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Status of vitamin D and the associated host factors in pulmonary tuberculosis patients and their household contacts: A cross sectional study

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ABSTRACT

Innate immunity plays an important role in pathophysiology of tuberculosis which is influenced by various host factors. One such factor is vitamin D which, along with its associated molecule, can alter the host defense against *Mycobacterium Tuberculosis (M.Tb.)* via altered production of cathelicidin and nitric oxide, both having bactericidal effect. Therefore, assessment of vitamin D and its associated molecules in tuberculosis patients and household contacts as compared to healthy controls were done and the implication of these findings in susceptibility to tuberculosis (TB) was studied. 80 active TB patients, 75 household contacts and 70 healthy controls were included. Vitamin D receptor (VDR), vitamin D binding protein (VDBP) and inducible nitric oxide synthase (iNOS) mRNA levels were studied using quantitative PCR. Serum VDR, cathelicidin, and iNOS levels were measured using ELISA. Vitamin D and NO levels were measured in serum using chemiluminescence based immunoassay and greiss reaction based colorimetry kit respectively. Decreased serum levels of vitamin D were observed in active TB patients as compared to healthy controls ($p < 0.001$). VDR and iNOS mRNA levels were found to be significantly lower in active TB patients compared to household contacts and healthy controls ($p < 0.0001$ and 0.005 respectively). VDBP mRNA expression was found to be lower in active TB group as compared to household contacts and healthy controls however the difference was not found to be significant ($p > 0.21$). Although, mRNA expression of VDR, VDR protein and iNOS along with vitamin D levels were significantly ($p < 0.05$) higher in household contacts compared to active TB group. However, levels of iNOS, NO and cathelicidin were found to be higher in TB patients as compared to household contacts and healthy controls ($p < 0.01$, 0.05 and 0.01 respectively). Higher levels of Vitamin D along with VDR and iNOS expression in household contacts as compared to active TB patients suggest vitamin D might have a protective role against TB plausibly decreasing disease susceptibility. Low vitamin D levels in active TB patients warrants further studies to determine the role of vitamin D supplementation in prevention and treatment of TB.

1. Introduction

Innate immunity plays an important role in pathophysiology of tuberculosis with several host factors influencing the disease outcome. One such factor is vitamin D along with its transport protein vitamin D binding protein (VDBP) and its nuclear receptor vitamin D receptor (VDR), that can play a potential role in altering the host defense against *Mycobacterium tuberculosis (M.Tb.)*

1, 25 dihydroxyvitamin D3 (1,25$(OH)_2$D3), active form of vitamin D, has been extensively used in medicine as a potent regulator of calcium homeostasis. In addition to its calcaemic activities, 1, 25$(OH)_2$D3 displays anti-inflammatory and immunomodulatory properties [1,2]. In 1986, Rook et al. provided the first evidence for the role of vitamin D in the immunological control of *M.Tb.* by showing reduced proliferation of *M.Tb.* in macrophages treated with 1, 25$(OH)_2$D3 [3]. Vitamin D brings about its nuclear function via binding with its nuclear receptor, vitamin D receptor (VDR) protein; further forming an active transcriptional complex, which increases the expression of proteins such as cathelicidin, a cationic antimicrobial peptide, which brings about *M.Tb.* killing [4]. Along with the antimicrobial peptide, it has been shown that vitamin D-mediated effects in human cells may involve other antimicrobial effector functions such as generation of inducible nitric oxide synthase (iNOS or NOS2) required for generation of nitric oxide (NO) in the presence of infection [5]. However, the activity of vitamin D

Abbreviations: TB, tuberculosis; *M.Tb.*, *Mycobacterium tuberculosis*; VDR, vitamin D receptor; VDBP, vitamin D binding protein; iNOS, inducible nitric oxide synthase; MDM, Monocyte Derived Macrophages; iNOS, inducible nitric oxide synthase; NO, nitric oxide; CLIA, chemiluminescence based immune assay

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depends upon its bioavailability in the host which is determined by another host factor called vitamin D binding protein (VDBP). VDBP is another major determinant to the free and total circulating vitamin D concentration [6]; it binds 85–90% of the vitamin D in circulation [7], increases its reservoir in circulation, and aids in reabsorbing vitamin D filtered in the kidney.

One-third of the world's population harbours *M.Tb.* in an asymptomatic, latent form [latent tuberculosis infection (LTBI)] but retains a lifelong risk of future disease. Tuberculosis control relies on the identification and preventive treatment of individuals who are latently infected with M.Tb. One of the high risk groups for harbouring infection with a higher chance of developing the disease are the household contacts of pulmonary tuberculosis patients who are living in close contact with the infected individuals. Therefore, it is important to study the role of these host factors in TB patients along with their household contacts.

With this background, levels of Vitamin D and NO were investigated along with their associated molecules (VDR, VDBP, iNOS and cathelicidin) in active TB patients and household contacts as compared to healthy controls along with the assessment of the implication of these findings in susceptibility to TB.

2. Materials and methods

2.1. Study participants

The cross sectional study was carried out in the Department of Biochemistry and Medicine, AIIMS, New Delhi. 80 active TB patients, 75 household contacts and 70 healthy controls aged between 18–60 years were recruited for the study after obtaining written informed consent. Newly diagnosed laboratory confirmed active pulmonary TB (positive for sputum smear and gene expert) who had not received more than 2 weeks of anti-tubercular treatment were recruited from DOTS Centre, AIIMS, New Delhi. HIV, diabetes, hypertension and other inflammatory diseases were excluded on the basis of baseline laboratory investigations (hemogram, liver function tests, kidney function tests, fasting glucose levels and screening for HIV) and patient history along with thorough clinical examination. Household contacts of TB patients were recruited who had spent at least 6 h per day for at least 2 months with the TB patients with no signs and symptoms of TB. The exclusion criteria were same as for the active TB patients. Mantoux test was also done in household contacts. Mantoux negative individuals with no other clinical symptoms of TB were directly recruited under household contacts group. Mantoux positive individuals were followed up by sputum test or chest X-ray. Individuals negative for these tests were recruited under household contacts and those with positive test were further investigated for active disease. However, individuals negative for mantoux test were not assessed for their anergic status. Individuals with altered clinical parameters like hemogram, liver function test, kidney function test, blood glucose, etc and those who were taking vitamin D supplementation were excluded from the study. Healthy controls were recruited from the general population with no history of known contacts with TB patients and any clinical signs and symptoms of tuberculosis. The study was approved by the Institutional Ethics Committee (Ref NO: IEC/NP-299/07.08.2015, RP-03/2015). Clinical information from all the patients was recorded on a predesigned questionnaire and their written informed consent was taken before sample collection. The demographic profile has been shown in Table 1.

2.2. Sample collection and storage

6 mL of peripheral venous blood was collected from study participants and the specimen was given a personal identifier number that was used to link and maintain the biological information derived. 4 ml blood in plain vial was used for serum separation and the serum was stored at −70 °C for further analysis. 2 ml blood was collected in EDTA vials for isolation of DNA and RNA for single nucleotide polymorphism and mRNA expression studies respectively.

2.3. Quantitative PCR (qPCR) for mRNA expression study

RNA isolation was done from whole blood using TRIzol method and cDNA was synthesized using Verso cDNA synthesis kit (AB1453A, Thermo). Real time detection of VDR, VDBP, and iNOS was done using specific primers as shown in Table 2 (designed using PRIMER-BLAST) using 2X Maxima SYBR Green qPCR master mix (Thermo scientific). The PCR amplification was performed in a 10 µL reaction volume with cycling parameters as follows: For VDR and VDBP: Denaturation at 95 °C for 5 min, 35 cycles at 95 °C for 30 s, 64 °C for 45 s and 72 °C for 30 s and one final cycle of extension at 72 °C for 5 min. For iNOS: denaturation at 95 °C for 5 min, 35 cycles at 95 °C for 30 s, 54 °C for 45 s and 72 °C for 30 s and one final cycle of extension at 72 °C for 5 min. GAPDH was used as a reference gene. A melting curve analysis was performed after every reaction to confirm the specific amplification of products.

2.4. Estimation of serum levels of VDR, iNOS and cathelicidin

Serum levels of VDR (SEA475Hu, USCN, Life Science Inc., Wuhan, Hubei), cathelicidin (HK321 Hycult Biotech, Netherlands), and iNOS (201-12-0929, Sun Red, Biotechnology company) were measured in active TB patients, household contacts and healthy controls using commercially available Sandwich ELISA kits as per manufacturer's instructions.

2.5. Estimation of serum levels of vitamin D and nitric oxide (NO)

Serum levels of 25-OH Vitamin D were estimated in all the samples using automated chemiluminescence based immunoassay system (VITROS ECi, Johnson and Johnson Ortho Clinical Diagnostics). Competitive immunoassay was used where the light signals read by the system is inversely proportional to the concentration of 25-OH vitamin D present in the sample.

The serum levels of NO were measured by Biovision's colorimetric assay kit, (K262-200, Biovision) which employs the indirect quantitative estimation of NO using Greiss reaction. This is an indirect process of measurement of NO as it is rapidly oxidized into nitrate and nitrite. By this assay kit, nitrite is measured in a two simple step. In first step, nitrate is converted into nitrite using nitrate reductase. The second step uses greiss reagent to convert nitrite to a deep purple azo compound (N-alpha-naphthyl-ethylenediamine). The amount of the azochromophore accurately reflects nitric oxide amount in serum.

2.6. Statistical analysis

All statistical analyses were performed on GraphPad Prism 6 (GraphPad Software Inc., San Diego, CA, USA). Non-parametric statistical analyses were performed throughout the study. Mann-Whitney U and Kruskal-Wallis test was used for comparison of variables between two and three groups respectively. Values were expressed as median ± SD. Graphs were presented in median ± interquartile range. A p-value of less than 0.05 was considered significant.

3. Results

3.1. Study population

A total of 80 newly diagnosed active tuberculosis patients and 75 household contacts of active tuberculosis patients were recruited. 70 healthy individuals with no known history of tuberculosis were recruited as controls. The mean age of active tuberculosis patients was 36.2 ± 7.1 years, household contacts were 35.5 ± 6.9 years and

Table 1
Socio demographic and clinical characteristics of study participants.

Demographic characteristics	Active TB patients (n = 80)	Household contacts (n = 75)	Healthy controls(n = 70)
Age (Mean ± SD)	36.2 ± 7.1	35.5 ± 6.9	30.1 ± 5.4
Male (%)	76.25	73.33	87.14
Female (%)	23.75	26.67	12.86
Race	North Indian	North Indian	North Indian
Recruited in Winter (%)	20.23	16.46	12.67
Recruited in Spring (%)	31.45	32.34	36.54
Recruited in Summer (%)	37.32	40.23	38.23
Recruited in Autumn (%)	11.00	10.97	12.56
Smoking (%)	43.56	30.02	21.82
Non- vegetarian (%)	73.17	63.77	60.26
Vegetarian (%)	26.83	36.32	39.74
Indoor Occupation (%)	32.43	44.23	34.36
Outdoor Occupation (%)	67.57	55.77	65.64
Body Mass Index (Kg/m^2) in mean ± SD	18.6 ± 4.1	23.1 ± 3.6	21.6 ± 3.2
Blood Glucose levels (mmol/L)	5.2 ± 1.2	5.4 ± 0.7	4.2 ± 0.6
Serum albumin levels (g/dl)	3.72 ± 0.71	4.80 ± 0.28	4.37 ± 0.41
Abnormal chest X ray (%)	96.36	None	None
[a]Gene Xpert positive (%)	94.5	Not done	Not done
[b]AFB positive (%)	72.34	Not done	Not done

[a] Gene Xpert positive: Positive for nucleic acid amplification (NAA) test for *M.Tb.* genome present in TB cases.
[b] AFB positive: Positive for acid fast bacilli staining.

Table 2
Specific primer sequence for *vdr, vdbp and nos2*.

Gene	Protein	Accession number	Primer Sequence
For genotyping of Fok1 VDR polymorphism			
vdr	Vitamin D receptor	NG_008731.1	Forward:5'-AGCTGGCCCTGGCACTGACTCTGCTCT-3' Reverse:5'-ATGGAAACACCTTGCTTCTTCTCCCTC-3'
For mRNA expression of *vdr,vdbp and nos2*			
vdr	Vitamin D receptor	NM_001364085.1	Forward:5'- GGCCGGACCAGAAGCCTTT-3' Reverse: 5'- GGTGAATAGTGCCTTCCGCT-3'
vdbp	Vitamin D binding protein	NM_000583.3	Forward:5'-ACTACAGTATCCCCAGGGTG-3' Reverse: 5'-AGCACCTCCTCTCTCCTGTAG-3'
nos2	Inducible nitric oxide synthase	NM_000625.4	Forward:5'-TCCCGAAGTTCTCAAGGCAC-3' Reverse: 5'- GTGTCACTGGACTGGAGGTG-3'

healthy controls was 30.1 ± 5.4 years. The body mass index and serum albumin levels were found to be lower in active TB patients (Table 1).

3.2. Low mRNA expression of VDR, iNOS and VDBP in active TB patients as compared to household contacts and healthy controls

VDR, iNOS and VDBP mRNA expression in leukocytes of active TB patients and household contacts were measured by quantitative real-time PCR and were compared to healthy controls. VDR expression was compared by determining GAPDH normalized C_t values. Significantly lower expression of VDR and iNOS mRNA was observed in active TB patientsas compared to household contacts and healthy controls (p value < 0.0001 and 0.005 respectively) (Fig. 1a and c). Expression of VDBP mRNA was lower in active TB patients as compared to household contacts and healthy controls (p > 0.21) (Fig. 1b).

3.3. Lower serum levels of vitamin D, VDR and higher iNOS protein in active TB patients compared to household contacts and healthy controls

The serum levels of 25-OH vitamin D were measured using chemiluminescence based immunoassay and vitamin D receptor and iNOS by sandwich ELISA. Serum vitamin D levels varied among all the groups with significantly lowest levels seen in active TB patient group (11.60 ± 5.1 ng/mL) as compared to household contacts (20 ± 6.5 ng/mL), and healthy controls (21.50 ± 7.5 ng/mL) (p < 0.0001) (Fig. 2a). The levels were comparable between household contacts and healthy controls. Similarly, VDR levels were lower in active TB patients (4.67 ± 2.2 ng/mL) as compared to household contacts (6.28 ± 2.2 ng/mL) and healthy controls (6.19 ± 4.2 ng/mL) with p < 0.02 and 0.0005 respectively (Fig. 2b). The serum levels of iNOS protein were significantly higher in active TB patients (7.83 ± 3.3 ng/mL) as compared to household contacts (6.74 ± 2.36 ng/mL) and healthy controls (6.86 ± 2.43 ng/mL) with p-value < 0.01 and 0.05 respectively (Fig. 2c).

3.4. Higher serum levels of cathelicidin (LL-37) and nitric oxide (NO) in active TB patients compared to household contacts and healthy controls

The serum levels of cathelicidin (LL-37) were significantly higher in active TB patients (8.83 ± 5.8 ng/mL) as compared to household contacts (6.34 ± 5.2 ng/mL) and healthy controls (3.75 ± 4.1 ng/mL) with p-value < 0.005 and 0.0001 respectively. Significantly higher levels of cathelicidin was observed in household contacts compared to healthy controls (p < 0.007) (Fig. 3a).Similarly, it was observed that active TB patients had significantly higher levels of nitric oxide (12.99 ± 5.9 nmol/μl) in serum as compared to household contacts (9.78 ± 5.12 nmol/μl) and healthy controls (10.56 ± 5.38 nmol/μl) (p-value < 0.05and 0.05 respectively) (Fig. 3b).

4. Discussion

Tuberculosis is a major public health problem and a cause of concern of morbidity and mortality worldwide. It is estimated that 5–10% of one third of the world's population infected with *M.Tb.* develop this clinical disease. Household contacts who might be harbouring latent tuberculosis are most vulnerable to develop TB. Hence, identifying such

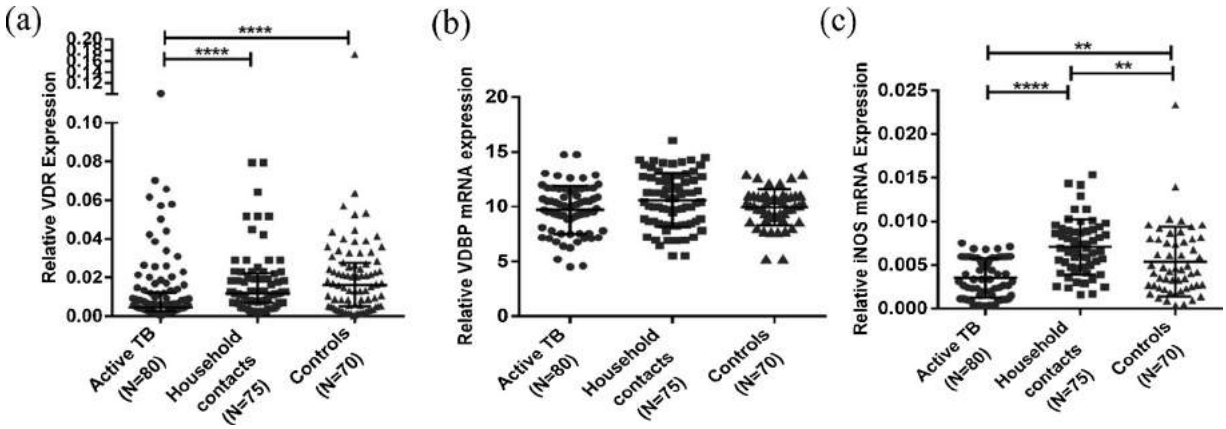

Fig. 1. mRNA expression of VDR, VDBP and iNOS in active TB patients, household contacts and healthy controls.1a, 1b and 1c show the mRNA expression of VDR, VDBP and iNOS respectively in active TB patients, household contacts and healthy controls. Data is represented as median ± interquartile range and each point represents individual sample value. Mann-Whitney U test was used for comparison of values between groups. One asterisk (*) indicates a p-value < 0.05; two asterisks (**) indicate a p-value < 0.01, three asterisks (***) indicate a p-value < 0.001 and four asterisks (****) indicate a p-value < 0.0001. Active TB = Naïve active pulmonary TB; Household contacts = contacts living with the pulmonary TB patients for minimum of 2 months with no sign and symptoms of TB; Control = Healthy controls with no history of TB.

cases and prevention of activation of TB in them will help bring down the incidence of new cases. So far, most of the studies have been performed on bacterial aspects and very few on host aspect. Thus, host factors responsible for the protection, pathogenesis and activation of the disease need to be studied thoroughly. Some of them include environmental, lifestyle and immunological risk factors which are likely to contribute towards susceptibility to the disease.

Vitamin D, an immunomodulator, is one such factor and the current study was designed to assess the role of vitamin D and its associated molecules in the pathophysiology of pulmonary tuberculosis. Decreased vitamin D levels were observed in active pulmonary TB patients as compared to household contacts and healthy controls. These results are in concordance with previous studies showing lower serum levels of vitamin D in tuberculosis patients [8,9]. In type 2 diabetes patients, when Monocytes Derived Macrophages (MDMs) with low VDR expression were supplemented with vitamin D, MDMs eliminated M.Tb. efficiently [10]. Some studies involving clinical trials have shown that vitamin D supplementation with phenyl butyrate improved the clinical symptoms especially in vitamin D deficient TB patients [11,12] providing evidence for protective role of vitamin D in TB patients.

Vitamin D circulates in the blood in protein bound form with vitamin D binding protein (VDBP) and albumin. Any deficiency of these transport proteins may affect vitamin D availability to the immune and other cells. Therefore VDBP and albumin levels were checked. Lower mRNA expression levels of VDBP along with significantly low serum albumin levels were seen in active TB patients as compared to household contacts and healthy controls. This points toward the fact that VDBP and albumin deficiency could play a role in vitamin D deficiency states. This could be attributed to overall nutritional deficiency in tuberculosis.

Since, vitamin D acts via binding to its nuclear receptor VDR, the expression levels of VDR at both mRNA and protein levels were compared. Significant lower expression of VDR (both mRNA and protein) in active TB patients compared to household contacts and healthy controls was observed. In one of the previous studies, expression of VDR mRNA was found to be significantly higher in *M.Tb.*-stimulated macrophage cultures compared to unstimulated cultures [13]. The results of the current study are in accordance with a study in which lower expression of VDR was observed in leprosy patients as compared to controls [14]. Another study based on microarray has shown a 3.3 fold

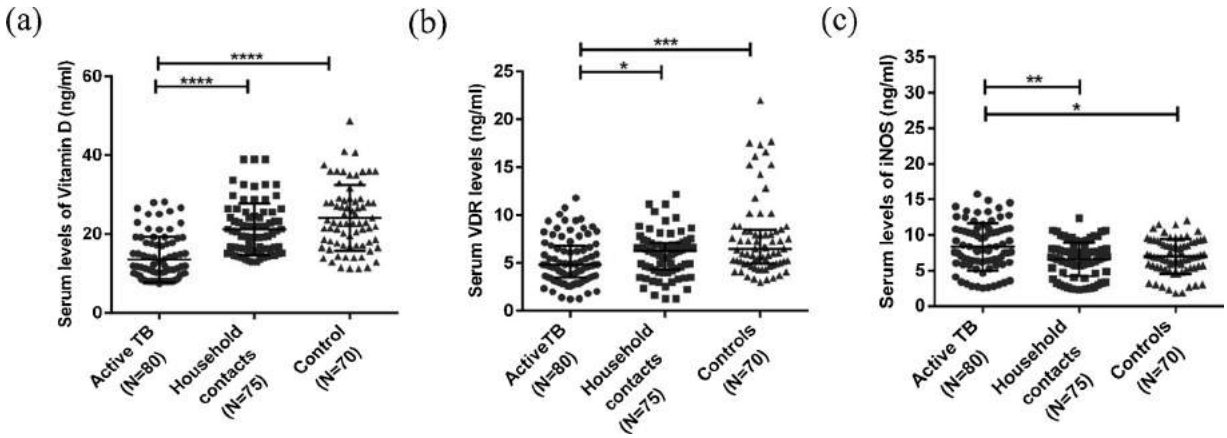

Fig. 2. Serum levels of Vitamin D, VDR, and iNOS in active TB patients, household contacts and healthy controls. 2a, 2b and 2c show serum levels of Vitamin D, VDR protein and iNOS protein respectively in active TB patients, household contacts and healthy controls. Data is represented as median ± interquartile range and each point represents individual sample value. Mann-Whitney U test was used for comparison of values between groups. One asterisk (*) indicates a p-value < 0.05; two asterisks (**) indicate a p-value < 0.01, three asterisks (***) indicate a p-value < 0.001 and four asterisks (****) indicate a p-value < 0.0001. Active TB = Naïve active pulmonary TB; Household contacts = contacts living with the pulmonary TB patients for minimum of 2 months with no sign and symptoms of TB; Control = Healthy controls with no history of TB.

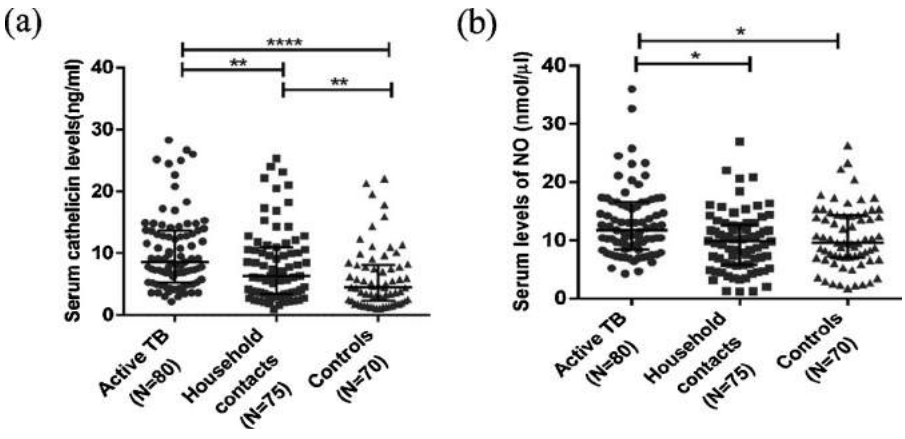

Fig. 3. Serum levels of Cathelicidin (LL-37) and Nitric Oxide in active TB patients, household contacts and healthy controls. 3a and 3c show serum levels of cathelicidin (LL-37) and Nitric oxide (NO) in active TB patients, household contacts and healthy controls. Data is represented as median ± interquartile range and each point represents individual sample value. Mann-Whitney U test was used for comparison of values between two groups. One asterisk (*) indicates a p-value < 0.05; two asterisks (**) indicate a p-value < 0.01, three asterisks (***) indicate a p-value < 0.001 and four asterisks (****) indicate a p-value < 0.0001. Active TB = Naïve active pulmonary TB; Household contacts = contacts living with the pulmonary TB patients for minimum of 2 months with no sign and symptoms of TB; Control = Healthy controls with no history of TB.

downregulation in VDR expression in cells upon infection with *M.Tb.* [15]. Lower expression of VDR in the active TB patients may be due to an attempt by *M.Tb.* to downregulate the expression of VDR along with the other host genes for its own advantage, however the exact mechanisms need to be studied. Additionally, low vitamin D levels in active TB patients might be one of the factors for decreased expression of VDR as vitamin D has been shown to regulate the expression of its own receptor [16,17].

Vitamin D along with VDR regulates expression of various genes including antimicrobial peptide cathelicidin. Intracellular cathelicidin levels could be a limiting factor in *M.Tb.* killing. Despite low vitamin D and VDR levels, cathelicidin levels in serum were found to be higher in active TB patients as compared to both household contacts and healthy controls. These findings are in line with previous report on cathelicidin levels being enhanced in active TB patients [18,19]. Alveolar macrophages (AMs) have been shown as most efficient cells at producing cathelicidin after infection with M. Tb. suggesting that cathelicidin from AMs may be an important participant in the innate immune response during early infection in humans [20]. Increase in cathelicidin levels in active disease could be explained by a study done in an *in vitro* model where multiplication of bacteria stimulated TLR by pathogen-derived ligand like bacterial lipopeptides, which acted as a direct trigger for upregulation of cathelicidin to exert its anti-microbial activity [4]. However, in the present study, the cathelicidin levels estimated in serum depicted systemic levels, although localized or intracellular cathelicidin (LL-37) concentrations need to be evaluated for better understanding of interaction between vitamin D status and localized LL-37 responses within immune cells and at localized immunologic barrier sites.

Additionally, higher levels of cathelicidin in household contacts were observed as compared to healthy controls. A study has shown that cathelicidin antimicrobial peptide gene expression was significantly higher in progressive TB, whereas in latent TB it was similar to the control group [21]. Based upon our findings, it can be hypothesized that household contacts of TB patients might have undetected latent infection which can be the reason for the increased level of cathelicidin compared to healthy controls, though lower compared to active TB patients where active multiplication of *M.Tb.*is going on. Increased levels of cathelicidin in active TB group might be the result of increased bacterial load through the inflammatory pathway [4,5], which may not have been enough to control overwhelming bacterial load.

As vitamin D has been shown to induce iNOS synthase [5], the decreased vitamin D levels observed in active TB patients might have contributed to decrease in iNOS mRNA expression in active TB patients as compared to household contacts and healthy controls. Also, our results are in accordance with one of the previous findings where *M.Tb.* was shown to downregulate iNOS expression [22]. However, the levels of iNOS protein and NO were seen to be higher in active TB patients as compared to household contacts followed by healthy controls. Lower iNOS mRNA levels with high iNOS protein levels in active TB patients might suggest potential alteration in mRNA levels or protein stability via IL-13 and L- arginine [23]. In an in vitro experimental model L-arginine supplementation has been shown to induce *H.pylori*-stimulated iNOS protein translation and NO production [24]. However, the mechanism behind potential alterations at the level of protein translation and stabilization is not fully understood and needs to be studied further in experimental models of *M.Tb.* infection.

This is the first study showing altered mRNA expression of iNOS in household contacts of TB patients that have shown higher expression of iNOS as compared to both active TB patients and healthy controls. Higher expression of iNOS in household contacts could be due to reactivation of latent infection or a fresh exposure from active TB patient. But the infection might be curtailed due to enough cathelicidin and NO as significantly higher levels of cathelicidin and NO was seen compared to normal healthy control group. This hypothesis is also supported by the fact that vitamin D levels required for synthesis of cathelicidin and NO were sufficient as household contacts were found to have significantly higher levels of vitamin D compared to active TB patients. However, this needs confirmation with follow up study of household contacts for development of disease.

In conclusion, higher levels of vitamin D along with VDR and iNOS expression in household contacts as compared to active TB patients suggest plausible protective role of vitamin D against TB. Based on the present findings, vitamin D could be implicated in innate immunity of the host via production of antimicrobial agents to contain bacterial multiplication. High risk household contacts despite being exposed to active TB patients did not develop active disease possibly due to vitamin D levels required for production of cathelicidin and NO levels were sufficient enough to contain the initial infection. However, in case of active TB patients, suboptimal vitamin D levels may have led to initial low production of cathelicidin and NO insufficient to contain the bacilli. However later, increased cathelicidin and NO levels in active TB patients could be attributed to overwhelming bacterial load and may serve as potential biomarkers in diagnosis of TB amongst high risk household contacts of TB. Also, the present study warrants further studies to determine the role of vitamin D supplementation in the prevention and treatment of tuberculosis.

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