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Plasma amino acid profiles in thalassaemia major with iron overload

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ABSTRACT

Introduction: Iron overload in thalassaemia major patients mainly occurs due to periodic transfusions. When iron exceeds transferrin capacity, non-transferrin bound iron accumulates and causes tissue damage, including in the gastrointestinal tract, resulting in impaired enterocyte function and amino acid absorption. The aim of this study was to evaluate amino acid profiles in patients with thalassaemia major after repeated transfusions and chelation. **Methods:** Whole blood amino acids were analysed from dried blood spots using liquid chromatography tandem mass spectrometry. This study consisted of two parts: a cross-sectional and a cohort study in thalassaemia-β-major patients. In the cross-sectional study, amino acid profiles were analysed in 219 thalassaemia patients who received routine transfusion and chelation therapy, and 60 healthy control subjects. The cohort study included 21 subjects, from whom blood samples were taken at pre-transfusion, 1-day post-transfusion, one and three months post-chelation to evaluate changes in amino acid levels. **Results**: There were significant differences between amino acid levels in thalassaemia subjects and controls. Positive correlations were found between serum iron and transferrin with age, also between transferrin with proline, valine, phenylalanine, aspartic acid, and glutamic. Phenylalanine and aspartic acid were significantly lower in subjects with transferrin lower than 180 µg/dL. Significant correlations were found between haemoglobin with essential and non-essential amino acid groups. From the cohort study, significant changes were observed in glycine, alanine, leucine, and aspartic acid. **Conclusion:** Amino acid profiles in thalassaemia patients differed compared to healthy controls, even after transfusion and chelation. Phenylalanine and aspartic acid were significantly lower in subjects with low transferrin levels.

Keywords: amino acid, chelation, iron overload, thalassaemia, transfusion

INTRODUCTION

Thalassaemia is a congenital disorder characterised by impaired synthesis of one or more globin chains of the haemoglobin protein. It is the most common genetic disorder in the world,

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including in Indonesia, with a global prevalence of 7%. Thalassaemia is prevalent in the thalassaemia belt, which stretches from the Eastern Mediterranean through the Middle East, India to Southeast Asia, and North to South Africa (Mehta & Keohane, 2012). The prevalence of carriers in India, Thailand, and Indonesia are among the highest in Asia and Southeast Asia (up to 15%). Thalassaemia's clinical manifestation is associated with its genetic mutations (Mehta & Keohane, 2012).

Data obtained from the Thalassaemia Center in Cipto Mangunkusumo Hospital (RSCM) showed 1,570 registered thalassaemia patients and the number of new patients per year was approximately 49-75 (Thalassaemia Center, 2016). Thalassaemia major patients may suffer from iron overload due to ineffective erythropoiesis and periodic transfusions, which may be accompanied by the dysfunction of multiple organs (Mariani, *et al.*, 2009; Patel & Ramavataram, 2012). The amount of plasma iron could exceed the binding capacity of transferrin and as a result, not all iron is bound because it has been fully saturated, generating toxic-free iron or non-transferrin bound iron (NTBI) (Brittenham, 2011; Gkouvatsos, Papanikolaou & Pantopoulos, 2012). Free iron stimulates reactions in body cells, generating oxygen radicals, which can affect tissues in the gastrointestinal tract, liver, pancreas, and small intestine; this can lead to the organ's dysfunction and damage (Mariani *et al.*, 2009; Patel & Ramavataram, 2012; Brittenham, 2011). Less transferrin availability will result in more NTBI and more damage.

Abnormalities in the enterocytes due to iron overload can also cause diminished protein digestion and amino acid absorption (Wu, 2013). Children with thalassaemia often have short stature and are undernourished. Iron overload in thalassaemia major patients can be reduced by chelation therapy and the many iron binding proteins in the body. Reductions in protein levels, such as transferrin, will cause increases in NTBI. Therefore, the level of amino acids in a person's body needs to be sufficient to sustain the level of proteins, including transferrin (Brittenham, 2011; Poggiali *et al.*, 2012).

Amino acids are organic substances containing amino and acid groups (Wu, 2013). The breakdown of dietary and endogenous proteins is a source of amino acids for protein synthesis. Dietary protein is digested by proteases in the gastrointestinal tract, which produces amino acids. Amino acids that cannot be synthesised *de novo* are called essential amino acids and these are isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine. These amino acids are obtained from dietary intakes of meat, milk, eggs, and fish. Amino acids that can be synthesised by the body are called non-essential amino acids, which include aspartic acid, asparagine, glutamic acid, cysteine, glycine, serine, tyrosine, citrulline, ornithine, arginine, proline, and alanine (Wu, 2013). Absorption of free amino acids and small peptides occurs in the enterocytes through several mechanisms: passive diffusion, the Na-independent system, and the Na-dependent system.

The aim of this study was to describe the plasma amino acid profile of thalassaemia major patients and to determine the profile changes after transfusion and chelation therapy. Differences in the amino acid levels of thalassaemia patients compared to healthy subjects may provide insights into the nutritional requirements for improving their quality of life.

MATERIALS AND METHODS

This study consisted of two parts: a cross-sectional study and a cohort study of thalassaemia-β major and thalassaemia-β/Hb E patients, as well as healthy controls. Control subjects were healthy patients with normal haematology results and normal liver and kidney function tests. The cross-sectional study included 219 patients who had received repeated transfusions and iron chelation therapy, and 60 healthy controls. The sample size for thalassaemia subjects was calculated based on the rule of thumb parameters, using a ratio of 1:10 and the samples were taken consecutively. The study was conducted at the Thalassaemia Center and the Central Laboratory of Cipto Mangunkusumo Hospital and Indonesian Medical Education and Research Institute (IMERI) Faculty of Medicine, University of Indonesia, from March 2018 to October 2019. The participants were children with thalassaemia major who visited the polyclinic, routinely received transfusion and iron chelation therapies, and were willing to participate in the study. Their parents signed a form of informed consent. The controls were all healthy subjects with no history of thalassaemia and they were unmatched.

For this study, haemoglobin, serum iron (SI), transferrin, and ferritin were extracted from the hospital medical records. Samples were collected on the first day of the polyclinic routine control schedule before transfusion (pre-transfusion). Haemoglobin was analysed using the Sysmex blood cell counter (XE 5000, Japan) and iron was analysed using the Roche autoanalyser (Cobas-311, USA). Transferrin was measured as total iron binding capacity (TIBC). Blood samples for amino acid analysis were collected using dried blood spot (DBS) samples and kept at –20°C until analysis.

The cohort study was performed with 21 thalassaemia subjects. Blood samples were taken at pre-transfusion; one day post-transfusion; one month post-transfusion; and three months post-transfusion. The purpose was to identify if their amino acid profiles would change during and after chelation and transfusion therapies. This study was approved by the Research Ethics Board of the Faculty of Medicine, University of Indonesia.

Amino acid concentrations from DBS samples were examined using the Waters Xevo TQD – liquid chromatography tandem mass spectrometry (LCMSMS, UK), with the Chrom-system kit for 13 amino acids (Chromsystem, 2019). To evaluate amino acid profile, the concentration of each amino acid was calculated using pre-transfusion data as baseline.

Statistical analyses of changes in the amino acid concentration after transfusion and after chelation were performed using IBM SPSS Statistics for Windows version 20.0 (IBM Corp, Armonk, New York, USA). Results with $p<0.05$ were considered statistically significant. Data with normal distribution data are shown as mean±standard deviation (*SD)* and data with abnormal distribution are shown as median (percentile 5-95).

RESULTS

In the cross-sectional study, there were 219 thalassaemia-β major and thalassaemia-β/Hb E subjects, consisting of 105 females and 114 males, who had their amino acid profiles analysed (Table 1). The results analyses in this study were not differentiated by gender, type of thalassaemia, or chelation received. There was no significant difference in the haemoglobin, SI, TIBC, transferrin

Table 1. Characteristics of thalassaemia subjects (*n*=219)

Characteristic of subject	*n* (%)	*Mean/Median*
Gender		
Female	105 (47.9)	
Male	114 (52.1)	
Age (year)		11.9 (0.3 – 32)
Haemoglobin (g/dL)†		8.7 (5.0 – 11.3)
SI (µg/mL)		158 (59 – 258)
TIBC (µg/dL)		183 (127–294)
Transferrin Saturation (%)		96 (32 – 100)
Ferritin (ng/mL)		1038 (434 – 11751)

SI: Serum iron; TIBC: Total iron binding capacity
Data are shown in median (min-max), except haemoglobin (g/dL)† in mean±*SD*

saturation, and ferritin levels between males and females (p>0.05). The median transferrin saturation and ferritin levels were very high, but transferrin measured as TIBC was mostly low. The analysis was compared to control subjects, comprising 30 healthy males and 30 healthy females aged 8-35 years old with no history of thalassaemia.

Haemoglobin levels in thalassaemia major subjects were between 5.6 and 11.3 g/dL, respectively, with 27.4% subjects having a haemoglobin level of below 8 g/dL. Significant, positive correlations were found between total essential and non-essential amino acids group levels and haemoglobin levels (p<0.050).

There were significant differences between the medians of each amino acid level in thalassaemia subjects and control subjects (p<0.05), except for aspartic acid (p=0.160), as shown in Table 2. Different patterns of amino acids can be observed, especially between the lower and upper boundaries. In subjects with thalassaemia, amino acid levels were mostly higher compared to the control group. The levels of amino acids in control subjects were compared to other countries, as shown in Table 3 (Tan & Gajra, 2006; Mayo Clinic

Table 2. Profile of amino acids in thalassaemia subjects compared to control group

Amino acids (µmol/L)	*Thalassaemia (Median; n=219)*	*Control subjects (Median; n=60)*	*p*
Glycine	349 (96-583)	194 (130-308)	0.001
Alanine	268 (89-446)	95 (74-159)	0.001
Proline	189 (97-312)	124 (62-191)	0.004
Valine	121 (56-196)	111 (53-285)	0.014
Leucine	155 (66-290)	117 ((57-277)	0.025
Ornithine	321 (78-627)	50 (30-93)	0.001
Methionine	244 (11-555)	15 (5-19.8)	0.007
Phenylalanine	77 (23-325)	36 (10-68)	0.002
Arginine	16 (2-34)	23 (7-40)	0.028
Citrulline	58 (13-164)	27 (17-46)	0.006
Tyrosine	31 (1-92)	31(28-65)	0.012
Aspartic acid	31 (2-182)	33 (6-138)	0.160
Glutamic acid	102 (39-266)	23 (6-42)	0.004

Table 3. Range of amino acids compared to other populations

Amino acids (µmol/L)	*Control subjects (n=60)*	*Reference Singapore*[†]	*Reference USA*[‡]
Glycine	130-308	135-342	149-417
Alanine	74-159	260-585	144-557
Proline	62-191	82-301	80-357
Valine	53-285	169-354	106-320
Leucine	57-277	96-203	51-196
Ornithine	30-93	36-92	22-97
Methionine	5-19.8	26-48	11-37
Phenylalanine	10-68	48-73	30-95
Arginine	7-40	61-132	31-132
Citrulline	17-46	14-61	11-45
Tyrosine	28-65	48-96	31-106
Aspartic acid	6-138	2-18	<11
Glutamic acid	6-42	4-68	22-131

[†] Tan IK & Gajra B (2006).
[‡] Mayo Clinic Laboratories (2020).

Laboratories, 2020). Some were lower compared to reference values from Singapore and USA, except for aspartic acid. The references are included here as a note that it is common to see differing reference levels by country as they can be reflective of many factors, including nutritional status.

There were positive, significant correlations between age and serum iron (r=0.189, p=0.005), transferrin as TIBC (r=0.149, p=0.027), and ferritin (r=0.461, p=0.001), but no correlations were found between age and transferrin saturation (Table 4). Transferrin was fully or nearly saturated (90-100%) in 54% of subjects and only 16% of subjects had a transferrin saturation below 55%. Total iron binding capacity (TIBC), which represents transferrin, was between 117-420.5, with 46.1% of subjects having low TIBC (below 180 µg/dL). Significant correlations were present between transferrin, expressed as TIBC, and proline, valine, phenylalanine, aspartic acid, and glutamic acid levels.

Using multivariate analysis, it was shown that TIBC was most influenced by the amino acids arginine and tyrosine (TIBC= 130.008 + 2.299 Arginine + 0.948 Tyrosine). Phenylalanine and aspartic acid were significantly lower in thalassaemia subjects, with a TIBC of less than 180 µg/mL compared to those with a TIBC of more than 180 µg/mL. Subjects with ferritin levels of more than 8000 ng/mL had significantly lower levels of aspartic acid (p=0.032) and glutamic acid (p=0.011) compared to subjects with lower ferritin levels.

Table 4. Correlations for TIBC to amino acids

Amino acids	*Correlation coefficient (r)*	*p*
Glycine	-0.007	0.923
Alanine	0.104	0.126
Proline	0.151	0.026
Valine	0.219	0.001
Leucine	0.129	0.570
Ornithine	-0.038	0.567
Methionine	-0.085	0.210
Phenylalanine	0.270	0.001
Arginine	0.092	0.175
Citrulline	0.046	0.502
Tyrosine	0.095	0.160
Aspartic acid	0.271	0.001
Glutamic acid	0.152	0.025

TIBC: Total iron binding capacity

Table 5. Cohort study results: changes in amino acid profiles during transfusion and chelation therapies

Amino acids (µmol/L)	*Pre-transfusion (n=21)*	*D-1 post-transfusion (n=21)*	*D-30 post-transfusion (n=21)*	*D-90 post-transfusion (n=21)*	*p*
Glycine	142 (97-206)	144 (115-225)	233 (161-308)	242 (161-326)	0.001*
Alanine	324 (160-142)	325 (214-478)	280 (168-394)	298 (164-486)	0.021*
Proline	218 (163-283)	234 (123-408)	226 (114-419)	248 (155-395)	0.813
Valine	161 (119-215)	173 (111-275)	174 (116-240)	176 (123-263)	0.306
Leucine	182 (123-247)	201 (125-334)	133 (100-235)	139 (100-235)	0.001*
Ornithine	246 (172-380)	246 (176-363)	220 (166-331)	218 (138-414)	0.488
Methionine	21 (14-43)	25 (14-42)	20 (11-28)	19 (13-42)	0.261
Phenylalanine	70 (40-181)	93 (50-264)	75 (42-148)	78 (50-251)	0.553
Arginine	9.8 (2.2-30)	13.3 (4-25)	8.6 (2.4-27)	11.0 (2.6-27)	0.261
Citrulline	27 (20-39)	29 (19-54)	33 (24-43)	31 (24-52)	0.261
Tyrosine	52 (32-89)	64 (35-92)	49 (33-87)	52 (46-118)	0.137
Aspartic acid	77 (47-99)	81 (50-112)	91 (56-145)	94 (69-144)	0.001*
Glutamic acid	189 (132-395)	164 (119-261)	160 (112-250)	153 (121-515)	0.634

Cohort study data of pre-transfusion; day 1 post-transfusion (D-1); 1 month post-transfusion (D-30); 3 months post-transfusion (D-90)
Data are shown in median (percentiles 5 & 95)
*$p<0.05$

In the cohort study, there were 21 thalassaemia major subjects (10 females and 11 males; aged 4-18 years). In this study, we also did not differentiate data by gender, type of thalassaemia, or chelation therapy. Data showed that following transfusion and chelation therapies, there were mostly no significant changes in amino acid levels, except for increases in glycine and aspartic acid, and decreases in alanine and leucine compared to pre-transfusion levels (Table 5). Transfusion of packed red cells still contained 20%-30% plasma, which can increase or decrease post-transfusion amino acid concentrations depending on the concentration of amino acids in the donor's plasma. These changes could also be caused by the food intake of the subjects. Thus, it is important to review all of the amino acid profiles, regardless of transfusion and chelation therapies, in order to determine the patient's nutritional needs.

DISCUSSION

Thalassaemia major is a congenital disorder that causes anaemia, which requires repeated transfusions and iron chelation therapy to prevent iron overload. In a state of iron overload, NTBI can stimulate reactions that produce oxygen radicals, which can damage tissues, including those in the gastrointestinal tract, consequently impairing nutrient digestion and absorption (Wu, 2013).

The mean haemoglobin levels of thalassaemia subjects in this cross-sectional study was 8.7 (range 5.0-11.3) g/dL, and 27.4% of subjects had a haemoglobin level of below 8 g/dL. Low haemoglobin causes lower metabolic body capacity, although some studies in sickle cell haemoglobinopathy have shown a hypermetabolic state requiring more micro- and macronutrients such as arginine for body metabolism (Umeakunne & Hibbert, 2019). In this

study, both essential and non-essential amino acid groups were positively correlated with haemoglobin, showing that more amino acids might improve the haemoglobin levels in thalassaemia patients. A study by Malluvalasa among thalassaemia children in India reported lower haemoglobin values than those reported in our study. In their study, mean haemoglobin was 7.73±1.10 g/dl; 52% had a pre-transfusion haemoglobin of less than 8 g/dL and there was no difference between genders. The mean ferritin level in those children was more than 3400 ng/mL, higher than our study (Malluvalasa, Sahoo & Kuppilli, 2018).

The analyses of age and iron status in thalassaemia subjects showed that body iron concentration, serum iron, and transferrin (measured as TIBC and ferritin) increased with age. Increased serum iron due to multiple transfusions can be a result of insufficient chelation therapy in these subjects, causing body iron to increase with age. Higher levels of iron increase the risk of oxidative stress caused by free iron not bound to transferrin. Transferrin, measured as TIBC, ranged from 127 to 294 mg/dL and showed a positive correlation, but 46.1% subjects had low transferrin (below 180 µg/dL). Impaired body metabolism and insufficient amino acid intake can result in low body production of transferrin. Less transferrin availability causes an increase in non-transferrin bound iron in the circulation and results in more damage to the organs.

Transferrin was fully or nearly saturated (90-100%) in 54% of subjects. Higher transferrin level decreases the saturation and also reduces the chance of producing NTBI. Table 4 shows significant, positive correlations between transferrin concentration (expressed as TIBC) and proline, valine, phenylalanine, aspartic acid, and glutamic acid; suggesting that the availability of these amino acids may be helpful in increasing the concentration of transferrin. Transferrin levels were most influenced by arginine and tyrosine. Arginine and tyrosine have a high influence on TIBC and haemoglobin production, although other amino acids also have a role (Papassotiriou *et al.*, 2018). Therefore, higher levels of arginine and other amino acids may be beneficial in increasing transferrin level, decreasing NTBI, and improving oxidative stress in high metabolic states (Malluvalasa *et al.*, 2018). Lower phenylalanine and aspartic acid levels may be caused by low intake, increased consumption by body metabolism, or gut microbiota metabolism (Umeakunne & Hibbert, 2019; Dodd *et al.*, 2017).

No correlation was found between age and transferrin saturation. This was due to the reaction principle of the reagents that were used. Transferrin as TIBC was measured indirectly by calculation of the unsaturated iron binding capacity and a high NTBI interferes with the reaction, causing error in the TIBC calculation (Roche, 2018).

The profiles of amino acids are important to be determined among thalassaemia patients as their levels were significantly different from controls. Most amino acids were significantly higher in thalassaemia patients, except for arginine and tyrosine. These differences in amino acid levels showed that thalassaemia subjects have a distinctly different metabolism and a need for more amino acids to improve their impaired body metabolism due to iron overload. Arginine has a role in cell division, stimulation of protein synthesis, hormone release, and cytokine production. Arginine is a conditionally essential amino acid in children and can become an essential amino acid under stressful states, such as haemolytic anaemia and thalassaemia (Umeakunne & Hibbert, 2019; Morris *et al.*, 2017). Tyrosine can be synthesised from

phenylalanine; it is a precursor of some hormones and has a role in metabolism in the adrenal, pituitary, and thyroid glands. These glands are also usually affected and damaged by the condition of iron overload. Different profiles of amino acids in thalassaemia subjects versus the control group may also reflect the higher body turnover of cell catabolism, increased need for amino acids as building blocks for protein, and increased absorption of some amino acids among the former.

Amino acid levels in the control group were somewhat different in comparison to the Asian population (Singapore) and in the USA. This could be due to different nutritional intakes, mostly proteins, in each country. Our hospital is the national referral hospital in Indonesia. Patients treated in this hospital come from different provinces in Indonesia, with different food habits. In the Thalassaemia Center in Bandung City, a study of 80 thalassaemia subjects aged 10-14 years showed that 81.2% had a short stature (Elizabeth *et al.*, 2018); improving their amino acid intakes may help to improve their body condition. In this study, very high ferritin levels were found in 35.6% subjects and levels of more than 10,000 ng/mL were also found in 10.5% subjects. A negative correlation between height-for- age *z-score* of <–2 and serum ferritin had also been reported by other studies (Moiz *et al.*, 2018; Abdulrazzaq *et al.*, 2005).

In the cohort study, 21 thalassaemia major subjects (10 females and 11 males; aged 4–18 years) were observed for three months. The fluctuations observed in amino acids before and three months after transfusion and chelation in this study mostly did not change significantly except for glycine, aspartic acid, alanine, and leucine. This showed that these amino acids were required for body metabolism. The results also indicated that the enterocyte function had remained relatively unchanged during the three-month period of transfusion and chelation. These changes could be caused by daily diet and the content of plasma in the transfused blood.

CONCLUSION

Not many research studies on the metabolomic analysis of amino acid levels are available that include patients with thalassaemia suffering from iron overload while receiving multiple transfusion and chelation therapies. This study showed that there was a different pattern of amino acid levels in the thalassaemia and control groups. Most of the amino acid levels were significantly higher in thalassaemia subjects. The differences were caused by high turnover of cells as the body requires more amino acids for its metabolism and regeneration of new cells. Nearly half of the thalassaemia subjects had low transferrin levels and it can cause more toxic NTBI. Half of the thalassaemia subjects also had very high ferritin levels, which showed that their chelation therapy was not adequate. Transferrin as TIBC was most influenced by the levels of arginine and tyrosine. Further study is required to determine if supplementation of these amino acids will improve the availability of transferrin. In the cohort study, it was found that some amino acid levels were significantly higher after three months of observation, which showed that these amino acids may be required for body metabolism.

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Authors' contributions

Timan IS, principal investigator, conceptualised and designed the study, prepared the draft of the manuscript and reviewed the manuscript; Wahidiyat PA, supervised the data collection of patients and reviewed the manuscript; Sjarif DR, led the data interpretation and reviewed the manuscript; Pasaribu MM, co-designed the study and supervised the data analysis; Putri F, collected the blood samples and analysed the samples; Widjaja L, reviewed the manuscript.

Conflict of interest

The authors declare that they have no potential conflict of interest.

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