ORIGINAL ARTICLE

Exploring Transferrin Gene Expression as A Biomarker of Ineffective Erythropoiesis and Iron Overload in HbE/β-Thalassaemia and β-Thalassaemia Trait

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ABSTRACT

INTRODUCTION: Iron overload is a major concern in transfusion-dependent thalassaemia patients, with soluble transferrin receptors (sTfR) playing a key role in iron regulation. This study aimed to evaluate the gene expression of *TfR1* and *TfR2* and their association with ineffective erythropoiesis (IE) and iron overload in HbE/β-thalassaemia patients. **MATERIALS AND METHODS:** A total of 2 ml whole peripheral blood was extracted for RNA from 6 subjects recruited from each HbE/β-thalassaemia patient, β-thalassaemia trait carriers, and healthy controls. TfR levels were measured using ELISA, while *TfR1* and *TfR2* gene expression were assessed using RT-qPCR. Data were analysed using ANOVA, Student's t-test, Kruskal–Wallis, and Mann–Whitney U tests with Bonferroni correction. **RESULTS:** Gene expression analysis revealed a significant downregulation of *TfR2* in HbE/β-thalassaemia patients and β-thalassaemia carriers *(P<0.001)* compared to healthy controls, while *TfR1* expression was significantly upregulated *(P<0.001)*. Additionally, sTfR levels were statistically higher in HbE/β-thalassaemia patients and parents compared to healthy controls (P<0.001). **CONCLUSION:** These findings suggest that *TfR1* and *TfR2* expression patterns may serve as potential biomarkers for assessing IE and iron overload in β-thalassaemia. Furthermore, elevated *sTfR* levels indicated that the transfusion regimen was insufficient to suppress ineffective erythropoiesis. In β-thalassaemia intermedia patients, haemoglobin levels may not be the most reliable marker for monitoring transfusion therapy, whereas *sTfR* could help in tailoring individualised transfusion regimens.

Keywords
Soluble Transferrin Receptor (sTfR), Iron-overload, Ineffective erythropoiesis, β-thalassaemia trait, HbE/β-thalassaemia patients.

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INTRODUCTION

The World Health Organisation (WHO) recognises thalassaemia as a major public health concern, particularly in tropical countries such as Malaysia, highlighting the need for systematic data collection and analysis to monitor disease prevalence, treatment outcomes, and related complications, including iron overload. Despite being the most common hereditary haematological disorder in Malaysia, comprehensive nationwide data on the geographical distribution of patients, as well as socioeconomic, clinical, and treatment outcome information, remain limited. The total cumulative number of registered thalassaemia patients increased by 9.0%, from 8,767 in 2020 to 9,554 in 2023, largely due to improved overall survival and enhanced data collection and cleaning efforts. HbE/β-thalassaemia is most prevalent among Malays, accounting for 3,101 patients (49.0%), whereas β-thalassaemia major occurs more frequently among Chinese, Indian, and Kadazan Dusun populations, comprising 353 (36.1%), 16 (25.4%), and 835 (83.9%) patients, respectively, based on the Annual Report of The Malaysia Thalassaemia Registry 2023.[1]

β-thalassaemia is an inherited autosomal recessive disorder characterised by reduced production of β-globin chains in haemoglobin A (HbA), which normally consists of two α- and two β-globin chains. HbE/β-thalassaemia results from the inheritance of a β-globin gene mutation alongside the HbE variant, leading to a spectrum of anaemia severity, ranging from mild to transfusion-dependent cases.[2] The imbalance in globin chain synthesis causes unpaired α-globin chains, resulting in ineffective erythropoiesis (IE) due to the premature destruction of erythroblasts at the polychromatic normoblast stage and the haemolysis of mature red blood cells.[3] This process contributes to erythroid expansion, extramedullary erythropoiesis, and bone deformities.[4] While disease severity can be influenced by additional genetic factors, such as α-hemoglobinopathies, HbE/β-thalassaemia remains highly variable in its clinical presentation.[5]

Chronic iron overload represents one of the most serious and challenging complications arising from long-term blood transfusions. Patients with Transfusion-Dependent Thalassaemia (TDT) inevitably develop clinical manifestations of iron overload over time unless timely and effective iron chelation therapy is administered. Transferrin receptors (TfRs) are critical for iron uptake and are predominantly expressed in erythroid precursor cells, with around 80% located in the erythroid marrow of adults. During erythroid maturation, truncated forms of TfR are released into circulation as soluble transferrin receptors (sTfR). Iron deficiency triggers an increase in TfR synthesis [6], leading to elevated sTfR levels, making them reliable indicators of iron deficiency anaemia (IDA).[7] Moreover, sTfR levels correlate with erythroid activity, serving as a useful marker of increased erythropoiesis.[8] Elevated sTfR levels have been observed in various haematologic disorders, including autoimmune haemolytic anaemia, hereditary spherocytosis, and β-thalassaemia major, all of which involve heightened erythropoietic demand. Therefore, the sTfR level may serve as a valuable adjunct marker for improving monitoring in non–transfusion-dependent thalassaemia (NTDT), where serum ferritin alone does not adequately reflect erythroid activity or the balance of iron metabolism in the management of β-thalassaemia.

Based on our current understanding of TfR regulation at the cellular level and the minimal biological and analytical variability observed in sTfR assays [9], this study aimed to assess the expression level of TfR and its association with iron overload and IE in patients with HbE/β-thalassaemia and β-thalassaemia trait. Additionally, the study explored the diagnostic and potential therapeutic significance of TfR in managing these conditions.

MATERIALS AND METHODS

Sample recruitment and ethical considerations

This study was conducted in accordance with the ethical standards outlined in the Declaration of Helsinki. All participants were provided with a Participant Information Sheet (PIS) and an Informed Consent Form (ICF) prior to their inclusion in the study upon approval from Universiti Sultan Zainal Abidin (UniSZA) Human Research Ethics Committee [(approval no. UniSZA.C/2/UHREC/628-2 J1d.2) (73)] and the Medical Research and Ethics Committee [(approval no. NMRR-19-855-45851 (IIR)].

A total of 6 subjects were selected from each group, HbE/β-thalassaemia patients and β-thalassaemia trait carriers, based on the severity of the disease. The patient samples were collected from the Haematology Department at Hospital Sultanah Nur Zahirah (HSNZ), while 6 healthy control samples were obtained from postgraduate students at Universiti Sultan Zainal Abidin (UniSZA), Gong Badak Campus, Terengganu, Malaysia.

Participants were included in the study if they met the following criteria: (i) transfusion-dependent HbE/β-thalassaemia patients and their biological parents (HbE trait and β-thalassaemia trait); and (ii) healthy individuals with normal haematological profiles, iron levels, and haemoglobin electrophoresis results. Exclusion criteria included: (i) presence of iron deficiency anaemia (IDA) or alpha-thalassaemia trait, confirmed by DNA analysis of the alpha-globin gene; and (ii) healthy participants with low haemoglobin levels, abnormal red cell indices, or the

presence of haemoglobin variants. The demographic data were obtained from patient's folder from the respective hospital.

Laboratory investigations

Biochemical assays

Serum soluble transferrin receptor (sTfR) level was quantitatively measured using ELISA employing monoclonal antibodies specific to sTfR. The assay was performed using a commercial kit (BioVendor Research and Diagnostic Products, Heidelberg, Germany), following the manufacturer's protocol. The reference range for sTfR was 1.0–2.9 μg/mL. Optical density (OD) readings were recorded at a wavelength of 450 nm using an ELISA reader, within 5–10 minutes of reaction completion. All samples were run as triplicate.

Quantitative RT-PCR

Total RNA was extracted from peripheral blood samples using the GeneJET RNA Purification Kit (Thermo Scientific, USA), following the manufacturer's instructions. RNA purity and concentration were determined using a NanoPhotometer® NP80 (Implen GmbH, Germany). Complementary DNA (cDNA) synthesis was performed using the GoTaq® 2-Step RT-qPCR Kit (Promega Corporation, USA).

Gene expression analysis of *TfR1* and *TfR2* was conducted using the SYBR Green-based GoTaq® 2-Step RT-qPCR System (Promega, Madison, WI, USA), adhering to the manufacturer's guidelines. Each 20 μL reaction mixture contained 10 μL of GoTaq® qPCR Master Mix (2X), 0.2 μL of CXR reference dye, 1 μL each of forward and reverse primers (20X), 6.8 μL of nuclease-free water, and 2 μL of cDNA template. A no-template control (NTC) was included, replacing the cDNA template with nuclease-free water. The analysis was conducted in technical triplicate for six biological samples for each group.

Amplification was performed using a StepOnePlus™ Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) with the following thermal cycling conditions: initial activation of GoTaq® DNA polymerase at 95°C for 2 minutes, followed by 40 cycles of denaturation at 95°C for 15 seconds, and annealing/extension at 60°C for 1 minute.

Gene expression levels were normalised to the endogenous control β-actin, and relative expression was calculated using the $2^{-}\Delta\Delta Cq$ method. Results were expressed as fold changes. The primer sequences used for amplification are detailed in Table I.

Table I. The primer sequences utilized for reverse transcription-quantitative PCR analysis

Gene name	Forward primer	Reverse primer
TfR 1	5′-GGCAAGTAGATGGCGATA -3′	5′-GCCCAAGTAGCCAATCTAA-3′
TfR 2	5′-GTGCGGAGACTCTGTGTT-3′	5′-GTTCCCGAAGGCTGGTTT -3′
β-actin	5′-GAGCGCGGCTACAGCTT-3′	5′-TCCTTAATGTCACGCACGATTT-3′

Statistical Analysis

Data were analyzed using the Statistical Package for Social Sciences (SPSS) version 20 (IBM Corp., Armonk, NY, USA). Group comparisons were performed using the Kruskal-Wallis test, with statistical significance set at $P<0.05$. For pairwise comparisons, the Mann-Whitney U test with Bonferroni correction was applied, adjusting the significance threshold to $P<0.017$. One-way analysis of variance (ANOVA) was used for comparisons involving multiple groups, while Student's t-test was employed to assess differences in sTfR levels between two groups.

RESULTS

Demographic data

Table 1 summarises the demographic, clinical, and biochemical characteristics of healthy controls, carrier parents, and HbE/β-thalassaemia patients. The gender distribution was balanced across groups. Patients exhibited markedly lower haemoglobin and BMI, with substantial increases in ferritin and sTfR levels, indicating anaemia with enhanced erythropoietic activity and iron overload. Hepcidin concentrations were significantly decreased in both patients and parents compared with controls, consistent with erythroid-driven hepcidin suppression. Splenomegaly was universally present in the patient group but absent in controls. Regular transfusion

and iron chelation therapy were reported only in patients. These results underscore the interplay between erythropoietic drive, iron dysregulation, and clinical severity across the thalassaemia spectrum.

Table II. Demographic, clinical, and biochemical characteristics of study participants (Mean ± SD).

Characteristics	Healthy Controls (n = 6)	Parents (n = 6)	HbE/β-thalassaemia Patients (n = 6)
Gender (M/F ratio)	3 / 3	3 / 3	3 / 3
Age (years)	25.3 ± 2.4	39.3 ± 2.1	12.5 ± 1.7
BMI (kg/m²)	22.8 ± 1.6	22.1 ± 1.5	17.9 ± 1.3
Hb (g/dL)	13.9 ± 0.3	11.9 ± 0.4	8.0 ± 0.3
Hepcidin (ng/mL)	20.2 ± 1.2	14.8 ± 1.5	8.9 ± 1.1
Ferritin (ng/mL)	92.7 ± 4.2	212.0 ± 15.6	686.0 ± 30.4
Serum Iron (µg/dL)	107.5 ± 4.1	115.5 ± 5.2	155.7 ± 6.1
sTfR (mg/L)	2.7 ± 0.2	4.1 ± 0.3	7.9 ± 0.4
Splenomegaly (mean size, cm)	None	Mild (1.5 ± 0.4)	Present (13.2 ± 1.6)
Transfusion status	None	None	Regular
Chelation therapy	No	No	Yes

Increased serum sTfR in HbE/β-thalassaemia patients

The analysis of the iron profile revealed a significant elevation in serum soluble transferrin receptor (sTfR) levels among HbE/β-thalassaemia patients and their parents compared to healthy controls *(P<0.001)* (Figure 1). Additionally, pairwise comparisons showed significant differences in sTfR levels between HbE/β-thalassaemia patients and their parents *(P<0.001)*, between patients and healthy controls *(P<0.001)*, and between parents and healthy controls *(P<0.001)*.

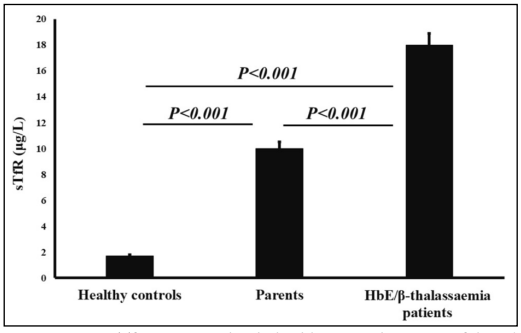

Figure 1. Serum sTfR concentration in healthy control, parents of the HbE/β-thalassaemia patients and HbE/β-thalassaemia patients.

Upregulated TfR1 in HbE/β-thalassaemia patients and their parents.

A significant increase in *TfR1* expression was observed, with levels more than five-fold higher in HbE/β-thalassaemia patients and over three-fold higher in their parents compared to healthy controls *(P=0.001)*. Furthermore, *TfR1* expression was significantly higher in HbE/β-thalassaemia patients than in their parents *(P=0.006)*. However, a significant decrease in TfR1 expression was observed in the parents (p=0.006) compared to the HbE/β-thalassaemia patients. (Figure 2).

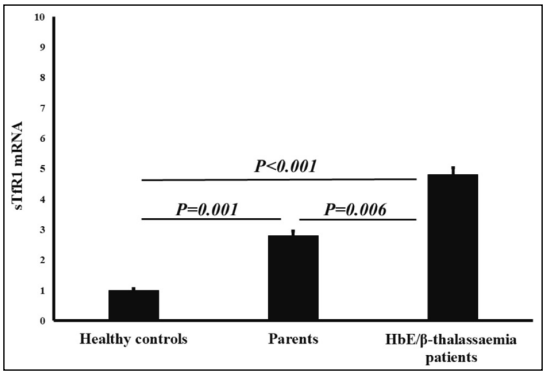

Figure 2. Gene expression analysis of sTfR1 in healthy control, parents of the HbE/β-thalassaemia patients and HbE/β-thalassaemia patients.

Downregulated sTfR2 in HbE/β-thalassaemia patients and their parents

The expression of *TfR2* was markedly reduced in both HbE/β-thalassaemia patients and their parents. Specifically, *TfR2* expression was 25-fold lower in patients and 11.5-fold lower in parents compared to healthy controls *(P<0.001)*. However, no statistically significant difference in *TfR2* expression was found between HbE/β-thalassaemia patients and their parents

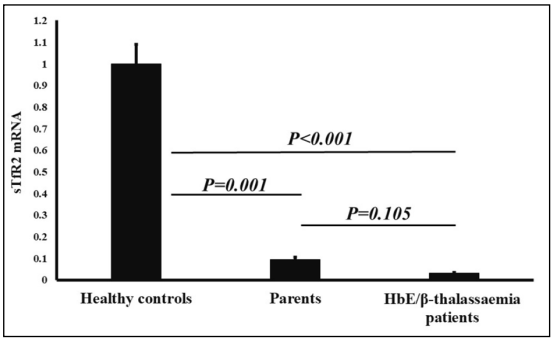

Figure 3. Gene expression analysis of *TfR2* in healthy control, parents of the HbE/β-thalassaemia patients and HbE/β-thalassaemia patients.

DISCUSSION

This study highlights the role of sTfR as a biomarker for IE, predominantly secreted by erythroid progenitor cells. Elevated sTfR levels are associated with erythrocyte hyperplasia and depleted iron reserves.[10] These findings underscore the importance of sTfR as a reliable marker for assessing the severity of IE in HbE/β-thalassaemia

patients. Additionally, understanding how sTfR levels reflect iron demand in erythropoiesis can further guide clinical decisions in managing thalassaemia patients, particularly in determining the need for transfusions.

sTfR2, a type II transmembrane glycoprotein, binds diferric-transferrin (holo-TF) with lower affinity than TfR1[11] and plays a regulatory role in iron homeostasis.[12] Unlike TfR1, which facilitates iron uptake, TfR2 is primarily involved in *hepcidin (HEPC)* regulation. Mutations in *TfR2* can lead to iron overload disorders, and its expression, predominantly in the liver, is essential for systemic iron sensing and erythropoiesis regulation.[13] Exploring the regulation of *TfR2* at the molecular level in the context of thalassaemia could provide valuable insights into how its downregulation contributes to the iron dysregulation observed in these patients. Given its potential to serve as a therapeutic target, further research on modulating *TfR2* expression could offer new avenues for iron overload management in thalassaemia.

TfR2 is stabilised on hepatic and erythroid cell membranes through differential TF binding, acting as a circulating iron sensor that modulates HEPC synthesis and red blood cell (RBC) production.[14] Aforementioned above, TfR2 plays a significant role in erythropoiesis, and its therapeutic modulation could help correct iron homeostasis in thalassaemia. Further studies should investigate the clinical implications of targeting TfR2 in thalassaemia patients, particularly in managing anaemia and iron overload.

Our findings revealed a significant elevation in serum sTfR levels *($p<0.001$)* in HbE/β-thalassaemia patients compared to their parents and healthy controls. These results align with previous studies that reported increased serum sTfR levels in individuals with β-thalassaemia trait (βTT), reinforcing its role in ineffective erythropoiesis. While haemoglobin levels alone may not be reliable indicators for guiding transfusion therapy in β-thalassaemia [15], sTfR offers valuable insights into transfusion needs. Elevated sTfR levels in βTT patients underscore ongoing erythropoietic stress, even in the absence of overt anaemia. Previous research has consistently reported higher sTfR levels in thalassaemia major compared to thalassaemia intermedia.[16] The use of sTfR as a clinical tool to predict transfusion requirements in thalassaemia patients is particularly promising, as it allows for better management of anaemia while avoiding unnecessary transfusions. This can improve the quality of life for patients and potentially reduce transfusion-associated complications.

Based on our previous study, HbE/β-thalassaemia patients and β-thalassaemia carriers showed increased serum ferritin, iron levels and transferrin saturation together with reduced serum hepcidin antimicrobial peptide (HAMP) and haemoglobin concentration levels compared to healthy controls ($P<0.001$).[17] In addition, we observed significantly higher reticulocyte counts in both groups compared with healthy controls ($P<0.001$), reflecting enhanced erythropoietic activity. This finding was consistent with elevated serum sTfR levels, further supporting the presence of ineffective erythropoiesis in thalassaemia.

In an integrative mechanism of thalassaemia, erythropoietin (EPO) is produced due to chronic anaemia and tissue hypoxia. This elevated EPO promotes massive expansion of erythroid precursors, which express abundant transferrin receptors (TfR1) to acquire iron for haemoglobin synthesis. Shedding of these receptors into circulation increases serum soluble transferrin receptor (sTfR) levels - a biomarker reflecting the magnitude of erythropoietic activity. Reticulocyte counts may also increase, reflecting heightened erythroid turnover, although many reticulocytes are prematurely destroyed due to ineffective maturation. The hyperactive yet ineffective erythropoietic marrow releases erythroferrone (ERFE) and growth differentiation factor-15 (GDF15), which suppresses hepatic HAMP expression. As a result, serum hepcidin levels (HAMP) become inappropriately low, even in the presence of iron overload. Reduced hepcidin removes its inhibitory effect on ferroportin, allowing continuous iron efflux from enterocytes and macrophages into plasma. Although total body iron is high, functional iron utilization is inefficient, as most erythroid precursors undergo apoptosis before producing

mature red cells. Consequently, haemoglobin (Hb) concentration remains low, and the anaemia persists, further stimulating EPO and worsening ineffective erythropoiesis.[18,19, 20]

Additionally, our study demonstrated a significant downregulation of *TfR2* expression in HbE/β-thalassaemia patients and their parents *(p<0.001)* compared to healthy controls, indicating altered iron regulatory signalling associated with ineffective erythropoiesis. The observed downregulation of *TfR2* expression in HbE/β-thalassaemia may reflect a disruption of the hepatic iron-sensing pathway, as TfR2 plays a critical role in mediating hepcidin (HAMP) induction in response to circulating holotransferrin. Reduced *TfR2* expression could therefore contribute to inappropriately low hepcidin levels, enhancing intestinal iron absorption and exacerbating systemic iron overload.[21] Understanding these regulatory mechanisms could lead to more targeted therapeutic approaches that address the underlying iron homeostasis issues in thalassaemia.

Furthermore, our results revealed significant upregulation of *TfR1* expression in HbE/β-thalassaemia patients and their parents *(p<0.001)* compared to healthy controls. These findings are consistent with previous studies demonstrating increased *sTfR1* expression in murine models of β-thalassaemia major and intermedia.[22] Elevated *TfR1* levels were particularly pronounced in patients requiring frequent transfusions and splenectomy, reinforcing its association with severe disease phenotypes.[23] However, some studies have reported reduced *TfR1* expression in specific β-thalassaemia models, highlighting potential context-dependent regulatory differences.[24]. This discrepancy in *TfR1* expression suggests that the regulation of *TfR1* might vary depending on disease stage, iron status, and treatment interventions. Future studies could explore how modifying TfR1 levels in different clinical settings could optimise patient management, especially in transfusion-dependent patients.

TfR1 is widely regarded as the "cellular iron gate" due to its role in iron uptake via ferric-transferrin internalisation. It is highly expressed in mammalian tissues and is critical for erythropoiesis, though its role in hematopoietic stem and progenitor cells remains unclear.[22] Prior research has shown that iron regulatory protein 2 (IRP2) binding at the 3' untranslated region enhances TfR1 translation during iron deficiency anaemia (IDA), leading to increased *TfR1* expression.[25] These findings suggest that *TfR1* plays a crucial role in erythroid precursor iron metabolism and iron transfer from erythroid cells to macrophages in the bone marrow. Consequently, targeting *TfR1* has been explored as a potential therapeutic approach to modulating iron metabolism and IE in thalassaemia.[26] Therapeutic strategies targeting *TfR1* could not only help control iron uptake but also improve erythropoiesis in thalassaemia patients, especially those with iron overload or transfusion dependency. Further clinical trials could explore the safety and efficacy of *TfR1* modulation in managing these conditions.

Excess iron disrupts IRP1 binding to iron-responsive elements, leading to TfR1 degradation and reduced cellular levels.[27] In contrast, *TfR1* expression is upregulated in conditions associated with increased erythropoiesis, such as β-thalassaemia major and IDA.[28] However, inconsistent patterns of *TfR1* expression have been observed in HepG2 cells treated with sera from β-thalassaemia major patients, underscoring the complexity of *TfR1* regulation in different cellular contexts.[22]

Taken together, our findings indicate significant upregulation of *TfR1 (p<0.001)* and marked downregulation of *TfR2 (p<0.001)* in HbE/β-thalassaemia patients and their parents compared to healthy controls. The altered expression of these genes correlated with increased serum sTfR concentrations, highlighting their role in erythropoiesis and iron metabolism. These results suggest that TfR1 may be a viable therapeutic target for managing thalassaemia. Given that *TfR* expression is tightly regulated by iron availability, its modulation could offer new treatment strategies to reduce red blood cell transfusion dependence and prevent iron overload in these patients. Future studies could investigate how modulating TfR1

levels can alleviate iron overload while promoting IE in transfusion-dependent thalassaemia patients.

The use of sTfR as a biomarker for assessing IE and iron overload in β-thalassaemia patients is further supported by these findings. Targeting TfR1 could provide a novel therapeutic avenue for reducing transfusion burden and mitigating systemic iron accumulation, ultimately improving patient outcomes. Moreover, longitudinal studies could track sTfR levels over time to evaluate disease progression and monitor therapeutic responses in patients undergoing treatment.

However, the sample size was considerably small (n= 6 per group) for sTfR level measurement due to COVID pandemic and lockdown was implemented during the time of subject recruitment. This limitation substantially limits the statistical power and may not be sufficiently robust to account for biological variability. The other limitation due to the same reason was the normal control group among the university students which might provide a selection bias who may not accurately represent the general population when comparing to the age, socioeconomic background or baseline health status.

CONCLUSIONS

In conclusion, this study provides suggestive evidence that the sTfR is a valuable biomarker for assessing IE and iron overload in patients with HbE/β-thalassemia. Elevated levels of sTfR were found to correlate with increased erythropoietic activity and iron demand, highlighting its significance in understanding the pathophysiology of thalassaemia. Our results also revealed a marked dysregulation in the expression of transferrin receptor isoforms, with upregulation of *TfR1* and downregulation of *TfR2*, which further emphasises their roles in erythropoiesis and iron metabolism in thalassaemia patients.

These findings suggest that sTfR could serve as a diagnostic tool to better assess the severity of IE and iron overload in thalassaemia patients. Moreover, the differential expression of *TfR1* and *TfR2* presents potential therapeutic targets. Modulating *TfR1* could help improve erythropoiesis and reduce transfusion dependency, while targeting *TfR2* could be key to managing iron overload, a common complication in thalassemia. Targeted therapies designed to regulate *TfR1* and *TfR2* expression hold promise for optimising iron homeostasis and alleviating the clinical burden of this disease.

Further research in a larger sample size is needed to better understand the complex regulatory mechanisms governing TfR expression, particularly in the context of varying disease states, and to evaluate the clinical efficacy of modulating sTfR levels in therapeutic interventions. Longitudinal studies are also crucial to track TfR levels as potential biomarkers for disease progression and response to treatment. Ultimately, the findings of this study open new avenues for improving patient outcomes in HbE/β-thalassaemia by enhancing our ability to manage IE and iron overload through targeted interventions.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest related to this manuscript.

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INSTITUTIONAL REVIEW BOARD (ETHICS COMMITTEE)

The study was approved by Universiti Sultan Zainal Abidin (UniSZA) Human Research Ethics Committee [(approval no. UniSZA.C/2/UHREC/628-2 J1d.2) (73)] and the Medical Research and Ethics Committee [(approval no. NMRR-19-855-45851 (IIR)].

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