PERIÓDICO TCHÊ QUÍMICA
ARTIGO ORIGINAL

ERITROFERRONA COMO UM NOVO BIOMARCADOR ASSOCIADO À ANEMIA EM PACIENTES IRAQUIANOS COM DRC

ERYTHROFERRONE AS A NEW BIOMARKER ASSOCIATED WITH ANEMIA IN IRAQI PATIENTS WITH CKD

الإرثروفيرون كمؤشر حيوي جديد يرتبط بفقر الدم لدى المرضى العراقيين المصابين بأمراض الكلى المزمنة

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RESUMO

Introdução: A eritroferrona (ERFE) é uma glicoproteína de síntese e liberação de hormônio pelos eritroblastos. Recentemente identificado como um regulador eritropoiético e ativado em resposta ao estímulo da eritropoietina (Epo). Nas doenças renais crônicas (DRC), a anemia é um distúrbio característico devido a uma diminuição da hipossensibilidade eritropoiética à Epo; esses pacientes recomendaram o uso de agentes estimuladores de eritropoiese (AEEs). **Objetivos**: Este estudo teve como objetivo avaliar o nível sérico de ERFE em pacientes com DRC e investigar os efeitos contínuos do uso de AEE anêmico em longo prazo associado a marcadores de eritropoiese e metabolismo do ferro. **Métodos**: Sessenta e cinco pacientes com DRC foram divididos em dois grupos, incluindo 30 pacientes em hemodiálise (HD) e 35 pacientes com DRC sem hemodiálise (não-HD), foram comparados a 25 voluntários saudáveis pareados por sexo e idade inscritos no estudo. O nível sérico de ERFE foi medido através de um ensaio imunoenzimático (ELISA).. **Resultados**: Um aumento significativo nos níveis séricos de ERFE em pacientes em HD da mediana (IQR) de 17,25 (13,4) ng/mL, razão de possibilidades (OR = 10,161), (AUC 0,996) maior do que CKD 4 (6,1) ng/ml, (OR = 6,295), (AUC = 0,984) p <0,001; também, estes estão positivamente correlacionados com o uso de AEE em HD e CKD (r = 1,00 e r = 0,95), respectivamente, em comparação com o grupo saudável 2 (2,1) ng/ml. Os níveis séricos de ERFE foram significativamente negativos em pacientes com DRC e HD. (p <0,05) relacionado à TFG (r = -0,396, e r = -0,68), saturação de transferrina (TS%) (r = -0,842 e r = -0,877), níveis séricos de ferritina (r = -0,865 e r = -0,866), e Ferro (r = -0,860 e r = -0,851), RBC (r = -0,841 e r = -0,843), hemoglobina (Hb) (r = -0,758 e r = -0,796) **Conclusões**: O presente estudo demonstra que os níveis séricos elevados de ERFE associados à atividade eritropoiética e anemia são maiores em pacientes com DRC com HD e não-HD tratados com AEE do que em pacientes sem AEE. Este estudo sugeriu o uso de ERFE como uma ferramenta de sucesso para a inspeção da atividade eritropoiética na DRC, especialmente aqueles que tomam AEEs para tratar anemia.

Palavras-chave: *Eritroferrona, Anemia, Eritropoiese, Iron Status.*

ABSTRACT

Background: Erythroferrone (ERFE) is a glycoprotein hormone synthesis and release by erythroblasts. Recently identified as an erythropoietic regulator and activated in response to stimulating erythropoietin (Epo). In chronic kidney diseases (CKD), anemia is a hallmark disorder due to a decrease in hyposensitive erythropoietic to the Epo; these patients recommended to use of Erythropoiesis-stimulating agents (ESAs). **The aim:** This study aimed to assess serum ERFE level in patients with CKD and investigate the continuing effects of long-term anemic ESA use associated with markers of erythropoiesis and iron metabolism. **Methods**: Sixty-five CKD patients divided in two groups, included 30 hemodialyses (HD) and 35 without hemodialysis (non-HD) CKD patients, were compared to 25 healthy voluntaries matched by gender and age enrolled in the current study. Serum ERFE level was measured by an enzyme-linked immunosorbent assay (ELISA). **Results:** Serum ERFE level was significantly elevated in HD patients median (IQR) about 17.25 (13.4) ng/mL, odds ratio (OR = 10.161), (AUC 0.996) greater than CKD 4(6.1) ng/ml, (OR = 6.295), (AUC = 0.984) p<0.001; also, these are positively correlated with the use of ESA in HD, and CKD (r = 1.00 and r = 0.95) respectively as compared to healthy group 2(2.1) ng/ml. Serum

ERFE levels were significantly negative (p<0.05) in both CKD and HD patients related to GFR (r = -0.396, and r = -0.68), transferrin saturation (TS%) (r = -0.842, and r = -0.877), serum levels of Ferritin (r = -0.865 and r = -0.866), and Iron (r = -0.860, and r = -0.851), RBC (r = -0.841, and r = -0.843), hemoglobin (Hb) (r = -0.758, and r = -0.796). **Conclusion:** The present study demonstrated that elevated serum ERFE levels associated with erythropoietic activity and anemia are higher in CKD with HD and non -HD patients treated with ESA than in non-ESA patients. This study suggested using ERFE as a successful tool for erythropoietic activity inspection in CKD, especially those taking ESAs to treat anemia.

Keywords: *Erythroferrone, Anemia, Erythropoiesis, Erythropoiesis-stimulating agents, hemodialysis, and CKD*

الملخص:

معلومات اساسية: الإرثروفيرون (ERFE) هرمون بروتيني سكري يتم تصنيعه وإفرازه من مولدات خلايا الدم الحمراءerythroblasts تم تحديده مؤخرا كمنظم لتكوين كريات الدم الحمر ويتم تنشيطه استجابة لتحفيز هرمون الأريثروبويتين (Epo) في أمراض الكلى المزمنة CKD يعد فقر الدم اضطرابًا مميزًا بسبب نقص الشديد في حساسية الكريات الحمر لهرمون الأريثروبويتين. لذلك كان هؤلاء المرضى يوصون باستخدام عوامل تحفيز تكوين الكريات الحمر (ESAs). **الهدف**: هدفت هذه الدراسة إلى تقييم مستوى الإرثروفيرون ERFE في مصل المصابين بأمراض الكلى المزمنة والتحقق من تأثير الاستخدام المستمر ل ESA كعلاج لفقر الدم على المدى الطويل وعلاقته بمؤشرات الدم وايض الحديد. **طرائق العمل**: شملت الدراسة الحالية 65 مريضا مقسَّمين إلى مجموعتين تضمنت 30 حالة غسيل كلى (الديلزة الدموية (HD و35 حالة أمراض الكلى مزمنة دون ديلزة مقارنة مع 25 من المتطوعين الاصحاء مع تطابق الجنس والعمر في الدراسة الحالية. تم تقييم مستوى الإرثروفيرون في المصل بالمقايسة الامتصاصية المناعية للإنزيم المرتبط (ELISA). **النتائج**: اظهرت ارتفاعا معنويا (P<0.05) في مستوى الدليل الحيوي الإرثروفيرون ERFE في مرضى الغسيل الكلوي HD بوسيط median ومجال ربيعي (IQR) مقداره 17.25 (13.4) نانوغرام/مل، ونسبة ارجحية (OR=10.161) ومساحة تحت المنحني (AUC= 0.996)، أكبر من مجموعة المرضى بدون غسيل كلوي بوسيط median ومجال ربيعي (IQR) مقداره 4 (6.1) نانوغرام/مل، ونسبة ارجحية (OR=6.295) ومساحة تحت المنحني (AUC=0.984)، p<0.001. كما أظهرERFE وجود علاقة ارتباطية معنوية ايجابية (P<0.05) مع استخدام علاج (ESAs) في كلا المجموعتين CKD وHD وبمعامل ارتباط (r=1.00, r=0.95) على التوالي. ان مستويات ERFE في مصل مرضى HD وCKD ترتبط ارتباطا معنويا عكسيا مع كل من معدل الترشيح الكلوي GFR (r=- 0.396, r=-0.68) نسبة تشبع الترانسفيرين TS% (r=-0.842, r=-0.877)، مستوى الفيريتين في المصل r=-0.865 r=-0.866) مستوى الحديد (r=- 0.860, r=-0.851)، اعداد كريات الدم الحمراء RBC (r=-0.841, r=-0.843) ومستوى الهيموغلوبين (Hb). (r=-0.758, and r=-0.796) على التوالي. **الاستنتاج**: الخلاصة: توضح الدراسة الحالية أن ارتفاع مستويات ERFE في المصل ترتبط بنشاط تكوين خلايا الدم الحمراء وفقر الدم وتكون أعلى في مرضى CKD الذين عولجوا بـ ESAs مقارنةً بالمرضى الذين لا يستخدمون ESAs. اقترحت هذه الدراسة الى امكانية استخدام الدليل الحيوي ERFE كأداة ناجحة للكشف عن نشاط خلايا الدم الحمراء في مرضى الكلى المزمنة، خاصة الذين يتعاطون محفزات تكوين الحمر ESAs كعلاج لفقر الدم.

الكلمات المفتاحية: الإريثروفيرون (ERFE)، فقر الدم ، تكوين كريات الدم الحمراء، عوامل المحفزة لتكوين كريات الحمر ESAs، الغسيل الدموي. وامراض الكلى المزمنة

1. INTRODUCTION:

Chronic kidney disease (CKD) is categorized by a frequent complication of anemia, especially in the end phases (5 stages) of the disease (Fishbane and Spinowitz, 2018). Anemia is a common feature of many patients with CKD and is associated with reduced quality of life, the mechanisms of anemia pathogenetic in CKD by decreased synthesis of erythropoietin and reticuloendothelial iron invasion caused by chronic renal inflammation, and recently that GDF-15 is a possible mediator of anemia through hepcidin in adult renal transplant recipients and stratification factor for achieving the end stage of diabetic kidney disease (Abass and Sharba, 2020; Mikhali *et al.*, 2017).

In CKD, deficiency of erythropoietin (EPO) synthesis and shorting of erythrocytes lifespan were played an important role in anemia pathogenesis (Kautz *et al.*, 2014; Sharba and Al-Zahid, 2016). Besides, patients of hemodialysis (HD) suffer from an iron deficiency for erythropoiesis. EPO exogenic or known by erythropoiesis-stimulating agents (ESAs), and iron supplementation are essential components of anemia administration in CKD patients (Atkinson and Warady, 2018).

Many studies have indicated the association between ESA and iron metabolism biomarkers in HD patients, such as hepcidin 25 a key regulator of stored iron release and the primary mediators of iron deficiency (Honda *et al.,* 2019; Webster *et al.*, 2017). ESA significantly suppresses levels of hepcidin 25 and ferritin, and the long treatment with acting ESA results in effective erythropoiesis and stored iron release (Honda *et al.,* 2019).

Erythroferrone (ERFE) is an erythropoietic-driven encoded by the FAM132B gene. It was a new regulator of iron homeostasis (Kautz *et al.,* 2014).

ERFE was expressed and released from erythroblasts in response to endogenous or exogenous erythropoietin (EPO) hormone (Kautz *et al.*, 2015). It regulated iron metabolism by inhibiting hepcidin and excess absorption with iron mobilization, making available a compensated supply during stress erythropoiesis such as rapid growth or hemostasis (Ganz, 2019). ERFE a newly identified cause of increasing anemia severity associated with a high iron and ferritin level in human patients with the major and intermediate beta-thalassemia, especially with splenectomized (Almousawi and Sharba, 2019).

This study aimed to assess serum ERFE levels in patients with CKD and investigate the continuing effects of long-term anemic ESA use associated with erythropoiesis and iron metabolism markers. Also, determine their potential in early erythropoietic activity prediction and anemia.

2. MATERIALS AND METHODS:

2.1. Subject of Patients

The study protocol was approved by the Ethics Committee of the faculty science, university of Kufa N0. 6097 on 26/11/2019. Moreover, it was conducted following the Declaration of Helsinki Principles and related ethical guidelines. All participants or their guardians involved in this research received verbal consent. A cross-sectional study was performed of Ninety participants in the present study; they have included sixty-five patients with CKD aged range (25-63) years, about 23 were males, and 12 females, 15 with Diabetic Mellitus, and 20 without Diabetic Mellitus who attended two Units for kidney disease at Al-Sadder Teaching Hospital and Al-Hakeem General Hospital in Al-Najaf, Iraq. This study was carried out from December 2019 via January 2020. CKD patients with different stages would include two independent groups: a group of 30 patients with end-stage (4-5) CKD eGFR <45 mL/min/1.73 m2, under hemodialysis (HD) for a time of median (IQR) 42 (17-85) months, about twice a week with standard bicarbonate dialysis in different types of non-cellulosic membrane filters. All patients were treated with intravenous iron and the individual dose of using 4000 units ESA. Our current study excluded patients with transplanted, cancer, chronic inflammatory diseases, and any end-stage complicated diseases. CKD Patients with diabetic mellitus were defined as type 1 (TDM1) or type 2 (TDM2).

Additionally, there are 35 stable clinical patients of CKD (1-3 stage) eGFR ≥45 mL/min/1.73 m2 with no acute or rapidly developing kidney disorders, intercurrent infections, or acute inflammatory processes. Such patients were not transplanted, not pregnant, and non-cancer. When These Patients did have laboratory evidence of iron deficiency were treated with oral iron supplements. All patients with renal transplant recipients are excluded from the study.

2.2. Healthy group

Twenty-five volunteers of both sexes (13 men and 12 women) as a control group were recruited from working in these hospitals and science faculty institution, which had no diseases such as diabetes, cardiovascular disorders, anemia, and kidney diseases.

2.3. Demographic Information and Laboratory Measurements

BMI (Body Mass Index) is performed by electronic balance and height device, for calculating the weight and height, and applied the Equation (Eq. 1).

$$BMI = \text{Weight (kg)} / \text{Height (m}^2\text{)} \quad \text{(Eq. 1).}$$

GFR can be calculated mathematically from the Modification of Diet in Renal Disease (MDRD) by Equation 2 (Levey *et al.*, 2009).

$$GFR\ (\text{ml/min/1.73 m}^2) = 186 \times (\text{creatinine concentration})^{-1.154} \times (\text{Age})^{-0.203} \times (0.742 \text{ if female}) \times (1.210 \text{ if black}) \quad \text{(Eq. 2).}$$

Five ml of blood was collected from all participants in the early morning and before one-hour of dialysis session for the HD patients, then divided into 2.5 ml put in EDTA-containing tubes for CBC, and 2.5 ml in gel tube and centrifuged for separated serum supernatants were stored at −deep freeze until batch analyses of ERFE and biomarkers of iron status.

2.4. Measurement of Hematologic Parameters

Complete blood counts were performed at the laboratory by an automatic blood analyzer device (Mythic 18, RINGELSAN CO., Turkey) using three reagents: a diluent, a lysis reagent, and a cleaning solution. A cyanide-free spectrophotometry method was used to measure hemoglobin by the formation of oxyhemoglobin at 555 nm (Van *et al.,* 1961). Serum phosphate, and calcium, depended on the standard laboratory procedure, were performed by the automatic biochemical analyzer (bt 35i) using the Direct

Method kit, which supplies from manufactures (Monobind Inc., USA). (Wassmuth *et al.,* 2011).

2.5. Measurement of TS%, Iron, and Ferritin

Serum ferritin levels quantification through the immunoenzymatic technique Enzyme-Linked Immunosorbent Assay (ELISA) using automated Elisa (Italy), testing of according to the manufacturing company, the Human Accu Bind Ferritin ELISA Kit was achieved (Monobind Inc., USA). Serum iron, transferrin, and total iron-binding capacity (TIBC) were measured by iron kit (using the chromogen ferrozine method and reagent Ferrozine 16.7 mM in Hydroxylamine hydrochloride). This processing with the advice of bt 35i (Turkey).TS % was measured from serum iron and total iron-binding capacity (TBIC). The Equation 3, (Horak and Sunderman, 1974).

Serum iron × 100/ TBIC. (Eq. 3)

2.6. Measurement of ERFE Biomarker

Serum ERFE level was estimated based on sandwich enzyme-linked immune-sorbent assay technology, by Automated Elisa (Italy) with using the human kit of (ELISA) was provided from (MyBioSource, San Diego, CA, USA) Depended on (Ganz et al., 2017).

2.7. Statistical Analysis

All data were analyzed by the SPSS software (V.24 Inc., Chicago, Illinois, USA) and GraphPad Prism (La Jolla, CA, USA, v.8.2.1). Kolmogorov-Smirnov test for variables distribution. Normally distributed Numerical Variables were compared between two groups by Independent t-test, and among two groups with ANOVA, all data expressed as (mean ± SD) standard deviation. The non-parametric Mann-Whitney U test was used to compare qualitative and quantitative values that were not normal, data expressed as median with inter-quartile range (IQR), and Kruskal-Wallis, respectively. Nominal variables were presented as frequency and percentage (%) were compared between studied groups using the Chi-square test. Correlation coefficient analysis was completed with Pearson's or Spearman rank. Whereas the point-biserial correlation coefficient is used in correlation analysis between continuous and binary variables. The receiver operating characteristic curve (ROC) analytical curve has been used to estimate the diagnostic efficiency of ERFE and TS% clinically viable by assay the ratio of area under the curve (AUC). ERFE has been recognized as an independent indicator of anemia in groups of patients through nominal regression data were expressed as odds ratios (OR), 95% confidence intervals (CI), and p values; Significance of differences was detected at $p<0.05$.

3. RESULTS AND DISCUSSION:

3.1. Demographic and clinical Characteristic of study populations

Demographic and laboratory clinical data characteristics of the CKD and HD patients are compared with Healthy subjects demonstrated in table 1. Sixty-five of CKD patients of different stages (1-5), and ranged in age (25-66) year were subdivided into thirty-five patients without hemodialysis, they are from (1-3) stages as CKD, and thirty patients with hemodialysis in (4-5) stage. These two populations of patients were compared with twenty-five healthy participation with matched age and gender.

The patients with CKD were a (mean ±SD) age of (50 ±10), range (25-63) years, about 23 (65.7%) were males, and 12 (34.3%) females, 4 (11.4%), and 11 (31.4) patients had TDM1 and TDM2, while 20 (57.1%) without Diabetic Mellitus respectively. The mean BMI (27.34±4.72) (kg/m^2), and the period of disease was (35.49±11.57) months. Only 12(34.3%) patients received ESA drugs, and 23 (65.7%) null ESA. The mean eGFR was (64.37±15.11) mg/mL/1.73 m^2, and the median (IQR) of Phosphate was 3.01(1.5) mg/dl, and calcium 8.9 (0.7) mg/dl.

In the HD patients had (mean±SD) age of (51.53±8.55), range (34-66) years, about 14 (46.7%) were male and 16 (53.3%) females, out of 30 HD patients, 5(16.7%) had TDM, 21(70.0%) TDM2, and 4(13.3%) without Diabetic Mellitus respectively. The mean BMI (27.88±3.64) kg/ m^2, and the disease period was (47.97±12.31) months. All patients of HD 30(100%) were treated with ESA. The mean eGFR was (12.21±6.16) mg/mL/1.73 m^2, and the median (IQR) of Phosphate and calcium were 4.65 (1.2) mg/dl and 7.3 (1.5) mg/dl, respectively.

Such patient outcomes were not significantly different $p>0.05$ in age (46.44±10.45), 13 (52%) males and 12 (48%) females compared to the healthy group. The Means of BMI (29.24±2.74) kg/m^2, but these were significant ($p<0.001$) decreased in variables eGFR (99.68±4.62) mg/mL/1.73m^2, and calcium were 9.1 (0.4) mg/dl, while a significant ($p<0.001$) increased of Phosphate 2.5 (1.0) mg/dl.

3.2. Relationship between Serum Erythroferrone Level and erythropoiesis status

In all study populations, after Tests of Normality (Kolmogorov-Smirnov), a skewed distribution of serum ERFE levels and the results represented as a median (IQR) value in Table 1 showed significantly ($p<0.001$) higher levels of ERFE 4, 6.1 ng/ml and a range of 2.2-10.5 ng/ml; mean 5.43 ng/ml for patients with CKD, and HD patients median (IQR) of 17.25 (13.4) ng/mL and a range (6.3-28.4) ng/ml; mean of 16.14 ng/ml when compared with a healthy group a median (IQR) of 2 (2.1) ng/ml and a rang (1.3-3.7) ng/ml; mean of 2.22 (ng/ml). Serum TS% levels showed a significantly increased in CKD (mean±SD) of (28.89±3.98) and (35.08±4.58) in HD patients as compared with the healthy group (mean±SD) of (24.4±3.56) ($p<0.001$).

Serum Ferritin levels indicated a significant increase in HD, a median (IQR) of 489 (118) ng/ml when compared with CKD, a median (IQR) of 121 (77) ng/ml; and a median (IQR) of 89 (25) ng/ml ($p<0.001$) for a healthy population. No significant difference in serum Iron levels between CKD and HD patients, but compared to healthy groups, rendered a highly significant difference ($p=0.02$). The results showed a significant decrease in mean of RBC_S (4.34±0.56) (10^6/ml), and Hb levels (11.97±1.53) g/dl in patients with CKD and (4.06±0.43) (10^6/ml), and (11.24±1.56) g/dl; compared with healthy group RBC_S of (4.68±0.42) ($X10^6$/ml), and Hb of (12.67±0.98) g/dl; ($p<0.001$, and $p=0.019$) respectively.

Person correlation coefficient or Spearman were estimated of ERFE with other Independent in both patients with CKD and HD (table 2). Serum ERFE level was a positively significant ($p < 0.001$) with the age ($r = 0.477$, and $r=0.6361$), treated with ESA ($r = 0.951$, and $r=1$), and DM ($r = 0.650$, and $r = 0/.41$) respectively. but it was inversely related with GFR ($r =-0.396$, and $r=-0.68$), TS% ($r =-0.842$, and $r=-0.877$), Ferritin ($r =-0.865$, and $r=-0.866$), Iron ($r =-0.860$, and $r=-0.851$), RBC ($r=-0.841$, and $r=-0.843$), hemoglobin ($r =-0.758$, and -0.796) respectively.

Despite the management of the fundamental disorders with anemia of chronic disease, and this was impracticable, persistent anemia accompanied symptoms and inaccurate diagnosis in many conditions; New therapeutic strategies to understand the pathophysiology of chronic disease anemia must be facilitated for this purpose. Recently, Dr. Kautz and colleagues in 2014 were discovered an important hormone in hematology (Kautz *et al.,* 2014). ERFE was the major role of iron supply as an erythropoietic regulator. It acts straight on liver cells to inhibit hepcidin expression (Kautz and Nemeth, 2014). Recent research has shown that ERFE plays a significant role in the increased severity of anemia in beta-thalassemia (Almousawi and Sharba, 2019; El-Gamal *et al.*, 2020). In the current study, ERFE has consistently been associated with mortality and progressive anemia in CKD and HD patients; However, publications that deal with these subjects in patients were very few studies in all world (Hanudel *et al.*, 2018; Honda *et al*., 2016; Spoto *et al*., 2019). This study was represented the modern study suggested in Iraq.

In The current study of CKD and patients under hemodialysis (HD) compared with the healthy group, I observed high levels of serum ERFE in both groups of patients CKD and HD as the comparison with healthy; this finding agreement with a recent study by author Ganz et al., ERFE level was measured in 97 HD patients (15.7 (7.9–32.5) ng/mL) but twice higher than in 51 patients with CKD 6.1 (2.6–15.0) ng/mL when compared with 161 healthy subjects 7.8 (4.7–13.2) ng/ml, (Ganz *et al.*, 2017). Moreover, another recent study in 2019 by Spoto *et al*. they have indicated a highly significant increase in serum levels of ERFE in both cohorts CKD, and HD with the mean value of (3.4–7.5, and 7.8 –10.5 ng/mL), respectively, with do not become significant in male and female patients. Who has advocated that ERFE was consistent with these patients and related to mortality and various severity with Cardiovascular events because these cohorts using ESA drugs lead to elevated serum ERFE level (Spoto *et al*., 2019). It has been extensively synthesized in the bone marrow and the spleen responsible for the erythropoietin hormone responsible for the erythroblasts to stimulate the survival and differentiation of the erythroid (Coffey *et al.*, 2018) or after exposures to blood loss and EPO injection (Kautz *et al.*, 2015; Rainville *et al.,* 2016). Because HD patients were persistent uptake of exogenous EPO hormone, it was stimulation and decreased renal clearance, thus elevated ERFE levels (Hanudel *et al.*, 2018). This was confirmed by the outcomes of the current study (Figure 2) showed a high level of serum ERFE in groups that deal with ESA more than a non-ESA group.

Additionally, the current study found correlations between ERFE levels and RBC, Hb, Ferritin, Iron, TS% inversely (Table 2). Agreement with recent studies of El Gendy *et al*. (El Gendy *et al.*, 2018). Who reported to that serum ERFE levels negatively correlated with serum iron (r=-

0.63; p=0.001), ferritin (r=-0.46; p=0.004), TS% levels (r=-0.66; p=0.001), and Hb (r =-0.39; p=0.01). Other studies (Honda *et al*., 2016; Sulovska *et al.*, 2016) also suggested that ERFE levels were negatively correlated with ferritin and hepcidin and positively correlated with sTfR in HD patients and that there was a positive correlation between hepcidin / ERFE ratio and hepcidin with those of ferritin.

These authors were attributed to the patients with IDA, ERFE as a physiological hepcidin suppressor, and elevated ERFE was an important role and injurious effects in clinical conditions with ineffective erythropoiesis such as β- thalassemia (Russo *et al.*, 2016). A study by (Kautz *et al*., 2015) indicated that significantly increased erythroblasts in ineffective erythropoiesis generate significant amounts of ERFE. On the other hence, patients with HD suffering from elevated iron accumulation as resultant, a major constituent of ineffective erythropoiesis, in which erythroblast count is greatly expanded, but the erythroblasts undergo intramedullary apoptosis before completing differentiation (Honda *et al.*, 2016; Mirciov *et al.*, 2017; Sulovska *et al*., 2016). Dysfunction of kidneys in patients with CKD and HD, inflammations, hypoxia, hemolysis, and iron status were affected by serum levels of hepcidin hormone (Wang *et al.*, 2017). This hormone regulates the iron absorption from the digestive canal. That iron stored out of the reticuloendothelial system is released. When it uses a recombinant Epo (rhEpo), this stimulated ERFE production suppresses hepcidin synthesized by inhibiting BMP/SMAD signaling in hepatocytes (Robach *et al.,* 2020).

On determining the critical point or threshold levels for serum TS% and ERFE levels to predict the development of anemia in CKD and HD patients, the results indicated that candidate TS% cutoff (>29.5) might not represent anemia. However, this result agrees with previous studies that describing that transferrin saturation (TS%) levels were assayed from fasting serum iron and transferrin concentrations, iron decreased in deficiency anemia highly changeable, accordingly, inappropriate for the iron depletion, and deficiency anemia; increased transferrin is synthesized associated with the iron store. As the TS% < 15%, the supply of iron for hemoglobin production in the bone marrow do not adequate; In that case, it is considered sensitive enough as screening tools of deficiency anemia (Clénin, 2017; Mikhali *et al.*, 2017). In other hence, we believed serum levels of TS%, if it is more than 15%, it is unsuitable tools for anemia; for example, most hereditary anemia such as thalassemia, have higher increased cutoff levels of TS% ≥75% and used to predict the iron overload, but these patients still suffering from severe anemia (Eissa and El-Gamal, 2014)

3.3. ROC curve analysis for ERFE, and TS%

By estimating the cutoff thresholds for parameters of erythropoiesis to identify patients with thresholds for serum ERFE and TS% levels to predict progressive anemia and emerging active erythropoiesis in patients. According the results which founded in Table 3, and Figure 1. In patients with HD the results of ROC curve analysis showed, (Figure.1A) Serum ERFE (ng/ml) was a highly positive cutoff ratio of > 3,650 (AUC= 0,996; sensitivity = 0,967; and specificity = 0,960) compared to the cutoff TS% of > 29,50 (AUC= 0,965; sensitivity = 0,867; and specificity = 0,96). But in patients with serum ERFE (ng/ml) was acknowledged as the superlative predictor of progressive anemia at a cutoff value of > 2.600 (AUC =0.894; sensitivity = 0.886 and a specificity = 0.760) in comparison with TS% levels at the cutoff > 29.50 (AUC =0.789; sensitivity =0.486; specificity = 0.960), (Figure.1B). Furthermore, ROC curve analysis of ERFE (ng/ml) was elevated of positive ratio in HD patients vs. CKD patients for a cutoff value of > 6.50 (AUC =0.919; sensitivity = 0.90, and a specificity = 0.68). (Figure.1C).

The results of Table 5 referred to Nominal Regression models to Estimates the predictors of ERFR in CKD and HD patients. The reference category is Healthy, in CKD patients, ERFE level of (OR=6.295, B=1.840, and Intercept=-5.159, p-value 0.001), as comparison high positive in HD patients of (OR=10.161, B=2.319, and Intercept=-9.569, p-value <0.001).

3.3. Comparison between with and without Erythropoiesis Stimulating Agents (ESA) in measurements of erythropoiesis indicator.

The results of the Box plot showed that ERFE and TS% high significantly (p<0.001) increased in HD patients with ESA treated (right green box) as a comparison with CKD patients with and without ESA (left green and blue box) (Figure 2A and B) respectively. The results indicated that both groups of HD and CKD patients with (ESA) a significant decrease (p<0.05) in the values of Hb and RBCs (green box plot) when compared to patients non -ESA treated (blue box plot). (Figure. 2C and D) respectively.

Th findings in the current study of Nominal Regression models and ROC analysis showed higher serum ERFE level (OR 10.161,95% CI

(3.281 to 31.467, and (AUC =0.991) in HD, more than (OR 6.295,95% CI (2.113 to 18.759), and (AUC=0.894) in CKD vs. healthy respectively. A recently study by Honda *et al.* (2019) that mentioned ERFE as an important biomarker in patients with CKD and the improvements following ESA management for this, the anemia in CKD patients requires a comprehensive assessment of ERFE levels with the attention of their best and physiological levels during erythropoiesis, as considering identifying variant ERFE as a specific biomarker of clonal erythropoiesis. (Bondu *et al.*, 2019). Despite these patients treated with ESA, significant numbers remain anemic (Atkinson and Warady, 2018). The current study supposed that associated with the prevalence of anemia in HD more than CKD because HD patients were persisted in ESA treatment lead to high expression of ERFE as a regulator of the erythroid mediator from erythroblast. It is a new sensitive biomarker in humans to estimate the relationship between iron metabolism and erythropoiesis and noticed that ESA might have a detrimental effect in the antidoping field (Kautz and Nemeth, 2014; Ramirez Cuevas *et al.*, 2020). As a result, hyperactivation of erythropoiesis and accelerated synthesis of the erythroid progenitor, but this undergo intramedullary apoptosis before the differentiation process is completed, therefore can cause anemia.

4. CONCLUSION:

Overall, the high levels of ERFE and the relationship with the hematological parameters of RBCs, HB, IRON, ferritin, and TS%, in HD patients WITH ESA treated more than CKD patients, has confirmed the suggested that regulatory role of ERFE in these parameters, and it possible that ERFE used as a successful tool for erythropoietic activity inspection in CKD and potential value in the identification of anemia risk, especially with ESAs treated. Future deep studies are needed to understand the role of ERFE as a biomarker of erythropoietic activity and exogenic erythropoietin (ESA) sensitivity at all levels of CKD-related anemia.

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Table 1. *Clinical demographic and laboratory data characteristics of CKD and HD patients with healthy subjects*

VARIABLES	CKD n=35		HD n=30		Healthy n=25		p-value
	Statistics value	95% CI	Statistics value	95% CI	Statistics value	95% CI	
Age (year) mean (SD)	50.86 (10.0)	47.42-54.29	51.53 (8.55)	48.34-54.73	46.44(10.45)	42.13-50.75	0.117
Male Female n (%)	23(65.7%) 12(34.3%)	-	14(46.7%) 16(53.3%)	-	13(52%) 12(48%)	-	0.279
No-DM n (%)	20(57.1%)		4(13.3%)		25(100%)		
TDM1 n (%)	4(11.4%)	-	5(16.7%)	-	0 0.0%	-	<0.001
TDM2 n (%)	11(31.4%)		21(70.0%)		0 0.0%		
Non-ESA n (%)	23(65.7%) ab	-	0 (0.0%) ab	-	25(100%)	-	<0.001
ESA n (%)	12(34.3%) ab		30(100%) ab		0(0.0%)		
BMI (kg/m2) Mean (SD)	27.34 (4.72)	25.72-28.96	27.88 (3.64)	26.53-29.24	29.24 (2.74)	28.11-30.37	0.245
Period diseases (mo.) mean (SD)	35.49 (11.57) a	31.51-39.46	47.97 (12.31) ab	43.37-52.56	0.00	0.00	<0.001
GFR (mL/min/1.73m2) Mean (SD)	64.37 (15.11) ab	59.18-69.56	12.21 (6.16) ab	9.91-14.51	99.68 (4.62)	97.77-101.59	<0.001
Phosphate (mg/dl) Median (IQR)	3.01 (1.5) ab	2.82-3.46	4.65 (1.2) ab	4.20-4.95	2.5 (1.0)	2.26-2.76	<0.001
Calcium (mg/dl) Median (IQR)	8.9 (0.7) b	8.48-8.86	7.3 (1.5) ab	7.12-7.76	9.1 (0.4)	8.87-9.17	<0.001
TS% mean (SD)	28.89 (3.98) ab	27.52-30.26	35.08 (4.58) ab	33.37-36.79	24.40 (3.56)	22.93-25.87	<0.001
Ferritin (ng/ml) Median (IQR)	121 (77) b	108.04-136.24	489 (118) ab	449.6-529.48	89 (25)	80.11-95.73	<0.001
IRON (mg/ml) mean (SD)	64.77 (21.56) a	57.36-72.18	61.18 (22.23) a	52.88-69.48	49.92 (15.33)	43.59-56.25	0.020
ERFE (ng/mL) Median (IQR)	4.0 (6.1) ab	4.41-6.46 ab	17.25 (13.4) ab	13.35-18.93	2.0 (1.2)	1.91-2.52	<0.001
RBC (X106/ml) Mean (SD)	4.34 (0.56) ab	4.15-4.53	4.06 (0.43) ab	3.99-4.32	4.68 (0.42)	4.50-4.85	<0.001
Hb (g/dl) Mean (SD)	11.97 (1.53) ab	11.45-12.50	11.24 (1.65) ab	11.03-12.62	12.67 (0.98)	12.26-13.07	0.019

a: Significant difference between groups of patients CKD, and HD with the healthy group at p-value <0.05. b: Significant differences between the groups of patients CKD, and HD at p-value <0.05. SD: Standard deviation, IQR: Inter Quartile range, CI: Confidence interval, TDM: Type Diabetic Mellites, ESA: Erythropoiesis-stimulating agents, BMI: Body Max Index, GFR: Glomerular Filtration Rate, TS: Transferrin saturate.

Table 2: *Correlation coefficient of ERFE with other variables in both CKD and HD patients.*

VARIABLES	CKD n=53			HD n=30		
	r	R squared	P-value (two-tailed)	r	R squared	P-value (two-tailed)
Age (year)	0.477	0.228	0.006 [ab]	0.636	0.405	0.001 [ab]
ESA	0.951	0.904	0.001 [ab]	1	1	0.001 [ab]
DM	0.650	0.422	0.001 [ab]	0.41	0.168	0.02 [a]
GFR (mL/min/1.73 m2)	-0.396	0.157	0.02 [a]	-0.68	0. 462	0.001 [ab]
TS%	-0.842	0.709	0.001 [ab]	-0.877	0.769	0.001 [ab]
Iron (mg/ml)	-0.865	0.7478	0.001 [ab]	-0.866	0.748	0.001 [ab]
Ferritin (ng/ml)	-0.860	0.740	0.001 [ab]	-0.851	0.724	0.001 [ab]
RBC ($X10^6$/ml)	-0.841	0.706	0.001 [ab]	-0.843	0.710	0.001 [ab]
Hb (g/dl)	-0.758	0.575	0.001 [ab]	-0.796	0.63	0.001 [ab]

[a] significant difference at p-value <0.05. [ab] significant differences at p-value <0.01

Table 3. *ROC curve analysis for the viability of ERFE (ng/ml), and TS% to use a clinically diagnostic of anemia between both patients' groups CKD and HD and with healthy subject*

CATEGORIES	CKD n=35 vs. Healthy n=25		HD n=30 vs. Healthy n=25		HD n=30 vs. CKD n=35	
	ERFE	TS%	ERFE	TS%	ERFE	TS%
AUC	0.894	0.789	0.996	0.965	0.920	0.8410
Std. Error	0703.040	0.059	0.005	0.022	0.036	0.047
95% CI	0.816-0.972	0.673-0.904	0.986-0.00	0.921-0.000	0.828 - 0.97	0.747-0.934
Youden index	0.647	0.446	0.927	0.827	0.676	0.490
Optimum Cutoff	> 2.600	> 29.50	> 3.650	> 29.50	> 7.80	> 33.1
Sensitivity	0.886	0.486	0.967	0.867	0.933	0.633
95% CI	0.740- 0.954	0.329-0.644	0.833-0.99	0. -0.947	0.786-0.99	0.455-0.781
Specificity	0.760	0.960	0.960	0.960	0.743	0.857
95% CI	0.5650.885	0.804-0.997	0.805-0.998	0.805-0.998	0.5790-0.858	0.706-0.937
Likelihood ratio	3.690	12.140	24.170	21.670	3.630	4.433
P- value	<0.001a	<0.001 a	<0.001 a	<0.001 a	<0.001 a	<0.001 a

AUC: Aera Under Curve. CI: Confidence interval a significant difference at p-value <0.05.

Table 4. *Nominal Regression models to Parameter Estimates ERFR as independent predictors in CKD and HD patients*

PATIENTS GROUPS [a]		B	Std. Error	Wald	df	Sig.	OR	95% CI for OR	
								Lower	Upper
CKD	Intercept	-5.159	1.544	11.173	1	0.001			
	ERFE (ng/mL)	1.840	0.557	10.907	1	0.001	6.295	2.113	18.759
	TS%	0.685	0.234	8.550	1	0.003	1.984	1.254	3.141
	RBC (X106/ml)	-2.085	1.450	2.068	1	0.150	0.124	0.007	2.131
	Hb (g/dl)	-.510	0.472	1.169	1	0.280	0.601	0.238	1.513
HD	Intercept	-9.569	1.957	23.909	1	0.001			
	ERFE ng/mL)	2.319	0.577	16.163	1	<0.001	10.161	3.281	31.467
	TS%	1.961	0.581	11.412	1	0.001	7.109	2.278	22.185
	RBC (X106/ml)	-6.500	2.635	6.086	1	0.014	0.002	8.603E-6	0.263
	Hb (g/dl)	-2.443	1.315	3.449	1	0.063	0.087	0.007	1.145

a. The reference category is Healthy. CI: Confidence interval. OR: Odds Ratio. df: degree freedom.

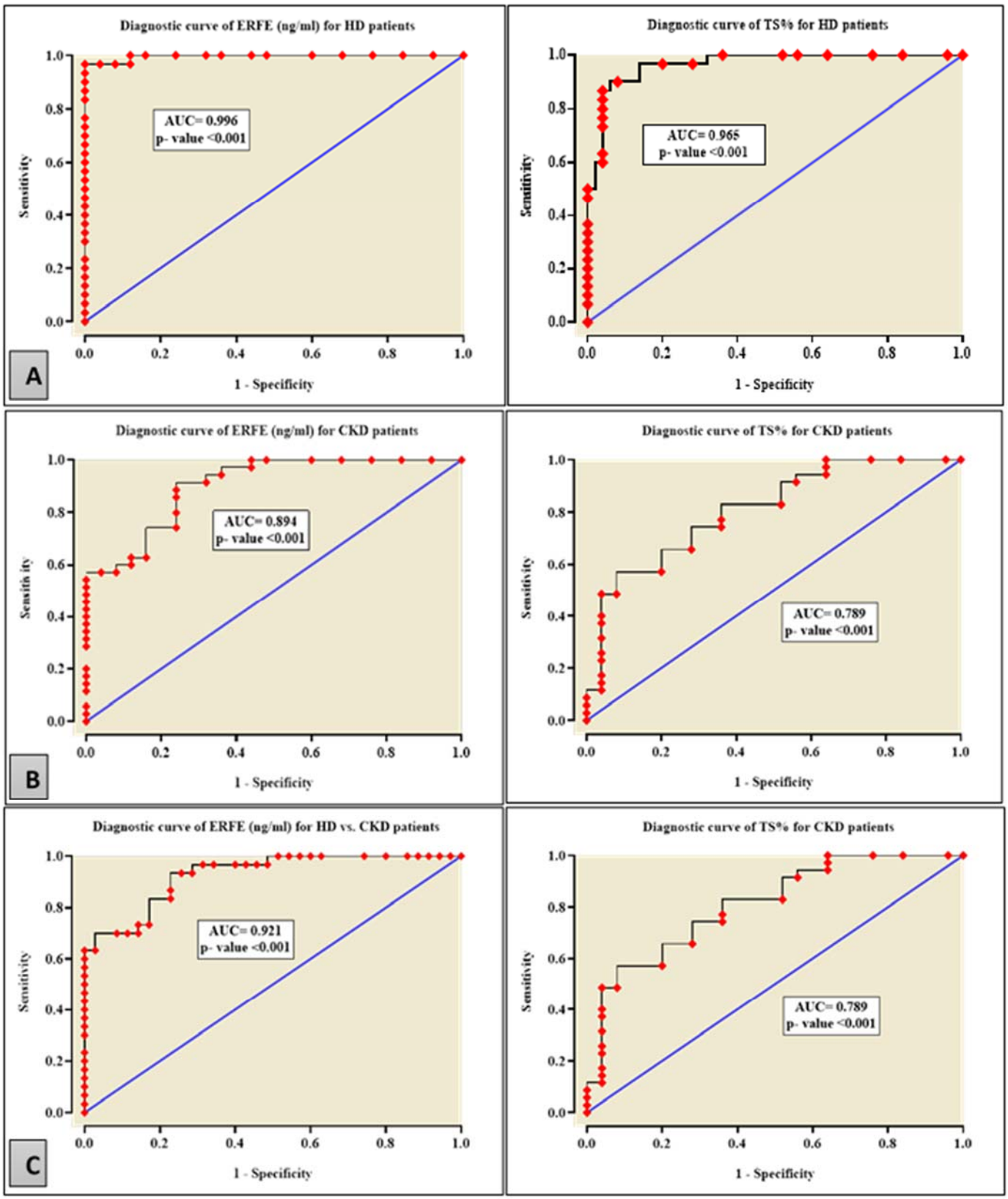

Figure 1*: Receiver operating characteristic curve (ROC) analysis of ERFE, and TS% for a viability diagnostic of (A) HD patients vs. healthy subjects by ERFE (upper), and TS% (lower). (B) CKD patients vs. healthy subjects by ERFE (upper), and TS% (lower). (C) HD vs. CKD subject by ERFE (upper), and TS% (lower).*

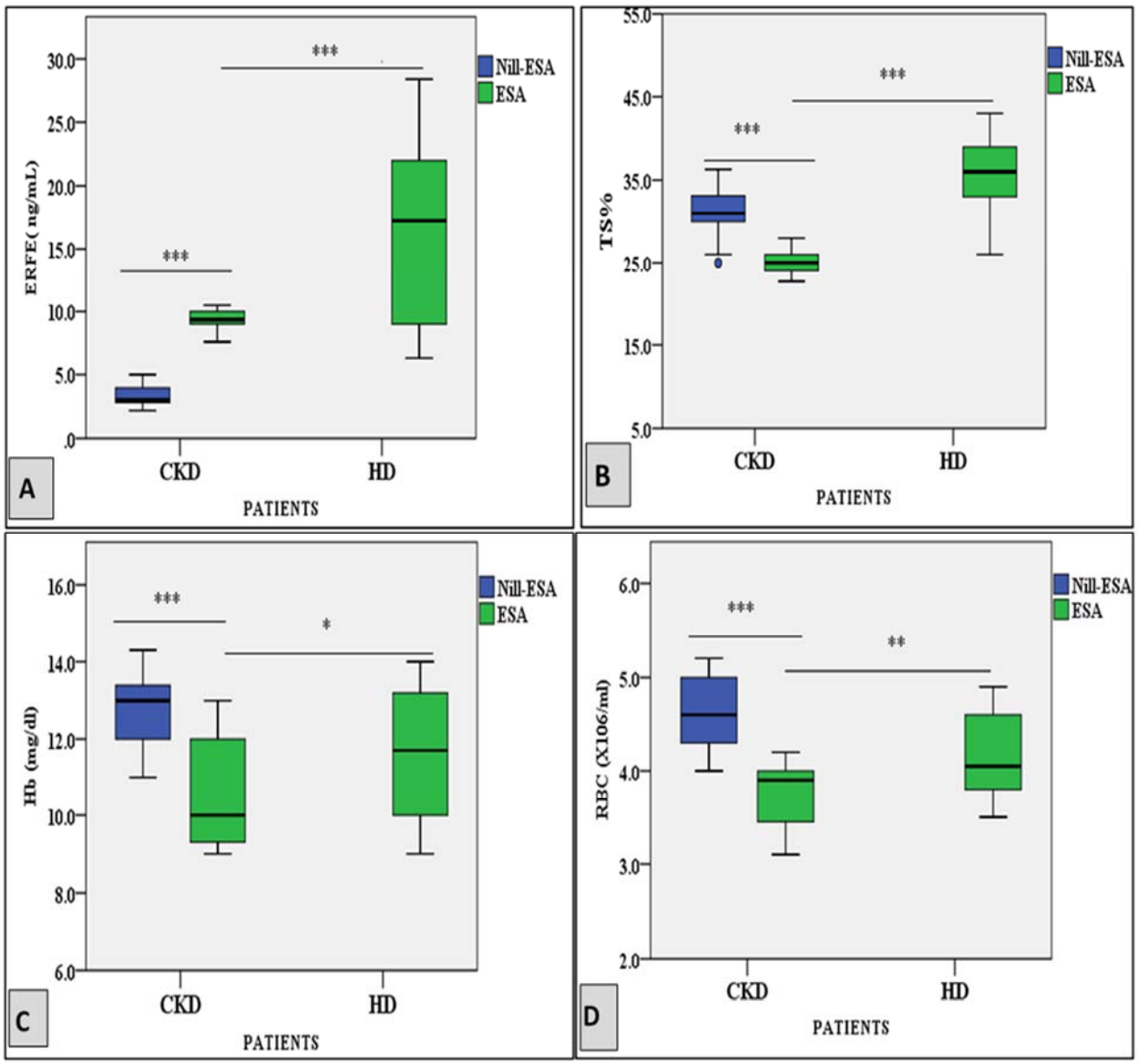

Figure.2*. Box plot showing a change of serum ERFE, TS%, HB, and RBC in CKD and HD patients with and without erythropoiesis-stimulating agents (ESA). A= Serum ERFE levels. B=TS%, C=HB, and D=RBC. Significant differences at three levels: * p-value <0.05, ** p-value<0.01, and *** p-value<0.001.*