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Development of a molecular-clinical predictive model for iron overload complications in beta-thalassemia major: Integrating transferrin receptor-2, ferroportin, soluble transferrin receptor, and toxic iron species (nontransferrin-bound iron)

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Abstract:

BACKGROUND: Beta-thalassemia major (β-TM) is a hereditary blood disorder requiring lifelong blood transfusions, which result in iron overload and progressive organ damage. Iron chelation therapy is therefore essential to reduce excess iron and its complications.

OBJECTIVES: This study aimed to evaluate the effect of different iron chelation therapies on the expression of iron-regulatory genes and on biochemical markers related to iron metabolism and inflammation in patients with β-TM.

MATERIALS AND METHODS: Thirty patients diagnosed with β-TM and 20 healthy controls were enrolled and stratified according to iron chelation therapy, along with a healthy control group for comparison. Expression levels of Transferrin receptor-2 (TFR2) and ferroportin (FPN) were evaluated by the polymerase chain reaction. Serum concentrations of interleukin-6 (IL-6), soluble TFR (sTFR), nontransferrin-bound iron (NTBI), cortisol, thyroid stimulating hormone (TSH), and ferritin were determined using the standard biochemical methods.

RESULTS: The expression of the TFR2 gene and serum IL6 was statistically significant, whereas FPN gene showed no statistically change. The treatment elevated cortisol levels; however, the alteration was not statistically significant. sTFR, NTBI, TSH, and ferritin all showed that there was too much iron in the body, however, some comparisons did not show any big differences.

CONCLUSIONS: It can be concluded that iron chelation therapy, particularly deferasirox, is related to the extent of TFR2 gene expression and IL-6 levels and changes in terms of iron metabolism and inflammation activity in β-TM patients. TFR2 and IL-6 showed significant alterations and may represent potential indicators of iron overload in β-thalassemia major patients.

Keywords:

Beta-thalassemia major, ferroportin, iron chelation therapy, iron overload, transferrin receptor-2

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Introduction

Beta-thalassemia major (β-TM) is a monogenic disorder. It is characterized

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by the mutations in the β globin gene (HBB), leading to ineffective erythropoiesis and hemolysis; hence, severe anemia develops.[1] Patients are transfusion dependent and therefore at risk of iron overload and secondary organ damage. The body controls iron absorption in the intestine and alters hepatic storage of iron.[2] However, chronic anemia and transfusion therapy causes excessive iron accumulation in the parenchymal organs. Leads to myocardial, hepatic and endocrine damage which contributes to morbidity and mortality in β-TM.[3] For the prevention of iron overload and its complications, patients receiving deferasirox, deferiprone, and deferoxamine therapy need to be treated with iron chelators to help remove excess iron from the body. Deferasirox was proved more effective than deferiprone to decrease liver iron concentration in patients with β-TM.[4] A number of genes related to iron homeostasis have been discovered, among them transferrin receptor-2 (TFR2) and ferroportin (FPN) (SLC40A1).[5]

TFR2 the TFR2 is critical for iron uptake through the transferrin and hepcidin expression. Disruption to TFR2 could result in low hepcidin levels and consequently enhanced intestinal iron absorption further activating iron loading in β-TM.[6] FPN is the only identified cellular iron exporter, which plays an important role in systemic iron homeostasis, and an aberrant FPN can cause pathological iron accumulation in β-TM.[6] Ferritin is the most frequently used, simplest metric of iron over load.[7] However, in the iron overload state observed in diseases such as nontransfusion-dependent thalassaemia, high levels of transferrin saturation may lead to the generation of toxic nontransferrin-bound iron (NTBI) that causes oxidative stress and tissue injury.[8] Soluble TFR (sTfR) represents erythropoietic process and cellular iron requirement, supplying additional information into iron metabolism beyond ferritin individually.[9] Inflammation furthermore has essential function in iron regulation. Interleukin-6 (IL-6) regulates iron metabolism through the stimulation of hepcidin and correlates with chronic inflammation, oxidative stress, and hormonal disorders in β-TM patients.[10] notwithstanding systemic iron overload, β-TM patients persist in absorbing iron, and multiple blood transfusions moreover contribute to oxidative injury and endocrine dysfunction.[11] thus, biomarkers such as TFR2, FPN, and IL-6 may possess potential clinical significance for assessing iron overload, inflammatory status, and response to chelation therapy in patients with β-TM.[12,13] Although iron excess markers and inflammatory parameters have been investigated separately, limited data exist regarding the joint evaluation of IL-6 levels and the expression of iron-homeostasis genes, such as TFR2 and FPN, in patients undergoing various iron chelation therapies.[14] Therefore, this investigation evaluates the impacts of different iron chelation treatments on iron-regulatory gene expression and biochemical markers of iron homeostasis and inflammation in patients with β-TM.

Materials and Methods

The study was conducted at the Erbil Center for Thalassemia in Erbil, Iraq and included 50 participants: 30 β-TM patients and 20 healthy controls. The study sought to examine the gene expression of TFR2 and FPN (SLC40A1) among thalassemia patients and a healthy cohort. Furthermore, it assessed other biochemical indicators, including ferritin, IL-6, sTFR, NTBI, thyroid stimulating hormone (TSH), and cortisol. The objective was to elucidate potential correlations among gene expression, inflammatory responses, and oxidative damage in the context of thalassemia. Subjects were divided into three groups depending on the organ-targeting approach of iron chelation therapy, with one control group.

- Group 1: Control group: 20 normal healthy subjects; 10 (50%) males and 10 (50%) females, their ages ranged from 18 to 40 years old
- Group 2: 5 β-TM patients receiving deferiprone in combination with deferoxamine and deferasirox therapy; 2 (40%) males and 3 (60%) females, their ages ranged from 18 to 26 years old. This group focused on deferiprone and heart organ targeting
- Group 3: 24 β-TM patients receiving deferasirox in combination with deferoxamine and deferiprone therapy; 15 (63%) males and 9 (37%) females, their ages ranged from 18 to 42 years old. This group focused on deferasirox and liver and spleen organ targeting
- Group 4: 12 β-TM patients receiving combination deferasirox and deferoxamine with deferiprone therapy; 7 (58%) males and 5 (42%) females, their ages ranged from 18 to 32 years old. This group focused on combined deferasirox and deferoxamine and liver and heart organ targeting.

Inclusion criteria

Patients ranging in age 18–42 years with a verified diagnosis of β-thalassemia major using hemoglobin electrophoresis and undergoing routine follow-up and treatment were enrolled.

Exclusion criteria

Patients were excluded if they had other hemoglobinopathies, known genetic disorders, chronic inflammatory diseases unrelated to β-thalassemia, pregnancy or lactation, acute infection, or had undergone surgery within 3 months prior to sampling.

Human ethical consideration

The study was approved by the Human Ethics Committee of the Erbil Health and Medical Technical College, Erbil Polytechnic University (26/0094; date:).

Blood sample collection

Blood samples were obtained from thalassemic patients and healthy controls utilizing two different types of tubes. A 5 mL gel tube was employed for serum collection. The blood was centrifuged at 3000 rpm for 15 min to isolate the serum, which was subsequently preserved at -80°C for future study. A second 5 mL EDTA tube was utilized exclusively for gene expression analysis, preserving the integrity of the genetic material for forthcoming molecular research.

Biochemical assays

Serum soluble sTfR, human IL6, and NTBI were quantitatively determined by enzyme-linked immunosorbent assay (ELISA) using the sandwich method specific for sTfR, IL6 and NTBI using the Sunlong Biotech Co. kit for Bio Vendor Research, product IDs SL1620Hu, SL100Hu and SL3533Hu, China (lot numbers 20250429, 20250606 and 20250429), respectively. Reference ranges were 26-1500 pg/ml for sTFR, 2–80 ng/L for IL6 and 0.2-8 µg/mL for NTBI; when using this assay, values increased in thalassaemia. Readings were done at a wavelength of 450 nm using an ELISA reader within 5–10 min.

Quantitative determination of serum ferritin, TSH, and cortisol was performed using the Cobas E411 full automated analyzer. The tests were conducted using Roche Elecsys Reagent kits, which are designed for use with the Cobas E411 analyzer, manufactured in Germany, for research and diagnostic purposes. The principle of this analyzer is electrochemiluminescence.

Gene expression

Total ribonucleic acid (RNA) was extracted from whole blood using the QIAamp RNA Blood Mini Kit (QIAGEN, Germany) to ensure high-quality RNA for downstream applications. RNA was measured with an OneDrop TOUCH NanoDrop spectrophotometer (Biometrics) for quantity and purity of RNA. To check the integrity of RNA was used Agarose gel electrophoresis. After successful RNA extraction, cDNA synthesis was performed using the UltraScript® Reverse Transcriptase and cDNA Synthesis Kit (polymerase chain reaction [PCR] BIOSYSTEMS) to generate high-quality complementary DNA for gene expression analysis.

Subsequently, the PCR technique is employed to amplify of the cDNA and then fluorescent dyes are used for detection. We performed quantitative PCR (qPCR) with the qPCRBIO SyGreen Blue Mix from PCR BIOSYSTEMS, and we used a BIO-RAD CFX96 Real-time Detection System to analyze the expression levels of selected target genes. The qPCR thermal cycling conditions included an initial denaturation at 95°C for 5 min, then 40 cycles of denaturation at 95°C for 15 s and annealing/extension at 60°C for 60 s Each qPCR assay was carried out in a final reaction volume of 20 µL, comprising qPCRBIO SyGreen Blue Mix, gene-specific primers, and cDNA template. Table 1 presents the specific primer sequences designed to amplify each gene. Beta-Actin served as the housekeeping gene for normalisation to ensure accuracy of gene expression data and to account for sample-to-sample variation. Comparative levels of gene expression were calculated using the $2^{-}\Delta\Delta Ct$ method. Primer efficiency was determined by serial dilutions of cDNA, and efficiencies ranging from 90% to 110% were considered acceptable.

Statistical analysis

Statistical analysis was performed using GraphPad Prism version 10.5.0 (GraphPad Software, San Diego, CA, USA). Data normality was assessed using the Shapiro–Wilk test. Comparisons between two groups were conducted using an unpaired two-tailed Student's *t*-test, while comparisons among more than two groups were performed using the one-way analysis of variance, followed by Tukey's *post hoc* test for multiple comparisons. $P < 0.05$ was considered statistically significant.

Results

This study investigated TFR2 and FPN gene expression with iron-related biomarkers in 30 people with beta thalassemia major compared to 20 healthy controls. The patient group showed significantly elevated IL-6 (113.3 ± 99.34 vs. 46.98 ± 13.63, $P < 0.01$), TFR2 (11.16 ± 2.42 vs. 7.83 ± 2.86, $P < 0.001$), sTFR (4134 ± 4462 vs. 1392 ± 256.2, $P < 0.05$), and NTBI (22.20 ± 27.90 vs. 5.58 ± 1.14, $P < 0.05$) compared to the control group, as illustrated in Figure 1, and thyroid stimulation hormone (TSH) (3.448 ± 1.919, 1.931 ± 0.9072, $P < 0.001$) and Ferritin (4488 ± 3305, 109.9 ± 63.70, $P < 0.0001$), indicating enhanced iron overload and inflammatory activity in affected individuals. In contrast, FPN and cortisol levels did not show any statistically significant differences between the groups, with

Table 1: Primer sequences used for qPCR

Gene	Forward primer	Reverse primer
TFR2	GCACCTCAAAGCCGTAGTGTAC	CCACCTGTTCATAGAGAGTCTGC
SLC40A1	GAGACAAGTCCTGAATCTGTGCC	TTCTTGCAGCAACTGTGTCACAG
ACTB	TGACAGGATGCAGAAGGAGA	GCTGGAAGGTGGACAGTGAG

TFR2=Transferrin receptor 2, SLC40A1=Ferroportin gene, ACTB=Beta-Actin

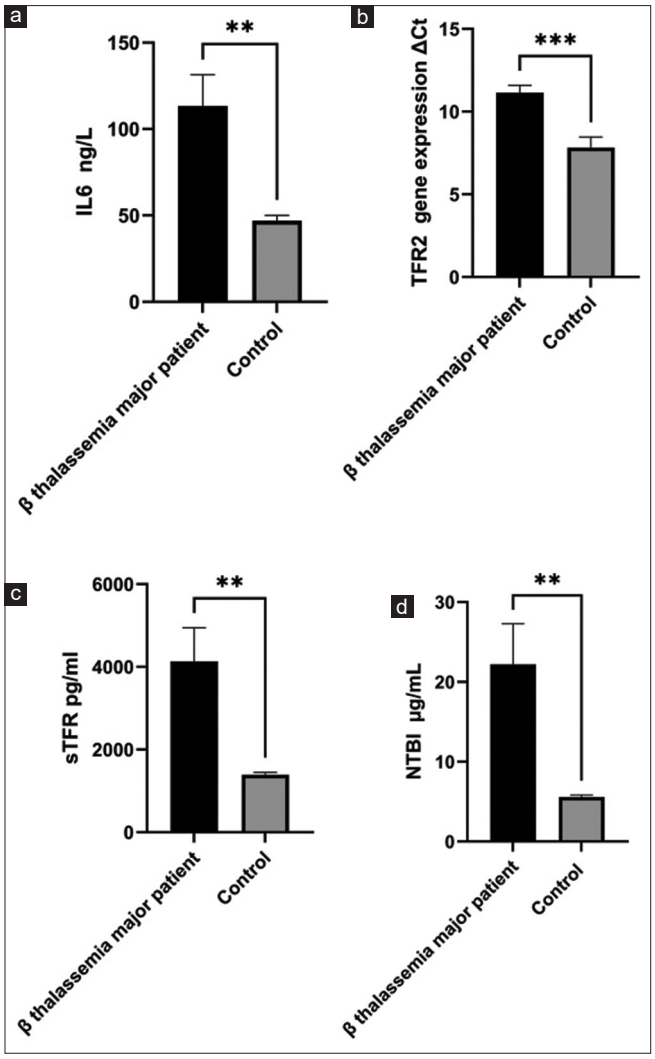

Figure 1: Juxtaposed analysis of biomarkers in β-thalassemia major patients and healthy controls. (a) Interleukin-6 levels, (b) Transferrin receptor-2 gene expression, (c) Soluble transferrin receptor levels, (d) Nontransferrin bound iron levels. ($P < 0.001$) (*) significant, (ns) nonsignificant, ($P < 0.0001$) (**) highly significant

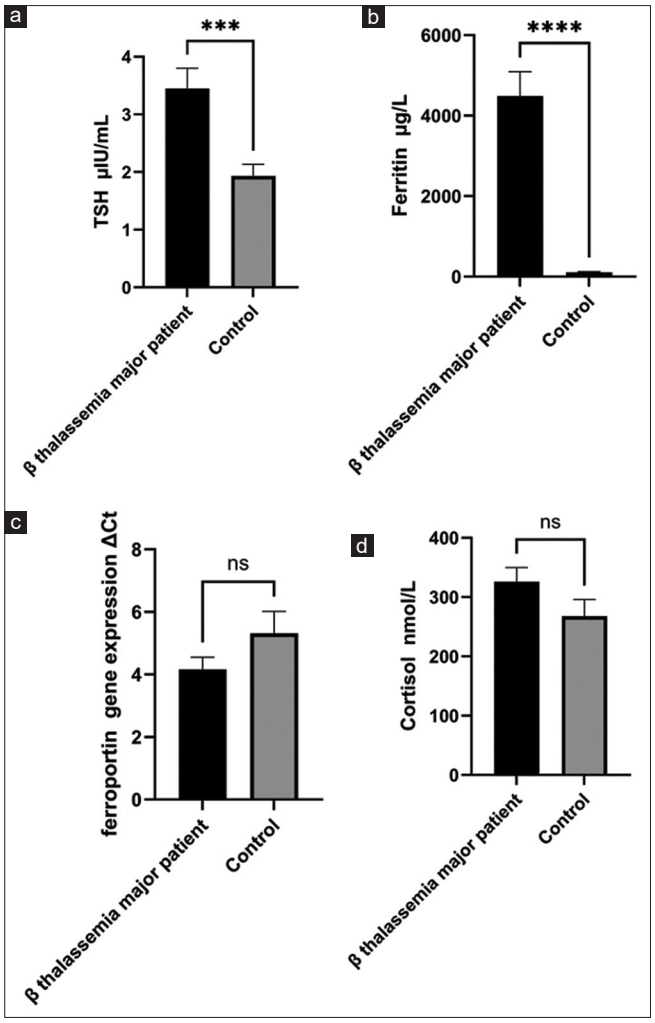

Figure 2: Comparison of biochemical and molecular markers between β-thalassemia major patients and healthy controls. (a) Thyroid stimulating hormone (TSH) levels, (b) Ferritin levels, (c) Ferroportin gene expression ΔCt. (d) Cortisol levels. ($P < 0.001$) (*) significant, (ns) nonsignificant, ($P < 0.0001$) (**) highly significant

$P = 0.1626$ (ns) and 0.1168 (ns), respectively, as shown in Figure 2.

On the other hand, the study investigated gene expression, including the TFR2 gene and the FPN gene. With six serum levels of the molecular and biochemical biomarker, including IL-6, soluble sTFR, NTBI, Cortisol, TSH, and Ferritin, in 30 patients with β-TM undergoing different iron chelation regimens to see how different therapies affected it. The therapeutic groups are the deferiprone combination (heart-targeted), deferasirox alone or in combination (liver and spleen-targeted), the deferasirox–deferoxamine combination (heart and liver-targeted), and the untreated control group.

The TFR2 gene expression showed significant variation between the groups; the mean TFR2 were (11.10 ± 2.66, 11.14 ± 2.42, and 11.10 ± 2.55 respectively) and 7.83 ± 2.86 in the healthy controls group. The deferasirox and deferasirox–deferoxamine groups differed significantly from the control ($P = 0.0006$ and $P = 0.0062$), while no differences were observed among the treatment groups ($P > 0.99$). As shown in Figure 3a, the FPN gene expression did not differ significantly between the four groups. The mean FPN was (4.69 ± 2.62, 3.98 ± 2.24, 4.10 ± 1.97, respectively) and 5.32 ± 3.15 in the control group. *Post hoc* analysis confirmed no meaningful variation between any pairs ($P > 0.32$), as shown in Figure 3b.

Serum IL-6 concentrations differed significantly among the treatment groups. The mean IL-6 concentrations were (103.4 ± 88.5, 118.1 ± 104.1, 100.5 ± 94.9, in the same order) and 46.98 ± 13.63 ng/L in the healthy control group. Deferasirox showed a significant increase versus the control ($P = 0.0289$*), while other group comparisons

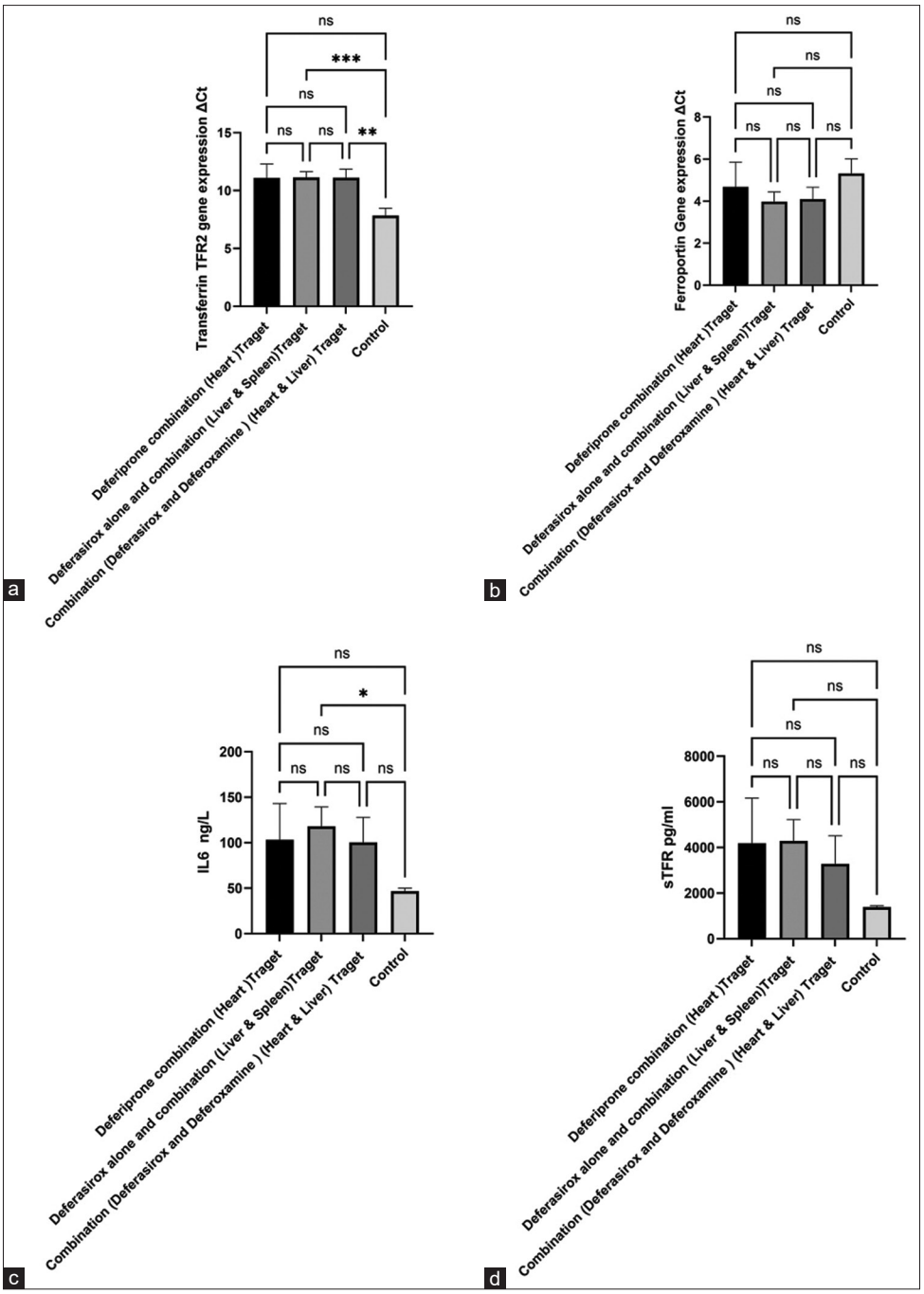

Figure 3: Comparison of molecular and biochemical parameters among β-thalassemia major patients receiving different chelation therapies and controls. (a) Transferrin receptor 2 gene expression, (b) Ferroportin gene expression, (c) interleukin 6 levels, (d) Soluble transferrin receptor levels. ($P < 0.001$) (*) significant, (ns) nonsignificant, ($P < 0.0001$) (**) highly significant

were not significant, indicating variable inflammatory responses among patients, as shown in Figure 3c.

The sTFR revealed no statistically significant differences among the chelation groups, but sTFR levels were higher in all chelation-treated groups compared to controls ($P = 0.072$, $P > 0.05$ for all comparisons), indicating a nonsignificant trend toward increased erythropoietic activity. The mean serum sTFR concentrations were (4192 ± 4408, 4289 ± 4590, 3292±, in the same order) and 1392 ± 256 pg/ml in the untreated control group, as shown in Figure 3d.

An NTBI concentration revealed no statistically significant differences among the study group. Mean values were higher in all patients who received therapy, particularly in the Deferasirox group (23.35 ± 28.74), compared to controls (5.58 ± 1.14). While the differences between treatment groups are not statistically significant ($P > 0.05$ for all), deferasirox-treated patients tended toward

increased NTBI (mean difference = 17.77 µg/mL, $P = 0.0601$), as shown in Figure 4a.

The cortisol levels did not reveal statistically significant differences among the studied groups. The mean cortisol concentrations were (382.4 ± 111.1, 314.6 ± 129.8, 315.5 ± 139.6, correspondingly) and 267.9 ± 124.4 nmol/l in the baseline group. There were slightly higher cortisol values in chelation-treated patients, especially in the deferiprone group compared to controls; however,

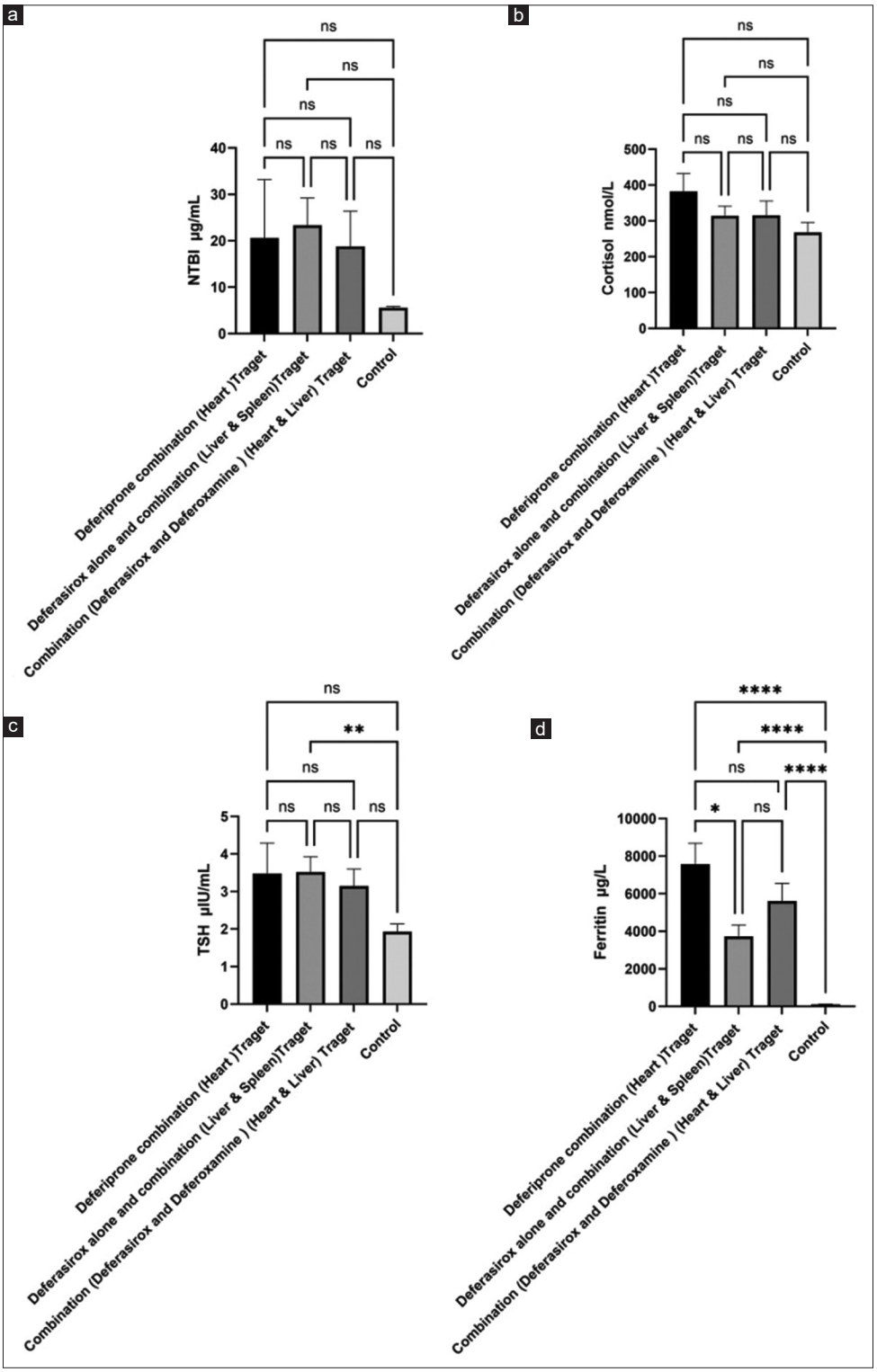

Figure 4: Illustrates group comparisons for selected clinical and molecular parameters in beta thalassemia major patients under different iron chelation therapies, (a) Shows NonTransferrin-Bound Iron levels, (b) Presents cortisol concentrations, (c) thyroid stimulation hormone (TSH) levels, (d) highlights ferritin levels. ($P < 0.001$) (*) significant, (ns) nonsignificant, ($P < 0.0001$) (**) highly significant

none of these differences reached statistical significance ($P > 0.29$), as shown in Figure 4b.

Serum TSH level varied significantly among the studied groups. The mean TSH amounts were (3.48 ± 1.82, 3.52 ± 1.99, 3.15 ± 1.55 correspondingly), and 1.93 ± 0.91 μIU/mL in the untreated control group. Deferasirox revealed a significant increase versus the control ($P = 0.0096$*), while other group comparisons were not significant ($P > 0.17$). As shown in Figure 4c.

The analysis of ferritin levels revealed a highly significant statistical difference between the studied groups. The mean serum ferritin concentrations were (7575 ± 2486, 3731 ± 2932, 5612 ± 3261, respectively) and 109.9 ± 63.7 ng/mL in the untreated control group. A *post hoc* multiple comparison test showed significantly higher ferritin concentrations in all chelation groups compared with the control. Deferasirox therapy resulted in significantly lower ferritin levels than deferiprone ($P = 0.0116$*), as shown in Figure 4d.

The receiver operating characteristic (ROC) curve analysis demonstrated an area under the curve (AUC) of 1.0 ($P < 0.0001$) for ferritin. TFR2 (AUC = 0.81, $P = 0.002$), TSH (AUC = 0.7767, $P = 0.001$), and IL-6 (AUC = 0.7333, $P = 0.0056$) showed statistically significant AUC values in contract, FPN, sTFR, NTBI, and cortisol showed lower AUC values with nonsignificant P values, as shown in Figure 5 a-h.

Discussion

In this study, 30 β-TM patients, compared to 20 healthy controls, showed significantly higher levels of including IL-6, TFR2 gene expression, sTFR, NTBI, Cortisol, thyroid-stimulation hormone (TSH), and ferritin, indicating inflammation, iron overload, and increased erythropoietic activity. The result is likeness with earlier studies revealing increased IL-6 and iron-related markers due to ineffective erythropoiesis and repeated blood transfusions.[15] High expression of TFR2 supports its role as an iron sensor in thalassemia.[16] Elevated ferritin and NTBI, which reflect iron overload, agree with other research.[17] In contrast, the FPN and cortisol showed no significant change, suggesting posttranscriptional regulation in humans, but unlike the findings in animal models.[18]

Moreover, the patients under different iron chelation regimens observed alterations in gene expression and serum biomarkers. There was significant variation in TFR2 across groups. Previous studies have highlighted the complex pathophysiology of iron overload in β-TM, including iron-regulatory genes.[19] The consistent TFR2 expression among chelation therapies notes a common

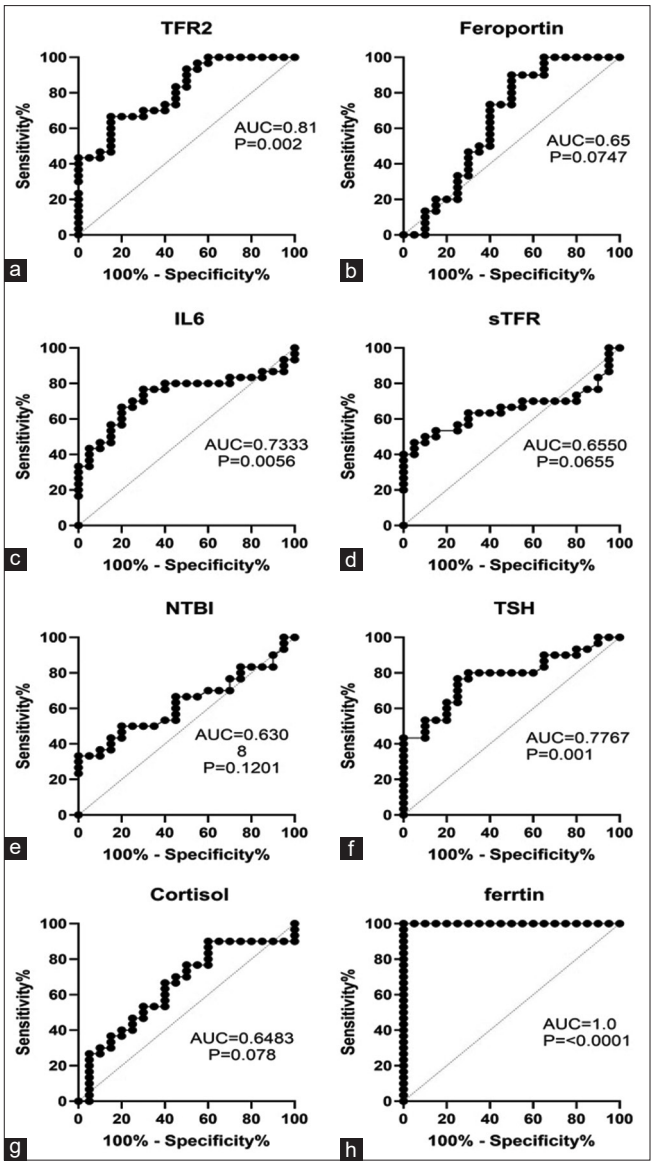

Figure 5: (a-h) (Receiver operating characteristic) curves demonstrate the diagnostic performance of the investigated biomarkers in β-thalassemia major. Ferritin showed excellent discrimination, whereas Transferrin receptor-2 (TFR2), interleukin-6, and TSH displayed strong and statistically significant diagnostic performance. Ferroportin, soluble TFR, nontransferrin-bound iron and cortisol showed low, nonsignificant area under the curve values, suggesting restricted clinical usefulness

mechanism for systemic iron regulation and its potential use as a molecular marker for severe iron overload.

The FPN gene expression showed no significant variations. Previous studies have highlighted the complexity of iron regulation changes in β-TM and the variable effects of chelation on FPN.[19,20] This finding implies the present results suggest that FPN reflects systemic iron homeostasis rather than chelation efficacy in β-TM.

A statistically significant difference in IL-6 levels was found among the groups. Previous studies reported

variable IL-6 results.[21] Raised IL-6 and hepcidin in this study suggest a mild pro-inflammatory response associated with chelation therapy.

The sTFR revealed no statistically significant differences among the chelation groups, but sTFR levels were higher in treated patients compared to controls. This is in contrast to the study conducted in Egypt, which found significantly higher sTfR levels in all β-TM patients.[9] This finding suggests that sTfR reflects compensatory marrow activity and iron demand, showing consistent levels across chelation regimens indicative of a shared homeostatic response.

The NTBI concentration showed no statistically significant differences among groups. However, patients on deferasirox-based regimens tended to have higher NTBI levels compared to the untreated group. As opposed to previous studies, it noted increased NTBI with certain chelation therapies.[22] The present results suggest that while chelation reduces systemic iron, residual NTBI persists, reflecting ongoing iron turnover and mild oxidative stress in β-TM.

Cortisol level also did not differ significantly among the groups, but the slightly higher mean levels in the deferiprone group suggest subclinical adrenal stress. Prior studies reported β-TM patients who adhered to chelation therapy had a significantly lower prevalence of short stature.[23]

The TSH measurements were significantly increased in treated patients, particularly those on deferasirox, consistent with an earlier study from Bangladesh showing a strong postchelation correlation,[24] indicating subclinical hypothyroidism as a common endocrine complication in β-TM.

Serum ferritin remained significantly elevated across all chelation groups, with deferasirox showing lower levels than deferiprone combination therapy. This aligns with a Bangladesh study that showed a strong postchelation correlation between ferritin levels and treatment response.[24] Highlighting serum ferritin as a commonly used indicator of body iron burden in β-TM patients.

The ROC analysis showed outstanding discrimination capacity for serum ferritin (AUC = 1.0), validating its reliability as a marker of iron excess in patients with β-thalassemia major.[25,26] TFR2, IL-6, and TSH further revealed statistically significant AUC values, corroborating recent findings associating iron homeostasis pathways, inflammation, and hormonal dysfunction with iron excess in β-TM.[27] By comparison, FPN, sTFR, NTBI, and cortisol exhibited reduced diagnostic performance, consistent with recent findings emphasizing their multifaceted regulation and heterogeneity in iron accumulation conditions.[12,28]

Several limitations of this study should be noted, including a modest sample size due to strict eligibility criteria and the few of β-thalassemia major patients recruited from one center, which may affect generalizability. The cross-sectional study design prevents establishing causality, and imbalanced chelation group sizes may have influenced statistical comparisons. Future studies with larger sample sizes are noted.

Conclusions

The following study demonstrates the significant effect of chelation therapy on iron metabolism indicators in patients with β-TM. The up-regulation of the TFR2 gene in the deferasirox and deferasirox-deferoxamine treatments attests to the increased mobilization and improved homeostasis. However, FPN and cortisol levels remain, while IL-6 and Thyroid Stimulation Hormone (TSH) rise, indicating inflammation phenomena due to iron being chronically toxic. Ferritin and NTBI remain over the ideal posttreatment, thus necessitating the development of improved therapies. Based on the research, TFR2, IL-6, and ferritin should be considered as early predictors of exacerbation of iron excess, and clinical practices should be re-evaluated employing larger patient groups with a longer period of observation. Further research into genetic aspects of patients is also recommended. This investigation enhances the understanding of immune responses related to diagnostics and treatment, clarifying the roles of molecular markers in iron overload and aiding the development of improved therapies.

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Nil.

Conflicts of interest
There are no conflicts of interest.

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