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# In transfusion-dependent thalassemia, increased iron overload is associated with lower serum alpha-klotho, which is strongly associated with lower total and ionized calcium concentrations.

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## Abstract

**Background.** Patients with transfusion-dependent thalassemia (TDT) show disorders in calcium metabolism. The α-klotho protein is predominantly expressed in tissues that are involved in calcium homeostasis, and lowered levels are associated with bone disease.

**Aim of the study.** To study the associations between low α-klotho status and calcium metabolism in relation to iron status in children with TDT.

**Methods.** α-klotho, calcium, parathyroid hormone (PTH), calcyphosin, vitamin D3, phosphorous, fibroblast growth factor receptor 2 (FGFR2), as well as iron and erythron biomarkers were measured in 60 children with TDT and 30 healthy control children.

**Results.** A meaningful part of TDT patients showed lowered α-klotho levels, and those children also showed low serum total and ionized calcium concentrations. TDT patients showed increased PTH, FGFR2, and calcyphosin and lowered vitamin D3 as compared with healthy children. The α-klotho levels were significantly correlated with total and ionized calcium (positively) and with iron overload biomarkers and the number of blood transfusions (inversely). Partial Least Squares path analysis showed that 40.1% of the variance in serum total calcium could be explained by the regression on α-klotho, vitamin D3 (both positively), and calcyphosin (inversely) and that the effects of the latter are mediated by iron overload and the number of blood transfusions.

**Conclusion.** In TDT, iron overload and its consequences may induce lowered levels of α-klotho which in turn may lead to lower calcium thereby explaining at least in part the effects of TDT on bone metabolism including spontaneous pathological fractures, osteoporosis, osteopenia, and skeletal deformities.

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**Keywords:** Calcium, α-klotho, inflammation, oxidative stress, antioxidants, biomarkers.

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## Introduction

Beta-thalassemia major (β-TM) is a hematologic disorder caused by absent or severely reduced synthesis of the β-globin chain in the hemoglobin A molecule resulting in damage to the erythrocyte membrane and subsequent anemia [1,2]. Patients with β-TM require lifelong blood transfusions to increase hemoglobin levels and minimize the detrimental effects of inefficient erythropoiesis [3]. Patients with the latter condition, denoted as transfusion-dependent thalassemia (TDT), are prone to many complications due to the frequent blood transfusions [4]. Chronic blood transfusions may cause severe iron overload, which may cause toxicity to various organs including the liver, heart, endocrine organs [5-7], bones and joints [8]. The latter may be associated with severe consequences including spontaneous pathological fractures, osteoporosis, osteopenia, skeletal deformities, and bone pain [9-11].

Many bone-related biomarkers may be used for the early detection of changes in bone metabolism and calcium disorders including parathyroid hormone (PTH) [12,13], vitamin D [14,15], calcitonin [13], and serum calcium, phosphorous, and alkaline phosphatase [10,15,16]. Alternative biomarkers used to examine changes in bone turnover and calcium homeostasis in TDT comprise insulin-like growth factor-1 (IGF-1) and osteocalcin [11], sclerostin [17], thyroid hormones [13], osteoblast differentiation inhibitors, namely Dickkopf-1 [18], tartrate-resistant acid phosphatase 5b, receptor activator of nuclear factor-kappa B ligand and osteoprotegerin [19,20].

Other biomarkers which play a role in bone disorders and are elevated in patients with TDT are calcyphosin (CAPS1) and fibroblast growth factor receptor 2 (FGFR2) [21]. Calcyphosin is a calcium-binding protein involved in both $Ca^{2+}$-phosphatidylinositol and

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cAMP signal cascades [22]. FGFR2 is expressed on preosteoblasts and osteoblasts during the later phase of bone formation [23]. Dysregulation of FGFR2 results in a spectrum of bone pathologies [24,25]. Other authors examined serum soluble α-Klotho in TDT, but could not find a difference between TDT and control groups [26]. Klotho is a β-glucosidase-like membrane-bound protein that displays a secreted splice form [27,28]. The α-Klotho gene is predominantly expressed in tissues that are involved in calcium homeostasis including the parathyroid glands, kidney and the choroid plexus [29,30]. α-Klotho regulates calcium and phosphate reabsorption in the kidney and indirectly, as a cofactor for FGF23, regulates vitamin D metabolism [31]. Moreover, α-Klotho promotes endothelial nitric oxide production and inhibits Wnt signaling and oxidative stress pathways [32,33], and inhibits intracellular insulin and IGF-1 signaling, which is an evolutionarily conserved pathway associated with an extended life span [34]. Also, in animal models, α-Klotho may delay the ageing process in association with suppressing insulin and IGF-1 signaling and oxidative stress toxicity [34,35]. However, there are no data whether α-Klotho in TDT is associated with aberrations in calcium homeostasis and iron overload.

Hence, the present study aims to examine whether TDT in children is accompanied by lowered serum α-Klotho and whether there are significant associations between serum α-Klotho and calcium concentrations or calcium-related biomarkers (Vitamin D3, PTH, calcyphosin, FGFR2, phosphate) and iron overload biomarkers (iron, ferritin, transferrin saturation).

## Subjects and methods

Participants

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This study recruited 90 participants, namely 30 healthy controls and 60 TDT children, aged 3-12 years old and of both sexes. The TDT patients were recruited at the Thalassemia Unit at Al-Zahra'a Teaching Hospital, Najaf, Iraq. Pediatricians and hematologists made the diagnosis of β-TM according to the criteria of 2019 ICD-10-CM Diagnosis Code D56.1. The diagnosis was based on the typical clinical symptoms (e.g. severe anemia, hepatosplenomegaly, and abnormal bone growth), hematological tests including hemoglobin <7g/dl and hypochromic microcytic RBCs with anisopoikilocytosis and high reticulocyte percentage, and by elevated HbA2 levels as assayed using HPLC (VARIANT TM β-Thalassemia Short Program). Thirty apparently healthy children were recruited as the control group. None of the controls was anemic or had an immune-inflammatory or systemic disease. We excluded any subject with splenectomy, systemic diseases such as renal failure, diabetes mellitus, or subjects with overt inflammation defined as serum C-reactive protein (CRP) levels > 6mg/l. The latter exclusion criterion was used to ascertain that the change in ferritin or other acute-phase reactant proteins is due to iron overload rather than to an acute phase response.

The frequency of administration of blood transfusions with packed RBCs at 2 or 4-week intervals was based on Hb levels that should be kept above 9 g/dL. Moreover, patients were on an iron-chelating therapy (3-5 times weekly) with deferoxamine mesylate USP (Desferal®) infusion at a dose range between 25-50 mg/kg/day over 8 hours/day depending on the ferritin levels. Folic acid was also given to most patients to reduce ineffective erythropoiesis. TDT patients were treated with vitamin C to assist the chelation of iron with deferoxamine through stimulation of iron release from the reticuloendothelial system. Written informed consent was obtained from the patient's first-degree relatives (mother or

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father) after appropriate oral explanation according to the Declaration of Helsinki. The study was approved by the IRB of the University of Kufa number 419/2018.

**Measurements**

Five mL of venous blood were drawn from all participants after an overnight fast. The patients' samples were collected just before their blood transfusion session. Blood was left at room temperature for 10 minutes for clotting, centrifuged 3000 rpm for 5 minutes, and then serum was separated and transported into Eppendorf tubes. Serum albumin, calcium, and phosphate were measured using a ready for use kit supplied by Biolabo® Co (Maizy France). Ionized calcium was calculated from the following formula**:** $I.Ca^{2+} = 0.813 \times T.Ca^{0.5} - 0.006 \times Albumin^{0.75} + 0.079$ [36], which give the best approximate result. The amount of iron in sera was determined by colorimetric kits supplied by Spectrum® (Cairo, Egypt). Transferrin saturation percentage (TS%) was calculated from the following equation: TS% = Iron * 100/TIBC [37]. TIBC was measured by saturation of serum transferrin with iron, and the unbound iron portion is precipitated with magnesium carbonate, and then the iron was remeasured in the supernatant. Serum PTH and soluble α-Klotho levels were measured using ELISA kits supplied by MyBioSource® (San Diego, USA). Serum ferritin levels were measured by using ELISA kit supplied by Elabscience® (Wuhan, China). Serum calcyphosin and FGFR2 were measured using an enzyme-linked immunosorbent assay (ELISA) using kits supplied by Bioassay Technology Laboratory (Shangai, China). These kits were designed for human samples depending on the biotin double antibody sandwich technology. Hematological parameters were measured by a five-part differential Mindray BC-5000 hematology analyzer (Mindray Medical Electronics

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Co., Shenzhen, China). Vitamin D was determined by a fluorescence immunoassay (FIA) using kits designed for the I-Chroma™ instrument (BioLabs Diagnostics, Italy) to estimate total 25(OH)D2/D3 level in human serum.

The inter-assay CV% of ferritin, PTH, and soluble α-Klotho kits were <15%, <10%, and <10%, respectively, and the sensitivities of the ferritin, PTH and α-Klotho assays were 10.0 ng/ml, 15.6 pg/ml, and <56.25 pg/ml, respectively. The inter-assay CV% of iron was <2.19%. The inter-assay CV of calcyphosin was <10%, and sensitivity= 0.026 mM, while the inter-assay CV% of FGFR2 was <10%, and sensitivity=0.09 ng/ml. For samples with highly concentrated analytes, we employed sample dilutions. We computed a z unit-weighted composite score which reflects iron overload as z iron + z transferrin saturation % + z ferritin (IO index). CRP was measured using a kit supplied by Spinreact®, Spain, which is based on latex agglutination.

Statistical analysis

Analysis of contingency tables ($\chi^2$ test) was employed to assess associations between nominal variables while analysis of variance (ANOVAs) was used to assess differences in continuous variables among diagnostic groups. Associations between scale variables were computed using Pearson's product-moment correlation coefficients. Multivariate general linear model (GLM) analysis followed by tests of between-subject effects and pairwise comparisons among treatment groups were used to examine the associations between TDT (versus controls) and the biomarkers. A false-discovery rate (FDR) procedure was employed to control for type I errors when performing multiple comparisons [38]. Simple boxplots with the minimum, Q1, median, Q3, and maximum

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values, and out- and far-out values were employed to display the results of α-Klotho assays. All tests were two-tailed, and a p-value of 0.05 was used for statistical significance. We used IBM SPSS 25 windows version to analyze the data.

Partial Least Squares (PLS) structural equation modelling was employed using the Smart PLS software [39] to assess the causal paths from the number of blood transfusions and iron overload to different calcium homeostasis-related molecules (α-Klotho, CAPS, vitamin D3, PTH and FGFR2) and the final output variable was total calcium. All variables were entered as single indicators except the iron overload index which was constructed as a latent vector (LV) extracted from iron, TS%, and ferritin [40]. Complete PLS analysis was performed when the outer model complied with quality data, namely the LV displays excellent composite reliability (> 0.7), and adequate Cronbach's alpha (> 0.7), rho_A (> 0.8) and average variance extracted (AVE > 0.5) values, and when all loadings on the LV are > 0.6 ($p<0.001$). Moreover, the model fit should be adequate with an SRMR value < 0.080 [39]. Consequently, we perform complete PLS path modelling on 5000 samples and compute path coefficients with exact p-values and direct and (specific) indirect effects.

## Results

*Demographic and Clinical data*

The socio-demographic and clinical data in TDT and healthy control children are presented in **Table 1.** The patient group was further divided into two groups, namely those with normal α-Klotho (n=30) concentrations and those with low α-Klotho (n=30) levels using the median split method (median=350.3 pg/mL). There were no significant differences in age, sex ratio, and rural/urban ratio between the three study groups.

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*Biomarkers and diagnostic groups*

Univariate GLM analysis showed that (after controlling for age and sex) α-Klotho was significantly (F=8.24, df=1/86, p=0.005) lower in TDT (mean ±SE=344.9 ±32.7 pg/mL) than in normal control children (508.9 ±46.5 pg/mL). **Figure 1** shows the box plot of the α-Klotho values in both TDT and control children and that there are no out- and far-out values in the data set. TDT patients showed lower total (F=9.77, df=1/86, p=0.002) and ionized (F=13.67, df=1/86, p<0.001) calcium levels than normal control children. Patients allocated to the low α-Klotho group showed significantly lower total and ionized calcium as compared with the two other groups. Both TDT subgroups also showed significant increases in serum iron, TS%, and ferritin and iron overload index. Patients with TDT also show increases in serum PTH, FGFR2, and calcyphosin as compared with control children, while vitamin D3, RBCs and Hb were decreased in TDT patients as compared with the control group. No significant differences in serum phosphate were detected between the study groups. A multivariate GLM analysis with age and sex as covariates did not change these results and showed no significant effects of these covariates on the biomarkers except phosphate (F=8.45, df=1/85, p=0.005), which was higher in girls than in boys.

*Intercorrelation matrix*

**Table 2** shows the intercorrelations between α-klotho, number of blood transfusion, the iron overload index, and the other biomarkers. In the whole study group, serum α-Klotho was significantly correlated with total and ionized calcium and negatively with PTH. There was a significant inverse correlation between α-Klotho levels and iron, TS%,

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and ferritin, and the iron overload index. α-Klotho levels were also significantly and positively correlated with Hb and negatively with the number of blood transfusions. In the control group, α-Klotho was significantly correlated with total calcium (r=0.380, p=0.039) and calcyphosin (r=0.523, p=0.003). In TDT patients, α-Klotho was significantly and positively correlated with total calcium (r=0.617, p<0.001) and ionized calcium (r=0.610, p<0.001), and inversely with Hb (r=-0.301, p=0.020), whereas no significant correlations with calcyphosin could be found (r=-0.066, p=0.617). In the whole study group, the number of blood transfusion and iron overload were strongly intercorrelated and showed similar correlations with the other biomarkers.

*Results of multiple regression analysis*

**Table 3** shows the results of different multiple regression analyses with total and ionized calcium levels as dependent variables and other biomarkers as explanatory variables while allowing for the effects of age and sex. Regression #1 shows that 40.1 % of the variance in total calcium could be explained by α-Klotho, vitamin D (both positively), and calcyphosin (inversely). **Figure 2** shows the partial regression of total calcium on α-Klotho after adjusting for the variables listed in Table 3, regression #1. We found that 42.5% of the variance in ionized calcium (Regression #2) was explained by α-Klotho, vitamin D (both positively), and calcyphosin (inversely). In the healthy children control group, 14.9% of the variance of total calcium could be explained by serum albumin (regression #3). In TDT patients, 38.1% of the variance in total calcium was explained by α-Klotho, and vitamin D. **Figure 3** shows the partial regression of total calcium on α-Klotho in TDT after adjusting for the variables listed in Table 3.

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*Results of PLS analysis*

**Figure 4** shows the results of the PLS analysis. The model quality data were more than adequate with an SRMR value of 0.012 while the LV showed composite reliability of 0.979, Cronbach alpha=0.968, rho_A=0.968, and AVE=0.939. We found that 40.1% of the variance in total calcium could be explained by the regression on α-klotho and vitamin D3 while calcyphosin was not significant at the alpha=0.05 level. The iron overload LV was a significant predictor of α-klotho, vitamin D3, calcyphosin, PTH, and FGFR2. There were significant specific indirect effects of number of blood transfusions on calcyphosin (t=9.94, p<0.001), FGFR2 (t=5.31, p<0.001), α-Klotho (t=2.63, p=0.008), PTH (t=8.13, p<0.001), and vitamin D3 (t=5.13, p<0.001), which were all mediated by the iron overload LV. Furthermore, there were significant specific indirect effects of blood transfusions on total calcium mediated by the path from iron overload to α-Klotho (t=2.54, p=0.011) and the path from iron overload to vitamin D3 (t=2.18, p=0.029). As such, there were strong effects of blood transfusions (t=4.35, p<0.001) and iron overload (t=4.48, t<0.001) on total calcium. All other paths were non-significant and thus deleted from the study, e.g. between calcium and PTH, and between α-Klotho and vitamin D3, PTH, FGFR2, and calcyphosin.

## Discussion

The first major finding of this study is that TDT patients have lower α-Klotho levels than controls and that a meaningful part (around 50%) of TDT patients show low α-Klotho levels. In one study, serum α-Klotho levels tended to be lower in TDT patients as compared with controls, although the difference was not statistically significant [26]. Our PLS analysis

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showed that the number of blood transfusions significantly predicted lowered α-Klotho and that this effect was mediated by iron overload. Previously, it was shown that serum iron overload is accompanied by decreased expression of α-Klotho in the kidneys and that iron chelation may attenuate the angiotensin-II-associated decreases in α-Klotho expression [41]. It is interesting to note that, in patients with chronic kidney disease, iron deficiency may lead to increased α-Klotho expression [41]. α-Klotho deficiency may cause activation of hypoxia-inducible factors (HIF) which regulate serum iron, which in turn negatively affect α-Klotho levels [42]. Nevertheless, the associations established in our study between α-Klotho and iron overload may, in theory, also be explained by the consequences of iron overload including chelation treatment, activated immune-inflammatory and oxidative stress pathways [40]. In this respect, it was shown that the type of chelation treatment did not affect α-Klotho levels [26]. In animal studies, iron overload may trigger down-regulation of α-Klotho expression while iron chelation may reverse this down-regulation, suggesting that abnormal iron metabolism is implicated [43]. TDT is associated with inflammation and oxidative stress toxicity as a direct consequence of iron toxicity [44]. Su and Yang concluded that α-Klotho might behave as an acute phase response since restraint stress is accompanied by a downregulation of α-Klotho mRNA and increased serum α-Klotho protein [45]. Importantly, α-Klotho acts as an anti-inflammatory modulator through regulation of the production of nuclear factor-κB associated inflammatory proteins thereby reducing the production of several pro-inflammatory cytokines and oxidative stress toxicity [34]. At the cellular and organismal level, α-Klotho confers protection against oxidative stress [46-48] whereby α-Klotho attenuates superoxide production, oxidative damage, and apoptosis

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through the cAMP/PKA pathway [49] while α-Klotho deficiency may increase endogenous generation of reactive oxygen species [50].

Moreover, lowered α-Klotho may have other detrimental effects which could play a role in TDT. For example, α-Klotho modulates hematopoietic stem cell differentiation and erythroid cell generation and development [51]. In mice, α-Klotho insufficiency may increase erythropoiesis through the HIF signaling pathway with consequent synthesis and secretion of renal erythropoietin [51]. Experimental deletion of α-Klotho results in stimulation of erythropoietin production in the kidney, which in turn induces abnormal generation of erythrocytes in the bone marrow and spleen [51]. α-Klotho-induced inhibition of the HIF pathway and erythropoietin expression may be associated with reduced osteoblast numbers and osteopenia [51]. Finally, loss of α-Klotho is known to cause endothelial dysfunction by promoting oxidative stress [52], which may adversely affect hematopoiesis [53].

The second major finding of this study is that α-Klotho levels are strongly associated with total/ionized calcium levels and that TDT children belonging to the low α-Klotho group show deficient calcium levels. Previous studies showed that, in β-TM patients, α-Klotho correlated with serum and urine calcium [54]. α-Klotho participates in the regulation of calcium homeostasis in cerebrospinal fluid and blood by effects in the choroid plexus, parathyroid glands, and distal tubules [55,56]. In this regard, α-Klotho is a critical player that integrates “a multi-step regulatory system of calcium homeostasis”, which continually adjusts calcium concentrations and maintains calcium within a narrow physiological range [57]. Reabsorption of calcium in the distal tubule of the kidney is facilitated by specific channels [58] which are activated by α-Klotho [59]. As such, α-Klotho

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expression responds to $Ca^{2+}$ concentration through $Na^{+}$, $K^{+}$-ATPase in the order of seconds, indicating that α-Klotho is a fast regulator of $Ca^{2+}$ absorption [60]. Moreover, α-Klotho regulates vitamin D3 production, which is a major regulator of intestinal calcium absorption [55].

Lowered α-Klotho expression may have some detrimental effects which are relevant to calcium metabolism and TDT. First, low α-Klotho may increase cytosolic $Ca^{2+}$ activity, which is associated with enhanced translocation of cell membrane phospholipids and shrinkage of RBCs membrane, suggesting that α-Klotho deficiency may accelerate eryptosis [61]. Second, in humans, α-Klotho deficiency or functional variants of α-Klotho are associated with the development of vascular calcification [62,63] and osteoporosis [64].

The third major finding of our study is that TDT is accompanied by lower total and ionized calcium, and vitamin D3, but increased PTH, FGFR2, and calcyphosin levels while there are no significant differences in phosphate levels. These results extend those of previous papers which reported reduced levels of serum calcium and vitamin D3 and increased levels of calcyphosin, FGFR2 and PTH in thalassemia [15,65,66]. One hypothesis is that some of those changes could be induced by the effects of lower α-Klotho since a deficiency in α-Klotho was proposed to induce high serum PTH, phosphate, and FGF23 levels [67-73]. In addition, α-Klotho is a significant regulator of vitamin D biosynthesis [56]. Nevertheless, in our study no significant associations between α-Klotho, on the one hand, and PTH, FGFR2 and vitamin D3, on the other hand, could be detected after considering the effects of iron overload. The latter was significantly associated with PTH, FGFR2, calcyphosin, (positively) and vitamin D3 (negatively), suggesting that mechanism related

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to iron overload may be involved. Previously, higher PTH levels were detected in β-TM patients, and these were positively associated with increased ferritin, one of the indicants of iron overload [74]. Chronic inflammation with increased levels of IL-1β and iron deficiency increase ferritin and FGF23 cleavage levels [75]. In thalassemia patients, increased iron overload and ferritin levels are associated with lowered vitamin D [76].

Furthermore, the strong effects of iron overload in our PLS analysis on all these biomarkers may suggest that, in TDT, the fine-tuning feedback systems between α-Klotho and calcium, vitamin D3, FGFR2 and PTH are overwhelmed by the iron overload (or its consequences). For example, vitamin D3 may upregulate α-Klotho expression [77] explaining that PTH may indirectly upregulate α-Klotho by mediating increases in vitamin D3 [78]. In addition, vitamin D may stimulate the expression of FGF23 and α-Klotho, while vitamin D3 formation is limited by a negative feedback regulation [79,80]. Also, the α-Klotho/FGF23 signaling pathway regulates the vitamin D/PTH signaling pathway and vice versa [77]. Although α-Klotho regulates intestinal phosphate absorption, thereby maintaining circulating phosphate in the physiological range [81], we could not detect hyperphosphatemia in TDT patients. This may be explained by the counterbalancing activities of iron chelators which increase renal phosphate excretion [82]. Future studies should investigate the effects of iron overload and accompanying inflammation and oxidative stress on PTH, calcyphosin, vitamin D3, phosphate, and FGFR2.

The results of our study should be interpreted with reference to its limitations. First, this is a case-control study and, therefore, no firm causal conclusions can be made. Second, it would have been more interesting if we had used advanced bone health imaging

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techniques, including Dual-energy X-ray absorptiometry (DEXA) to measure bone density in association with α-Klotho levels.

**Conclusion**

α-klotho and total/ionized calcium levels are significantly lower in TDT than in healthy control children. TDT patients show increased PTH, FGFR2, and calcyphosin and lowered vitamin D3 as compared with healthy children. α-klotho levels are significantly and positively associated with total/ionized calcium, the iron overload index, and the number of blood transfusions. A large part of the variance in serum calcium may be explained by the regression on α-klotho, vitamin D3 (both positively), and calcyphosin (inversely). The effects of the three latter biomarkers on total calcium are mediated by iron overload and the number of blood transfusions.

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Declaration of interest

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Authorships.

All authors contributed significantly to the paper and approved the final version.

References

1. Giardine B, Borg J, Viennas E, Pavlidis C, Moradkhani K, Joly P, Bartsakoulia M, Riemer C, Miller W, Tzimas G. Updates of the HbVar database of human hemoglobin variants and thalassemia mutations. Nucleic acids research 2014;**42**: D1063-D9.
2. Wahidiyat PA, Sastroasmoro S, Fucharoen S, Setianingsih I, Putriasih SA. Applicability of a clinical scoring criteria for disease severity of ß-thalassemia/hemoglobin E in Indonesia. Medical Journal of Indonesia 2018;**27**: 26-32.
3. Galanello R, Origa R. Beta-thalassemia. Orphanet journal of rare diseases 2010;**5**: 11.
4. Malik S, Syed S, Ahmed N. Complications in transfusion–dependent patients of ß-thalassemia major: A review. Pak J Med Sci 2009;**25**: 678-82.
5. Hamed AA, Elguindy W, Elhenawy YI, Ibrahim RH. Early cardiac involvement and risk factors for the development of arrhythmia in patients with β-thalassemia major. Journal of pediatric hematology/oncology 2016;**38**: 5-11.
6. Daher R, Manceau H, Karim Z. Iron metabolism and the role of the iron-regulating hormone hepcidin in health and disease. La Presse Médicale 2017;**46**: e272-e8.
7. Abdulzahra MS, Al-Hakeim HK, Ridha MM. Study of the effect of iron overload on the function of endocrine glands in male thalassemia patients. Asian journal of transfusion science 2011;**5**: 127.
8. Dhawan P, Kanojia RK, Chandra J, Kumar A, Anand R, Gupta S. Wrist Joint Skeletal Changes in Children With Transfusion-dependent Thalassemia. J Pediatr Orthop 2020;**40**: e473-e8.
9. Salama OS, Al-Tonbary YA, Shahin RA, Sharaf Eldeen OAJH. Unbalanced bone turnover in children with β-thalassemia 2006;**11**: 197-202.
10. Saboor M, Qudsia F, Qamar K, Moinuddin MJJHTD. Levels of calcium, corrected calcium, alkaline phosphatase and inorganic phosphorus in patients' serum with β-thalassemia major on subcutaneous deferoxamine 2014;**2**: 2.
11. Merchant R, Udani A, Puri V, D'cruz V, Patkar D, Karkera AJTIJoP. Evaluation of osteopathy in thalassemia by bone mineral densitometry and biochemical indices 2010;**77**: 987-91.
12. Angelopoulos NG, Goula A, Rombopoulos G, Kaltzidou V, Katounda E, Kaltsas D, Tolis G. Hypoparathyroidism in transfusion-dependent patients with β-thalassemia. Journal of bone and mineral metabolism 2006;**24**: 138-45.
13. Pirinççioğlu AG, Akpolat V, Köksal O, Haspolat K, Söker M. Bone mineral density in children with beta-thalassemia major in Diyarbakir. Bone 2011;**49**: 819-23.
14. Fung EB, Aguilar C, Micaily I, Haines D, Lal A. Treatment of vitamin D deficiency in transfusion-dependent thalassemia. American journal of Hematology 2011;**86**: 871.
15. Al-Hakeim HK, Ridha MAS, Muhammed ZH. Calcium status in severe iron overload Iraqi thalassemia major patients

Biochem. Cell. Arch 2018;**18**: 22-32.

16. Aslan I, Canatan D, Balta N, Kacar G, Dorak C, Ozsancak A, Oguz N, Cosan RJIjoe. Bone mineral density in thalassemia major patients from Antalya, Turkey 2012;**2012**.
17. Voskaridou E, Christoulas D, Plata E, Bratengeier C, Anastasilakis A, Komninaka V, Kaliontzi D, Gkotzamanidou M, Polyzos S, Dimopoulou MJH, Research M. High circulating sclerostin is present in patients with thalassemia-associated osteoporosis and correlates with bone mineral density 2012;**44**: 909-13.
18. Voskaridou E, Christoulas D, Xirakia C, Varvagiannis K, Boutsikas G, Bilalis A, Kastritis E, Papatheodorou A, Terpos EJh. Serum Dickkopf-1 is increased and correlates with reduced bone mineral density in patients with thalassemia-induced osteoporosis. Reduction post-zoledronic acid administration 2009;**94**: 725-8.
19. Abd El-Moneim ES, Zolaly MA, Al-Hawsawi ZM, Abdelmoneim AA, Abosdera MM. Age-related changes in biochemical bone profile in thalassemic children. Pediatr Neonatol 2018;**59**: 189-97.
20. Salah H, Atfy M, Fathy A, Atfy M, Mansor H, Saeed J. The clinical significance of OPG/sRANKL ratio in thalassemia patients suffering from osteopenia or osteoporosis in Egyptian patients. Immunol Invest 2010;**39**: 820-32.
21. Al-Hakeim HK, Alhillawi ZH. Effect of serum fibroblast growth factor receptor 2 and CAPS proteins on calcium status in β-thalassaemia major patients who are free from overt inflammation. Growth Factors 2018;**36**: 178-85.
22. Clément S, Dumont JE, Schurmans S. Loss of calcyphosine gene expression in mouse and other rodents. Biochemical and biophysical research communications 1997;**232**: 407-13.
23. Eswarakumar VP, Monsonego-Ornan E, Pines M, Antonopoulou I, Morriss-Kay GM, Lonai P. The IIIc alternative of Fgfr2 is a positive regulator of bone formation. Development 2002;**129**: 3783-93.
24. Katoh M. FGFR2 abnormalities underlie a spectrum of bone, skin, and cancer pathologies. J Invest Dermatol 2009;**129**: 1861-7.
25. Wilkie AO. Bad bones, absent smell, selfish testes: the pleiotropic consequences of human FGF receptor mutations. Cytokine & growth factor reviews 2005;**16**: 187-203.
26. Stefanopoulos D, Nasiri-Ansari N, Dontas I, Vryonidou A, Galanos A, Psaridi L, Fatouros IG, Mastorakos G, Papavassiliou AG, Kassi E, Tournis S. Fibroblast Growth Factor 23 (FGF23) and Klotho Protein in Beta-Thalassemia. Horm Metab Res 2020;**52**: 194-201.
27. Saghiv M. The klotho gene and soluble klotho in health and disease: From 1997-2018; A Review. Ann cardiol Vasc Med 2018;**2**: 1007.
28. Lewin E, Olgaard K. Klotho, an important new factor for the activity of Ca2+ channels, connecting calcium homeostasis, ageing and uraemia. Nephrology Dialysis Transplantation 2006;**21**: 1770-2.
29. Kuro-o M, Matsumura Y, Aizawa H, Kawaguchi H, Suga T, Utsugi T, Ohyama Y, Kurabayashi M, Kaname T, Kume E, Iwasaki H, Iida A, Shiraki-Iida T, Nishikawa S, Nagai R, Nabeshima YI. Mutation of the mouse klotho gene leads to a syndrome resembling ageing. Nature 1997;**390**: 45-51.
30. Urakawa I, Yamazaki Y, Shimada T, Iijima K, Hasegawa H, Okawa K, Fujita T, Fukumoto S, Yamashita TJN. Klotho converts canonical FGF receptor into a specific receptor for FGF23 2006;**444**: 770-4.
31. Quarles LD. Role of FGF23 in vitamin D and phosphate metabolism: implications in chronic kidney disease. Experimental cell research 2012;**318**: 1040-8.
32. Xu Y, Sun Z. Molecular basis of Klotho: from gene to function in aging. Endocrine reviews 2015;**36**: 174-93.
33. Kuro-o M. Klotho. Pflugers Arch 2010;**459**: 333-43.

34. Kurosu H, Yamamoto M, Clark JD, Pastor JV, Nandi A, Gurnani P, McGuinness OP, Chikuda H, Yamaguchi M, Kawaguchi H, Shimomura I, Takayama Y, Herz J, Kahn CR, Rosenblatt KP, Kuro-o M. Suppression of aging in mice by the hormone Klotho. Science 2005;**309**: 1829-33.
35. Yamamoto M, Clark JD, Pastor JV, Gurnani P, Nandi A, Kurosu H, Miyoshi M, Ogawa Y, Castrillon DH, Rosenblatt KP, Kuro-o M. Regulation of oxidative stress by the anti-aging hormone klotho. J Biol Chem 2005;**280**: 38029-34.
36. Mateu-de Antonio J. New predictive equations for serum ionized calcium in hospitalized patients. Medical Principles and Practice 2016;**25**: 219-26.
37. Elsayed ME, Sharif MU, Stack AG. Chapter Four - Transferrin Saturation: A Body Iron Biomarker. In: Makowski GS, ed. Advances in Clinical Chemistry: Elsevier, 2016:71-97.
38. Benjamini Y, Hochberg Y. Controlling the false discovery rate: a practical and powerful approach to multiple testing. Journal of the Royal statistical society: series B (Methodological) 1995;**57**: 289-300.
39. Ringle C, Da Silva D, Bido D. Structural equation modeling with the SmartPLS. Bido, D., da Silva, D., & Ringle, C.(2014). Structural Equation Modeling with the Smartpls. Brazilian Journal Of Marketing 2015;**13**.
40. Al-Hakeim HK, Najm AH, Al-Dujaili AH, Maes M. Major Depression in Children with Transfusion-Dependent Thalassemia Is Strongly Associated with the Combined Effects of Blood Transfusion Rate, Iron Overload, and Increased Pro-inflammatory Cytokines. Neurotoxicity research 2020;**38**: 228-41.
41. Xu Y, Peng H, Ke B. α-klotho and anemia in patients with chronic kidney disease patients: A new perspective. Experimental and therapeutic medicine 2017;**14**: 5691-5.
42. Xu Y, Peng H, Ke B. α-klotho and anemia in patients with chronic kidney disease patients: A new perspective. Experimental and Therapeutic Medicine 2017;**14**: 5691-5.
43. Saito K, Ishizaka N, Mitani H, Ohno M, Nagai R. Iron chelation and a free radical scavenger suppress angiotensin II-induced downregulation of klotho, an anti-aging gene, in rat. FEBS Lett 2003;**551**: 58-62.
44. Al-Hakeim HK, Najm AH, Al-Dujaili AH, Maes M. Major Depression in Children with Transfusion-Dependent Thalassemia Is Strongly Associated with the Combined Effects of Blood Transfusion Rate, Iron Overload, and Increased Pro-inflammatory Cytokines. Neurotox Res 2020;**38**: 228-41.
45. Su X-M, Yang W. α-Klotho is an acute phase protein and altered by restraint stress in mice. International journal of clinical and experimental pathology 2014;**7**: 5922.
46. Yamamoto M, Clark JD, Pastor JV, Gurnani P, Nandi A, Kurosu H, Miyoshi M, Ogawa Y, Castrillon DH, Rosenblatt KP, Kuro-o M. Regulation of oxidative stress by the anti-aging hormone klotho. The Journal of biological chemistry 2005;**280**: 38029-34.
47. Ravikumar P, Ye J, Zhang J, Pinch SN, Hu MC, Kuro-o M, Hsia CC, Moe OW. α-Klotho protects against oxidative damage in pulmonary epithelia. American Journal of Physiology-Lung Cellular and Molecular Physiology 2014;**307**: L566-L75.
48. Sun S, Cheng B, Sun P-G, Wu X-H, Wu Q-Q, He P. RTEF-1 protects against oxidative damage induced by H2O2 in human umbilical vein endothelial cells through Klotho activation. Experimental Biology and Medicine 2015;**240**: 1606-13.
49. Wang Y, Kuro-o M, Sun Z. Klotho gene delivery suppresses Nox2 expression and attenuates oxidative stress in rat aortic smooth muscle cells via the cAMP-PKA pathway. Aging Cell 2012;**11**: 410-7.
50. Izbeki F, Asuzu DT, Lorincz A, Bardsley MR, Popko LN, Choi KM, Young DL, Hayashi Y, Linden DR, Kuro-o M, Farrugia G, Ordog T. Loss of Kitlow progenitors, reduced stem cell

factor and high oxidative stress underlie gastric dysfunction in progeric mice. J Physiol 2010;**588**: 3101-17.
51. Vadakke Madathil S, Coe LM, Casu C, Sitara D. Klotho deficiency disrupts hematopoietic stem cell development and erythropoiesis. Am J Pathol 2014;**184**: 827-41.
52. Kuro-o M. Klotho as a regulator of oxidative stress and senescence. Biological chemistry 2008;**389**: 233-41.
53. Hosokawa K, Arai F, Yoshihara H, Nakamura Y, Gomei Y, Iwasaki H, Miyamoto K, Shima H, Ito K, Suda T. Function of oxidative stress in the regulation of hematopoietic stem cell-niche interaction. Biochemical and biophysical research communications 2007;**363**: 578-83.
54. Baldan A, Giusti A, Bosi C, Malaventura C, Musso M, Forni GL, Volpato S, Zuliani G, Borgna-Pignatti C. Klotho, a new marker for osteoporosis and muscle strength in β-thalassemia major. Blood Cells Mol Dis 2015;**55**: 396-401.
55. Nabeshima Y-i, Imura H. α-Klotho: a regulator that integrates calcium homeostasis. American journal of nephrology 2008;**28**: 455-64.
56. Nabeshima Y-i. Discovery of α-Klotho unveiled new insights into calcium and phosphate homeostasis. Proceedings of the Japan Academy, Series B 2009;**85**: 125-41.
57. Nabeshima Y. The discovery of alpha-Klotho and FGF23 unveiled new insight into calcium and phosphate homeostasis. Cell Mol Life Sci 2008;**65**: 3218-30.
58. Nijenhuis T. Hoenderop JG, and Bindels RJ. TRPV5 and TRPV6 in Ca 2005;**2**: 181-92.
59. Lu P, Boros S, Chang Q, Bindels RJ, Hoenderop JG. The β-glucuronidase klotho exclusively activates the epithelial Ca2+ channels TRPV5 and TRPV6. Nephrology Dialysis Transplantation 2008;**23**: 3397-402.
60. Drüeke TB. Klotho, FGF23, and FGF receptors in chronic kidney disease: a yin-yang situation? Kidney Int 2010;**78**: 1057-60.
61. Kempe DS, Ackermann TF, Fischer SS, Koka S, Boini KM, Mahmud H, Föller M, Rosenblatt KP, Kuro OM, Lang F. Accelerated suicidal erythrocyte death in Klotho-deficient mice. Pflugers Arch 2009;**458**: 503-12.
62. Hu MC, Shi M, Zhang J, Quiñones H, Griffith C, Kuro-o M, Moe OW. Klotho deficiency causes vascular calcification in chronic kidney disease. J Am Soc Nephrol 2011;**22**: 124-36.
63. Lim K, Lu T-S, Molostvov G, Lee C, Lam F, Zehnder D, Hsiao L-LJC. Vascular Klotho deficiency potentiates the development of human artery calcification and mediates resistance to fibroblast growth factor 23 2012;**125**: 2243-55.
64. Riancho JA, Valero C, Hernández JL, Ortiz F, Zarrabeitia A, Alonso MA, Pena N, Pascual MA, González-Macías J, Zarrabeitia MTJB. Association of the F352V variant of the Klotho gene with bone mineral density 2007;**8**: 121-7.
65. Mirhosseini NZ, Shahar S, Ghayour-Mobarhan M, Banihashem A, Kamaruddin NA, Hatef MR, Esmaili HA. Bone-related complications of transfusion-dependent beta thalassemia among children and adolescents. Journal of bone and mineral metabolism 2013;**31**: 468-76.
66. Sultan S, Irfan SM, Ahmed SI. Biochemical Markers of Bone Turnover in Patients with β-Thalassemia Major: A Single Center Study from Southern Pakistan. Advances in Hematology 2016;**2016**: 5437609.
67. Hu MC, Kuro-o M, Moe OW. Renal and extrarenal actions of Klotho Seminars in nephrology: Elsevier, 2013:118-29.
68. Hu MC, Kuro-o M, Moe OW. Secreted klotho and chronic kidney disease Endocrine FGFs and Klothos: Springer, 2012:126-57.

69. Hu MC, Kuro-o M, Moe OW. The emerging role of Klotho in clinical nephrology. Nephrology Dialysis Transplantation 2012;**27**: 2650-7.
70. Yamada S, Giachelli CM. Vascular calcification in CKD-MBD: Roles for phosphate, FGF23, and Klotho. Bone 2017;**100**: 87-93.
71. Villanueva LS, González CS, Tomero JAS, Aguilera A, Junco EO. Bone mineral disorder in chronic kidney disease: Klotho and FGF23; cardiovascular implications. Nefrología (English Edition) 2016;**36**: 368-75.
72. Kuro-o M. The FGF23 and Klotho system beyond mineral metabolism. Clinical and experimental nephrology 2017;**21**: 64-9.
73. Takenaka T, Inoue T, Miyazaki T, Hayashi M, Suzuki H. Xeno-klotho inhibits parathyroid hormone signaling. Journal of Bone and Mineral Research 2016;**31**: 455-62.
74. Pirinççioğlu AG, Gökalp D, Söker M. Parathyroid functions in thalassemia major patients. Ann Clin Endocrinol Metab 2017;**1**: 015-9.
75. David V, Martin A, Isakova T, Spaulding C, Qi L, Ramirez V, Zumbrennen-Bullough KB, Sun CC, Lin HY, Babitt JL. Inflammation and functional iron deficiency regulate fibroblast growth factor 23 production. Kidney international 2016;**89**: 135-46.
76. Tharwat RJ, Balilah S, Habib HM, Mahmoud NH, Beek FS, Almadani FK, Elmaghraby SA, Al-Loqmani DD, Al-Mahdi HA. Ferritin and Vitamin D levels and its relation to bone diseases in thalassemic adults: A hospital-based retrospective cohort study. Journal of Applied Hematology 2019;**10**: 15.
77. Forster RE, Jurutka PW, Hsieh J-C, Haussler CA, Lowmiller CL, Kaneko I, Haussler MR, Kerr Whitfield G. Vitamin D receptor controls expression of the anti-aging klotho gene in mouse and human renal cells. Biochemical and biophysical research communications 2011;**414**: 557-62.
78. Lips P. Vitamin D physiology. Prog Biophys Mol Biol 2006;**92**: 4-8.
79. Kuro-o M. Klotho, phosphate and FGF-23 in ageing and disturbed mineral metabolism. Nat Rev Nephrol 2013;**9**: 650-60.
80. Alesutan I, Feger M, Pakladok T, Mia S, Ahmed MS, Voelkl J, Lang F. 25-Hydroxyvitamin D3 1-α-hydroxylase-dependent stimulation of renal klotho expression by spironolactone. Kidney Blood Press Res 2013;**37**: 475-87.
81. Bian A, Xing C, Hu MC. Alpha Klotho and phosphate homeostasis. J Endocrinol Invest 2014;**37**: 1121-6.
82. Cheddani L, Leblanc T, Silve C, Tabibzadeh N, Prié D, Haymann J-P, Péraldi M-N, Daudon M, Meria P, Letavernier E. Iron Chelation Resulting in Renal Phosphate Wasting. Kidney international reports 2018;**3**: 1.

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**Table 1.** Sociodemographic and biomarkers data in children with transfusion-dependent thalassemia (TDT) with (TDT+Klotho<median) or without (TDT+Klotho>median) lowered α-Klotho and in healthy control children (HCC).

| Variables | HCC [A] N=30 | TDT+Klotho > median [B] (n=30) | TDT+Klotho < median [C] (n=30) | $F/\chi^2$ | df | p |
|---|---|---|---|---|---|---|
| Age (years) | 7.13(2.49) | 7.83(3.50) | 8.07(2.79) | 0.81 | 2/87 | 0.448 |
| Sex (Female/Male) | 13/17 | 18/12 | 13/17 | 2.22 | 2 | 0.329 |
| Residency (Rural / Urban) | 4/26 | 11/19 | 5/25 | 5.53 | 2 | 0.063 |
| Number of blood transfusions | - | 93.00(66.40) | 102.00(51.98) | 0.34 | 1/57 | 0.564 |
| Iron (µM) | 15.24(3.43) [B,C] | 44.32(9.08) [A] | 42.29(6.79) [A] | 169.06 | 2/87 | <0.001 |
| Transferrin saturation % | 26.66(9.63) [B,C] | 85.82(12.08) [A,C] | 80.06(10.74) [A,B] | 270.37 | 2/87 | <0.001 |
| Ferritin (ng/ml) | 153.43(44.21) [B,C] | 3214.32(1488.48) [A] | 3381.98(2159.44) [A] | 43.21 | 2/87 | <0.001 |
| Iron Overload Index (z scores) | -1.36(0.31) [B,C] | 0.72(0.21) [A] | 0.64(0.22) [A] | 655.21 | 2/87 | <0.001 |
| Albumin (g/dL) | 3.91(0.36) [B,C] | 4.31(0.56) [A] | 4.47(0.61) [A] | 9.03 | 2/87 | <0.001 |
| Total Calcium (mM) | 2.26(0.21) [C] | 2.20(0.24) [C] | 1.99(0.22) [A, B] | 12.39 | 2/87 | <0.001 |
| Ionized Calcium (mM) | 1.21(0.06) [C] | 1.18(0.06) [C] | 1.12(0.07) [A, B] | 16.00 | 2/87 | <0.001 |
| Phosphate (mM) | 1.54(0.31) | 1.60(0.29) | 1.59(0.33) | 0.31 | 2/87 | 0.738 |
| α-Klotho (pg/ml) | 512.78(313.28) [C] | 511.45(167.13) [C] | 174.41(77.83) [A, B] | 25.89 | 2/87 | <0.001 |
| Vitamin D3 (ng/ml) | 8.85(2.38) [B,C] | 7.15(1.34) [A] | 6.91(1.37) [A] | 10.85 | 2/87 | <0.001 |
| Parathyroid hormone (pg/ml) | 111.65(63.82) [B,C] | 240.03(98.69) [A] | 211.71(100.12) [A] | 17.18 | 2/87 | <0.001 |
| FGFR2 (ng/ml) | 4.08(2.67) [B,C] | 7.72(4.31) [A] | 9.11(4.12) [A] | 14.24 | 2/87 | <0.001 |
| Calcyphosin (mM) | 2.45(1.31) [B,C] | 9.59(3.92) [A] | 9.26(5.91) [A] | 28.13 | 2/87 | <0.001 |
| Red blood cells ($10^6$/µl) | 4.49(0.62) [B,C] | 3.69(0.57) [A] | 3.86(0.57) [A] | 15.54 | 2/87 | <0.001 |
| Hemoglobin (g/dl) | 14.11(1.41) [B,C] | 7.67(1.58) [A] | 8.18(1.11) [A] | 201.91 | 2/87 | <0.001 |

Results are shown as mean (SD). [A, B, C]: Pairwise comparison among group mean differences.
Iron overload index: computed as z iron + z TS% + z ferritin, FGFR2: Fibroblast growth factor receptor 2.

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**Table 2**. Correlation matrix between α-Klotho, number of blood transfusions, iron overload and calcium-associated biomarkers.

| Biomarkers | α-Klotho | Number of blood transfusions | Iron overload |
|---|---|---|---|
| Total Calcium | 0.555** | -0.297** | -0271** |
| Ionized Calcium | 0.552** | -0.365** | -0.322** |
| Phosphate | 0.059 | 0.094 | 0.078 |
| Vitamin D3 | 0.068 | -0.381** | -0.421** |
| Parathyroid hormone | -0.219* | 0.399** | 0.480** |
| FGFR2 | -0.180 | 0.422** | 0.499** |
| Calcyphosin | -0.176 | 0.510** | 0.591** |
| Hemoglobin | 0.228* | -0.802** | -0.872** |
| Red blood cell number | 0.076 | -0.480** | -0.476** |
| Iron | -0.301** | 0.789** | 0.979** |
| Transferrin saturation % | -0.265* | 0.788** | 0.976** |
| Ferritin | -0.285** | 0.802** | 0.953** |
| Number of blood transfusions | -0.269* | - | 0.818** |
| Iron overload index | -0.293** | 0.818** | - |

All n=90, *p<0.05, **p<0.01
FGFR2: Fibroblast growth factor receptor 2, Index of iron overload index: computed as z iron + z transferrin saturation % + z ferritin).

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**Table 3**. Multiple regression analysis with total or ionized calcium levels as dependent variables.

| Dependent variables | Explanatory variables | β | t | p | $F_{model}$ | df | p | $R^2$ |
|---|---|---|---|---|---|---|---|---|
| **#1. Total Calcium** | **Model** | | | | 19.23 | 3/86 | <0.001 | 0.401 |
| | α-Klotho | 0.510 | 6.02 | <0.001 | | | | |
| | Vitamin D3 | 0.199 | 2.24 | 0.027 | | | | |
| | Calcyphosin | 0.179 | -2.00 | 0.049 | | | | |
| **#2. Ionized Calcium** | **Model** | | | | 21.18 | 3/86 | <0.001 | 0.425 |
| | α-Klotho | 0.502 | 6.05 | <0.001 | | | | |
| | Vitamin D3 | 0.236 | 2.72 | 0.008 | | | | |
| | Calcyphosin | 0.191 | -2.18 | 0.032 | | | | |
| **#3. Total Calcium in controls** | **Model** | | | | 4.91 | 1/28 | 0.035 | 0.149 |
| | Albumin | 0.386 | 2.22 | 0.035 | | | | |
| **#4. Total Calcium in TDT patients** | **Model** | | | | 24.05 | 2/57 | <0.001 | 0.381 |
| | α-Klotho | 0.551 | 5.50 | <0.001 | | | | |
| | Vitamin D3 | 0.285 | 2.85 | 0.006 | | | | |

TDT: transfusion-dependent thalassemia

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**Figure 1**. Box plot of α-Klotho values in children with transfusion-dependent thalassemia (TDT) and healthy control children (HCC)

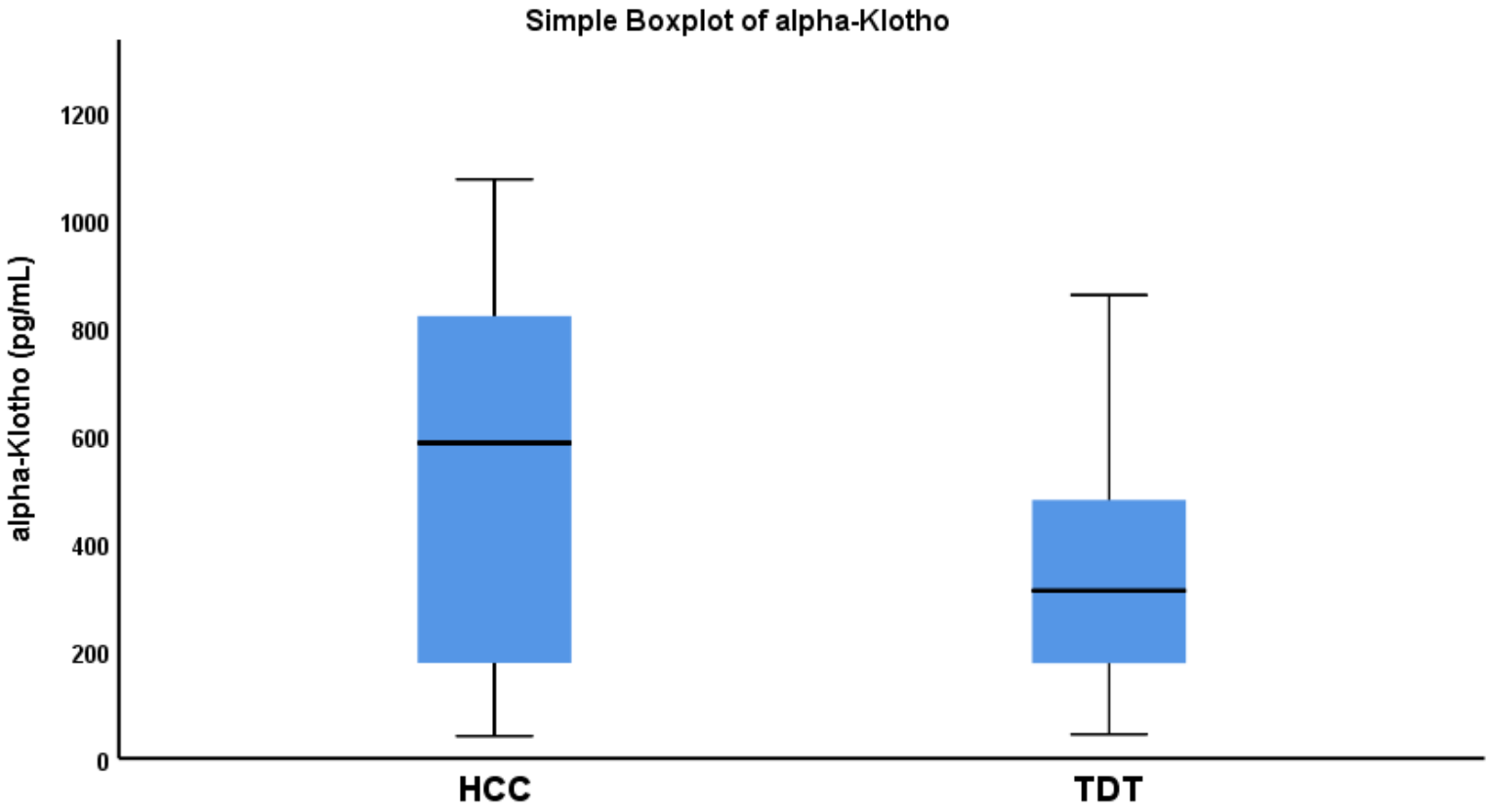

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**Figure 2**. Partial regression of total calcium on α-Klotho in the total sample of patients and controls after adjusting for the effects of vitamin D3 and calcyphosin.

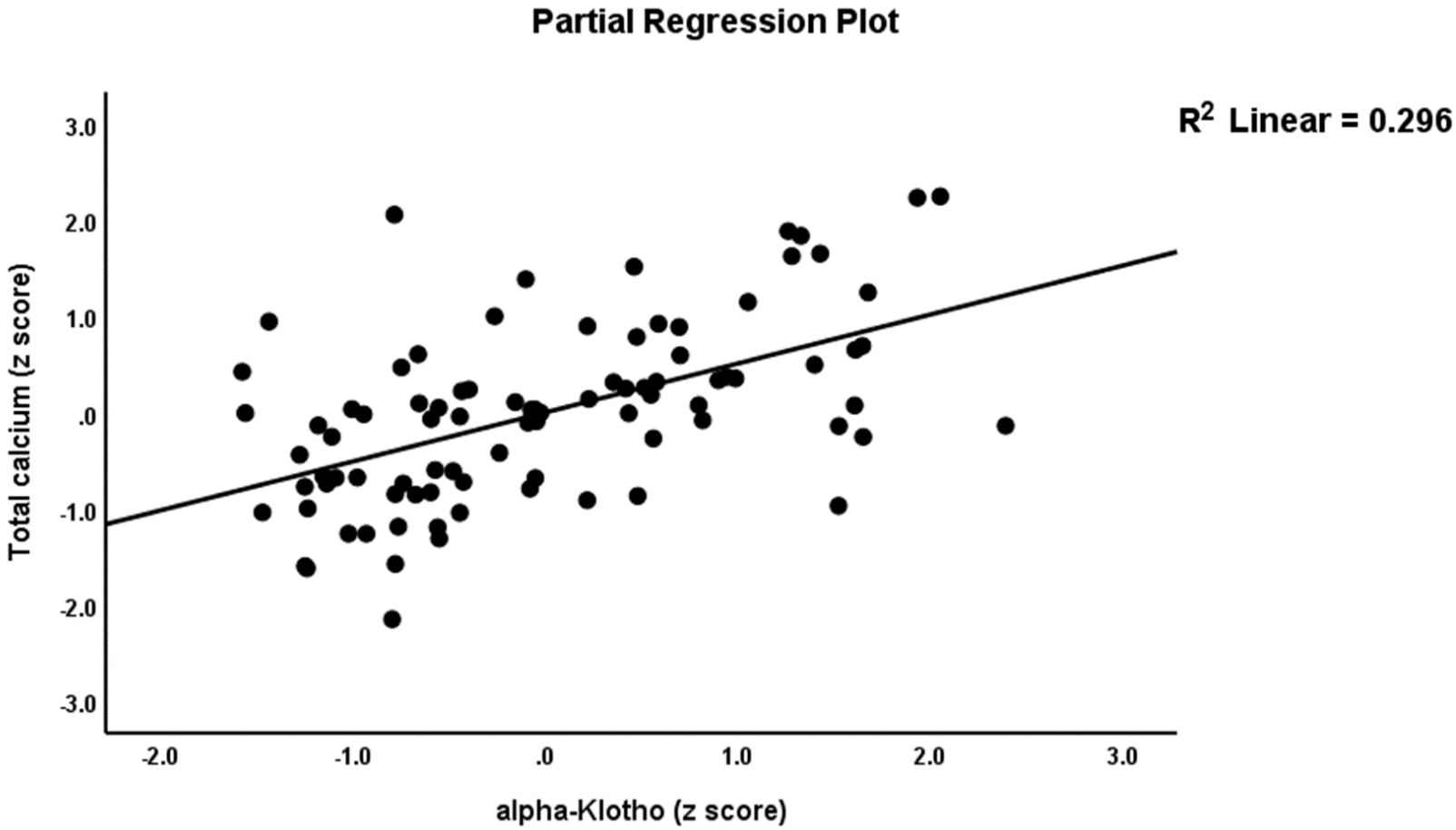

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**Figure 3**. Partial regression of total calcium on α-Klotho in patients with transfusion-dependent thalassemia after adjusting for the effects of vitamin D3 and calcyphosin.

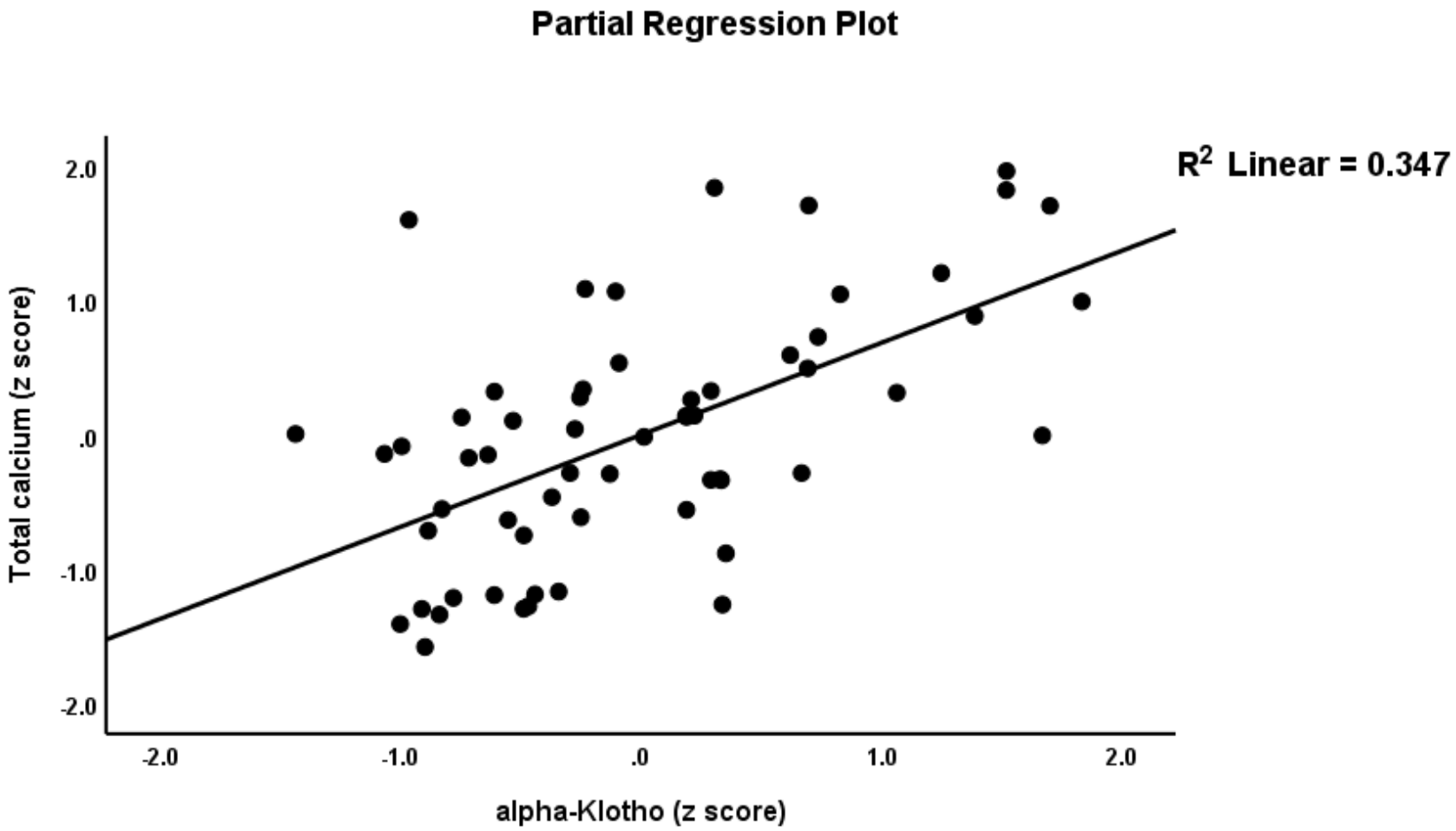

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**Figure 4**. Results of Partial Least Squares (PLS) path analysis. Total calcium (output variable) and α-Klotho, calcyphosin, vitamin D3, parathyroid hormone (PTH), Fibroblast Growth Factor Receptor 2 (FGFR2) (input variables) were entered as single indicators, while iron overload was constructed as a latent vector extracted from iron (Fe), ferritin and transferrin saturation percentage (TS%). A complete PLS path modelling on 5000 samples was conducted. Shown are path coefficients with exact p-values for the inner model and loadings with p-values for the outer model. The figures in the blue circles denote the explained variance.

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