RESEARCH ARTICLE

# Insulin-like Growth Factor-1 and Calcium Ion Levels are Negatively Associated with Serum β-Cross Laps in Multi-transfused β-thalassemia Major Patients

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## Abstract

**BACKGROUND:** Thalassemia patients with repeated transfusions (multi-transfusion) are often at risk of experiencing early osteoporosis. Several studies have demonstrated that osteoporosis in these individuals was associated with altered bone remodeling, characterized by decreased insulin-like growth factor-1 (IGF-1) and serum calcium ions, as well as increased serum β-Cross Laps (β-CTx) levels. Despite the prevalence of this condition, there is limited literature on the relationship between IGF-1 levels and calcium ions with β-CTx. Therefore, this study was conducted to examine the relationship between IGF-1 and calcium ions levels with serum β-CTx in multi-transfused β-thalassemia major patients.

**METHODS:** A cross sectional study involved 29 thalassemia patients with multiple transfusions, aged 2-18 years, that were selected from the electronic medical records. Calcium ion levels were examined using ion selective electrode method. Subsequently, IGF-1 and serum β-CTx levels were examined by enzyme-linked immunosorbent assay (ELISA), and the data were analyzed using the Pearson correlation test.

**RESULTS:** The results showed that the mean serum IGF-1 levels, calcium ion, and β-CTx were 20.11±20.763 ng/mL, 1.26±0.07 mmol/L, and 9330.40±1696.76 ng/mL, respectively. Statistical analysis showed a significant relationship between the levels of IGF-1 (r=-0.573; $p$=0.001) and calcium ion (r=-0.373; $p$=0.046) with serum β-CTx. A moderate negative relationship was found between IGF-1 levels and β-CTx, while calcium ion levels and β-CTx showed a weak negative relationship.

**CONCLUSION:** A moderate negative correlation between IGF-1 and serum β-CTx, and a weak negative correlation between calcium ion and serum β-CTx suggest that IGF-1 and calcium ions may serve as potential indicators of bone turnover and osteoporosis risk in multi-transfused β-thalassemia major patients, underscoring their potential role in routine clinical evaluations.

**KEYWORDS:** miR-200a expression, NO level, early-onset preeclampsia, late-onset preeclampsia

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## Introduction

Thalassemia is a genetic disorder of the globin gene, leading to partial or complete deficiency of globin chain production.(1,2) World Health Organization (WHO) data in 2017 showed that 70% of the world population are carriers. Several studies have also shown that the prevalence of the disorder is one per thousand births in Southeast Asia. In 2018, its prevalence in Indonesia was 9,028, which increased to 25,000 by 2020. The disorder has been reported to be more common in males than females, with the highest age group of 2-18 years.(1–3) According to previous studies, thalassemia is classified into 3 groups, namely major,

intermediate, and minor. In addition, complications with multi-transfusion range from moderate to severe, including hemosiderosis and hemochromatosis, infections, bone deformities, growth impairments, endocrine issues, cardiac disorders, alloimmunization, and allergic or hemolytic reactions.(4,5) On the other hand, liver and pancreas have the highest iron storage capacity and play important role in metabolism. Detecting metabolic abnormalities at a young age is crucial for thalassemia major patients. One of the strategies in the last decades to improve survival rate of thalassemia major patient are intervention of new oral iron chelator and better adherence with iron chelation treatment, starting since early age.(6)

β-thalassemia major patients often depend on regular blood transfusions for survival and the urgency increases with age and secondary conditions.(7,8) Each packed red cells (PRC) unit contains 250 mg of iron, but iron excretion is limited to 1 mg per day. Without chelation therapy, patients who receive 25 PRC units per year typically accumulate 5 grams of excessive iron, leading to hemochromatosis. (9) This complication has been reported to cause anterior pituitary damage, altered growth hormone pathway, and impaired liver production of insulin-like growth factor-1 (IGF-1).(8,10) Additionally, as the combination of blood transfusion and iron chelation therapy increasing, the endocrine complication has also risen and interfere with the growth and impaired bone growth in thalassemia patient. (11) Decreased serum IGF-1 and calcium ions alter bone mineral density (BMD) and bone tissue architecture, which are highly correlated with abnormal bone turnover. This turnover can be assessed using a reliable marker, β-Cross Laps (β-CTx). Bone matrix mainly consists of type I collagen, and during bone resorption, osteoclasts degrade the collagen fibrils into β-CTx. Therefore, β-CTx serves as a specific marker for resorption and can be used to evaluate turnover abnormalities. As a marker for metabolic bone disorder, β-CTx was found to be more sensitive and specific to detect turnover than alkaline phosphatase (ALP).(12)

Excessive erythropoiesis activities in β-thalassemia major promote unbalanced bone homeostasis, leading to bone structure abnormalities both in quality and quantity. Calcium levels are tightly regulated by parathyroid hormone, and its presence is essential in the bone mineralization process. Altered calcium levels are frequently observed in β-thalassemia major patients, and these conditions subsequently aggravate the impairment of osteoblast and osteoclast formation. Multi-transfusion leads to ferritin accumulation, as toxic accumulation of ferritin triggers the formation of free radicals and leads to calcium sensing receptor (CaSR) damage in parathyroid and liver cells. (1,3,13) The β-CTx are often released into the bloodstream during bone resorption and serve as a specific marker for mature collagen type I degradation.(14,15) IGF-1 and β-CTx are well-established as independent risk factors for poor fracture prognosis in elderly with osteoporosis. However, there was a lack of studies examining their association with calcium ion levels, particularly in the pediatric population, especially among those with β-thalassemia major.(16) Therefore, this study was conducted to determine the effect of the relationship between IGF-1 and calcium ion with β-CTx on BMD in multi-transfused β-thalassemia major patients. Knowledge of the relationship is needed to ensure the 3 parameters are equally used in the decreased bone density risk's clinical evaluation and to support osteoporosis diagnosis in multi-transfused thalassemia patients.

## Methods

### Study Design and Settings

This was a retrospective analytic observational study with a cross-sectional approach conducted from July to September 2020 on thalassemia major patients in Grobongan and Rembang, Central Java. Ethical clearance was obtained from the Health Study Ethics Commission of the Faculty of Medicine, Universitas Diponegoro, Semarang (No. 205/EC/KEPK/FK-UNDIP/VIII/2020).

### Subject Recruitment

This study included male and female patients receiving more than 10 transfusions, aged 2 to 18 years, who had normal body temperature (36.5-37.2°C), normal leukocyte count (4000-11,000/µL), normal liver function (Aspartate Aminotransferase (AST) = 3-45 µL and Alanine Aminotransferase (ALT) = 5-35 µL), and normal kidney function (urea = 3-25 mg/dL and creatinine = 0.6-1.3 mg/dL). Subjects using calcium ions supplements, corticosteroid drugs, and anti-epileptic drugs (AED), as well as subjects with hematological malignancies were excluded from the study. Consent was obtained from parents or patients' guardians. The data on age, sex, body weight, duration of thalassemia, and volume of blood transfusions received were recorded.

### Hematological Examination

Venous blood samples were collected with a total of 10 mL from the median cubital vein. Of this, 7 mL was placed into a plain tube (without anticoagulant) and the remaining

volume into an EDTA tube. The blood in the EDTA tube was analyzed for routine hematological parameters using an automated hematology analyzer. The blood in the plain tube was allowed to clot for 30 minutes and then centrifuged at 1000 rpm for 15 minutes. The resulting serum was separated and stored at -20°C. The obtained serum was aliquoted into three portions of 500 µL each for the measurement of serum IGF-1 levels, serum calcium ion levels, and serum β-CTx levels. The hematological examination was conducted using the Sysmex XN 1000 tool (Sysmex, Kobe, Japan) with the Flow Cytometry method using cell pack and cleaning reagents, as well as a stromalyzer.

**Calcium Ion Examination**

Calcium ion examination was performed with the ion selective electrode method using the EXIAS e|1 Analyzer, (EXIAS Medical GmbH, Graz, Austria), and O-cresolphthalein complexone (CPC) reagent. Reagent preparation involved mixing 2 reagents in a small beaker glass at a 1:1 ratio. The mixture was homogenized and covered with parafilm, then incubated for 10 minutes at a temperature of 20°C to 25°C. For sample preparation, each blood specimen was labeled and centrifuged at 3000 rpm for 10 minutes. Following centrifugation, the serum was separated and transferred into sample cups, which were subsequently stored in a freezer at a temperature of -20°C to -50°C. Then, 1000 µL of blank reagent was added to a blank tube, while 20 µL of standard reagent and 1000 µL of reagent were added to the standard tube. The same procedure was followed for the serum sample tube. The mixtures were then incubated for 5 minutes at 20°C to 25°C. Absorbance of the sample and standard was measured against the blank within 50 minutes using a photometer at a wavelength of 578 nm.

**Enzyme-linked Immunosorbent Assay (ELISA) Analysis for IGF-1 and β-CTx Measurement**

IGF-1 and β-CTx were examined using the Biotek ELX800 device (BioTek Instruments, Winooski, VT, USA) with ELISA method. Human IGF-1 (Insulin-like Growth Factor 1) ELISA Kit (Cat. No. E-EL-H0086; Elabscience, Houston, TX, USA) was used for IGF-1 measurement. Using a double antibody sandwich technique, a monoclonal antibody specific to IGF-1 was coated onto a microplate and incubated with a biotin-conjugated antibody. A color change occurs through an avidin-based enzymatic reaction. The enzyme-substrate reaction was stopped by the addition of sulfuric acid solution, and the intensity of the resulting color change was measured using a spectrophotometer at a wavelength of 450 nm. The concentration of IGF-1 in the sample was determined by comparing the optical density (OD) of the sample to a standard curve.

Meanwhile, the kit for Human β-CTx (Beta Cross Laps) ELISA Kit (Cat. No. E-EL-H0960; Elabscience) was used to measure β-CTx. The procedure was requiring 100 µL of serum per sample and involved incubating standards, controls, and patient samples at room temperature. The reaction was stopped with a stop solution, resulting in a color change from blue to yellow. The absorbance was measured at 450 nm using a spectrophotometer, and β-CTx concentrations were calculated by comparing the optical density values of the samples with those on the standard curve.

**Statistical Analysis**

The results were analyzed to determine the relationship of IGF-1 and calcium ion levels to β-CTx. Data were presented in mean±standard deviation (SD) and statistically analyzed using the SPSS program (IBM Corporation, Armonk, NY, USA). Normal data distribution was verified using the Shapiro-Wilk test, while the Pearson test was used to determine correlation between both IGF-1 and calcium ion levels to β-CTx. A $p<0.05$ with 95% confidence intervals were considered significant.

## Results

**Characteristics of Study Subjects**

The basic characteristics of these samples were shown in Table 1. A total of 29 patients with multi-transfused β-thalassemia major were included. In this study, most of the subjects were male as much as 16 subjects (55.2%), and the average age was 11.1±3.71 years. Calcium levels were in the normal range (1.27±0.08 mmoL/L), but IGF-1 and β-CTx levels were in the abnormal range, with IGF-1 level that was lower than normal while β-CTx level was higher than normal.

**IGF-1 was Negatively Correlated with β-CTx**

Figure 2 illustrated that IGF-1 was significantly related to β-CTx. The IGF-1 level had a median (min-max) of 14 (1.5-103.9) ng/mL (Table 1). In addition, the data normality test showed that the IGF levels were abnormal. As a result, the data transformation was carried out. Afterwards, IGF-1 data were normally distributed, and data analysis was performed using the Pearson test. it was found that there was a moderate negative relationship between IGF-1 levels and β-CTx level ($p$=0.001; r=-0.573).

**Table 1. Characteristics of research subjects.**

| Variables | Value |
|---|---|
| Gender, n (%) | |
| Male | 16 (55.2%) |
| Female | 13 (44.8%) |
| Age (years), mean±SD | 11.1±3.71 |
| Body Weight (kg), median (min-max) | 25 (11-53) |
| Height (cm), mean±SD | 133.48±18.01 |
| Body Mass Index (kg/m$^2$), median (min-max) | 15.75 (11.63-48.07) |
| Hemoglobin (g/dL), mean±SD | 9.38±1.53 |
| Hematocrit (%), mean±SD | 27.61±4.75 |
| Erythrocytes (x10$^6$/uL), mean±SD | 3.96±0.67 |
| MCV (fL), mean±SD | 69.95±5.79 |
| MCH (pg), median (min-max) | 23.9 (17.0-27.9) |
| MCH Concentration (g/dL), mean±SD | 34.03±0.98 |
| Leukocytes (x10$^3$/uL), mean±SD | 7.29±1.97 |
| Platelets (x10$^3$/uL), median (min-max) | 219 (103-574) |
| IGF-1 (ng/mL), median (min-max) | 14 (1.5-103.9) |
| Calcium Ion (mmol/L), mean±SD | 1.27±0.08 |
| β-CTx (ng/mL), mean±SD | 9330.41±1696.76 |

SD: Standard deviation; MCV: Mean corpuscular volume; MCH: Mean corpuscular hemoglobin; IGF-1: Insulin-like growth factor-1; β-CTx: β-Cross Laps.

### Calcium Ion was Negatively Correlated with β-CTx

Figure 2 illustrated that calcium ion levels were significantly related to β-CTx. The calcium ion levels had a mean of 1.27±0.08 mmol/L (Table 1). The data normality test indicated that calcium ion levels were normal. Therefore, data analysis in this study was carried out with the Pearson test. A weak negative relationship was found between calcium ion level and β-CTx level ($p$=0.046; r=-0.373).

## Discussion

Most of the subjects in this study were male (55.2%). This result contradicted with a study held in Indonesia in 2017, which revealed that 74.3% thalassemia patients were female. (17) On the other hand, the data of hematology examination showed characteristics of microcytic hypochromic anemia. This result was in line with the specification of thalassemia. This study also identified the abnormal levels of IGF-1. In the physiologically normal pediatric population, IGF-1 levels are both age and sex-dependent. Serum IGF-1 levels typically increase as the child grows, reach a peak value at puberty, and decrease with aging.(18) In this study, with a mean age of 11.1±3.71 years, the mean serum of IGF-1 concentration was only 20.11±20.74 ng/mL, which was even lower if presented as median (14 (1.5-103.9) ng/mL). However, according to IGF-1 pediatric reference, the expected IGF-1 level for 11 year-old children range from 111 to 551 ng/mL. This finding suggests that children with thalassemia exhibit significantly reduced IGF-1 levels compared to the healthy children population (19), indicating a potential impairment in growth factor regulation associated with the disease.

IGF-1 not only served as an endocrine growth hormone mediator that could affect metabolic and anabolic actions, but also an important regulator of skeletal homeostasis, including bone modelling and re-modelling, contributes to maintenance of bone mass and linear-radial bone growth. Low IGF-1 serum level has been proven to be associated with increased risk of low BMD. This result showed that IGF-1 level was a useful tool to evaluate the risk of fractures

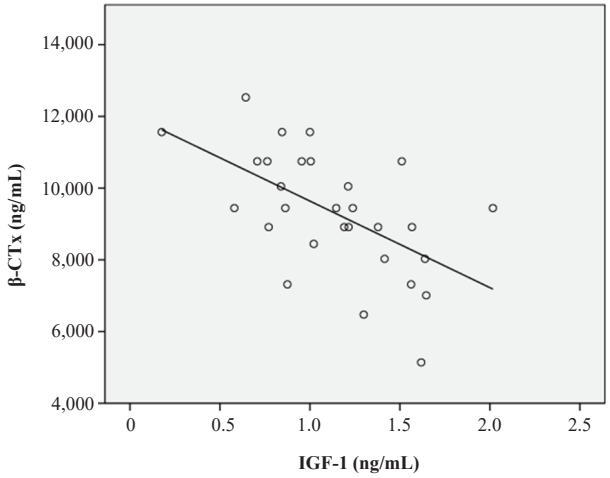


**Figure 1. Scatter plot graph of the relationship between IGF-1 levels and serum β-CTx in multi-transfused β-thalassemia major subjects.**

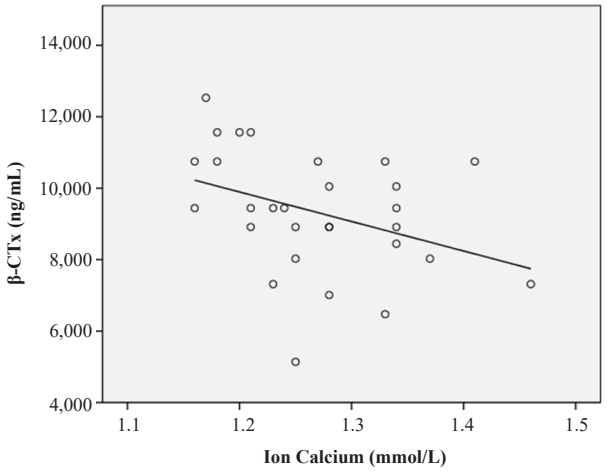


**Figure 2. Scatter plot graph of the relationship between calcium ion levels and serum β-CTx in multi-transfused β-thalassemia major subjects.**

in adults and children with osteoporosis.(20) Therefore, IGF-1 deficiency in multi-transfused thalassemia patients was characterized by growth impairment.

This result of this study revealed a moderate negative relationship between IGF-1 levels and serum β-CTx, with r=-0.573. According to previous studies, low levels of serum IGF-1 were observed in chronic anemia and hypoxia, which further impaired hepatic protein synthesis. Phosphorylated insulin-like growth factor binding protein-1 (IGFBP-1) inhibited IGF-I action, bone formation, and osteoblast activity, led to growth impairment and the children grew smaller compared to their normal counterparts of the same age. Severe hypoxia stimulated erythropoiesis continuously. However, since erythropoiesis was ineffective, it consequently caused progressive marrow expansions and extramedullary erythropoiesis, which occurred primarily in the spleen, resulting in hypersplenism.. As a result, ferritin levels were increased and aggravated pre-existing anemia, which negatively affected serum IGF-1 levels.(21) In addition, improper deferoxamine administration in multi-transfused thalassemia patients inhibited DNA synthesis, osteoblasts and fibroblasts proliferation, osteoblast differentiation, interfered with collagen formation, and increased osteoblast apoptosis.(10,21)

A weak negative relationship was observed between calcium ion levels and β-CTx in subjects. Relationship between these variables may occurred due to high activity of parathyroid hormone (PTH) as a response mechanism for decreased calcium absorption in the intestine. (22) Deficiency of vitamin D stimulated 1,25(OH)2D3 production, which in turn enhanced calcium absorption from the intestine to maintain normal serum calcium levels within the physiological range (approximately 8.5–10.5 mg/dL) at the adaptive stage. This regulation reflects the body's adaptive response to maintain calcium homeostasis, which is essential for bone mineralization and overall metabolic stability. High levels of PTH maintain serum calcium levels with the concomitant effect of hypophosphatemia, decreased IGF-I secretion, and restricted bone growth that limited calcium utilization within bones.(13) Moreover, the mean level of β-CTx in children with thalassemia was higher than control group, serving as a signal in the development of osteoporosis.(23)

The ability of calcium ions to increase serum β-CTx levels was explained by a hypothesis that sufficient serum calcium levels inhibited calcium ion absorption in the intestine. These events further increased the levels of β-CTx by reducing the production of PTH, which served as a calcium serum regulator. Patients with multi-transfused thalassemia often present endocrinopathy as a complication, due to excess iron accumulation that affected IGF-1 and calcium metabolism.(24) However, despite all of the homeostatic adaptation, the weak correlation between serum calcium and β-CTx observed in our results suggests that calcium levels alone may not reliably reflect dynamic changes in bone resorption activity. Therefore, future studies should incorporate additional bone formation and regulatory markers, such as PTH and 25(OH) vitamin D, to provide a more comprehensive evaluation of bone turnover and endocrine function. These markers are especially relevant given the risk of iron-induced endocrine dysfunction and impaired bone health in this population.

The growth disorder in pediatric with thalassemia was due to bone remodeling imbalance between resorption activity by osteoclasts and the formation activity by osteoblasts. Increased resorption by osteoclasts led to higher production of receptor activator of receptor activator of nuclear factor kappa-B (RANK) ligand (RANKL). Osteoclastogenesis was enhanced by RANK-RANKL signaling pathway and macrophage colony-stimulating factor (M-CSF). As a result, osteoclast activity exceeded osteoblasts (bone-forming cells) activity. This condition led to a decrease in bone mass. The binding of RANK-RANKL signaling also induce tumor necrosis factor (TNF) receptor associated factor (TRAF) and leads to activation of several cascades, such as p38 mitogen-activated protein kinase (MAPK), which has an important roles in osteoimmunology.(25)

In addition, β-CTx were expressed in various body tissues, including cartilage during the embryonic stage, bone tissue (osteoblasts, mesenchymal stem cells), and their marrow (B lymphocytes). The β-CTx expression was affected by several cytokines, peptides, hormones, and drug administration. This expression increased following depletion of calcium ion, vitamin D, interleukin (IL)-1α, IL-1β, IL-6, IL-11, IL-17, IL-18, TNF-α, TNF-β, bone morphogenetic protein (BMP)-2, transforming growth factor (TGF)-β, and 17β-estradiol. The concentrations also decreased by prostaglandin E2 (PGE2), PTH, fibroblast growth factor (FGF), glucocorticoids, IGF-1, and cyclosporine. Moreover, osteoclasts were down-regulated due to iron chelation therapy along with endocrine changes. (23,26,27) Elevated β-CTx levels were associated with increased osteoblast precursors, an indication of increased bone turnover.(28) A study in normal populations to predict subclinical atherosclerosis revealed that β-CTx levels provided a better predictor than other risk factors, such as age and glomerular filtration rate. This also served as a better

screening marker compared to other bone markers such as osteopontin, ALP, and osteoprotegerin. A metabolism or bone demolition product directly used as a marker for bone resorption had a sensitivity of >70% and an 80% specificity. In addition, increased levels of extracellular calcium ion provided the ability to induce apoptosis and necrosis, leading to calcification.(29–31)

Previous study found that selective androgen receptors modulators (SARMs) may offer potential benefits as a therapy for osteoporosis, as the higher dose of SARMs were tended to decrease the number of osteoclast cells, as well as increasing the osteoblast cells. Vegetables including onion, parsley, and garlics also were reported to inhibit bone resorption, and caffeic acid successfully suppresses osteoclastogenesis by suppressing the nuclear factor-kappaB (NF-κB) activity. These findings may have potential role in developing treatment to prevent excessive altered bone remodeling in pediatric with multi-transfusion, especially due to thalassemia.(32)

In this study, there are wide age range of participants (2 to 18 years), no participants classifications based on the presence of complications, and relatively small number of the sample size of multi-transfused β-thalassemia major patients. For future studies, we suggest narrowing the age range or stratifying participants based on the total number of blood transfusions received to better clarify the cumulative effects of transfusions on bone growth. Additionally, classifying patients based on the presence of complications and increasing the sample size may contribute to more accurate and reliable results.

## Conclusion

The results indicated a moderate negative relationship between serum IGF-1 and β-CTx levels as well as a weak negative relationship between calcium ion and serum β-CTx levels in multi-transfused β-thalassemia major patients. Therefore, these parameters might be potential to be used clinically in determining osteoporosis risk and supporting its diagnosis, which tended to prevent osteoporosis, fractures, and bone abnormalities.

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## Authors Contribution

NSW, ED, YMN and SB were involved in concepting and planning of the research. SB, NSW, DR and A performed the data acquisition/collection. SB, A, ED and MH calculated the experimental data and performed the analysis. SB, NSW, A and ED drafted the original manuscript and designed the figures and table. NSW, DR, YMN, IB and ED aided in interpreting the results. All authors took part in giving critical revision of the manuscript.

## Conflict of Interest

The authors declare no conflicts of interest or competing interests related to the content of this manuscript.

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