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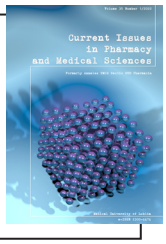

Assessment of the potential role of Trefoil Factor-3 marker as a predictive marker of complication in splenectomized and non splenectomized patients with beta thalassemia major

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ABSTRACT

The study's goal is to appraise the immunological inflammatory marker Trefoil Factor 3, which interacts with thalassemia pathogenesis particularly following splenectomy, and may offer new therapy options for the illness and its repercussions.

This is a case-control study design that included 60 patients identified as β-thalassemia major as participators in this study, in addition to 30 seemingly healthy subjects with age and sex close to the patients group who served as a control group. The participants were distributed into four groups: control group, splenectomized patients, non-splenectomized patients, and total patients. Suitable statistical techniques were employed to investigate the results.

The study's findings demonstrated that there was a significance increase in the serum levels of TFF3 when comparing between (splenectomized, non-splenectomized and total patients) with healthy group (322.16±51.241, p-value=0.01, 317.20±42.449, p-value=0.01, 320±46.6, p-value=0.01), *vs* (309.38±21.94), respectively. Moreover, a comparison between splenectomized and non-splenectomized showed a significantly decrease in TFF3 (322.16±51.241) *vs* (317.20±42.449), (p-value=0.043).The presented study also revealed significant positive correlation between TFF3 level with ferritin, iron, total iron binding capacity, transferrin saturation, transferrin, fasting serum glucose, insulin and homeostasis model assessment-insulin resistance. Furthermore, unsaturated iron binding capacity and homeostasis model assessment-beta found a significant negative correlation with TFF3 level.

High serum levels of TFF3 in beta thalassemia patients, especially in splenectomies patients, are downregulated by inflammatory cytokines, which are primarily regarded as traditional inflammatory cytokines and are related to insulin resistance. Hence, TFF3 level can serve as a potential predictive for the early detection of beta thalassemia in the development and progression of complications.

INTRODUCTION

Thalassemia syndrome, which is affected by reduced expression of any of the hemoglobin double globin chains, results in a genetic ailment that is thought to be the most prevalent genetic condition in the world, α (HB-A) and β (HB-B) [1]. β-thalassemias are forms of thalassemia induced by the reduction or absence of synthesis of the hemoglobin beta chains (HBB). The β-thalassemia condition results in various outcomes, including severe anemia. Untreated β-thalassemia major results in death. β-thalassemia major is currently described via pronounced inefficient erythropoiesis and severe hemolysis. Due to anovulation after hemosiderin deposition, these patients frequently have delayed sexual development and diminished fertility [2].

Splenectomy is traditionally used as an alternative to blood transfusions in individuals with beta-thalassemia [4,5]. Researches have indicated that splenectomized patient Hb concentration and Quality of Life significantly improve

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after treatment, yet, a number of observational studies have found that those patients experienced severe adverse effects. According to reports, having a splenectomy raises the chance of thrombosis by 4-5 times and raises the hazard of infections and sepsis. Likewise, extra vascular problems such leg ulcers, pulmonary hypertension and stroke are more common [6,7]. Additionally, it decreases the body›s capacity to scavenge harmful free iron species. Hence, only those with hypersplenism that causes cytopenia should have this surgery. According to the indications that have been made, splenomegaly should be undertaken in those with left upper quadrant ache as a symptom, premature satiation and elevated need for blood [8,9] Moreover, patients who are at high risk should receive additional treatment as necessary, such as the appropriate vaccinations, antibiotics, and thrombo protection, using heparin or aspirin [8,9].

Insulin resistance is a condition where a particular insulin concentration has a lower impact on target cells than expected. This might result in reduced glucose tolerance before type 2 diabetes mellitus manifests itself [3]. Insulin resistance in common in beta-thalassemia patients [3].

Trefoil factor family proteins (Tff1, Tff2, Tff3) consist of tiny, as well as tight peptides comprising single (Tff1, Tff3), or double (Tff2) trifoil domains. These secretory proteins that are abundantly expressed in the digestive system and assist in the operation of restoration after mucosal injury by maintaining the integrity of the epithelial barrier [10]. TFF3's motogenic and antiapoptotic actions are essential for mucosal preservation and restitution [11]. In addition, TFF3 has been demonstrated to be crucial in encouraging epithelial regrowth following injury, and discontinuity of the mucosal surface causes a topical rise in TFF expression [12].

The presence of TFF 3 is distinct in the intestine's goblet cells, likewise in the hypothalamus, breast, salivary glands, respiratory tract, and other organs, but at a lower level [12]. The central nervous system and the ocular tissues of people generate TFF peptides [13]. The structure of human trefoil factor 3 consist of six cysteine residues and are found in an amino acid sequence of hTFF3. These residues are successively linked in couples through disulfide bonds to create three ring construction [14]. Due to its stability, this structure offers resistance to both acidic and basic environments, besides protease hydrolysis.

Human TFF3 is shielded from harm by its structural stability in the complex environment of the digestive system. Several investigations have revealed that hTFF3 is crucial for maintaining and repairing the intestinal mucosa [15,16]. Both intricate, as well as accurate controls are used to normalize human TFF3, in addition, a wide diversity of compounds are implicated in the regulation of hTFF3 expression. According to numerous studies, proteins of the trefoil factor family demonstrate a behavior known as "self-induction" so as to raise their level of expression [17].

MATERIALS AND METHODS

Our study is of a case-control study design, and includes 60 patients identified as β-thalassemia major. They are dependent on blood transfusion and are without any chronic diseases. In addition, 30 seemingly healthy (without anemia or any immune or chronic diseases) subjects served as a control group, and their ages and sex were matched to the patients group. The participants were distributed into four groups: control group (Group 1: n=30, 11 males, 19 females), splenectomized patients (Group 2: n=20, 10 males, 10 females), Non-splenectomized patients (Group 3: n=40, 22 males, 18 females), and total patients (Group 4: n=60, 32 males, 28 females). The study lasted from January 2022 to March 2022.

Presence of thalassemia disease was recorded in the "Thalassemia Unit" in "Al Zahra Teaching Hospital" in Al-Najaf, Iraq, Children or adolescents who were part of the study were receiving regular monthly or bimonthly blood transfusions (transfusion-dependent). Their ages ranged from 7 to 20. Children who had undergone alteration in chelation therapy medication during the previous six months or who had a history of a chronic condition supplementary thalassemia were excluded from the experiment.

Ethical approval and consent to participate the research was approved by the University of Kufa's institutional ethics board (8298/2022). All controls and patients, as well as their guardians (parents or other close family members) gave written informed consent prior to participation in this study.

Fasting serum glucose samples were collected in a serum separator tube, and samples were allow to clot (15 min) at room temperature before centrifugation for 15 min at 3000Xg. The serum specimens were then reserved at -20°C before they were assayed. The concentrations of serum glucose and total iron were measured by means of standard enzymatic methods (kits). Serum Ferritin, Fasting insulin and Trefoil factor 3, were detected via enzyme-linked immunosorbent assay (ELISA kits).

The homeostasis model assessment (HOMA-IR), which uses the equation of fasting insulin concentration (IU/L) glucose (mmol/L)/22.5, was applied to measure insulin resistance. Insulin resistance was recognized in people with HOMA-IR > 2.7, and HOMA-β levels were calculated by the formula: HOMA-β = 360×Insulin/(Glucose-63)% [18]. The anthropometric measurements of body mass index (BMI) was also calculated via equation: the relation of weight to height squared, by unit kg/m^2 [19].

All data are expressed in the form of means ± standard deviations. Microsoft Excel 2016 and SPSS 26 were employed to analyze statistical information. The statistical significance threshold was set at p-value≤0.05. To gauge how closely the parameters were correlated, Pearson's correlation coefficient was applied. We also employed one-way ANOVA and Fishers Least Significant Difference (LSD) to compare the differences amongst groups. Trefoil factor 3 level effects were assessed by using receiver operating characteristic (ROC)-area under curve for diagnosis.

RESULTS

Table 1 provides an overview of the patient characteristics and TFF3 expression levels. The patients with β-Thalassemia major had significantly increased ferritin, iron,TIBC, TS%, FSG, insulin, HOMA-IR, TFF3 (p-value=0.001, 0.005, 0.001, 0.001, <0.001, <0.001, 0.01, 0.01, respectively), in the non-splenectomy group (Group 3), compared with

Table 1. Comparison between biochemical parameters of Splenectomy and Non-Splenectomy patients with control group

Parameters	Control Group 1 Mean±S.D n=30	Splenectomy Group 2 Mean±S.D n=20	Non-splenectomy Group 3 Mean±S.D n=40	Total Patients Group 4 Mean±S.D n=60	p-value
Age (Years)	16.27±4.118	16.80±4.444	14.23±5.066	15.515±4.755	P1=0.046 P2=0.728 P3=0.57 P4=0.06
BMI (kg/m²)	23.85±4.059	17.87±5.557	16.27±4.062	17.07±4.8095	P1=0.190 P2=0.001 P3≤0.001 P4=0.001
Ferritin (ng/mL)	122.26±45.23	3987±1519.4	2855.1±1416.3	3421±1467	P1=0.001 P2=0.001 P3≤0.001 P4≤0.001
IRON (µmol/L)	22.15±5.16	53.89±17.11	39.09±7.88	46.4±12.49	P1=0.005 P2=0.005 P3=0.001 P4=0.001
TIBC (µmol/L)	70.01±9.35	60.98±9.98	84.05±11.13	72.5±10.5	P1=0.001 P2=0.001 P3=0.003 P4=0.069
UIBC (µmol/L)	46 ±12.87	32.08±16.10	27.75±15.43	29.9 ±15.8	P1=0.05 P2=0.001 P3=0.04 P4=0.001
TS (%)	36.33±12.06	64.74±17.03	61.11±12.45	63.1±14.5	P1=0.05 P2=0.001 P3=0.001 P4=0.001
Transferrin (g/L)	0.18±0.033	0.3±0.01	0.16±0.03	0.21±0.02	P1= 0.01 P2=0.65 P3=0.01 P4=0.04
FSG (mg/dL)	88.35±11.33	107.35±33.87	125.44±22.90	115.5±28.48	P1=0.01 P2≤0.001 P3=0.001 P4=0.001
Insulin (µlu/mL)	6.12±3.13	15.92±1.95	11.63±5.71	13.8±3.88	P1=0.001 P2≤0.001 P3≤0.001 P4≤0.001
HOMA IR	1.79±1.59	3.99±1.55	2.90 ±1.88	3.55±1.75	P1=0.065 P2=0.01 P3=0.003 P4=0.003
HOMA-β	94.11±11.12	61.56±12.74	68.88±13.30	65.54±13.3	P1=0.01 P2≤0.001 P3≤0.001 P4≤0.001
Trefoil factor 3 (pg/mL)	309.38±21.94	322.16±51.241	317.20±42.449	320±46.6	P1=0.043 P2=0.01 P3=0.01 P4=0.01

Data presented as mean ± SD, SD - Standard deviation, n - number of subjects, NS=non-significant at the >0.05 level

P1 - represents the comparison between splenectomy and non-splenectomy group, P2 - represents the comparison between non-splenectomy patient and control group, P3 - represents the comparison between splenectomy group and control group, P4 - represents the comparison between total patients and control group

the control group (Group 1), as well as lower BMI, UIBC and HOMA-β (p-value=0.001, 0.001, <0.001, respectively). We found a non-significance in age and transferrin (p-value=0.728, 0.65, respectively) (Table 1).

The same table demonstrates the presence of significantly elevated TIBC, FSG and HOMA-β (p-value=0.001, 0.01, 0.01, respectively), but showed a significant decrease in age, ferritin, iron, UIBC, TS%, transferrin, insulin and TFF3 (p-value=0.046, 0.001, 0.005, 0.05, 0.05, 0.01,0.001,0.043, respectively). BMI and HOMA-IR (p-value=0.190, 0.065, respectively) was non significant in the splenectomy group (Group 2), compared with the non-splenectomy group (Group 3).

A comparison between the splenectomy group (Group 2) and control group (Group 1) showed a significance decrease in BMI, TIBC, UIBC and HOMA-β (p-value≤0.001, 0.003, 0.04, <0.001, respectively), However, ferritin, iron, TS%, transferrin, FSG, insulin, HOMA-IR and TFF3 (p-value≤0.001, 0.001, 0.001, 0.01, 0.001, <0.001, 0.003, p=0.01, respectively) were found significantly increased, while age (p-value=0.57) was non-significant.

This present study displayed a significant elevation in serum levels of ferritin, iron, TS%, transferrin, FSG, insulin, HOMA-IR and TFF3 (p-value≤0.001, 0.001, 0.001, 0.04, 0.001, <0.001, 0.003, 0.01, respectively), while BMI, UIBC and HOMA-β (p-value=0.001, 0.001, <0.001, respectively) were found significantly decreased. There was non-significance regarding age and TIBC (p-value=0.06, 0.069, respectively) in the total patients (Group 4), as compared with control group (Group 1).

Regarding clinicopathological features for Beta Thalassemia major patients and TFF3 expression in relation to each other, as shown in Table 1, a significant positive correlation is seen between TFF3 level with ferritin, iron, TIBC, TS%, transferrin, FSG, insulin and HOMA-IR, while UIBC and HOMA-β displayed a significant negative correlation with TFF3. Age and BMI had, however, non-significant correlation with TFF3 (p-value=0.829, 0.132, respectively).

Figure 2 and 3 illustrate ROC for TFF3, with area under curve of 0.815 in the splenectomized group and 0.785 in the non splenectomized group. Moreover, the sensitivity and specificity of TFF3 was 0.75 and 0.733 in the splenectomized group, and 0.7 and 0.7 in the non splenectomized group. The cut off value for TFF3 in the splenectomized group was 322.28 pg/mL and that in the non splenectomized group was 321.76 pg/mL.

Table 2. TFF 3 and concentrations of biochemical parameters in the total patients group

Variables	r	p
Age (years)	-0.023	0.829
BMI (kg/m²)	-0.160	0.132
Ferritin (ng/mL)	0.512**	<0.001
IRON (µmol/L)	0.415**	<0.001
TIBC (µmol/L)	0.208*	0.049
UIBC (µmol/L)	-0.336**	0.001
TS %	0.391**	<0.001
Transferrin (g/L)	0.208*	0.049
FSG (mg/dL)	0.506**	<0.001
Insulin (µU/mL)	0.433**	<0.001
HOMA-IR	0.468**	<0.001
HOMA-β	-0.339**	0.001

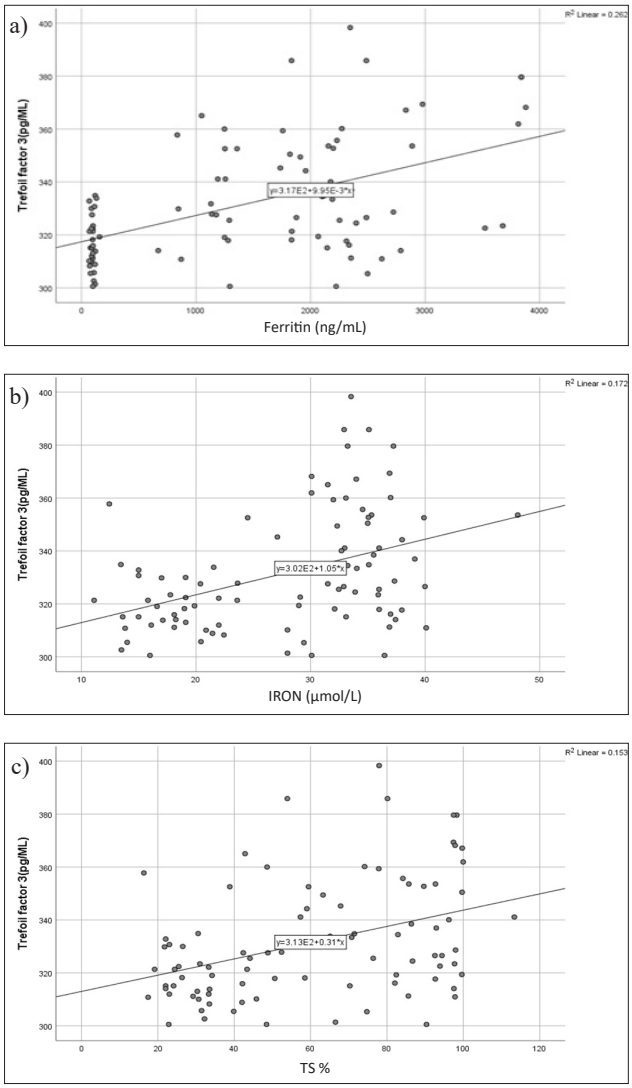

Figure 1. Serum TFF3 level and the biochemical parameters of: a) Ferritin, b) IRON, c) TS%. Here, a higher significant positive correlation is seen

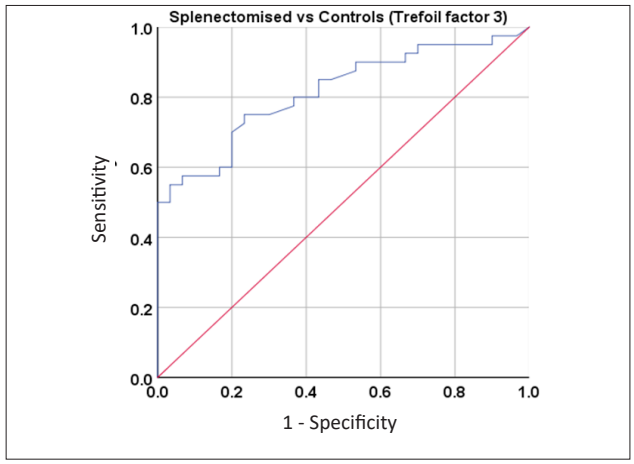

Figure 2. ROC curve of TFF3 exhibiting recognition of splenectomised patients group

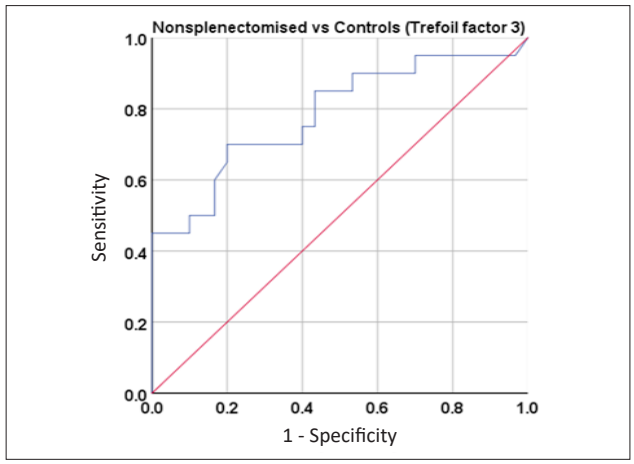

Figure 3. ROC curve of TFF3 exhibiting recognition of non-splenectomised patients group

DISCUSSION

This study focused on four groups: healthy group and three patients group: splenectomized, non-splenectomized and total patients. As inflammatory marker TFF3 increased for all groups of patients and decreased in the healthy group, this indicates that the TFF3 is a pathological cytokine. In the present study, the lower body mass index under normal values for patients groups, the increased ferritin, iron, total iron binding capacity, transferrin saturation percentage and transferrin is due to the destruction of red blood cells and lack of mechanisms to remove iron. This leads to iron overload, which accumulates in the body, and the patient requires treatment with iron chelation therapy to keep the body from being poisoned from iron overload. Furthermore, the three patients groups display insulin resistance, hence these groups are susceptible to diabetes in the near future, as well as possible malfunction of pancreatic gland or receptors.

The thalassemias represent one of the most prevalent inherited monogenic sickness in the world, and, indeed, are defined by inherited autosomal recessive abnormalities in hemoglobin synthesis [20]. Beta-thalassemia syndromes are a set of inherited blood illnesses described via diminished or missing beta globin chain formation, leading to lowered

Hb in red blood cells (RBC) and diminished RBC output, in addition to anemia. The majority of thalassemia are recessively inherited characteristics [21].

Iron is a necessary nutrient for bodily functions, particularly those of the brain [22], and iron is a versatile nutrient with several functions that include transporting oxygen, electrons and numerous other essential physiological activities [23]. The manufacture of hazardous reactive oxygen species (ROS) by free iron, however, causes cellular toxicity [24]. Hence, in the extracellular fluid and blood, iron has to attach to specialized carrier proteins such as transferrin [25,26].

Iron overload can manifest in beta-thalassemia major as an outcome of inefficient erythropoiesis and excessive iron absorption from blood transfusions and consequent problems. Beta-thalassemia major is usually associated with hemochromatosis [27]. The erythroid regulator task modifies the intestinal iron intake reply to erythron requirements, while the role of the storage regulator is to regulate iron collection. These have been suggested as diverse routes to regulate iron metabolism [28,29]. Erythroid regulator enhances iron absorption in these patients because their erythropoiesis is poor, but there is debate regarding how mutations affect iron capacity in beta-thalassemia major patients [30].

Insulin resistance weakens glycogen production and protein catabolism in skeletal muscles, as well as in lipid metabolism, As a result, the liver secretes more very low-density lipoproteins, resulting in raised triglyceride levels [31,32]. Research have shown that endothelial dysfunction, hypertension, dyslipidemia, and MetS may all be linked to insulin resistance [33-35].

According to what we know, ours is the first study of Iraqi beta-Thalassemia major patients for assessment of TFF3 level. The increase level of TFF3 may be related to the pathogenesis of beta-thalassemia major complication as diabetes mellitus and inflammation.

TFF3 is recognised as a crucial protein implicated in regulating lipid and glucose metabolisms [10,36]. TFF3, the regulator pathway for β-cell reproduction, also holds the ability to increase or maintain functioning islet β cells mass [37]. TFF3 has a wide range of functionalities, and is assumed to interact with mucins directly via elevated viscidness and resilience of mucin containing fluids, such as respiratory mucus, gastrointestinal mucus and the tear film [38]. It has been long believed that this might be its main mechanism of operation. Still, recent research has demonstrated that TFF3's motogenic influence acts significantly in epithelial restitution after injury so as to guarantee that the epithelial layer seals off quickly [39]. TFF peptides have been shown in numerous studies to have a variety of advantageous *in vivo* and *in vitro* impacts on a variety of processes, including epithelial restoration, cell motility, wound healing and apoptosis [40].

CONCLUSION

These data show that elevated TFF3 levels might be a mediator that is involved in the pathogenesis and progression of beta thalassemia complications. TFF3 is considered to be the major link between insulin resistance and related inflammatory disorders. However, increased TFF3 levels is seen in patients with and without splenectomies. More work is therefore needed to further characterize the functions of TFF3 in thalassemia as it appears to be an interesting candidate for further research in metabolic relevant conditions.

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CONFLICT OF INTEREST

The authors have no conflicts of interest with any industrial or other association concerning the submitted article.

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