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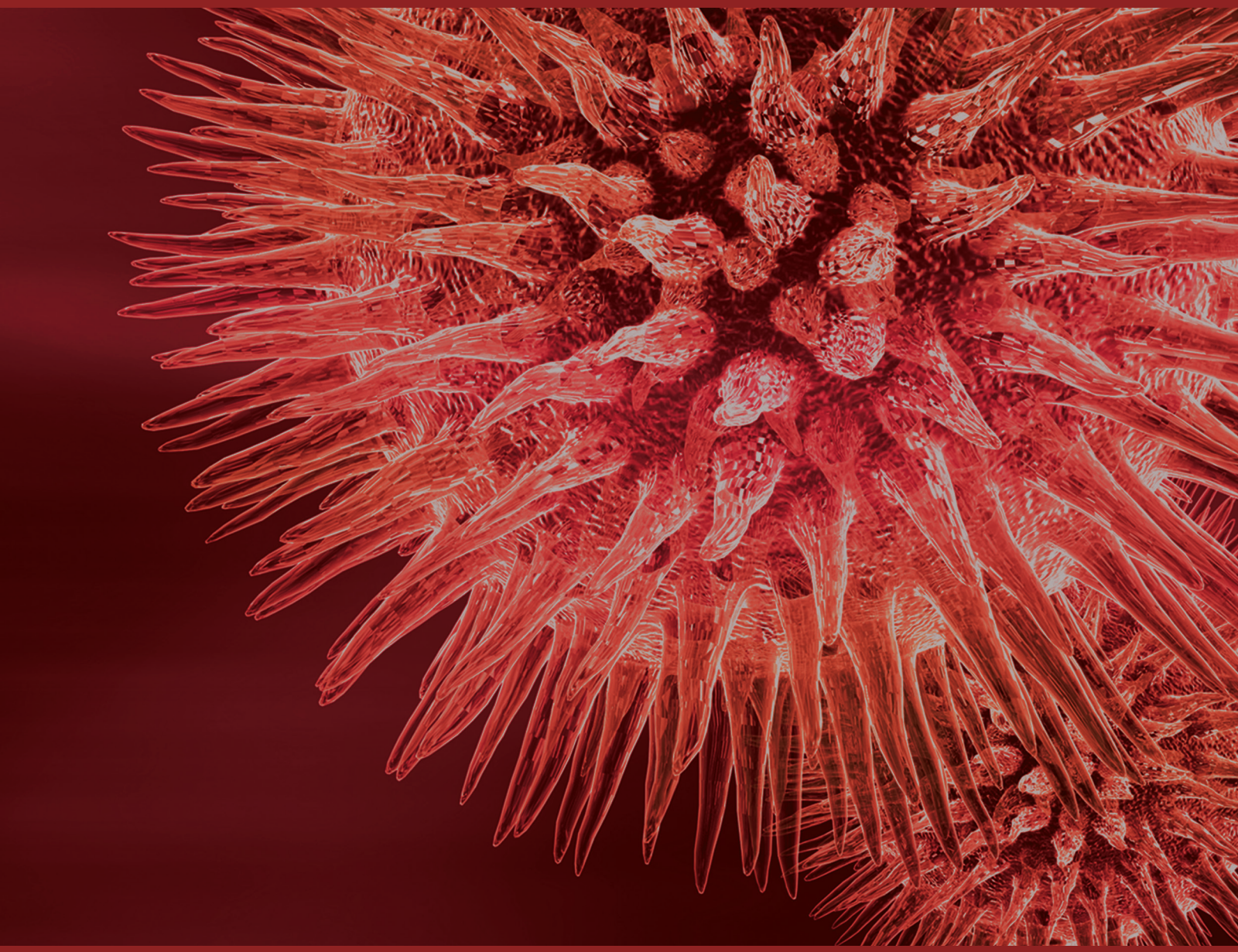
Guest Editors: Paolo Ricchi, Aldo Filosa, Aurelio Maggio, and Suthat Fucharoen



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Editorial

Non-Transfusion-Dependent Thalassemia: A Complex Mix of Genetic Entities Yet to Be Fully Discovered

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The management of patients with non-transfusion-dependent thalassaemia (NTDT) has been a challenging task: in fact, within this conventional definition, clinicians have to deal with a great variety of syndromes mixed in terms of their molecular background, clinical course, and severity which share only the characteristics that are not entirely dependent on transfusions [1, 2]. In fact, NTDT phenotypes include patients with β-thalassemia intermedia, hemoglobin E/β-thalassemia, and Hemoglobin H disease (α-thalassemia intermedia) but also those with structural variant of hemoglobin associated with "α" or "β" thalassemia in heterozygous condition which often have analogous characteristics [3]. However, among different genetic entities of NTDT, the lack of a clear genotype-phenotype relationship further complicates this complex and extensive scenario in clinical practice [4]. Thus, despite the availability of recent guideline from Thalassemia International Federation, the strength of several treatments and follow-up recommended strategies should be confirmed in selected population [5]; in fact, most of these recommendations arise from retrospective and cross sectional studies where different patients were heterogeneously treated along their life with occasional transfusions, iron chelation, and splenectomy. Furthermore, in clinical practice, most of these recommendations are difficult to transfer into the "wide-spectrum" of phenotypes particularly in the case of patients first diagnosed in adult life.

This special issue was launched to provide some insights into this wide subject, which is continually evolving. Out of the five articles published in this special issue on NTDT, two are updated reviews. It is well worth it to note that one focused on the role of oxidative damage by reactive oxygen species (generated by free globin chains and labile plasma iron) as a potential additive contributor to cell injury, tissue damage, and hypercoagulability observed in patients with NTDT; the other was devoted to expanding the emerging setting of endocrine and bone disease in NTDT. We collected also three original articles, two describing the profile of HbH disease in Taiwan and its incidence in Lebanon, respectively, and one evaluating again the endocrine and bone disease in β-thalassemia intermedia patients. Obviously, some of this epidemiological information could be only of regional interest but certainly adds some more clinical useful information.

In conclusion, despite the great effort and benefit to encompass such a variety of syndromes in the acronyms NTDT, we should be always prepared to face the limitation of mixing heterogeneous patients within a single category. Further studies are needed to better define and compare at clinical and molecular level the different genetic entities of NTDT. The recent finding of similar degrees of anemia but diverse patterns of the GDF15-hepcidin-ferritin axis between β-thalassemia intermedia and HbH disease in a Sardinian series should stimulate extending such comparative evaluation among all different classes of NTDT [6]. We hope that

readers of this special issue will find our articles accurate and updated and will focus on the need of developing more studies in this intricate and not fully explored setting.

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Review Article

Endocrine and Bone Complications in β-Thalassemia Intermedia: Current Understanding and Treatment

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Thalassemia intermedia (TI), also known as nontransfusion dependent thalassemia (NTDT), is a type of thalassemia where affected patients do not require lifelong regular transfusions for survival but may require occasional or even frequent transfusions in certain clinical settings and for defined periods of time. NTDT encompasses three distinct clinical forms: β-thalassemia intermedia (β-TI), Hb E/β-thalassemia, and α-thalassemia intermedia (Hb H disease). Over the past decade, our understanding of the molecular features, pathophysiology, and complications of NTDT particularly β-TI has increased tremendously but data on optimal treatment of disease and its various complications are still lacking. In this paper, we shall review a group of commonly encountered complications in β-TI, mainly endocrine and bone complications.

1. Introduction

β-thalassemia, one of the most common monogenetic diseases worldwide, constitutes a group of hereditary blood disorders resulting from a defect in the synthesis of the β-globin chain [1, 2]. This defect causes a disproportionate ratio of alpha- and beta-globin chain synthesis leading to ineffective erythropoiesis (IE) and a chronic hemolytic anemia. Based on genetic and clinical features, β-thalassemia is divided into 3 distinct categories: thalassemia major, thalassemia intermedia, and thalassemia minor. Patients with β-thalassemia major (β-TM) harbor two defective copies of the *β-globin* chain and present during the first 2 years of life with a severe lifelong transfusion dependent microcytic anemia. People with thalassemia minor or the carrier state have one defective copy of the β-globin chain (heterozygous) and are usually clinically silent [1–3].

Beta thalassemia intermedia (β-TI) is a disease of intermediate severity where affected patients usually present with a later onset of microcytic anemia and milder clinical symptoms compared to β-TM. It belongs to the nontransfusion dependent thalassemia (NTDT) group which also includes α-thalassemia intermedia (hemoglobin H disease) and hemoglobin E/β-thalassemia (mild and moderate forms). It arises from a homozygous or a compound heterozygous mutation leading to partial suppression of beta-globin protein production. Three mechanisms are responsible for the milder phenotype of β-TI: inheritance of a mild or silent beta-chain mutation, coinheritance of α-thalassemia, and hereditary persistence of HbF, $\delta\beta$-thalassemia, and Gγ XMN1 polymorphism [2–4].

The clinical manifestations and complications of β-TI are unique and different from well treated β-TM patients but are similar to β-TM patients who are poorly transfused. Hence, it is plausible that the manifestation of β-TI are due to dyserythropoiesis. The disease is characterized by marked phenotypic heterogeneity with some patients remaining asymptomatic and maintaining a baseline hemoglobin range of 7–10 g/dL, while some others requiring transfusions due mostly to suboptimal growth and development, skeletal

deformity, exercise intolerance, and declining hemoglobin levels because of progressive splenomegaly. Typical physical exam findings include growth retardation, thalassemic bone deformities, splenomegaly, and moderate to severe hepatomegaly [2, 3, 5].

The triad of chronic anemia, ineffective erythropoiesis, and iron overload characterizes β-TI and is mostly responsible for its clinical sequelae. Other disease complications include endocrinopathies, bone disorders, and end organ damage. Some complications as extramedullary hematopoiesis, leg ulcers, gallstones, thrombosis, and pulmonary hypertension, are rarely encountered in β-TM, but frequently seen in β-TI [2–4, 6, 7]. Older age and splenectomy have been shown to be independently associated with an increased risk of most β-TI disease-related complications [8, 9]. Much of the disease associated morbidity and mortality can be reduced with regular surveillance, early treatment, and follow-up in a comprehensive multidisciplinary setting.

Treatment of β-TI needs to be individualized and tailored to the patient's clinical scenario. Conventional treatment consists of transfusions, iron chelation, splenectomy, supportive therapies, and psychological support. Nonconventional treatment includes hematopoietic stem cell transplantation which remains to be the only curative treatment, fetal hemoglobin modulation, and gene therapy [2, 4, 5, 9].

In this review, we shall cover two of the major complications encountered in β-TI: endocrine and bone complications. We will also shed light on iron overload in β-TI as well as other mechanisms that may lead to these complications.

2. Iron Overload in β-TI

Iron overload in β-TI is multifactorial and attributed primarily to increased gastrointestinal iron absorption [10]. It can also result from chronic hemolysis and occasional blood transfusions required to treat disease complications [10, 11]. In response to chronic anemia and ineffective erythropoiesis, levels of growth and differentiation factor 15 (GDF 15), twisted gastrulation factor 1, and hypoxia-inducible transcription factors (HIFs) increase leading to hepcidin suppression and ferroportin and erythropoietin upregulation [11–14]. The outcome is an increase in duodenal iron absorption and release of iron from the reticuloendothelial system [11]. Increased gastrointestinal iron absorption, coupled with transfusions, leads to iron overload. Even though iron overload occurs in patients with β-TI at a much slower rate than that in those with regularly transfused β-TM, with advancing age, it can reach levels much higher than normal thresholds with markedly elevated liver iron concentration (LIC) and high levels of circulating toxic non-transferrin-bound iron (NTBI).

Serum ferritin levels and LIC determined by R2 and R2* magnetic resonance imaging (MRI) positively correlate in β-TI patients [15–17] where 800 and 300 ng/mL of serum ferritin correspond to 5 mg and 3 mgs Fe/g dry weight (DW) [18]. Vascular, endocrine, and bone morbidity in β-TI has been shown to be significantly associated with serum ferritin more than 800 ng/mL and LIC more than 6-7 mg Fe/g DW [18–21]. Spot ferritin measurements, however, may underestimate the burden of iron overload and subsequently delay therapy [20]. LIC, which is the reliable and noninvasive gold standard, approximates iron overload better than serum ferritin [16, 20, 22, 23].

If untreated, iron overload will lead to organ dysfunction involving mostly the liver, heart, and endocrine organs and a wide spectrum of complications and clinical outcomes. A recent study (THALASSA) on 95 patients with NTDT showed efficacy of the once daily oral iron chelator deferasirox in patients at least 10 years of age with LIC $\geq$ 5 mg Fe/g DW and serum ferritin of at least 800 ng/mL [18]. Despite the availability of chelation therapy including oral agents, iron overload remains a problem because of poor adherence to chelation regimens and high cost of such treatment.

3. Endocrine Complications in β-TI

Endocrine complications are amongst the most common complications in β-TI and are mostly attributed to iron overload and suboptimal chelation [2–4, 6, 24]. They are associated with splenectomy, increasing age, severe ineffective erythropoiesis, and low fetal hemoglobin levels [9, 24]. The frequency of these complications is lower than that in β-TM and varies greatly according to severity of the anemia and iron overload [25]. Earlier onset of these complications is observed with higher LIC compared to lower concentrations [21] and a lower frequency has been observed in patients on iron chelation therapy and or on hydroxyurea [9]. However, no relationship could be established between endocrine dysfunction and serum ferritin level, age of start of desferrioxamine, and hemoglobin level [26, 27].

The most frequent endocrine complications reported in β-TI are growth retardation, delayed puberty, hypogonadism, diabetes, impaired thyroid, parathyroid and adrenal functions, and dyslipidemias. Early recognition and treatment of endocrine complications is important in order to prevent late irreversible sequelae and increase the chances of successful reproduction. Patients with established endocrine disease should be referred to an endocrinologist and managed according to recommendations in β-TM patients [2, 4, 6, 24, 26, 27].

The sections below will offer a detailed up to date review of the most frequent endocrine complications encountered in β-TI. They will also summarize important recommendations for screening and management of these complications and highlight the 2013 Thalassemia International Federation (TIF) Guidelines for the Management of NTDT [24].

3.1. Growth Retardation. The prevalence of short stature in children and adults with thalassemia is approximately 25% regardless of the type of the thalassemia and serum ferritin concentration [25]. 20%–30% of thalassemic patients have growth hormone (GH) deficiency and 70–80% have peak growth hormone (GH) levels on provocative tests lower than those seen in patients with constitutional short stature [28]. In β-TI, growth hormone deficiency is seen in 31% of patients (28) while the prevalence of short stature (height more than 2 SD below man height for age (below 3rd percentile))

varies between reports ranging from 7 to 46% [26, 27]. In children and adolescents, hypogonadism has been shown to be associated with short stature and GH deficiency to be a significant negative predictor of height. In adults, GH deficiency was the only significant predictor of short stature [25].

The pathogenesis of growth failure in thalassemia is multifactorial and is mainly due to transfusional iron overload and resulting endocrinopathies (GH deficiency, hypothyroidism diabetes), nutritional deficiencies, and intensive use of chelating agents particularly desferrioxamine. Other aetiologies particularly in suboptimally treated children are increased metabolism, chronic anemia, and hypoxia. The anterior pituitary is particularly sensitive to iron associated free radical oxidative stress. Even a modest amount of iron deposition in the anterior pituitary by MRI can interfere with its function. Dysregulation of the GH insulin like growth factor axis leads to growth hormone deficiency and growth deceleration [25, 28].

All patients with NTDT including those with β-TI who are ≥10 years should undergo standing and sitting height every 6 months, bone age, growth hormone stimulation, insulin-like growth factor (IGF)-1 level, and IGF-BP3 level (in patients who fall-off the growth curve (5%) and have decreased height velocity or delayed bone age, desferrioxamine toxicity, and other hormonal and nutritional imbalances) [24].

Frequent transfusions should be considered in patients with growth failure (height is more indicative of growth pattern than weight) with reassessment for tapering or withdrawal when a sustained clinical benefit is achieved [24].

3.2. Delayed Puberty and Hypogonadism. Delayed puberty and hypogonadism are the most common endocrine common complications in β-TI and are attributed to iron-mediated damage leading to dysregulation of the hypothalamic-pituitary axis [9, 25, 26, 28–30]. Delayed puberty is defined as no puberty in girls by 13 years and in boys by 14 years. Hypogonadism is defined as absence of testicular development in boys and breast development in girls by 16 years. Hypogonadic hypogonadism is the most frequent and often undertreated endocrine complication of β-TI, seen in 24% of patients, affecting females more than males [9, 25, 30]. It has been shown to be correlated with early onset of transfusion therapy and serum ferritin levels of approximately 2000 ng/mL in TM patients [31] and high LIC [21], increasing age [25] and HU treatment [9] in β-TI patients. It can lead to maternal and fetal problems even when iron chelation therapy is employed [32–35]. However, gonadal function of women with this complication is not affected and fertility can be salvaged [36].

Routine early screening for delayed puberty and hypogonadism is warranted to initiate treatment and prevent complications. Tanner staging every 6 months for prepubescent children and annual evaluation of luteinizing hormone, follicular stimulating hormone, insulin-like growth factor (IGF), and IGF-binding protein-3 for children between 8 and 10 years are recommended [37]. Testosterone, estradiol, pelvic ultrasound, nutrient levels, and thyroid function are also indicated for patients with evidence of pubertal delay. Routine assessment for infertility, secondary hypogonadism, and impotence needs to be performed in all adults [24].

Literature still lacks accurate management guidelines for delayed puberty and hypogonadism in β-TI. Gonadal steroids (ethinyl estradiol, estrogen-progesterone hormone, and testosterone esters) and gonadotropin releasing hormone should be initiated for females >13 and males >16 years of age not showing pubertal change [7, 25, 37]. Human chorionic gonadotropin (hCG), human menopausal gonadotropin (hMG), and intracytoplasmic sperm injection (ICSI) can be offered to males with spermatogenesis problems to assist in attaining pregnancy in the partner [7]. In females with chronic anovulation, stimulation with gonadotropins can still increase estradiol and produce ova, but global assessment of the patient is necessary before induction of pregnancy [38].

3.3. Fertility and Pregnancy. In most β-TI patients, fertility is not affected and most pregnancies can be achieved spontaneously [38, 39]. Pregnancy in this patient population is, however, associated with intrauterine growth retardation (IUGR) in more than half of cases, higher risk of abortion, preterm and cesarean section delivery, and thromboembolic events [40, 41]. This is attributed to multiple factors including anemia, hypoxia, acute hypersplenism, splenomegaly, and hypercoagulability state [40]. Splenectomy may be required after or even during gestation. With advancing pregnancy, anemia increases and blood transfusion therapy may be needed but it is often limited by the risk of alloimmunization in previously untransfused women [42]. Increased risk of thromboembolism may necessitate short-term anticoagulation with low-molecular weight heparin and platelet anticoagulants followed by a long-term oral anticoagulant [5, 40]. Folic acid deficiency due to increased erythropoiesis, poor absorption, and low dietary intake is common and folic acid supplements are recommended to prevent fetal neural tube defects [5, 11, 43]. Optimal management of pregnant β-TI women, therefore, requires a multidisciplinary approach with close maternal and fetal surveillance. Assessment of iron overload, cardiac, endocrine, liver, viral, and red blood cell antibodies status before pregnancy and ensuring adequate management are recommended [24].

3.4. Diabetes Mellitus and Glucose Intolerance. Glucose tolerance abnormalities and diabetes mellitus are common complications in thalassemia patients. While glucose intolerance occurs at an earlier stage during adolescence in β-TI patients, diabetes frequently occurs at later stages and is usually secondary to iron overload and subsequent chronic liver disease [34, 44]. The prevalence of diabetes and glucose intolerance is 9.4% [34] and 7.1% [34, 44] in β-TM and 2% and 24% in β-TI [26, 27].

The development of diabetes in thalassemia is attributed to impaired insulin excretory function secondary to chronic iron overload in the pancreas [45], selective immune system activation against pancreatic β-cells leading to cell damage [46], and/or pancreatic cell death due to fat transformation

[47]. In addition, Noetzli et al. (2012) showed that impaired insulin sensitivity was associated with inflammation markers and somatic iron overload [48]. Even though several studies correlated elevated LIC with development of diabetes [49, 50], a single measurement of LIC is a poor predictor of endocrine failure, especially pancreatic iron deposition [51, 52]. A pancreatic MRI $T2^*$ coupled with a gradient echo sequence is recommended for detecting pancreatic fat and predicting the incidence of diabetes [52]. A serum ferritin level of around 3000 ng/mL has been shown to be associated with a higher risk of developing diabetes [31].

Iron-mediated diabetes can be partially reversed if treated earlier [53]. High doses of insulin are required to correct blood glucose levels in these diabetic patients [50]. Experts recommend early screening and detection of glucose impairments and insulin resistance in all thalassemia patients starting at age of 8 to 10 years as the disease can be halted before developing into overt-diabetes in adulthood [25, 43]. According to TIF NTDT guidelines, patients with β-TI who are ≥10 years should undergo annual fasting blood sugar and if indicated oral glucose tolerance test [24].

3.5. Dyslipidemia. Lipid profile is altered in patients with β-TM and β-TI [54]. Serum total cholesterol (TC) and low-density lipoprotein cholesterol (LDL-C) levels are lower in thalassemia patients than normal controls, while triglycerides (TG) levels are not significantly different or elevated compared to normal controls [54–58]. In β-TI patients, lipid profile variations are common but not a troubling feature of the disease. A study conducted by Hartman et al. has revealed that the levels of TC, HDL-C, and LDL-C were significantly lower among children and adolescents with β-TI compared to β-TM and healthy controls [54]. These values were independent of age, sex, ferritin, and hemoglobin levels [54]. The mechanism and pathophysiology behind such findings are still unclear and it is hypothesized that in β-TI, lipid profile changes are secondary to continuous erythropoietic activity and elevated cholesterol consumption [51, 59]. In β-TM, on the other hand, oxidative stress and high iron overload appear to be the main contributors [56, 60]. Liver damage may also play an important role in determining the altered lipoprotein pattern in beta-thalassemia [57]. In addition, vitamin E plays an important role in the reduction of LDL oxidation as they were both correlated. A study conducted on 30 individuals with TI has shown that the content of plasma and LDL α-tocopherol was significantly lower as compared to the control group [61]. While the studies showing the efficacy of vitamin E are limited to 15 patients with TI that were treated with 600 mg/day for a period of 9 months, the levels of vitamin E started to increase within 3 months as well as the levels of LDL [62].

Routine lipid profile investigation in young β-TI patients is not recommended [54]. More investigations are, however, required to show the basic mechanism of the dyslipidemia in this thalassemia subgroup and whether calibrating serum lipid levels may affect the disease outcome.

3.6. Hypothyroidism and Hypoparathyroidism. Hypothyroidism is a late consequence of iron deposition in the thyroid gland that ultimately leads to parenchymal fibrosis [63]. Prevalence of hypothyroidism ranges from 4% to 24.4% [27, 31, 43, 63, 64] in β-TM and 2 to 3% in β-TI [11]. Splenectomy is a specific risk factor for hypothyroidism in β-TI. In TM, contributing factors include poor compliance with desferrioxamine and elevated serum ferritin levels reaching 3000 ng/mL, which is correlated with hypoparathyroidism as well [31]. The risk of hypothyroidism is significantly increased with every 1 mg Fe/kg DW elevation in LIC. According to the TIF NTDT guidelines, free thyroxine (FT4) and thyroid-stimulating hormone need to be performed annually on all β-TI patients ≥10 years [24]. Hypothyroidism must be treated promptly with L-thyroxine [7].

Hypoparathyroidism, seen in up to 6.7% of TM patients, is not well studied in β-TI [65]. β-TI patients ≥10 years need to be screened for this complication by calcium, phosphate, and vitamin D every year and by parathyroid hormone level if indicated [24]. Calcitriol is recommended for mild hypocalcemia and intravenous calcium administration followed by oral vitamin D for severe cases with tetany [7].

Early iron chelation to prevent hypothyroidism and hypoparathyroidism is recommended by the Thalassemia Clinical Research Network. Thyroid dysfunction, if detected early, can also be reversed with combined desferrioxamine (DFO) and deferiprone (DFP) chelation [65]. In a study from North America, ninety-one percent of chelated patients showed no thyroid dysfunction and 96% were devoid of hypoparathyroidism [32]. In the Optimal Care Study, hydroxyurea (HU) treatment was found to be protective against hypothyroidism in β-TI when compared to transfusion therapy, which was found to be a risk factor [9, 66]. However, low doses of HU (8–15 mg/kg/day) did not alter thyroid function in β-TI patients in one study [66]. More studies are required to elucidate the role of HU in the thyroid function of β-TI patients.

3.7. Hypoadrenalism. Contradictory data exist regarding the pituitary-adrenal dysfunction in thalassemia patients. Some studies described normal basal concentrations of aldosterone, cortisol and adrenocorticotropic hormone (ACTH) and normal response to stimulation by ACTH and metyrapone [67–69]. Other studies reported adrenal hypofunction, elevated basal ACTH levels, and increased response to insulin-induced hypoglycemia [70–73]. In their study, Huang et al. (2014) found that 61% of TM patients had adrenal insufficiency, predominantly among males (92%). Ten out of eleven subjects possibly had insufficiency attributed to a hypothalamic origin [74]. Impaired adrenal function is attributed to iron loading of the pituitary and the adrenal and may play an important role in determining the delayed sexual maturation almost always present in the thalassemic patients [72, 73, 75]. All β-TI patients ≥10 years should undergo annual adrenocorticotropic hormone stimulation test [24]. Studies on the pituitary-adrenal axis function specific to β-TI as well as treatment guidelines for those with adrenal insufficiency are still lacking.

3.8. Bone Abnormalities. Bone abnormalities in β-TI are quite frequent and range from a decrease in the bone mineral density (BMD) and consequent osteoporosis to spinal cord

compression and increased risk of the development of fractures. They are similar to those seen in β-TM but often more marked due to enhanced ineffective erythropoiesis. The underlying cause of the bone disease in β-TI is attributed to several factors including bone marrow expansion, ineffective erythropoiesis, vitamin D deficiency, genetic factors, endocrine dysfunctions secondary to iron overload, and reduced physical activity [9, 76, 77].

The majority of patients with β-TI have decreased levels of IGF-1 that usually plays an essential role in the bone remodeling cycle that stimulates osteoclasts and the differentiation and proliferation of osteoblasts. An increased level of the receptor activator of nuclear factor KB ligand (RANKL) leads to decreased bone thickness followed by bony deformities, osteopenia, and ultimately fractures [78]. Additionally, the *Bsml* Vitamin D receptor (VDR) gene has been shown to be linked with the osteopenia that develops in thalassemia [79]. The urinary excretion of urinary N-telopeptide cross-linked collagen type I (NTx) has been shown to be a sensitive and reliable index of the hip BMD Z-score in patients with thalassemia [79].

3.9. Osteoporosis. Osteoporosis is defined by WHO as a decrease in the bone mineral density and disruption of the bone architecture leading to an increased risk of fractures [80]. A decrease in bone mass can occur due to increased bone resorption or decreased bone formation both of which can lead to osteopenia/osteoporosis in thalassemia [81]. Factors that have been associated with increased rates of osteoporosis in β-TI patients include female gender, iron overload, splenectomy, and low fetal hemoglobin levels [9, 19–21]. A recent study has shown that there is a significantly higher rate of osteoporosis in β-TI as compared to β-TM, accounting for 81.6% and 59.8%, respectively [82]. The prevalence of osteopenia was lower in β-TI as compared to β-TM accounting for 8% and 22.6%, respectively [82]. Furthermore, a reduction of BMD was present in the spine, femoral neck, and distal radius in more than 2/3 of the patients with both β-TM and β-TI [81]. The decrease that was detected in the lumber region was significantly linked with the level of hemoglobin suggesting that the lumbar region is mostly affected among thalassemia patients [83].

Several procedures have been used to describe the extent of bone loss in patients with thalassemia including the Dual energy X-Ray absorptiometry (DXA) to estimate the BMD and the peripheral Quantitative Computer Tomography (pQCT) to assess the regional changes of BMD [84–86]. In a study comparing the use of pQCT with DXA in patients with thalassemia, a reduced pQCT of the trabecular and cortical parameters was found in β-TI and was more severely affected than in β-TM [85]. The recent TIF NTDT guidelines recommend that β-TI patients $\geq$10 years be screened for osteoporosis by annual BMD of the spine, hips, radius, and ulna (dual-energy X-ray absorptiometry) and undergo hormonal and nutritional profile and spine imaging for back pain or neurological findings [24].

Studies on the prevention and management of osteoporosis in β-TI are scarce. Lower rates of osteoporosis have been reported in β-TI patients treated with iron chelation and hydroxyurea than in those who have not [9]. Adherence to daily exercise programs can help maintain bone strength and improvement of the bone status aiding in prevention of the bone complications. Despite transfusion normalizing hemoglobin levels, iron chelation, and adequate hormonal replacement therapy, patients with thalassemia can continue to have progressive bone disease and BMD loss over time [82, 85, 87].

Vitamin D and calcium are often prescribed to patients with β-TI with careful renal function monitoring in the hope of improving mineral density. The efficacy and exact treatment regimen for these supplements have not yet been defined [5, 42]. Bisphosphonates, which are potent osteoclast inhibitors, constitute the treatment of choice in thalassemia associated osteoporosis as these drugs modify the biochemical markers of bone formation and resorption. They have been shown to be safe and efficacious in improving BMD and reducing bone complications and pain in both β-TM and β-TI [5, 42, 88–92]. Dental surveillance is necessary during treatment with bisphosphonates, because this treatment has been associated with jaw necrosis. The 3 most well studied bisphosphonates in thalassemia are zoledronic, pamidronate, and neridronate [88–92]. Further studies are still, however, required to establish the long-term efficacy and outcome of bisphosphonates. The continuous increase in erythropoietin activity, despite treatment with zoledronic acid and the increase in BMD, contributes to the bone loss in β-TI suggesting that blood transfusions may be capable of controlling bone loss more efficiently than bisphosphonates [93]. There is a definite need for more research studies to determine the value of medications such as parathyroid hormone treatment, denosumab, and sotatercept for the treatment of osteoporosis in β-TI patients [94].

3.10. Fractures. Fractures are more frequently seen in β-TM compared to β-TI and the site of fractures differs with arms and forearms affected in β-TM and metacarpal bones in β-TI [95]. The prevalence rate of fractures in β-TI is 12.2% and is likely to increase with age and in patients with a lower lumbar bone mass at a rate almost similar to that seen in β-TM. A decreased BMD is a major risk factor for the development of fractures in β-TI. Other independent risk factors are the use of sex hormone replacement therapy and hypogonadism [96].

3.11. Extramedullary Hematopoiesis. Extramedullary hematopoiesis (EMH), defined as the development of erythropoietic tissue outside the marrow cavity, is a phenomenon that compensates for the decreased efficiency of the bone marrow in providing RBCs for the circulation [2]. Many body sites can be involved in EMH including the spleen, lymph nodes, liver, breast, spinal canal, prostate, heart, thymus, kidney, and adrenal glands [2, 4, 9]. The incidence of EMH is highest among individuals with a chronic hemolytic anemia, specifically NTDT patients [2], thus contributing to the development of osteoporosis and deformities of the facial bones, obliteration of maxillary sinuses, and protrusion of the upper jaw, along with increased risk for fractures of long bones and spinal compression [6, 97, 98]. The incidence of EMH in patients with β-TI may reach up to 20% compared

to poly-transfused TM patients where the incidence remains less than 1% [2]. However, more than 80% of cases may remain asymptomatic and the lesions are usually discovered incidentally by radiologic techniques [2, 4, 6, 97].

One of the devastating complications of EMH is its progression to a spinal cord compression, secondary to a paraspinal mass that can manifest as paraplegia or cauda equina syndrome [99–101]. Among all imaging modalities, MRI is the method of choice for the diagnosis and follow-up evaluation of a spinal cord compression due to an EMH [99, 101–103]. Establishing an early diagnosis of β-TI and instituting optimal management of the underlying anemia and associated symptoms are important steps to prevent EMH and its complications.

The treatment for spinal cord compression involves blood transfusions, surgical decompression, and radiotherapy either singly or in combination [2, 4, 6]. Though hypertransfusion is effective in relieving compression symptoms, HU may replace transfusions and limit expansion of the ectopic hematopoietic tissue [104]. While surgical decompression is an effective method that allows improvement of symptoms, radiotherapy alone remains to be the treatment of choice. The latter cannot be performed until a diagnosis is made. Radiotherapy hinders the hematopoietic activity thereby triggering the decline in the size of the mass and improvement of the associated symptoms [100].

4. Conclusion and Recommendations

Recent research has revealed that β-TI is not a mild disease and is associated with greater morbidity and a wider spectrum of organ dysfunction and complications than previously thought. Endocrine and bone complications are highly prevalent in this disease and necessitate close monitoring, treatment, and follow-up. Early recognition of these complications, institution of appropriate treatment including transfusion regimen and chelation therapy, and specific treatment of each complication are the keys to successful management. Meticulous follow-up by a multidisciplinary team in a comprehensive thalassemia center as well as strict compliance with tailored treatment protocols are major prerequisites for achieving and maintaining an excellent prognosis. The main challenges that remain are the under recognition and underestimation of the morbidity of such complications as well as the absence of affordable worldwide-established treatment protocols.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Incidence of Alpha-Globin Gene Defect in the Lebanese Population: A Pilot Study

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Background. It is well established that the Mediterranean and Arab populations are at high risk for thalassemias in general and for alpha-thalassemia in particular. Yet, reports on alpha-thalassemia in Lebanon are still lacking. In this study, we aim at assessing the incidence of alpha-thalassemia in the Lebanese population. *Methods*. 230 newborns' dried blood cards remaining from routine neonatal screening at the American University of Beirut Medical Center were collected for DNA extraction. Samples were screened for the 21 most common α-globin deletions and point mutations reported worldwide, through multiplex Polymerase Chain Reaction (PCR) and Reverse-Hybridization technique. *Results*. Upon analyses, the carrier rate of α-thalassemia was found to be 8%. Two mutations detected the $-\alpha^{3,7}$ single gene deletion found in 75% of cases and the nongene deletion α2 IVS1 [−5nt] in the remaining samples. *Conclusion*. This study is the first dedicated to investigate α-thalassemia trait incidence in Lebanon. Data obtained demonstrates a high carrier rate in a relatively, highly consanguineous population; it also highlighted the presence of two common mutations. These results may be of an important impact on premarital and newborn screening policies in our country.

1. Introduction

Alpha-thalassemia is one of the most common hemoglobin gene defects. It is considered as a severe, life-shortening autosomal recessive disease [1] which is caused by downregulation of α-globin synthesis with underproduction of fetal (HbF, $\alpha_2\gamma_2$) and adult (HbA, $\alpha_2\beta_2$) hemoglobin. The clinical phenotype in alpha-thalassemia varies according to the number of α-genes affected [2].

Four clinical conditions of variable severity are recognized: the silent carrier state (−1 gene), the alpha-thalassemia trait (−2 genes), the intermediate form of hemoglobin H disease (−3 genes), and the hemoglobin Bart hydrops fetalis syndrome which is lethal in utero or soon after birth (−4 genes) [2].

The Mediterranean and Arab countries are considered high risk areas for thalassemia in general and for α-thalassemia in particular where the frequency exceeds by far that of beta-thalassemia in some of these populations. Alpha-thalassemia carrier frequency can vary among countries to reach the highest in UAE, Oman, and Saudi Arabia with a 50% carrier rate [3] (Figure 1). While no effective treatment for thalassemia has been reported to date, studies have shown that carrier screening and genetic counseling are the most effective solutions for reducing its incidence. Indeed, prenatal as well as premarital screening programs in countries with high incidence have already been established. In Lebanon; accurate epidemiological data on alpha-thalassemia is still lacking.

In this study, we screened for α-globin gene defects in a sample of Lebanese newborns at the American University of Beirut Medical Center over a period of one year.

2. Methods

Two hundred and thirty Lebanese newborns (age 1–3 days) at the AUBMC from the period of August 2012 to July 2013 were included in this research. The study was approved by the Institutional Review Board (IRB) of the AUB. AUBMC is a quaternary care center that receives referrals from

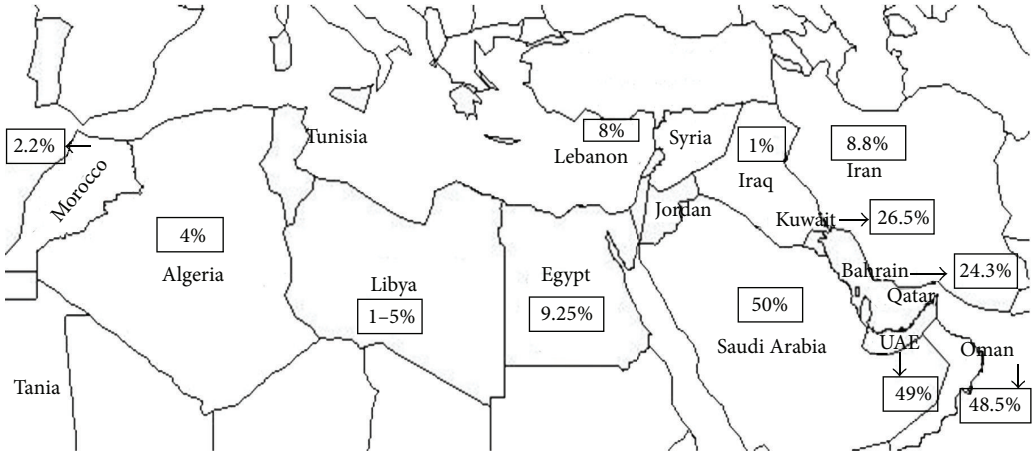

Figure 1: α-thalassemia carrier frequency in some Eastern Mediterranean countries.

all around Lebanon. Parental approval was obtained after explaining to them the risks and benefits of participation in this research study that serves as a basis to design systematic study to assess the incidence of disease causing α-globin gene alleles in our population. Remaining blood samples from the newborn's blood cards that are received routinely in the Chemistry Department of laboratory medicine for routine G6PD screening in neonates were collected. DNA was extracted from the blood collection cards, using the QIAamp DNA Micro Kit (Qiagen) and as described by the manufacturer. DNA analysis for the identification of the following 21 α-globin mutations, 3.7 and 4.2 single gene deletions, MED, SEA, THAI, FIL, and 20.5 double gene deletions, anti-3.7 triplication, α1 cd 14, α1 cd 59, α2 init cd, α2 cd 19, α2 IVS1 [−5nt], α2 cd 59, α2 cd 125, α2 cd 142 (Hb Constant Spring), α2 cd 142 (Hb Icaria), α2 cd 142 (Hb Pakse), α2 cd 142 (Hb Koya Dora), α2 poly A-1, and α2 poly A-2, was performed by means of Polymerase Chain Reaction (PCR) followed by Reverse-Hybridization techniques. Three multiplex PCR reactions were performed for each sample following the manufacturer's instructions (Viennalab, GmbH Vienna, Austria) and as previously described [4].

Data analysis was performed using Statistical Package for Social Sciences (SPSS) version 19. Descriptive statistics was done using frequencies and percentages.

3. Results and Discussion

Alpha-thalassaemia occurs at high frequencies in parts of the world extending from sub-Saharan Africa through the Mediterranean region and Middle East, to South East Asia with a carrier frequency reaching as high as 80–90% in some of these populations such as Nepal and Andhra Pradesh province of India [5].

Although numerous studies have been carried out to assess the carrier rate of β-thalassemia in Lebanon (2-3%) [6], data on α-thalassemia is still incomplete.

In this pilot study a total of 230 neonates were recruited in total: 30 of them were rejected due to inadequate blood amount. Of the 200 DNA samples analyzed, 8% were found to be carriers of one α-gene defect. This is much higher compared to carrier rate of beta-thalassemia in our population (2%) [6]. Our finding is comparable to many Middle Eastern and Mediterranean countries such as Israel (5–9%) [7], Turkey (7.5%) [8], and Greece (7%) [9] but lower than that reported from the countries in the Arabian Peninsula (Figure 1).

Table 1: The type and frequency of α-gene mutations detected by ethnicity.

	Muslim	Christian	Druze
Total *n* (%)	136 (68%)	57 (25.8%)	7 (3.5%)
Mutations (%)	13 (9.6%)	3 (5.3%)	0 (0%)
$-\alpha^{3,7}$	*10 (77%)*	*2 (67%)*	—
α2 IVS1 [−5nt]	*3 (23%)*	*1 (33%)*	—

Only two alpha-thalassemia gene defects have been observed in our newborn carriers. These are $-\alpha^{3,7}$ (75%) and α2 IVS1 [−5nt] (25%). $-\alpha^{3,7}$, the most common, is a well described founder mutation in the Mediterranean and Middle Eastern countries [3, 7, 10–12]. α2 IVS1 [-5nt] is also highly prevalent in the Mediterranean countries [10] but is rarely detected in the Arabian Peninsula, whereas the α-thalassemia determinants $--^{20.5}$ and $-^{MED}$, relatively common among both Middle Eastern and Mediterranean countries, were not detected in our sample (Table 1). In addition, αpolyA1α, an equally common mutation in the Arabian Peninsula [10], was not detected in our population.

Studies have shown that the incidence of alpha-thalassemia vary among different ethnic groups and communities [13]. Lebanon is composed of a population with heterogeneous background and where intercommunity mating is still a rare occurrence. In our study, out of the 200 newborns, 136 were Muslim (68%), 57 were Christian (28.5%), and 7 were Druze (3.5%). We found that the carrier rate of alpha-thalassemia in the Muslim community (9.6%) is almost twice that of the Christian community (5.3%). No deleterious alpha-thal gene was found in the Druze families analyzed. The small number of Druze families that were

recruited ($n = 7$) reflects our population's constitution with the Druze community representing less than 5% of the total Lebanese population [14].

Allelic distribution did not differ between communities with −3,7del single gene deletion being the most common in both Muslims and Christians.

4. Conclusion

This study is the first dedicated to investigate α-thalassemia trait incidence in Lebanon. The high incidence of alpha-globin gene defect found should steer attention to the importance of hemoglobinopathies in our population and may justify contemplating social preventive measures such as establishing premarital, screening for α-thalassemia especially in consanguineous mating.

Also based on our findings, inclusion of molecular diagnosis for $\alpha^{3.7}$ and $\alpha 2$ IVS1 [−5nt] determinants in the general newborn screening should be envisaged. This will serve for an early, adequate management of hemoglobinopathies in our population.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Clinical Features and Molecular Analysis of Hb H Disease in Taiwan

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Thalassemia is highly prevalent in Taiwan, but limited data are available about the association between genotypes and clinical manifestations in Taiwanese patients with Hb H disease. Here, we studied α-globin gene abnormalities and clinical features in Taiwanese patients with Hb H disease. Of the 90 patients, sixty-four (71.1%) were deletional and twenty-six (28.9%) were nondeletional Hb H disease. The (- -^{SEA}) type of α^0-thalassemia mutation was detected in the majority of patients (>95%). The most common genotype was (- -^{SEA}/-$\alpha^{3.7}$), followed by (- -^{SEA}/$\alpha^{cs}\alpha$). After further investigation of the genotype-phenotype correlation in 68 patients, we found that patients with nondeletional Hb H disease had more severe clinical features than those with deletional Hb H disease, including younger age at diagnosis, more requirement of blood transfusions, and larger proportion of patients with splenomegaly, hepatomegaly or jaundice. This is probably a consequence of the lower hemoglobin levels and the higher Hb H levels. The clinical severity was highly variable even among patients with an identical genotype, and the diversity was much more profound among patients with (- -/$\alpha^{cs}\alpha$) genotype. Therefore, predicting the phenotype directly from the genotype in Hb H disease remains relatively difficult in Taiwan.

1. Introduction

The α-thalassemia, arising from deletions or mutations of α-globin genes, is the most common inherited disease of hemoglobin synthesis in the world, especially in the Far East [1]. Three of the four α-globin genes are affected in patients with Hb H disease, which can show a wide spectrum of clinical phenotypes, ranging from no symptoms, to mild anemia with only occasional transfusions, to severe anemia and hemolysis with hepatosplenomegaly needing frequent transfusions, and even to fatal hydrops fetalis syndrome [2–4]. Due to the high carrier rate of approximately 5–7% of α-thalassemia [5], Hb H disease is not rare in Taiwan.

Due to the wide clinical spectrum of Hb H disease and the increased life expectancy in these patients, an individualized treatment approach is needed. Information about the genotype-phenotype correlation is thought important to assess patients with Hb H disease and beneficial for optimal patient management [6–8]. However, comprehensive clinical and molecular analysis in patients with Hb H disease in Taiwan is limited. Therefore, we assessed α-globin genotypes in 90 patients with diagnosis of Hb H disease in this study.

The correlation between genotype and phenotype was further analyzed in 68 patients who received regular follow-ups at our hospitals.

2. Materials and Methods

From 1999 to 2013, ninety patients with diagnosis of Hb H disease by DNA analysis in the China Medical University Hospital were enrolled. These patients were referred to our laboratory for DNA analysis due to abnormal results of hematological studies and hemoglobin electrophoresis. Age of these patients ranged from 1.7 to 63.4 years. There were 47 males and 43 females. The majority of patients came from the middle part of Taiwan. The study was approved by the Institutional Review Board of the China Medical University Hospital.

All of the patients were initially presented with microcytic anemia, and hematological data were determined with an automated blood cell counter (Sysmex XE-2100 with SP-1000i series; Sysmex, Kobe, Japan). Hemoglobin analysis was performed with electrophoresis by automated high-performance liquid chromatography (PRIMUS CLC385; Primus Corporation, Kansas, MO, USA) according to manufacturer's instructions.

Genomic DNA was extracted from peripheral blood leukocytes using a commercially available kit (GE Healthcare, Buckinghamshire, UK). Identification of α-globin gene was done using allele-specific polymerase chain reaction (PCR) and direct sequencing analysis of α-globin gene. We designed primer sets on HBA1 (NM_000558.3) and HBA2 (NM_000517.3) DNA sequences. Mutations were detected by 11 pairs of primers using gap-PCR and PCR-restriction fragment length polymorphism-based methods, which were developed by Liu el al. [9, 10]. Certain α-globin gene mutations which are highly prevalent in Taiwan can be determined, including α^0-thalassemia mutations ($--^{SEA}$, $--^{Fil}$, and $--^{THAI}$), α^+-thalassemia mutations ($-\alpha^{3.7}$ and $-\alpha^{4.2}$), the Constant Spring variant ($\alpha^{CS}\alpha$; TAA $\rightarrow$ CAA at codon 142 of α_2 gene), and the Quong Sze variant ($\alpha^{QS}\alpha$; CTG $\rightarrow$ CCG at codon 125 of α_2 gene). Unknown mutations were characterized by direct sequencing of the PCR-amplified product of α_2- and α_1-globin genes.

Clinical features were reviewed from medical records in 68 patients who received regular followups at the China Medical University Hospital or the Chung Shan Medical University Hospital, which are the main hospitals for the care of thalassemic patients in Taiwan. Complete physical examination and laboratory analysis of complete blood count and serum biochemical values were done every 3 months. In patients with abnormal results, such as splenomegaly, hepatomegaly, or jaundice, abdominal ultrasonography was performed every year thereafter. The size of spleen and liver was further measured by ultrasonography, and an age and gender matched reference range was used for the diagnosis of splenomegaly and hepatomegaly [11]. Clinical data were collected up to the last followup on May 31, 2014. Statistical analysis was performed with the SPSS 16.0 software program. Student's t-test was used to compare the continuous data, and Pearson chi-square or Fisher exact test was for categorical variables. The statistical value of $P < 0.05$ was considered to be significant.

Table 1: α-Globin genotypes of the 90 patients with Hb H disease in Taiwan.

α-Globin genotype	Number of patients	%
Deletional Hb H disease		
$--^{SEA}/-\alpha^{3.7}$	43	47.8
$--^{SEA}/-\alpha^{4.2}$	10	11.1
$--^{SEA}/-\alpha^{4.2\ G\text{-}Taichung}$	7	7.8
$--^{Fil}/-\alpha^{3.7}$	3	3.3
$--^{Fil}/-\alpha^{4.2\ G\text{-}Taichung}$	1	1.1
Nondeletional Hb H disease		
$--^{SEA}/\alpha^{CS}\alpha$	22	24.5
$--^{SEA}/\alpha^{QS}\alpha$	4	4.4
Total	**90**	**100**

3. Results

3.1. Genotypes of Hb H Disease. Table 1 shows the frequencies of various α-globin genotypes among the 90 Taiwanese patients with Hb H disease. Deletional Hb H disease is defined as a deletion removing both α-globin genes on one chromosome 16 plus a deletion removing only a single α-globin gene on the other chromosome 16, and nondeletional Hb H disease is a deletion removing both α-globin genes on one chromosome 16 plus an α^+-thalassemia point mutation or a small insertion/deletion involving either the α_2- or α_1-globin gene on the other chromosome 16 [2, 12, 13]. Sixty-four (71.1%) were deletional Hb H disease and twenty-six (28.9%) were nondeletional Hb H disease. The ($--^{SEA}$) type of α^0-thalassemia mutation was detected in the majority of patients (>95%), and the ($--^{Fil}$) type of α^0-thalassemia mutation was detected in the remaining patients. There was no patient with the ($--^{THAI}$) deletion in the present study. Among patients with deletional Hb H disease, the rightward deletion of 3.7 kb ($-\alpha^{3.7}$) was the most common type of mutation (71.2%), followed by the leftward deletion of 4.2 kb ($-\alpha^{4.2}$) and the G-Taichung variant with the 4.2 kb deletion ($-\alpha^{4.2\ G\text{-}Taichung}$). Among patients with nondeletional Hb H disease, most patients had the Constant Spring variant (84.6%), and the remaining patients had the Quong Sze variant. There was no patient with a compound heterozygosity for a nondeletional α^0-thalassemia mutation and a nondeletional α^+-thalassemia mutation in the present study.

3.2. Clinical Features and Their Association with Genotypes. A total of 90 patients with Hb H disease were enrolled for genotype study. Among them, sixty-eight patients who received regular follow-ups at our hospitals were further investigated the correlation between genotype and phenotype. The other 22 patients were excluded because of no sufficient clinical information available. Compared with patients

Table 2: Comparison of clinical features between patients with deletional and nondeletional Hb H disease in Taiwan.

	Deletional Hb H disease ($n = 44$)	Nondeletional Hb H disease ($n = 24$)	P value
Initial manifestations			
Age at diagnosis (year)	1.7–63.4 (median 24.0)	3.0–36.9 (median 10.4)	0.040*
Gender	21 males, 23 females	15 males, 9 females	0.261
Laboratory data at diagnosis			
Hb (g/dL)	5.5–11.5 (median 9.1)	4.6–12.1 (median 8)	0.001*
MCV (fL)	43.9–75.1 (median 60)	63.0–78.1 (median 68.8)	<0.001*
MCH (pg)	15.1–28.0 (median 17.5)	16.8–22.4 (median 18.3)	0.568
MCHC (g/dL)	15.3–33.7 (median 29.0)	19.1–29.4 (median 25.9)	0.740
Hb H (%)	0.7–29.7 (median 5.9)	6.3–35.5 (median 12.3)	<0.001*
Follow-up clinical features			
History of blood transfusions	10 (22.7%)	14 (58.3%)	0.003*
Splenomegaly	9 (20.5%)	14 (58.3%)	0.002*
Hepatomegaly	3 (6.8%)	7 (29.2%)	0.013*
Jaundice	4 (9.1%)	13 (54.2%)	<0.001*
Gallstones	2 (4.5%)	5 (20.8%)	0.035*
Cholecystitis	2 (4.5%)	0	0.289
Splenectomy	4 (9.1%)	5 (20.8%)	0.172
Cholecystectomy	2 (4.5%)	0	0.289
Thrombotic event	1 (2.3%)	1 (4.2%)	0.659
Leg ulcers	0	0	—
Growth retardation	0	0	—
Delay of pubescence	0	0	—
Serum ferritin level (ng/mL)	40.2–699.0 (median 142.5)	40.3–1619.0 (median 210.7)	0.001*

*$P < 0.05$.

with deletional Hb H disease, patients with nondeletional Hb H disease had more severe clinical features, including a younger age at diagnosis, more requirement of blood transfusions, and a larger proportion of patients with splenomegaly, hepatomegaly, or jaundice. Additionally, we found that the occurrence of splenomegaly could be an important indicator of clinical significance (see Table S1 in Supplementary Material available online at http://dx.doi.org/10.1155/2014/271070). In the present study, patients with hepatomegaly or jaundice always had splenomegaly concomitantly. Among the 14 patients with nondeletional Hb H disease who had splenomegaly, nearly all also had jaundice and half of them had hepatomegaly. Two patients with deletional Hb H disease suffered from cholecystitis with gallstones, and both received cholecystectomy during the episodes. Asymptomatic gallstones were accidentally found by abdominal ultrasonography in 5 patients with nondeletional Hb H disease, but none received cholecystectomy. Two patients experienced thrombotic events of lower legs in the fourth decade of life, and their genotypes were ($--^{SEA}/-\alpha^{4.2\ G\text{-}Taichung}$) and ($--^{SEA}/\alpha^{CS}\alpha$). None of the 68 patients had leg ulcers. Growth and pubescence were normal in all patients.

At diagnosis, patients with nondeletional Hb H disease had lower hemoglobin levels, higher MCV levels, and higher proportions of Hb H. They also had higher serum ferritin levels. In all patients with deletional Hb H disease, serum ferritin levels were not more than 800 ng/mL. Because glucose-6 phosphate dehydrogenase deficiency is also highly prevalent in Taiwan and can aggravate clinical manifestations in patients with Hb H disease, all patients were screened for the deficiency. Only 3 patients were positive for the deficiency, but none of them exhibited more severe anemia. Table 2 summarizes the clinical and hematological features of these patients according to their genotypes.

4. Discussion

The incidence of genetic subtypes of Hb H disease varies greatly in different ethnic groups. The proportion of nondeletional Hb H disease was as high as more than 50% in Thailand, but as low as less than 20% in Cyprus and Sardinia [12, 14–16]. Of the 90 Taiwanese patients with Hb H disease in the present study, the nondeletional genotype accounted for 28.9%. The incidence of the Constant Spring variant was higher among patients with nondeletional Hb H disease in this study (84.6%) compared with Chen et al.'s report from Hong Kong [13]. The majority of patients with Hb H disease had the ($--^{SEA}$) type of α^0-thalassemia mutation, and the most common genotype was ($--^{SEA}/-\alpha^{3.7}$), followed by ($--^{SEA}/\alpha^{CS}\alpha$). In addition to the 90 patients with Hb H disease, we detected 12 patients with β-thalassemia associated with Hb E, which is the second common cause of thalassemia intermedia in Taiwan.

The diversity of clinical and hematological features was noted. We confirmed that patients with nondeletional Hb H disease had more severe clinical manifestations. Several possible mechanisms have been proposed to explain why synthesis of normal α-globin chains decreases in patients with nondeletional Hb H disease, including loss of compensation from the "healthy" α-globin gene and interference of transcription by the mutant α-globin gene [12, 17]. More than half of these patients with nondeletional Hb H disease had a history of blood transfusions, and some of them even were transfusion dependent, whereas many patients with deletional Hb H disease were first diagnosed after infection-induced hemolysis or during health assessment or pregnancy, therefore older in age at diagnosis among these patients. Less than one-quarter of patients with deletional Hb H disease had history of transfusions, and the majority received less than 3 transfusions. None were transfusion dependent. Splenomegaly, hepatomegaly, and jaundice were also more common in patients with nondeletional Hb H disease. It is probably due to the severity of anemia and propensity towards hemolysis in these patients as a consequence of the lower hemoglobin levels and higher Hb H levels. Additionally, it was interesting to find that two patients with deletional Hb H disease received cholecystectomy due to acute cholecystitis, whereas about 20% of patients with nondeletional Hb H disease had silent gallstones, but this did not develop into acute or chronic cholecystitis. However, we did not detect any factors contributing to the high prevalence of gallstones, but low incidence of symptomatic cholecystitis in these patients with nondeletional Hb H disease.

In contrast to the report of Chen et al. which found that the G-Taichung variant would aggravate the clinical symptoms in patients with Hb H disease [18], the 8 patients with $(--/-\alpha^{4.2\ \text{G-Taichung}})$ genotype in this study were clinically similar to those with deletional Hb H disease, such as $(--/-\alpha^{3.7})$ or $(--/-\alpha^{4.2})$ genotypes. These patients had less severe clinical features compared with those with nondeletional Hb H disease. Patients with the Quong Sze variant are thought to be more prone to hemolysis because the intracellular aggregates of hyperunstable variant of α-globin chains might cause additional membrane damage and dysfunction [2]. But the severity of clinical features in the 4 patients with $(--^{\text{SEA}}/\alpha^{\text{QS}}\alpha)$ genotype was similar to that with $(--^{\text{SEA}}/\alpha^{\text{CS}}\alpha)$ genotype in this study. Of importance, we found that the clinical severity was highly variable even among patients with an identical genotype. It may relate to the complex interaction between environmental and genetic factors. The diversity was found much more profound among those with $(--/\alpha^{\text{CS}}\alpha)$ genotype. Therefore, predicting the phenotype directly from the genotype in Hb H disease remains relatively difficult in Taiwan.

Conflict of Interests

The authors declare that they have no conflict of interests regarding the publication of this paper.

Authors' Contribution

Yu-Hua Chao and Kang-Hsi Wu contributed equally to this work.

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Review Article

Novel Approach to Reactive Oxygen Species in Nontransfusion-Dependent Thalassemia

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The term Nontransfusion dependent thalassaemia (NTDT) was suggested to describe patients who had clinical manifestations that are too severe to be termed minor yet too mild to be termed major. Those patients are not entirely dependent on transfusions for survival. If left untreated, three main factors are responsible for the clinical sequelae of NTDT: ineffective erythropoiesis, chronic hemolytic anemia, and iron overload. Reactive oxygen species (ROS) generation in NTDT patients is caused by 2 major mechanisms. The first one is chronic hypoxia resulting from chronic anemia and ineffective erythropoiesis leading to mitochondrial damage and the second is iron overload also due to chronic anemia and tissue hypoxia leading to increase intestinal iron absorption in thalassemic patients. Oxidative damage by reactive oxygen species (generated by free globin chains and labile plasma iron) is believed to be one of the main contributors to cell injury, tissue damage, and hypercoagulability in patients with thalassemia. Independently increased ROS has been linked to a myriad of pathological outcomes such as leg ulcers, decreased wound healing, pulmonary hypertension, silent brain infarcts, and increased thrombosis to count a few. Interestingly many of those complications overlap with those found in NTDT patients.

1. Introduction to NTDT and Iron Overload

Thalassemia is an entity involving a collection of inherited diseases caused by defective or absent hemoglobin chain synthesis leading to anemia due to ineffective erythropoiesis. The severity of the disease depends on the genotype inherited [1–6]. Patients who carry the trait are often asymptomatic and continue to live a normal life, while β-thalassemia major patients suffer from many complications that may be ameliorated due to lifelong transfusions.

According to the WHO, the carrier rate of β-thalassemia is around 1.5% of the world population. It was also suggested that the incidence of individuals born with the severe form of the disease is 60,000 per year. Most of these patients are from regions around the tropical belt, including the Mediterranean, Middle East, central Asia, India, and southern China [7]. However, with the era of globalization and easier travel methods, migration is now facilitating the spread of the disease towards the Western countries.

Nontransfusion-dependent thalassemia (NTDT), as its name implies, is a term coined to describe those patients that do not require lifelong transfusions who instead may need emergent transfusions for specific clinical settings [8]. The primary forms of NTDT include β-thalassemia intermedia, hemoglobin E (HbE) β-thalassemia, and hemoglobin H disease [9]. These 3 clinical entities are the ones suggested such that reactive oxygen species are an integral player in the development of disease specific complications.

As opposed to thalassemia major, where transfusional induced iron overload is targeted towards the reticuloendothelial system and parenchyma, iron is amassed in patients with NTDT that differ, primarily occurs in hepatocytes [10–13]. The rate of iron loading is significantly different in thalassemia major ranging between 0.30 and 0.60 mg/kg/day versus 0.01 mg/kg/day in NTDT [14]. Iron overload in NTDT is a slow process; nevertheless, patients with the disease start experiencing iron-related morbidity beyond 10 years of age

[14, 15]. The pattern of iron accumulation and the predilection of iron to target organs in NTDT is markedly different from transfusion-dependent thalassemia (TDT). Cardiac siderosis is of integral importance in management decisions in TDT as it is a major cause of morbidity and mortality; however, its importance is less pronounced in NTDT patients, even those with relatively elevated total body iron [16–19].

The master regulator of iron balance in humans is hepcidin, a peptide produced by the liver [20]. Hypoxia downregulates the expression of hepcidin, which leads to both increased intestinal iron absorption and increased release of recycled iron from the reticuloendothelial system [21, 22]. This in turn causes depletion of macrophage iron, relatively low levels of serum ferritin, and preferential portal and hepatocyte iron loading [13, 23].

The pathophysiology of iron loading in NTDT appears to be similar to that observed in patients with hereditary forms of hemochromatosis [13] and is different from that seen in thalassemia major where there is predilection for nontransferrin bound iron (NTBI) accumulation.

NTBI is a powerful catalyst for the formation of hydroxyl radicals from reduced forms of O_2 [24]. Labile or "free" iron can convert relatively stable oxidants into powerful radicals. Iron concealed in proteins, as in catalytic sites of enzymes or stored in ferritin, is not exposed to oxygen radicals and cannot participate in this chemistry [25].

ROS are capable of causing oxidative damage to macromolecules leading to lipid peroxidation, oxidation of amino acid side chains (especially cysteine), formation of protein-protein crosslinks, oxidation of polypeptide backbones resulting in protein fragmentation, DNA damage, and DNA strand breaks [26, 27].

The liver, another concern, is also affected gravely in NTDT patients with the spectrum of injury ranging from fibrosis to hepatocellular carcinoma in hepatitis negative, chelation naïve NTDT patients [11, 12, 28–31]. Although NTDT is a nontransfusional disease, iron overload toxicity occurs in targeted organs that have specific complications in NTDT including pulmonary hypertension, leg ulcers, extramedullary hematopoiesis, endocrinopathies, and thromboembolic diseases.

In a recent study addressing pulmonary hypertension in thalassemia, patients with β-thalassemia intermedia (TI) had a 5-fold increased prevalence of pulmonary hypertension on right heart catheterization than patients with β-thalassemia major (5.7% versus 1.2%). Another common complication in NTDT, namely, leg ulcers, is more common in older patients with TI. The mechanism by which this complication is brought about is still unclear as some patients who are maintained on relatively low hemoglobin levels and have the same amount of fetal hemoglobin in TI patients do not develop ulcers. One explanation could be due to the fragility of the subcutaneous tissue of the skin of elderly TI patients due to reduced tissue oxygenation making healing more difficult after minimal trauma. Blood transfusions may provide some form of relief to the painful and indolent ulcers [15]. Yet another complication in NTDT is osteoporosis as a result of vitamin D deficiency and bone marrow expansion, which is quite common among TI patients [32, 33]. This may lead to bone pain and more importantly pathologic fractures.

There are several endocrine complications in patients with TI due to iron overload and anemia [15, 34]. Such complications include delayed puberty; however, fertility is usually preserved in these patients. In special clinical situations such as in pregnant women with TI, there is an increased risk of preterm delivery, intrauterine growth restriction, abortion, Cesarean section delivery, and thromboembolism [35]. A hypercoagulable state such as that seen in pregnancy warrants the need for anticoagulation in pregnant women especially if they have additional prothrombotic risk factors [36].

NTDT is associated with a hypercoagulable state, and patients with β-thalassemia syndromes have a pronounced risk starting childhood [37–39]. The mechanism that brings about this state of hypercoagulability in patients with NTDT is thought to be due to abnormalities in platelets along with pathological red blood cells among many other factors that are thought to contribute to clinically evident thrombotic events (Figure 1) [8, 40–44]. The largest epidemiological study to date which analyzed data from 8860 thalassemia patients (6670 thalassemia major and 2190 TI) demonstrated that thromboembolic events occurred 4.38 times more frequently in TI patients than in thalassemia major patients [45].

Renal damage is an emerging issue in TI. It is apparent that chronic hypoxia causes proximal tubular cell dysfunction and interstitial fibrosis, which, in the presence of other renal risk factors, may lead to progressive renal disease [46–48]. Early proximal tubular markers such as NAG, β_2-microglobulin, phosphaturia, and uricosuria should be evaluated in these patients to detect early tubular abnormalities, which can salvage the kidney ultimately [49]. Although strokes are uncommon in TI patients, one study showed that 37.5% of patients with TI have evidence of silent brain infarction on magnetic resonance imaging (MRI) [50].

2. Background

Based on previously discussed data, it is clear that reactive oxygen species (ROS) are heavily implicated in the pathophysiology of NTDT. Many previous studies tested the effect of antioxidants on the treatment of NTDT. One study on fermented papaya preparation (FPP), a natural health food product obtained by biofermentation of Carica papaya, has been shown to limit oxidative stress both in vivo and in vitro [51]. Administration of FPP to patients with β-thalassemia major and intermedia and to patients with β-thalassemia/HbE disease for 3 months yielded a decrease in ROS generation, in membrane lipid peroxidation, and in externalization of phosphatidyl serine residues. There was a concomitant increase in glutathione (GSH) levels. However, no changes were observed in hematological parameters such as RBCs and hemoglobin (Hb) [52]. Curcumin, a natural herb used as food additive, contains polyphenol compounds. An extract derived from dried rhizomes of curcumin was given to patients with β-thalassemia/HbE disease as antioxidants [53]. It showed a decrease in iron-catalysed lipid peroxidation

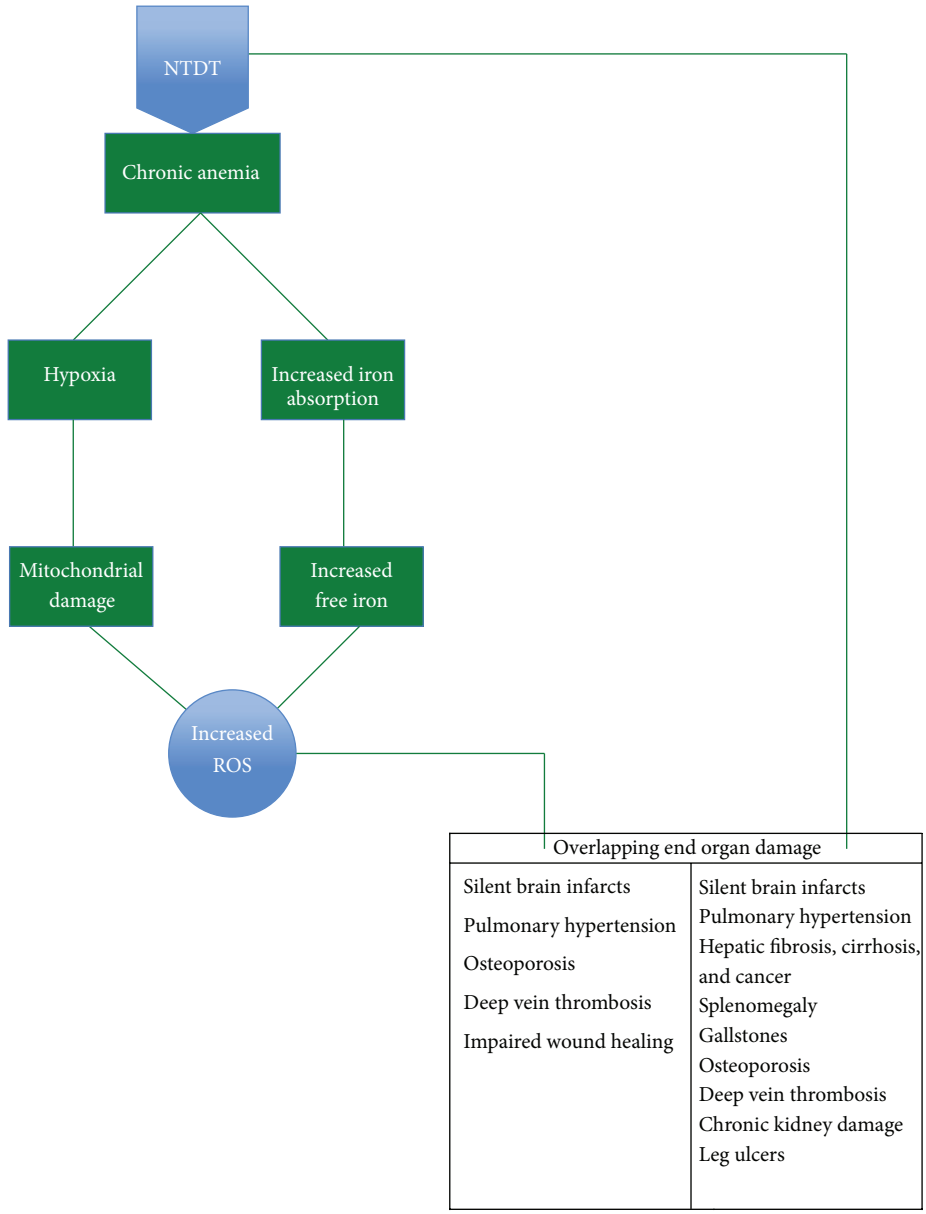

Figure 1: Mechanism of end organ damage in NTDT.

in vitro [54]. The results in patients treated with curcumin for one year demonstrated a significant decrease in oxidative parameters concomitant with a decrease in methemoglobin and NTBI. These changes were observed throughout the administration of curcumin. However, there were no changes in Hb levels throughout the period of treatment [55]. Vitamin E has well-established antioxidant properties. Since vitamin E is frequently deficient in homozygous β-thalassemia patients [56], its supplementation was studied extensively. The results showed that in heterozygotes patients, high dose of oral vitamin E decreased lipid peroxidation in RBCs and increased their survival [57]. Other studies showed improvement in the plasma antioxidant/oxidant balance, in the oxidation of low-density lipoproteins [58], and in the impaired osmotic fragility of RBCs [59]. Parenteral administration of vitamin E was more effective than oral administration [60]. Most of these studies, however, did not show a significant improvement in clinical parameters, that is, Hb concentration and transfusion requirement. All studies done on the effect of antioxidants inNTDT show a decrease in oxidative stress. However, the previous literature suggests that this decrease in oxidative stress in those patients was being tested against improvement in RBC indices. No study to date tried to link decrease ROS burden with improvement of end organ damage, in which ROS are implicated in NTDT patients.

3. Tissue Hypoxia and ROS

Many of the clinical manifestations of NTDT can be attributed to the chronic hypoxic environment created by

the pathologic red blood cells. Another major source of ROS formation is the underlying irregular and insufficient supply of oxygen which creates a disturbed cellular physiology [61]. There is no consensus over the definition of tissue hypoxia. This is clouded further by the fact that partial oxygen pressures differ between tissues, which made many experts adopt the definition of tissue hypoxia as a condition in which the cells of a tissue have abnormal oxygen utilization such that the tissue experiences anaerobic metabolism [62]. The strongest contributor to hypoxia induced ROS is the mitochondria. In cellular hypoxia, a more reductive state is present. Reducing substances such as NADH and FADH2, which participate in the electron transport chain where oxygen is an integral part, accumulate due to the disruption in the chain. This buildup makes electrons readily available for production of ROS. Another postulated theory for the increase of mitochondrial ROS is that under hypoxic conditions nitric oxide radicals may be produced. These radicals bind and inhibit cytochrome oxidase resulting in an increased affinity towards oxygen and an increase in reduction of electron carriers upstream from the terminal oxidase. This will lead to the formation of ROS [61]. Chronic tissue hypoxia is a source of oxidative damage. This was shown by two studies, one in mice exposed to high altitude and the other by observations in hypoxic chronic obstructive pulmonary disease patients [61, 63]. The electrochemical gradient across the mitochondrial membrane (Dcm) is indicative of an active proton gradient that drives ATP synthesis [64]. The Dcm collapse observed in thalassemic patients, particularly in those who are nontransfused, shows the energetic failure under hypoxic conditions due to the metabolic switch from oxidative phosphorylation to anaerobic glycolysis. The mitochondrial impairment is coupled with endogenous ROS overproduction [65]. The hypoxia effect is highlighted by the higher lymphocytic ROS in nontransfused compared to transfused patients, despite their lower iron overload [66–68]. Redox imbalance causes an increased lipid peroxidation. Lipid peroxidation causes hemolysis, which worsens the already severe anemia and further worsens redox imbalance due to hemoglobin release [69]. Apart from hypoxia, lipid peroxidation may induce Dcm decrease with ROS overproduction, as observed in senescent cells [70] and after exposure to an environmental metal mixture [71]. Such findings strengthen the central role played by mitochondrial impairment in thalassemia. Cumulative oxidative damage, produced by iron and hypoxia, triggers a vicious cycle that may lead to organelle collapse [72].

4. ROS, NOX Family, and End Organ Damage

4.1. NOX Family. Superoxide generation by an NADPH oxidase was considered as an oddity only found in professional phagocytes. However, over the last years, six homologs of the cytochrome subunit of the phagocyte NADPH oxidase were found: NOX1, NOX3, NOX4, NOX5, DUOX1, and DUOX2.

The homologs are now referred to as the NOX family of NADPH oxidases. These enzymes share the capacity to transport electrons across the plasma membrane and to generate superoxide and other downstream ROS [72]. Of particular interest to our discussion is NOX4, which is highly expressed in the kidney, osteoclasts, endothelial cells, smooth muscle cells, hematopoietic stem cells, fibroblasts, keratinocytes, melanoma cells, and neurons. Induction of NOX4 mRNA expression is observed in response to endoplasmic reticulum stress, shear stress, hypoxia, and ischemia [73–76].

4.2. Oxidative Stress and Pulmonary Hypertension. Nonphagocytic NADPH oxidases have recently been suggested to play a major role in the regulation of physiological and pathophysiological processes, namely, hypertrophy, remodeling, and angiogenesis in the systemic circulation. Moreover, NADPH oxidases have been suggested to serve as oxygen sensors in the lungs. Chronic hypoxia induces vascular remodeling with medial hypertrophy leading to the development of pulmonary hypertension. NOX4 has been shown to be a major player in the vascular remodeling associated with development of pulmonary hypertension [77]. Most of the available animal models of pulmonary hypertension (PHT) exhibit the two principal pathological features in the pulmonary vasculature common to most forms of PHT. These include excessive vasoconstriction and remodeling of the pulmonary arteriolar wall, primarily by a mechanism of smooth muscle proliferation within the medial layer. Because ROS may promote vasoconstriction, smooth muscle cell proliferation, and vascular remodeling, they are likely to play a critical role in many forms of PHT [78–81]. It is known that ROS can stimulate release of arachidonic acid, the substrate used for production of all arachidonic acid metabolites, including the potent constrictor, thromboxane. Therefore, ROS mediate constriction due to impaired acetylcholine (Ach) responses observed in hypoxic pulmonary arteries (Figure 2 and Table 1). This is caused by stimulating production of thromboxane [82–84].

4.3. Oxidative Stress and Wound Healing. Temporary hypoxia after injury triggers wound healing, but prolonged or chronic hypoxia delays wound healing. In normally healing wounds, ROS such as hydrogen peroxide (H_2O_2) and superoxide (O^2) are thought to act as cellular messengers to stimulate key processes associated with wound healing, including cell motility, cytokine action, and angiogenesis. However, an increased level of ROS transcends the beneficial effect and causes additional tissue damage [85, 86]. Various damaging effects of ROS/reactive nitrogen species (RNS) can be seen in chronic wounds. An overproduction of ROS/RNS results in inactivation of epidermal enzymatic antioxidants, despite increased enzymatic antioxidant expression in the wound and significantly depletes nonenzymatic antioxidant levels in wound tissues. This results in sustained elevation and survival of ROS/RNS in chronic wounds [87]. Sustained oxidative and nitroxidative stress prolongs the inflammation in chronic wounds as both ROS and RNS stimulate neutrophil and macrophage chemotaxis and migration and also induce the expression of adhesion molecules in the capillaries. Direct cellular effects of ROS/RNS include impaired migratory,

Table 1: Mechanism of ROS damage in specific complications.

Pulmonary hypertension (PHT)	Chronic hypoxia-vascular remodeling with medial hypertrophy due to NADPH oxidases
Delayed wound healing	(i) ROS/reactive nitrogen species (RNS) overproduction prolongs the inflammation in chronic wounds as both ROS and RNS stimulate neutrophil and macrophage chemotaxis and migration (ii) Direct cellular effects of ROS/RNS include impaired migratory, proliferative and extracellular matrix (ECM) synthetic properties of dermal fibroblasts, and keratinocytes
Thrombosis	(i) Propagation of platelet activation by inactivating nitric oxide (ii) Release of platelet agonists such as ADP, giving formation of isoprostanes and ox-LDL causing the release of proatherogenic molecules such as CD40L which are mainly produced by NADPH oxidase
Osteoporosis	NOX1, NOX2, and NOX4 (NOX family of NADPH oxidases) play role in bone resorption due to activation of mature osteoclasts
Silent brain infarcts	NOX2 imbalance causes brain injury/stroke

proliferative, and extracellular matrix (ECM) synthetic properties of dermal fibroblasts and keratinocytes [88]. There is excess iron deposition in the skin of patients with venous ulceration that increases the chances of free radical production by Fenton reaction [89]. There is no record to date that measures iron deposition in the skin of NTDT patients.

In summary, hypoxia stimulates wound healing such as the release of growth factors and angiogenesis [85]; however, oxidative stress is thought to play a detrimental role in the healing process.

4.4. Oxidative Stress and Thrombosis. The first study demonstrating that platelets were able to generate ROS was published in 1977. Currently, we know that ROS are implicated in platelet activation by (1) propagation of platelet activation by inactivating nitric oxide, (2) releasing platelet agonists such as ADP, giving formation of isoprostanes and ox-LDL, and (3) releasing proatherogenic molecules such as CD40L, which are mainly produced by NADPH oxidase [90]. ROS formation is functionally relevant for platelet activation; the role of NADPH oxidase was also studied in a model of thrombus formation upon blood perfusion at high shear. Platelet thrombus formation in samples where NADPH oxidase inhibitors were added was significantly reduced [91]. NADPH oxidase activation is also implicated in platelet mediated LDL oxidation [89]. Generally, ROS have been suggested to act as second messengers in platelet activation. Specific proposed functions of NOX-derived ROS in platelets include regulation of platelet aggregation, adhesion, and recruitment [92–96].

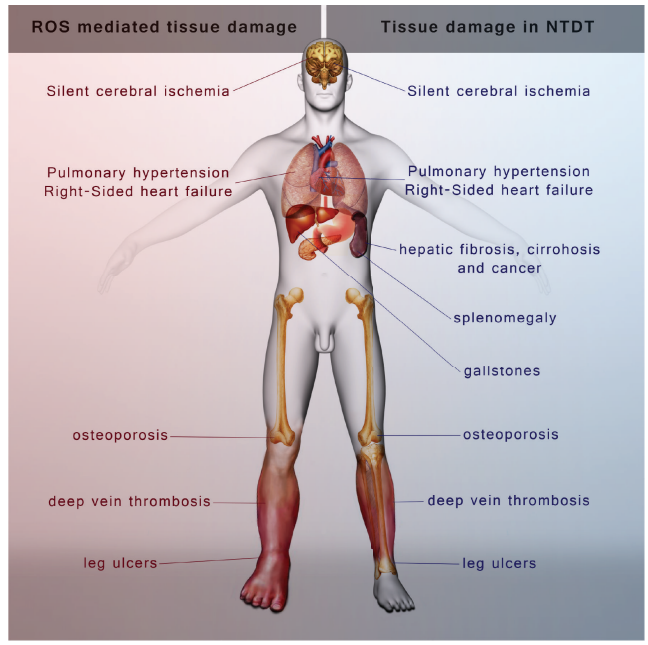

Figure 2: ROS mediated organ damage versus NTDT mediated tissue damage.

4.5. Oxidative Stress, Osteoporosis, and Brain Infarcts. NOX1 appears to be required for the differentiation of precursor into mature osteoclasts in response to the receptor activator of the NFkB ligand RANKL [97]. Experiments indicate that both NOX4 and NOX2 participate in bone resorption by activating mature osteoclasts [98]. NOX also plays a role in the CNS where stroke size was markedly reduced in NOX2-deficient mice, while increased NOX2 expression in diabetic rats was associated with an aggravated ischemic brain injury [99, 100].

5. Conclusion

Increased ROS formation is already proven in TDT patients. It is more pronounced in NTDT patients due to their chronic hypoxemic state resulting from less blood transfusions and a different pattern of iron accumulation that results in "free iron" ready for generating oxidative material. ROS excess has been linked to numerous pathological processes most of which coincide with those present in NTDT patients.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Beta-Thalassaemia Intermedia: Evaluation of Endocrine and Bone Complications

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Objective. Data about endocrine and bone disease in nontransfusion-dependent thalassaemia (NTDT) is scanty. The aim of our study was to evaluate these complications in β-TI adult patients. *Methods*. We studied retrospectively 70 β-TI patients with mean followup of 20 years. Data recorded included age, gender, haemoglobin and ferritin levels, biochemical and endocrine tests, liver iron concentration (LIC) from $T2^*$, transfusion regimen, iron chelation, hydroxyurea, splenectomy, and bone mineralization by dual X-ray absorptiometry. *Results*. Thirty-seven (53%) males and 33 (47%) females were studied, with mean age 41 ± 12 years, mean haemoglobin 9.2 ± 1.5 g/dL, median ferritin 537 (range 14–4893), and mean LIC 7.6 ± 6.4 mg Fe/g dw. Thirty-three patients (47%) had been transfused, occasionally (24/33; 73%) or regularly (9/33; 27%); 37/70 (53%) had never been transfused; 34/70 patients had been splenectomized (49%); 39 (56%) were on chelation therapy; and 11 (16%) were on hydroxyurea. Endocrinopathies were found in 15 patients (21%): 10 hypothyroidism, 3 hypogonadism, 2 impaired glucose tolerance (IGT), and one diabetes. Bone disease was observed in 53/70 (76%) patients, osteoporosis in 26/53 (49%), and osteopenia in 27/53 (51%). *Discussion and Conclusions*. Bone disease was found in most patients in our study, while endocrinopathies were highly uncommon, especially hypogonadism. We speculate that low iron burden may protect against endocrinopathy development.

1. Introduction

Beta-thalassaemia intermedia (β-TI) is a form of NTDT encompassing patients who do not require regular transfusions throughout life, although they may be needed occasionally or even frequently in certain clinical conditions [1, 2]. These patients spontaneously maintain Hb levels 7–10 g/dL and show different levels of splenomegaly and iron overload.

Beta-TI patients show clinical pictures of intermediate severity between the asymptomatic carrier (Thalassaemia Minor) and the transfusion-dependent thalassaemia patients (Thalassaemia Major). The association of mild, moderate, and severe β-mutations as well as association of triplication/multiplication of α-globin genes can be responsible for wide spectrum of genotypes, and a genotype/phenotype correlation has been observed.

However, several genetic as well as environmental factors can modify clinical expression. The genes affecting the globin chain production are recognized as primary and secondary modifiers, but several other conditions, called tertiary modifiers, can affect the clinical expression, such as the coinheritance of hereditary haemochromatosis or Gilbert syndrome, or alterations of genes involved in iron absorption, bone metabolism, or susceptibility to infections [3]. There is increasing evidence that bone disease may be modified by polymorphisms at loci that are involved in bone metabolism, including the genes for the vitamin D receptor, collagen, and the oestrogen receptor [4–7]. Moreover, varying availability of medical care can influence the outcome of β-TI patients.

Endocrine complications are less commonly seen in patients with NTDT than in those with more severe forms

of thalassemia (β-TM and/or severe forms of haemoglobin E/β-thalassaemia) [1, 8–12].

Nonetheless, β-TI patients remain at risk of morbidity from endocrine gland and bone pathology, associated with bone pain and fractures [3]. An increased risk of developing several complications, including diabetes mellitus, hypothyroidism, hypoparathyroidism, hypogonadism, and osteoporosis, has been reported in association with higher liver iron concentration (LIC) values or serum ferritin levels as the patients advance in age [13–17]. Conversely, from observational studies lower rates of the same morbidities were associated with iron chelation therapy [11].

As in patients with β-TM [18], the pathophysiology of endocrinopathies and low bone mineral density in patients with β-TI is probably multifactorial; ineffective erythropoiesis and expansion of the erythron in the bone marrow are directly implicated [1, 19, 20] as well as low foetal haemoglobin, splenectomy, and hydroxyurea therapy [14, 19, 21].

In view of the limited data on endocrinopathy and bone disease prevalence in NTDT, we evaluated these complications in a group of β-TI adult patients.

2. Patients and Methods

In a retrospective study, 70 β-TI adult patients with a mean followup of 20 years were investigated. All patients were followed up at the Hereditary Anaemia Centre of Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico of Milan. The study was in adherence to the tenets of the Declaration of Helsinki and approval from the local ethics committee was obtained.

Disease history and systemic treatment, if any, were recorded for all patients including age, gender, haemoglobin and ferritin levels, biochemical and endocrine tests, bone metabolism indices, transfusion regimen, chelation, hydroxyurea therapy, bone specific treatment, and splenectomy.

Patients were considered occasionally transfused when the number of transfusions was <6 per year.

Liver iron concentration (LIC) was derived from T2* from magnetic resonance imaging (Scanner MRI 1,5 Tesla Avanto Siemens, Enlargen - software CMR Tools, Imperial College, London) according to the formula $[1/(T2^*/1000)] \times 0.0254 + 0.202$ [21].

All patients underwent bone densitometry scan performed by dual X-ray photon absorptiometry (Hologic Bone Densitometer QDR Discovery A, Version 12.7.3.1 WALTHAM, MA, USA) at the lumbar spine and femur. The bone mineral density values were expressed as T- and Z-scores; the T-score was calculated as a standard deviation score (SDS) from a normal reference population database, while the Z-score was calculated as an SDS from an age- and sex-matched population. Data were classified according to the WHO report (WHO Technical Report, ISCD Official Position Paper 2007): T-score > −1: normal, T-score between −1 and −2.5: low bone density (osteopenia), and T-score < −2.5: osteoporosis.

Data are presented as means and standard deviation or median and range as appropriate.

3. Results

Among 70 patients, 37 (53%) were males and 33 (47%) were females; mean age was 41±12 years and mean of haemoglobin levels was 9.2 ± 1.5 g/dL. Median ferritin was 537 (range 14–4893) ng/mL and mean LIC 7.6 ± 6.4 mg Fe/g dw. Eleven patients (16%) were anti-HCV antibody positive, and among them 6 (55%) were HCV-RNA positive. Splenectomy had been performed in 34 out of 70 patients (49%) and 13 patients (18%) had a history of bone fractures (2 spontaneous and 11 following traumas).

Regarding patient treatment, 37/70 (53%) had never been transfused and 33 (47%) had been occasionally (24/33; 73%) or regularly (9/33; 27%) transfused. The patients in the latter group were considered NTDT patients because of the regular transfusion regimen that did not start in childhood but starts later, after an occasional event (pregnancy, surgery, infection, ...), or because of clinical complications, such as heart failure and fatigue with very low Hb levels leading to poor quality of life. Moreover, the mean transfusional range was 40 days.

Thirty-nine patients (56%) were on chelation therapy and 11 (16%) on hydroxyurea. Hormone defects were corrected with replacement therapy in all cases except one, due to clinical contraindications to sex steroid treatment. Four patients (6%) were on bisphosphonate, 5 (7%) on calcium + vitamin D supplementation, 2 (3%) on calcium alone, and 16 (23%) on vitamin D alone.

Characteristics of the population are summarized in Table 1.

Only 15 out of 70 patients (21%) were diagnosed with endocrinopathy: 10 had hypothyroidism (5 of them were subclinical and did not require therapy), 3 hypogonadism, 2 impaired glucose tolerance (IGT), and one diabetes. Among them, only one patient presented two endocrine complications associated (hypogonadism and diabetes). None of the patients presented with hypoparathyroidism. Vitamin D deficiency was present in 49 patients (70%).

Bone disease was observed in 53 (76%) patients, osteoporosis in 26, and osteopenia in 27 β-TI (Table 2).

In the group of patients with osteoporosis, 7 of them (10%) had involvement of both vertebral and femoral sites, 18 (26%) presented only vertebral osteoporosis, and one patient (1%) only had femoral osteoporosis. In the group of patients with osteopenia, 15 (21%) had involvement of both sites; 8 (11%) only had involvement of the vertebral site and 4 (6%) only had involvement of the femoral site. Among the 5 patients with selective involvement of the femoral site (cortical bone disease), BMD was reduced both at the femoral neck and at total femoral scan.

4. Discussion

Endocrine data in β-TI patients is scanty in the literature. The biggest problem with β-TI patients is the wide heterogeneity of the genotypes and of clinical picture, covering a broad

Table 1: Characteristics of the population.

Parameter	Values
Male (number (%))	37/70 (53)
Female (number (%))	33/70 (47)
Age (years ± SD)	41 ± 12
Hb (g/dL ± SD)	9.2 ± 1.5
Ferritin (ng/mL (range))	537* (14–4893)
LIC (mg Fe/g dw ± SD)	7.6 ± 6.4
Anti-HCV positive (number (%))	11/70 (16)
HCV-RNA positive (number (%))	6/11 (55)
Splenectomy (number (%))	34/70 (49)
Transfusion therapy (number (%))	
Never	37/70 (53)
Occasionally	24/70 (34)
Regularly	9/70 (13)
Chelation therapy (number (%))	39/70 (56)
Hydroxyurea therapy (number (%))	11/70 (16)
Hormone replacement therapy (number (%))	2/70 (3)
Levothyroxine therapy (number (%))	5/70 (7)
Bisphosphonate (number (%))	4/70 (6)
Calcium + vitamin D (number (%))	5/70 (7)
Calcium alone (number (%))	2/70 (3)
Vitamin D alone (number (%))	16/70 (23)

*median and range.

Table 2: Osteopenia/osteoporosis in 70 β-TI adult patients.

Osteoporosis	26/70	Vertebral	18/26		
		Femoral	1/26	Total	0
				Neck	0
				Both	1/1
		Both	7/26	Total	1/7
				Neck	2/7
				Both	4/7
Osteopenia	27/70	Vertebral	8/27		
		Femoral	4/27	Total	0
				Neck	0
				Both	4/4
		Both	15/27	Total	3/15
				Neck	3/15
				Both	9/15
Normal	17/70				

spectrum from good general health even in adult age to a compromised condition with early growth retardation and skeletal deformities, due to intense ineffective erythropoiesis and erythroid bone marrow hypertrophy [22, 23]. In such a complex scenario, which also comprises differing genetic patterns and organ damage, the identification of genetic and/or clinical risk factors for endocrinopathy or osteoporosis is a real challenge. Further, bone and endocrine state have been less studied in β-TI than in β-TM patients.

In our patients with β-TI, the prevalence of endocrinopathy does not appear significantly different from that expected in the general nonthalassaemic population [24–26], while bone demineralization, even severe, is a highly common finding, affecting more than two thirds of patients. The prevalence of osteopenia and osteoporosis is very similar, and trabecular bone is predominantly involved regardless of bone disease severity. These findings are in line with previous literature, as bone abnormalities typical of β-TM have been described as often present in β-TI, and sometimes more marked, starting from the first decade of life. An increased risk of fractures, even spontaneous or following minor traumas, has also been reported; this has been ascribed to osteoporosis as well as to medullary overgrowth due to erythroid stress, aiming to compensate for anaemia [27]. When fractures are recurrent, some authors recommend transfusion therapy [28]. In a large thalassaemic population from North America, Vogiatzi et al. described a 12% prevalence of fractures in β-TI patients, increasing with age and with use of sex hormone replacement [29], that is, with hypogonadism.

An additional negative influence on bone condition can be caused by the association between β-TI and 25-OH vitamin D deficiency, frequently reported in thalassaemic populations [12, 29]. In our group, vitamin D deficiency was demonstrated in the majority of the β-TI patients, 35% of whom were compliant with cholecalciferol supplementation prescribed at the usually recommended dose of 800 IU/day [30]. Nonetheless, bone demineralization of varying severity and low 25OH-vitamin D levels were present in most cases. The persistence of inadequate vitamin D status despite supplementation and the possibility of reduced skin production or impaired liver metabolism of the vitamin have been previously described in β-TM patients [31, 32]. If β-TI patients had similarly increased vitamin D requirements in order to produce adequate 25-OH D serum levels, they would need high dose supplementation, as indicated by Soliman et al., recommending repletion therapy with 50000 IU of vitamin D weekly for 8 weeks, followed by maintenance with doses up to 50000 IU per month [32].

Unlike β-TM, endocrinopathies play a minor role in bone condition in β-TI. In a previous study from our group, 78.4% out of 111 β-TM patients were hypogonadic, 17.1% were hypoparathyroid, and 16.2% were both hypogonadic and hypoparathyroid, while glucose metabolism was impaired in 40% of patients (diabetes mellitus in 18% and IGT in 22%) [33]. Conversely, in our β-TI patients a single endocrine defect was present in merely 15 out of 70 patients (21%), and only one patient (0.01%) had more than one defect.

The low prevalence of endocrine complications in β-TI has been previously described; however, in our patients the prevalence of single defects differs from previous reports. More precisely, hypogonadism was exceptional in our group; there were no cases of verified infertility, and more than 50% of our patients had offspring in all cases without induction. In the literature, hypogonadism has been described as the most common endocrine complication in β-TI patients, with predominant involvement of females [13, 34]. Moreover, puberty has been described as not infrequently delayed and irregular menses as not unusual, even if generally fertility appeared not to be compromised, with spontaneous conception [35–37]. Diabetes and hypothyroidism appear less common in the

literature than hypogonadism; the Optimal Care study found prevalence of 1.7%, 5.7%, and 17% for diabetes, hypothyroidism, and hypogonadism, respectively [13]. By contrast, the thyroid was the most frequently affected gland among our patients (10 cases, 14%), and 24% of them had antithyroid antibodies positivity; this percentage is in line with the high prevalence of autoimmune hypothyroidism in Italy [38]. The prevalence of glucose metabolism impairment in our patients was similar to values described in the literature, while no patient was affected by hypoparathyroidism.

It is well known that the different endocrine involvement between β-TI and β-TM highlights the different impact of iron overload on the two diseases. In effect, severe iron-overload related endocrine dysfunction is universally described in β-TM, while in β-TI the pattern of iron overload is preferentially hepatic and it develops gradually throughout life. Compared to the reported data in NTDT [39], our β-TI patients showed a very low iron burden, expressed as LIC (mean values 7.6 mg Fe/g dw); this could explain the low prevalence of endocrinopathies, especially regarding the most iron-sensitive cells (gonadotrophs). On the other hand, these LIC values in our series can be ascribed to the nontransfusion-dependent status *per se*, considering that >50% had never been transfused (mean Hb 9.8 g/dL), and all were compliant with iron chelation therapy when prescribed. This is in agreement with the reported association between transfusion therapy (either intermittent or regular) and increased risk of endocrinopathy [13]. In fact the 3 patients with hypogonadism in the present group all had high LIC values and had been transfused.

A further important consideration is the good tissue oxygenation due to the satisfactory Hb levels in our patients, with the mean Hb in the whole series being 9.2 g/dL (evaluating pretransfusion values in regularly or occasionally transfused patients).

Regarding bone mineralization, the group of transfused (both regularly and occasionally) thalassaemic patients showed lower BMD values compared to ones that were not transfused. This finding suggests that when bone damage is established, it persists despite transfusion therapy. In fact, in the group of transfused patients haemoglobin levels had been low for many years before starting transfusional therapy.

We recognize that one limit of our study is the lack of statistical analysis, but this is due to the low prevalence of endocrinopathies, which does not allow any correlation to be drawn. For example, it would be interesting to evaluate if endocrine deficiency can be associated with splenectomy or with hydroxyurea treatment, but this analysis would require a larger population.

For the same reason, we did not divide patients into groups according to transfusion status to avoid an excessive fragmentation of the population.

In view of the scarcity of endocrinological and densitometric data on the β-TI population in the literature, unlike the well-studied β-TM patients, our findings warrant further studies to evaluate the impact of transfusion and iron-chelation therapy on the outcome of the patients.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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