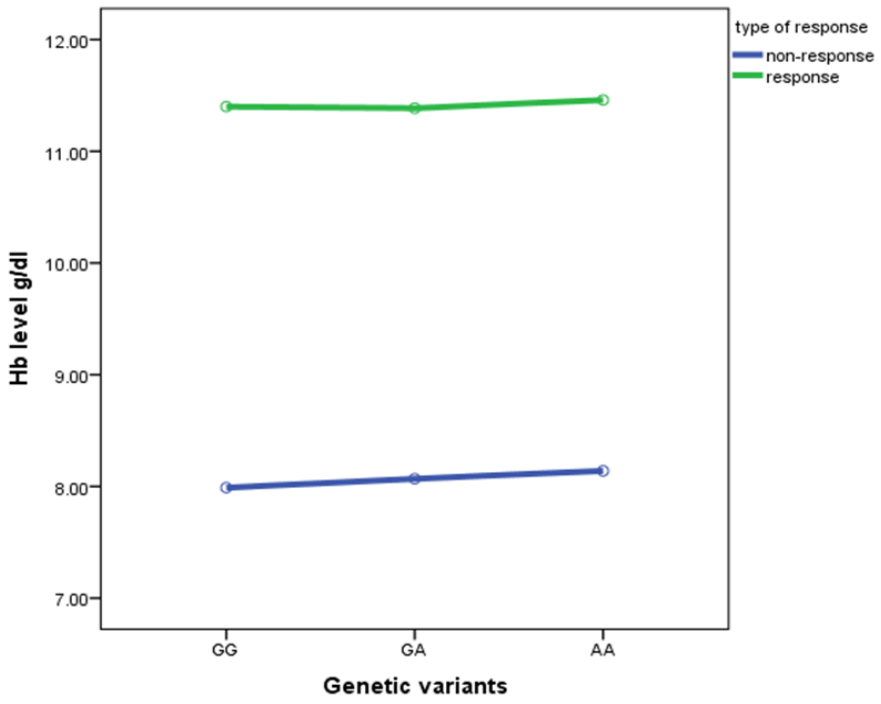

Figure 3.8 The Combined Effect of Genetic Variants and Type of Response on Hb Level

3.6 Distribution of ORAI1 rs6486795 and STIM1 rs1561876

Cross-tabulation of the two SNPs is illustrated in Table 3-18 and Figure 3.9. The study showed that the sample that carries the genetic variant TTAA for both genes respectively has the highest prevalence among the population (23.2%), while CCGG and TTGG are the lowest (4.5%).

Table 3-18 Cross-tabulation of ORAI1 rs6486795 and STIM1 rs1561876 No.(%)

Groups	GG	GA	AA	Percent
TT	5 (4.5)	13 (11.6)	26 (23.2)	44 (39.3)
TC	9 (8.0)	10 (8.9)	24 (21.4)	43 (38.4)
CC	5 (4.5)	10 (8.9)	10 (8.9)	25 (22.3)
Total	19 (17.0)	33 (29.5)	60 (53.6)	112 (100.0)

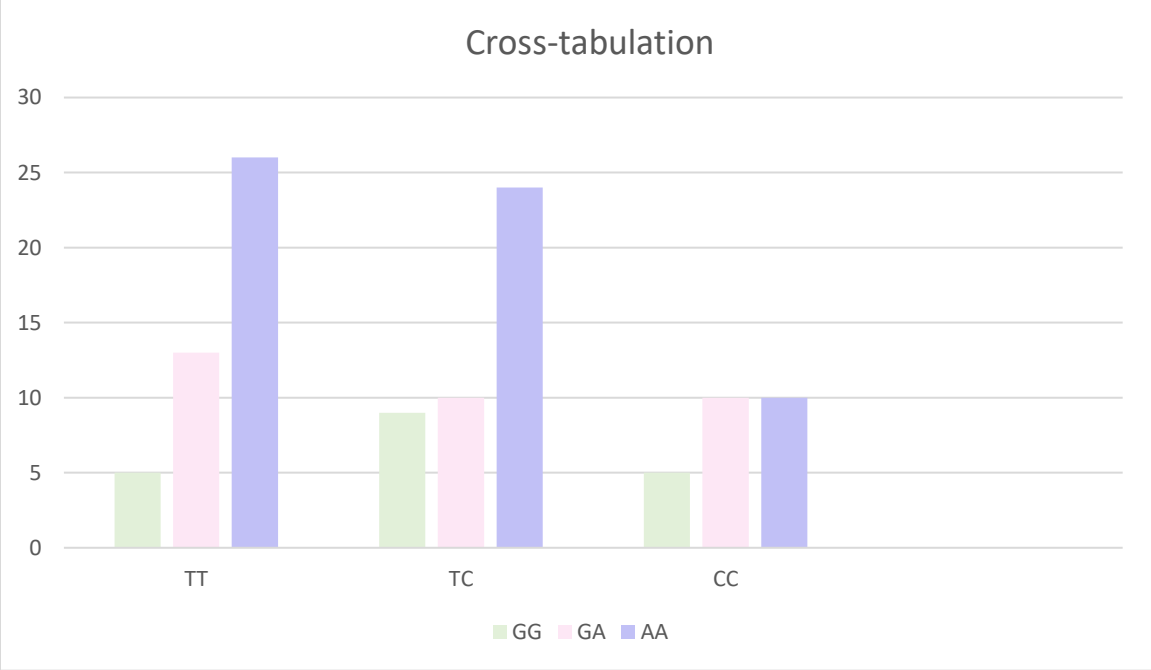

Figure 3.9 Cross-tabulation of ORAI1 rs6486795 and STIM1 rs1561876

3.7 Interaction of ORAI1 rs6486795 and STIM1 rs1561876 on Hb and EPO levels

No significant effects on Hb level were seen by the interaction of ORAI1 rs6486795 and STIM1 rs1561876 in Table 3-19 and Figure 3.10 (p-value <0.05).

Table 3-19 Interaction of ORAI1 rs6486795 and STIM1 rs1561876 on Hb Level

ORAI1 rs6486795	STIM1 rs1561876	Mean	SE	P value
TT	GA	8.785	0.499	0.677 NS
CC	GG	10.700	0.805	0.431 NS

[NS]= Non significant

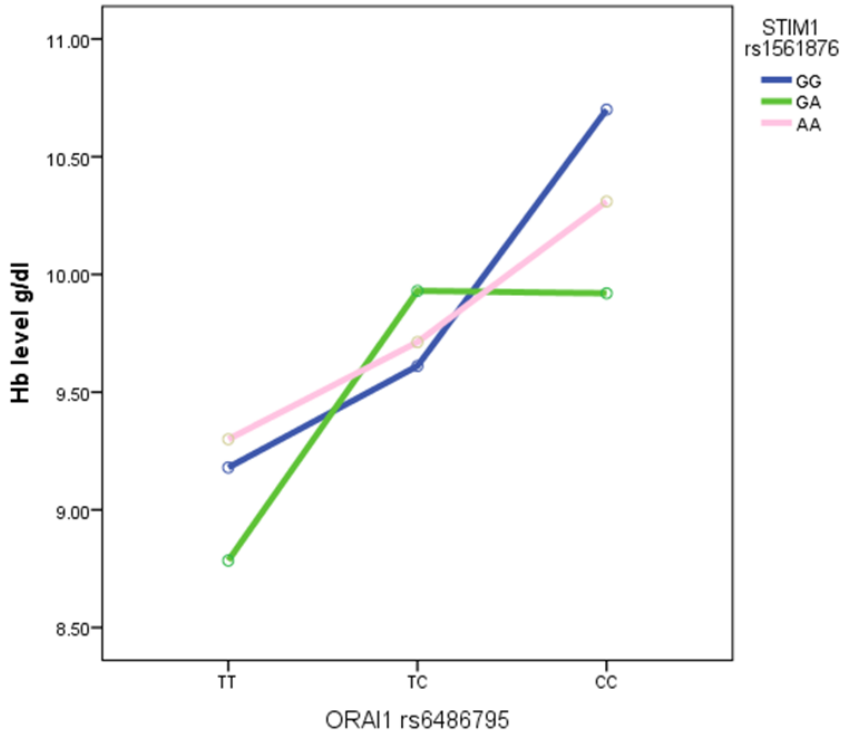

Figure 3.10 Interaction of ORAI1 rs6486795 and STIM1 rs1561876 on Hb level

The interaction of the two SNPs and their impact on Hb level showed that TTGA genotype has the lower Hb level and CCGG genotype has the higher Hb level among other groups that are considered good responders but represent only 4.5% of the patients' population as shown by Table 3-18.

No significant effects on EPO level were seen by the interaction of ORAI1 rs6486795 and STIM1 rs1561876 in Table 3-20 and Figure 3.11 (p-value <0.05).

Table 3-20 Interaction of ORAI1 rs6486795 and STIM1 rs1561876 on EPO Level

ORAI1 rs6486795	STIM1 rs1561876	Mean	SE	P value
TT	GA	12.585	1.269	0.747 NS
CC	AA	15.303	1.447	0.386 NS

[NS]= Non significant

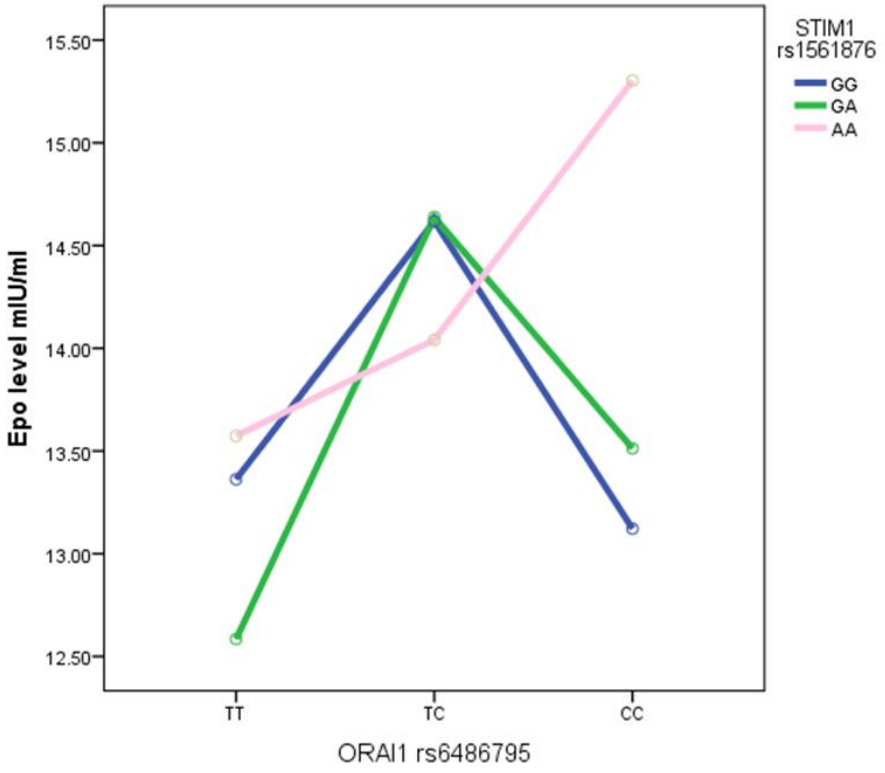

Figure 3.11 Interaction of ORAI1 rs6486795 and STIM1 rs1561876 on EPO Level

The interaction of the two SNPs and their impact on EPO level showed that TTGA genotype has the lower EPO level and CCAA genotype has the higher EPO level among other groups.

Chapter Four
Discussion

4. Discussion

Chronic renal failure is a progressive incurable disease with considerably high rates of morbidity and mortality, it is insidious and most of patients have no symptoms during the early stages of the disease, serious symptoms appear in advanced stages (Kalantar-Zadeh et al., 2021). One of the common complications of CRF is anemia which contributes to decreased quality of life, increased hospitalization, cognitive impairment, and higher risk of negative cardiovascular events. Anemia of CRF is treated by ESAs together with iron supplementation (Portolés et al.,2021; Shaikh et al.,2019), these medications result in the correction of anemia and enhanced quality of life with no need for blood transfusion (Ng et al., 2010).

The response to the treatment is different between individual patients and this difference is related to some factors that affect drug response, some of these factors can be handled while others will remain stable during a patient's lifetime like genetic determinants, there is limited information about its potential in erythropoietin responsiveness (Brown, 2006).

Pharmacogenetic associations that can affect drug response have increased in number as time goes by, proteins that are significant in clinical pharmacology including transporters, enzymes, drug receptors, and targets have genetic polymorphisms identified in many of them, and these polymorphisms can result in changes in the amount, binding, structure, and/or function of these proteins, affecting how drugs interact with them. Pharmacokinetics and pharmacodynamics of drugs can be altered by genetic variation and can affect both drug efficacy and toxicity. It is established that 20% to 95% of drug metabolism and response can related to genetic factors (Ventola, 2013).

4.1 ORAI1 and STIM1 Genetic Polymorphism

ORAI1 and STIM1 are the main components of store-operated calcium channels that mediate a particular way of Ca^{2+} influx which contributes to the function of many cell types, ORAI1 proteins located in the plasma membrane form the channels and are activated by STIM1 that present in the endoplasmic reticulum, any mutation in the genes of these components can result in alteration of their functions (Lacruz & Feske,2015).

ORAI1 mutation can lead to a decrease in SOC signaling pathway, and a defect in STIM1 gene can result in a lack of store-operated Ca^{2+} entry, contributing to different health problems (Chou et al., 2011; Picard et al., 2009).

When SOCs lose their function leading to a lack of store-operated Ca^{2+} influx due to mutations the patients will suffer from immunodeficiency distinguished by life-threatening bacterial, fungal, and viral infections (Feske, 2009).

Both the pore-forming proteins (ORAI) and the calcium store sensor (STIM) have a crucial role in cell signaling process by interacting with G protein-coupled receptors and protein tyrosine kinase coupled receptors, this role explains the importance of these channels as drug targets for pharmacological therapeutic intervention (Rubaiy, 2023).

This is the first study of its type to investigate the impact of ORAI1 rs6486795 (T> C, A) gene polymorphism and STIM1 rs1561876 (G > A, C, T) gene polymorphism on erythropoietin resistance in Iraqi patients with CRF taking erythropoietin injection and on maintenance hemodialysis.

4.1.1 ORAI1 rs6486795 Gene Polymorphism

In the distribution of allele frequencies of ORAI1 rs6486795 SNP among the patients enrolled in this study the homozygous wild genotype TT represents 39.3 % of the population while the heterozygous mutant TC genotype and the homozygous mutant CC genotype represent 38.4% and 22.3% respectively, TT and TC groups have an almost similar percentage and CC group was the lower, some similarity can be found when these results were compared with the results of a Taiwanese study in 2011 in which they represent that the frequency of TT, TC, and CC were 39.6%, 46.1%, 14.3% respectively (Kuo et al., 2011). A study in which 290 normal controls were included also found that the two prominent groups were TT and TC (41.72% and 43.45% respectively) and the CC group was the lowest in percentage (14.83%) (Chang et al., 2014).

Another Taiwanese study of 579 chronic kidney disease patients showed the genetic distribution as follows TT genotype 42.9 %, TC genotype 42.9 %, and CC genotype 14.2 % (Hwang et al., 2014).

Hardy–Weinberg equilibrium for ORAI1 rs6486795 gene polymorphism was used to predict the expected frequencies of genotype, this test was first described in the early twentieth century and its expectation appears to hold for most human populations (Wigginton et al., 2005).

According to this study, the results of Hardy–Weinberg equilibrium test were statistically significant, showing that the expected predominant group will be the heterozygous TC group while both homozygous wild TT genotype and mutant CC genotype will decrease in frequencies.

4.1.2 STIM1 rs1561876 gene polymorphism

Regarding the distribution of allele frequencies of STIM1 rs1561876 SNP in this study among the enrolled patients the predominant group is the homozygous mutant genotype AA which represents 53.6 % of the population followed by the heterozygous mutant genotype GA 29.5 % while the homozygous wild genotype GG takes the last place between groups with 17% of the population, these results are approximately consistent with a 2020 Chinese study in the frequencies of AA, GA, and GG of 300 healthy Chinese Han individuals (54.0%), (36.33%), (9.67%) respectively (Lou et al., 2020).

In a 2020 study of Han Chinese breast cancer (early stage) patients, the distribution of STIM1 rs1561876 SNP was 49.8% AA genotype, 42.9% GA genotype, and 7.3% GG genotype (Huang et al., 2020). In another Taiwanese study of patients with Kawasaki disease, the distribution of STIM1 rs1561876 gene polymorphism is as follows AA genotype represents 52.8%, GA genotype 42.5%, and GG genotype 4.7% (Hsu et al., 2013).

By comparison of the results of this study with the above previous studies all of them showed that the AA group is the predominant and the GG group is the lowest in frequencies.

Hardy–Weinberg equilibrium for STIM1 rs1561876 gene polymorphism was used to show the expected frequencies of different genotypes, the statistically significant results of this test regarding this SNP showed that the heterozygous mutant GA group will increase in frequency and percent, while both the homozygous wild GG group and mutant AA group will decrease in frequency and percent.

4.2 Associations of demographic data with biochemical parameters

The ages of the 112 enrolled patients in this study range from 20 to 79 years with a mean of 50.94 ± 13.42 years, about one-third of the patients were in the age group (60-79) these elderly patients have higher erythropoietin serum levels than young patients, in 2017 a study in Canada showed that in ESA therapy with higher Hb targets the adverse outcomes is restricted to sick elderly patients, and the healthier younger patients without resistance to the treatment may benefit from the health-related quality of life perspective (Collister et al., 2017) which may explain the significantly higher erythropoietin levels in elderly patients compared to the young group as presented in Table 3-3 .

According to the findings, male patients in the age group (20-39) have a statistically significant rise in Hb level over female patients in the same age group Table 3-5 this resembles the finding of a study done in New York in which the results showed that the female patients with ESRD on hemodialysis need a higher Epo dose to gain a response equivalent with male patients (Ifudu et al., 2001).

In this study, more than half of the patients had poor responses to epoetin alfa, and the total prevalence of erythropoietin resistance was 54.5% this percentage is considered to be high when compared to other studies, in 2007 a study of epoetin hypo-responsiveness in patients with CRF done in Australia, Canada, and Europe showed that the prevalence of erythropoietin resistance was 15% (Rossert et al., 2007). While a study done in 2015 in Brazil showed that the prevalence of the resistance reaches 34% (Alves et al., 2015).

4.3 Association of demographic parameters with genetic variations

4.3.1 Association of demographic parameters with ORAI1 rs6486795 gene polymorphism

The results showed that there was a significant association between genetic variants and the type of response Table 3-10, this is agreed with a previous study in Taiwan in which ORAI1 rs6486795 gene polymorphism was significantly correlated with the risk of EPO resistance in dialysis patients (Kao et al., 2021).

Table 3-11 Represents that there was no significant association between gender and different genotypes of ORAI1 rs6486795 gene polymorphism.

4.3.2 Association of demographic parameters with STIM1 rs1561876 gene polymorphism

According to the results, there was no significant association between different genotypes and response represented in Table 3.13 this disagreed with a previous study about STIM1 genetic polymorphism in which the results showed a significant correlation between STIM1 rs1561876 gene polymorphism and risk of erythropoietin resistance (Kao et al., 2021).

Regarding gender differences, unlike ORAI1 gene polymorphism, the results show that there was a significant association between gender and genetic variations of STIM1 rs1561876 gene polymorphism represented in Table 3-12

4.4 Association of biochemical parameters with different genotypes

In ORAI1 gene polymorphism our results showed that patients of CC genotype have a statistically significant rise in Hb levels over patients of TT genotype this is inconsistent with the representation of a 2021 Taiwanese study which illustrated that CC/CT genotype has a higher risk of EPO resistance (Kao et al., 2021).

Regarding STIM1 gene polymorphism in this study, there were no significant associations between genetic variants and biochemical parameters, representing no impact of the gene polymorphism on erythropoietin resistance and this is controversially disagreeing with the 2021 Taiwanese study in which AA genotype has increased risk of EPO resistance (Kao et al., 2021).

In this study the genetic groups has a statistically significant decrease in Hb level and a statistically significant rise in S. Cr and BU levels compared to the healthy control group in both ORAI1 and STIM1 gene polymorphism, represented by Tables 3-14 And 3-15.

In Table 3-16 and Figure 3.7 the results show that there is a statistically significant mean difference in Hb levels between responders and non-responders in all genetic variants of ORAI1 gene polymorphism, TT responders group has a statistically significant rise in Hb level over TT non-responders with a mean difference of 3.281±0.233, the same way in TC and CC genotypes with mean differences of 3.371±0.210 and 3.314±0.287 respectively between responders and non-responders the p-value is 0.001.

Regarding STIM1 gene polymorphism there is also a statistically significant mean difference in Hb levels between responders and non-responders in all genetic variants represented by Table 3-17 and Figure 3.8 GG responders group has a statistically significant rise in Hb level over GG non-responders with a mean

difference of 3.411±0.316, the same way in GA and AA genotypes with mean differences of 3.317±0.243 and 3.320±0.179 respectively between responders and non-responders the p-value is 0.001.

4.5 Cross-tabulation of ORAI1 rs6486795 and STIM1 rs1561876 and their interaction on Hb and EPO levels

A cross-tabulation of rs6486795 in ORAI1 gene and rs1561876 of STIM1 gene showed that TTAA group has the highest prevalence among the population (23.2%), while CCGG and TTGG are the lowest (4.5%) as represented by Table 3-18.

Depending on the interaction of rs6486795 in ORAI1 gene and rs1561876 of STIM1 gene and their impact on Hb level and EPO level the results showed that there were no significant effects on both, but also represent that TTGA genotype has the lower Hb level and EPO level among other groups, CCGG genotype has the higher Hb level while CCAA genotype has the higher EPO level as illustrated in Table 3-19 and Table 3-20.

As CCGG group who have the higher Hb level and are considered good responders represent only 4.5% of the patients' population based on this study, this might explain the commonness of erythropoietin resistance among these patients.

4.6 Conclusions

- ORAI1 gene and STIM1 gene were detected in Iraqi patients with CRF on hemodialysis. The distribution of ORAI1 gene polymorphism rs6486795 T> C, A the homozygous wild type (TT) and the heterozygous mutant type (TC) have approximately the same percentage while the homozygous mutant type (CC) has the lower percentage, regarding STIM1 gene polymorphism rs1561876 G > A, C, T the homozygous mutant type (AA) is more predominant than (GA) and (GG) genotype.
- Both of the SNPs in the two genes that were detected in Iraqi patients taking erythropoietin were stated to be non-significantly associated with erythropoietin resistance but they cannot be excluded from the factors that contribute to erythropoietin resistance as we noticed that CC genotype in ORAI1 rs6486795 gene polymorphism has a statistically significant rise in Hb levels over TT genotype.
- Cross-tabulation of ORAI1 rs6486795 and STIM1 rs1561876 and their interaction on Hb and EPO levels estimated that the TTAA genotype represents the highest prevalence of (23.2%) while CCGG who have the higher Hb level and are considered good responders represent only (4.5%) of the patients' population.

4.7 Recommendations and Future Work

- A larger number of patients is required to give more accurate information about the role of ORAI1 and STIM1 genetic polymorphisms in erythropoietin resistance.
- Study of other SNPs in both ORAI1 and STIM1 genes and their impact on erythropoietin resistance
- Investigating the effects of the genetic polymorphisms on other types of ESA therapy and comparing the new results with those of this study

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Appendices

Questionnaire for patients enrolled in the study

Name:	age:	gender:
Weight:	phone number:	address:
Occupational status:	marital status:	educational level:

Duration of disease:

Duration of being on dialysis:

Duration of treatment with erythropoietin:

Concomitant disease:

Family history:

Drug history:

Notes:

الخلاصة

المقدمة:يعد الفشل الكلوي المزمن مرض تقدمي يعرف بأنه تلف في بنية الكلية أوخلل في عمل الكلى لأكثر من 3 أشهر, يصنف الفشل الكلوي المزمن الى خمس مراحل تبعاً لمعدل الترشيح الكبيبي. يبدأ ظهور فقر الدم في المراحل المبكرة من الفشل الكلوي ويزداد سوءًا مع تقدم مرض الكلى نتيجة انخفاض إنتاج الإريثروبويتين. يعتبر كل من العوامل المنشطة للإريثروبويتين ومكملات الحديد معايير ذهبية لعلاج فقر الدم في الفشل الكلوي المزمن، إلا أن عدم الاستجابة للعلاج تمثل عقبة أمام تحقيق هدف العلاج لدى العديد من المرضى. يُعدّ كلا الجينين ORAI1 و STIM1 من جينات قنوات الكالسيوم المعتمدة على المخازن، وهي إحدى المسارات المنشطة للإريثروبويتين ومن المحتمل أن يساهم تعدد الاشكال الجيني في تلك الجينات في عدم الاستجابة لعقارالإريثروبويتين.

هدف الدراسة: هدفت الدراسة لمعرفة العلاقة بين تعدد الأشكال الجيني في جين ORAI1 rs6486795 T>C,A و جين STIM1 rs1561876 G > A, C, T عدم الاستجابة لعقارالإريثروبويتين.

المرضى و الاساليب: أجريت دراسة رصدية مقطعية في مدينة الإمام الحسين الطبية / مركز الدكتور عادل السَّباح لغسيل الكلى في كربلاء . 112 مريضًا من الذكور والإناث، تتراوح أعمارهم بين 20 إلى 79 عامًا، يخضعون لغسيل الكلى ويتلقون جرعة أسبوعية موصى بها من حقن إيبوتين ألفا لأكثر من 4 أشهر، وتم تسجيل 62 شخصًا من الأصحاء في الدراسة كمرجع لنتائج الفحوصات البيوكيميائية. تم إجراء الفحوصات البيوكيميائية والتحاليل الدموية على كل مشارك لتحديد مستويات الهيموجلوبين والإريثروبويتين والكرياتينين في مصل الدم واليوريا في الدم ومستويات الحديد. حيث أُستخدم تقنية تفاعل البوليميراز المتسلسل النوعي للألليل للكشف عن تعدد أشكال النوكليوتيدات المفردة rs6486795 T> C, A في جين ORAI1 وتعدد أشكال النوكليوتيدات المفردة rs1561876 G > A, C, T في جين STIM1.

النتائج: أظهرت النتائج التي تم الحصول عليها من الدراسة الحالية أنه فيما يتعلق بتوزيع النمط الجيني rs6486795 لجين ORAI1 بين المرضى المسجلين، فإن النمط الجيني المتماثل الطبيعي TT يمثل 39.3٪ من السكان بينما يمثل النمط الجيني المتغاير الطفرة TC والنمط الجيني المتماثل الطفرة CC نسبة 38.4٪ و 22.3٪ على التوالي. أما بالنسبة لتوزيع النمط الجيني rs1561876 لجين STIM1 بين المرضى المسجلين، فإن المجموعة السائدة هي النمط الجيني المتماثل الطفرة AA الذي يمثل 53.6٪ من السكان يليها النمط الجيني

المتغاير الطفرة GA بنسبة 29.5٪ بينما النمط الجيني المتماثل الطبيعي GG يأخذ المركز الأخير بين المجموعات بنسبة 17٪ من السكان.

الاستنتاجات: وفقا لنتائج الدراسة الحالية فإن تعدد الأشكال الجيني لجيني قنوات الكالسيوم المعتمدة على المخازن ORAI1 و STIM1 ليس لها تأثير إحصائي ذو دلالة إيجابية على عدم الاستجابة للإريثروبويتين ولكن لا يمكن استبعاد أن لهما دورًا مساهمًا في هذه الحالة. إذ لاحظنا ارتفاعًا إحصائيًا ذو دلالة في مستويات الهيموغلوبين لدى الأفراد الحاملين للنمط الجيني CC في rs6486795 لجين ORAI1 مقارنةً بالحاملين للنمط الجيني TT. أظهر التحليل الترددي لعلاقة جيني ORAI1 rs6486795 و STIM1 rs1561876 وتأثيرهما على مستويات الهيموغلوبين و الارثروبويتين أن النمط الجيني TTAA يمثل أعلى معدل نسبة انتشار (23.2٪) بينما يمثل CCGG الذي يتميز بمستويات أعلى من الهيموغلوبين ويعتبر من المستجيبين الجيدين للعلاج، نسبة أقل بكثير(4.5٪) من إجمالي عدد المرضى وهذا قد يفسر شيوع عدم الاستجابة لعقار الإريثروبويتين بين هؤلاء المرضى.

جمهورية العرا ق

وزارة التعليم العالي والبحث العلم ي

جامعة كربلاء

كلية الصيدلة

فرع الادويه والسمو م

تأثير تعدد الأشكال الجيني لجينَيّ ORAI1 وSTIM1 على مقاومة الارثروبويتين في المرضى العراقيين الذين يعانون من الفشل الكلوي المزمن و المعتمدين على الديلزة الدموية

رسالة

مقدمة الى مجلس كلية الصيدلة / جامعة كربلاء

كجزء من متطلبات نيل درجة الماجستير في علم الادوية والسموم

بواسطة

فرح فلاح ذياب

بكالوريوس صيدلة (جامعة كربلاء 2015)

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