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Study of Erythroferone Hormone in Children with Beta Thalassemia Major

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ABSTRACT

Background: Iron overload is a principal reason for morbidity and death in transfusion-dependent beta-thalassemia cases. Erythroferrone hormone (ERFE), a member of tumor necrosis family- alpha (TNF-α) superfamily produced by erythroblasts and stimulated by endogenous or exogenous erythropoietin (EPO), Under these conditions, ERFE inhibits the formation of hepcidin, restoring the functionality of ferro-portin, that is accountable for enhancing intestinal iron absorption and mobilizing iron reserves. **Objective:** The aim of the currents study is to analyze the role of ERFE in children suffering from beta thalassemia major.

Patients and methods: A prospective case-control study was conducted at Tanta University Hospital during the period from March 2021 to November 2021. The study included 40 children previously diagnosed with beta thalassemia major, and 40 healthy children matched in sex and age as a control group. Serum ERFE was calculated utilizing enzyme linked immunosorbent assay (ELISA) technique. **Results:** Serum ERFE level was significant increase in the beta thalassemia patients' group than control group. In the patients group, serum ERFE was higher in non spleenectomized patients, and in patients receiving blood transfusion more than once/ month, also, ERFE was higher in those with serum ferritin >1000 ng/ml. There is significant positive correlation between ERFE level and serum levels of ferritin and transferrin saturation (T.SAT %). Receiver Operating Characteristic (ROC) curve analysis demonstrated that ERFE level above 1.6 ng/ml is the cutoff value indicating iron overload with high diagnostic efficacy. **Conclusion:** ERFE is a possible diagnostic tool in predicting iron overload with sensitivity 95%, specificity 62 %, and accuracy 78%.

Keywords: Beta thalassemia major, Erythroferrone, Iron overload, case control study, Tanta University.

INTRODUCTION

Thalassemia is a heterogeneous hereditary hemoglobinopathy defined by globin chain abnormalities and autosomal recessive inheritance. Homozygous or compound heterozygous formulae have a disturbance in the formation of - and non-globin chains, leading to inefficient erythropoiesis and a decrease in normal hemoglobin A production [(1)].

Iron overload is the primary cause for morbidity and mortality in transfusion-dependent -thalassemia cases. As the body doesn't have action for excreting extra iron, iron accumulation leads to organ malfunction, especially in the heart, liver, and the endocrinal system, if left untreated [(2)].

The role of laboratory investigations in expecting the incidence of tissue iron accumulation, as determined by measurement of hepatic iron content using biopsy or noninvasive imaging techniques, was exhaustively investigated. Some investigations have identified serum ferritin as an acceptable prediction factor of the severity of iron overload, and its predictive value has been demonstrated in thalassemia and is connected with cardiac-related death [(3)]. However, its ability to forecast iron accumulation has been deemed inadequate by other researchers. Moreover, its benefit was reduced because of being an acute phase reactant increasing in some concomitant conditions like inflammatory states, hepatic disorder, rapid cell turnover, and deficiency in vitamin C [(4,5)].

Transferrin saturation (T.SAT %), labile plasma iron (LPI), and non-transferrin-bound iron (NTBI) are shown indicators of iron toxicity risk. Though, since NTBI and LPI assays aren't generally accessible or completely standardized, and because inter-laboratory variation is significant, the publicly available T.SAT percent is frequently used to infer the likelihood of higher concentrations of these two iron-toxic chemicals. T.SAT percentage values should be interpreted with caution during chelation therapy or inflammations [(6)].

Erythroferrone (ERFE) was suggested to be an erythroid hormone that FAM132B encodes a protein that regulates iron metabolism in humans. It is the most important negative mechanism of hepcidin under stressing condition or inadequate erythropoiesis, being formed by erythroid precursors in the bone marrow and spleen under the control of renal erythropoietin (EPO) [(7)]. Under these conditions, ERFE inhibits the formation of hepcidin, restoring the role of ferro-portin, that is accountable for enhancing the absorption of iron in the intestines and mobilising iron reserves. In settings needing an increase in erythropoietic activity, ERFE could be reasonably seen as playing a crucial function in enhancing iron accessibility for haemoglobin production [(8)].

There are two components to the raised ERFE synthesis because of anemia: as erythropoietic cells are activated by the effect of EPO, the number of erythroid precursor cells increases. Second, each individual cell in this population produces more ERFE. In anemias accompanied by inefficient erythropoiesis, EPO dramatically expands and stimulates the erythroid precursor population, but the majority of these cells do not produce mature erythrocytes. These deadend erythroid precursors secrete elevated quantities of ERFE, which persistently suppresses hepcidin and

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causes iron overload. In these iron-loading anemias, high quantities of unbound iron are considered to cause damaging in tissues by accelerating the creation of reactive oxygen species. Iron toxicity is characterized by destruction of or damaging hepatocytes, cardiomyocytes, and endocrine glands, in addition to a higher risk for infections [9].

Erythroferrone is currently regarded as one of the promising clinical indicators for measuring erythropoiesis activity in patients with iron-imbalance-related blood diseases [10].

Considering these results, we aimed to research serum level of erythroferrone hormone in Egyptian children suffering from β-Thalassemia major (TM), and its association with iron overload.

PATIENTS AND METHODS

A prospective case control study included 80 children who were classified into two groups; *Group I* included 40 children previously diagnosed with beta thalassemia major, with age ranging from 2.5 to 18 years. They were selected from Hematology Unit, Pediatrics Department, Tanta University Hospital during the period from March 2021 to November 2021. They were 19 (47.5%) males and 21 (52.5%) females. *Group II* included 40 healthy children matched sex and age as a control group. Their ages ranged from 3 to 17 years. They were 21 (52.5%) males and 19 (47.5%) female.

Clinical evaluation: All cases involved in this study underwent detailed history taking like family history of thalassemia, onset, and course of the disease, number of blood transfusions, compliance to chelation treatment, and medical records of splenectomy operation. Thereafter, careful clinical examinations were conducted especially the presence of pallor, jaundice, organomegaly and facial abnormalities.

Sampling: All participants in the study provided venous blood samples by conventional venipuncture under strict aseptic conditions, then carried in VACUETTE blood collecting tubes with K2EDTA for CBC on ERMA PCE-210N cell counter (Tokyo, Japan) with inspection of Giemsa-stained smears, and for reticulocytes count, and tube with clot activator/Sep to be utilized for serum obtaining. One portion of the blood samples was utilized directly for estimate of liver and renal function tests and LDH on Konelab 60i, and serum ferritin, and T.SAT percent on BECKMAN COULTER; AU480; Brea, Calif., USA, while the other portion was frozen at 20°C for estimation of serum erythroferrone.

Serum erythroferrone determination Erythroferrone hormone level was measured by a commertially available quantitative sandwich ELISA kit provided by SunRed Company, China. Catalog number: 201-12-5785 in accordance with the manufacturer's instructions. The sensitivity is 0.045ng/mL. The standard stock concentration of 6.4ng/mL was serially diluted. The colorimetric detection on the Tecan Spectra II Microplate Reader was detected at 450 nm (Switzerland). A logit-log standard curve was presented, from that the concentration of the sample was determined.

Ethical Consent:

The study was approved by the Local Ethical Committee of the Faculty of Medicine, Tanta University; Institutional Review Board (IRB) for human studies (Approval Code: 34334/12/20). Guardians of every patient signed an informed written consent for acceptance of participation in the study. This work has been carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for studies involving humans.

Statistical analysis

The collected data were coded, processed and analyzed using the SPSS (Statistical Package for Social Sciences) version 22 for Windows® (IBM SPSS Inc, Chicago, IL, USA). The data were presented as numbers and percentages for the qualitative data, mean, standard deviations, and ranges for the quantitative data with parametric distribution and median with interquartile range (IQR) for the quantitative data with non-parametric distribution.

Chi-square test and Fisher's exact test were used in the comparison between two groups with qualitative data. Independent t-test and Mann-Whitney U test were used in the comparison between two groups with quantitative data. Spearman coefficient was applied to correlate between quantitative variables. Univariate and multivariate logistic regression analyses were used to determine the related co-variables associated with iron overload. Receiver operating characteristic curve (ROC) was used to determine the diagnostic performance of the markers, area more than 50% gives acceptable performance and area about 100% is the best performance for the test. P value $\leq$0.05 was considered significant.

RESULTS

The laboratory information of the two studied groups is summarized in table 1. There were insignificant differences as regard white blood cells count, direct bilirubin, and renal function test among the two studied groups. While there were significantly different in hemoglobin level (Hb), platelets count, reticulocytes count, liver enzymes, total and indirect bilirubin, LDH, ferritin, and T.SAT% between the two studied groups.

Table (1): Comparison between the two studied groups regarding basic laboratory data.

Variable		Group (I) (Patients group)			Group (II) (Control group)			t-test	P-value
Hemoglobin (gm/dl)	**Mean ± S.D**	**7.17**	**±**	**0.40**	**12.21**	**±**	**0.49**	**50.055**	**0.001***
Platelets count (PLTs) (×10^3/Cmm)	**Mean ± S.D**	**566.63**	**±**	**124.63**	**271.15**	**±**	**8.34**	**12.510**	**0.001***
White blood cells count (WBCs) (×10^3/Cmm)	**Mean ± S.D**	**9.55**	**±**	**2.06**	**8.71**	**±**	**1.92**	**1.458**	**0.149**
Reticulocytes count (%)	**Mean ± S.D**	**3.03**	**±**	**0.46**	**1.01**	**±**	**0.17**	**19.367**	**0.001***
AST (IU/L)	**Mean ± S.D**	**48.73**	**±**	**4.92**	**20.65**	**±**	**5.20**	**24.813**	**0.001***
ALT (IU/L)	**Mean ± S.D**	**35.98**	**±**	**4.74**	**16.13**	**±**	**4.32**	**19.580**	**0.001***
Total Billirubin (mg/dl)	**Mean ± S.D**	**2.17**	**±**	**0.40**	**0.45**	**±**	**0.05**	**25.297**	**0.001***
Indirect Billirubin (mg/dl)	**Mean ± S.D**	**1.98**	**±**	**0.40**	**0.34**	**±**	**0.04**	**24.502**	**0.001***
Direct Billirubin (mg/dl)	**Mean ± S.D**	**0. 19**	**±**	**0.03**	**0.18**	**±**	**0.05**	**1.097**	**0.284**
Urea (mg/dl)	**Mean ± S.D**	**24.40**	**±**	**6.29**	**23.20**	**±**	**5.30**	**0.788**	**0.433**
Creatinine (mg/dl)	**Mean ± S.D**	**0.62**	**±**	**0.10**	**0.56**	**±**	**0.15**	**1.397**	**0.166**
Lactate dehydrogenase (LDH) (U/L)	**Mean ± S.D**	**554.08**	**±**	**70.61**	**137.75**	**±**	**12.84**	**136.687**	**0.001***
Ferritin (ng/ml)	**Mean ± S.D**	**1293.95**	**±**	**188.60**	**76.79**	**±**	**6.41**	**8.656**	**0.001***
Transferrin saturation (T. SAT)(%)	**Mean ± S.D**	**77.28**	**±**	**15.27**	**38.88**	**±**	**4.14**	**15.353**	**0.001***

As illustrated in table (2), there was a significant increase in serum erythroferrone in the case group than the controls.

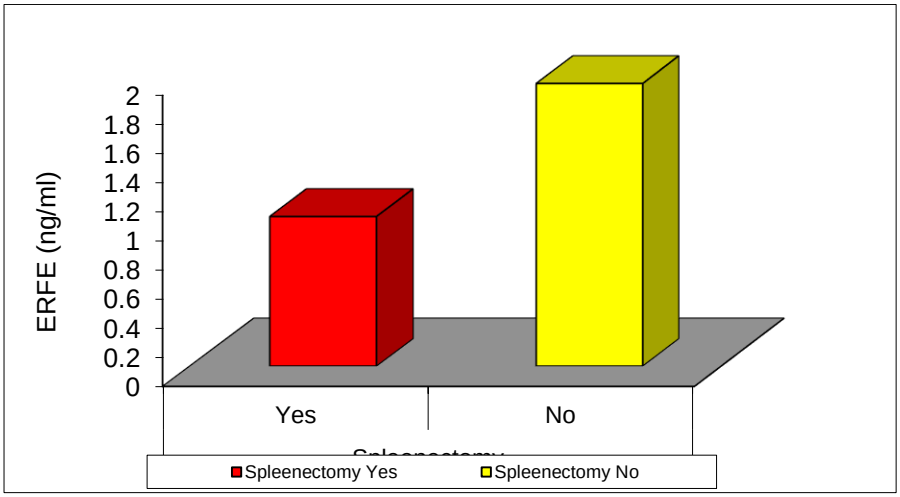
Table (2): Comparison between the two studied groups regarding the serum erythroferrone level.

Variable		Group (I) (Patients group)			Group (II) (Control group)			t-test	P-value
Erythroferrone (ERFE) (ng/ml)	Mean ± S. D	1.62	±	0.1	0.63	±	0.10	6.040	0.001*

Also, there was a substantial higher level of serum erythroferrone level in non spleenectomized cases than spleenectomized ones; (P-value 0.04). Also in patients receiving blood transfusion more than once/month than those receiving once/month; (P-value =0.001), as shown in Figures 1a and 1b.

As regards iron overload markers, patients were further subdivided into two groups according to serum ferritin concentration as follows; *Group Ia*: Serum ferritin <1000 ng\ml (19 patients) and ***G****roup Ib*: Serum ferritin >1000 ng\ml (21 patients). Table 3 showed that serum erythroferrone level and transferrin saturation was significant increase in cases with ferritin level >1000 ng\ml than those with ferritin level lower than1000 ng/ml.

A



B

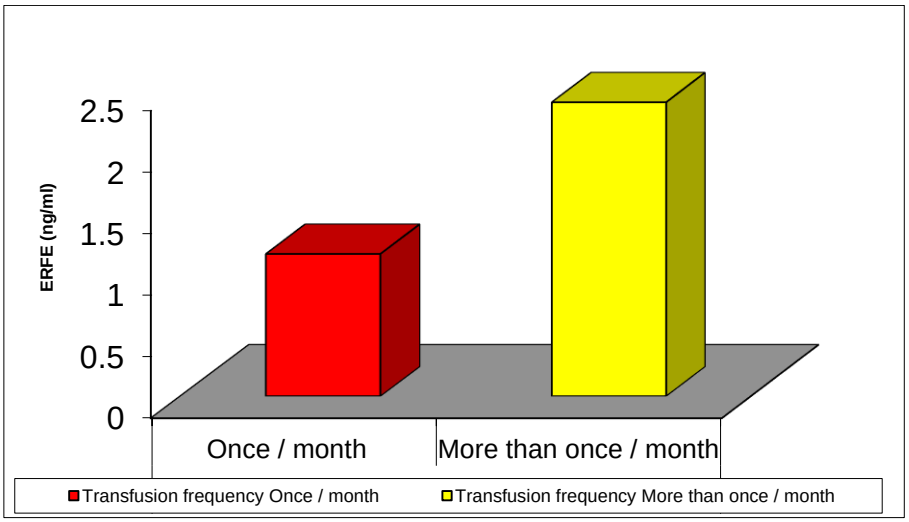


Figure (1): Comparison between ERFE mean value regarding history of splenectomy, and blood transfusion frequency.

Table (3): Comparison between patient subgroups regarding iron overload markers.

Variable		Group (Ia) (Ferritin <1000 ng\ml) (n=19)			Group (Ib) (Ferritin >1000 ng\ml) (n=21)			t-test	P-value
ERFE (ng/ml)	Mean ± S.D	1.07	±	0.14	2.12	±	0.13	3.916	0.001*
T. SAT (%)	Mean ± S.D	72.26	±	4.25	81.81	±	15.04	2.955	0.017*

As shown in table (4), a significantly positive association was found among erythroferrone levels and both ferritin and T.SAT, while no significant correlation was detected between erythroferrone level and Hb, LDH, and reticulocytes count in both of the studied groups

Table (4): Correlation study between serum erythroferrone level and different laboratory parameters in the studied groups.

ERFE (ng\ml)	Group (I) (Patients group)		Group (II) (Control group)	
	r	P-value	r	P-value
Ferritin (ng\ml)	0.394	0.012*	0.494	0.001*
T.SAT (%)	0.493	0.001*	0.731	0.001*
Hb (gm\dl)	0.233	0.108	- 0.136	0.401
Reticulocytes count (%)	- 0.185	0.254	- 0.036	0.826
LDH (U\L)	0.125	0.374	0.183	0.259

/Using ferritin as a dependent variable, both univariate and multivariate analyses, indicated that there was a significantly association among ERFE level and T.SAT (%) and Iron overload (Table 5).

Table (5): Univariate and Multivariate models for linear regression to establish the best independent predictors of iron overload in β-TM patients using ferritin as a dependent variable.

Variable	Univariate		Multivariate	
	OR (95% CI)	P-value	OR (95% CI)	P-value
ERFE(ng/ml)	0.385 (0.107– 0.585)	0.001*	0.574 (0.217 – 0.853)	0.007*
T. SAT(%)	0.456 (0.198 – 0.745)	0.015*	0.745 (0.418 – 0.974)	0.042*

For determination of iron overload, and discrimination between β-TM cases had iron overload from those without iron overload, Roc curve examination was done and presented that serum erythroferrone at level higher than 1.6 ng/ml, had a sensitivity 95%, and a specificity 62%, accuracy 78%, with AUC 0.926, positive predictive value (PPV) 69%, and negative predictive value (NPP) 93% (**Figure 2**).

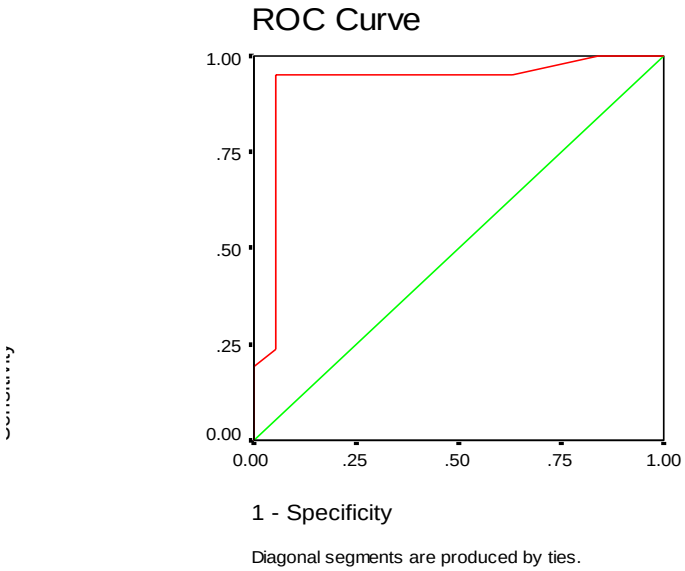


Figure (2): ROC curve analysis for discriminating β-TM cases with iron overload from those with non-iron overload.

DISCUSSION

In the present study we studied serum erythroferrone in Egyptian children had beta-thalassemia major, and its association with iron overload indicators serum ferritin and transferrin saturation, searching for its possible role as a predictive indicator of iron excess to prevent further consequences.

Accordingly, 40 children previously diagnosed with beta thalassemia major were from the Hematology unit; Pediatrics department, Tanta University Hospital and their serum levels of erythroferrone hormone were estimated and compared with that of 40 healthy children matched in sex and age as a control group

Our work showed that β -thalassemia cases had significant decrease in hemoglobin level than controls. This agreed with **El-Gamal *et al.* [3], Saad *et al.* [11], Sulovska *et al.* [12] and Kheansaard *et al.* [13]**. Also, platelets and reticulocytic showed significant increase in cases than controls while insignificant difference as regards WBCs was found. This agreed with results of **El-Gamal *et al.* [3], Saad *et al.* [11] and Kheansaard *et al.* [13]** but reported insignificant difference regarding platelets count between beta thalassemia major cases and controls.

Our results showed that beta thalassemia major cases had significant increase in indirect billirubin and LDH compared to controls; and this was in accordance with **El- El-Gamal *et al.* [3] and Tantawy *et al.* [14]**. Also, liver enzymes were significant increase in beta thalassemic cases than controls; this came in harmony with the results reported by **Karim *et al.* [15]** in their comparative research regarding hematology and biochemical results showed beta thalassemia major cases with normal control.

There was insignificant difference as regard urea and creatinine levels between beta thalassemia patients and control group. This came in conflict with the results of **Karim *et al.* [15]** that showed significant decrease in serum creatinine level in beta thalassemia major cases than control group .

In the present work, ferritin values were determined by spot evaluation of pre-transfusion serum ferritin because we are not able to locate previous ferritin data for the majority of cases. We utilized the 1000 ng/ml ferritin cutoff to classify the iron overload status of -TM cases since, based on the majority of guidelines and in accordance with usual practice, this is the threshold for iron overloaded, this is the threshold at which chelation should be initiated in an effort to prevent iron overload **Shander *et al.* [16], Wang *et al.* [17]** and **Shander and Sazama [18]**. Furthermore, this range of concentration of blood ferritin was recognized in multiple investigations as significant with therapeutic and prognostic significance; serum ferritin below this level could be classified as an accurate marker for the absence of hepatic cirrhosis, regardless of the disease's period **Wang *et al.* [17]**

Serum ferritin concentrations were significantly higher in β-TM cases (mean 1293.95 ng/ml) than controls , this coincides with **Karim *et al.* [15], El-Gamal *et al.* [3]** and **Saad *et al.* [11]** About 52.5% of β-TM cases results showed serum ferritin >1000 ng/ml. Also, Transferrin saturation was significant increase in-case group than controls . This agreed with **Saad *et al.* [11]** who stated that transferrin saturation in beta thalassemia major cases was significant increase than controls.

With respect to the principal study biomarker ERFE, our findings showed significantly higher cocentrations of ERFE in β-TM cases' group, compared to healthy children . Thus, our findings were in accordance with the findings of the earlier research of **Kautz *et al.*** [7], **Kautz *et al.*** [19], **Jiang *et al.*** [20] and **Makis *et al.*** [12] on ERFE, despite the fact that their experiments were conducted on thalassemic mouse models and not on humans. Meanwhile, and in agreement with **Ravasi *et al.*** [21], **Aboul-Enein *et al.*** [22], **El-Gamal *et al.*** [3] and **Saad *et al.*** [11] which were applicated on human subjects and reported significantly elevated the frequency for ERFE gene expression in β-TM cases (indicating increased erythroferrone level), compared to normal subjects.

There was significantly positive association among serum erythroferrone and ferritin levels ,this came in line with **Saad *et al.*** [11]who reported significantly positive association among serum erythroferrone and ferritin levels in β-TM patients , but this was conflicting with the results reported by **El-Gamal *et al.*** [3] as they reported insignificant correlation between erythroferrone gene expression and ferritin level. Also this was in conflict with **Almousawi** [23] as they reported negative correlation between erythroferrone and ferritin levels**.**

Our study also revealed significantly positive association among erythroferrone and transferrin saturation in both cases and control groups, this kept on track with **El-Gamal *et al.*** [3] and **Saad *et al.*** [11] regarding patients' group as they revealed insignificant correlation between erythroferrone and transferrin saturation in control group.

There was insignificant correlation between erythroferrone and Hb level and reticulocytes count in patients and control groups, this came in accordance with **El-Gamal *et al.*** [3]. Also, there was insignificant correlation between erythroferrone and LDH in both patients and control groups.

Our results showed that ERFE level was significant increase in non spleenectomized cases than spleenectomized ones (**P= 0.004**) this came in accordance with results of **Almousawi** [23] who reported significantly increased erythroferrone level in beta thalassemia non-spleenectomized patients than spleenectomized ones**.**

Also, our results denoted that erythroferrone level was significant increase in beta thalassemia cases receiving transfusions more than once\month than that receiving once\ month.

There was a significant rise in serum erythroferrone level and transferrin saturation in cases with serum ferritin >1000 ng/ml than those with insignificant iron overload (ferritin < 1000 ng/ml)**,** this was in accordance with **Kautz *et al.*** [7] and **El-Gamal *et al.*** [3] who reported that β-TM cases with serum ferritin level > 1000 ng\ml have significantly increased level of ERFE gene expression compared to those with ferritin level < 1000 ng/ml.

On performing multiple linear regression analysis using ferritin as dependent variate and erythroferrone and transferrin saturation as independent variates, it was revealed that both erythroferrone & transferrin saturation were independent predictors of Iron overload, this was in accordance with **El-Gamal *et al.*** [3].

Also, by using Roc curve to evaluate the sensitivity and specificity of erythroferrone for detection of Iron overload, our results showed that serum erythroferrone at cutoff higher than (1.6 ng/ml) had AUC 0.926 for cultivated β-TM patients with Iron overload from those with non-Iron overload with sensitivity 95% and specificity 62%.

CONCLUSION

The association between ERFE and iron overload in Egyptian -TM cases verified the postulated regulatory role of ERFE on iron status in -thalassemia, designating it as a potential diagnostic tool for predicting iron overload conditions with 95% sensitivity, 62% specificity, and 78% accuracy. We think that adding ERFE testing into the evaluation of -TM patients in the early phases of iron transfusions reliance would give a promising, sensitive, and predictive technique for early iron accumulation, thereby mitigating this unavoidable and potentially detrimental process.

Limitations of the study

The sample size was small as it is a single-center study.

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