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Association of Vitamin D Deficiency with Cathelicidin (LL-37) Level in Drug Resistant Tuberculosis Patients

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The authors declare that there is no conflict to interest.

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ABSTRACT

Background: Antimicrobial peptide LL-37 is produced in response to active vitamin D and produces immunomodulatory effects and inhibits Mycobacterium tuberculosis replication in vitro. To date, no such clinical studies have been conducted to show relationship between 25hydroxyvitamin D and drug resistant tuberculosis patients in Pakistan.

Objective: This study was conducted to find the association between insufficiencies of vitamin D with Cathelicidin LL-37 in drug-resistant tuberculosis patients and compare them with the control group.

Methodology: Newly diagnosed drug-resistant TB patients and healthy controls ages 15-50 years old (mean 27.17,SD ± 10.37 years) were enrolled. Plasma LL-37 and serum 25 (OH) D levels were measured by ELISA technique in 46 patients and 94 controls.

Results: There was a significant difference (p-value <.001) in the level of Vitamin D in both case and control group. Vitamin D insufficiency prevalence was 57.8% and 42.2% with the mean baseline concentrations of 34.39ng/mL (SD ± 15.28) and 39.41ng/mL (SD ± 18.28) in cases and control respectively. In the mean serum concentrations 52.95ng /mL (SD ± 19.67) and 39.00ng /mL (SD ± 18.02) of LL-37 in cases and control respectively, there was a significant difference of P-value <.001 was found. However, concentrations of vitamin D did not associate with serum LL-37 concentrations (p-value>0.05).

Conclusion: Cathelicidine LL-37 is a potential biomarker of TB disease. However, the relationship between vitamin D & LL-37 requires further studies in TB patients.

Key words: Vitamin D; Cathelicidin LL-37; Drug resistant tuberculosis.

Introduction

Drug-resistant tuberculosis (DR-TB) is major threat to control tuberculosis (TB) as recommended first line drugs are no more effective against it.[1] Inappropriate treatment, retreatment of TB, hospitalization for treatment, compliance to treatment and inadequate supervision of treatment are some of the contributing factors that lead to the development of drug-resistant strains.[1-4] In the pre-antibiotic era Vitamin D was used as treatment for TB. In the mid-19th century, Williams reported that cod liver oil can be used for the management of patients with tuberculosis[5] (Mashhadi, Fawad et al.).[7] In the late 1800s in sanatoria ultraviolet radiation (UVB) therapy was used as a treatment for *Mycobacterium tuberculosis* (*Mtb*) infections. There exist conveyed rates of Vitamin D deficiency (VDD) TB patients fluctuating from 75% to 98%.[8]

Multiple studies have highlighted relations between VDD and increased risk of developing TB.[8] VDD is one of the possible aspects for the progression of TB reported by Shao *et al*.[9] Gibney *et al* reported lower VD level in tuberculosis cases andlatent infection patients as compared to those with no infection of *Mtb*in African immigrants in Australia, Victoria and Melbourne.[10,11] Mehr *et al* reported that in newly diagnosed TB patients, 42.27% patients were Vitamin D deficient and female patients were significantly deficient in Vitamin.[12] Fahad *et al* reported 18 female and 13 male Vitamin D deficient out of eighty newly diagnosed TB patients.[13] A study by Iftikhar *et al* also showed significantly lower Vitamin D level in female patients as compared to male.[14] A study conducted in Korea among individuals with a history of tuberculosis, deficiency of Vitamin D was 71.7% and this deficiency was highly prevalent in a female. The control group with no history of TB show prevalence of 72.1%.[15] A study showed a severe deficiency of Vitamin D 83.3% in TB patients, 80.7% in household contacts and 67.1% in community controls.[15] Another study showed that low VD level is connected with the progression of TB in household contacts.[16] Vitamin D deficiency was seen in African immigrants developed TB factors that may change as a result of migration are diet and sunlight contact and thus the level of VD. This deficiency of VD and risk of TB were also seen in foreign-born in UK and London.[17] But it is still uncertain whether vitamin D deficiency leads to TB or malnutrition has been seen in the disease.[18]

All these relations are normally credited to the effects of VD metabolites on host defense, which has been well considered in vitro. However, in humans, few studies have simultaneously assessed VD levels along with indicators of the host defense system in seriously ill patients.[19] VD has an important part in the macrophage activation to stop*Mtb* growth.[20-23] The well-known relationship between VD metabolites and the immune system related to the VD metabolites have special effects on the production of Cathelicidin LL-37, a perilous family of antimicrobial proteins, AMP and defensins.[19,24] They are present in epithelial cells with lungs and white blood cells.[24]

VD has no direct anti-*Mtb* action, but its active metabolites modify immune response against *Mtb*.[25] It plays main role in immunomodulation, it stimulates synthesis of Cathelicidin not only monocytes but also in colonic cancer cells, keratinocytes, leukaemia and bone marrow cells.[26-30] Initially, Cathelicidin is found in pieces of neutrophils and also can be secreted through epithelial cells monocytes and macrophages when activated by vitamin D.[31-33] In this study we investigated the association of VDD with the level of cathelicidin (LL-37) in the DR-TB patients.

Objective

This study was conducted to find the association between insufficiencies of vitamin D with Cathelicidin LL-37 in drug-resistant tuberculosis patients and compare them with the control group.

Methodology

This research study was designed as a case-control study to assess the association of Vitamin D deficiency with Cathelicidin LL-37 level in cases and compare them to the control group. This case-control design was taken as 1:2, taking control double to the size of the cases. Total 140 participants joined this study and the calculation of this sample size was done through Open Epi by assuming the power of 80% and confidence interval of 95 based on the prevalence of 57% and 33% of Vitamin D deficiency in TB patients and in general population respectively.[14] Study protocol was approved by Ethical Board (EB) of Khyber Medical University (KMU), (DIR/KMU-EB/AV/000556).

This study was done between June 2018 and January 2019 in Programmatic Management of Drug Resistant Tuberculosis (PMDT) Unit, Lady Reading Hospital (LRH) Peshawar. During that time 727 patients coming to the OPD (Out Patient Department) were screened. The screening was done through sputum smear microscopy (SSM), Drug susceptibility Testing (DST), culture and Line Probe Assay (LPA) in accordance with WHO guideline.Through screening 290 were found as newly diagnosed DR-TB patients. Those 290 were assessed through designed questionnaire; among them 65 meet the inclusion criteria of the study were recruited and 225 were excluded in accordance with the exclusion criteria. The recruited patients were asked for consent, among them 46 agreed to be the part of the study. They were called at morning and in the fasting state, their sample collections (blood) and their height, weight, body mass index in kg/m2

(BMI) measurement were carried out. Blood (6ml) were taken by applying aseptic technique through the sterilized syringes. Each 3 ml of blood were transferred into Gel and clot activator tube. Each sample within an hour was taken into the pathology lab of LRH for centrifugation. After centrifugation samples were stored at -20°C in Biochemistry Lab of LRH. On the same side a healthy control, age and gender-matched to the patients were taken from the general population. Both cases and control with renal, hepatic and endocrine system disorder were excluded from the study. Lactating and pregnant women and those taking any Vitamin D supplementation were excluded. Health care workers, lab staff and those with family history and exposure to TB were excluded from the control group. After the completion of the required sample size, all the samples were transferred into the physiology Lab of IBMS KMU in ice packs. Serum concentrations of 25(OH) D and LL-37 were assessed by

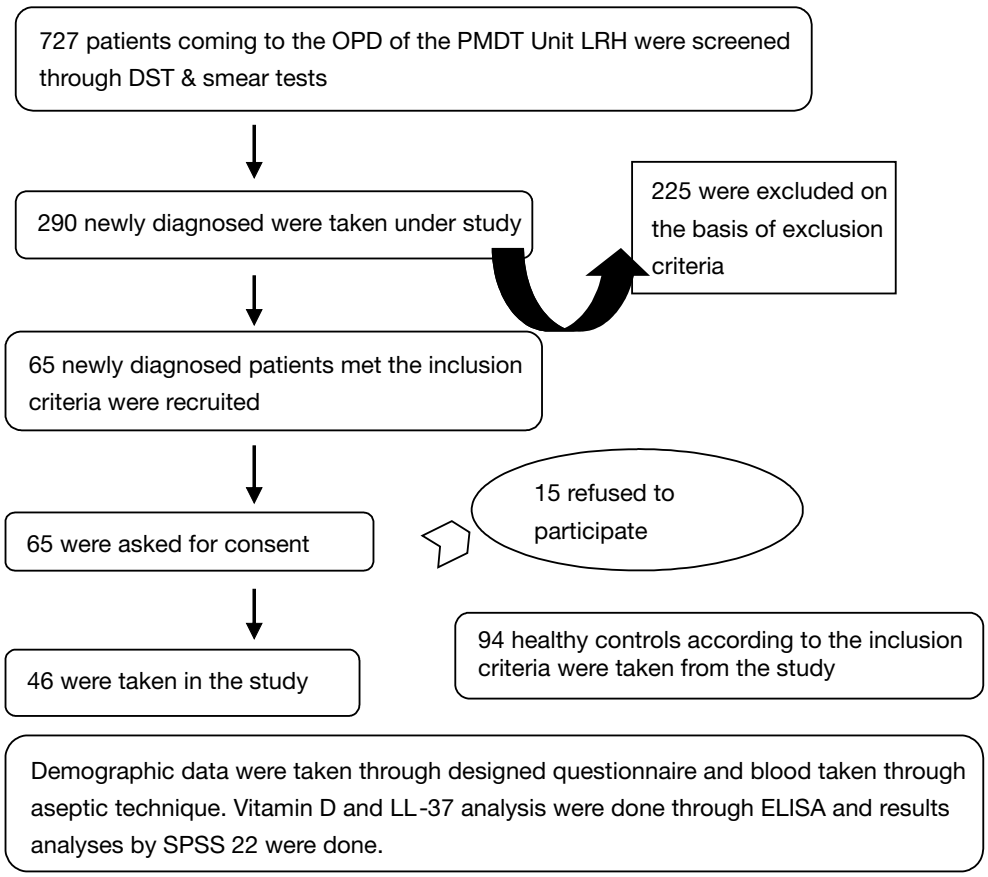

Figure 1. Flow chart shows study protocol

specific enzyme-linked immunosorbent assay (ELISA) kits. For both assays, protocol was followed as per the manufacture manual. The intra and interassay CVs for 25(OH) D measurement was <5% and < 6% respectively. The intraassay CV for LL-37 measurement was <6%.

Statistical Analysis

Results of the study were analyzed using SPSS.22. Normality of the data was checked by using the ShapiroWilk test of normality. For the numerical data i.e. serum Vitamin D and LL-37, mean and standard deviation were calculated and categorical data representation was done using frequency and percentages. Independent sample t-test was used to evaluate the difference of Vitamin D and LL-37between the two groups. Univariate analysis was done using chi square statistics by taking age, weight, height, BMI and sunlight exposure as independent variables with Vitamin D and LL-37 as outcome variables. Multivariate analysis was performed to find the associated risk factors for vitamin D deficiency using logistic regression between independent variables and vitamin D levels. Further Multivariate analysis was performed through multiple regression between different variables and Cathelicidin LL-37 to find out factors associated with its level. A p-value less than 0.05 were considered statistically significant.

Results

Forty six patients and ninety four healthy controls were included in the study. There was an insignificant difference in the age of both groups and majority of the study participants were in the younger age (15-25 years) in both the groups and female to male ratio was 2 to 1 (Table1). There was a significant difference P < .001 between underweight, normal, overweight and obese in both case and control group. In the cases group, 73.9% were underweight and 4.3% were obese while in the control group 13.8% were underweight and 7.4% were obese. Both the groups were having significant difference in their educational status. In cases, majority of the patients were having education status upto middle and secondary level while in control group majority were grade 12 and above. Significant differences in the professional status of both groups were found. Unemployment status was 80.4% in cases, 37.3% in control, while 19.6% were employed in cases and the control group has total 62.8% employed. Cases from rural residence were (52.2%) while in control group (43.6%) was residing in the rural areas. Signicant differences in the family living structure of the groups were found. Thirty-four (73.9%) of the cases were living in the joint family system and sixty-one 33.1% of the controls were having joint family structure. A significant difference in the sunlight exposure per week was found. Among cases, 41.3% were having adequate sunlight exposure per week while in controls, it was 77.7%. Only 42.3% of the cases had sufficient level of vitamin D and while in controls it was 78.9%.(Table 2)

There wasn't any statistically significant difference between mean Vitamin D levels of the two groups. Mean± SD of vitamin D was 34.39±15.28 and 39.41±18.28 incases and controls groups respectively. But there was a significant difference between the LL-37 levels in two groups. Mean ±SD of cases and controls were 52.95±19.67 and 39.00±18.02 respectively with a mean difference of 13.95. In multivariate analysis urban residence was significantly associated with the Vitamin D status in reference to the rural residence; Age, gender, professional status and housing system with or without sunlight exposure have no association with the vitamin D status.(Table 3)

Association of Vitamin D with Cathelicidin in study groups

Linear regression was performed to find out the association between Vitamin D with Cathelicidin in both the study groups separately and combine. A slight insignificant but weak linear upward correlation of 6.3% variation in the Cathelicidin with Vitamin D level was observed. A highly insignificant and very weak downhill relationship was observed between Cathelicidin and

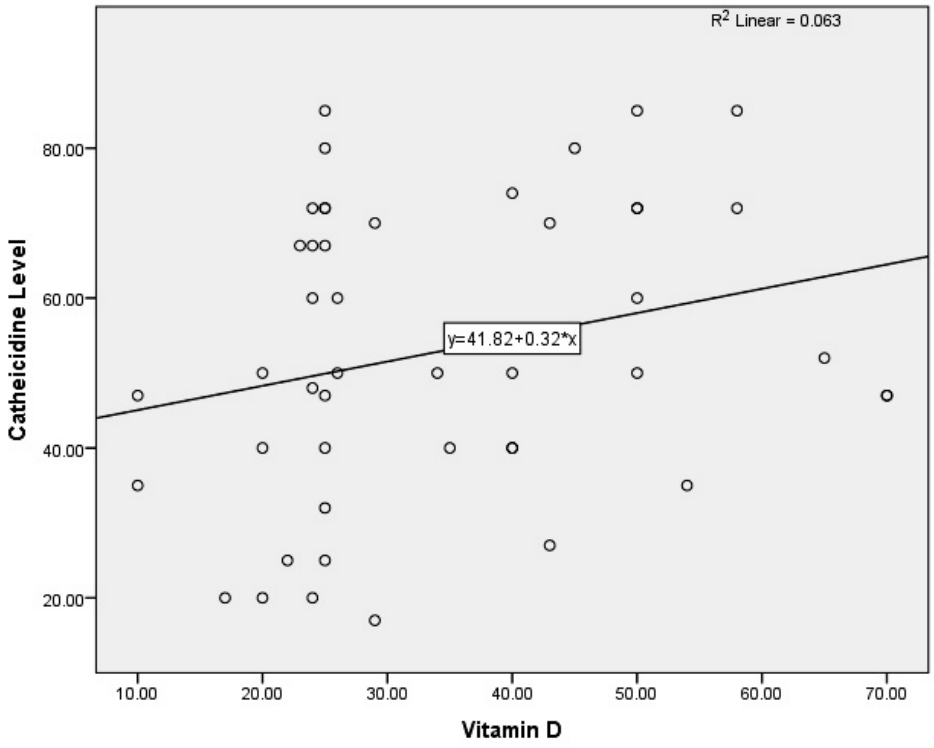

Figure 2. Scatter plot for the correlation between Cathelicidin and Vitamin D level in cases (r= .252, R2 = 0.063, P= .092).

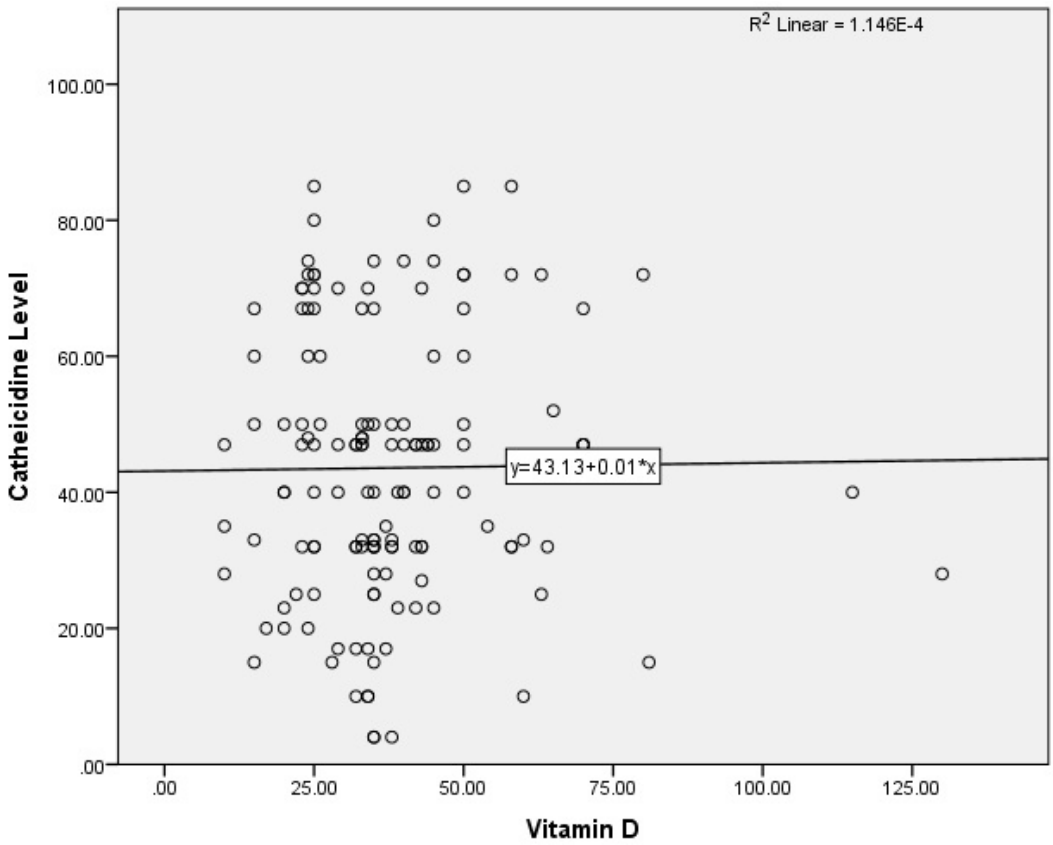

Figure 3. Scatter plot for the correlation of Vitamin D with Cathelicidin LL-37 in study groups.

Table 1. Tests results of cases: Clinical characteristics of cases

TB History	Frequencey (%)
History of TB	30(65.2)
No history of TB	16(34.8)
Family history of TB	27(58.7)
No family history of TB	19(41.3)
History of MDR -TB	21(45.7)
No history of MDR -TB	25(54.3)
The family history of MDR -TB	33(71.7)
No family h istory of MDR -TB	13(28.3)
Type of DR-TB	
DR-TB & MDR-TB	41(89.2)
XDR-TB	5(10.9)

Table 2. Mean difference between the Vitamin D of groups at baseline (n=140)

Overall Cathelicidin LL-37 Difference between the Case and Control groups at baseline			
Group	**Mean**	**Std. Dev.**	**P-value**
Case	52.95	19.67	<.001
Control	39.00	18.02	
Difference	13.95		

Table 3. Chi square test between categorical variables (n=140)

Variables	Vitamin (insufficiency)	Vitamin D (normal)	P-value
Gender of study			
Male (n%)	17(37.8%)	30(31.6%)	0.468
Female (n %)	28(62.2%)	65(68.4%)	
BMI of study groups			
Underweight (n%)	21(46.7%)	26(27.4%)	0.149
Normal (n%)	15(33.3%)	39(41.1%)	
Overweight (n%)	7(15.6%)	23(24.2%)	
Obese (n%)	2(4.4%)	7(7.4%)	
Housing system			
Open house with sunlight (n%)	26(57.8%)	81(85.3%)	<.001
Close house with no sunlight (n%)	19(42.2%)	14(14.7%)	
Sunlight exposure per week			
Exposure (n%)	0(0.0%)	92(100.0%)	<.001
No exposure (n%)	45(93.8%)	3(6.2%)	
Residence status			
Rural	14(21.5%)	51(78.5%)	0.012
Urban	31(41.3%)	44(58.5%)	
Professional status			
Employed	15(22.1%)	53(77.9%)	0.013
Unemployed	30(41.7%)	42(58.3%)	

Vitamin D in the control group. Also, no clear cut correlation and the highly insignificant correlation were observed in the scatter plot between Vitamin D and Cathelicidin. All the data distributed away from the linear line with only some of the data near to the line.

Discussions

Tuberculosis is the serious public health problem although it is treatable but the rise of drug resistant tuberculosis is the major threat to control TB, as first line drugs are no more helpful due to the development of resistance to some major drugs like RIF and INH.[34,35] So there is need for an alternative pathway other than the treatment through antibiotics. Much literature documented lower vitamin D level in the TB patients along with its role on the treatment outcome[21] (Iftikhar et al., 2013).[11-14, 33,36] Vitamin D is one of the important fat soluble vitamins synthesized in the human skin whenever exposed to UVB radiations, along with that it can also be obtained from some diet i.e. dairy products, milk, eggs and green vegetables.[37] Its functions are broad; not only having role in the bone mineralization along with its growth and calcium transport regulation in the muscles but also regulates: calcium and phosphorus homeostasis, control the insulin secretion, regulation of proliferation of cells, stimulations of differentiation of cells, initiation of apoptosis and an important role in the immune system.[38,39] It through immune system pathway by stimulating the production of defensin and Cathelicidin LL-37, which are important antimicrobial peptides, will fight against *Mtb*. So for the production of these antimicrobial peptides, an adequate level of vitamin D is necessary.[40] This study was designed with the objectives to find the association of vitamin D with the Cathelicidin LL-37 level in drug-resistant TB patients and compare them to the control group.

In this study, we have got an insignificant weak linear uphill relationship between Cathelicidin LL-37 and Vitamin D with a 6.3% variation in the Cathelicidin level with that to vitamin D by r = .252, P = .09 in the cases. While the study conducted by Alexandra *et al.,* reported an insignificant and a weak downhill relationship for LL-37 level with Vitamin D level. Though both the levels were analyzed using ELISA technique (kits [IDS Ltd, Fountain Hills, AZ for Vitamin D and Hycult Biotechnology, Uden, Netherlands for LL-37] but the study design was a multi-centre randomized and open-label phase III clinical trial and the study subjects were only sensitive TB patients and 95 cases were included in the study and conducted in US, was different from our study in which the study participants were DR-TB 46 cases and a single centre study.[7] Similarly in another study no significant correlation (r = -0.132) between the Cathelicidin and Vitamin D level was reported. The ELISA technique [for vitamin D kit of IDS, Fountain Hill, AZ, USA and for LL-37 kit of Hycult Biotechnology, AA Uden The Netherlands] were used for

analysis of 22 samples.[8] In our study and the above two studies noted no significant correlation was observed between vitamin D and LL-37 but the difference between the results of correlation between our study i.e. weak linear upward and in the above two studies mentioned which was weak linear downward may also be due to the same models of ELISA kits were used for assay and sensitive TB cases by the two studies mentioned above, though both were in two different countries. While in our study ELISA kits [CALBIOTECH a life science company &Elabscience (USA)] for vitamin D and LL-37 were used, the difference may be due to the reliability of different companies ELISA kits. Further by Kyungho Park *et al*ER stress increases CAMP expression via NF-_B-C/EBP_ activation, independent of VDR, illuminating a novel VDR-independent role for ER stress in stimulating innate immunity and also ER stress increases LL-37 mRNA expression in keratinocytes.[41]

Moreover in univariate analysis the mean serum concentration of vitamin D was significantly correlated with the age of the studied groups however, the mean serum concentration of LL-37 was not significantly correlated with that of the age but significantly correlated with the weight and BMI of studied groups. This result of vitamin D significant correlation with age is similar to the study reported the same significant association but in the same study, a significant association was also reported for LL-37 with age which is in contradiction to our study.[7]

Furthermore, in multivariate analysis, the possible risk factors for vitamin D deficiency were an urban residence and close house system with no sunlight exposure for cases. While in multivariate analysis the mean serum LL-37 concentration was significantly associated with open housing system with sunlight exposure in the case.

Our study reported mean serum concentration of 25(OH) D 34.39±15.28ng/ml for 46 cases which are higher than the results reported 20.4±9.7ng/ml for ninety-five cases.[7] Moreover, our study reported the mean concentration of 39.41±18.28ng/ml for the control group which is fairly different from the study at Sargodha reported the very low mean level of 9.3±1.38ng/ml for the control group and also the very low mean level of 13.9± 1.68ng/ml in the patient's group.[42] A study in the tertiary care hospital Rawalpindi reported the significant mean level of 20.68±14.06ng/mL between the patient's group and 57.91±18.19ng/mL for the control group, conducted the study for one-year duration from January to December in the OPD of Pulmonology department.[43] The variations in our results may be due to the different factors including diet, study in different areas with different sun exposure. Another important reason may also be due to the study samples collection in the summer season and the patients belong to the rural areas of Khyber Pakhtunkhwa (KPK) and 91.3% patients were living in the open houses with sunlight exposure and 41.3% of the total patients were having sunlight exposure for every week.

A significant mean serum Cathelicidin level was 52.95±19.67 in the cases and control have 39.00±18.02 in our study was noted. This is near to the mean serum concentration of 49.5 ng/ml with SD of 23.8 ng/ml but the number of cases is this study was 95, which are greater than from our study (n= 46).[7]

Moreover, our study reported 62.2%, 93.8%, 41.3%, 41.7% of insufficiency of vitamin D in the female; those have no sunlight exposure, urban and unemployed respectively.

Conclusion

Cathelicidine LL-37 is a potential biomarker of TB disease. However, the relationship between vitamin D & LL-37 requires further studies in TB patients.

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