Justification of Universal Iron Supplementation for Infants 6-12 months in Regions with a High Prevalence of Thalassemia

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Abstract

Background: With a possible increased risk of iron absorption for those with thalassemia, many clinicians hesitate to adopt a universal iron supplementation program for all infants. Therefore, we aimed to determine thalassemia prevalence in 6 to 12-month old infants, along with the iron status of those with and without thalassemia. Procedures: We performed a cross-sectional descriptive study of healthy infants attending the Well Baby Clinic at Thammasat University Hospital. All enrolled were evaluated for complete blood count, hemoglobin electrophoresis, iron parameters, and molecular genetics for common α- and β-thalassemia. Results: Overall, 97 of 206 (47%) participants had thalassemia minor, Hb E traits being the majority; none had thalassemia intermedia or major. Familial history of anemia or thalassemia was an increased risk for detecting thalassemia minor in offspring (OR 5.18; 95% CI 2.60-10.33, P=0.001). Between normal and iron-replete thalassemia minor infants, there were no statistical differences in transferrin saturation, serum ferritin, and hepcidin. However, one-third of those with thalassemia minor (31/97) also had iron deficiency anemia (IDA) with a similar risk of having iron deficiency to infants without thalassemia. There was no hepcidin suppression in our infants with thalassemia minor compared to healthy controls. Conclusions: In Southeast Asia, thalassemia and IDA are endemic, and infants with thalassemia minor, particularly Hb E and β-thalassemia trait, are also at risk of IDA; thus, our short-term universal iron supplementation program for 6 to 12-month old infants should not increase the risk of those with thalassemia minor developing iron overload in the future.

Introduction

Anemia is a global health problem; an estimated 300-million children worldwide had anemia in 2011.[1] Iron deficiency anemia (IDA) is the most common cause of anemia in childhood. Thus, the WHO has recommended an international anemia control guideline: all children and women living in settings where the prevalence of anemia exceeds 20% should receive supplemental iron.[2] In Thailand, this recommendation has been adopted by the Department of Health, Ministry of Public Health (MOPH) and incorporated into our routine childcare. It is fully subsidized by the Thai government through the National Health Security Office (NHSO). The MOPH recommends a universal iron supplement in Thai infants over 6 months old for IDA prevention when these babies come for routine vaccination. Iron supplementation continues until 24 months of age, with 12.5 mg of elemental iron weekly.[3]This age group has been selected as infants over 6 months who have a high prevalence of IDA, which can impair physical, behavioral, and cognitive functions and result in persistent neurocognitive defects, despite later iron therapy.[4] However, local practitioners, in particular pediatricians, are concerned about this policy since there is a high prevalence of thalassemia and hemoglobin disorders in Thailand (P. Surapolchai et al., manuscript in preparation). It is widely accepted thalassemia disease could significantly increase the risk of iron overload (IOL), leading to iron toxicity in later life.[5-6]

Thalassemia (thal) is characterized by inherited mutations of α and β globin genes causing decreased globin synthesis. At least 5.2% of the world population carries one allele of globin gene variants (carrier or trait).[7]

For α-thal, there are two types based on molecular defects: α^0-thal caused by deletions of two linked α-globin genes *in cis* (–/αα) and α^+-thal caused by deletions of one α-globin gene (-α/αα) or nucleotide mutations ($\alpha^T\alpha/\alpha\alpha$ or $\alpha\alpha/\alpha\alpha^T$). Coinheritance of two affected alleles (in autosomal recessive mode) leads to chronic hemolytic anemia and ineffective erythropoiesis known as thalassemia disease (thal disease).[8] Hemoglobinopathy, on the other hand, is mainly caused by mutations of coding sequences, leads to qualitative defects. Several hemoglobinopathies are innocuous and do not lead to any clinical consequences.[9] However, some mutations such as hemoglobin E (Hb E) at codon 26 of the β-globin genes (GAG >AAG) also have quantitative effects and interaction of Hb E with β-thal mutations leads to Hb E/β-thalassemia (Hb E/β-thal) syndrome with heterogeneous clinical severity. In Thailand, 30 to 40 percent of Thais possess thal carriers including α-thal, β-thal and Hb E. Due to the high prevalence of all genotypes, it is not uncommon to find individuals with combined α and β-globin abnormalities.[10-13] Collectively, these thal traits, either simple or in combination, are asymptomatic and do not require specific treatment; they are then classified as thalassemia minor (thal minor). In addition, individuals with homozygous Hb E (Hb E/E), although carry two defective β-globin genes, they are benign with milder forms of anemia without hepatosplenomegaly or blood transfusion required.[14]

Several previous studies have extensively examined iron status in thal patients[15-18], but little is known about thal minor in comparison to normal populations[19], particularly in infants. Iron overload is one of the most common complications in thal patients due to blood transfusions and increased iron absorption[5-6]. Intestinal iron intake in thal has been shown to be enhanced due to hepcidin suppression by the upregulation of erythropoietic markers, such as GDF-11, GDF-15, and Erfe, in response to chronic anemia and erythropoietin drive.[20-22] Hepcidin generally controls iron intake through duodenal enterocytes by limiting the expression of ferroportin: an intestinal iron gateway into our circulation. Recent studies have consistently shown hepcidin suppression in thal patients.[23,24] More recently, a study from Sri Lanka has demonstrated β-thal carriers had mildly suppressed hepcidin concentrations out of proportion to their iron stores. It has been suggested that widespread distribution of iron supplementation could possibly increase the risk of harmful iron overload in β-thal carriers.[25] In Thailand, there has been no data on the iron status and hepcidin levels in the young Thai population with our common thal traits; α-thal and Hb E and homozygous HbE, especially infants who would receive supplementation through our national program.

Our main objective in this study was to determine the iron status in infants aged 6 to 12 months at our Well Baby Clinic and identify the prevalence of iron deficiency (ID) and IDA among those with or without thal. In addition, we evaluated the clinical and laboratory characteristics of each group to identify which factors, including hepcidin levels, would significantly influence iron status. Our work aimed to illustrate whether infants with thal are at similar or lower risk of ID or IDA compared to the general population at this age and supply evidence regarding safe universal iron supplementation for Thai infants in the future.

Methods

Study population

This is a cross-sectional descriptive study approved by the Human Ethics Committee of Thammasat University No. 1 (Faculty of Medicine). Infants aged 6 to12 months who attended the Well Baby Clinic at Thammasat University Hospital, from June 2016 to June 2017, were recruited randomly after informed consent was granted by their parents or legal guardians. Our inclusion criteria were term newborns (38-42 weeks gestation) with a birth weight between 2500-4000 grams having no prenatal and perinatal complications such as severe birth asphyxia, severe respiratory distress, or neonatal intensive care unit admission. Infants with chromosome abnormalities/syndromes, infectious/inflammatory diseases, and any acute health problems were excluded. All clinical samples from enrolled subjects were collected before routine universal iron supplementation. We collected demographic/clinical data through direct interviews with two investigators (PSi and PSu). Weight and length of participants were measured and evaluated by Z-score, according to WHO guidelines.[26] The Z-scores of weight-for-lengths below or above two standard deviations (SD) are categorized as underweight and overweight, respectively.

Hematological and biochemical evaluation

All participants underwent complete blood count evaluation using automated cell count (UniCel®DxH 800, Beckman Coulter, Brea, USA), hemoglobin typing by automated capillary electrophoresis analyzer (MINICAP, Sebia, Lisses, France), iron parameters including serum iron (SI), total iron-binding capacity (TIBC) using a fully automated quantitative assay, and serum ferritin by electrochemiluminescence immunoassay (ECLIA or Elecsys® technology, Roche Diagnostics, Penzberg, Germany). The assays of SI, TIBC, and serum ferritin were performed using ROCHE COBAS BIO centrifugal analyzer according to the manufacturer's instruction.[27] Serum hepcidin was determined by a competitive inhibition enzyme-linked immunosorbent assay (cELISA)[28,29], with detection ranges of 2.47-200 ng/mL, according to the manufacturer's instructions (Catalog No. CEb979Hu, Cloud-Clone Corp., Uscn Life Science Inc., Wuhan, China), using afternoon blood sampling to prevent diurnal variation.[28,30] In the assay, a monoclonal antibody specific to hepcidin was pre-coated onto a microplate. A competitive inhibition reaction was launched between biotin-labeled hepcidin and unlabeled hepcidin (standards or samples) with the pre-coated antibody specific to hepcidin. After incubation, the unbound conjugate was washed off. Next, avidin conjugated to horseradish peroxidase (HRP) was added to each microplate well and incubated. The amount of bound HRP conjugate was reversely proportional to the concentration of hepcidin in the sample. After the addition of the substrate solution, the intensity of color developed was reversely proportional to the concentration of hepcidin in the sample.

Molecular analysis

Genomic DNA was extracted from peripheral blood leukocytes using a standard protocol of phenol-chloroform extraction. Alpha-globin genotyping was performed by a single-tube multiplex gap polymerase chain reaction (Gap-PCR) for detecting seven common α-globin deletions ($-^{SEA}$, $-^{THAI}$, $-(\alpha)^{20.5}$, $-^{FIL}$, $-^{MED}$, $-\alpha^{3.7}$, $-\alpha^{4.2}$) and a single-tube multiplex amplification refractory mutation system (ARMS-PCR) for screening six common non-deletional α-globin mutations in Thailand: initiation codon (ATGA-G), codon 30 (ΔGAG), codon 59 (GGCGAC), codon 125 (CTGCCG) or Hb QuangSze, termination codon (TAACAA) or Hb Constant Spring, and a termination codon (TAATAT) or Hb Paksé.[31] Beta-globin genotyping was performed by ARMS-PCR for detecting 16 common beta-globin mutations (-28, CD8/9, CD17, CD19, CD26 (Hb E), CD26 G>T (stop codon), CD27/28, IVSI-I, IVSI-5, CD35, CD41, CD41/42, CD43, CD71/72, CD95, and IVSII-654).[32] A single-tube multiplex Gap-PCR and enzymatic amplification was used for common beta-globin gene deletions (3.48 kb, 619 bp, Filipino (β)°, SEA HPFH (β)°, Chinese$^{G}\gamma(\ ^{A}\gamma\delta\beta)^{\circ}$, Thai (δβ)°, Hb Lepore, HPFH-6 $^{G}\gamma(\ ^{A}\gamma\delta\beta)^{\circ}$, Siriraj-thal$^{G}\gamma(\ ^{A}\gamma\delta\beta)^{\circ}$, Asian Indian type A, and Asian Indian type B).[33,34] Hemoglobin E testing was studied by restriction fragment length polymorphism (RFLP)-PCR utilizing *Mnl* I restriction enzyme.[35]

Definitions

Hemoglobin (Hb) <11 g/dL was used to define anemia, according to WHO criteria.[36] Participants were classified as having ID if their serum ferritin (SF) was <30 ng/mL or transferrin saturation (TS) was <16% (TS = SI/TIBC x100).[37] IDA was diagnosed if there was compatibility with either laboratory criteria or therapeutic response to iron therapy.

The diagnosis of β-thal trait was based on Hb A2 level >3.5%. Infants with Hb F>10% were investigated for common beta-globin gene deletions to diagnose δβ-thal traits and hereditary persistence of fetal hemoglobin (HPFH) (Viprakasit V's personal communication).[38]

Statistical analysis

The sample size was calculated from this formula: $N=Z^2pq/d^2$, Z=1.96, p=0.4, q=0.6, d=0.07. The study population used, according to the prevalence of thal in Thailand (30-40%),[8,9] was 188, approximating 200 participants (with 10% dropout). Demographic data were summarized as frequency and percentage for qualitative data and as mean and SD for quantitative data. Student's t or Mann-Whitney U test was used to compare continuous variables; the chi-square or Fisher's exact test was used for categorical variables, as appropriate. Univariate and multivariate logistic regression analysis was performed to identify risk factors.

A P of <0.05 was considered statistically significant.

Results

A total of 206 infants, consisting of 114 males (55.3%), with the mean age of 8.2 months (SD 2.0, range 6-12 months), were enrolled randomly after informed consent. Interestingly, we found 97 individuals (47%) with some form of thal minor, 39 with α-thal (18.9% of the total population), 45 with β-globin mutation mainly Hb E (21.8%), and 13 with combined α and β globin abnormalities (6.3%). None of these individuals had the genotype found in thal diseases such as HbH disease or Hb E/β-thal; therefore, they were all classified as thal minor and were subsequently used as a group for further analysis. Details of all comprehensive genotype data are shown in **Table 1** . Infants with thal minor had no history of blood transfusion and no hepatosplenomegaly.

We found no significant difference in all clinical characteristics: age, gender, growth and nutrition parameters, and the iron markers such as serum ferritin, TS, and hepcidin levels. The exception was having a family history of anemia or thal being more common in infants with thal minor (43.3%) than those without thal (12.8%), as shown in**Table 2.** In our logistic regression analysis, having a history of anemia or thal showed an increased risk of thal minor in infants: odd ratio 5.18 (95% CI: 2.60-10.33), P =0.001. Interestingly, the number of infants with thal minor with IDA at first diagnosis was higher than those with normal globin genotypes (32.0% *vs.* 20.2%); moreover, the number of infants with normal iron status and ID were significantly different among infants with and without thal minor (P =0.037) **(Table 2).** To determine the effects of iron status (normal iron, ID, or IDA) on hematological parameters, we performed subgrouping analysis within the groups of infants with and without thal minor (**Table 3**). There were significant differences in Hb, hematocrit (Hct), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), and red blood cell distribution width (RDW) within both groups suggesting the significant role of iron in determining Hb, Hct and red blood cell indices. With the exception of increased RBC counts and RDW, other RBC parameters were lowest in IDA, followed by ID and normal iron. Of note, infants with thal minor who had IDA (N=31) displayed the statistically lowest values in MCV, MCH, and highest RDW **(Table 2),** suggesting co-inheritance of globin mutations can have epistatic effects on hematology on top of the primary effects of iron status.

In this regard, we further compared the group of infants with normal iron and IDA to see the effects of thal minor (see **Supplementary Table 1**). Coinheritance of thal had significant effects on Hb, Hct, RBC, MCV, MCH, RDW but not MCHC **(suppl. Table 1)** only in those with normal iron status. However, we found only RBC and MCV to be significantly different in infants who already had IDA, suggesting this epistatic effect was operative on those only two parameters. In addition, we found an effect of iron on significantly decreased levels of Hb A2 in infants without thal minor but not in those with thal. On the other hand, the basal Hb F in infants with thal minor was generally higher than those without thal minor, suggesting a delay in globin switching, one of the consequences of globin abnormality.[39,40] Finally, the iron status was not significantly associated with the levels of persistent Hb F expression within the groups of both infants with and without thal minor (**Table 3**).

To determine the effects of thal minor on hepcidin expression, we compared serum hepcidin, serum ferritin, and TS among healthy infants (no thal and normal iron status) with those having different types of thal minor as shown in **Figure 1** . Details of measurements in each group are shown in **Supplementary Table 2A** (with iron-replete) and **2B** (with iron deplete). The levels of hepcidin tended to be slightly lower in those with thal minor and lowest in those with combined α and β globin mutations (**Figure 1C).**This finding was consistent with a slightly increasing trend of serum ferritin **(Figure 1A)** and TS **(Figure 1B)** in those with combined thal minors, although there were no statistically significant differences. We also found no differences in these parameters as a group (normal *vs* . thal minor) and by different genders (male *vs* . female) (**see Supplementary Figure 1**).

The primary diagnosis of infants with IDA using Hb levels, SF and TS values in our study was further confirmed in the majority of those by the therapeutic response to iron therapy. Thirty-four out of 53

IDA infants with (n=31) and without thal minor (n=22) who can be reached by telephone appointments have received iron therapy (4-6 mg/kg/day) for 8-12 weeks, and all showed therapeutic response. Their hematology during fellow up visits revealed a significant increase in all RBC parameters compared to the baseline within their groups (P <0.05). Interestingly, IDA infants without thal minor (n=18) had slightly greater increment for Hb and MCV after iron therapy than IDA infants with a thal minor (n=16); however, there were no statistically significant differences between the groups (P =0.099 and 0.278, respectively). The mean Hb increment after iron therapy was 1.7 g/dL (SD 1.1, range 0.1-4.1) vs 1.1 (SD 0.9, range 0.2-3.1 g/dL) and mean MCV increment 4.9 fL (SD 3.7, range 0.3-11.8 fL) vs 3.5 (SD 3.4, range 0-9.5) fL, in IDA infants without and with thal minor respectively. This result has confirmed our diagnosis of IDA in both groups at the baseline. (**Supplementary Table 3**)

Discussion

Our study found the prevalence of thal minor (carrier) to be nearly half of the infant population (47%). It appeared to be higher than previously reported, which had been ranges of 30-40%.[8,10] One of the main reasons was the DNA testing used in our current study was far more comprehensive than the approach used (cord blood hemoglobin studies, hemoglobin typing, etc.) some 20 years ago. This finding was consistent with the recent thal prevalence reported by Viprakasit V *et al* . in 2009[41] with Hb E trait being the most common type of thal minor[9,10,41]. In Thailand with its frequency up to 50-60% in Southeast Asia,[9] we found no individuals with thal disease and this might result from the effectiveness of our prevention and control program that screens for thal carriers in pregnant women and their partners in order to identify couples with genetic risk of severe thal syndromes.[11] Therefore, our studied population would simply represent 'healthy' infants who had received routine care in our health system and were a primary target for the iron supplementation program endorsed by the MOPH.

A study of β-thal traits showed mildly increased erythropoiesis, evidenced by elevated erythropoietin levels.[42]Besides, adults with α- or β-thal traits have shown increases in soluble transferrin receptors or erythropoietin concentrations, indicating ineffective erythropoiesis and increased erythropoietic drive leading to hepcidin suppression and upregulated iron absorption.[17] Previous studies in India and Iran examining the iron status of adults with β-thal traits concluded that β-thal traits had higher serum ferritin than the controls, representing an advantage in iron balance.[43,44] These findings were discordant with others, which had stated that ID might commonly coexist with thal traits.[17,45,46] These conflicting results caused uncertainty in iron supplementation strategies for areas with a high prevalence of hemoglobinopathy. A universal iron supplementation program has raised concern since it might increase the risk of iron overload in individuals with thal minors.

A recent community study of 1821 Sri Lanka schoolchildren aged 8-18 years (48.3% males) from the Oxford group has shown that this might be the case for those with β-thal traits.[25] Eighty-two β-thal carriers with iron-replete had evidence of increased erythropoiesis, a slight but significant reduction in hepcidin, and suppression of hepcidin out of proportion to their iron stores: hepcidin-ferritin ratio compared with non-carrier controls (n=176 with normal MCV and MCH). In another recent cross-sectional study of 2273 children (aged 12–19 years) from a total of 7526 students, also in Sri Lanka, this effect was also observed in the iron-replete α-thal carriers as compared to the non-iron deficient controls without thal minor (4.8 ng/mL vs. 5.3 ng/mL, P = 0.02).[47] However, they did not identify such findings in those with Hb E traits from both cohorts.[25,47] Based on these results, it has been proposed the hepcidin cutoff of < 3.2 ng/mL might be used to select cases for iron supplementation in countries with high rates of thal carriers.[47] Both studies were conducted in primary and secondary school students; this is the age group in which iron supplementation is given in Sri Lanka. However, the effect of thal carriers on hepcidin suppression and risk of iron accumulation in younger thal minor remains unclear.

Our study, for the first time, determined this iron supplement issue in infants with thal minor. While we could not find a significant hepcidin suppression in our infant thal minors compared to previous studies, our results were somewhat in line with such findings. Most of our thal minors were Hb E traits, and this condition did not show a significant enough globin imbalance leading to ineffective erythropoiesis and subsequent hepcidin

suppression. Moreover, even for individuals with homozygous Hb E, we found no evidence of such an effect. Our infants with α-thal carriers also demonstrated no effects of hepcidin suppression, differing from the previous study.[47] It might be possible our studied population was younger with remaining Hb F expression (**Table 1 and 2**) and have less globin imbalance and ineffective erythropoiesis *per se* . It is, therefore, possible the erythropoietic drive that suppresses hepcidin was not fully operative yet.

In addition, the normal physiology of hepcidin expression, especially within the first year of life, might be more dynamic. A recent study in late preterm infants (32-36 weeks gestation) described a physiologic decrease of hepcidin levels during the first 4 months of life to increase iron availability.[48] A recent longitudinal study that followed 140 Spanish healthy and full-term infants found hepcidin levels to increase from 6 to 12 months of age with the levels of hepcidin positively correlated with iron status.[49]These findings suggested that, in normal babies, a regulation of hepcidin production is under development during the first year of life; this might also be true for infants with thal. Therefore, the effects of ineffective erythropoiesis on hepcidin suppression in thal traits might not be fully apparent during the first year of their life. This result warrants further study to define at what age this effect would be first identified.

As a result, we still found our infants with thal minor having a high proportion of iron depletion (57.7%), similar to infants without thal (61.5%); the number of thal infants with IDA was even significantly higher than infants without thal minor (32 *vs.* 20.2%). Thus, the likely causes and possible risk factors of ID need to be further identified (P. Surapolchai, manuscript in preparation). Nevertheless, infants with thal minor who have IDA or ID would benefit from proper iron supplementation. Interestingly, infants with a coexisting thal minor and IDA had significantly reduced Hb, MCV, MCH, and MCHC with increased RDW versus those having thal minor with normal iron or with ID (**Table 2**). These findings were consistent with previous studies in India where MCV and MCH were significantly lower in adults with combined thal traits and IDA than with either of these conditions.[45] To the best of our knowledge; this is the first time RBC indices have been comprehensively analyzed in thal carriers at this age group (6-12-month-old). Our findings could be used for future reference.

Among 36 thal minor infants with anemia, we found 5 cases who did not have coexisting IDA, including infants with two α-thal traits ($-\alpha^{3.7}/\alpha\alpha$ and $-^{SEA}/\alpha\alpha$), one β-thal trait, one Hb E trait and one homozygous Hb E. This suggested that α- and β-thal traits might be the cause of mild anemia in some infants. Accordingly, anemic infants unresponsive to oral iron therapy certainly should be investigated for thal, rather than continuously undergoing long-term iron therapy by default, as toxicity or other side effects may develop. Familial history of anemia or thal as shown herein, was found to be strongly associated with thal minor in offspring and could be used to diagnose future cases early.

In conclusion, our study showed infants (aged 6-12 months) with thal minor in Thailand, in which the majority had Hb E and α-thal traits, are at similar risk of having IDA as the general population and this may partially be due to a lack of hepcidin suppression at this age or the type of mutations found in our study. Therefore, a universal short-term period of iron supplementation in infants would be not too harmful since more than half of the population could benefit from this strategy. However, beyond this age group, particularly for school children, a proper measurement of serum hepcidin and using a cut-off as described earlier would be an alternative approach for the selection of those who should genuinely receive iron supplementation; this would minimize the chance of overtreating individuals with thal minor in areas of a high prevalence of thal and hemoglobinopathies.[47]

Conflict of interest

The authors declare no conflict of interest.

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Author contributions

PSi and PSu were the co-principle investigators of the project, evaluated all study participants, collected data, performed analysis and drafted the manuscript. VV, as a senior author, developed the concept of this study, analysis plan, and overall interpretation of the results and revised the manuscript. All authors read and approved the final version of the manuscript.

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FIGURE 1 Comparison of serum ferritin (A), transferrin saturation (B) and serum hepcidin (C) between normal infants (NL) and infants with three subgroups of thalassemia minor; α-thalassemia trait, β-thalassemia trait or hemoglobin (Hb) E trait, and combined α- and β-globin mutations

Legend to Figure: #All infants shown herein were determined as having normal iron status. The difference between each parameter of infant groups was analyzed using a one-way analysis of variance (P). Note: NL: normal; a: α-thalassemia trait, b: β-thalassemia trait or hemoglobin (Hb) E trait, and c: combined α- and β-globin mutations. O: infants with thalassemia minor who had iron deficiency (ID); X: infants with thalassemia minor who had iron deficiency anemia (IDA).

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