SUBMITTED 23 JUL 24
REVISION REQ. 9 SEPT 24; REVISION RECD. 22 SEPT 24
ACCEPTED 9 OCT 24
ONLINE-FIRST: OCTOBER 2024
DOI: https://doi.org/10.18295/squmj.10.2024.054

Vitamin D Status and Oxidative Stress in Children with Sickle Cell Anaemia in Sagamu, Nigeria

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Abstract

Objectives: Sickle cell anaemia (SCA) is characterized by nutritional deficiencies and oxidative stress. Vitamin D possesses antioxidant properties but its role in SCA in sub-Saharan Africa has not been fully understood. The relationship between Vitamin D status, oxidative stress and antioxidants status in children with SCA was investigated for its possible role in reducing complications arising from oxidative stress in SCA. ***Methods:*** Case-control study involving 100 HbS genotype and 100 HbA genotype children (control) of comparable age (5-12 years). Baseline characteristics were obtained and serum vitamin D, calcium, CAT, SOD, GPX, GST and XO levels were quantified by standard laboratory methods. ***Results:*** Serum levels of vitamin D, calcium, CAT, SOD, GPX and GST were significantly lower in SCA group compared to the control, while the XO level was significantly higher in the HbS group compared with control. There was positive correlation between vitamin D, CAT and SOD (r = 0.821, 0.869), weak positive association between vitamin D and Ca (r = 0.545) and no significant relationship between vitamin D and other

measurands in the SCA group. ***Conclusion:*** Sufficient vitamin D status might impact positively on the antioxidant status in SCA individuals thereby reducing associated complications.
Keywords: Sickle Cell Anemia; Vitamin D Status; Oxidative Stress; Antioxidant Status; Vaso-Occlusive Crisis.

Advances in Knowlegde

-This study emphasizes that sufficient vitamin D levels in children with sickle cell anaemia might be protective against oxidative stress related complications.

Application to Patient Care

-This study shows that there is a positive association between vitamin D levels and measured enzymatic antioxidants in children with sickle cell anaemia.

Introduction

Sickle cell anaemia (SCA) is an inherited chromosomal disorder caused by the substitution of valine for glutamic acid in the 6th position of the adult haemoglobin ß-globin chain resulting in the generation of mutated haemoglobin; haemoglobin S genotype (HbS) which has the propensity to polymerize under conditions of low oxygen saturation, acidosis and dehydration.[1] Repeated cycles of polymerization of the HbS lead to increase mechanical fragility and later cause irreversible damage to red blood cells (RBC) deformability. Loss of RBCs fragility can progress to vaso-occlusion and endothelial injury with concurrent haemolysis, systemic inflammatory response and oxidative stress.[1]

Africa has the highest prevalence of SCA with high mortality and morbidity rate, though it is also common in many part of the world including Asia, America and Europe. Approximately 20% of African general population is carrier of the mutated haemoglobin gene. Nigeria has about 33% of the global burden of SCA with 100,000-150,000 newborns living with the disorder year in year out. Report from a meta-analysis showed that 50-80% of children with SCA living in Africa die before the age of five.[2]

Nutritional deficiencies are common findings in individuals with SCA probably due to reduced nutrient absorption or decreased desire for food resulting from repeated illness. Other possible

causes include high basal metabolic rate to compensate for the chronic haemolysis in them, renal impairment and decreased binding protein levels in the inflammatory state of the disorder.[3]

Previous studies have reported that the oxidative stress seen in SCD is caused by the chronic redox imbalance within the RBCs mitochondria which is often associated with constant generation of reactive oxygen species (ROS).[1]

Reactive oxygen species (ROS) also referred to as free radicals, are produced by a variety of cellular processes, including: enzymatic reactions, mitochondrial electron transport chain, exposure to environmental pollutants, toxic metals, and certain medications like cyclosporine[4]. These ROS include nitric oxide (•NO), alkoxy (-OR), peroxyl (ROO·), hydroxyl (·OH), hydrogen peroxide (H_2O_2), and superoxide ($O2^{\bullet-}$). They are highly reactive because of their unpaired electrons and their generation gets multiplied many folds during pathological complications. Accumulation of these ROS often results in lipid peroxidation, DNA fragmentation, cell death, DNA damage, protein modification, and membrane damage. To reduce or prevent free radical-directed oxidative damage, the human body makes use of antioxidants like glutathione peroxidase (GPX), catalase (CAT), and superoxide dismutase (SOD) for free radical scavenging and metal chelating in order to reduce ROS load[5]. However, when there is an imbalance between the antioxidants and ROS, oxidative stress arises. As mentioned earlier oxidative stress is also a feature of SCA which develops as a result of the imbalance between the levels of generated free radicals [ROS and reactive nitrogen species (RNS)] and antioxidants activity or concentration in the RBCs. There are few proposed possible mechanisms by which free radicals are constantly generated in SCA individuals. Constant generation of these free radicals in SCA individual might be from one of the possible mechanisms which include the auto-oxidation process of deformed HbS, continuous heme iron release post- haemolysis and decreased circulating nitric oxide (NO) level. Another proposed source of ROS is the production of reduced NADPH oxidase, altered regulation of metabolism of xanthine enzymes, chronic pro-inflammatory state and increased expression of oxidative enzymes.[6] Thus chronic haemolysis is often complicated with vaso-occlusion, severe pain crises, multiple organ ischaemia, and other systemic complications seen in SCD. Also the resultant oxidative stress could worsen the complications of SCA including hypoxia, inflammation, infection, dehydration, vaso-occlusive and haemolytic crisis.[7]

It is necessary to have enough antioxidants to protect against the deleterious effects of oxidative stress in SCA individuals, and these antioxidants include non-enzymatic and enzymatic substances.[6] Non-enzymatic antioxidants include vitamins C, E, B2,A, glutathione, and microelements such as zinc, selenium etc while enzymatic antioxidants include superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX), glutathione S-transferase (GST), glutathione reductase, glutaredoxin (Grx), thioredoxin/thioredoxin reductase system and peroxiredoxins (Prx) etc.[6]

Research on vitamin D has increased in the recent times because of its possible numerous roles in many disease conditions. Recently, there have been reports that vitamin D has high antioxidant and anti-inflammatory properties even among type 2 diabetic patients, or following complicated pregnancies and ageing.[8] Vitamin D has been shown to play its antioxidant role by facilitating balanced mitochondrial activities and prevention of oxidative stress-related protein oxidation, lipid peroxidation and DNA damage.[9] The association between hypovitaminosis D and musculoskeletal disorders in SCA is well known, the relationship between vitamin D deficiency and worsening oxidative stress and haemolysis in SCA has also been suggested.[9]

As submitted earlier there are reports on vitamin D status in SCA, vitamin D and inflammatory markers in SCA but in this present study the association between Vitamin D status and some enzymatic antioxidants and marker of oxidative stress in children with SCA was investigated for its possible role in reducing complications arising from oxidative stress in SCA.

METHODS

Study design and study population.

This is a case-control study and ethical approval was obtained from the Olabisi Onabanjo University Teaching Hospital Health Research Ethics Committee (OOUTH/HREC).This present study involved 100 (hundred) children aged 5-12 years with HbS genotype in their steady state (i.e. without active vaso-occlusive crisis) and 100 (hundred) apparently healthy children with hemoglobin A genotype (HbA).The participants were selected from the children attending the Paediatric Haematology clinic and the Well-child clinic of the Hospital using a non-random

convenient sampling technique. The study was conducted within a period of nine (9) months between November 2022 and July 2023.

Inclusion/Exclusion Criteria

Children with HbS genotype in stable state (i.e without active vaso-occlusive crisis) and apparently healthy HbA genotype children were included in the study but those on vitamin D containing supplements in the previous 6 (six) months were excluded. Vitamin D supplements can significantly impact serum vitamin D levels in the participants making it difficult to interpret results and potentially masking the relationship with other variables. Those with clinical history or on medication for any known cardiovascular diseases (CVD), chronic kidney diseases (CKD), or any known endocrinopathies were excluded from the study because of their influence on oxidative stress and vitamin D metabolism.

Sample size

The minimum sample size for the study was calculated with the formula proposed by Bolarinwa.[10] With a $Z\alpha = 1.96$, $Z\beta=1.28$, prevalence of vitamin D deficiency in HbS and HbA of 20 % and 5% respectively, margin of error of 5%, and a confidence level of 95%, the minimum sample size was 34 for each group.

Data collection

Inform consent form was signed and received from each of participants' parents or guidance after educating them on the benefits and relevance of the study. The demographic characteristics and clinical measurements were obtained using a structured interviewer-administered questionnaire. Clinical measurements included blood pressure, weight, height and body mass index (Kg/m^2) which was calculated.

Blood specimen collection

Ten milliliters (10mls) of venous blood was withdrawn from each participant and dispensed into Gel clot activator tube (SST) and were allowed 30 minutes for clot retraction. Then each specimen tubes were centrifuged at 3000g for 15 minutes using Uniscope Laboratory centrifuge, model SM 112 (Surgifriend Medicals, England) and the obtained serum decanted into their respective well

labeled plain tubes for the estimation of serum vitamin D, calcium, catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPX), glutathione S transferase (GST) and xanthine oxidase (XO) levels. The serum samples were stored at -20°C using the freezer compartment of the SCANFROST fridge/freezer model SFVFFF 350 prior to analysis, which was done within 3 months of specimen collection.

Laboratory investigations

The HbS and HbA genotype was determined by haemoglobin electrophoresis using cellulose acetate in alkaline buffer, on digital Axiom Electrophoresis machine.[11] Serum vitamin D level was measured by Enzyme Linked Immunosorbent Assay (ELISA) on Stat Fax 4000 ELISA reader as described by Wallace et al.,[12] using Calbiotech Vitamin D ELISA kit. Serum calcium was assayed by Arsenazo III colorimetric method on Spectrum Lab spectrophotometer as described by Young et al., [13] Glutathione Peroxidase activity was evaluated by colorimetric method as described by Wendel et al.,[14] Superoxide Dismutase activity was determined by spectrophotometric method as described by Kuthan et al.,[15] Xanthine oxidase was determined by catalytic spectrophotometric method, conjugating xanthine oxidase with horseradish peroxidase as described by Li et al.,[16] Glutathione S Transferase was assessed by spectrophotometric method as described by Habdous et al.,[17] Serum Catalase activity was assessed by colorimetric method as described by Sinha et al.,[18] The colorimetric measurements were done on Spectrum Lab spectrophotometer. Vitamin D was classified based on the ELISA kit manufacturers' manual as follows; levels <20ng/ml, 21ng/ml-29ng/ml, 30ng/ml-150ng/ml, >150ng/ml were considered as deficiency, insufficiency, sufficiency and intoxication respectively.[12]

Data analysis

Data obtained was subjected to statistical analysis using SPSS version 25.0. The results obtained were grouped and expressed as mean ± Standard Deviation (SD). Students T-test was used to compare variables between the two groups. Correlation between the analytes and vitamin D was determined using Pearson correlation. Significant difference was set at $p<0.05$.

RESULTS

The results are expressed as mean ± SD. The mean of the weight, height and body mass index (BMI) were significantly lower ($p<0.05$) in the SCA children (HbS genotype) than in the HbA genotype children, while the mean of the age, systolic blood pressure (SBP) and diastolic blood pressure (DBP) were not statistically different as shown in **Table 1**.

The mean concentrations/activities of the serum vitamin D, calcium, CAT, SOD, GPX and GST were significantly lower ($p<0.05$) in SCA children compared to the HbA genotype children, while the mean serum activities of XO was significantly higher in the SCA children than in the HbA genotype children ($p<0.05$); **Table 2**.

The correlation between serum vitamin D and other analytes in the SCA children is shown in **Table 3**. Serum vitamin D was positively correlated with calcium, SOD and CAT ($r = 0.545, 0.755, 0.799$respectively). There was no significant relationship between vitamin D and other measurands.

Figures 1, 2 and **3** respectively show the relationship between serum vitamin D and calcium, SOD and CAT ($r = 0.545, 0.755, 0.799$ respectively).

20% of the SCA children are vitamin D deficient (< 20ng/ml), 60% had vitamin D insufficiency (21-29ng/ml) and the remaining 20% are vitamin D sufficient (30-150ng/ml).

5% of the controls (children with HbA genotype) are vitamin D deficient (< 20ng/ml), 15% had vitamin D insufficiency (21-29ng/ml) and the remaining 80% are vitamin D sufficient (30-150ng/ml).

DISCUSSION

It is known that continuous ROS and RNS generation in excess of protective antioxidant leads to oxidative stress which is a major feature of SCA. The oxidative stress often is a result of repeated haemolysis and auto-oxidation of sickled haemoglobin and in turn could worsen haemolysis and other complications in SCA individuals.[6] Understanding the relationship between the levels of

vitamin D, which is now known to possess high antioxidant properties and some markers of oxidative stress, could be beneficial in improving common complications resulting from SCA.

In this present study the finding of significant lower weight in children with SCA compared with those with HbA genotype of comparable age and social status, is similar to the work of Olawale et al., where it is reported that both wasting and stunting were more common in children with SCA in Nigeria.[19] However, these findings contradicts a previous retrospective study by Chawla et al., who reported that nearly 25% of children and adolescents diagnosed with SCA in certain countries including New England are overweight or obese.[20] These discrepancies in the reports between New England, a developed community and a developing community, like Nigeria might be as a result of readily available advanced treatment methods (hydroxyurea, chronic transfusions), early diagnostic techniques, less severity of illness and obesogenic lifestyle in the developed countries. It has also been reported that social class have a modifying influence on growth and nutritional status in SCA, such that the SCA children in the upper social class have a better growth status than those of lower social class even in Nigeria.[21] Thus, the lower weight reported in children with SCA in this present study might be explained by the increase susceptibility to infections, malnutrition due to poor dietary intake and lack of access to balanced diet, higher basal metabolic rate and endocrine dysfunction, common in many under-developed Africa communities.[22]

Though the concentration of serum calcium is maintained within a fairly narrow range, this present study reported low serum vitamin D and calcium among SCA children compared to HbA genotype children; these findings is not surprising because vitamin D is known to be a major factor for the absorption and reabsorption of this bone forming mineral, from the gastrointestinal tract and renal tubules respectively.[1] In order to maximize the effectiveness of calcium absorption, optimal vitamin D levels are required. In the absence of sufficient vitamin D, the body absorbs only 10% to 15% of dietary calcium, however in the presence of adequate vitamin D; the intestinal calcium absorption rises to 30% to 40%.[23] A number of studies have shown the critical importance of vitamin D receptor (VDR) in the tissues for the regulation of calcium and bone metabolism in healthy individuals[24] and as reported in a study by Mobarki et al, a strong positive correlation exists between serum vitamin D and calcium in healthy young adults.[25] In rodent models, vitamin D receptor knockout mice developed “soft bone disease”, which was treated with a rescue diet

containing high calcium and lactose content.[26]This might explain the positive relationship between the serum vitamin D and serum calcium in this present study. Hence, the lower calcium level in SCA children compared to the apparently healthy controls in this study could be as a result of vitamin D insufficiency/deficiency found in the SCA children which also might contribute to their lower BMI (lower weight, short stature). These findings are similar to the report submitted by Adekunle et al.[27] The deficient/insufficient vitamin D levels found in the SCA children in this present study might be as a result of the characteristic high resting metabolic rate present in them which is not matched by adequate nutritional intake due to reduced/decreased appetite associated with recurrent illness common in them. Also, the frequent microinfarctions found in SCA often disrupts the renal system's structure and function, hence the kidneys might produce less 1, 25 dihydroxy vitamin D.[28]

High serum antioxidant levels is essential in protecting against the effect of excess circulating free radicals generated during series of pathological events that is common in SCA. For example, it is known that CAT is responsible for the detoxification of hydrogen peroxide by catalyzing its conversion to water and oxygen, protecting cells such as RBCs from cellular activities generating these free radicals. Findings from this present study show that antioxidants activities such as that of CAT, GPX, GST and SOD were lower in SCA children compared to HbA genotype children, this is in agreement with the work of Engwa et al., where it was found that individuals with HbS vaso-occlusive crisis had low serum concentrations/activities of SOD and CAT.[29] XO is also a known marker of oxidative stress, as it functions by oxidizing hypoxanthine to xanthine and xanthine to uric acid, a process in purine degradation pathway leading to generation of several ROS.[30] In this present study it was found that serum activity of XO in SCA children is higher than that of compared apparently healthy controls, this finding is similar to that of Al-Balushi et al.[31] Thus it is important to note that the increased production of oxidants by high levels of XO may be associated with damage to the endothelium and painful vaso-occlusive crisis characteristic of SCA patients. So, in most cases of SCA as also reported in our study, antioxidant status are often reduced due to continuous sickled haemoglobin auto- oxidation and release of heme iron which enhances the fenton reaction, just to mention few causes.[6] Therefore, the oxidative stress which is characteristic of SCA as a result of the depletion in the circulating antioxidants levels often results in worsening vasculopathy complications seen in SCA individuals.[32]

Vitamin D is recently considered to have antioxidant properties which might be as a result of its influence in enhancing the expression of several genes in the antioxidant defense system and its possible role in suppressing activities of oxidants such as NADPH oxidase in the human body.[33] In this present study it was found that deficient/insufficient vitamin D concentration was positively correlated with low serum levels of CAT and SOD, which is similar to the report of Zakhary et al., a review study in which vitamin D3 supplementation was found to be associated with reduction in ROS generation and improved antioxidant status of type 2 diabetic patients.[34] It is known that SOD helps to mop up excess superoxide anions in the body thereby reducing their concentration in the circulation, thus protecting against structural damage to mitochondria DNA and other proteins. Therefore the positive relationship found between vitamin D deficiency/insufficiency and low SOD activities in this study is not surprising and might be because of the role of vitamin D in enhancing the expression of NRF2 gene which is an important regulator of SOD1 and SOD2 gene expression.[34] Also it was found in this study that vitamin D deficiency/insufficiency positively correlated with the low activities of CAT in SCA which is in agreement with submission of Bhat et al.,[35] this finding is in order because of the possible role of vitamin D in upregulating antioxidants gene expression in human body.[36]

In this study, vitamin D deficiency/insufficiency was observed in 80% of SCA children that participated, and in comparison, only 20% of children with HbA genotype are vitamin D deficient/insufficient, similar to the reports of Yesim et al.[37] Vitamin D deficiency has been found to be prevalent among SCA patients and is often associated with acute and chronic bone pain and bone fracture.[38] Also lower 25(OH) vitamin D levels has been shown to increase pain frequency in SCA children .[39]

Conclusion

The findings of this present study agree with the submission of previous studies that nutritional deficiencies and oxidative stress is a major feature of SCA. Oxidative stress as demonstrated in this study by the elevated XO levels, and the decreased levels of enzyme antioxidants such as CAT, SOD, GPX and GST, could predispose SCA individuals to vascular endothelial damage resulting in complications including painful vaso-occlusive crisis. The strong association between

serum vitamin D, CAT and SOD found in this study, indicate that sufficient vitamin D level might impact positively on the antioxidant status in SCA individuals thereby reducing associated complications. Regular screening of children with SCA for vitamin D deficiency/insufficiency is important. Supplementation with vitamin D analogs and intake of vitamin D rich diets like egg, cheese, milk, and tuna fish should be encouraged in SCA patients. Research investigating how genetic variations in vitamin D and antioxidant metabolism could impact sickle cell disease severity and treatment is worth carrying out.

Authors' Contribution

OOO conceptualized the study, conducted the research and supervised the work. AAS, OOS and SPO collected and analyzed the data. AAS and OOS drafted the manuscript. All authors reviewed and edited the manuscript and approved the final version of the manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

Funding

No funding was received for this study.

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Table 1: Baseline characteristics of children with Sickle cell anaemia (HbS genotype) and HbA genotype (Control)

Parameters	HbS (n=100)	HbA (n=100)	p-value
Age (yrs)	8.94±0.11	9.21 ± 1.05	0.110
Weight (Kg)	21.75±0.45	31.19±0.66	0.001***
Height (m)	1.19 ± 0.01	1.32 ± 0.02	0.003***
BMI (Kg/m^2)	15.36 ± 0.14	17.9 ± 0.56	0.001***
SBP (mmHg)	110.9±0.18	116±0.22	0.340
DBP (mmHg)	88.1±0.24	90.2 ±1.22	0.060

*BMI: Body Mass Index; SBP: Systolic blood pressure, DBP: Diastolic blood pressure; *** p value <0.05*

Table 2: Serum concentrations of vitamin D, calcium and the antioxidants status in children with Sickle cell anaemia (HbS genotype) and HbA genotype (Control)

Parameters	HbS (n = 100)	HbA (n = 100)	p-value
Vit. D (ng/ml)	22.44 ±1.46	51.67 ±1.76	0.001***
Ca (mg/dl)	8.06± 0.65	9.53 ± 0.73	0.001***
CAT (µmol/ml)	72.95 ± 5.4	155.2 ± 3.8	0.001***
SOD (µmol/ml)	55.41 ± 1.5	97.44 ± 1.5	0.001***
GPX (IU/gHB)	91.54 ± 1.8	121.4 ± 0.3	0.001***
GST (U/ml)	4.4±0.29	7.68±0.31	0.001***
XO (IU/L)	169.8 ± 3.2	70.55±5.8	0.001***

*CAT: Catalase, SOD: Superoxide Dismutase, GPX: Glutathione Peroxidase, GST: Glutathione S Transferase, XO: Xanthine Oxidase; *** p value<0.05*

Table 3: Correlation between vitamin D, calcium and measures of antioxidant status in SCA children.

Parameters	r	p value
Vit D vs Ca	0.545	0.022*
Vit D vs SOD	0.755	0.001***
VitD vs CAT	0.799	0.001***
Vit D vs GPX	0.156	0.452
Vit D vs GST	0.251	0.441
Vit D vs XO	- 0.226	0.277

*CAT: Catalase, SOD: Superoxide Dismutase, GPX: Glutathione Peroxidase, GST: Glutathione S Transferase, XO: Xanthine Oxidase; *** p value<0.05*

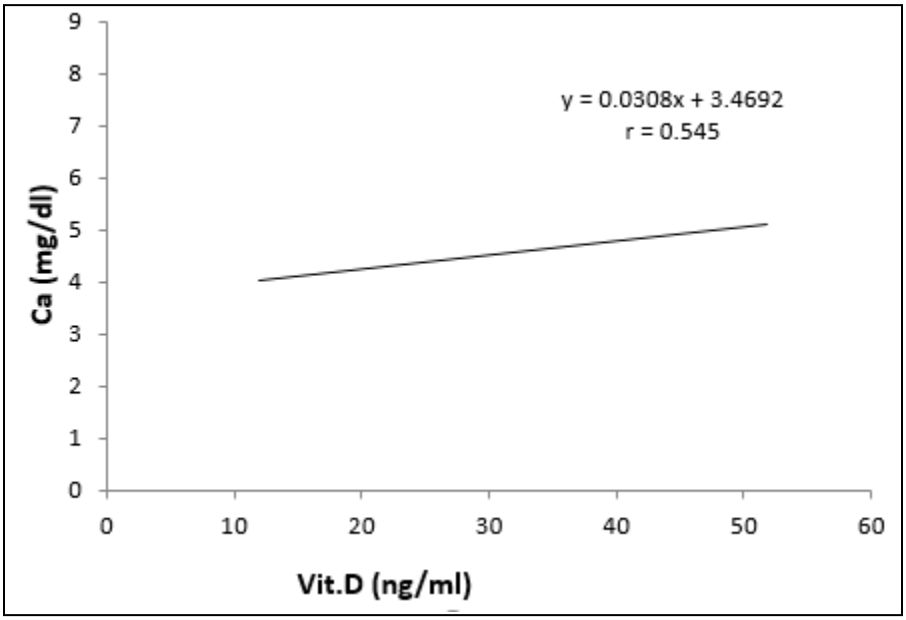
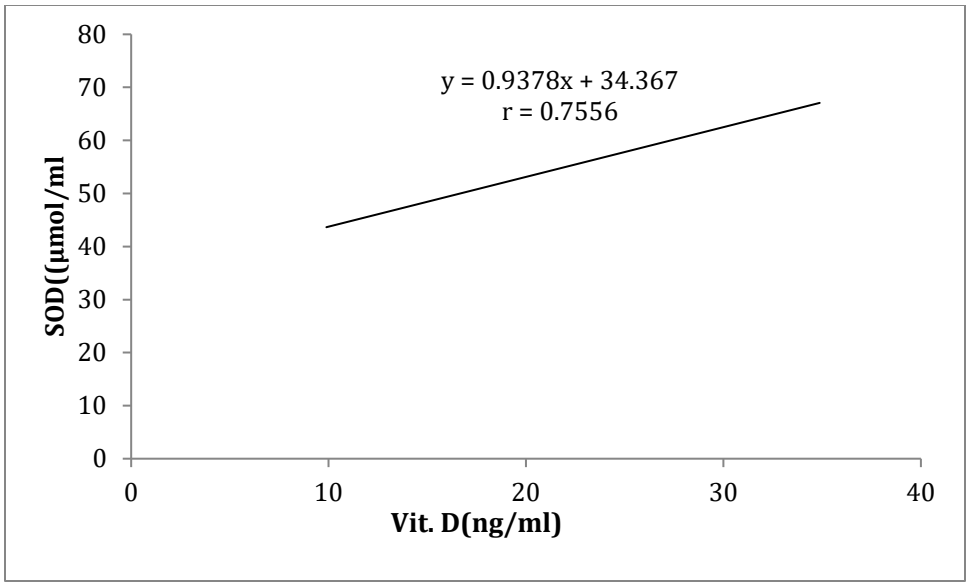


Figure 1: Correlation between serum vitamin D and calcium in SCA children



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495 **Figure 2:** Correlation between serum vitamin D and SOD in SCA children

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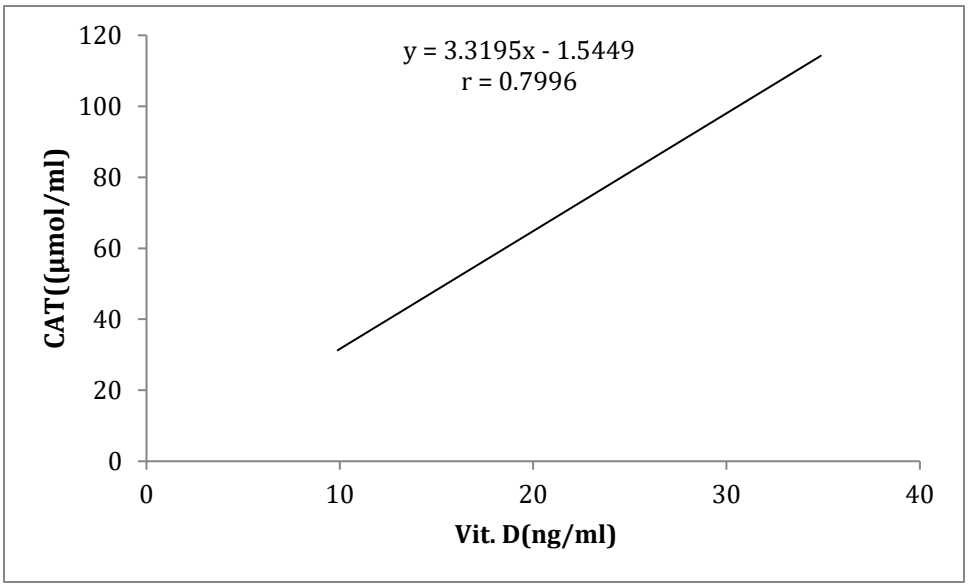

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498 **Figure 3:** Correlation between serum vitamin D and CAT in SCA children