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Plasma Vitamin D levels in correlation with circulatory proteins could be a potential biomarker tool for pulmonary tuberculosis and treatment monitoring

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ABSTRACT

Background: Tuberculosis (TB), a life-threatening immune challenging disease to the global human community has to be diagnosed earlier and eliminated in the upcoming era. Vitamin D, a fat-soluble micronutrient, mainly from epidermal cells of the skin and a few dietary sources, is associated with the immune system in various disease management. Therefore, a better understanding of vitamin D metabolism and immune function in tuberculosis should be studied for the consideration of biomarkers.

Methods: The study consist of Pulmonary Tuberculosis (PTB) patients (n = 32) at two-time points: Baseline (PTB BL) and after 6 months of anti-TB treatment (ATT) (PTB PT), latently *Mtb* infected (IFNγ +) group (n = 32) and a non-LTB healthy control (IFNγ-) group (n = 32). Vitamin D levels were measured using High-performance liquid chromatography (HPLC). The cytokine data from the same participants assayed by ELISA from our earlier investigations were used to correlate it with serum Vitamin D levels.

Results: The assayed serum Vitamin D levels between the groups showed significantly lowered levels in PTB BL when compared with IFNγ + and IFNγ- groups. And, the Vitamin D levels in the PTB group after ATT were significantly lower than the baseline levels. The Vitamin D data were compared with pro- and anti-inflammatory cytokines and adipokines levels by performing a principal component regression analysis. Based on the PC scores, the study group showed distinct clusters for the TB group and control group. And, the correlation analysis between the study group and immunological indices showed significant correlations. Vitamin D significantly correlated with IFNγ, TNFα, IL17A, IL-4 and Resistin in the TB group, whereas IL-6 and G-CSF in the control group.

Conclusion: The baseline measurement of Vitamin D levels was significantly decreased in the PTB group when compared with IFNγ + and IFNγ- groups showing the importance of Vitamin D as a preventive factor against the TB disease progression. The six-month post-treatment of TB showed a further decrease in Vitamin D levels in PTB. The significantly correlated immunological indices with Vitamin D levels are the biomarker profile that could predict TB.

1. Introduction

Tuberculosis (TB) is a communicable respiratory disease caused by a single infectious agent. *Mycobacterium tuberculosis (Mtb)* is one of the top 10 deadliest diseases worldwide in 2021. Global reports about 10 million people were newly developed for TB with 1.5 million death rates

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[1]. To end TB worldwide, there is a great need for a point of care (POC) rapid diagnostic methods to shorten the gap between diagnosis and treatment. The WHO suggests high-prioritized target product profiles (TPPs) under POC for the diagnosis of TB, which includes some biomarkers –non-sputum-based TB diagnostic tests [2,3]. Moreover, we focused on a blood-based biomarker signature for TB disease and for monitoring anti-TB treatment.

Vitamin D is also known as 25-hydroxyvitamin-D (25(OH) D), a steroid hormone mostly synthesized from 7-dehydrocholesterol in adipose tissue of the skin, when exposed to UV irradiation, and few are obtained from dietary source [4]. Vitamin D from dietary sources are mostly low and also stored in the tissues. During metabolism, Vitamin D is secreted from tissues as a prohormone, and then peripherally circulated in the host [5]. Vitamin D metabolism has a major role in the hosts as a co-factor enzyme that binds to the Vitamin D receptors (VDR) of cells. VDRs are highly expressed in all kinds of nucleated cells and tissues of organs, such as immune cells, keratinocytes and other cells like β-islet cells, prostate, and small intestine, through which the Vitamin D is absorbed into the cells. Therefore, the multiple roles of Vitamin D have been accomplished through receptor transportation to maintain homeostasis in the host [4,5]. Vitamin D has a significant role in the host's protection from various factors that affect the homeostasis of the host [6]. Therefore, circulating Vitamin D helps in host protection from wounds and various infectious diseases like psoriasis, skin cancer and Miscellanea through immune mechanisms [7].

According to the Institute of Medicine (IOM), the optimal level of Vitamin D in humans is $\geq$ 20 ng/mL, and below the range is considered deficient. Many clinical and experimental research says Vitamin D deficiency disturbs the immune homeostasis that leads to many pulmonary diseases including tuberculosis [8]. But, the role of Vitamin D metabolism and its peripheral circulation in the host protection against *Mtb* infection is not clear. Also, impaired immune mechanism with lowered Vitamin D was observed in multiple studies, where the immune cells of innate and adaptive immunity were deficient in the presentation of antigen [9,10]. Many investigations proclaim Vitamin D deficiency could be an influential factor in the cause of Pulmonary TB disease in humans [11]. Therefore, if the circulatory Vitamin D levels overlap with TB disease and its immune mechanism, then it can be studied for the biomarker for tuberculosis, which comes under the high-prioritized target product profiles (TPPs) [2,3]. Hence, our study deals with assaying the peripherally circulating Vitamin D levels in Pulmonary TB (PTB) patients at baseline and six-month after anti-TB treatment were controlled with interferon-gamma assay positives (IFN-γ +) as latently infected individuals (LTB) and the healthy controls (HC) who are all negative for the interferon-gamma assay (IFN-γ -). Then, the measured Vitamin D levels corresponded to the immunological response during the TB disease to suggest how Vitamin D could be a prognostic biomarker for tuberculosis.

2. Methods

2.1. Ethics statement

All the individuals recruited for the study were examined under the clinical protocol designed by the Institutional Review Board of the National Institute for Research in Tuberculosis (NCT01154959). Written consent was obtained from the study participants before sample collection.

2.2. Study group, population and sample collection

The plasma samples were collected after the consent from Pulmonary Tuberculosis (PTB) patients at two-time points of baseline and after 6-month treatment of anti-TB treatment (ATT), latently *Mtb* infected (IFN-γ +) group and non-LTB healthy controls (IFN-γ -). The PTB patients (n = 32) were microscopically sputum smear-positive for *Mtb* at the time of diagnosis with X-ray positive for TB disease. The LTB group (n = 32) were positive for interferon-gamma (IFN-γ) test when diagnosed for 3rd generation Quantiferon-TB gold tube assay. The LTB-ve HC group (n = 32) were negative for IFN-γ test and not symptomatic of TB. The biochemical and hematological profiles were assayed for all the participants in each group (Table 1).

2.3. Vitamin D estimation

Vitamin D levels were assayed using high-performance liquid chromatography (HPLC) from the serum samples. The whole blood collected from the study groups was centrifuged to separate the serum, from which 250 μL was taken in a 5 mL conical tube. To which, 60 μL of 25 (OH) D2 was added as internal standard and mixed well for 30 s, then left at ice-cold temperature for 15 min. Next, a 250 μL of methanol and isopropanol mixture (90:10) was added and vortexed for 15 s, then further incubated on ice for protein precipitation. Thereafter, 500 μL of n-Hexane was added to the mixture and vortexed for 1 min and centrifuged at 2000 rpm for 10 min. Then the upper hexane layer was separated and evaporated to dryness under nitrogen and reconstituted with 60 μL of methanol, then vortexed for 30 s and centrifuged at 1000 rpm for 1 min to get the clear solvent. From the solvent, an aliquot of 25 μL was taken to the reverse phase (C_{18}) HPLC system to elute 25(OH) D3 at the rate of 2.5 mL/minute with a guard column by using Methanol & Water (85:15 V/V). The Vitamin D3 levels in ng/mL were estimated based on the peaks raised at the wavelength of 265 nm which was compared with the commercial standards [12].

2.4. Other immune assays

Vitamin D levels were correlated with circulatory levels of cytokines and other soluble proteins, which are the altered factors of TB disease. Hence, to correlate Vitamin D with circulatory proteins, we have taken the cytokine and other soluble protein data that were generated from the same study individuals for other investigations into TB disease [13,14]. Therefore, pro-and anti-inflammatory cytokine data and adipokine data were taken as the immunological indices, which were altered upon TB disease to correlate with Vitamin D levels.

Table 1
Study demography.

Study Demographics	PTB	LTB	HC
No. of subjects recruited	32	32	32
Gender (Male / Female)	24/8	17/15	12/20
Median Age (Range)	40 (18 - 55)	36 (21 - 59)	36 (21-58)
Median Height, cm	164 (147 - 182)	162 (146 - 175)	163 (143 - 179)
Median Weight, kg	45 (33 - 68)	59 (37 - 80)	61 (41 - 95)
White Blood Cells (WBC) 10^3/μL	7.7 (3.6 - 14.1)	8.1 (4.1 - 12)	7.4 (4.5 - 10.7)
Lymphocytes 10^3/μL	1.9 (0.9 - 4.2)	3.4 (2.1 – 5.6)	3.5 (2.1 - 4.6)
Neutrophils 10^3μL	6.3 (4.4 - 8)	5.3 (3.3 - 6.6)	5.1 (3.7 - 6.5)
Eosinophils 10^3μL	0.3 (0.09 - 1.3)	0.4 (0.09 – 2.64)	0.4 (0.1 - 3.4)
Hemoglobin (g/dL)	12.2 (7.9 - 17)	13.4 (7.8 - 18.1)	13 (6.5 - 18.8)
RBC (mill/cmm)	4.4 (2.9 - 6.1)	4.7 (3.9 - 6.5)	4.8 (3.3 - 6.3)
Hematocrit (%)	35 (25 - 45)	40 (26 -55)	40 (25 - 60)
Platelets 10^3μL	340 (113 - 551)	269 (138 - 423)	275 (140 - 416)
Fasting Blood Glucose, mg/dL	102 (76 - 163)	92 (64 - 174)	90 (73 - 159)
Urea, mg/dL	17 (9 - 33)	20 (9 - 39)	20 (12 - 42)
Creatinine, mg/dL	0.7 (0.5 - 0.9)	0.8 (0.5 - 1.2)	0.7 (0.4 - 1.3)
SGOT, U/l	23 (11 - 96)	21 (12 - 56)	21 (12 - 59)
SGPT, U/l	16 (6 - 69)	16 (7 - 47)	16 (9 - 53)
Alkaline Phosphatase, U/l	78 (37 - 201)	-	-

2.5. Statistical analysis

The study results were statistically analyzed with the geometric means (GM) of each group as a central tendency. The significant differences between the three groups were analyzed by using Kruskal-Wallis with Dunn's multiple comparisons test and between the two groups were analyzed by using the non-parametric Mann-Whitney *U* test. The baseline and post-treatment data were analyzed for the significant difference using a non-parametric Wilcoxon matched-pairs signed-rank test. An ordinary one-way ANOVA test for linear trend was performed for significant differences between multiple groups. A Principal Component Regression (PCR) analysis was performed to predict the significant difference between the study groups according to the PC scores after the data normalization. A heat map was developed for the correlation matrix analysis using the Spearman r test between the multiple parameters. The above analyses were performed using GraphPad PRISM version 9.2.0.

3. Results

3.1. PTB individuals show a diminished level of Vitamin D than LTB and non-LTB healthy individuals

The assayed plasma levels of Vitamin D were compared between PTB, LTB positives (IFN-γ +) and LTB negative (IFN-γ -) healthy individuals. The Vitamin D levels in PTB patients were significantly diminished when compared with IFN-γ + ve individuals and IFN-γ -ve healthy group (Fig. 1A). Therefore, Vitamin D metabolism is associated with TB disease (PTB). To determine the effect of ATT on Vitamin D levels in PTB, we compared the measured levels of Vitamin D in PTB patients at Baseline with Vitamin D levels after 6 months treatment against TB. The data shows significantly decreased Vitamin D levels after anti-TB treatment than before treatment of TB (Fig. 1**B**). Thus, alteration of Vitamin D levels in PTB individuals after ATT, further reveals the association of Vitamin D metabolism with TB disease.

3.2. Association of Vitamin D metabolism with TB disease, bacterial burden and correlation with BMI

The examination of Vitamin D levels of PTB patients with unilateral lung disease and compared with PTB patients having bilateral disease relates Vitamin D metabolism with TB disease. The data showed significantly higher levels of Vitamin D in PTB patients having unilateral disease than the PTB patients with bilateral disease (Fig. 2**A**). Whereas, when we associated disease severity with Vitamin D using the lung cavity status of PTB patients, the data showed no significant difference between severe and non-severe PTB patients (Fig. 2**B**).

Then, to understand the association between Vitamin D and burden of *Mtb* in the host, we analyzed the Vitamin D levels within PTB patients, according to their sputum smear grade. The linear trend analysis revealed a significant trend showing Vitamin D levels decrease upon higher the *Mtb* smear grade in the PTB group (Fig. 2**C**). Finally, to relate Vitamin D metabolism with the BMI of study individuals, we did a Spearman correlation analysis between the Vitamin D levels of PTB, LTB and healthy donors with their BMI. The data showed a positive correlation between Vitamin D and BMI (Fig. 2**D**).

3.3. Vitamin D metabolism impacts immunological indices in tuberculosis

The above clinical data revealed an association of Vitamin D metabolism with TB, To understand the relationship between Vitamin D metabolism and the immune system, we examined cytokines and other circulatory proteins as the immunological factors; which are altered while the host immune system defending against TB disease. Hence, previously measured circulatory levels of cytokines and adipokines data from the same study individuals were taken as the immunological indices to compare with Vitamin D metabolism [13,14]. Principal Component Regression Analysis (PCRA) was performed by pooling all the study groups together with their Vitamin D levels data as dependent variables against independent variables such as cytokines and adipokines data. The whole data sets were statistically normalized before performing PCR analysis and plotted a heat map for data visualization (Fig. 3**A**).

The resulted PCR analysis shows a significant distinct cluster based on the PC scores upon their Vitamin D levels among the study groups (PTB BL, PTB PT, IFNγ + and IFNγ-) respectively (Fig. 3**B**). The PC plots were showing PTB BL and PTB PT in the negative region and in different locations of the plot, whereas IFNγ + and IFNγ- were in the positive region of the plot without differentiating their location for Vitamin D levels. Therefore, based on this result we characterized the study groups PTB BL and PTB PT as TB groups, whereas IFNγ + and IFNγ- as a control groups. Further analyses were performed based on the above characterizations to profile a prognostic biomarker for TB.

Likewise, a loading plot was plotted according to the PC scores, generated after PC Regression (PCR) analysis between the Vitamin D levels as the dependent variable and the various immunological indices as independent variables among the study groups (Fig. 3**C**). Next, merging the Vitamin D PC plot and PCR plot for immunological indices,

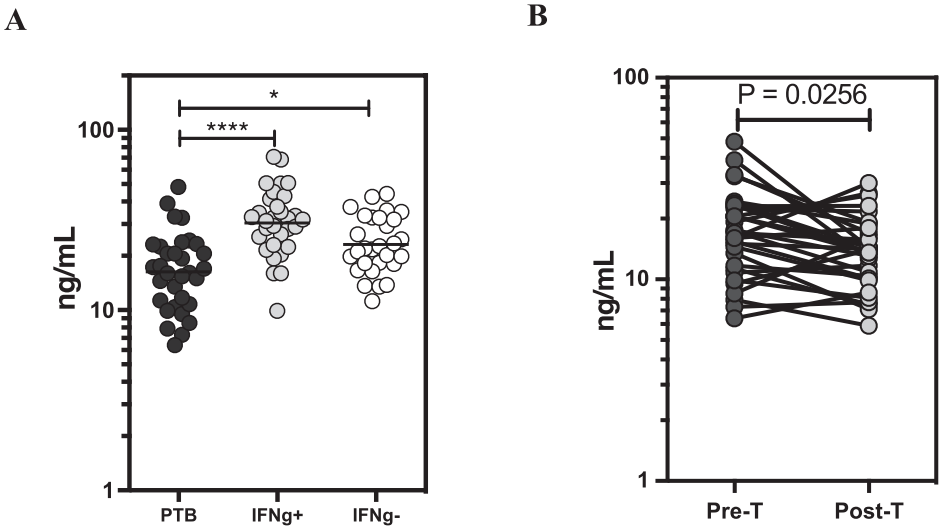

Fig. 1. Vitamin D metabolism has a significant role in tuberculosis pathogenesis. (A) The Vitamin D levels were compared between PTB, IFNg + and IFNg- individuals using the Kruskal-Wallis test with Dunn's multiple comparisons and the P-value for * and **** is 0.0329 and < 0.0001 respectively. Each circle in the scatter plot represents a single, and the line at the centre of the scatter plot is the geometric mean. (B) The systemic Vitamin D levels in PTB individuals at baseline (Pre-T) and six months after TB treatment (Post-T) were compared by the Wilcoxon test for matched pairs. Each circle in the first column connected by a line with another circle in the second column in the plot represents single individuals at two different time points that were matched for the analysis.

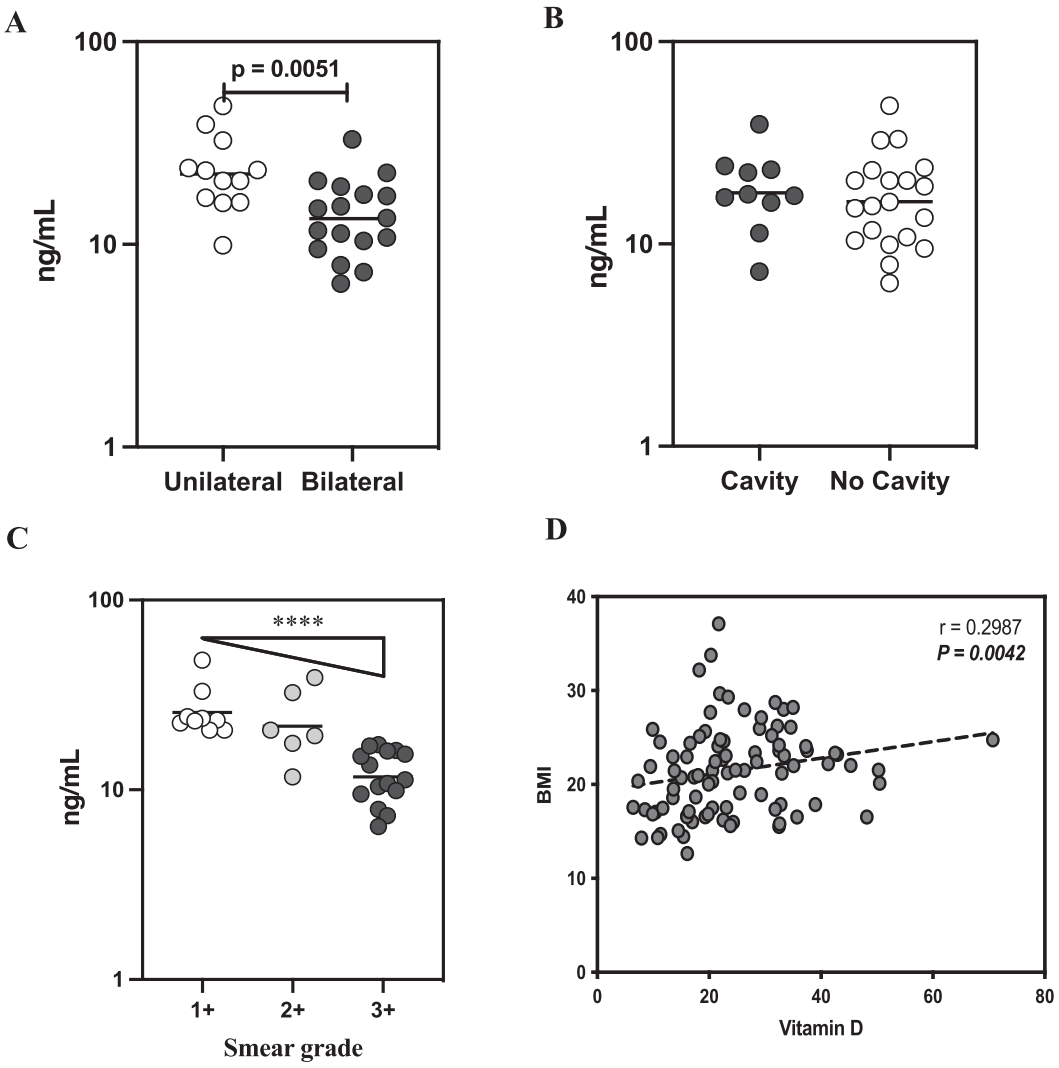

Fig. 2. Vitamin D metabolism impacts TB disease, Mtb bacterial burden in lungs and BMI, but not in the severity of TB. (A) The measured circulating Vitamin D levels were compared among PTB individuals with Unilateral lung disease or Bilateral lung disease. (B) The circulatory levels of Vitamin D in PTB individuals were compared with cavitary disease or non-cavitary disease. (C) The vitamin D levels were compared among PTB individuals upon bacterial burdens. Each circle in the scatter plots (A-C) represents a single individual and the line at the center of the scatter plot is the geometric mean. The P values were calculated using the Mann-Whitney *U* test (AB) and Linear trend analysis (C), where **** is < 0.0001 respectively. (D) Spearman correlation was performed between Vitamin D levels and BMI of each individual in the study group.

we plotted a biplot to show the association of Vitamin D metabolism with the host immune system in the study group (Fig. 3**D**).

3.4. Correlation of immunological indices with Vitamin D metabolism and consideration of TB biomarker profile

The association of Vitamin D metabolism with immunological indices has to be understood through correlation analyses. Therefore, based on the classification among the study group from the PC analysis, the Spearman correlation matrix was performed in the TB group (PTB BL and PTB PT) and the Control group (IFNγ + and IFNγ-) respectively (Fig. 4**A & 4B**). The TB group showed a significant positive correlation of Vitamin D with pro-inflammatory cytokines such as IFNγ, TNFα, and IL17A, also with anti-inflammatory cytokine IL-4 and adipokine protein Resistin. While in the Control group, the Vitamin D positively correlated with IL-6 and G-CSF.

Next, to validate the whole analysis, we performed ROC based on their normal levels. Globally, individuals having below 20 ng/uL of Vitamin D levels are deficient. Hence, both the study groups were classified into Vitamin D deficient and Vitamin D sufficient based on the global range of Vitamin D levels (Table 2). Then, the estimated levels of IFNγ, TNFα, IL17A, IL-4 and Resistin in the TB group and IL-6 and G-CSF among the Control group were allowed to perform AUROC based on their sufficiency and deficiency Vitamin D classification. Therefore, the immunological indices were showing significant Areas under the receiver operating curve (AUROC) except IL-6 of the Control group (Fig. 5 **A to F**).

Accordingly, we conclude Vitamin D in the combination of IFNγ, TNFα, IL17A, IL-4 and Resistin as a prognostic biomarker profile for TB diagnosis, while Vitamin D along with G-CSF as a biomarker profile for latent TB infection.

4. Discussion

Though, there are so many findings on Vitamin D (D2 & D3) having a vital role in the immune system, our investigation supports Vitamin D has a relationship with tuberculosis (TB). Evidently, an Iranian study reports Vitamin D deficiency rate was higher among the patients diseased with tuberculosis than the healthy individuals [15]. Globally, some meta-analysis shows the association of Vitamin D with TB infection

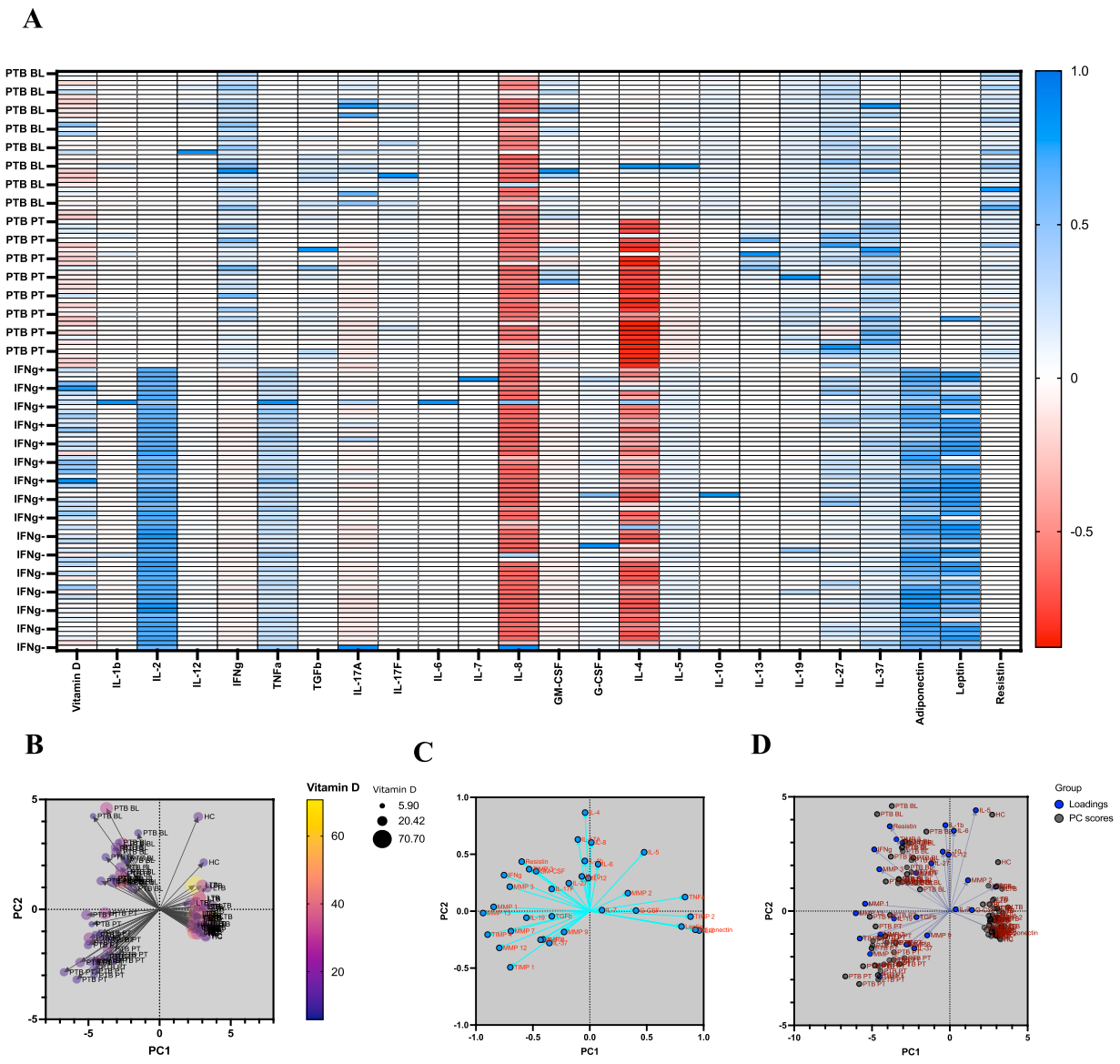

Fig. 3. Association of Vitamin D levels with circulatory immunological indices. (A) The Vitamin D and other circulatory immunological indices data were normalized and compared between the groups. (B) Principal Component Regression (PCR) analysis was performed for Vitamin D levels against immunological indices between the study groups. Each circle in the plot represents each individual, size and colour of each circle show the level of Vitamin D among the study group. Based on the PC scores, the study groups were shown as different clusters in the quadrants of the plot. (C) PCR plot showing the spread of immunological indices among the study group. (D) Loading plot showing Vitamin D metabolism in the study group and their association with immunological indices.

and disease. But, a recent clinical trial resulted in the failure of Vitamin D supplements in the prevention of tuberculosis [16,17]. Hence, it is a need of understanding Vitamin D metabolism in humans and its role in the immune system. Vitamin D deficiency impairs the immune system; therefore, the resulted immune-modulations cause routes for various diseases. Several investigations reports, that the lack of Vitamin D hormonal absorbance by cells through VDR was observed in various disease conditions [18]. Thus, Vitamin D metabolism is deemed to be very essential in human health; and the poor metabolism influences the demineralization of bone, cardiovascular disorders, cancer, diabetes and other infectious diseases [19]. Besides, immunological studies manifest the association of Vitamin D deficiency with respiratory diseases and disorders [20].

Correspondingly, there is a relationship between Vitamin D deficiency and the pathogenesis of *Mtb* in humans [21]. Certain in-vitro studies found an increased IFN-γ release was reported against *Mtb* infection upon Vitamin D supplement [22,23]. Likewise, reduced *Mtb* growth was observed with the sufficiency of Vitamin D was also shown in a few investigations [9]. Globally, TB research groups reveal the association of Vitamin D deterioration among active TB cases. A meta-analysis on Vitamin D status in Tb disease by Aibana concludes that the risk of TB development was high among Vitamin D deficient individuals, thus Vitamin D could predict TB disease [24]. In this context, our study shows significantly decreased circulatory levels of Vitamin D in pulmonary TB patients when compared with latently infected (IFNγ +) individuals and non-TB exposed health groups (IFNγ-). This observation underpins various research claims showing inadequate Vitamin D levels associated a high risk of TB disease. The clinical examinations such as chest X-ray, sputum grades and body mass index (BMI) among the pulmonary TB group manifest the severity of the disease is corresponding to the improvised Vitamin D metabolism. Even though the deficiency of Vitamin D levels upon TB disease when compared with control groups was seen, it is unfair to say Vitamin D alone is a biomarker for TB, since Vitamin D deficiency has been seen in other diseases also. Hence, our study forwarded to analyze the immunological alteration of Vitamin D deficiency among the study groups.

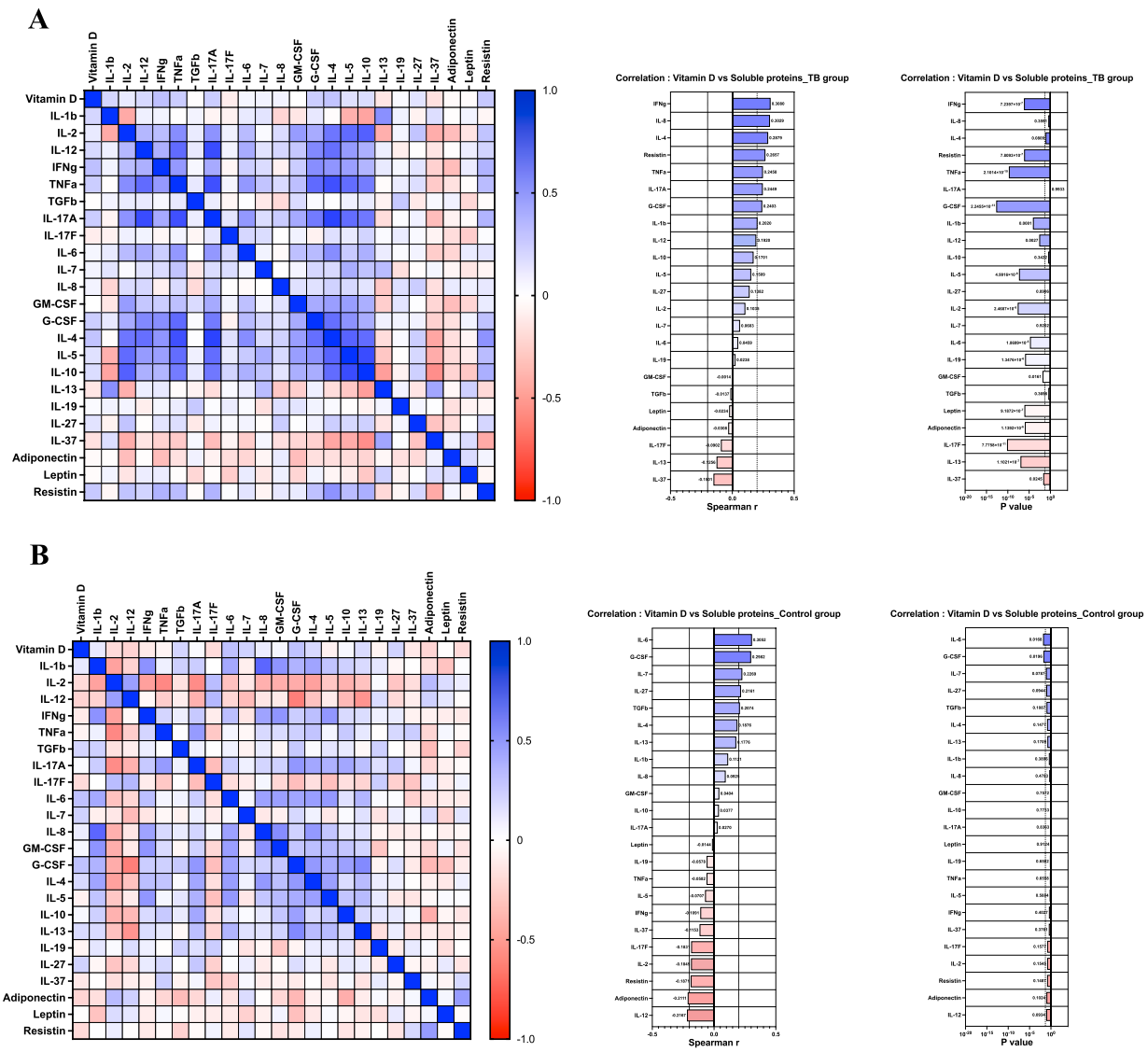

Fig. 4. Correlation of Vitamin D metabolism with Immunological indices. (A) Spearman Correlation matrix analysis was performed among the TB group. The bar graph shows the correlation between Vitamin D and immunological indices in the TB group. (B) Spearman Correlation matrix analysis was performed among the Control group. The bar graph shows the correlation between Vitamin D and immunological indices in the Control group.

Table. 2
Status of Vitamin D levels in the study groups.

Vitamin D	PTB (32)	IFNg+ (32)	IFNg- (32)
Deficient	20	4	12
Sufficient	12	28	20

Several investigations evidently depict the physiological impact that circulating Vitamin D and its metabolism must have on the homeostasis of immune system [10]. To understand the association of an impaired immune system with Vitamin D deficiency, our study analyzed the immune factors that are contributing to host protection against *Mtb* during infection and disease with respect of serum Vitamin D levels. Hence, various circulating levels of cytokines and adipokines are the soluble immune index proteins from the same study individuals were taken for further analysis. Principal component regression (PCR) analysis was performed for the multiple comparisons of immunological indices upon Vitamin D levels among the study individuals. Since PC analyses is the preferred statistical method for the investigation of multi-factorial analysis in biological discovery or diagnosis [25,26], we have applied PCR for our multi-parameters which resulted in the study groups showing two-distinct clusters for PTB BL, PTB PT, IFNγ + and IFNγ- respectively. Cluster in the negative region for Vitamin D levels are TB groups consisting of PTB BL and PTB PT, whereas the cluster in the positive region is IFNγ + and IFNγ- as the control group. Based on the same PC scores, the immunological indices were shown in the PC plot as factors that regress on Vitamin D levels depicting which cluster of the immunological indices lie on. Therefore, the PCR analysis shows the dynamics in the association of immune indices across the study groups.

The significance of Vitamin D association with the immune regulators was analyzed by performing a Spearman correlation matrix analysis. The correlation between Vitamin D and immunological indices was shown for the TB group (PTB BL & PTB PT) and control group (IFNγ+ & IFNγ-) separately. Next, the correlated parameters were taken for AUROC to identify the significant parameters having more sensitivity and specificity for Vitamin D. Therefore, Vitamin D deficiency in a combination of increased IFNγ, TNFα, IL17A, IL4 and Resistin as the biomarker profile for TB disease, whereas Vitamin D deficiency with increased IL6 and G-CSF is for latent TB infection. Likewise, a group from Spain reported IFNγ, IP-10, Ferritin and Vitamin D as a biomarker profile for the discrimination of latent infection from active TB among

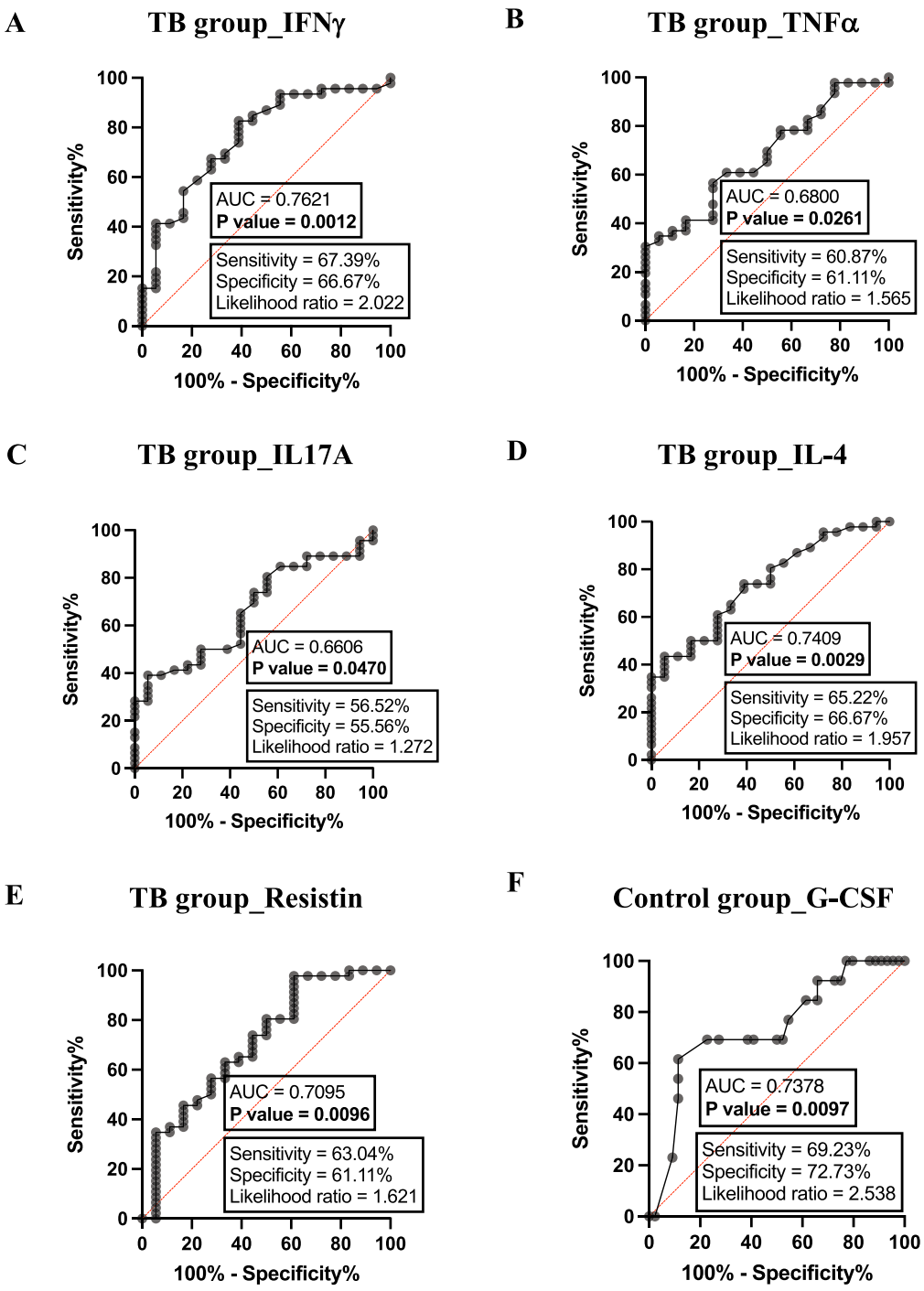

Fig. 5. A significant validation of immunological indices that impacts on Vitamin D metabolism. (A - E) Area under receiver operating curve (AUROC) was performed for IFNg, TNFa, IL17A, IL-4 and Resistin levels between Vitamin D level sufficient and deficient among TB group. (F) Area under receiver operating curve (AUROC) was performed for G-CSF levels between Vitamin D level sufficient and deficient among Control group. The ROC graph showing each dot in the curve was plotted for immunological indices between Specificity % and Sensitivity %.

children. They have assayed the released cytokines and ferritin levels from the blood cells upon in-vitro stimulation by TB antigens in Quantiferon assay tubes and concluded by ROC analysis [27].

Though, there are several non-sputum-based biomarkers were studied globally for TB as biomarker signatures, inclusive of MTB DNA biomarkers, plasma level soluble protein biomarkers, cellular biomarkers using flow cytometer, differentially expressing gene biomarkers and micro-RNA biomarkers [28,29]. Fortunately, the resultant data from our study coordinates with major global research, showing the association between Vitamin D and TB disease. Hence, our finding showcases the nutritional index impacts on immune homeostasis in the host upon TB disease could be a biomarker signature. Further validation studies may precise the biomarker profile for point of care (POC) TB diagnosis.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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