Association of circulating serum free bioavailable and total vitamin D with cathelicidin levels among active TB patients and household contacts

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Abstract

The free hormone hypothesis postulates that the estimation of free circulating 25(OH)D may be a better marker of vitamin D status and is of clinical importance compared to total vitamin D levels because it is the fraction involved in biological activities. Studies have shown that cathelicidin inhibits the growth of *Mycobacterium Tuberculosis* in a vitamin D-dependent manner and therefore adequate vitamin D is required for its expression. The aim of the study was to determine the association between serum-free and bioavailable and total vitamin D with LL-37 levels in ATB patients, LTBI and individuals with no TB infection. This was a cross sectional study and free and bioavailable vitamin D and LL-37 levels were measured. 95 specimens were further selected to estimate total vitamin D levels. The median free and bioavailable vitamin D levels of study participants were 3.8 ng/mL. The median LL-37 levels were 318.8 ng/mL. The mean total vitamin D levels were 18.9 ng/mL. Significantly weak inverse associations were found and vitamin D is involved in the regulation of LL-37 expression and low vitamin D levels can alter this relationship.

Background

Vitamin D deficiency is a prominent risk factor for TB disease worldwide (1–5). Vitamin D can be obtained in two forms, D2 is obtained through diet and D3 is obtained through skin biosynthesis (6). Its main circulating active metabolite 1, 25(OH)D is involved in regulation of antimicrobial activity and therefore important in TB therapy (7). So far, total vitamin D or 25(OH)D has been considered a better index for determining vitamin D status due to its longer half-life (6, 8–11). However, the free hormone hypothesis postulates that the estimation of free circulating 25(OH)D may be a better marker of vitamin D status and is of clinical importance compared to total vitamin D levels because it is the fraction involved in biological activities (10, 12–14). Bioavailable 25(OH)D is used to represent free vitamin D and the 10–15% fraction is loosely bound to albumin (8, 15). About 85–90% of total 25(OH)D is bound to VDBP and 10–15% is loosely bound to albumin and a small fraction remains unbound (13, 16). Free 25(OH)D is increased and readily available to cells when DBP levels are at low concentrations Previous studies report that changes in DBP levels and 25(OH)D binding affinity can lead to higher levels of free 25(OH)D, even in the absence of total vitamin D levels (17, 18). According to the Endocrine Society, total vitamin D status is classified into three groups: <20 ng/mL deficient, 21–29 ng/mL deficient, and > 30 ng/mL optimal; or sufficient amounts (19). In vitro and in vivo studies have shown that LL-37 inhibits the growth of *MTB* in a vitamin D-dependent manner (20, 21). Accordingly, studies have reported that adequate levels of 25(OH)D are required for expression of LL-37(22, 23). According to our systematic review, six studies reported that vitamin D regulates LL-37 expression and that vitamin D deficiency alters this function (24). Because the free fraction of vitamin D, which enters cells to cause biological effects, has not been studied with the LL-37 molecule, we hypothesize that there is no relationship between free and bioavailable vitamin D and total vitamin D with the LL-37 levels among the ATB patients, LTBI and individuals with no TB infection. This study aimed to determine the association between serum-free and

bioavailable and total vitamin D with LL-37 levels in ATB patients, LTBI and individuals with no TB infection.

Results

Social demographic characteristics

A total of 148 participants consisting of 56 newly diagnosed ATB patients, 49 individuals with LTBI and 43 individuals with no TB infection were included in the study. Of these 95 samples 56 ATB patients, 16 LTBI and 21 individuals with no TB infection were further selected according to specimen availability for total vitamin D analysis. The median age of the study participants was 28 (IQR 20.0–35.0) years with majority being females. Both HIV positive and negative individuals were included in the study. Details of the social demographic characteristics and clinical factors are found elsewhere(25, 26).

Serum Free and bioavailable and total vitamin D levels among ATB LTBI and those with no TB infection

The overall median (IQR) of free and bioavailable vitamin D levels of the study participants was 3.8 (1.10.6.20) ng/mL. According to the reference ranges used in this study, 53 (35.8%) participants had < 1.92 ng/mL and the majority 46 (31%) were ATB patients. Eighty-one (54.7%) were between 1.92 and 8.82 ng/mL and those with values >8.82 were 14 (9.5%) participants. The ATB patients had the lowest median free and bioavailable serum vitamin D levels with statistical significance of $p < 0.001$ as shown in Table 1. No statistically significant difference was noted in the free and bioavailable vitamin D between the male and female participants. Statistical significance was observed in free and bioavailable vitamin D levels in HIV-positive and HIV-negative subjects, those with BCG scars and subjects without a scar, and in alcohol users and non-users, Table 1 provides further details. Among age categories, age groups up to 18 years had higher free and bioavailable levels compared to other categories, although no statistical significance was observed. The mean total vitamin D levels were 18.9 ng/mL. Statistically lower total vitamin D levels were found among the ATB patients s shown in Table 2. The details of total vitamin D analysis have previously been reported elsewhere.

Table 1
Showing free and bioavailable, vitamin D median levels among social and clinical factors characteristics

Participant characteristic	Free and bioavailable vitamin D Median(IQR)	*P-value*
Age(years)	4.05(2.50, 5.30)	0.44
18 and below	2.65(1.30, 5.30)	
19–30	2.70(1.20, 6.20)	
31–40	2.65(1.75, 4.05)	
Above 40		
Sex	3.05(1.40, 5.30)	0.97
Female	2.95(1.50,5.30)	
Male		
TB status	5.30(3.20, 6.20)	< 0.001
No TB infection	4.20(2.50, 6.20)	
Latent TB infection	1.30(1.10, 1.80)	
Active TB		
Alcohol consumption	2.50(1.35, 4.35)	0.01
No	5.00(1.80, 6.30)	
Yes		
Smoking	3.20(1.40, 5.30)	0.73
No	2.50(1.40, 5.10)	
Yes		
HIV status	3.40(1.70, 5.50)	< 0.01
Negative	1.45(1.30, 3.20)	
Positive		
BCG scar	1.90(1.30, 5.10)	0.02
No	3.55(1.80, 5.70)	
Yes		

Free vitamin D in ng/mL, p-value is < 0.05

Table 2
Shows the mean free and bioavailable vitamin D levels among TB patients, LTBI, and those with no TB infection

TB status	Frequency (n)	Free and bioavailable (Vitamin D ng/mL) Mean SD	*P* value	Total vitamin D ng/ml Mean SD	*P* value
ATB	56	1.93 (1.8)	*P*< 0.0001	16.61 7.6	p < 0.001
No TB infection	43	5.1 1 (2.3)		21.65 7.1	
LTBI	49	4.69 (2.5)		22.9 9.4	
TOTAL	148	3.76 (2.6)		18.95 8.3	

SD is the standard deviation LTBI = latent TB infection

Correlation of free and bioavailable and total vitamin D levels in ATB, LTBI, and those with no TB infection

An analysis of the relationship between free and bioavailable vitamin D and total vitamin D levels was performed and found a significantly weak positive association, rho 0.22. Figure 1 shows the correlation analysis.

Serum Ll-37 Levels Among Atb Patients, Ltbi And Individuals With No Tb Infection

An analysis of LL-37 levels was performed and the median (IQR) were 318.8 ng/mL (157.9, 547.1). Higher LL-37 levels were found among the ATB the compared LTBI and those with no infection TB groups, p = 0.002 as shown in Fig. 2. Other details of the LL-37 analysis have been reported elsewhere(25).

Median serum concentrations were significantly higher among the ATB patients compared to the LTBI and those with no TB infection

Correlation of LL-37 with free and bioavailable vitamin D levels in ATB patients, LTBI, and those with no TB infection

A correlation of LL-37 with free and bioavailable vitamin D levels between the three groups was performed and a significantly weak negative association was observed Fig. 3 shows the details. When a correlation was performed between LL-37 and free and bioavailable vitamin D levels a significant negative association was observed, r=-0.2, *p* = 0.27.

Correlation of LL-37 and total vitamin D levels in ATB patients, LTBI, and those with no TB infection

A correlation between LL-37 and total vitamin D was performed and overall a statistically significant weak negative association was found as shown in Fig. 4. When the analysis was performed between the two molecules in the group with adequate vitamin D levels, this was a very weak positive result and an insignificant association was observed r = 0.01, $p = 0.98$.

Discussion

The present study found low levels of free and bioavailable vitamin D in TB patients compared to other groups. Similarly, low levels of total vitamin D were found in ATB patients compared to the other groups. High level of LL-37 was found in the ATB patients compared to the LTBI patients and those with no TB infection. These results are comparable to our systematic review, which found low levels of vitamin D and high levels of LL-37 in tuberculosis(24). We performed a correlation between total vitamin D levels with free and bioavailable levels and found a significantly weak positive correlation. This result is comparable to a study that performed the same correlation and found a stronger association than our study (27).

The association performed between free and bioavailable vitamin D levels with LL-37 and that of total vitamin and LL-37 levels had the same significance. We found significantly weak negative associations in both cases. Our result is similar to a recent study that performed the same correlation in pregnant women (10). In addition, another study found the same correlation in postmenopausal women in the American and African American populations, and no difference was found by race (28). According to Naweed et al. (2016) reported the same finding in relation to race for both free and bioavailable and total vitamin D (13). All of these studies concluded that free vitamin D levels are not superior to total vitamin D and may not be a better index of vitamin D status. On the contrary, two studies reported that free vitamin D levels may be a better predictor of vitamin status than total levels based on their association with (29, 30). The study by Bhan et al. found a positive association with vitamin D levels above 30 ng/mL (15). On the contrary, our study found a weak association between the sufficient groups. However, a stronger association was observed in the correlation analysis of free and bioavailable vitamin D with LL-37 levels in the adequate group. This finding is possibly caused by the action of free 1,25-dihydroxyvitamin D, the bioactive molecule that regulates LL-37. Although the free and bioavailable levels may not be a better index of vitamin D status compared to total vitamin D this scenario can suggests that the free fraction of vitamin D may be more efficient in the production of free 1,25-dihydroxyvitamin and therefore better in regulating LL-37 expression compared to total levels. A study by Johnsen et al. (2019) found a stronger correlation between free vitamin D than total vitamin D levels and bone mineral density (31). According to Aloia et al. (2015), reference ranges for free vitamin D levels may not be relevant due to racial differences, and also these levels may depend on vitamin D status rather than hormonal control (28). All of these variable findings necessitate further research in this area in order to unfold substantial insights. To our knowledge, this was the first study to perform an analysis between free and bioavailable vitamin D levels

containing the LL-37 molecule in TB patients. The differences between the male and female free vitamin D levels were not statistically significant although the female had higher levels.

Regarding age, the younger participants had higher free and bioavailable vitamin D levels compared to the other age groups although no statistical significance was noted. As reported earlier the total vitamin D also reported no significance with age in the study groups(26).

According to the free hormone hypothesis, the effective and clinically important fraction of vitamin D is the 10–15% that enter the cells (8). This part may be responsible for vitamin D immunomodulation in numerous disease states, including TB. However, vitamin D bioavailability can be controlled by numerous factors involved in its absorption, transport and metabolism (32). Furthermore, according to Mendel, movement of the hormone into the cell depends on the separation of this hormone from its binding protein, blood flow rate and absorption into the cell (33). According to our systematic review, previous studies have performed analyses between total vitamin D and LL-37 levels among TB patients (23, 34–37). To our knowledge, this is the first to examine the relationship between free and bioavailable vitamin D and LL-37 levels in TB patients. The few studies found have evaluated bioavailable vitamin D and LL-37 levels in other disease states (38–40).

In general, an accurate interpretation of free and bioavailable vitamin D levels may require an estimate of DBP levels, which can act as confounders. According to Bhan (2014) found lower levels of DBP in a healthy black population, and another study in pregnant women found the same (41). Consequently, free vitamin D levels in the black population are expected to be higher than in other populations with higher DBP levels. We recognize that one of the limitations of the present study is the lack of estimation of serum DBP levels, which represent the main transport of 25(OH) D. Another limitation was that the correlation of free and bioavailable vitamin D with bone mineral density was not measured. We were unable to measure PTH in our study. According to previous studies, a correlation between PTH and vitamin D levels is an indicator of good bone mineral density.

The strength of this study is the direct measurement of free and bioavailable vitamin D using the ELISA method, which gives accurate results compared to the indirectly calculated methods. The strength of this study is the direct measurement of free and bioavailable vitamin D using the ELISA method which gives accurate results compared to the indirectly calculated methods.

Conclusion

Significantly weak inverse associations were found between free and bioavailable and total vitamin D with LL-37 levels. Therefore vitamin D is involved in the regulation of LL-37 expression and low vitamin D levels can alter this relationship. Studies on the correlation of free and bioavailable vitamin D and 1,25dihydroxivtamin D and LL-37 are warranted to confirm our results.

Methods

Study design study site and study participants

A comparative cross-sectional study of newly diagnosed ATB patients, LTBI and individuals with no TB infection aged between 12 and 65 years was conducted. ATB patients were enrolled between the periods July 2019 to August 2020 and the LTBI, and samples from non-TB infected individuals from the KTB project were used.

Laboratory analysis

Measurement of free and bioavailable vitamin D using ELISA method

Free serum 25(OH)D was measured using a 96-well competitive (ELISA) kit catalogue, abx570015 (abbexa) Ltd., Cambridge, UK. The inter-assay and intra-assay CVs were less than 10%. The sensitivity of the assay was 1.88 ng/mL and the minimum detection range was between 3.125 n/mL and 200 ng/mL. The diluted standards and the control were pipetted into the standard and control wells. The plate was placed on a shaker to mix gently. The detection reagent working solution was added to each well and the plate placed on the shaker to mix. The plate was covered with a seal and incubated at 37°C for 45 minutes. The solution was discarded. Using a 300 L multichannel pipette, the plate was filled with wash buffer and washed three times. After washing, the remaining wash buffer was removed by decantation. The working solution of Detection Reagent B was added to each well. The plate was sealed and incubated at 37°C for 30 minutes. The solution was discarded and the wash step repeated as before. The 3,3,5,5-tetramethylbenzidine (TMB) substrate was added to each well. The plate was covered with a seal and placed on a shaker to mix and incubated for 10 minutes at 37°C, forming a blue color. A stop solution was added to each well and mixed thoroughly, the solution turned yellow in color. The OD was immediately measured at 450 nm using a spectrophotometer. The intensity of the yellow color was inversely related to the amount of vitamin D bound on the plate. A standard curve was constructed and a best-fit trend line was fitted through the standard points with an R2 of 0.97. A reference range of 1.92–8.82 ng/mL, adopted from Pathology Associates Medical Laboratories (PAML), was used.

Measurement of total vitamin D using electrochemiluminescence

Total vitamin D levels were analyzed by the electrochemiluminescence using Elecsys vitamin D3 assay according to the manufacturer’s instructions. The assay was performed in three incubation steps.

Measurement of LL-37 using ELISA method

A human 96-well competitive enzyme-linked immunosorbent assay (ELISA) kit catalog (CAMP), abx150919 (abbexa Ltd, Cambridge, UK) was used to determine LL-37 according to the manufacturer's instructions.

Statistical analysis

Data were analyzed using STATA software (Stata Corp. STATA Version 16.0, College Station, Texas, USA and Graph Pad Prism (Version 8). Normal distribution was calculated using the Shapiro-Wilk, Anderson-Darling, D'Agostino and Pearson tests tested and Kolmogorov-Smirnov tests. Continuous data were analyzed in medians and interquartile range (IQR), confidence interval (CI) at 95% and alpha of $p < 0.05$ was considered significant and power of 80%. Categorical variables were summarized as n(%) Man-Whitney U test was used for variables with two categories and Kruskal-Wallis test for 3 or more categories. Correlations between LL-37 and vitamin levels D levels were performed using pairwise correlation. Linear regression analysis was performed to determine the association between vitamin D levels andTB disease. Reference range of 1.92–8.82 ng/mL adopted by Pathology Associates Medical Laboratories (PAML) were used in the study.

Abbreviations

ATB
Active TB
DBP
D binding Protein
ELISA
Enzyme-linked immunosorbent assay
HIV
Human Immunodeficiency Virus
IOR
Interquartile Range KTB:Kampala TB Cohort
LL37
Cathelicidin
LTBI
Latent TB infection
MTB
Mycobacterium Tuberculosis
PTH
Parathyroid hormone
TB
Tuberculosis

Declarations

Ethics approval and consent to participate

The study was approved by Makerere University School of Biomedical Sciences Higher Degree Research and Ethics Committee (SBS HDREC) (#SBS-637), Research and Ethics Committee Mulago Hospital, Kiruddu Referral Hospital, and the National council of Science, and Technology (HS2639). Waiver of

consent was sought to use the KTB samples. Written informed consent was obtained from the active TB patients. Patients' personal information was kept confidential by using serial codes with no names recorded on the questionnaire. All adult participants in the study gave written informed consent for participation and parents or guardians consented for the minors. All experimental protocols were approved by Makerere University SBS HDREC (#SBS-637), and the National Council of Science and Technology (HS2639) as guided by the Helsinki declaration.

Data availability:

All data and reagents are available on request by the corresponding author.

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Authors' contributions

Conceptualization: E.L.A, Data curation: E. L. A, Formal analysis: E. L. A, O.R, M.B, A.A. Methodology: E L A, Provided KTB samples: I.A.B, Software: O.R, M.B, Supervision: W W, DP K, I A B, M L. J, Writing – original draft: E. L. A. Writing – review & editing: E L A, D P K, W.W, O.R, A.A, M L. J, I.A. B

Additional information

Competing interest

The authors declare no competing interests

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Figures

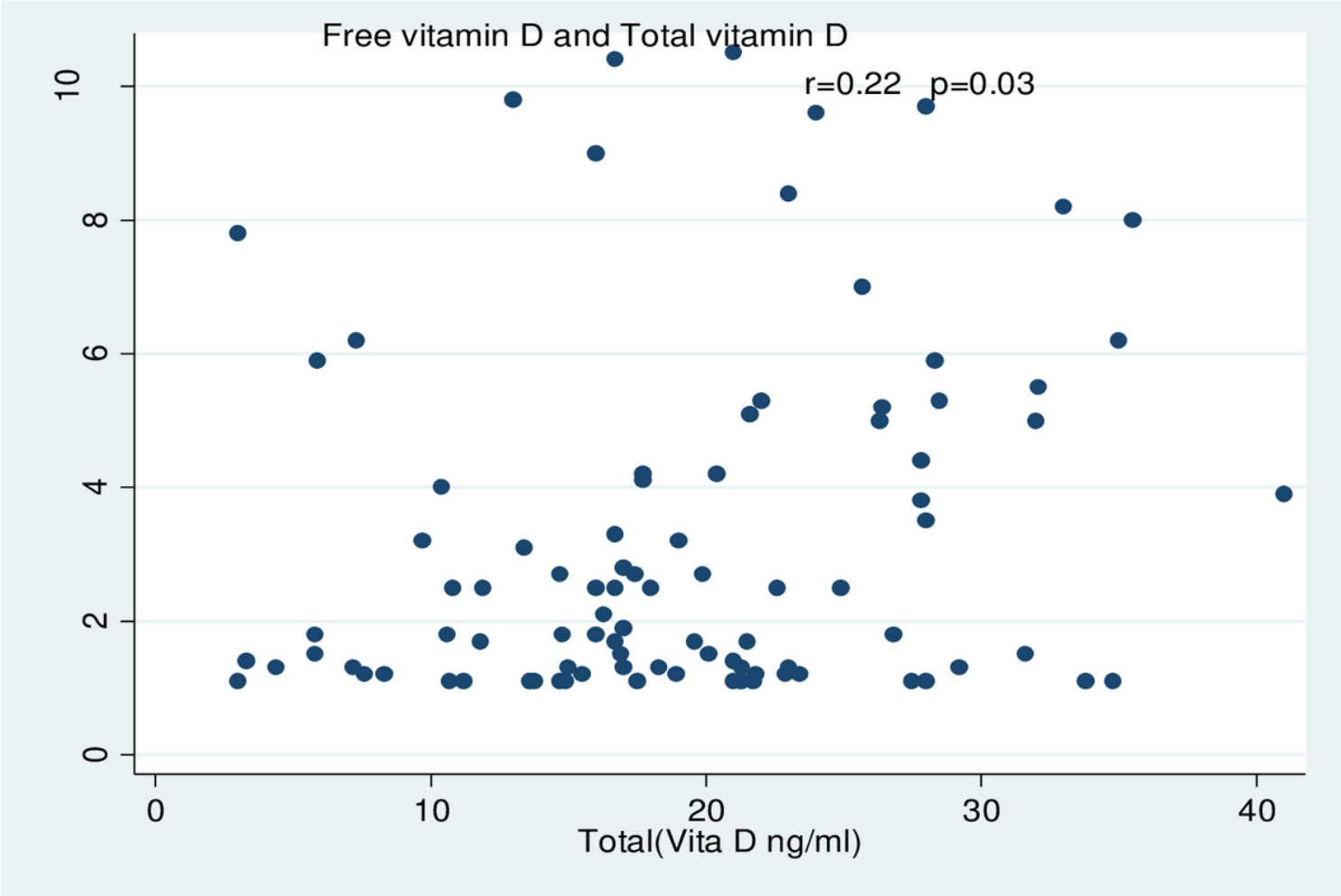

Figure 1

Correlation of free and bioavailable with total vitamin D levels in ATB, LTBI, and individuals with no TB infection

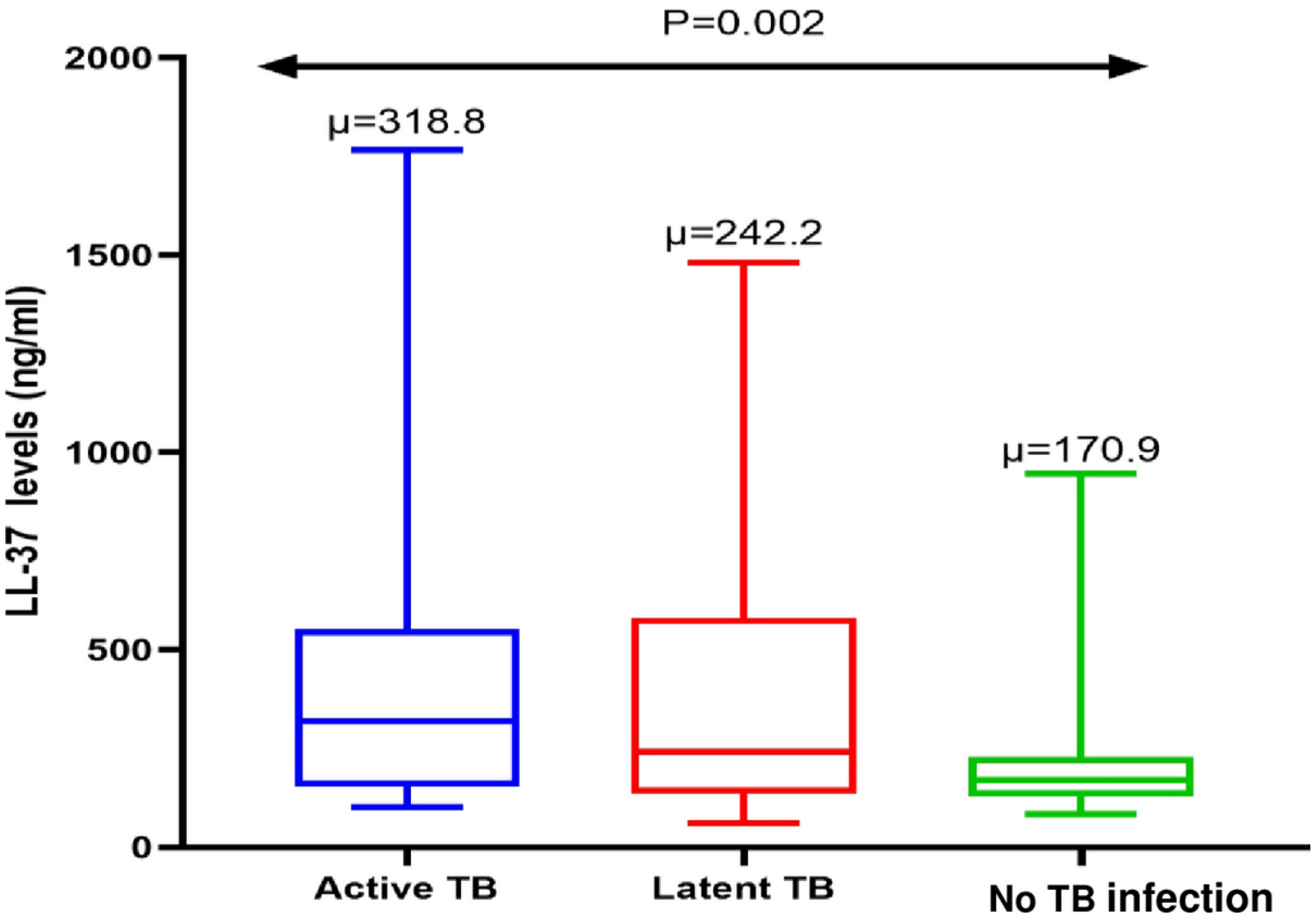

Figure 2

Comparison of serum LL-37 levels among ATB patients, LTBI individuals, and individuals with no TB infection. The boxes represent medians, and the upper and lower ends of the box represent the 75th and 25th percentiles, while the whiskers are the 5th and 95th percentiles with significance at< 0.05. Median serum concentrations were significantly higher among the ATB patients compared to the LTBI and those with no TB infectioThe figure was generated using STATA version12, https://www.stata.com by O.R

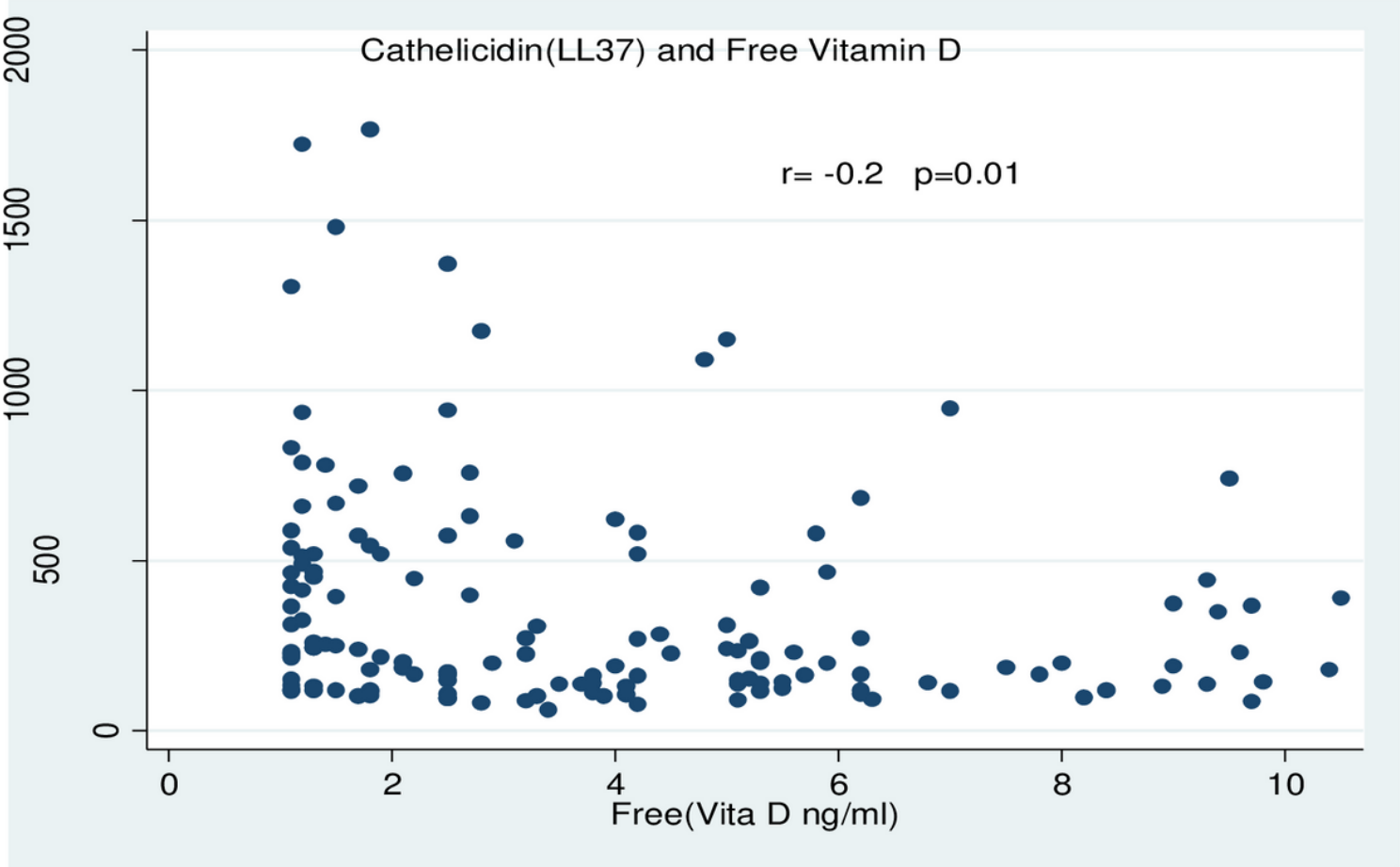

Figure 3

Shows the correlation of LL-37 with free and bioavailable vitamin D levels of ATB patients, LTBI, and individuals with no TB infection

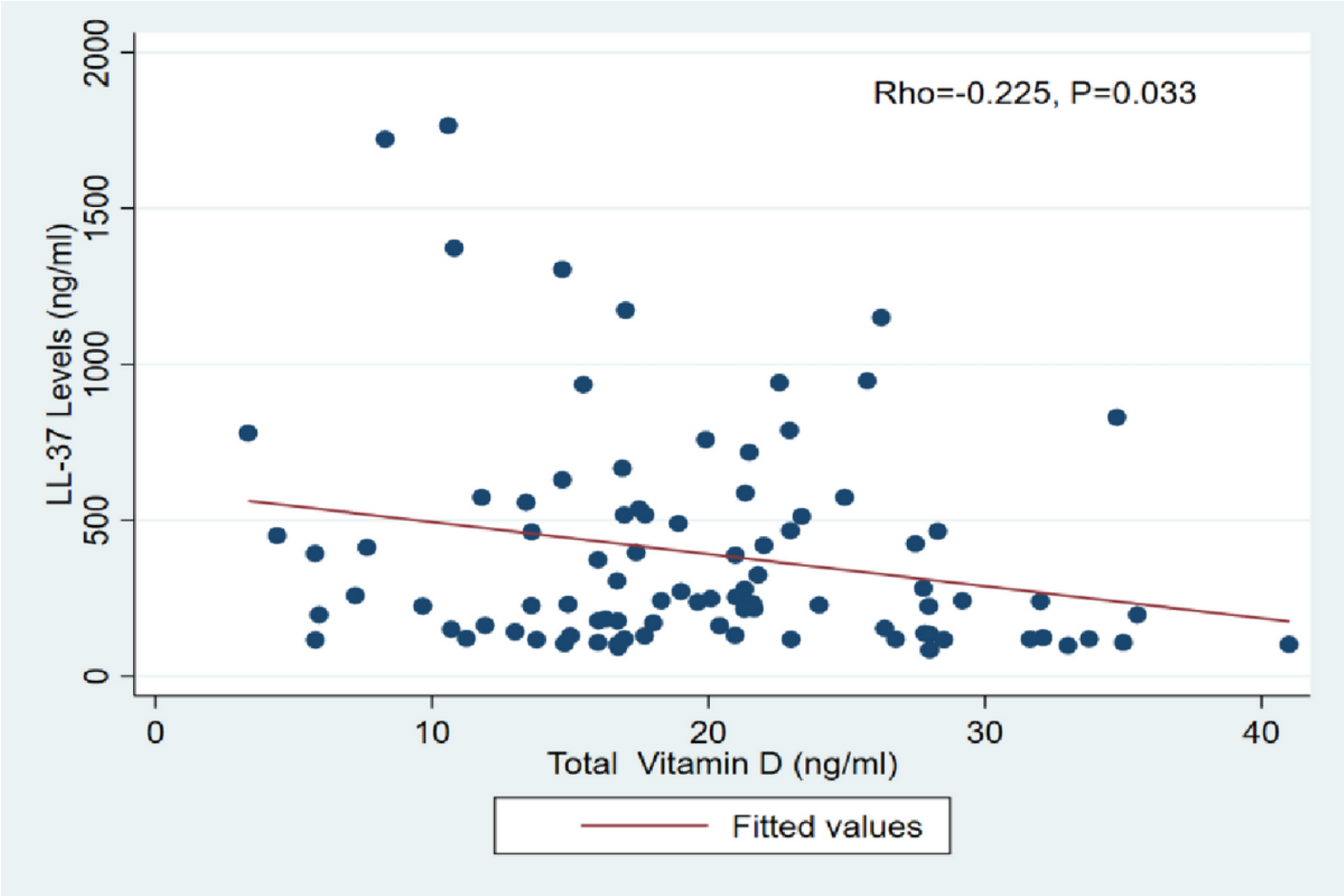

Figure 4

Correlation of free and bioavailable with total vitamin D levels in ATB, LTBI, and individuals with no TB infection

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- DATASETofsocialdemographicandotherfactors.xlsx