

# Amino acid

*by* Ina S Timan

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## 3 Amino acid profile in patients with thalassemia major analyzed by liquid chromatography-tandem mass spectrometry

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**Abstract.** Patients with thalassemia major develop secondary iron overload due to ineffective erythropoiesis and recurrent transfusions. Excessive iron overload results in increased non-transferrin-bound iron, which induces tissue injury as it catalyzes the formation of free radical ions. Iron accumulation can impair the structural and functional integrity of the intestines and reduce amino acid availability. Iron overload can be alleviated by iron chelation. Transferrin is the body's natural chelator that predominantly comprises amino acids glycine and aspartic acid. Research has shown lower transferrin levels in patients with iron overload. The present study aimed to assess the changes in iron status and the amino acid profile in patients with thalassemia major. Serum iron; unsaturated iron-binding capacity; total iron-binding capacity; and serum ferritin, glycine, and aspartic acid were assessed in a cohort of 21 patients (13 patients with beta thalassemia major and 8 with beta Hb E thalassemia). The results showed significant changes in iron status: elevated ferritin post-transfusion and decreased ferritin and elevated glycine levels after chelation. Future studies are required to assess the changes in other amino acids.

### 1. Introduction

Thalassemia is a congenital disorder characterized by defective synthesis of one or more globin chains of the tetramer hemoglobin. It is the most common genetic disorder in humans. The condition is associated with various genetic mutations, and the clinical spectrum ranges from asymptomatic (no anemia) to severe cases leading to fetal death. The subtypes of thalassemia are named based on the predominantly affected  $\alpha$  or  $\beta$  chains (decreased or complete lack of synthesis) [1].

Approximately 7% of the world's population is affected by thalassemia. The condition is commonly found in the so-called "thalassemia belts," which stretch from the Eastern Mediterranean through the Middle East, India to Southeast Asia, and North to South Africa. Sardinia, Cyprus and Greece have the highest frequency of beta thalassemia carriers in Europe (6%–19%), whereas India, Thailand, and Indonesia have the highest frequency of beta thalassemia carriers among the Asian and Southeast Asian countries (0.3%–15%) [1]. At the end of 2015, a total of 1543 patients with thalassemia major were registered (or receiving treatment) at the Thalassemia Center of the Cipto Mangunkusumo Hospital Children's Health Department. The incidence of new cases of thalassemia major at this institution is 49–75 persons/year [2].



Patients with beta thalassaemia exhibit increased erythropoiesis owing to anemia. Upregulation of erythropoiesis triggers an increase in iron absorption in the intestines, which in turn leads to excess of iron in the body. Periodical blood transfusion in patients with thalassemia major exacerbates the iron overload in tissues such as hepatic parenchyma, endocrine tissues, gastrointestinal tract, and myocardium [3].

Owing to this sequence of events, the plasma iron concentration exceeds the iron-binding capacity of transferrin, which manifests as high levels of non-transferrin-bound iron (NTBI). NTBI catalyzes the conversion of hydrogen peroxide into free radical ions, which cause injury to the cell membranes, proteins, deoxyribonucleic acid, and tissues. In the absence of adequate preventive and therapeutic measures, excess iron in the body can lead to complications of iron accumulation in tissues [4–7]. Iron precipitation in various organs such as the gastrointestinal tract can impair the structural and functional integrity of various organs [8]. Laboratory tests used to assess the iron status include transferrin saturation, serum ferritin level, and transferrin index [9,10]. Secondary iron overload refers to the condition wherein the transferrin saturation exceeds 60%. Serum ferritin levels maintained at <1000 ng/mL are associated with a low incidence of complications due to iron overload [7]. Transferrin index above 100% reflects the presence of NTBI [11].

Secondary iron overload in patients with thalassemia major can be prevented or alleviated by iron chelation [6]. Transferrin is a strong chelator that reversibly binds to iron. Transferrin consists of 678 particular amino acid compounds. The predominant amino acid residues of the transferrin protein (in that sequence) are 58 lysine, 58 leucine, 57 alanine, 50 glycine, and 44 aspartic acid [12]. The present study aimed to determine the changes in iron status and amino acid profile post-transfusion and post-chelation in patients with thalassemia major.

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## 2. Methods

### 2.1. Study design

This study used a cohort design and was conducted at the Thalassemia Center and Central Laboratory of RSCM between October 2016 and December 2016. Children with a diagnosis of thalassemia major who attended the Kiara polyclinic and underwent blood sampling were eligible for inclusion. The inclusion criteria were as follows: children diagnosed with thalassemia major who regularly received transfusion and iron chelation therapy, documented history of iron overload (defined as transferrin saturation > 60% and/or serum ferritin > 1,000 ng/mL) in the medical records, and written informed consent of parents for participation in the research. The exclusion criterion was patients who did not come for examination before the next transfusion. The sample size was determined by paired sample t test, which indicated a minimum sample size of 18; however, 21 subjects were enrolled in the study. Blood samples were obtained from all patients on three occasions, i.e., pre-transfusion, 1 day post-transfusion, and 1 month post-chelation.

### 2.2. Measurements

The iron status was determined based on serum iron, unsaturated iron-binding capacity (UIBC), total iron-binding capacity (TIBC), and serum ferritin levels. For the purpose of this study, the amino acid profile refers to levels of glycine and aspartic acid. Secondary iron overload was defined as iron overload status based on ferritin levels > 1000 µg/L.

A dried blood spot sample was used for amino acid analysis with Xevo TQD (Xevo TQD Waters Indonesia, PT Kromtekindo Utama, Indonesia) using liquid chromatography-tandem mass spectrometry and reagent kit from recipe [13].

Serum iron levels were determined using Cobas 501 via colorimetric method. UIBC was determined using the photometric principle, whereas TIBC was calculated from the serum iron level plus UIBC [10]. Ferritin levels were determined using Cobas e411 device based on the principle of sandwich-type electrochemiluminescence immunoassay [13].

### 2.3. Statistical analysis

Within-run tests were conducted for all laboratory parameters. The test was performed with control material 5 times in a row on the same day at the time of the examination. The coefficient of variation (CV) is the percentage of the standard deviation (SD) divided by the mean, whereas deviation is the percentage ranged from the smallest to the largest difference between the test value and the middle value of the control divided by the middle value of the control. All data were recorded and processed using Statistical Package for the Social Sciences (SPSS; IBM Corporation) ver 20.

Pre-transfusion, post-transfusion, and post-chelation iron status and amino acid levels were compared. The distribution of variables was assessed using the Shapiro–Wilk test. Between-group differences with respect to normally distributed variables were assessed using the paired *t*-test, whereas those with respect to non-normally distributed variables were assessed using the non-parametric Wilcoxon test.  $P < 0.05$  was considered statistically significant.

### 3. Results

The parameters assessed in this study were not categorized by sex, age, patient characteristics, type of thalassemia, chelation therapy, and food intake. Characteristics of the study subjects are presented in Table 1. A total of 21 subjects were enrolled in this study (10 women and 11 men; age range, 4–18 years).

**Table 1.** Demographic and clinical characteristics of subjects (n = 21)

Subject Characteristic	n (%)	Mean (range)
Sex		
• Male	10 (47.6)	
• Female	11 (52.4)	
Age (years)		11.95 (4–18)
Weight (kg)		31.55 (17–46)
Height(cm)		135 (58–157)
IMT (kg/m <sup>2</sup> )		17.99 (14.64–51.12)
Diagnosis		
• Thalassemia-β major*	13 (62)	
• Thalassemia-β/Hb E	8 (38)	

\*One of the patients underwent splenectomy

Table 2 presents a comparison of the coefficient of variation (CV) of the within-run test of iron status of the study with that shown in the brochure.

**Table 2.** Comparison of the CV value (%) of the within-run iron status with that shown in the reagent brochure

Parameter	Researcher CV		Brochure CV	
	N Control	P Control	N Control	P Control
Serum iron (μg/dL)	0.8	0.84	0.9	0.8
UIBC (μg/dL)	1.6	0.8	2.1	0.8
Ferritin (ng/mL)	2.1	0.99	2.2	1.1

The within-run accuracy test of glycine and aspartic acid using sample material is presented in Table 3.

**Table 3.** Comparison of CV values for within-run amino acid rate with that reported from another study

Parameter	Researcher CV	Other study CV using MSMS methods
Glycine ( $\mu\text{mol/ Liter}$ )	6.74%	<10%
Aspartic Acid ( $\mu\text{mol/ Liter}$ )	21.19%	<10%

The results of iron status and amino acid profile analyses are presented in Tables 4 and 5, respectively.

**Table 4.** Iron status at pre-transfusion, post-transfusion, and 1 month post-chelation

Parameter	Pre-transfusion	Post-transfusion	1 month post-chelation	p value (a vs. b)	p value (a vs. c)
	Mean $\pm$ SD <sup>a</sup>	Mean $\pm$ SD <sup>b</sup>	Mean $\pm$ SD <sup>c</sup>		
Serum iron ( $\mu\text{g/dL}$ )	171 (59–231)	185.9 (37–232)	197 (68–344)	0.741	0.118
UIBC ( $\mu\text{g/dL}$ )	23 (0–237)*	9.9 (0–171)	15 (0–177)	0.305	0.135
TIBC ( $\mu\text{g/dL}$ )	201 (141–453)	210.56 (112–266)	210 (131–462)	0.181	0.578
Ferritin level ( $\text{ng/dL}$ )	3724 (1367–8938)	3980 (1456–9057)	3254 (1010–7061)	<b>0.002</b>	<b>0.046</b>

a = pre-transfusion, b = post-transfusion, c = post-chelation ;  $p < 0.05$  (Statistically Significant)

Reference values of serum iron: 37–145  $\mu\text{g/dL}$  (female), 59–158  $\mu\text{g/dL}$  (male); UIBC: 112–346  $\mu\text{g/dL}$ ; TIBC : 228–428  $\mu\text{g/dL}$ ; ferritin 30–400  $\text{ng/mL}$  (male), 15–150  $\text{ng/mL}$  (female)

**Table 5.** Results of amino acid profile analysis

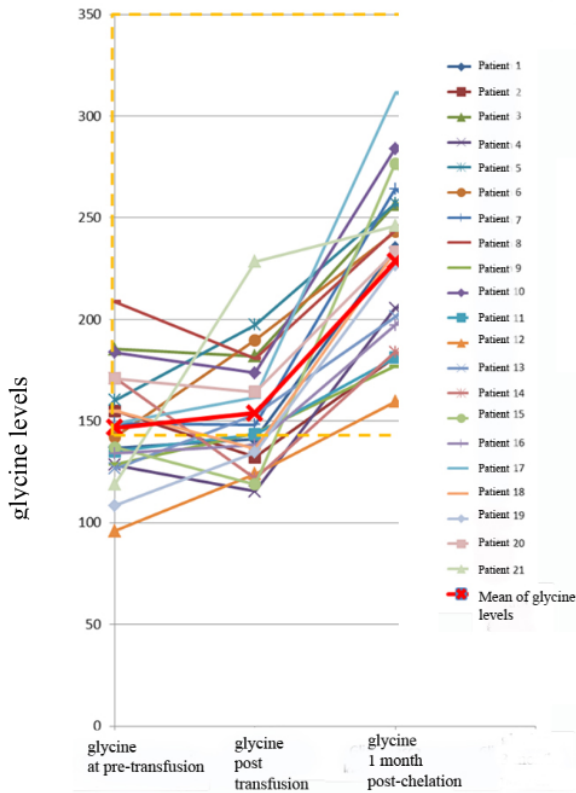
Parameter	Pre-transfusion Mean $\pm$ SD <sup>a</sup>	Post-transfusion Mean $\pm$ SD <sup>b</sup>	1 month post-chelation Mean $\pm$ SD <sup>c</sup>	p value (a vs. b)	p value (a vs. c)
Aspartic Acid ( $\mu\text{mol/L}$ )	83.08 (47–2032)	78.27 (5–1928)	106.96 (5–3416)	0.639	0.322
Glycine ( $\mu\text{mol/L}$ )	146.69 $\pm$ 27.01	144.03 (115–229)	233.56 (160–311)	0.337	<b>0.000</b>

a = pre-transfusion; b = post-transfusion; c = post-chelation ;  $p < 0.05$  (Statistically Significant)

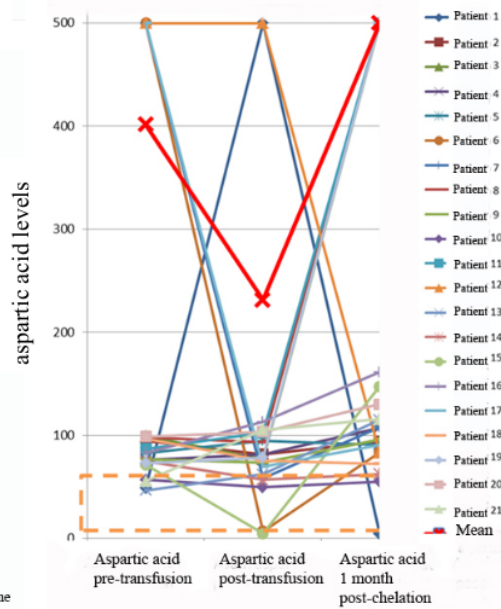
Reference level of aspartic acid: 29–87  $\mu\text{mol/L}$ ; glycine: 149–417  $\mu\text{mol/L}$

Figure 1 shows that the mean glycine level at pre-transfusion was 146.68  $\mu\text{mol/L}$ , which increased to 153.80  $\mu\text{mol/L}$  post-transfusion; At 1 month post-chelation, the mean glycine level further increased to 228.49  $\mu\text{mol/L}$ .

A similar trend was observed with respect to levels of aspartic acid as shown in Figure 2. The mean aspartic acid level at pre-transfusion was 401.4  $\mu\text{mol/L}$ , which increased to 231.27  $\mu\text{mol/L}$  post-transfusion. At 1 month post-chelation, it further increased to 616.11  $\mu\text{mol/L}$ .



**Figure 1.** Changes in the mean glycine levels Reference level of glycine: 149–417  $\mu\text{mol/L}$



**Figure 2.** Changes in the mean aspartic acid levels Reference level of aspartic acid: 29–87  $\mu\text{mol/L}$

**4. Discussion**

The precision and accuracy of tests for serum iron, UIBC, and ferritin in this study showed lower coefficient variation (CV) than that listed in the brochure. The results of the amino acid accuracy tests showed that the CV for glycine and aspartic acid was 6.74% and 21.19%, respectively. Our study population comprised patients with thalassemia major (13 patients with major  $\beta$ -thalassemia and 8 patients with  $\beta$ -thalassemia/HbE). One of the patients with major beta thalassemia underwent splenectomy.

The serum iron levels at post-transfusion (median: 185  $\mu\text{g/dL}$ ) were higher than those at pre-transfusion (median: 171  $\mu\text{g/dL}$ ); however, the increase was not statistically significant ( $p = 0.741$ ). Increased serum iron levels at post-transfusion can be attributed to the iron content in the transfused blood. According to previous study, patients with thalassemia may experience post-transfusion increase in serum iron levels by as much as 1.2 mg/kg body weight [14]. The serum iron levels at 1 month post-chelation (median: 197  $\mu\text{g/dL}$ ) were higher than those at post-transfusion, although the increase was not statistically significant ( $p = 0.118$ ). This suggests that the chelation therapy did not adequately facilitate binding of excess iron in patients with thalassemia major.

UIBC reflects the amount of apotransferrin that can still bind to iron. The median UIBC at pre-transfusion [23  $\mu\text{g/dL}$  (range, 0–237)] was higher than that at post-transfusion [9.9  $\mu\text{g/dL}$  (range, 0–171)], although the difference was not statistically significant ( $p = 0.305$ ). This decrease was attributable to the iron content in the transfused blood, which resulted in excess iron in serum

compared to the availability of apotransferrin. The body cannot meet the needs of carrier proteins. Excess iron in the serum will affect the results of the examination because the tool cannot detect excess iron.

The median UIBC [5] at post-chelation [15  $\mu\text{g/dL}$  (range, 0–177)] was lower than that at pre-transfusion, although the difference was not statistically significant ( $p = 0.135$ ). This reflects the relative lack of apotransferrin, which is liable to aggravate iron overload in the future.

TIBC is an indirect measure of the total capacity of transferrin to bind to iron. There was no significant difference between the median TIBC at pre-transfusion (201  $\mu\text{g/dL}$ ) and at post-transfusion (201.56  $\mu\text{g/dL}$ ) ( $p = 0.181$ ). The median TIBC at 1 month post-chelation was 210  $\mu\text{g/dL}$  ( $p = 0.578$ ). The TIBC measurements did not reflect the actual circumstances owing to UIBC examination errors, because if the result is negative, it will be considered as 0.

The median serum ferritin level was 3724 ng/dL at pre-transfusion and increased to 3980 ng/dL at post-transfusion ( $p = 0.002$ ). The increase is likely attributable to blood transfusion. The median serum ferritin level at 1 month post-chelation was 3254 ng/dL ( $p = 0.046$ ). The decrease in serum ferritin level is attributed to the binding of ferritin to the chelator in the tissues, although the serum ferritin level is still high.

Plasma levels of amino acids are affected by dietary intake, their rate of synthesis and utilization in the body, and the intracellular protein turnover. The median glycine level was 142.16  $\mu\text{mol/L}$  at pre-transfusion and increased to 144.03  $\mu\text{mol/L}$  at post-transfusion ( $p = 0.337$ ). Packed Red Cell (PRC) transfusion contains 20%–30% plasma that increases the glycine level. The glycine levels significantly increased at 1 month post-chelation (median: 233.56  $\mu\text{mol/L}$ ;  $p = 0.000$ ). Increased glycine levels are likely attributable to decreased glycine degradation in the gastrointestinal tract, which can result from iron overload in the gastrointestinal tract, increased glycine synthesis, and increased dietary intake of glycine.

Aspartic acid is an important amino acid for neurotransmitters. Marshmallow extract, conglutin, and legume extracts found in seeds and nuts are rich sources of aspartic acid [15]. The median aspartic acid level at pre-transfusion was 83.08  $\mu\text{mol/L}$ , whereas that at post-transfusion was 78.27  $\mu\text{mol/L}$  ( $p = 0.639$ ). The aspartic acid [31] is increased at 1 month post-chelation (median: 106.96  $\mu\text{mol/L}$ ), although the increase was not statistically significant ( $p = 0.322$ ). Increase in aspartic acid levels may occur because of decreased aspartic acid degradation in the gastrointestinal tract due to iron overload in the gastrointestinal tract, increased aspartic acid synthesis, and increased dietary intake of aspartic acid. It is worth noting that the aspartic acid assay showed a fairly high CV of 21.19%, and because this study is the first amino acid study conducted on whole human blood with Xevo TQD tool from Waters, it may require special treatment according to tool usage procedure.

In this study, patients with thalassemia major were found to have increased glycine and aspartic acid levels. This indicates that the function of enterocytes in transporting amino acids to the circulation is [15] maintained.

To the best of our knowledge, the present study is the first amino acid research with whole human blood sample using Xevo TQD tool from Waters. However, this study did not take into account food intake and iron chelation therapy received by the patients.

## 5. Conclusion

This study involved 21 [28] patients with thalassemia major who received recurrent transfusion and chelation therapy. We documented significant changes in iron status, i.e., increased post-transfusion ferritin and decreased ferritin at 1 month post-chelation. The significant changes observed in their amino acid profiles were increased glycine levels at 1 month post-chelation.

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