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Original Article

Analysis of Hepcidin and Interleukin-6 Levels among Transfusion-Dependent Thalassemia Patients With and Without Alloimmunization/Autoimmunization

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ABSTRACT

Background: Transfusion-dependent thalassemia (TDT) necessitates regular transfusions, resulting in complications such as iron overload, hemolytic anemia, and the emergence of alloantibodies/autoantibodies. This situation poses challenges in obtaining compatible transfusions. Excessive iron and chronic hemolysis impact the elevation of Interleukin-6 (IL-6), initiating an inflammatory process that triggers hepcidin formation and influences antibody development. This study aims to analyze disparities in IL-6 and hepcidin levels and establish the correlation between IL-6 and hepcidin in TDT patients with and without alloimmunization/autoimmunization. **Methods:** Forty whole blood samples were collected from TDT patients with and without alloimmunization/autoimmunization, centrifuged, and the serum extracted, then stored in a refrigerator at -80°C. IL-6 and hepcidin levels were assessed using the ELISA method. The Mann-Whitney U test was employed to evaluate differences in hepcidin and IL-6 levels between the two groups. In contrast, the Spearman Correlation test was utilized to analyze the correlation between hepcidin and IL-6 levels. **Results:** IL-6 levels in the TDT group with alloimmunization/autoimmunization (3.64 pg/mL) were significantly higher compared to the TDT group without alloimmunization/autoimmunization (1.41 pg/mL; $p < 0.05$). Hepcidin levels in the TDT group with alloimmunization/autoimmunization (2,950.6 pg/mL) were significantly higher compared to the TDT group without alloimmunization/autoimmunization (1,599.6 pg/mL; $p < 0.05$). The Spearman correlation test revealed a significant positive correlation between hepcidin and IL-6 levels in TDT patients with alloimmunization/autoimmunization ($r = 0.764$; $p = 0.000$). Additionally, a significant positive correlation was observed between hepcidin and IL-6 levels in TDT patients without alloimmunization/autoimmunization ($r = 0.559$; $p = 0.010$). **Conclusion:** IL-6 and hepcidin levels were elevated in TDT patients with alloimmunization/autoimmunization compared to those without. Interleukin-6 and hepcidin exhibited a positive correlation in both transfusion-dependent thalassemia groups.

Keywords: Anemia, Antibodies, Hepcidin, Interleukin-6, Transfusion-Dependent Thalassemia.

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INTRODUCTION

Thalassemia, a group of hereditary diseases with a global prevalence of approximately 7-8%, results from a reduction in the synthesis of normal globin chains, impairing hemoglobin and red blood cell production.[1,2] In Indonesia, as many as 20 million individuals possess this hemoglobin gene disorder, with cases rising from 4,896 in 2012 to 10,793 in June 2021, particularly in Java.[3]

Thalassemia is categorized based on transfusion requirements into transfusion-dependent thalassemia (TDT), which necessitates regular blood transfusions, and non-transfusion-dependent thalassemia (NTDT), which does not require lifelong transfusions.[4] Although iron overload from routine blood transfusions in TDT patients can lead to growth and development disorders, iron-chelating drugs, such as deferasirox and deferiprone, may not effectively reduce excess iron, as observed in a study by Sutrisnanigsih et al.[3,5,6]

Hemolysis and routine transfusions in TDT patients can rapidly increase iron overload over an extended period, prompting macrophages to elevate free radicals and interleukin-6 (IL-6) levels, consequently enhancing hepcidin production. As a key molecule, IL-6 stimulates hepcidin transcription via the STAT pathway, influencing the immune system balance between Th17 cells (IL-17 production) and Treg cells (Foxp-3 production).[7] Elevated IL-6 alters the Treg/Treg cell equilibrium, causing an inflammatory state, conversion of B lymphocyte cells into plasma cells, and increased antibody formation.[8-10]

Hepcidin, synthesized by hepatocytes, functions as a regulator of iron balance in both acute and chronic inflammatory conditions. IL-6, elicited in response to inflammation, plays a pivotal role in upregulating hepcidin expression.[11-16] Iron accumulation initiates an elevation in IL-6 levels, subsequently leading to an augmentation in hepcidin production. Hepcidin attempts to diminish iron absorption from the intestine and excretion from macrophage cells. However, regular transfusions in TDT contribute to elevated iron levels and an increase in IL-6.[17,18] Comparative studies between TDT and NTDT patients indicate higher hepcidin levels in TDT.[2,19] This circumstance, coupled with heightened IL-6, hepcidin, and a shift in the Th17 cell balance towards inflammation, initiates the formation of antibodies.[8,17,18,20]

To categorize TDT patients based on antibody presence, we divided them into two groups: TDT with alloimmunization/autoimmunization and TDT without alloimmunization/autoimmunization. No studies have explored IL-6 and hepcidin levels in

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these groups. This study aims to compare IL-6 and hepcidin levels in TDT patients with and without alloimmunization/autoimmunization and examine the relationship between IL-6 and hepcidin levels across the two groups.

MATERIALS AND METHODS

Research Sample

This study employed an observational analytical approach with a case-control design involving 40 blood samples from TDT patients. The samples were stratified into two groups: 20 TDT patients with alloimmunization/autoimmunization and 20 TDT patients without alloimmunization/autoimmunization. The participants received treatment at the Outpatient Clinic of Dr. Soetomo Regional Public Hospital (hereinafter referred to as RSUD Dr. Soetomo) from June to August 2023. Inclusion criteria encompassed TDT patients undergoing routine transfusions at RSUD Dr. Soetomo who were willing to participate in the research. Exclusion criteria included thalassemia patients concurrently experiencing other conditions such as infection or inflammation and those unwilling to participate. Ethical approval for this research was obtained from the Health Research Ethics Committee of RSUD Dr. Soetomo, Surabaya, with the ethical certificate number 0699/KEPK/VI/2023.

Examination

A total of 5 mL of whole blood, collected consecutively in a BD SST II Advance tube, underwent centrifugation to obtain serum and was subsequently stored in a refrigerator at -80 °C until the completion of sample collection. Serum IL-6 levels were assessed using a sandwich ELISA (E-EL-H6156, Elabscience Biotechnology Inc., Houston, TX, USA), while serum hepcidin levels were measured with a sandwich ELISA (E-EL-H6013, Elabscience Biotechnology Inc., Houston, TX, USA). For IL-6 examination, a serum sample was introduced into an ELISA microplate containing specific antibodies against human IL-6. Biotinylated detection antibody and NeutrAvidin™ horseradish peroxidase (HRP) conjugate were added to each microplate, followed by incubation. Subsequently, a substrate reagent was added, resulting in a color change from the initial mixture to blue. The enzyme reaction was halted by the addition of a stop solution, causing the color to shift to yellow. Optical density (OD) was measured using a spectrophotometer at a wavelength of 450 nm, and the detection range of IL-6 was 1.56-100 pg/mL. For hepcidin analysis, the serum sample was placed in an ELISA microplate coated with a specific antibody against human hepcidin. Biotinylated detection antibody and NeutrAvidin™ horseradish peroxidase (HRP) conjugate were added to each microplate, followed by incubation. Subsequently, a substrate reagent was added, resulting in a color change from the initial mixture to blue. The enzyme reaction was halted by the addition of a stop solution, causing the color to shift to yellow. OD was measured at 450 nm using a spectrophotometer, and the detection range for hepcidin was 62.5-4,000 pg/mL.

Data Analysis

Differences in interleukin-6 and hepcidin levels between TDT patients with and without alloimmunization/autoimmunization were assessed utilizing the Mann-Whitney U test. The correlation between hepcidin levels and Interleukin-6 in TDT patients with and without alloimmunization/autoimmunization was examined through the Spearman Correlation test. Data distribution was scrutinized using the Shapiro-Wilk test. Statistical significance was determined at a p-value < 0.05.

RESULTS

General Characteristics of Research Subjects

The research subjects exhibited a higher representation of women in the TDT group with alloimmunization/autoimmunization. In contrast, the TDT group without alloimmunization/autoimmunization showed an equal gender distribution, with the majority of patients in both groups being ≤ 18 years old, spanning an age range of 5-39 years. Blood type O positive was the most prevalent blood type in both groups. The highest frequency of transfusion per month in both groups ranged from 1-2 times, with the TDT group with alloimmunization/autoimmunization showing the highest frequency of 3-4 times per month (Table 1).

Differences in Hepcidin and IL-6 Levels among TDT Patients with and without Alloimmunization/ Autoimmunization

Hepcidin levels in the TDT group with alloimmunization/ autoimmunization (2,950.6 pg/mL) were significantly higher compared to the TDT group without alloimmunization/ autoimmunization (1,599.6 pg/mL) (Table 2). A statistically significant difference in hepcidin levels was observed between the TDT patients with alloimmunization/autoimmunization and those without alloimmunization/autoimmunization (p-value = 0.001).

IL-6 levels in the TDT group with alloimmunization/autoimmunization (3.64 pg/mL) were significantly higher compared to the TDT group without alloimmunization/autoimmunization (1.41 pg/mL) (Table 2). A statistically significant difference in IL-6 levels was observed between the TDT patients with alloimmunization/autoimmunization and those without alloimmunization/autoimmunization (p-value = 0.005).

Analysis of the Correlation between Hepcidin and IL-6 Levels in TDT Patients with and without Alloimmunization/Autoimmunization

In the TDT group with alloimmunization/autoimmunization, a positive correlation between IL-6 and hepcidin levels was identified, yielding a significant p-value of 0.000 ($p < 0.05$) (Figure 1). Additionally, a Spearman correlation coefficient value of 0.764 was obtained for the correlation between IL-6 and hepcidin in the TDT group with alloimmunization/autoimmunization. This value suggests that the correlation between IL-6 and hepcidin levels in TDT patients with alloimmunization/autoimmunization falls within the strong correlation category.

Table 1: General Characteristics of Research Subjects.

Characteristics	TDT	
	With Alloimmunization/ Autoimmunization n = 20 (%)	Without Alloimmunization/ Autoimmunization n = 20 (%)
Sex		
Male	8 (40)	10 (50)
Female	12 (60)	10 (50)
Age (years)		
≤ 18	15 (75%)	18 (90%)
> 18	5 (25%)	2 (10%)
Median (min-max)	9.5 (5-39)	11 (6-27)
Blood Type		
A	6 (30%)	4 (20%)
B	4 (20%)	6 (30%)
AB	0 (0%)	2 (10%)
O	10 (50%)	8 (40%)
Resus Positive	20 (100%)	20 (100%)
Transfusion Frequency/Month		
1-2	13 (65%)	15 (75%)
3-4	7 (35%)	5 (25%)

Table 2: Differences in Hepcidin and IL-6 Levels among TDT Patients with and without Alloimmunization/Autoimmunization.

Parameters	Groups	Median (pg/mL)	Range (pg/mL)	p-value
Hepcidin	Without Alloimmunization/Autoimmunization	1,599.6	826.3–3,882.4	**0.001***
	With Alloimmunization/Autoimmunization	2,950.6	1,633.4–5,220.7	
IL-6	Without Alloimmunization/Autoimmunization	1.41	0.27–6.00	**0.005***
	With Alloimmunization/Autoimmunization	3.64	0.76–11.89	

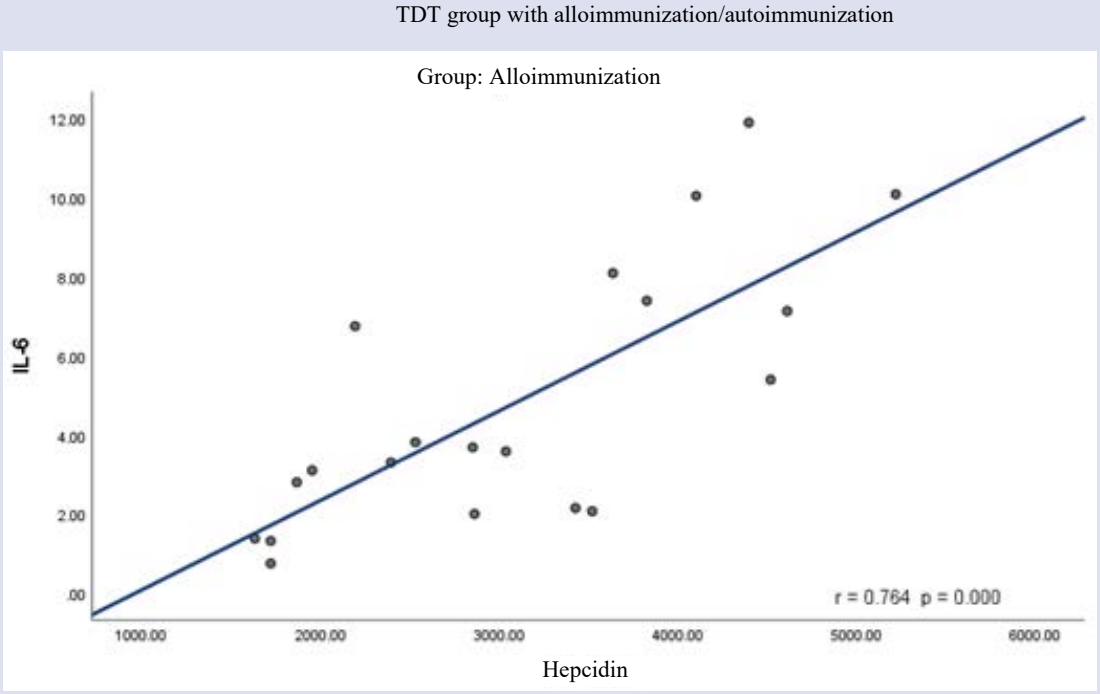

Figure 1: Scatter Plot Analysis of the Correlation between IL-6 and Hepcidin Levels in TDT Patients with Alloimmunization/Autoimmunization.

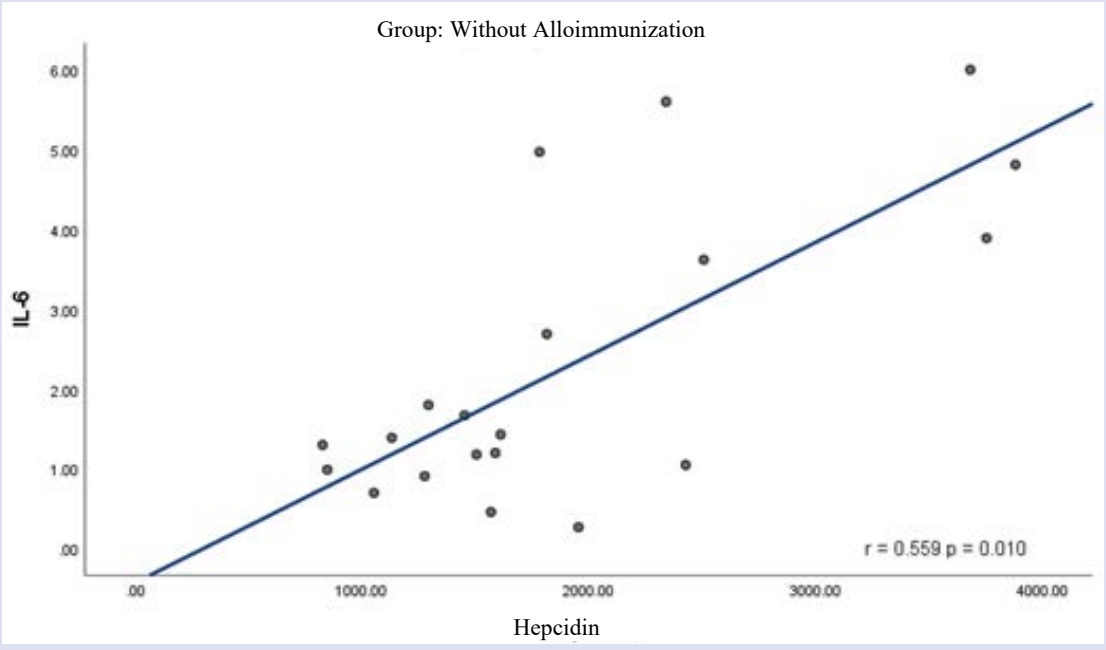

Figure 2: Scatter Plot of the Correlation between IL-6 and Hepcidin Levels in TDT Patients without Alloimmunization/Autoimmunization.

Similarly, in the TDT group without alloimmunization/autoimmunization, a positive correlation between IL-6 and hepcidin levels was observed, accompanied by a Spearman correlation coefficient value of 0.559 and a significant p-value of 0.010 ($p < 0.05$) (Figure 2). The correlation coefficient value of 0.559 indicates that the correlation between IL-6 and hepcidin levels in TDT patients without alloimmunization/autoimmunization falls within the strong correlation category.

DISCUSSION

Characteristics of Research Subjects

The predominant age group in both studied cohorts was ≤18 years, reflecting the common trend in TDT cases to manifest early in life due to the swift onset of iron overload, with thalassemia diagnoses often occurring during infancy and leading to the initiation of blood transfusion therapy between the ages of 6 and 24 months.[4,21,22] This finding aligns with research conducted in Egypt, where the highest prevalence was observed in individuals under 12 years of age.[23] Another study in Jakarta by Djumhana reported that a majority of TDT cases occurred before the age of 18.[24]

The average age of TDT subjects with alloimmunization/autoimmunization was 9.5 years, while those without were 11 years. Although these results are consistent with a study in Malaysia,[25] the mean age is lower than that observed in a study in Bangkok, where TDT patients with and without alloantibodies had mean ages of 35 and 31 years, respectively.[26] A study in Indonesia by Tambunan et al. demonstrated a prevalence of women, with the highest mean age in the thalassemia group with positive allo-immunization being 13 years,[27,28] which aligns with findings at RSUD Dr. Soetomo, reporting a similar mean age of 11.7 years.[5] This aligns with the thalassemia prevalence study by Hernaningsih et al. in East Java, covering the age range of 5-17 years,[29] and with the study by Fatmasyithah et al. in Aceh, which focuses on the age range of 5-12 years.[30]

Blood type O positive was predominant in both groups, consistent with findings in Indonesia and India.[27,28,31] This aligns with the results of the Indonesian Red Cross's study in Sidoarjo, indicating that the majority of patients requiring blood transfusions have type O blood.[32] The most common transfusion frequency in both groups was 1-2 times per month, although the TDT group with alloimmunization/autoimmunization had a higher proportion of patients transfused 3-4 times per month. This concurs with a study at RSUD Dr. Soetomo, Surabaya, which reported a frequency of 20-50 times per year.[28] The study also echoes findings by Rismayanti et al., which identified the highest frequency of transfusions at 10-15 times per year in thalassemia patients.[33] Increased transfusion frequency correlates with a heightened risk of alloimmunization/autoimmunization due to frequent exposure to alloantigens, explaining the higher transfusion frequency in the TDT group with alloimmunization/autoimmunization.[34] This comprehensive analysis of TDT patient profiles, encompassing factors such as age, sex, blood type, and transfusion frequency, provides valuable insights into the direction of thalassemia treatment and management.

Differences in Hepcidin and IL-6 Levels among TDT Patients with and without Alloimmunization/Autoimmunization

This study categorized research subjects into two groups: TDT with and without alloimmunization/autoimmunization. The disparity in hepcidin and IL-6 levels between the two groups revealed a significant difference ($p < 0.05$), with the alloimmunization/autoimmunization TDT group exhibiting elevated hepcidin and IL-6 levels compared to the group without alloimmunization/autoimmunization. These findings align with research conducted in Australia and other studies that reported differences and increased levels of hepcidin[19,21,35-37] and IL-6[38-40] in TDT patients compared to NTDT patients. This discrepancy arises from alloantigen stimulation resulting from repeated blood transfusions, showcasing a substantial difference and higher levels in the TDT group.[35,36] Research on sickle cell disease also indicates an elevated risk of alloimmunization with an increasing number of transfusions,[41] while TDT, known for requiring routine lifelong transfusions, shares a similar risk profile.[4]

Elevated IL-6 and hepcidin levels in TDT patients with alloimmunization/autoimmunization stem from frequent transfusions, leading to increased exposure to alloantigens. This antigen exposure triggers a cascade involving TCR introduction, macrophage activation producing IL-6, and ultimately, IL-6, inducing the differentiation of B cells into plasma cells that generate antibodies.[17,35,36] Another contributing mechanism is the proinflammatory cytokine function of IL-6.[20] The inflammatory process in TDT initiates with excess iron accumulation from routine transfusions or chronic hemolysis.[4] This excess iron prompts the generation of free radicals, leading to chronic inflammatory conditions that induce the proinflammatory cytokine IL-6 and alterations in the immune system. The rise in IL-6 levels is intricately linked to increased hepcidin levels in the TDT process, as IL-6 serves as the primary molecule for augmenting hepcidin transcription via the STAT pathway.[42] Consequently, IL-6 triggers an upsurge in hepcidin production, while immune system modifications, such as heightened macrophage activity, increased B lymphocyte activity, and diminished Treg cell function, bolster antibody formation.[35,43-46] Notably, macrophage cells exhibit hyperactivity in conditions like thalassemia, inflammation, sickle cell disease, and autoimmune hemolysis.[46] Additionally, the concentration of iron, the quantity of free radicals produced, and the degree of impairment to the body's immune system demonstrate a positive correlation.[35]

The heightened levels of IL-6 and hepcidin can serve as marker parameters for predicting antibody appearance in TDT patients without alloimmunization/autoimmunization or forecasting the severity of the alloimmunization/autoimmunization process. This significance arises from the acknowledged complications introduced by the presence of antibodies in crossmatching examinations, leading to potential delays in blood transfusions for TDT patients.[17]

Analysis of the Correlation between Hepcidin and IL-6 Levels in TDT Patients with and without Alloimmunization/Autoimmunization

This study revealed a positive correlation between hepcidin and IL-6 levels in both TDT patient groups, irrespective of alloimmunization/autoimmunization status, indicating that elevated IL-6 levels correspond to higher hepcidin levels. These findings align with the established theory that an increase in IL-6 stimulates hepcidin expression through the JAK/STAT3 signaling cascade, promoting hepcidin transcription and regulating iron availability in the body. This regulatory mechanism aims to prevent the accumulation of free iron resulting from routine transfusions and hemolysis.[43, 47-49]

Furthermore, the study demonstrated that IL-6 and hepcidin levels were notably higher in the TDT group with alloimmunization/autoimmunization (3.64 pg/mL and 2,950.6 pg/mL) compared to the group without alloimmunization/autoimmunization (1.41 pg/mL and 1,599.6 pg/mL). This disparity suggests a more severe inflammatory process in TDT patients with alloimmunization/autoimmunization. The intensified inflammatory response arises from excess iron accumulation and repetitive chronic hemolysis, triggering the activation of macrophages and subsequent IL-6 production.[4,20] Interleukin-6, in turn, induces the differentiation of Th17 cells, leading to IL-17 production, a process implicated in antibody formation.[10] The regulatory role of Treg cells, intended to maintain balance, appears compromised in this scenario. Research by Ni et al. suggests that an

imbalance in iron and ROS can lead to Treg cell death, perpetuating proinflammatory conditions and antibody formation.[50]

Another supporting mechanism for the antibody formation process persists, as iron accumulation induces changes in the immune system, notably an augmented role of macrophages in IL-6 production due to macrophage stimulation.[51,52] Additionally, cell death resulting from free radicals during the iron accumulation process identifies innate immunity receptors (macrophage cells, neutrophil cells, and dendritic cells) as DAMPs (Danger-associated molecular patterns), which are recognized by innate immunity and trigger immune system activation, subsequently leading to antibody formation.[53] Iron loss serves as a negative regulator for the antibody response mediated by Th17 cells. Elevated iron levels increase the differentiation of Th17 cells, perpetuating the differentiation of B lymphocyte cells into plasma cells that produce antibodies.[33] The study also observed an increase in the number and activity of B lymphocyte cells in TDT, accompanied by heightened immunoglobulin secretion.[51] This condition further supports the formation of antibodies in the presence of high levels of IL-6 and hepcidin. Conversely, the mechanism preventing antibody formation despite elevated IL-6 and hepcidin levels is attributed to iron's inhibitory effect on the differentiation of Th17 cells, which results in the non-formation of antibodies.[50] This finding is supported by another study, which found that only 30% of a group formed antibodies over a 2-year transfusion period. This phenomenon is linked to the underlying disease nature and patient genetics influencing alloimmunization.[54] The patient's HLA II genotype, such as the presence of the HLA-DRB1*0901 allele, plays a crucial role in preventing the alloimmunization process, impeding antibody formation.[55] Additionally, heme has an inhibitory effect on B lymphocyte cell proliferation, suppressing antibody production.[56]

The study underscores significant differences in IL-6 and hepcidin levels and a positive correlation in both TDT patient groups, with and without alloimmunization/autoimmunization. Therefore, we posit that the assessment of the IL-6 parameter alone could serve as a practical marker for predicting increased hepcidin levels. This is particularly valuable in settings where hepcidin assessment may not be readily available. Moreover, the heightened levels of IL-6 and hepcidin in the TDT group with alloimmunization/autoimmunization indicate an ongoing inflammatory process. We believe these two parameters can serve as predictive factors for increased antibody formation in TDT patients without alloimmunization/autoimmunization or exacerbate the existing alloimmunization/autoimmunization process. Although the study does not comprehensively explain why some patients develop antibodies while others do not, it establishes a foundation for further research to unravel the intricacies of the immune system in TDT.

CONCLUSION

IL-6 and hepcidin levels were significantly different and had a positive correlation in both TDT patients with alloimmunization/ autoimmunization and those without, with values of IL-6 and hepcidin levels being higher in TDT patients with alloimmunization/ autoimmunization.

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DISCLOSURE

The authors report no conflict of interest in this work.

AUTHOR CONTRIBUTIONS

Each author contributed equally to all parts of the research. All authors have critically reviewed and approved the final draft and are responsible for the content and general aspects of the manuscript.

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