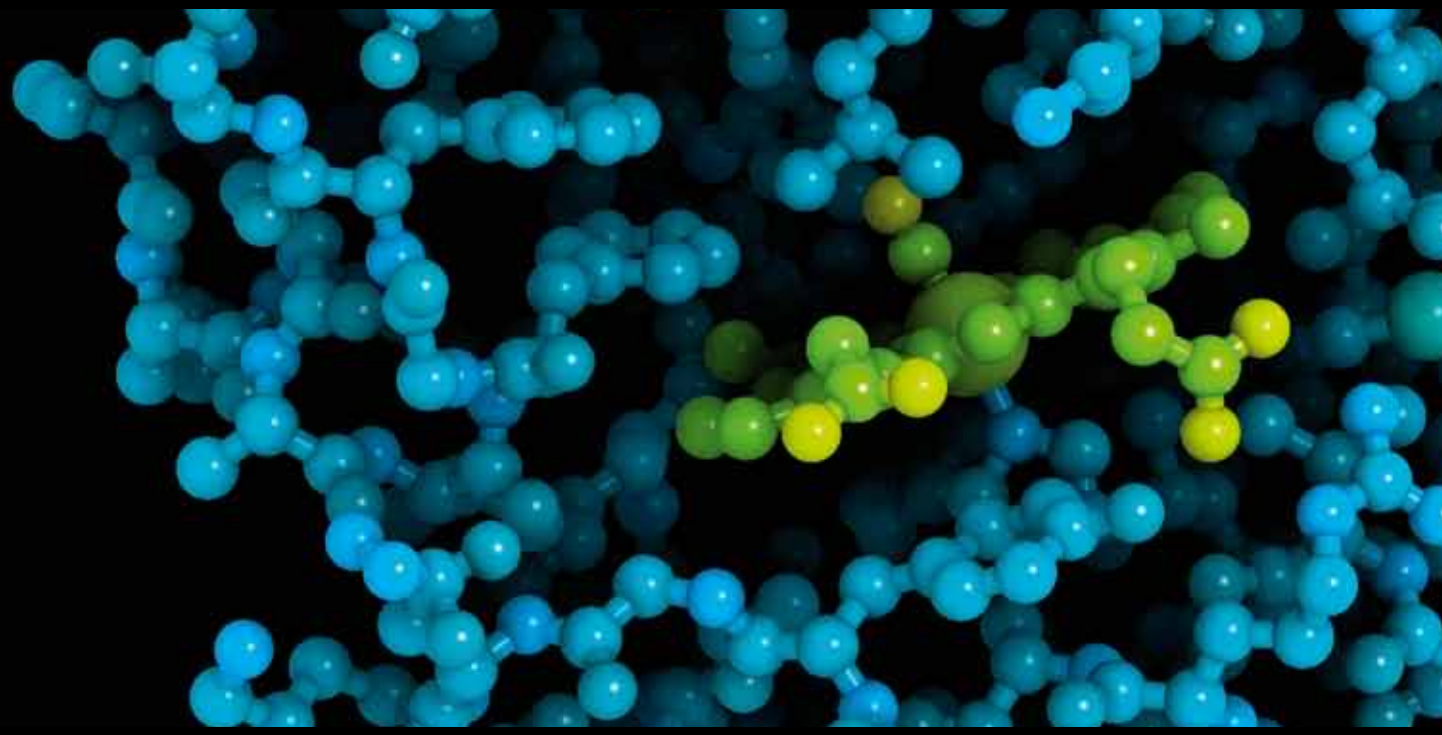
# β-Thalassemia: New Therapeutic Modalities, Genetics, Complications, and Quality of Life

Guest Editors: Mehran Karimi, Sezaneh Haghpanah, Ali T. Taher, and Maria Domenica Cappellini



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# Editorial Board

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*Editorial*

# $\beta$-Thalassemia: New Therapeutic Modalities, Genetics, Complications, and Quality of Life

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Beta-thalassemia is considered one of the most common genetic disorders which mass migration is introducing to countries worldwide and challenging them with its management. Advanced and improved medical care has allowed us to prolong the lives of thalassemia patients, simultaneously uncovering novel complications and outlining new challenges. We herein present in this special issue of *Anemia* some of the most cutting-edge research outcomes pertaining to the latest complications and the novel treatment strategies in iron-chelation therapy. We also study the effects of these interventions on the quality of life of patients. It is studies like these that serve as the basis of evidence-based medicine and guide clinicians through their process of decision making.

One article of this special issue addresses the new method of using transactional Doppler ultrasonography for measuring intracranial blood flow velocity in patients with beta-thalassemia intermedia. This study shows higher blood flow velocity in these patients compared to the control group, which may point to a higher risk of ischemic events in the future.

Another paper discusses that thalassemic DNA-containing red blood cells are under oxidative stress, which induces externalization of phosphatidylserine. This mechanism is involved in the removal of these cells from the circulation by the spleen, similar to that of the removal of senescent red blood cells.

This special issue also includes a paper explaining the pathophysiology of bone modifications in beta-thalassemia. Imbalance in mineral turnover resulting in abnormal regulation of bone metabolism may be related to hormonal and genetic factors, iron overload, and iron chelation therapy. These factors and their contribution are addressed.

Moreover, a review article that addresses the mechanism of tissue damage arising from iron overload in patients with $\beta$-thalassemia major is presented. It is the result of oxidative stress from free radical production, altered antioxidant enzymes, and its interaction with other essential trace element levels.

The last paper presented is a study comparing the quality of life in patients with $\beta$-thalassemia major and myelodysplastic syndrome with iron-overload treated either with deferasirox or deferoxamine injections. The results show that deferasirox can improve health-related quality of life treatment satisfaction and adherence compared to subcutaneous deferoxamine injection. This issue is crucial and often neglected in the long-term treatment of patients with iron overload.

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*Research Article*

# Health-Related Quality of Life, Treatment Satisfaction, Adherence and Persistence in $\beta$-Thalassemia and Myelodysplastic Syndrome Patients with Iron Overload Receiving Deferasirox: Results from the EPIC Clinical Trial

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Treatment of iron overload using deferoxamine (DFO) is associated with significant deficits in patients' health-related quality of life (HRQOL) and low treatment satisfaction. The current article presents patient-reported HRQOL, satisfaction, adherence, and persistence data from $\beta$-thalassemia ($n = 274$) and myelodysplastic syndrome (MDS) patients ($n = 168$) patients participating in the Evaluation of Patients' Iron Chelation with Exjade (EPIC) study (NCT00171821); a large-scale 1-year, phase IIIb study investigating the efficacy and safety of the once-daily oral iron chelator, deferasirox. HRQOL and satisfaction, adherence, and persistence to iron chelation therapy (ICT) data were collected at baseline and end of study using the Medical Outcomes Short-Form 36-item Health Survey (SF-36v2) and the Satisfaction with ICT Questionnaire (SICT). Compared to age-matched norms, $\beta$-thalassemia and MDS patients reported lower SF-36 domain scores at baseline. Low levels of treatment satisfaction, adherence, and persistence were also observed. HRQOL improved following treatment with deferasirox, particularly among $\beta$-thalassemia patients. Furthermore, patients reported high levels of satisfaction with deferasirox at end of study and greater ICT adherence, and persistence. Findings suggest deferasirox improves HRQOL, treatment satisfaction, adherence, and persistence with ICT in $\beta$-thalassemia and MDS patients. Improving such outcomes is an important long-term goal for patients with iron overload.

## 1. Introduction

Regular blood transfusions are essential for the management of haematological conditions such as $\beta$-thalassemia major and myelodysplastic syndromes (MDS). As a result, however, patients with these conditions are susceptible to the development of transfusion-dependent iron overload (hemosiderosis or secondary iron overload). In the absence of a naturally occurring physiological mechanism for the removal of excess iron in the body, life-long treatment and

adherence to iron chelation therapy (ICT) are necessary to prevent the morbidity and mortality that may result if excess iron is allowed to accumulate [1, 2].

Deferoxamine (DFO), most commonly delivered by continuous subcutaneous infusion over 8 to 12 hours a day, is the oldest available form of ICT used by patients with transfusion-dependent disorders. Prior research, albeit in small sample sizes, has indicated significant deficits in health-related quality of life (HRQOL) among patients receiving DFO for the treatment of transfusion-dependent iron overload, compared to values from age-matched normative populations [3, 4]. In particular, the time-consuming nature of DFO regimens and side effects associated with this form of ICT (including local site reactions) [5–7] can have a detrimental impact on numerous facets of patients' lives, including work; social activities; sex life; sleep; emotional well-being [8]. As a result, patient satisfaction with DFO treatment regimens is low and suboptimal adherence is common among patients [3, 4]. Improvements in ICT administration convenience and tolerability are expected to improve patient's satisfaction with ICT and HRQOL, thus promoting adherence to ICT regimens and potentially reducing iron overload-related morbidity/mortality and associated healthcare costs [1, 9, 10].

Deferasirox (Exjade) is an oral ICT first approved in 2005 and is the most widely prescribed ICT today [11]. Deferasirox has been shown to be an efficacious and generally well-tolerated therapy for the treatment of iron overload in $\beta$-thalassemia and MDS patients [12, 13]. Findings from randomised control trials comparing outcomes in patients with iron overload treated using either deferasirox or DFO have also suggested the superiority of deferasirox in terms of treatment satisfaction and adherence [14, 15]. However, additional research using validated patient-reported outcome (PRO) measures is needed in order to better understand the added benefits of deferasirox over DFO in terms of reducing HRQOL burden and improving treatment satisfaction, adherence, and persistence among patients with transfusion-dependent iron overload. The current work seeks to address these needs by presenting and discussing PRO findings from the Evaluation of Patients' Iron Chelation with Exjade (EPIC) study (NCT00171821), a large-scale prospective study designed to investigate the efficacy and safety of deferasirox in patients diagnosed with transfusion-dependent iron overload [12, 13].

## 2. Methods

*2.1. Study Design.* The EPIC study was a prospective, 1-year, multicentre, open-label phase IIIb trial conducted by 136 investigators across 23 countries [12, 13]. A PRO substudy within the EPIC trial was conducted to assess self-reported HRQOL and treatment satisfaction, adherence, and persistence in patients with transfusion-dependent iron overload. Based on the availability of validated questionnaire translations, the PRO substudy included participating study sites in Australia, Belgium, France, Germany, Greece, Italy, the Netherlands, and the UK. Study findings reported here focus specifically on data from adult patients ($\geq$16 years of age) with $\beta$-thalassemia and MDS. Based on the inherent differences in the underlying disease and patient profiles, study findings for patients with $\beta$-thalassemia and MDS will be reported separately. Of note, however, PRO data were also collected from patients with a variety of other transfusion-dependent disorders (including sickle cell disease, aplastic anemia, and other rare anemias) but sample sizes were considered too small ($n < 30$) for evaluable analysis as separate subgroups.

In accordance with EPIC trial selection criteria, all patients enrolled in this study were required to have transfusion-related iron overload, evident by a serum ferritin level of $\geq$1000 ng/mL or with LIC > 2 mg Fe/g dw, as determined by R2-Magnetic Resonance Imaging (MRI) [12, 13]. Patients unsuitable for participation in a clinical study from a clinical perspective (e.g., presence of systemic diseases which would prevent the patient from undergoing treatment) or a practical perspective (e.g., history of non-compliance to medical regimens) were excluded from the study. Assessments of HRQOL and satisfaction, adherence, and persistence to ICT were collected at baseline and at the end of the study (EOS; week 52 or at time of early study discontinuation), where appropriate, using the Medical Outcomes Short-Form 36-item Health Survey (SF-36v2) and the Satisfaction with ICT Questionnaire (SICT). Questionnaires were provided to patients in the native language of the respective country in which the patient was enrolled. Both questionnaires have been linguistically validated for use in the respective countries, ensuring the cross-cultural equivalence of the questionnaires and enabling data collected from different countries to be considered in a single-pooled dataset.

*2.2. Ethics.* The EPIC study was conducted in accordance with the Declaration of Helsinki; the International Conference on Harmonization (ICH) Tripartite Guidelines for Good Clinical Practice 1996; the Rules Governing Medicinal Products in the European Community (Directive 91/507/EEC); the US 21 Code of Federal Regulations dealing with clinical studies. Written informed consent was obtained from all patients prior to participation in the PRO substudy.

*2.3. PRO Measures*

*2.3.1. The Medical Outcomes Short-Form 36-Item Health Survey (SF-36v2).* The SF-36v2 is a self-administered questionnaire comprising 36-items measuring eight dimensions of general HRQOL: physical functioning (10 items), role limitation due to physical health problems (4 items), bodily pain (2 items), general health perceptions (5 items), vitality (4 items), social functioning (2 items), role limitations due to emotional problems (3 items), and general mental health (5 items). In addition to scores for individual dimensions, two summary scores assessing physical and mental dimensions of health and well-being can also be calculated: Physical Component Summary (PCS) score and the Mental Component Summary (MCS) score, respectively.

Although specific "tools" for the assessment of HRQOL have been developed for thalassemia [16], the SF-36 has the advantage of having been used extensively within clinical

trials and academic studies, across a wide range of disease areas, including $\beta$-thalassemia and MDS [3, 17–20]. The psychometric validity and reliability of the instrument as a generic measure of health-related functional status and well-being is well established [21–23]. In this study, the SF-36v2 was collected from patients at both baseline and EOS. All data were handled and scored in accordance with the developer's instructions: item scores for each dimension were coded, summed, and transformed to a scale from 0 (worst possible health state) to 100 (best possible health state), whereby higher values indicate better HRQOL. Domain scores were only calculated if at least half of all items comprising a domain were completed by the patient; missing data was not imputed [22].

*2.3.2. Satisfaction with ICT Questionnaire (SICT).* The SICT is a questionnaire designed specifically to assess patient satisfaction with ICT regimens [24]. It comprises 19 items assessing four domains of patient satisfaction: perceived effectiveness of ICT (PE), burden of ICT (BD), acceptance of ICT (AC), and side effects of ICT (SE). Patients rate all items on a response scale from 1 "very dissatisfied" to 5 "very satisfied". Domain scores are calculated as the mean score across constituent items and a higher score indicates greater satisfaction with respect to the questionnaire domain. As with the SF-36v2, domain scores were calculated if at least half of all items comprising a domain were completed by the patient; no missing data was imputed [24].

In addition, the SICT also includes three individual items designed to assess adherence to ICT "How often did you follow the chelation therapy regimen exactly as recommended by your doctor?", ICT persistence "How often did you think about stopping your chelation therapy?", and difficulties remembering to take ICT "How often did you have trouble remembering to take your chelation therapy?". All three items are assessed on a 5-point Likert scale from 1 "Always" to 5 "Never" and are designed to be interpreted as standalone items of the respective concepts.

Previous studies in patients with a variety of transfusion-dependent haematological disorders have provided evidence that the SICT is a reliable and valid measure of iron overload patients' satisfaction, adherence, and persistence to ICT regimens [24, 25]. All patients participating in the PRO substudy completed the SICT at EOS. Only patients with prior history of ICT were required to complete the SICT at baseline; the SICT was not relevant at this timepoint for those patients with no prior history of ICT.

*2.3.3. Statistical Analyses and Data Interpretation.* Descriptive statistics for subscale domains and summary component scores of the SF-36v2 were computed at baseline and EOS. To highlight the HRQOL burden associated with iron overload, mean SF-36 domain, and summary scores at baseline, and EOS were compared to published data of patients with $\beta$-thalassemia or MDS and age-matched norms derived from the UK general population [3, 17, 22, 26]. Confidence interval estimates were used to evaluate the significance of differences in observed study means relative to other reference groups. SICT domain scores and responses to questions regarding patient-reported adherence and persistence with ICT therapy utilization were also summarized at baseline and EOS.

Relevant differences in group means between study and other reference populations for SF-36 domain scores (e.g., disease-specific and UK general population) were evaluated using a distribution-based approach for establishing clinically meaningful difference. In this regard, differences that are 0.5 standard deviation (SD) units of a baseline score were characterized as clinically meaningful [27–29].

Analysis of questionnaires at baseline and EOS (e.g., week 52 or at time of early study discontinuation) was undertaken only in cases where sample sizes were large enough ($n > 30$) for statistical analyses. Data were presented separately for patients with underlying $\beta$-thalassemia and MDS due to inherent differences in disease populations and patient profiles.

## 3. Results

*3.1. Demographic and Clinical Characteristics.* The demographic and clinical characteristics of $\beta$-thalassemia ($n = 274$) and MDS ($n = 168$) patients evaluated in this PRO substudy are displayed in Table 1 and are generally similar to those of the overall $\beta$-thalassemia and MDS populations enrolled in the EPIC trial [12, 13]. As expected, the mean age of patients with MDS was considerably higher than the mean age of patients with $\beta$-thalassemia. Almost all $\beta$-thalassemia patients ($n = 270$; 98.5%) had a history of prior ICT, with 66.4% ($n = 184$) having previously received DFO monotherapy and 30.3% ($n = 84$) having previously received DFO and deferiprone. Only 51.8% ($n = 87$) of MDS patients had a history of prior ICT, however, with 37.5% ($n = 63$) of the MDS sample having previously received DFO monotherapy and 8.3% ($n = 14$) having previously received DFO and deferiprone. As such, where relevant, differences between MDS patients with a history of ICT and ICT-naïve patients are highlighted. Note, however, that sample sizes of evaluable data do not allow for statistical comparison of differences between these two groups.

*3.2. Changes in HRQOL following Treatment with Deferasirox*

*3.2.1. $\beta$-Thalassemia Patients.* At baseline, mean scores for 6 of the 8 SF-36 domains among patients with $\beta$-thalassemia were notably lower than equivalent scores derived from UK norms for persons aged 25 to 34 years (the exceptions being vitality and mental health). Of these domains, differences in scores for physical functioning, role-physical, and general health at baseline among patients with $\beta$-thalassemia, compared to UK general population norms, were at a level considered to be clinically meaningful; indicating significant burden within this population. However, baseline SF-36 domain scores among these patients were similar to historical reference patients previously receiving infused chelation therapy as reported by Payne et al. and in which 82% of patients were $\beta$-thalassemia patients [3] (Figure 1).

Mean SF-36 PCS and MCS scores for $\beta$-thalassemia patients at baseline ($\bar{x} = 45.64$ [SD = 9.25] and $\bar{x} = 47.72$

Table 1: Demographic and clinical characteristics of $\beta$-thalassemia and MDS patients.

| | $\beta$-thalassemia ($N$ = 274) | MDS ($N$ = 168) |
|---|---|---|
| Mean age (SD) | 26 (11.5) | 68 (10.3) |
| Males, $n$ (%) | 127 (46.4) | 96 (57.1) |
| Age groups, $n$ (%) | | |
| <6 yrs | 17 (6.2) | 0 (0) |
| 6 to <12 yrs | 25 (9.1) | 0 (0) |
| 12 to <16 yrs | 15 (5.5) | 0 (0) |
| 16 to <50 yrs | 213 (77.7) | 4 (2.4) |
| 50 to <65 yrs | 4 (1.5) | 55 (32.7) |
| ≥65 yrs | 0 (0) | 109 (64.9) |
| Prior chelation therapy, $n$ (%) | | |
| No | 4 (1.5) | 81 (48.2) |
| Yes | 270 (98.5) | 87 (51.8) |
| Prior chelation therapy, $n$ (%) | | |
| None | 4 (1.5) | 81 (48.2) |
| DFO only | 184 (66.4) | 63 (37.5) |
| Deferiprone only | 2 (0.7) | 9 (5.4) |
| DFO and deferiprone* | 84 (30.3) | 14 (8.3) |
| Other ICT | 3 (1.1) | 1 (0.6) |

* Patients may have received both DFO and deferiprone as prior chelation therapies, but these may not have been in combination.

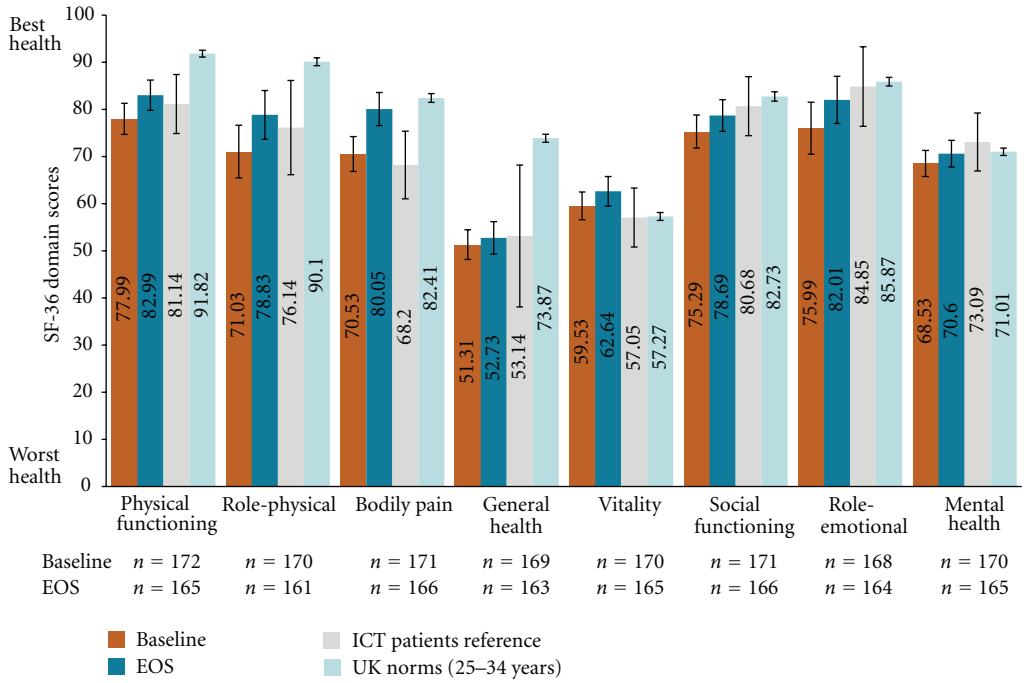


Figure 1: Mean SF-36 domain scores for $\beta$-thalassemia population versus disease-related and general population references at baseline and EOS. Data illustrated in the above figure are representative of the mean and 95% confidence interval estimates of the mean as calculated using the formula: 1.96 $*$ standard deviation $\div\sqrt{n}$. Reference data for ICT patients were based on Payne et al. [3] (where 82% of patients in this study were thalassemia patients); UK norms from Jenkinson et al. [42] and direct email communications from Dr. Jenkinson on Oct 24, 2011 with age-specific norms.

[SD = 10.63], resp.) were also lower compared to UK norms (by −6.94 and −0.35 points, resp.); however, only the PCS score was substantially different and considered clinically meaningful. Relative to other patients with a history of receiving infusional ICT, mean summary component scores at baseline for patients with $\beta$-thalassemia in the EPIC study were similar.

Mean SF-36 domain scores at EOS were generally higher following treatment with deferasirox and closer to population norm scores for the UK general population and

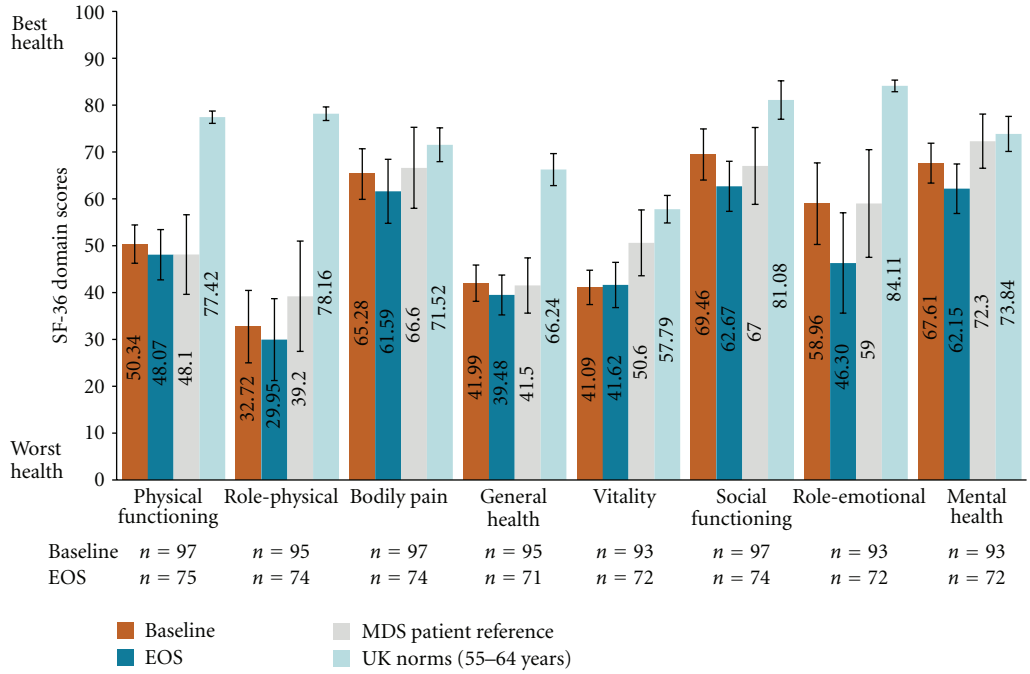


Figure 2: Mean SF-36 domain scores for MDS population versus MDS and general population references at baseline and EOS. Data illustrated in the above figure are representative of the mean and 95% confidence interval estimates of the mean as calculated using the formula: 1.96 ∗ standard deviation $\div\sqrt{n}$. Transfusion-dependent MDS patient reference data reported by Jansen et al. [17]; UK norms from Jenkinson et al. [42] and direct email communications from Dr. Jenkinson on Oct 24, 2011 with age-specific norms.

patients previously receiving infusional chelation therapy [3]. Meaningful changes in scores from baseline to EOS were observed for bodily pain, role-physical, and role-emotional in patients with $\beta$-thalassemia. Mean SF-36 domain scores at EOS were lower compared to UK population norms for physical functioning, role-physical and general health domains, but only the general health domain score at EOS was lower than population norms to a clinically meaningful degree. Mean SF-36 PCS and MCS scores were also higher at EOS compared to baseline ($\bar{x}$ = 48.15 [SD = 9.03] and $\bar{x}$ = 48.67 [SD = 9.52], resp.). PCS scores were, however, still substantially lower than population norms such that the difference can be considered clinically meaningful.

*3.2.2. MDS Patients.* In patients with MDS, mean baseline scores for all SF-36 domains were lower than age-matched UK norms for persons aged 65–74 years but were similar to other patients with MDS (Figure 2) as previously reported by Jansen et al. [17]. Differences between MDS and the UK normative sample were also at the level considered to be clinically meaningful for physical functioning, role-physical, general health, vitality, social functioning, and role-emotional domains. Mean SF-36 PCS and MCS scores for MDS patients at baseline ($\bar{x}$ = 35.47 [SD = 8.58] and $\bar{x}$ = 47.16 [SD = 11.29], resp.) were also lower than those derived from the UK normative sample (−9.00 and −5.12, resp.), however, only differences in PCS were of a clinically meaningful magnitude. Relative to other MDS patients as reported by Jansen et al. (2003), mean summary component scores at baseline for patients with MDS in the EPIC study were similar [17].

In patients with MDS, mean SF-36 domain scores were lower at EOS compared to age-matched UK population norms for all SF-36 domain scores. Mean scores at EOS were no different from means of MDS patients as reported by Jansen et al. [17]. Except for the bodily pain domain, all other functional and well-being domains of the SF-36 scale were lower than UK norms to a clinically meaningful degree. Compared to baseline, PCS scores remained relatively stable ($\bar{x}$ = 35.71 [SD = 9.64]), but MCS scores declined at EOS ($\bar{x}$ = 43.56 [SD = 11.79]). At EOS, deficits for both PCS and MCS were lower than UK norms to a substantial and clinically meaningful degree.

Further analysis of SF-36 scores for MDS patients with and without prior ICT history suggest that ICT-naïve patients had at baseline lower PCS scores and lower domain scores for physical functioning, role-physical, bodily pain, and general health domains, compared to patients with prior experience of ICT. However, ICT naïve patients had higher MCS scores and scores for all constituent domains, except for vitality (Table 2). Scores for all SF-36 domains and summary components were lower at EOS compared to baseline among patients with prior history of ICT. SF-36 domains for ICT-naïve patients were higher for physical functioning, role-physical, general health, vitality, and PCS but were lower for other domains and MCS at EOS.

Table 2: Mean (SD) SF-36 domain and summary scores among MDS patients according to prior ICT history.

| | Reference MDS population ($n$ = 50): mean (SD) | Age-matched UK norms: mean (SD) | Baseline mean (SD) | | EOS mean (SD) | |
|---|---|---|---|---|---|---|
| | | | Prior ICT ($N$ = 59–62) | ICT Naïve ($N$ = 34-35) | Prior ICT ($N$ = 38–40) | ICT Naïve ($N$ = 32–35) |
| Physical functioning | 48.1 (30.6) | 77.42 (25.38) | 53.60 (21.35) | 44.56 (17.94) | 47.43 (24.39) | 48.79 (23.29) |
| Role-physical | 39.2 (42.5) | 78.16 (28.11) | 36.39 (39.21) | 26.43 (36.85) | 30.21 (40.90) | 29.66 (35.60) |
| Bodily pain | 66.6 (31.2) | 71.52 (26.46) | 65.61 (27.43) | 64.69 (27.16) | 63.23 (28.53) | 59.77 (31.85) |
| General health | 41.5 (21.3) | 66.24 (22.57) | 42.70 (18.94) | 40.72 (20.00) | 37.78 (20.01) | 41.55 (15.92) |
| Vitality | 50.6 (25.3) | 57.79 (21.28) | 43.25 (19.02) | 37.35 (15.92) | 42.89 (24.10) | 40.20 (16.81) |
| Social functioning | 67.0 (29.6) | 81.08 (26.14) | 68.55 (29.75) | 71.07 (23.24) | 62.18 (25.57) | 63.21 (21.21) |
| Role-emotional | 59.0 (41.4) | 84.11 (24.41) | 57.18 (44.05) | 61.90 (41.34) | 41.23 (43.45) | 51.96 (45.83) |
| Mental health | 72.3 (20.9) | 73.84 (19.35) | 66.78 (22.34) | 69.06 (18.54) | 60.07 (22.85) | 64.47 (23.01) |
| Physical component summary | 35.7 (11.7) | 44.47 (12.32) | 36.97 (8.48) | 33.10 (8.31) | 36.21 (8.70) | 35.11 (10.80) |
| Mental component summary | 48.9 (12.6) | 52.28 (9.89) | 46.32 (12.02) | 48.49 (10.04) | 42.14 (11.61) | 45.27 (11.97) |

Transfusion-dependent MDS patient reference data reported by Jansen et al. [17]; UK norms from Jenkinson et al. [42] and direct email communications from Dr. Jenkinson on Oct 24, 2011 with age-specific norms.

### 3.3. Changes in ICT Satisfaction following Treatment with Deferasirox

*3.3.1. β-Thalassemia Patients.* At baseline, patients with β-thalassemia and prior history of ICT were generally satisfied with the perceived effectiveness of their ICT prior to initiation of deferasirox in the EPIC study but were neither satisfied nor dissatisfied with side effects of ICT, acceptance of ICT and burden of ICT as measured by SICT domains (Figure 3). Compared to baseline, patient-reported satisfaction associated with side effects of ICT, acceptance of ICT, and burden of ICT SICT domains increased with deferasirox by ≥1.4 points at EOS.

*3.3.2. MDS Patients.* At baseline, patient-reported satisfaction with ICT was high (>3.5) among MDS patients with prior history of ICT as measured by SICT domains of perceived effectiveness of ICT, side effects of ICT, and burden of ICT (Figure 4). Patients were neither satisfied or dissatisfied on SICT domain of acceptance of ICT at baseline. Compared to baseline, patient-reported satisfaction with ICT-related side effects, acceptance, and burden were higher at EOS following treatment with deferasirox based on SICT domain scores. Scores for the perceived effectiveness of ICT SICT domain remained stable between baseline and EOS.

SICT data were also evaluated at EOS for those MDS patients with no prior history of ICT. Notably, the SICT domain scores reported were comparable to EOS scores for patients with prior ICT for SICT domains: side effects of ICT ($\bar{x}$ = 4.18, SD = 0.17), acceptance of ICT ($\bar{x}$ = 4.17, SD = 0.69), and burden of ICT ($\bar{x}$ = 4.51, SD = 0.55). Patient-reported satisfaction for the perceived effectiveness of ICT domain was also high at EOS ($\bar{x}$ = 4.25, SD = 0.60).

### 3.4. Changes in ICT Adherence following Treatment with Deferasirox.

Following treatment with deferasirox, the proportion of β-thalassemia patients who reported always following their ICT regimen as recommended by their doctor increased from 32.4% ($n$ = 58/179) at baseline to 67.1% ($n$ = 116/173) at EOS. Patient-reported adherence was also high among patients with MDS who had a prior history of ICT at baseline where 62.5% ($n$ = 35/56) of patients reported having always followed their ICT regimen as they were told by their doctor. Following treatment with deferasirox, patient's self-reported adherence to ICT increased to 85.7% ($n$ = 36/42) at EOS. Similarly, patient-reported adherence among MDS patients with no prior history of ICT at EOS was high, with 82.9% ($n$ = 29/35) of patients reporting that they always followed their ICT regimen as they were told by their doctor.

### 3.5. Changes in ICT Persistence following Treatment with Deferasirox.

Following treatment with deferasirox in the EPIC study, the proportion of patients with β-thalassemia who

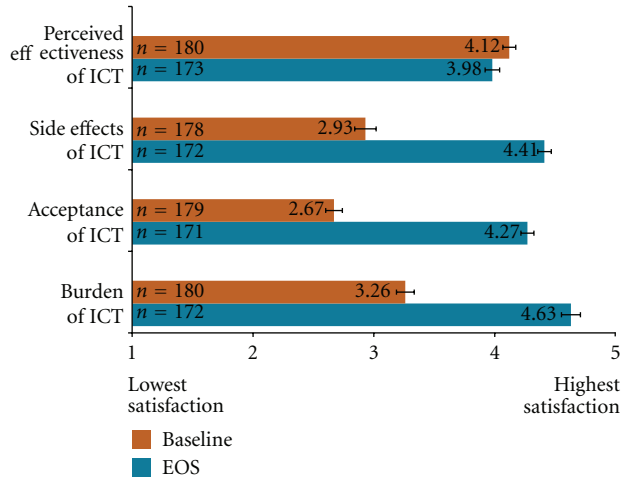


Figure 3: Mean SICT domain scores at baseline and EOS for overall $\beta$-Thalassemia population.

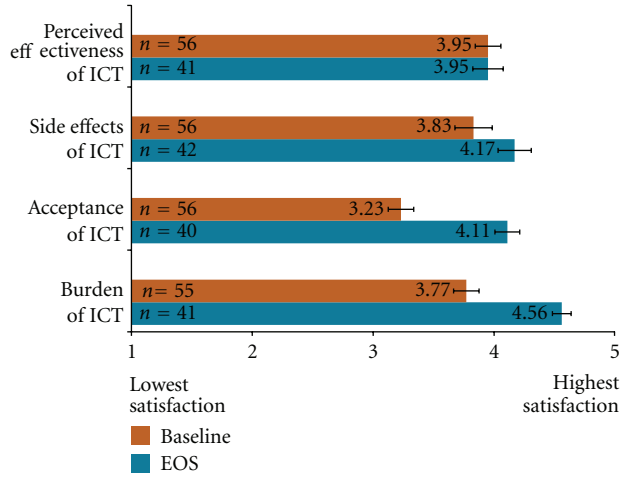


Figure 4: Mean SICT domain scores at baseline and EOS for MDS population with prior history of ICT.

never thought about stopping ICT increased from 40.8% ($n$ = 73/179) at baseline to 76.3% ($n$ = 132/173) at EOS. Among patients with MDS and a prior history of ICT, the proportion of patients who never thought about stopping ICT was high at baseline (75.9%, $n$ = 41/54), but decreased slightly at EOS following treatment with deferasirox (69.0%, $n$ = 29/42). Persistence to ICT, however, was high at EOS among MDS patients with no prior history of ICT; 77.1% ($n$ = 27/35) of this population never thought about stopping ICT at EOS.

## 4. Discussion

The EPIC study is the largest prospective evaluation of any iron chelation therapy conducted to date. Findings from this study have demonstrated that deferasirox is an efficacious and generally well-tolerated treatment for the treatment of iron overload in patients with transfusion-dependent disorders such as $\beta$-thalassemia and MDS [12, 13]. In addition, this study provided a unique opportunity to collect data concerning the role that deferasirox can play in addressing HRQOL concerns associated with iron chelation therapies and how deferasirox may help address issues related to treatment satisfaction, adherence, and persistence among patients with iron overload.

SF-36 data collected for $\beta$-thalassemia patients at baseline suggest that these patients have lower HRQOL compared to age-matched norms on almost all facets of HRQOL assessed within the SF-36. The greatest deficits were seen in related "physical" domains of the SF-36 (e.g., physical functioning, role-physical, bodily pain, and general health). These findings are supportive of prior investigations among smaller samples of $\beta$-thalassemia patients and confirm assertions that $\beta$-thalassemia is a debilitating disease [3, 4, 18]. Similarly, as reflective of prior research, deficits on all facets of HRQOL were also observed among patients with MDS [17, 30]. Notably, however, HRQOL deficits appeared greater among patients with MDS compared to those with $\beta$-thalassemia.

The burdensome nature of infused ICT (DFO) has been cited as a contributory factor to diminished HRQOL domains observed in patients with transfusion-dependent disorders [3, 4, 8]. As such there is an expectation that, compared to DFO, oral ICT such as deferasirox will have a less detrimental effect on patient's HRQOL. Although the EPIC study does not provide a direct head-to-head comparison of deferasirox versus DFO, the prospective evaluation of HRQOL over time following initiation of deferasirox provides unique insight into the differential impact of both infused and oral ICT on HRQOL among patients with $\beta$-thalassemia and MDS. This is especially true for $\beta$-thalassemia patients, many of whom had been receiving DFO prior to enrolment in the EPIC study. The prospective assessment of HRQOL over time in MDS patients also addresses the limitations of prior research in MDS patients which, in collecting HRQOL data at one time point, give little indication of changes in HRQOL in this population over time.

Observations from the EPIC study suggest that deferasirox was associated with directional improvements in all facets of HRQOL as assessed by the SF-36 among patients with $\beta$-thalassemia which were similar to prior reported studies of similar populations [3, 17]. Of most note were improvements in bodily pain; a key but often overlooked symptom of $\beta$-thalassemia [31]. In contrast to patients with $\beta$-thalassemia, patients with MDS in the EPIC study had slightly lower mean HRQOL domain scores at EOS, however, mean scores were similar to observations reported by Jansen et al. [17] and may be representative of declining prognosis and disease progression with transfusion dependence and the need for supportive care [32, 33].

Observed differences in HRQOL between the two populations must be interpreted in the context of the demographic and clinical characteristics of the respective populations. In contrast to the $\beta$-thalassemia which is a genetic condition typically diagnosed in early years of life, MDS is an acquired disorder with the majority of cases occurring in patients over

the age of 60 [34]; a difference reflected in the substudy samples for the EPIC trial (mean age of 26 versus 68 years, resp.). In addition, modern-day therapy has increased life expectancy for $\beta$-thalassemia patients, such that patients can now live for decades [35, 36]. Past research has also indicated that HRQOL in patients with $\beta$-thalassemia remains relatively stable over time; adding confidence that changes observed in this study are a result of the study treatment (e.g., switch to deferasirox) as opposed to statistical artifact [37]. By contrast, the prognosis of patients with MDS is generally poor due to disease progression, deterioration, and increasing transfusion dependence [38]. The presence of comorbid conditions (e.g., diabetes, coronary heart disease, or chronic pulmonary obstructive disease) which are increasingly prevalent among elderly populations also complicate the management of MDS and contribute to poor risk among these patients [39]. As such, HRQOL may be expected to deteriorate among MDS populations over time, independent of the form of ICT that is received by the patient.

The EPIC study provides unique insight into the impact that deferasirox may have on patients HRQOL over a one-year period. However, the key to minimising long-term morbidity and mortality in patients with transfusion-dependent disorders is ensuring patient's adherence to recommended ICT regimens. The burdensome nature of infusional ICT regimens or iterative ICT combination regimens may complicate compliance and result in suboptimal adherence, and persistence [3, 4]. This was also evident in the present study, particularly among patients with $\beta$-thalassemia where approximately 40% of patients at baseline self-reported that they always followed their treatment regimen as recommended by their doctor (e.g., adherence) and never thought about stopping ICT treatment (e.g., persistence). However, self-reported adherence and persistence among patients with $\beta$-thalassemia increased at EOS following treatment with deferasirox (67.1% and 76.3%, resp.). Likewise, the proportion of MDS patients with prior history of ICT who reported always following their ICT regimen as recommended by their doctor increased at EOS compared to baseline. Self-reported persistence with ICT was slightly lower at EOS compared to baseline (75.9% and 69.0% never thought about stopping their ICT at baseline and EOS, resp.) among MDS patients with prior history of ICT which may be associated with underlying disease progression.

Patients who are less satisfied with prescribed treatment are expected to be less likely to adhere to recommended treatment protocols. Consistent with this, patients with $\beta$-thalassemia in the EPIC study demonstrated a higher level of satisfaction with ICT at EOS following treatment with deferasirox, particularly in relation to practical aspects of ICT such as side-effects, burden, and acceptability of treatment regimen which coincided with higher self-reported adherence and persistence with deferasirox at EOS. In MDS patients with prior history of ICT, notable improvements in satisfaction with ICT were recorded between baseline and EOS and higher self-reported adherence. This observation suggests that the directional changes in HRQOL in patients with MDS may be associated with other factors such as progressive underlying disease, complications of older age, among others, and the may not be related to study treatment. Furthermore, levels of satisfaction among patients with MDS and no prior history of ICT were also high at EOS, offering further support that the directional changes in aspects of HRQOL between baseline and EOS may be attributable to other factors.

In reflecting on potential limitations of the present study, the number of MDS patients for whom there was evaluable data should be considered. The high rate of study discontinuations among MDS patients participating in the EPIC study is acknowledged as source of potential bias in these data and is a key factor limiting the availability of evaluable data in this substudy. As detailed by Gattermann et al. (2010), of 341 patients with MDS enrolled, 175 patients completed the study (median duration of treatment: 50.6 weeks) whereas 166 discontinued (rate: 48.7%) with the primary reason for study discontinuation being due to adverse events ($n = 78$) [13]. Nonetheless, when interpreting findings from the current study, it is necessary to consider that HRQOL, treatment satisfaction, adherence, and persistence may be negatively affected in those patients discontinuing treatment due to adverse events.

In interpreting the findings of this study, it is also important to appreciate that the defining features of controlled clinical studies (e.g., study selection criteria, predetermined assessment schedules, study treatment) may introduce bias to the evaluation of health outcomes (e.g., HRQOL, treatment satisfaction, adherence, persistence, etc.). In particular, the exclusion of participants with a history of poor compliance to medical regimens may have affected ratings of adherence, and persistence at baseline and EOS. As such, further consideration of naturalistic studies would help to establish the real-world validity of observed changes in HRQOL, treatment satisfaction, adherence and persistence associated with deferasirox observed in the EPIC study. A recent retrospective assessment of iron chelation adherence from the Thalassemia Clinical Research Network (TRCN) also documents high patient-reported adherence with deferasirox [40, 41]. With this end in mind, the present study provides further evidence of the validity of the SF-36 and SICT as measures of HRQOL and treatment satisfaction/adherence/persistence, respectively, and supports their use in future studies of transfusion-dependent conditions and ICTs. Standardisation of PRO assessments in future studies (using the SF-36 and SICT, e.g.) is particularly important for enabling meaningful comparisons to be made between varying ICT regimens.

## 5. Conclusions

This substudy represents the largest prospective evaluation of patient-reported outcomes with deferasirox to date. Findings indicate improvements in patient-reported HRQOL, ICT satisfaction, adherence, and persistence following treatment with deferasirox particularly among $\beta$-thalassemia patients, the majority of whom had been using infused ICT prior to enrolment in the study. Patient satisfaction with deferasirox is high and patients receiving deferasirox report being more likely to adhere and persist with ICT. Such evaluations are

vital for improving both the long-term health outcomes and survival of patients with transfusion-dependent iron overload and minimising future health resource use.

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*Review Article*

# Physiopathology of Bone Modifications in β-Thalassemia

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β-thalassemia major (βTM) or Cooley anemia is characterized by significantly reduced or absent synthesis of β-globin chains, which induces important pathologic consequences including hemolytic anemia, altered erythropoiesis, and bone marrow overstimulation. The pathogenesis of bone changes in patients with βTM is not yet completely understood. However, an unbalance in bone mineral turnover resulting from increased resorption and suppression of osteoblast activity has been detected in βTM patients. The abnormal regulation of bone metabolism may be related to hormonal and genetic factors, iron overload and iron chelation therapy, nutritional deficits, and decreased levels of physical activity. Here, we review the most recent findings on the physiopathology of bone abnormalities in βTM. Clinical presentation and radiological features of βTM-related bone changes are also discussed.

## 1. Introduction

β-thalassemia, firstly described by Cooley and Lee [1], comprises a group of inherited, autosomal, recessive, and hematologic disorders characterized by decreased or absent synthesis of β-globin chains. The mature hemoglobin (Hb) molecule is a tetramer composed of two α-globin and two β-globin chains, along with a heme prosthetic group. β-globin synthesis is controlled by one gene located on each chromosome 11 [2]. Defects are usually secondary to point mutations and rarely occur as a consequence of deletions [2]. In β-thalassemia, β-globin chain production can range from near to normal to completely absent, leading to varying degrees of excess α-globin chains and disease severity [2]. β-thalassemia trait (minor), resulting from heterozygosity for β-thalassemia, is clinically asymptomatic and manifests with microcytosis and mild anemia. β-thalassemia intermedia comprises a clinically and genotypically heterogeneous group of disorders, ranging in severity from the asymptomatic carrier state to severe, transfusion-dependent disease. β-thalassemia major (βTM) or Cooley anemia is characterized by severely reduced or absent synthesis of β-globin chains from both genes, with symptoms and signs beginning at about six months of age (abdominal swelling, growth retardation, irritability, jaundice, pallor, skeletal abnormalities, and splenomegaly) [2].

In βTM, the defective synthesis of β chains, together with excess α chains, leads to hemolytic anemia, altered erythropoiesis, reduced erythrocyte survival, and bone marrow overstimulation [1–4]. Patients need blood transfusions to correct anemia and iron-chelating therapy to control iron overload [1–4]. Anemia, excess body iron, and iron-chelation therapy can result in endocrine disorders (e.g., diabetes mellitus, hypogonadism, hypothyroidism, hypoparathyroidism, hypopituitarism, and Addison's disease), growth retardation, liver and cardiac failure, and splenomegaly. The latter can worsen anemia and occasionally causes thrombocytopenia and neutropenia, thereby increasing the risk of infections and hemostatic disorders [2–5]. Heart failure is the leading cause of death in patients with βTM [3, 6].

## 2. Epidemiology

The worldwide prevalence of α- and β-thalassemia trait is 1.7% [4]. Males and females are equally affected. The

incidence of thalassemia trait is 4.4 per 10,000 live births [4]. $\beta$-thalassemia in its various presentations is more common in the Mediterranean area, Africa, and Southeastern Asia.

## 3. Pathogenesis of Bone Changes in $\beta$-Thalassemia

The pathogenesis of bone changes in $\beta$TM patients is not yet completely understood [7]. In spite of the improved treatment of the hematologic disorder and its complications, $\beta$-thalassemia patients exhibit an unbalance in bone mineral turnover with increased resorptive rates and suppression of osteoblast activity, resulting in diminished bone mineral density (BMD) more evident in the lumbar spine [8, 9]. Putative mechanisms involved in the pathogenesis of bone abnormalities in $\beta$TM are discussed in the following subsections.

*3.1. Impairments in Osteoblast Activity.* Mahachoklertwattana et al. [7, 10] reported growth retardation and delayed bone age, reduced BMD (especially of the lumbar spine), and low serum IGF-I levels in children and adolescents with $\beta$TM. In these patients, bone histomorphometry revealed increased osteoid thickness and delayed osteoid maturation and mineralization, indicating impaired bone matrix maturation and defective mineralization [7]. In addition, iron depots were detected along mineralization fronts and osteoid surfaces, while focal-thickened osteoid seams were found together with iron deposits. Dynamic bone formation studies revealed reduced bone formation rates. These findings indicate that delayed bone maturation and focal osteomalacia contribute to the pathogenesis of bone disease in suboptimally blood-transfused $\beta$TM patients with iron overload. Iron depots within bones and low circulating IGF-I levels may partly contribute to skeletal abnormalities [7].

Morabito et al. [11] showed that $\beta$TM patients displayed an unbalanced bone turnover, characterized by enhanced resorption rates (indicated by high levels of pyridinium cross-links) and a decreased neoformation phase (evidenced by low levels of osteocalcin, an osteoblast-derived protein) [11]. Voskaridou and colleagues [12] found increased serum levels of Dickkopf-1 (Dkk1), a soluble inhibitor of wingless type (Wnt) signaling, and sclerostin [13], a Wnt inhibitor, specifically expressed by osteocytes, in $\beta$TM patients. Higher circulating levels of Dkk1 and sclerostin correlated with reduced bone mineral density of lumbar spine and distal radius as well as with increased bone resorption and reduced bone formation markers. These findings indicate that disruption of Wnt signaling in patients with thalassemia and osteoporosis leads to osteoblast deregulation. Therefore, sclerostin and Dkk-1 have been proposed as potential targets for treatment in patients with thalassemia-induced osteoporosis [13].

*3.2. Abnormal Osteoclast Activity.* Besides impairments in osteoblast activity, which are thought to be a major cause of osteopenia/osteoporosis in $\beta$TM, an enhanced activation of osteoclasts is also invoked as a contributing factor [14]. This provides the rationale for the use of bisphosphonates, which are potent inhibitors of osteoclast function, for the management of $\beta$TM-induced osteoporosis [15].

An association between increased circulating levels of proresorptive cytokines and altered bone turnover has been detected in $\beta$TM patients [16]. The receptor activator of nuclear factor-kappa B (RANK)/RANK ligand (RANKL)/osteoprotegerin (OPG) pathway has recently been recognized as the final, dominant mediator of osteoclast proliferation and activation [9]. The OPG/RANKL system acts as an important paracrine mediator of bone metabolism also in thalassemic patients. Indeed, these patients showed no differences in plasma levels of OPG in the presence of higher circulating levels of RANKL, with consequent lower OPG/RANKL ratio and increased osteoclastic activity [16]. Urinary levels of pyridinium cross-links, a marker of bone resorption, were higher in $\beta$TM patients than controls and were positively correlated with plasma levels of RANKL, pointing to a central role of the OPG/RANKL system in development of bone abnormalities in $\beta$TM. It is suggested that the OPG/RANKL pathway may be involved in mediating the skeletal actions of sex steroids in $\beta$TM patients, as indicated by the negative correlation existing between serum levels of RANKL and sex hormones [16]. Therefore, the improvement of patient compliance to hormonal replacement therapy could correct the alterations of the OPG/RANKL system, potentially normalizing bone turnover. Furthermore, since the degree of bone resorption depends on Hb levels and the severity of hypogonadism, adequate hormonal replacement therapy and annual monitoring of bone conditions may be of benefit in young adult $\beta$TM patients [17].

The negative relationship between Hb and RANKL levels as well as between erythropoietin and OPG/RANKL ratio also suggests that medullary expansion may act through enhanced RANKL levels in increasing bone resorption. Indeed, anemia, by continuously stimulating erythropoietin synthesis and hence determining bone marrow hyperplasia, may increase bone resorption through enhanced RNAKL levels [11]. In addition, the expansion of bone marrow can cause mechanical interruption of bone, cortical thinning, bone distortion, and increased fragility [14].

*3.3. Hormonal Factors.* Hormonal abnormalities, including diabetes, thyroid/parathyroid dysfunction, and hypogonadism, are believed to underlie the altered bone turnover observed in $\beta$TM [14]. In female $\beta$TM patients, low estrogen and progesterone levels enhance osteoclast activity and reduce bone formation, while in males, low testosterone levels result in a decrease in its stimulatory effects on osteoblast proliferation and differentiation [14]. In addition, insufficiency of the GH-IGF-1 axis leads to impaired osteoblast proliferation and bone matrix formation, while increasing osteoclast activation [14].

*3.4. Genetic Factors.* Genetic factors have been shown to play a role in the pathogenesis of osteopenia/osteoporosis in $\beta$TM

patients [14]. For instance, a polymorphism G → T or TT in the regulatory region of COLIA1 at the recognition site for transcription factor Sp1 is associated with the presence of osteoporosis [18]. Sp1 polymorphism occurs more frequently in females but is not specific to any ethnic group. In $\beta$TM male patients, the presence of the Sp1 mutation is associated with more severe osteoporosis of the spine and the hip compared with female patients [18]. In addition, male $\beta$TM patients who are heterozygous or homozygous at the polymorphic Sp1 site have lower BMD than females and no improvements in spinal osteoporosis in response to treatment with bisphosphonates [18]. Another study showed a consistent association between Sp1 polymorphism and vertebral osteoporosis in a sample of Italian $\beta$TM patients, suggesting the possibility that genotyping of the Sp1 site could be of clinical value for the identification of thalassemic patients at risk for osteoporosis and fractures [19].

Vitamin D receptor (VDR) polymorphisms at exon 2 (FokI) and intron 8 (BsmI) may be involved in determining the stature and BMD at femoral neck (FBMD) and lumbar spine (LBMD) in $\beta$TM patients [20]. Indeed, significantly shorter stature and lower LBMD and FBMD were observed in patients harboring the CC VDR genotype, while significant shorter height and lower LBMD have been reported in prepubertal and pubertal female patients with the BB VDR genotype [20].

*3.5. Iron Overload and Iron-Chelation Therapy.* Iron overload impairs osteoid maturation and inhibits local mineralization, resulting in focal osteomalacia [14]. In addition, the incorporation of iron in calcium hydroxyapatite affects the growth of crystals, leading to defective mineralization [14].

Deferoxamine, the most commonly used iron chelator, inhibits DNA synthesis, osteoblast and fibroblast proliferation, osteoblast precursor differentiation, and collagen formation, while enhancing osteoblast apoptosis [14].

*3.6. Miscellanea.* Nutritional deficits are commonly observed in $\beta$TM patients and may contribute to bone abnormalities. In particular, vitamin C deficiency can lead to impaired osteoblast activation and reduced collagen synthesis. Low vitamin D levels are associated with alterations in calcium/phosphate homeostasis, reduced osteoblast activity, and increased bone resorption rates [14]. Finally, decreased levels of physical activity, due to disease complications and/or overprotection, negatively influence bone turnover, leading to reduced bone formation and enhanced resorption [14].

## 4. Clinical Features

Bone marrow expansion and extramedullary hemopoiesis can result in the classical enlargement of cranial and facial bones with mongoloid appearance, as originally described by Cooley [1, 3]. Novel transfusion regimens and early iron-chelating therapy have improved the survival of $\beta$TM patients [21] and have substituted the marked bone abnormalities previously described [1] with less severe skeletal lesions. Yet, sequelae of osteopenia and severe osteoporosis represent the leading cause of morbidity in $\beta$TM patients [14, 22]. Indeed, the prevalence of osteoporosis in these patients is as high as 50%, with higher rates in males [23, 24].

In $\beta$TM patients, bone fractures range incidence between 38 and 41% and occur as a consequence of falls in over 50% of cases [14, 25]. Fractures more frequently involve the upper limb, while spine, hips, and pelvis are affected in 10% of cases [14, 25]. Due to the high bone fragility of $\beta$TM patients, fractures of long bones, especially those involving the femur, should be treated as pathological fractures and require the stabilization of the entire bone with intramedullary nailing [26].

$\beta$TM patients may also develop the so-called thalassemic osteoarthropathy, a nonerosive seronegative osteoarthropathy of varying severity, characterized by soft tissue swelling and pain, usually localized at the ankle joints [27]. Other skeletal abnormalities relatively common in $\beta$TM patients include lower and upper limb length discrepancy due to premature fusion of the epiphyseal line [28], axial deviation of the limbs, osteochondrosis, and short stature [14, 29, 30]. Involvement of the spine is frequent and can manifest as spinal deformities (e.g., scoliosis, kyphosis), vertebral collapse, cord compression, or intervertebral disc degeneration [9, 31–35].

## 5. Radiological Features

In $\beta$TM patients, the most evident radiological changes are those caused by intense marrow hyperplasia [36]. Such abnormalities include bone cortex thinning and widening of intratrabecular spaces, usually seen in hands, but also in the pelvis and ribs [36]. Extramedullary hemopoietic tissue sometimes grows beneath the periosteum, producing a scalloped cortex edge in hands, feet, tibiae, fibulae, knees, radii, and ulnae. In other cases, extramedullary hemopoietic tissues can appear as large intrathoracic masses, simulating paravertebral tumors. In the skull, significant thickening of the cranium can take place, and overgrowth of the facial bones can impede pneumatization of sinuses [36].

## 6. Conclusions

Bone changes are frequent in $\beta$TM patients and occur as a consequence of the hematological disorder and its complications as well as iron overload, iron-chelation therapy, nutritional deficits, and sedentarism. The sequalae of osteoporosis, especially vertebral and long bone fractures, represent a major cause of morbidity in these patients. A better understanding of the pathogenetic mechanisms underlying bone abnormalities in $\beta$TM is needed to develop targeted treatments. As of now, the early detection of osteoporosis and the eventual institution of bisphosphonate treatment are the most effective strategies to reduce the incidence and severity of skeletal complications. The use of new-generation iron chelators may avoid the negative effects of deferoxamine on bone metabolism. Finally, the identification and correction of nutritional and hormonal deficits and the engagement in physical training programs

should be pursued in $\beta$TM patients to reduce the incidence of osteoporosis and increase overall bone strength.

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*Review Article*

# Correlation of Oxidative Stress with Serum Trace Element Levels and Antioxidant Enzyme Status in Beta Thalassemia Major Patients: A Review of the Literature

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Beta thalassemia major is an inherited disease resulting from reduction or total lack of beta globin chains. Patients with this disease need repeated blood transfusion for survival. This may cause oxidative stress and tissue injury due to iron overload, altered antioxidant enzymes, and other essential trace element levels. The aim of this review is to scrutinize the relationship between oxidative stress and serum trace elements, degree of damage caused by oxidative stress, and the role of antioxidant enzymes in beta thalassemia major patients. The findings indicate that oxidative stress in patients with beta thalassemia major is mainly caused by tissue injury due to over production of free radical by secondary iron overload, alteration in serum trace elements and antioxidant enzymes level. The role of trace elements like selenium, copper, iron, and zinc in beta thalassemia major patients reveals a significant change of these trace elements. Studies published on the status of antioxidant enzymes like catalase, superoxide dismutase, glutathione, and glutathione S-transferase in beta thalassemia patients also showed variable results. The administration of selective antioxidants along with essential trace elements and minerals to reduce the extent of oxidative damage and related complications in beta thalassemia major still need further evaluation.

## 1. Introduction

Beta thalassemia is one of the most common inherited single gene disorder caused by about 200 mutations in the beta globin genes. In beta thalassemia where there is no or reduced production of beta globin chains, the alpha chain production will continue to occur. This increased synthesis of alpha chains makes the developing erythrocytes more fragile leading to early damage, ineffective erythropoeisis and anemia. Beta thalassemia exists in different forms depending upon the beta globin chains deficit. The most severe form among them is beta thalassemia major which occurs as a result of inheritance of two beta globin chain mutations either in homozygous or compound heterozygous states. Patients with beta thalassemia major need repeated blood transfusions for survival due to severe anemia. The beta globin chain deficit for beta-thalassemia trait (minor) is 50%, while that for beta-thalassemia major is 100% and between 50–80% for beta-thalassemia intermediate [1]. Malaysia has a multiracial population of 27.7 million, consisting of 50.8% Malays, 23.0% Chinese, and 6.9% Indians, indigenous people of Sabah and Sarawak (11.0%), and other minority groups (8.3%) [2]. According to the report of Malaysian Ministry of Health, one (1) out of twenty (20) Malaysians is a carrier of thalassemia. In Malaysia, there are about 600,000–1 (one) million thalassemia carriers. They are common among Malays, Chinese, and Sabahans but rarely among Indians and native Sarawakians. Thalassemia major is considered to be one of the life-threatening genetic disorders in Malaysia with the gene frequency of 3.4–4.5% [3]. Recurrent blood transfusions in beta thalassemia major lead to accumulation of excess iron in the body tissues. This secondary iron overload is responsible for peroxidative damage by increased production of reactive oxygen species within the erythrocytes leading to oxidative stress. This oxidative stress will cause growth failure as well as liver, cardiovascular, endocrine,

and neurological complications in beta thalassemia major. It has been evident from previous studies that iron overload is the main causative agent responsible for increased production of free radical and reactive oxygen species and subsequent oxidative stress which is compensated by various antioxidants present in the body. These antioxidants are complex molecules that protect important biological sites from oxidative injury [4] in a retrospective study involving 123 thalassemia major children found that the most common complication among these beta thalassemic children was growth failure (57.8%) which may be due to neurosecretary disturbance and insensitivity of growth hormone. They further concluded that chronic anemia and hemosiderosis may also be the contributing factors to growth failure. The next is the liver problems (21.1%), heart diseases (13.8%), and endocrinopathies (4.2%).

## 2. Oxidative Stress

Oxidative stress is defined as the interruption of balance between oxidants and reductants within the body due to the excess production of peroxides and free radicals. This imbalance will cause damage to cellular components and tissues in the body leading to oxidative stress. In patients with beta thalassemia major where frequent blood transfusions are required due to severe anemia, oxidative stress occurs as a result of increased levels of lipid peroxides and free-radical intermediates, as well as the decrease in total antioxidant capacity. Use of iron chelatory agents in combination with antioxidants can be helpful in the regulation of the antioxidant status in patients with beta thalassemia major. Oxidative stress and disturbance in antioxidant balance in beta thalassemia major has been studied extensively [5, 6]. Seventy-two children with beta thalassemia major on iron chelation therapy and 72 age-matched healthy controls irrespective of sex were included in the study. They found a significant increase in the levels of lipid peroxide and iron and significant decrease in levels of vit E and total antioxidant capacity. Serum zinc was significantly increased while copper levels decreased and there is a nonsignificant increase in erythrocyte superoxide dismutase. The results suggested that the oxidative stress and decreased antioxidant defence mechanism play an important role in the pathogenesis of beta thalassemia major.

It is concluded that repeated blood transfusions in beta thalassemia major patients causes secondary iron overload and this makes erythrocytes vulnerable to peroxidative injury [7]. Iron overload leads to peroxidative damage in beta-thalassemia major and antioxidant systems try to reduce tissue damage by lowering lipid peroxidation. They found that the markers of lipid peroxide damage such as melondialdehyde, antioxidant enzyme superoxide dismutase, and nitric oxide levels were significantly raised in thalassemia major children while mean glutathione peroxidase (GPx) levels were reduced in patients as compared to controls. These markers significantly correlated with serum ferritin levels. There was no significant difference in Glutathione (GSH) levels but it correlated with serum iron levels.

## 3. Oxidative Stress and Serum Trace Elements

Trace elements and the minerals play a vital role in the body to perform its functions properly. These elements and the minerals should present in the body in appropriate amounts and must be available for reacting with other elements to form critical molecules as well as to participate in various important chemical reactions. A number of trace elements are found in human plasma and here we are interested to discuss the correlation of trace elements like selenium, copper, zinc, and iron with oxidative stress in beta thalassemia major.

*3.1. Selenium.* One of the essential trace elements in human plasma is selenium. Selenium was first discovered as a byproduct of sulfuric acid production. It is a well-known electrometalloid and is mostly famous due to its anti cancerous properties. It is an essential constituent of the enzyme glutathione peroxidase and also incorporates in various important proteins such as hemoglobin and myoglobin. Selenium is also a component of the unusual amino acids selenocysteine which is essential for the production of various useful enzymes in the body. It helps in preventing free radical damage caused by ferrous chloride, and heme compounds. Its deficiency may affect the iron binding capacity of transferrin which leads to increase iron stores and subsequent tissue damage. An age- and gender-matched case control study has been conducted on patients with beta thalassemia major on iron chelation therapy [8]. The study indicates a significant decrease in plasma concentrations of the essential element selenium as well as decreased plasma activity of selenium-dependent antioxidant enzyme glutathione peroxidase (GPx). They also found significantly increased concentrations of all measures of body iron in beta-thalassemia patients as compared to healthy controls: another study on relationship between iron overload and antioxidant micronutrient status among 64 transfusion-dependent beta thalassemia major children on chelation therapy and 63 age- and sex- matched controls [9]. They measured serum levels of vitamins A and E, zinc, selenium, and copper and found significantly decreased levels of all these elements in beta thalassemia major children as compared to controls. There is a study done to evaluate the *in vitro* effects of vitamin C and selenium on natural killer cell activity of beta thalassemia major indicates a significant decreased in natural killer cell activity in all thalassemic patients as compared to control. The NK activity is increased by low-dose selenium treatment but no change is observed in control group. High-dose selenium decreased NK activity significantly in splenectomised patients. The result indicates the careful use of selenium dosage in thalassemic major patients [10].

*3.2. Copper.* Copper is the other essential trace element present in our bodies. It mostly forms metalloprotiens which act as enzymes. Copper is the major component of hemoglobin which is a protein responsible for oxygen transport in blood cells. Along with vitamin C, it is responsible for the production of protein called elastin thus maintaining the elasticity of the skin, blood vessels, and lungs. It is antibacterial and bears important antioxidant properties.

Copper is a central component of the antioxidant superoxide dismutase molecule and also helps in the formation of protein called ceruloplasmin thereby protecting the cells from free-radical injury. Copper is also required for the production of hormones like nor adrenaline and prostaglandins which are hormone-like chemicals involved in the regulation of blood pressure, pulse, and healing. Deficiency of this trace element will lead to anemia, neutropenia, and growth impairment, abnormalities in glucose and cholesterol metabolism, and increased rate of infections. On the other hand, an accumulation of copper in body leads to Wilson's disease with copper accumulation and cirrhosis of liver. A prospective study was performed to determine the serum levels of zinc and copper in beta thalassemia major children [11]. This cross-sectional study revealed that hypozincemia is common in thalassemic patients, but there is no copper deficiency. Another study was carried out to evaluate the level of some essential elements in one hundred and five thalassemic blood-transfusion-dependent patients and 54 healthy controls [12]. They found lower serum zinc and magnesium levels and higher copper and potassium levels in thalassemic major patients as compared to controls. Zinc deficiency may be due to hyperzincuria resulted from the release of zinc from hemolyzed red cells while hypercupremia occurs in acute and chronic infections and hemochromatosis which is the principal complication of thalassemia. A study done on status of thyroid function and iron overload in patients with beta thalassemia major on Deferoxamine in Jordan concluded that there is significantly high ($P < 0.05$) levels of serum ferritin, FT3, zinc, and copper in patients with beta thalassemia major as compared to controls [13].

*3.3. Zinc.* The next essential trace element present in the body is zinc. It takes part in various important body functions including protein synthesis, DNA synthesis, and cellular growth. It is found almost in every cell and plays a vital role in body's immune system affecting innate and acquired immunity. Zinc also has significant antioxidant properties thereby protecting the cells from damage due to free radicals. It is the active site for a number of metalloenzymes which are required for nucleic acid synthesis and also important for other host defense mechanisms like production of monocytes and macrophages and chemotaxis of granulocytes [14]. Zinc is absorbed from small intestine and found in the blood bound to albumin. Impaired growths, alopecia, loss of weight are few of the associated complications due to deficiency of zinc which is one of the factors responsible for growth and puberty disorders in thalassemic patients [15]. Frequent blood transfusions can lead to iron overload which may result in various endocrine abnormalities. They have studied two hundred twenty patients with beta thalassemia major on chelation therapy. They found that there is an association between the duration of chelation therapy and abnormalities in lumbar bone mineral density (BMD). Low serum zinc and copper was observed in 79.6% and 68% of the study population, respectively. There is significant association of serum zinc levels with lumbar but not femoral BMD. Another study was carried out to evaluate the serum copper and zinc in Jordanian thalassaemic patients. Forty two patients with $\beta$-thalassemia major on periodical blood transfusion and Deferoxamine were included in this study [16]. Forty age- and gender-matched healthy controls were included in the study. The results indicate that copper and zinc levels were significantly increased in beta thalassemia major patients compared with controls. These finding may be explained by the decreasing rate of glomerular filtration of zinc seen in chronic hemolysis and the disturbance in the metabolism of zinc and copper in thalassaemic patients due to the increasing serum zinc. The high level of copper could be due to increase absorption of copper from gastrointestinal tract. It is shown that a case control prospective study including 100 beta thalassemia major patients with heights within 3rd to 10th percentile [17]. They randomly divide patients in two groups ach comprising of 50 patients. Group 1 was given oral zinc supplements while group 2 is a control group with no zinc supplements. The patients were observed for 18 months. They found out that there is no significant difference in height between the two groups after 18 months of observation and concluded that oral zinc sulphate has no significant effect on linear growth of beta thalassemia major patient.

*3.4. Iron.* Iron is another essential trace element present in almost all cells of the body. Human body requires iron for the synthesis of oxygen carrying protein called haemoglobin found in red blood cells, and myoglobin which is also a protein found in muscles. It also takes part in the production of other important proteins in the body such as for DNA synthesis and cell division. Furthermore, iron is used in the connective tissues in our body, some of the neurotransmitters in our brain, and to maintain the immune system. Iron is transported through the blood by the serum protein, called transferrin. Transferrin is normally 30% saturated with iron. The total iron-binding capacity (tibc) reflects the status of iron in the body and is defined as the amount of iron needed for 100% transferrin saturation. The levels of TIBC are raised when the levels of iron are low thus will be helpful in the diagnosis and monitoring of iron deficiency anaemia. When iron is present in excess amounts in the body it will lead to hemochromatosis, which may be primary or secondary. Primary hemochromatosis is a genetic disorder characterized by increased iron absorption and consequent iron overload in the body. Secondary hemochromatosis occurs in diseases like thalassemia due to iron overload especially in thalassemia major where repeated blood transfusions are required. Beta thalassemia major patients require frequent blood transfusions which lead to iron overload in the absence of effective chelation therapy. This iron deposits in thalassemic patients can exceed from the storage and detoxification capacity of ferritin and also fully saturates transferrin and leads to the formation of free iron which accumulates in blood and tissues. This free iron will cause the formation of very harmful compounds, such as hydroxyl radical (OH). The hydroxyl radicals are highly reactive and attacks lipids to form lipid peroxides which contribute to oxidative stress [18]. Regular blood transfusions along with chelation therapy in beta thalassemia patients drastically improve the quality and duration of life

to third and fourth decades. Iron overload is serious complication of long-term blood transfusion. It requires adequate treatment in thalassemics so that the early deaths especially from iron-induced cardiomyopathies will be prevented. It has been shown that the cardiovascular involvement in beta thalassemia major patients without cardiac iron overload [19]. They involved twenty six patients with beta thalassemia major on chelation therapy without cardiac iron overload and thirty age- and gender-matched healthy controls in the study. The results indicated aortic stiffening associated with increased left ventricular mass and left atrial enlargement in the beta thalassemia patients as compared to controls. These changes may represent the signs of early cardiovascular involvement in beta thalassemia patients without cardiac iron overload. A retrospective chart view study revealed that three hundred and sixty transfusion-dependent beta thalassemic patients treated with Deferoxamine [20]. All patients were followed and treated from 1990–2004, disease complications were assessed by the measuring iron overload and mean serum ferritin concentrations yearly for all patients. The result showed that cardiac complications being the first most important cause of death followed by infections. Complications and deaths among these beta thalassemic major patients is iron-related organ dysfunction and age related. Furthermore, serum ferritin levels were found significantly higher in patients who died as compared to those who survived. It was also found that majority of the complicated patients were on nonoptimal chelation therapy and noncompliance. Early detection of iron overload on the heart is crucial in the management of beta thalassemia major. Serum ferritin is the poor indicator of myocardial iron deposition during early iron overload stage [21].

*3.5. Magnesium.* Magnesium is another trace element which is essential for maintaining proper body functions. It is vital for body's immune system, cardiovascular, and musculoskeletal systems. Deficiency of this element will lead to hypertension, diabetes, and cardiovascular diseases. A study was carried out to evaluate the level of some essential elements in one hundred and five thalassemic blood-transfusion-dependent patients and 54 healthy controls [12]. They found lower serum zinc and magnesium levels and higher copper and potassium levels in thalassemic major patients as compared to controls. Zinc deficiency may be due to hyperzincuria resulted from the release of zinc from hemolyzed red cells while hypercupremia occurs in acute and chronic infections and hemochromatosis which is the principal complication of thalassemia.

*3.6. Iodine.* The other vital trace element present in the body is iodine and is one of the powerful antioxidants present in the body. Bernard Courtois, a French chemist, was first discovered iodine in 1811. Iodine is present in almost every body tissue but found in greater quantities in thyroid, breast, stomach, liver, lungs, heart, adrenals, and ovaries. Iodine is important for mental and physical development and maintaining healthy immune system. It takes part in the production of thyroid hormones including thyroxin and triiodothyronine. These hormones are of primary importance in maintaining the body metabolism and brain development. Deficiency of this trace element may lead to cancer, diabetes, heart diseases, and multiple sclerosis. A study revealed increased sensitivity to the inhibitory effect of excess iodide on thyroid functions in 25 beta thalassemia major patients with normal thyroid functions [22]. The patients were given 20 mg of iodine three times daily for three weeks. They found significant decrease in concentration of thyroid hormones and significant increase in TSH concentrations with 56% of the patients reached to hypothyroid levels. They concluded that beta thalassemia major patients should not be given excess iodide due to increased sensitivity to inhibitory effects on thyroid functions as it may lead to permanent hypothyroidism. A study was carried out on long-term intensive combined chelation therapy on thyroid function in 51 beta thalassemia major patients after they achieved negative iron balance [23]. While on Deferoxamine monotherapy, eighteen patients required thyroxin but after combined therapy with deferrioxamine and deferiprone there is significant ($P < 0.0001$) decrease in iron overload and a significant increase in mean FT4 and FT3 concentrations with mean decrease in TSH. They concluded that negative iron balance can be achieved rapidly with combination chelation therapy than with monotherapy as well as there is reversal of hypothyroidism with this regime.

*3.7. Calcium.* Calcium is one of the most abundant trace elements present in the body. It is important for regulating cardiovascular, musculoskeletal, and nervous systems of the body. Deficiency of calcium may lead to rickets, osteomalacia, and osteoporosis. Excess of this trace element may lead to kidney stones, impaired renal functions, and prostate cancer. Studies have shown that calcium may activate the enzymes involved in the production of reactive oxygen species and free radicals by the mitochondria. It has been shown that seventy-five percent of patients had a low calcium level and 72.5% of patients had hypothyroidism [24]. The low calcium level was probably caused by a combination of hypoparathyroidism and osteomalacia resulting from deficient calcium intake. A case control study done on the effects of intramuscular injection of a megadose of cholecalciferol involving 40 beta thalassemia major patients and 40 nonthalassemic controls [25]. They found that among thalassemia major patients, two had hypoparathyroidism and low 25-OH D, and two had hypocalcaemia with hypophosphatemia, high alkaline phosphatase (ALP), high PTH, and serum 25-OH D below ng/mL. The remaining patients had low 25-OH D concentrations with normal serum Ca and PO4 concentrations. Vitamin D deficiency is present in 100% of thalassemia major patients and treatment with megadose injection of cholecalciferol is effective for hypovitaminosis D for 3 months. A case study done on 14-year-old girl with beta thalassemia major diagnosed since the age of 9 months came to their center with generalized tonic clonic seizure [26]. The investigations revealed diffuse intracranial calcifications in deep white matter, posterior fossa, basal ganglia, and both thalami. The laboratory and neuroimaging also indicate hypoparathyroidism. They recommend periodic assessment and control of serum calcium in all patients with thalassemia major

and prompt treatment with oral calcium and active form of vitamin D can prevent hypoparathyroidism and neurological complications in beta thalassemia major patients.

## 4. Oxidative Stress and Antioxidant Enzymes

Oxidative stress in beta thalassemia major patients activates various antioxidant enzyme systems to protect the body tissues from its damaging effects. A large number of antioxidant enzymes present in the body, here we are interested to determine the antioxidant status of the following enzymes in beta thalassemia major:

(i) superoxide dismutase,
(ii) glutathione peroxidase (GPx),
(iii) glutathione (GSH),
(iv) glutathione S transferase,
(v) catalase.

*4.1. Superoxide Dismutase.* One of the most important antioxidant enzymes present in the human body is superoxide dismutase. It exists in several different forms and was first discovered by two biochemist named Irwin Fridovich and Joe McCord. Superoxide dismutases are the proteins cofactor with copper, zinc manganese, iron, or nickel. In humans, it exists in three different forms including SOD1 found in cytoplasm, SOD2 present in cytoplasm, and SOD3 is extracellular. Superoxide is the main reactive oxygen species which react with nitric oxide radical and forms peroxynitrite thereby causing oxidative stress and cellular damage. SOD is the essential antioxidant that decreases the formation of reactive oxygen species and oxidative stress thus protecting the cells from damage. Erythrocyte superoxide dismutase protects the erythrocyte from being damaged during oxidative stress. A study revealed higher levels of erythrocyte superoxide dismutase and glutathione peroxidise (GPx) as well as higher plasma melanoyldialdehyde (MDA) in thalassemia major patients as compared to healthy controls [27]. They suggested that increased levels of malondialdehyde may be due iron overload through repeated blood transfusions and subsequent oxidative stress produced by reactive oxygen species. The rise in superoxide dismutase and glutathione peroxidase may occur as a result of compensatory mechanisms in response to oxidative stress.

*4.2. Glutathione Peroxidase.* Other important antioxidant enzymes found in the humans is glutathione peroxidase. It belongs to a group of antioxidant selenoenzymes that protects the cells from damage by catalyzing the reduction of lipid hydroperoxides. This action requires the presence of glutathione. Glutathione peroxidase levels in the body are in close relation with the glutathione which is the most important antioxidant present in the cytoplasm of the cells. The stability of the cellular and subcellular membranes depends mainly on glutathione peroxidase and the protective antioxidant effect of glutathione peroxidase depends on the presence of selenium. Glutathione peroxidase (GPx) also protects the heart from damage by oxidative stress due to oxygen free radicals through its antioxidant effect. A study was conducted on fifty six beta thalassemia major patients and fifty one healthy controls. The findings of the study confirm the peroxidative status generated by iron overload in beta thalassemia major patients and the significant increase in serum ferritin, iron, plasmatic thiobarbituric acid reactive substances (TBARS), and plasmatic superoxide dismutase (SOD) and glutathione peroxidase (GPx) activity, but vitamins E and zinc concentrations were significantly decreased in beta thalassemia major patients [28].

*4.3. Glutathione (GSH).* The next vital antioxidant enzyme in the body is glutathione which is a tripeptide containing three amino acids. It is present in almost all living cells and is considered to be the most powerful and most important antioxidant produced in the human body. It prevents damage to the cellular components by reactive oxygen species including free radicals and peroxides. It also exhibits strong anticancer and antiviral properties. Glutathione is important for the protection of proteins involved in the synthesis of nucleic acid and also helps in DNA repair. It plays an important role in the body's immune function through white blood cells as well as maintains the red blood cells integrity [29]. Glutathione is found exclusively in its reduced form (GSH). The oxidized glutathione or glutathione disulphide (GSSH) is converted to its reduced sulfhydryl form (GSH) which is a potent antioxidant, by the enzyme glutathione reductase, which becomes activated upon oxidative stress. Ratio of reduced glutathione to oxidized glutathione can be used to determine the cellular toxicity. In a study, researcher analysed glutathione reductase, glucose-6-phosphate dehydrogenase, and glutathione peroxidase in twenty five cases of homozygous beta thalassaemia, twenty cases of heterozygous beta thalassaemia and ten controls. The results indicate that significant elevation of these enzymes in homozygous beta thalassemia shows the presence of enzyme regulated glutathione turnover system in the overt state to overcome the red cell membrane damage due to autooxidant threat [30].

*4.4. Glutathione S Transferase.* Glutathione S transferase belongs to the group of enzymes that catalyze a number of reactions in the body. It catalyzes the conjugation of reduced glutathione through sulphydryl group to electrophilic centres. This activity is responsible for detoxification of compounds like lipid peroxides. It has been observed that GSTM1 which is the member of glutathione S-transferase family plays an important role in detoxification of metabolites of xenobiotics involved in cancer. Homogenous deletion of this GSTM1 results in a lack GSTM1 enzyme activity and is associated with lung, bladder, prostate, and other tumors. Genetic variations of GSTM1 enzyme are associated with patients receiving regular chelation therapy [31].

*4.5. Catalase.* Catalase was first discovered by Louis Jacques Thenard in 1818. It is an intracellular enzyme made up of four polypeptide chains with four porphyrin heme groups. Catalase is responsible for detoxification of hydrogen peroxide in the cells. Alteration in gene expression of this enzyme

will lead to increased risk of cancer. A study revealed increased levels of antioxidant enzymes superoxide dismutase, catalase, and glutathione peroxidase in red blood cells of beta thalassemia minor and near normal values of these enzymes in red blood cells of beta thalassemia major patients. They concluded that the red cells in beta thalassemia minor react to increased oxidant threat with augmented antioxidant enzyme activities while in beta thalassemia major patients normal antioxidant enzyme levels are due to presence of normal red cells because of to multiple blood transfusions [32].

## 5. Conclusion

This comprehensive review of literature indicates that oxidative stress in patients with beta thalassemia major is mainly caused by peroxidative injury due to secondary iron overload. Production of free radicals by iron overload, alteration in serum trace elements, and antioxidant enzymes status play an important role in the pathogenesis of beta thalassemia major. Impairment of the antioxidant status is associated with elevated plasma levels of lipid peroxidation. There is limited data available concerning oxidative stress, antioxidant status, degree of peroxidase damage, and role of trace elements in beta thalassemia major patients. Studies on trace elements like selenium, copper, iron, zinc, magnesium, iodine, and calcium reveal significant change in plasma concentration of these trace elements in beta thalassemia major patients. Zinc levels in beta thalassemia major patients were significantly decreased in most of the studies as compared to the controls. The reason proposed being hyperzincuria due to the release of zinc from hemolysed red cells. The patients suffering from beta thalasemia major do not survive for more than 5 years without blood transfusion [33]. A contrary study showed significantly reduced levels of serum zinc in beta thalassemia major patients [9]. Copper, another essential trace element, was found to be significantly decreased [9, 15] on thalassemia major patients but high levels of copper as compared to controls. This increased level of copper may be due to acute or chronic infections and hemochromatosis that occurs as complications in thalassemia major [12]. There is one prospective study indicating no change in serum copper levels in thalassemia major patients. Iron being the most important of all minerals was found to be significantly increased in beta thalassemia major patients [11]. Probably due to repeated blood transfusions and increased iron absorption from gastrointestinal tract. Studies also showed significantly decreased plasma concentrations of selenium in thalassemia major patients. Another important trace element is magnesium that plays an essential role in maintaining body's immune system as well as cardiovascular and musculoskeletal system found to be significantly higher in patients with beta thalassemia major as compared to controls [12]. Studies have shown that excess of iodine which is vital for the production of thyroxin and tri-iodothyronine may cause permanent hypothyroidism in beta thalassemia patients [22]. In addition, hypocalcaemia was found in beta thalassemia major patients than in controls [24, 25].

On reviewing the studies published on antioxidant enzymes status in beta thalassemia, major patients also showed variable results. A significant increase in superoxide dismutase was found in beta thalassemia major patients [27] but another study showed no significant change in superoxide dismutase, catalase, and glutathione peroxidase with possible explanation proposed to be due to the presence of normal red cells owing to multiple blood transfusions [32]. Another important antioxidant enzyme glutathione reductase found to be significantly increased in beta thalassemia major patients may be due to the presence of enzyme regulated glutathione turnover system to overcome red cell damage. In one study, Glutathione peroxidase was found to be significantly increased [28] but opposite results with significantly decreased levels of glutathione peroxidase in another study [7]. The important antioxidant enzyme glutathione S-transferase was found to have genetic variations associated with patients on chelation therapy [31].

The administration of selective antioxidants along with essential trace elements and minerals in order to reduce the extent of oxidative damage and the related complications in beta thalassemia major still need further evaluation.

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*Research Article*

# Thalassemic DNA-Containing Red Blood Cells Are under Oxidative Stress

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We studied the nature of enucleated RBCs containing DNA remnants, Howell-Jolly (HJ) RBCs and reticulocytes (retics), that are characteristically present in the circulation of thalassemic patients, especially after splenectomy. Using flow cytometry methodology, we measured oxidative status parameters of these cells in patients with $\beta$-thalassemia. In each patient studied, these cells had higher content of reactive oxygen species and exposed phosphatidylserine compared with their DNA-free counterparts. These results suggest that oxidative stress in thalassemic developing erythroid precursors might, through DNA-breakage, generate HJ-retics and HJ-RBCs and that oxidative stress-induced externalization of phosphatidylserine is involved in the removal of these cells from the circulation by the spleen, a mechanism similar to that of the removal of senescent RBCs.

## 1. Introduction

The development of red blood cells (RBCs) from their progenitors in the bone marrow includes the process of enucleation in which the final stages of nucleated erythroid precursors (orthochromatic normoblasts) expel their nuclei to generate enucleated reticulocytes (retics), which leave the marrow and mature into RBCs [1]. Normally, nucleated RBCs (normoblasts) are undetectable in the circulation, but in some hematological pathologies (e.g., thalassemia and sickle cell disease) they can be found in large numbers [1]. These diseases are also characterized by mature RBCs and retics that contain DNA remnants, that are called Howell-Jolly (HJ) bodies [1]. The frequency of these cells, which is very low, has been quantified using a flow cytometry technique [2–4].

The spleen is the major site of the reticuloendothelial system where senescent RBCs at the end of their life-span are removed by erythrophagocytosis [5]. It also removes from the circulation normoblasts and HJ-cells; thus, in thalassemia and sickle cell disease, the number of these cells in the patients' circulation increases considerably following splenectomy [1].

The removal of senescent RBCs has been attributed to various mechanisms [5], including exposure (externalization) of phosphatidylserine (PS) on their surface [6]. The macrophages of the reticuloendothelial system carry surface receptors that specifically bind PS, by which they internalize senescent RBCs [7]. The mechanism by which normoblasts and HJ-cells are removed from the circulation is unknown.

We have previously shown that in hemolytic anemias, including thalassemia and sickle cell disease, RBCs are under oxidative stress [8], and they generate more reactive oxygen species (ROS) and contain less reduced glutathione than normal RBCs, which results in membrane changes such as lipid peroxidation and externalization of PS.

Using flow cytometry, in the present study we show that HJ-RBCs and retics are under oxidative stress and carry exposed PS, which may present the trigger for their phagocytosis by macrophage and removal in the spleen.

## 2. Materials and Methods

*Blood Samples.* Peripheral blood (PB) samples were obtained from normal donors and splenectomized and nonsplenectomized patients with $\beta$-thalassemia intermedia and major. The samples were obtained from the counting vials after all diagnostic laboratory tests were completed. The research was approved by the Hadassah-Hebrew University Medical Centre Human Experimentation Review Board. The patients'

mutations and some relevant clinical parameters (e.g., transfusion and chelation therapy, splenectomy) were previously summarized [9]. In polytransfused patients, blood samples were obtained before transfusion, that is, at least 3 weeks following the previous transfusion. Informed consent was obtained in all cases.

*Flow Cytometry Measurements of Oxidative Stress Markers.* Cells were stained for transferrin-receptor by incubating with 5 $\mu$L of APC-conjugated antibodies (Ab) to CD71 at 4°C for 30 minutes. The sample was washed and then divided into two aliquots: one aliquot was stained for ROS with 2′-7′-dichlorofluorescin diacetate (DCFH, Sigma, St, Louis, MO), at final concentration of 0.1 mM, at 37°C for 15 minutes, then washed three times with $Ca^{++}$- and $Mg^{++}$-free Dulbecco's phosphate-buffered-saline (PBS) (Biological Industries, Beit-HaEmek, Israel). A stock solution of 20 mM DCF was prepared in methanol (Bio Lab, Jerusalem, Israel). The other aliquot was stained for external phosphatidylserine (PS), by suspending the cells in 100 $\mu$L of calcium buffer ((10 mM HEPES, 140 mM NaCl and 2.5 mM $CaCl_2$ (pH 7.4)) and 2 $\mu$L of FITC-conjugated Annexin-V (IQ Products, Groningen, The Netherlands). After 15 minutes at room temperature, in the dark, the cells were washed three times with calcium buffer and resuspended in 0.5 mL of the same buffer.

For every assay, 2 $\mu$L of propidium iodide (PI, Mallinckrodt Chemical Works, St. Louis, MO), dissolved in 0.1% sodium citrate, was added before analysis. Cells stained with anti-CD71 Ab alone, cells stained with anti-CD71 Ab and annexin-V, or cells stained with anti-CD71 Ab and DCF were used as controls to set the compensation levels. Following treatment as indicated above, the cells were analyzed with a Fluorescence Activated Cell Sorter (FACS-calibur, Becton-Dickinson, Immunofluorometry systems, Mountain View, CA). Instrument calibration and settings were performed using CaliBRITE-3 beads (Becton-Dickinson). The cells were passed at a rate of ~1,000 per second, using saline as the sheath fluid. A 488 nm argon laser beam was used for excitation. Threshold was set on forward light scatter (FSC) to exclude platelets and cell debris. Gates were set on RBCs, HJ-RBCs, retics, HJ-retics, normoblasts, and WBCs. Cells labeled with DCF and annexin-V were detected by the FL-1 PMT, and cells labeled with APC-conjugated anti-CD-71 Ab and PI were detected by the FL-4 and FL-2 PMT, respectively. All PMTs were set on log amplification. The Mean Fluorescence Intensities (MFIs) and the percentages of positive cells were calculated using the FACS-equipped CellQuest software (Becton-Dickinson). The results are expressed as the average ± standard deviation (SD) and compared using the two-sample Student's $t$-test for differences in means.

## 3. Results and Discussion

PB cells were simultaneously stained with an anti-CD71 Ab and PI, and either DCF or annexin-V. The anti-CD71 Ab marks the transferrin receptor, and PI the nucleic acid content. To evaluate the contribution of RNA (particularly in retics which contain small amounts of residual RNA) to the PI staining, PB cells were stained with PI in the presence or absence of RNase (0.4 mg/mL, Invitrogen, Carlsbad, CA). No difference was noted in the pattern of PI staining between these samples. The staining procedure identified cells as RBCs (CD71-PI-), HJ-RBCs (CD71-PI+), WBCs (CD71-PI++), retics (CD71+PI-), HJ-retics (CD71+PI+), and normoblasts (CD71+PI++). Figure 1(a) shows a flow cytometry dot-plot (PI versus CD71) analysis of a blood sample derived from a representative splenectomized $\beta$-thalassemic patient, indicating the various cell populations. The fluorescence distribution histograms of each cell population with respect to DCF-fluorescence, indicating generation of ROS, and annexin V-fluorescence, indicating exposed PS, with their MFIs, are shown in Figures 1(b) and 1(c), respectively. The results indicate higher ROS and PS in retics than in mature RBCs, and, more critically, in HJ-cells compared with their non-HJ counterparts: in the experiment presented in Figure 1(b), showing ROS results, the MFI of HJ-RBCs was 2.3-fold higher than that of RBCs, and the MFI of HJ-retics was 2.4-fold higher than retics. In Figure 1(c), showing PS results, the MFI of HJ-RBCs was 15.3-fold higher than that of RBCs, and the MFI of HJ-retics was 12.1-fold higher than retics.

Figure 2(a) depicts the frequency of HJ-RBCs in the PB of normal donors and in thalassemic patients. The results show no HJ-RBCs in normal donors and much higher frequency of HJ-RBCs in splenectomized patients compared with non-splenectomized patients. Figures 2(b)-2(c), which summarize the average ROS generation and percentage of PS-exposing cells, show that both parameters were significantly higher in HJ-RBCs versus RBCs and in HJ-retics versus retics. The results also show that both parameters are higher in cells from splenectomized versus nonsplenectomized patients, suggesting that the spleen removes the most damaged cells.

Although the process of nuclear expulsion from developing RBC precursors has been studied extensively [10, 11], the reasons for nuclear remnants (HJ-bodies) leftover in enucleated retics and RBCs in certain diseases have not been studied before. We now report that in $\beta$-thalassemia the generation of ROS and the externalization of PS, both parameters of oxidative stress, are elevated in HJ-retics and HJ-RBCs compared with their no-HJ-containing counterparts. ROS may be the cause of HJ formation. They are known to cause DNA breaks [12] that may generate micronuclei in various cell types [13], including lymphocytes and neutrophils. The occurrence of micronuclei has been used as a biomarker for cytogenetic damage [14, 15]. These micronuclei are equivalent to the HJ bodies in RBCs. The mechanism of HJ bodies' formation must occur prior to nuclear expulsion. We have previously demonstrated that thalassemic erythroid precursors, including orthochromatic normoblasts, are at higher oxidative status than their normal counterparts [9]. It might be hypothesized that DNA/nuclear breaks induced by oxidative stress might result in incomplete expulsion of the nuclear material, resulting in nuclear remnants which remain in retics and mature RBCs.

Several studies [16], including our own [9], indicated that ROS stimulate PS externalization on RBCs. Exposed

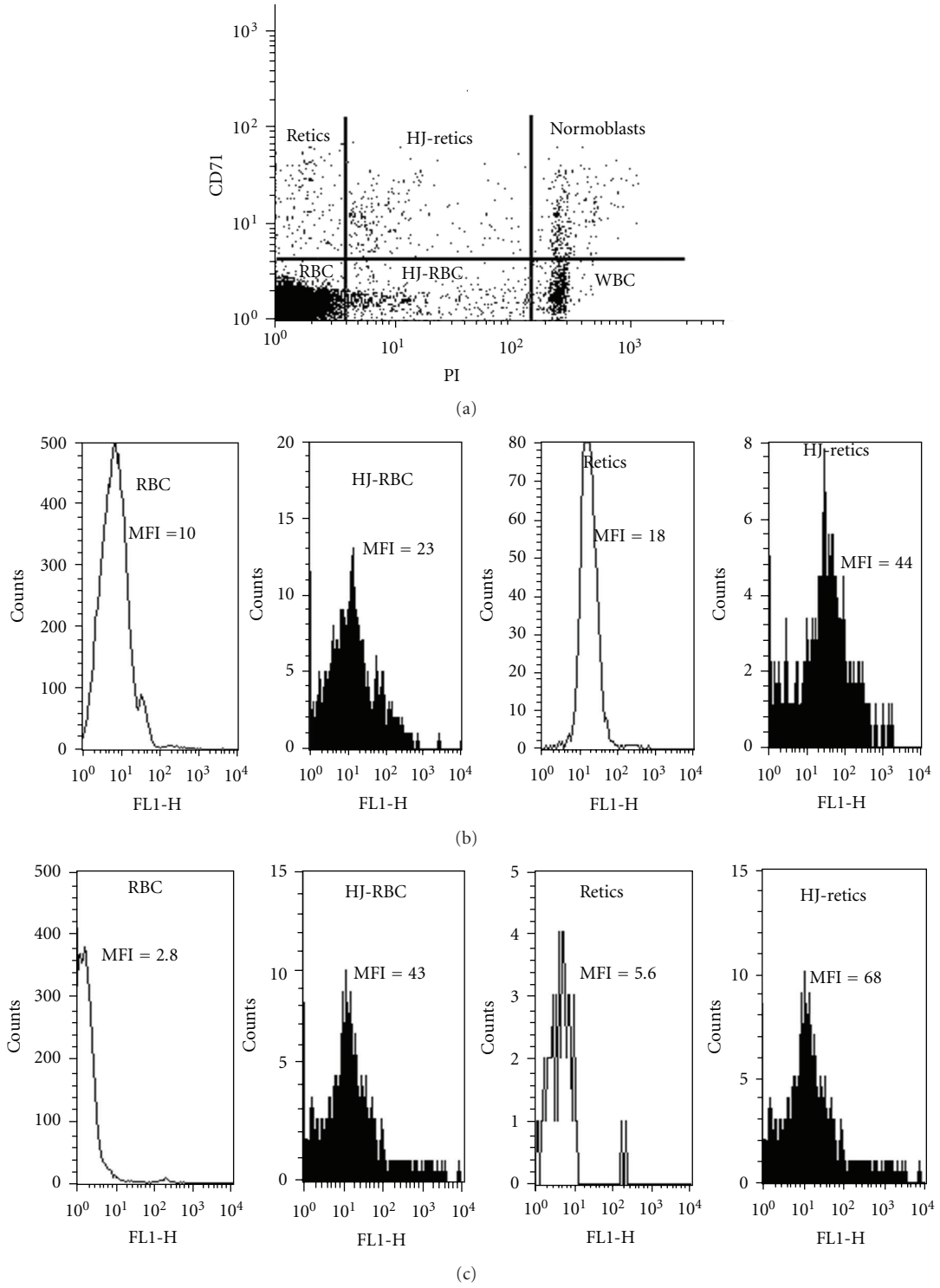


Figure 1: Flow cytometry analysis of ROS and PS in blood cells. Blood cells from a splenectomized $\beta$-thalassemic patient were simultaneously stained with an anti-CD71 antibody and propidium iodide (PI), and either DCF for measurement of ROS or annexin-V for measurement of external PS. (a) A CD71 versus PI dot-plot identifying cells as RBCs (CD71-PI-), HJ-RBCs (CD71-PI+), WBCs (CD71-PI++), retics (CD71+PI-), HJ-retics (CD71+PI+), and normoblasts (CD71+PI++). ((b)-(c)) Fluorescence distribution histograms of each cell population with respect to ROS (b) and PS (c). The results expressed as the mean fluorescence index (MFI) are presented for each cell population.

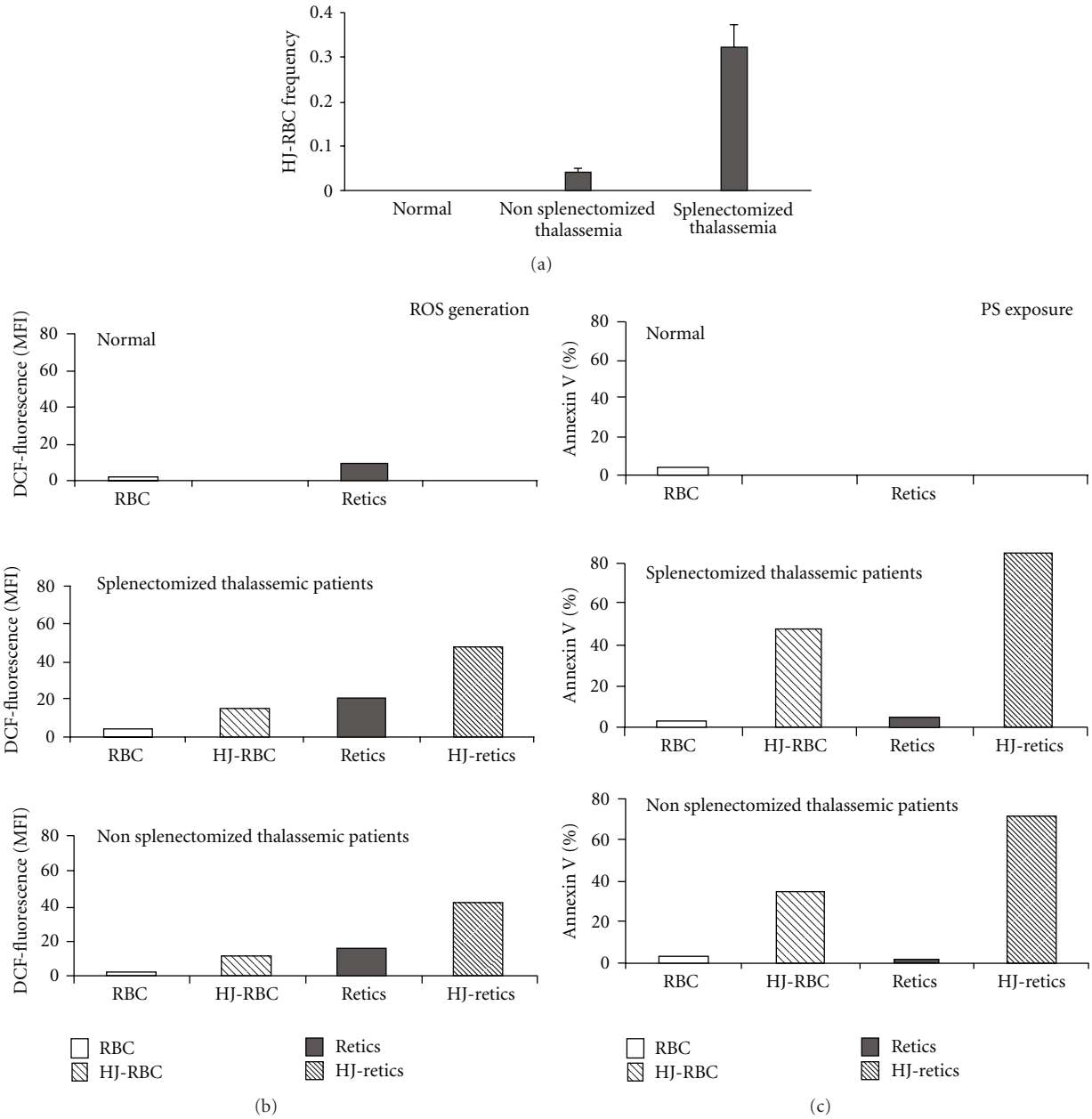


Figure 2: The frequency of HJ-cells and their oxidative status in normal donors and thalassemic patients. Cells obtained from the blood of normal donors and splenectomized and nonsplenectomized thalassemic patients ($N = 6$ in each group) were stained and analyzed as in legends to Figure 1(a). (a) The frequency of HJ-RBCs. (b) ROS generation. (c) PS exposure. The results are expressed as the percentage in the RBC population (a), the average ± S.D of the mean DCF-fluorescence index (MFI) for ROS (b) and the percentage of cells positively stained with annexin-V for PS (c).

PS was suggested, in addition to other mechanisms such as reduced expression of CD47 [17] and binding of autologous immunoglobulins and opsonins [18, 19] to signal erythrophagocytosis and removal of senescent RBCs from the circulation. To our knowledge, the signals for phagocytosis and removal of peripheral blood normoblasts or HJ-cells have not been studied. Our findings of enhanced exposure of PS on HJ-cells might suggest that exposed PS might participate in the removal of such cells by the spleen, although other signals cannot be ruled out.

In conclusion, the results of the present study suggest that oxidative stress in developing erythroid precursors might generate HJ-retics and HJ-RBCs and that oxidative stress-induced externalization of PS might be involved in their removal from the circulation by the spleen, a mechanism similar to that of the removal of aging (senescent) RBCs.

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*Research Article*

# Intracranial Blood Flow Velocity in Patients with $\beta$-Thalassemia Intermedia Using Transcranial Doppler Sonography: A Case-Control Study

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*Introduction*. Patients with $\beta$-thalassemia intermedia have a higher incidence of thromboembolic events compared to the general population. Previous studies have shown that patients with sickle cell disease, who are also prone to ischemic events, have higher intracranial arterial blood flow velocities measured by transcranial Doppler sonography (TCD). The aim of this study is to evaluate intracranial arterial flow velocities in patients with $\beta$-thalassemia intermedia and compare the results with those found in healthy subjects. *Methods*. Sixty-four patients with $\beta$-thalassemia intermedia and 30 healthy subjects underwent transcranial Doppler sonography. *Results*. Significantly higher flow velocities were found in intracranial arteries of patients compared to controls ($P = 0.001$). Previously splenectomized patients with thrombocytosis showed higher flow velocities than nonsplenectomized patients without thrombosis. *Conclusion*. The increased flow velocities in patients with $\beta$-thalassemia intermedia may point to a higher risk of ischemic events. Preventive measures such as blood transfusion or antiplatelet treatment may be beneficial in these patients.

## 1. Introduction

Patients with $\beta$-thalassemia intermedia (B-TI) seem to show higher rates of thromboembolic events than individuals without thalassemia or patients with $\beta$-thalassemia major, in particular if they have been splenectomized [1]. It is estimated that 4% of patients with $\beta$-thalassemia intermedia will experience a thromboembolic event [2]. Previous splenectomy and thrombocytosis and/or platelet abnormalities are major factors associated with thromboembolic events in patients with $\beta$-thalassemia intermedia [1, 3, 4], and ischemic stroke is increasingly recognized as one of the most devastating complications of this disease [5].

Ischemic stroke is also a known complication of sickle cell disease [6]. In a prospective study in patients with this condition, higher blood flow velocity in the intracranial arteries was associated with a higher risk of ischemic stroke [7]. The stroke prevention trial in sickle cell anemia (STOP) has indicated a role for transcranial Doppler sonography (TCD) in measuring intracranial arterial flow velocities to identify sickle cell patients at a high risk of ischemic stroke [8]. TCD measurement of intracranial flow velocities is of aid in deciding when to start blood transfusion in these patients to reduce the risk of ischemic stroke [8, 9].

An association between TCD findings and stroke risk has been confirmed in sickle cell disease; however, as far as we know, no studies have been conducted to evaluate TCD findings in patients with $\beta$-thalassemia intermedia, another high-risk group for ischemic stroke. In the present study, we compared the intracranial arterial flow velocities of $\beta$-thalassemia intermedia patients with those of healthy subjects.

## 2. Patients and Methods

This is a case-control study conducted in a tertiary outpatient clinic affiliated with Shiraz University of Medical Sciences, Southern Iran, for a period of one year during 2009.

Consecutive patients older than 15 years of age with confirmed $\beta$-thalassemia intermedia by complete blood count and hemoglobin electrophoresis who were referred to an outpatient thalassemia clinic enrolled in the study. Diagnosis of B-TI was based on complete blood count, hemoglobin electrophoresis, and initial hemoglobin (Hb) level of 7 gr/dL, and age of diagnosed anemia was after 2. All of them were transfusion independent. Patients were recruited at a routine follow-up visit with a hematologist in the clinic. Exclusion criteria were a history of diabetes mellitus, hypertension, ischemic heart disease, thrombosis, previous cerebrovascular disease, sickle cell anemia, or inadequate temporal window for TCD. The study was approved by the Ethics Committee of Shiraz University of Medical Sciences (no. 2885), and written informed consent to participate was obtained from all patients or their first-degree families. All patients were receiving folic acid (5 mg/day) and hydroxyurea (8–15 mg/kg/day).

For each patient, we completed a data collection form that included age, sex, place of residence, prior splenectomy, prior transfusion, history of thrombosis, previous stroke or transient ischemic attack, and laboratory information (complete blood cell count, ferritin, blood urea nitrogen, creatinine, alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, and albumin). Thrombocytosis was defined as a platelet count >500,000/dL.

Transcranial Doppler ultrasound was carried out in all patients. All TCD studies were performed by one investigator using a Legend TC22 transcranial Doppler ultrasound unit (Bristol, UK) with a 2-MHz transducer. The two middle cerebral arteries (MCAs), anterior cerebral arteries (ACAs), posterior cerebral arteries (PCAs), and the terminal internal carotid arteries were insonated using a temporal window approach (Eleven patients had poor temporal window who were excluded from the study). The basilar artery (BA) and vertebral arteries were examined through a suboccipital approach in sitting position. The highest mean flow velocity of each artery was recorded separately.

The control group consisted of 30 sex/age-matched subjects with no known hematologic disease. The same exclusion criteria as for the patients were used for the controls. All the control subjects underwent TCD examination with the same protocol.

The Mann-Whitney $U$ test, Pearson correlation coefficient ($r$), and $t$-test were used for comparison of the variables between groups. Results are expressed as percentages and absolute frequencies, where appropriate. Descriptive results are presented as the mean ± standard deviation (SD). $P$ values <0.05 were considered significant. Statistical analyses were performed in SPSS version 15.0 (SPSS, Chicago, Ill, USA).

## 3. Results

After applying the inclusion and exclusion criteria, 64 patients with $\beta$-thalassemia intermedia and 30 healthy subjects were recruited. There were no significant differences between patients and controls according to sex (male: 40.6% versus 53.3%; $P = 0.251$) or age (23.6 ± 5.2 versus 25.4 ± 5.4; $P = 0.001$). Mean velocities of all mentioned vessels were measured in all patients and control subjects, and no missing vessel was detected. Among the patients, 54.7% (35/64) had undergone splenectomy, and 9.4% (6/64) had received a blood transfusion once or twice a year before the study. None of the control subjects had received transfusion. In patients, mean Hb level was 9.3 ± 1.2 g/dL (range 6.9–12.3), mean white blood cell count was 8873 ± 2004/dL (range 4900–13800), and mean platelet count was $523 \times 10^3 \pm 219 \times 10^3$/dL (range $188 \times 10^3$–$1035 \times 10^3$). There were no significant differences in age, white blood cell count, or Hb level between splenectomized and nonsplenectomized patients ($P > 0.05$). Mean platelet count was significantly higher in patients who had undergone splenectomy ($696 \times 10^3 \pm 129 \times 10^3$/dL versus $315 \times 10^3 \pm 78 \times 10^3$/dL; $P = 0.001$). All the splenectomized patients had thrombocytosis, and none of the nonsplenectomized patients had thrombocytosis. Platelet count correlated with blood flow velocity in the right MCA ($r = 0.291$, $P = 0.020$), left MCA ($r = 0.366$, $P = 0.003$), left ACA ($r = 0.258$, $P = 0.040$), right PCA ($r = 0.270$, $P = 0.031$), left PCA ($r = 0.267$, $P = 0.033$), and BA ($r = 0.300$, $P = 0.016$). There were no correlations between platelet count and blood flow velocities of the other arteries ($P > 0.05$). Mean intracranial arterial flow velocities in $\beta$-thalassemia intermedia patients with or without splenectomy are shown in Table 1. There were no significant differences in white blood cell count, Hb levels, or platelet count between patients who had undergone transfusion and those who had not ($P > 0.05$).

Table 1: Mean intracranial arterial flow velocities in $\beta$-thalassemia intermedia patients with or without splenectomy.

| | Without splenectomy | With splenectomy | $P$ value |
|---|---|---|---|
| Left ICA | 84.6 ± 24.4 | 94.1 ± 20.7 | 0.097 |
| Right ICA | 83.1 ± 25.6 | 86.5 ± 25.1 | 0.538 |
| Left MCA | 66.6 ± 26.9 | 87.9 ± 23.9 | 0.001 |
| Right MCA | 71.3 ± 28.2 | 85.8 ± 22.7 | 0.026 |
| Left ACA | 58.2 ± 19.7 | 73.4 ± 18.3 | 0.002 |
| Right ACA | 57.3 ± 19.0 | 69.2 ± 18.2 | 0.013 |
| Left PCA | 35.1 ± 11.7 | 42.1 ± 11.6 | 0.021 |
| Right PCA | 34.8 ± 10.8 | 41.7 ± 14.3 | 0.037 |
| Left vertebral artery | 52.2 ± 15.0 | 58.3 ± 14.5 | 0.102 |
| Right vertebral artery | 55.6 ± 15.3 | 62.4 ± 17.5 | 0.106 |
| Basilar artery | 66.1 ± 17.2 | 76.7 ± 16.6 | 0.016 |

ICA, internal carotid artery; MCA, middle cerebral artery; ACA, anterior cerebral artery; PCA, posterior cerebral artery.

Comparison of the mean intracranial artery flow velocities between patients and controls showed significantly higher velocities in all intracranial arteries of patients ($P = 0.001$). Mean flow velocities recorded in patients and controls are shown in Table 2.

Table 2: Mean intracranial arterial flow velocities in $\beta$-thalassemia intermedia patients and healthy controls.

| | Patients | Healthy subjects | $P$ value |
|---|---|---|---|
| Left ICA | 89.8 ± 22.8 | 52.1 ± 8.6 | 0.001 |
| Right ICA | 84.9 ± 21.7 | 50.0 ± 8.4 | 0.001 |
| Left MCA | 78.3 ± 27.3 | 49.4 ± 9.1 | 0.001 |
| Right MCA | 79.3 ± 26.2 | 48.7 ± 7.6 | 0.001 |
| Left ACA | 66.5 ± 20.3 | 44.4 ± 9.5 | 0.001 |
| Right ACA | 63.8 ± 19.4 | 42.5 ± 7.8 | 0.001 |
| Left PCA | 38.9 ± 12.1 | 29.7 ± 6.6 | 0.001 |
| Right PCA | 38.6 ± 13.2 | 27.3 ± 6.3 | 0.001 |
| Left vertebral artery | 55.5 ± 14.9 | 29.5 ± 4.6 | 0.001 |
| Right vertebral artery | 59.3 ± 16.8 | 29.3 ± 4.6 | 0.001 |
| Basilar artery | 71.9 ± 17.6 | 47.1 ± 8.2 | 0.001 |

ICA, internal carotid artery; MCA, middle cerebral artery; ACA, anterior cerebral artery; PCA, posterior cerebral artery.

## 4. Discussion

In this study, higher blood flow velocities were found in all intracranial arteries of our patients. Flow velocity was higher in most arteries of splenectomized patients compared to nonsplenectomized patients, especially in the anterior circulation. In addition, there was a correlation between platelet count and flow velocities.

Numerous studies in sickle cell anemia have evaluated the role of TCD as a screening tool to identify and followup patients at high risk of ischemic stroke [7–10]. TCD can also detect asymptomatic cerebrovascular disease in patients with sickle $\beta$-thalassemia [11], and the findings on TCD study show a good correlation with cerebral angiography [12]. However, no such studies have been performed in $\beta$-thalassemia intermedia, another hematologic disease in which there is a substantial associated risk of ischemic stroke. Screening of asymptomatic $\beta$-thalassemia intermedia patients by TCD has not been reported previously, and the impact of this measure on further stroke remains to be defined. TI patients are prone to thromboembolic event, especially those patients associated with splenectonomy and thrombocytosis [2, 3].

There is growing evidence that increased intracranial arterial flow velocities associate with a higher risk of ischemic stroke [7, 13]. The recommendation for chronic red blood cell transfusion in patients with high stroke risk is based on findings from the STOP trial, in which patients with high intracranial arterial flow velocities showed a 90% reduction in stroke rate following this treatment [8]. Patients with $\beta$-thalassemia intermedia do not regularly receive blood transfusion, and they have a higher risk of ischemic stroke than $\beta$-thalassemia major patients, who are often treated with transfusion [3]. In addition, a higher rate of thromboembolic events occurs in splenectomized $\beta$-thalassemia intermedia patients [3, 14], who usually have a higher platelet count. The presence of a high flow velocity in patients with $\beta$-thalassemia intermedia may herald a cerebrovascular event and indicate a need for special attention. The association of flow velocity with splenectomy and platelet count suggests that these two factors should also be taken into account when interpreting stroke risk in a patient with $\beta$-thalassemia intermedia.

This study has some limitations. Patients were not prospectively followedup to clarify the impact of higher blood flow velocities on the risk of ischemic stroke. The interpretation presented here is based on previous studies, mainly in sickle cell disease, which showed a higher risk of stroke in association with higher flow velocities measured by TCD. Furthermore, we did not measure flow velocities before and after blood transfusion to estimate the impact of this measure on reducing velocities.

In conclusion, in this first study evaluating TCD findings in $\beta$-thalassemia intermedia patients, higher intracranial arterial blood flow velocities were found in comparison to normal subjects. These findings may indicate that these patients are at a higher risk of ischemic events and that preventive measures, such as blood transfusion or antiplatelet drug administration, could be beneficial. Nonetheless, prospective randomized clinical trials would be needed to establish recommendations in this regard.

## Conflict of Interests

All the authors declare that they have no conflict of interests.

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