CHAPTER 3

TO ASSESS THE IMPACT OF HOST SERUM VITAMIN D LEVEL
ON MANIFESTATION OF PROBABLE FGTB SUSCEPTIBILITY
3.1 Introduction

Tuberculosis (TB) remains a great burden and major global health problem and the concerned mechanism of pathogenesis involved in the progression of disease needs to be explored (Sayama R et al., 2015). TB exists in two forms: pulmonary tuberculosis and Extra-Pulmonary Tuberculosis (EPTB). Genital tuberculosis is one form of EPTB and it represents 12% of patients with pulmonary tuberculosis and 15%–20% of EPTB (Saraswat P et al., 2010). Transmission of disease occurs via the respiratory tract, and approximately 80% of those with active TB develop pulmonary disease. Some patients develop concurrent (secondary) EPTB, while in others the infection develops primarily in an extra-pulmonary organ. Symptoms of EPTB are non-specific and depend on the affected sites (Padberg J et al., 2015). The term ‘vitamin D’ refers to the parental vitamin D produced endogenously by the action of sunlight on 7-dehydrocholesterol in skin (also known as vitamin D3, or cholecalciferol) (Salahuddin N et al., 2013). Antimicrobial activity of vitamin D has been accomplished by inhibiting the growth of M.tb and up-regulating innate host responses (Azam F et al., 2016). Intracellular M.tb bacilli killed through the production of Nitric Oxide (NO) as well as antimicrobial peptide human cathelicidin (LL-37). Serum level of 25-hydroxyvitamin D up to 30ng/ml or more is sufficient for the conversion of its inactive form to bioactive form, which enhances the expression of cathelicidin (antimicrobial peptide). Simultaneously, if levels of 25(OH)D found to be less than 20 ng/ml, patient is immunocompressed (Nilay S et al., 2014). Addition of vitamin D to standard Anti Tubercular Therapy (ATT) drug regimen results in faster clearance of
the infection (Eisabeth L et al., 2012). Previous evidence suggests that hypovitaminosis D is associated with the susceptibility for cancer, autoimmune disease, diabetes and cardiovascular disease, which indicates the importance of sufficient vitamin D level (Farnik H et al., 2013). A therapeutic value of the immunomodulatory effect of vitamin D on tuberculosis patients has already been proven in randomized, controlled clinical trials (Alica K G et al., 2014). Vitamin D is supposed to be functionally important factor in susceptibility to bacterial as well as viral infection. It shows the significance of vitamin D, in the treatment of TB (Nava A E et al., 2009). Low serum Vitamin D is premeditated to be correlated with tuberculosis while the “dangerous” level was still unclear (Zeng J et al., 2015). Perceptivity to TB is also influenced by environmental and genetic factors or by gene-environment interactions (Azam F et al., 2015). The aim of this study was to identify the association between female genital tuberculosis and serum Vitamin D levels via synthesis of available evidence.

3.2 Materials and Methods

This case-control study was done in Department of Obstetrics and Gynaecology at Department of Microbiology, King George’s Medical University, Lucknow, India, Subjects were recruited with previous ethical approval and informed consent form was taken from each outdoor patient. The selection criterion of subjects (150 Cases & 150 controls) has been previously described in methodology section of chapter no 2. Study
was approved by from the Institutional Ethical Committee (No.6139/Ethics/R.Cell-15) of KGMU, Lucknow, India.

### 3.2.1 Sample Collection & Serum Separation

Blood will be collected in duplicate from both cases and controls according to the standard research protocol. One Blood samples will be kept at room temperature for 30 minutes after collection to allow clotting another sample kept in -80°C for molecular analysis. Samples will be centrifuged at 1200 × g for 10 minutes. The fresh serum will be stored at −80°C until analyzed. Serum samples will be stored for serum vitamin D level analysis.

### 3.2.2 Serum Vitamin D level Estimation

One milliliter of blood sample was used quantification for serum vitamin D level in cases as well as in control. Vitamin D level was measured by active human vitamin D ELISA kit (Bio-Detect cat#071) using an ELISA reader. The quantitative determination of circulating vitamin-D was done by using human plasma or serum as sample. ELISA methodology used for estimation of serum vitamin-D level. Assay procedure of ELISA was given in Figure 3.37.
Figure 3.36 ELISA ready to use kit with reagents, & 96 well plate of ELISA

Table: 3.16 Different concentration of serum Vitamin-D level

<table>
<thead>
<tr>
<th>S.no.</th>
<th>Vitamin D concentration (ng/ml)</th>
<th>Expected outcome in body</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>5-10</td>
<td>Severe vitamin D deficiency</td>
</tr>
<tr>
<td>2.</td>
<td>10-20</td>
<td>Vitamin D deficiency</td>
</tr>
<tr>
<td>3.</td>
<td>20-30</td>
<td>Suboptimal vitamin D provision</td>
</tr>
<tr>
<td>4.</td>
<td>30-50</td>
<td>Optimal Vitamin D level</td>
</tr>
<tr>
<td>5.</td>
<td>50-80</td>
<td>Upper normal</td>
</tr>
<tr>
<td>6.</td>
<td>&gt;80-100</td>
<td>Overdose but not toxic</td>
</tr>
<tr>
<td>7.</td>
<td>&gt;100</td>
<td>Vitamin D intoxication</td>
</tr>
</tbody>
</table>
**Figure: 3.37 Assay procedures for Vitamin-D level estimation**

Serum sample and calibrators added in antibody coated 96 micro wells plate

Vitamin-D enzyme conjugate 100µl added in each well
(All reagents are ready to use)

Incubated for 75 minutes at room temperature (RT)

Washed 3 times with wash buffer

100µl substrate reagent was added

Mixed well and incubated it for 10 min at RT

Stop reaction by added 50µl of stop solution

Read absorbance at 450nm (ref- 610to640nm) by micro plate Elisa reader
3.3 Statistical Analysis

Categorical variables were presented in number and percentage (%) and continuous variables were to be presented as mean±SD. Quantitative variables was compared using unpaired t-test. Qualitative variables were compared using Chi-square test/Fisher’s-exact test as appropriate. A p-value of <0.05 was considered statistically significant. The data was entered in MS EXCEL spreadsheet and analysis was done using SPSS version 21.0. Pearson correlation coefficients were used to determine the relationship of BMI, with serum vitamin D level concentrations in these subjects (case and control) groups, while correlation was defined as a measure of the strength of a linear relationship between two variables. The statistical measure of linear association is known as the correlation coefficient, denoted by the symbol r, and measures how close the points lie to a straight line. Its value always lies between –1 and +1. The value +1 indicates a perfect positive relationship between the two variables and the value –1 indicates a perfect negative relationship.

3.4 Results

During the study, 150 confirmed FGTB cases and healthy control enrolled for study following inclusion criteria. For the confirmation of M.tb in enrolled women, number of test was done as TB-PCR, AFB, LJ Culture, MGIT Culture. Out of 150 cases Acid-fast Bacilli (AFB) smear examination 14(93.3%), LJ culture 5(3.3%), MGIT culture 5(3.3%), PCR 147(98.0%) were found positive respectively. All these 150 FGTB Cases
were further confirmed by Diagnostic Laparoscopy (DL). Of 150 cases 61 were normal i.e., Level-1 and 59 were suspected i.e., Level-2.

The demographic variables (Age and BMI) and biochemical parameters (haemoglobin, thyroid, prolactin) between cases and controls are summarized in Table. Comparing the mean demographic value of age and BMI were (29.03±3.127, 27.03±3.00) and (22.92±3.33, 24.15±3.97) respectively with the p=0.012 and p=0.010 found to be significant among cases and controls Table 3.17. However, in biochemical investigation (haemoglobin, thyroid, prolactin) there was no significant correlation found between cases and controls. The mean serum vitamin D level was 14.96±8.81 in cases and 23.00±8.83 in controls with p-value <0.001. Statistical analysis showed a significant association between FGTB cases and controls Figure 3.39.

Levels of serum 25(OH)D observed in different socio demographic parameter are shown in Table 3.15. There was positive correlation found between BMI, and serum vitamin D level with p=0.012 and p=0.010 respectively Table 3.17. The prevalence of low 25(OH)D3 level in FGTB patients than in controls Figure 3.39. To demonstrate the role of Thyroid, serum prolactin, PPD, and tubercular test like TB-PCR, AFB, LJ-Culture and reduction of serum vitamin D level among FGTB case, it was found that there was no obvious correlation between above parameters.
Table 3.17 Demographic distribution of FGTB cases and Healthy Controls serum Vitamin D level (Mean±SD) of two groups

<table>
<thead>
<tr>
<th>Variables</th>
<th>Cases(N=150)</th>
<th>Controls (N=150)</th>
<th>p-value</th>
</tr>
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<tbody>
<tr>
<td>Age</td>
<td>29.03 ± 3.127</td>
<td>27.03 ± 3.00</td>
<td>0.06</td>
</tr>
<tr>
<td>Height (cm.)</td>
<td>155.50 ± 6.17</td>
<td>154.28 ± 6.78</td>
<td>0.147</td>
</tr>
<tr>
<td>Weight(Kg.)</td>
<td>55.37 ± 7.97</td>
<td>58.66 ± 18.71</td>
<td>0.078</td>
</tr>
<tr>
<td>BMI(kg/m2)</td>
<td>22.92 ± 3.33</td>
<td>24.15 ± 3.97</td>
<td>0.010*</td>
</tr>
<tr>
<td>Haemoglobin</td>
<td>11.49 ±1.63</td>
<td>11.29 ± 1.30</td>
<td>0.297</td>
</tr>
<tr>
<td>Vitamin-D Level</td>
<td>14.96 ± 8.81</td>
<td>23.00 ± 8.83</td>
<td>&lt;0.001*</td>
</tr>
</tbody>
</table>

*Unpaired t-test applied for significant. *significant

A positive linear correlation shows between serum vitamin D level and age of FGTB cases in Figure 3.38 (A). As the age increases level of serum vitamin D also decreases in females. Figure 3.38 (B) showed negative correlation between serum vitamin D level and BMI among FGTB cases. There was no significant correlation found between low serum vitamin D level and BMI.
Figure: 3.38 Correlation of vitamin D level among FGTB case

A) Vitamin D with Age B) Vitamin D with BMI
Discussion

The current study was carried out to evaluate the role of vitamin D cases in North Indian population. In our study we found a significant association between FGTB cases and controls. The low serum vitamin D level significantly associated to FGTB. The first report about the possibility of relationship between vitamin D and tuberculosis surfaced twenty years ago (Grange J M et al., 1985), but since then there have been conflicting reports about any such association in the subsequent studies (Azam F et al., 2016). A meta-analysis supported to our results showed that low serum vitamin D level was a risk for active TB and further verified the precise range of low serum vitamin D posing high risk of TB (Zeng J et al., 2015). Another meta-analysis reported by Nnoaham in (2008) found that serum vitamin D levels were lower in TB patients compared to controls.
Vitamin D also plays an important role in reducing the no. of risk chronic diseases as cancers autoimmune and infectious disease (Ho-Pham L T et al., 2010). Vitamin D modulates the immune system to fight against *M. tb* by promoting phagosomes maturation and enhancing the production of antimicrobial peptides (Chocano B P et al., 2009). Many other studies have investigated the association between vitamin D deficiency and TB. Similarly a number of studies that supported our results in Vietnam, Tanzania (Tostman A et al., 2010), West Africa (Wejse C et al., 2007), Gujarati Asians (Wilkinson R J et al., 2000), Sub-Saharan Africa (Gibney K B et al., 2008) had reported higher levels of vitamin D deficiency in patients with TB. Again, study conducted in Dow University of Health Sciences, Pakistan population have reported higher prevalence of vitamin D deficiency as lowers the vitamin D level in TB patients than non-TB individuals (Azam F et.al., 2016). Thus, our results were consistent with the results of most previous studies. On the other hand, there was contrasting evidence in studies conducted in Tanzania and Vietnam, which showed no considerable difference in 25(OH)D levels between TB cases and matched controls (Fris H et al., 2013) Demographic and biochemical factors like (thyroid, serum prolactin, haemoglobin) were found to have no association between FGTB and low serum vitamin D level. However, our study declared a positive correlation between BMI of cases and vitamin D level and this positive correlation is an indicator of major health nutritional condition of the subjects, which is supported by the study conducted by (Mrinal et al., 2014). In addition, the presence of TB and a history of TB were independently associated with vitamin D deficiency as well as low BMI. Vitamin D is known to have
numerous functions in response to infection, involving the innate and acquired immune systems. All of these functions are involved in the antimicrobial response to TB. Vitamin D insufficiency/deficiency is a worldwide, public health problem in both developed and developing countries (Joshi L et al., 2014). Vitamin D deficiency is associated with many chronic diseases, such as cardiovascular disease, autoimmune disease, cancer and chronic infections, PCOS and this has been depicted in several previous studies. Overall, studies suggested that supplementation of vitamin D through diet improves the immune response to TB. To the best of our knowledge, it is the first study that shows the effects of vitamin D level on FGTB cases in North Indian females.

Considering the combinational and synergistic effects of environmental factors with genetic factors such as the combination of $1, 25-(OH)2D3$ and interferon-$\gamma$ (which is more effective than either agent alone) in restricting the growth of $M.tb$ in monocytes,18 we investigated the plasma 25-OHD concentrations with VDR gene polymorphisms in susceptibility to tuberculosis.

3.6 Conclusion

In conclusion, vitamin D deficiency is highly prevalent among FGTB cases. Serum vitamin D level is low as compare to healthy control, and also shows a strong association between some of the demographical parameters, which indicates that vitamin D is essential in routine diet. Thus, serum vitamin D level can be used as a risk factor for FGTB. Further studies with large population group needed to establish a strong association between FGTB, serum vitamin D level and other demographic parameters.