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Interplay of Chemo Attractant Peptides (Cathelicidin and Chemerin) with Vitamin- D in Patients with Pulmonary Tuberculosis

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Authors' contributions

This work was carried out in collaboration between all authors. Author NTI Principal Investigator of grant, optimized assays and carried out bench testing, analyzed the data and wrote the manuscript. Author SSF contributed in lab experiments and assist in manuscript writing. Author RH co-principal Investigator performed, scientific contribution in data analysis and manuscript writing. Author NAR identified TB patients assisted PI in recruitment of TB patients. Author NV performed all experiments and recruited TB patients and healthy controls. Author BJ collaborator of our study, contributed in patient recruitment and classification of TB. Author MI consultant pulmonologist helped PI in diagnosis and classification of TB patients. All authors read and approved the final manuscript.

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ABSTRACT

Aim: Both Cathelicidin and Chemerin are chemo-attractant proteins and possess antimicrobial activity. Sufficient level of Vitamin D is important for optimum response of Cathelicidin for its anti-mycobacterial activity. Studies on the role of these antimicrobial peptides and their relationship with Vitamin D level are limited in tuberculosis. The aim of this study was to investigate an association of Vitamin D with antimicrobial peptide (Cathelicidin) and an adipokine (Chemerin) in patients with pulmonary tuberculosis (TB).

Methods: In a case control study we estimated level of Vitamin D, Chemerin, Cathelicidin and TNF α in pulmonary TB patients (n=22) and healthy endemic controls (n=17) using sandwich ELISA methodology. The study was conducted at Aga Khan University Karachi during 2011.

Results: TB group had higher proportion of subjects above median level of Cathelicidin (median test; p=0.034) and fewer number of subjects with Chemerin (median test; p=0.001). Pairwise comparison also showed significant differences between average ranks of Vitamin D vs. Cathelicidin (p<0.0001), Chemerin vs. Cathelicidin (p=0.04) and Vitamin D vs. TNF α (p<0.0001). Cathelicidin was identified as most discriminatory marker between TB disease and healthy group (ROC, AUC 0.780; p=0.007).

Conclusion: Our results highlight the role of Cathelicidin as a potential biomarker of active TB disease. The role of Cathelicidin and Chemerin as plausible biomarkers requires further studies in both inflammatory and non-inflammatory conditions.

Keywords: Tuberculosis; vitamin D; cathelicidin; chemerin.

1. INTRODUCTION

Tuberculosis (TB) is the leading cause of mortality and morbidity in developing world. In 2012, 0.5 million new incident cases were reported in Pakistan with an incidence of 231/100,000 population [1]. Pakistan ranks 6th among 22 highest TB burden countries [1]. Profound Vitamin D deficiency has been reported among new incident TB cases [2] and healthy volunteers in Pakistan [3]. Sufficient level of Vitamin D is important for macrophage activation for antimicrobial response [4]. The mechanism of activation of macrophages via Vitamin D pathway for anti-mycobacterial activity has already been delineated [5]. The stimulation of Toll like receptors (TLRs) on macrophages results in activation of Cathelicidin gene (*hCAP18*) by 1- α hydroxylase enzyme gene (*CYP27B1*) [6] to convert inactive Vitamin D [25(OH)D₃] in to active form [1,25-(OH)₂D₃]. A limited number of studies have shown relationship of circulating levels of inactive Vitamin D [25(OH)D₃] and Cathelicidin in clinical studies, such as TB [7] and sepsis [8]. Cathelicidin family of peptides are 23-40 amino acid long chain with α helical structure. Cathelicidin possess antimicrobial property because of its amphipathic topology and presence of negatively charged residue. Cathelicidin also up-regulates chemokine receptors on macrophages [9] and is involved in migration of immune cells to the site of infection.

Antimicrobial effect of Cathelicidin is very well studied against *M. tuberculosis* (M.tb) [10]. However, the anti-mycobacterial effect of Chemerin is not fully discovered. Chemerin is a non-glycosylated 16 kDa protein secreted as precursor of 143 amino acid residues known as Prochem 163S. This multifunctional protein primarily known for its adipokine and chemotactic activity for immune cells [Plasmacytoid (PDCs), NK and Macrophages]. Proteolytic cleavage of pro-chemerin molecule by cathepsin results in formation of Chem 156 isoform which possess greater affinity for chemokine receptor like 1 (CMKLR1). Chemerin is structurally similar to N terminus Cathelin domain of human Cathelicidin. The predominant function of Chemerin is glucose homeostasis [11,12]. Due to its involvement in regulation of immune cells and antimicrobial activity, it is intriguing to address the association of Chemerin with chronic inflammation such as TB. Its association in diabetes and obesity has already been established [13]. In countries with high burden of TB, the rate of pre-diabetes and Diabetes Mellitus (DM) is 24-25% among newly diagnosed TB patients [14]. Animal models of DM have also shown phenomenon of delayed inflammatory response in granuloma formation after aerosol challenge of M.tb [15,16]. The relationship of low serum Vitamin D with incidence of DM [17] has significantly move the field to study the association of DM co-infected with TB [18,19]. It is hypothesized that low serum Vitamin D may promote pro-inflammatory

cytokines milieu which is favorable for progression of TB with or without comorbidity of DM. It is therefore interesting to understand the complex relationship of Chemerin (marker of obesity and Diabetes) with Cathelicidin and Vitamin D in TB patients. This relationship is described in a conceptual diagram in Fig. 1.

The aim of our study is to assess the relationship of two related chemoattractant peptides, Cathelicidin and Chemerin with Vitamin D and pro-inflammatory cytokine (TNF α) in TB cases and healthy controls.

2. MATERIALS AND METHODS

2.1 Study Design

Twenty two (n=22) pulmonary TB patients (TB) were recruited from The Aga Khan University and Abbasi Shaheed Hospital outpatient clinics in this case control study. The chest physicians at both hospitals identified and referred the pulmonary TB patients as per study inclusion and exclusion criteria. Pulmonary disease was

confirmed by chest X-ray, Acid Fast Bacilli (AFB) sputum microscopy and history of TB symptoms. Majority of TB patients had moderate (PMD) (n=9) and, advanced (PAD) (n=7) pulmonary disease, two patients were characterized as minimal disease (PMD) and information was not available for 3 patients. The severity of disease classification was based on radiological findings, and extent of lung tissue involvement as per defined criteria [20]. Healthy endemic controls (EC; n=17) had no history of TB exposure and were recruited from administrative staff and research technologists of AKU research and teaching lab.

Study protocol was approved by Ethical Review Committee (ERC) of Aga Khan University (1689-Path-ERC-10). All participants gave written informed consent.

2.1.1 Inclusion criteria

The inclusion criteria for TB group (n=22) were active pulmonary TB diagnosed as per WHO guidelines [21] within one month of treatment.

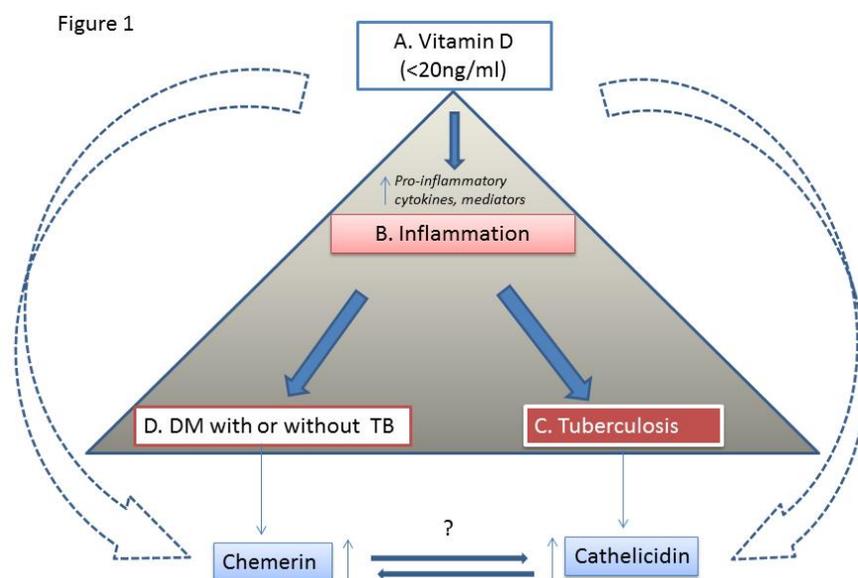


Fig. 1. Conceptual diagram of hypothetical relationship between vitamin D, Cathelicidin and Chemerin

Low vitamin D i.e less than 20 ng/ml (A) triggers a pro-inflammatory cytokine milieu, which leads to chronic inflammatory state (B). Reactivation of latent TB infection due to immunosuppression and disturbance of Th1 and Th2 cytokines axis may lead to progression of active disease (C), which is represented by elevated level of Cathelicidin. Chronic inflammatory state may lead to diabetes mellitus with or without co-incident TB (D) which is indicated by increase Chemerin level. The dashed arrow shows the relationship of Vitamin D to Chemerin and Cathelicidin

2.1.2 Exclusion criteria

Patients with treatment relapse or past TB history, extra-pulmonary TB and known MDR TB were not included in the study. TB patients with prior or current Vitamin D supplementation/injections, co-morbid conditions such as DM, malignancies or auto immune diseases were also excluded from the study.

Seventeen healthy controls were employees of AKU. The inclusion criteria for healthy controls (n=17) were no current or past TB diagnosis/DM/chronic infections and no current or prior intervention with Vitamin D. Healthcare workers and lab staff directly exposed to TB were also excluded.

2.2 Collection of Blood for Plasma and Whole Blood Stimulation Assays

Five ml blood was collected in 15 ml of conical tube (BD Falcon) containing sodium heparin (20 U/ml; Leo Pharmaceuticals, Ballerup, Denmark). Heparinized blood was diluted 1:4 with RPMI 1640 (supplemented with Pen/Strep, 2 mM L-Glutamine) for use in stimulation assays. Two ml of blood was collected separately in EDTA tube and centrifuged for 5 minutes at 2000 x g to separate clear portion i.e. plasma within 2hrs of collection. Plasma was stored in small aliquots and kept at -70°C until further use.

2.2.1 Preparation of active vitamin D [1, 25-(OH)₂D₃] for *In vitro* experiments

The 1, 25-(OH)₂D₃ Vitamin D was purchased from Sigma Chemicals (Sigma, St. Louis, USA). The crystallized form of Vitamin D was dissolved in reagent grade ethanol (EtOH) (Merck laboratories). A ten-fold higher working stock for each of the dilution was prepared to obtain a final concentration 0.1 μM in 500μl of whole blood cultures. The concentration of 1,25-(OH)₂D₃ for invitro experiments were consistent with previous reports for stimulation of PBMCs [22] and lympho-proliferative responses [23].

2.2.2 Stimulation of whole blood with active vitamin D [1, 25-(OH)₂D₃] for cytokine secretion in supernatants

Stimulation of whole blood cultures with Vitamin D [1, 25-(OH)₂D₃]. The whole blood cultures were stimulated with 0.1μM concentration of 1, 25-(OH)₂D₃ (Sigma, St, Louis USA) in 24 well tissue culture plates (Flow laboratories, UK).

Tissue culture plates (24 wells) were preloaded with 1, 25-(OH)₂D₃ in 50μl volume. Ethanol was allowed to evaporate, leaving 1, 25-(OH)₂D₃ in 0.1μM concentration in a well followed by 500 μl of diluted whole blood. The control well for Vitamin D was EtOH (vehicle control).

Supernatants collected at day 1 and day 3 post stimulation were stored as 4 x 100 microliters aliquots in -80°C until use.

2.2.3 Assessment of cytokines (TNFα, IFNγ and IL10) in stimulated whole blood cultures and plasma

Cytokine assessment was carried out using pairs of monoclonal antibodies from Endogen, Rockford IL, USA. Protocols for cytokine assessment have been described in detail elsewhere [24]. Briefly, Immulon4 plates were coated with capture antibodies overnight at 4°C. Next day plates were blocked with BSA and subsequently incubated with 100 μl of culture supernatant. Revealing and probing antibodies were added after appropriate incubation. All probing antibodies were labeled with biotin and revealing antibodies were labeled with avidin bound to horse radish peroxidase (HRP). Finally plates were developed with HRP substrate. All plates were read on plate reader (Biorad, CA, USA). Optical densities were read against known concentration of standards (1000 pg-7.8 pg.) of cytokines to determine the concentrations in test samples [24].

2.2.4 Estimation of inactive form of vitamin D [25(OH)D₃] in plasma

Baseline Vitamin D levels were assessed in plasma samples of TB patients and healthy controls. Circulating Vitamin D was measured by ELISA using the Immuno Diagnostic System Ltd [IDS, Fountain Hill, AZ, USA]. All protocols were followed according to manufacturer instructions. Each test was run in duplicate, with mean absorbance of test samples computed from the average for two wells normalized for 0 calibrator well. Concentrations of Vitamin D in test samples were derived by fitting a 2 parameter logistic curve to 6 standard concentrations, and was expressed as ng/ml (1 nmol/L * 0.4= 1 ng/ml) [2].

2.2.5 Estimation of Cathelicidin peptide by ELISA

Estimation of Cathelicidin peptide was carried out by ELISA (Hycult Biotechnology, AA Uden The

Netherlands) assay in plasma samples. All protocols were followed according to manufacturer instructions. Each sample was run in duplicate. The minimum measurable concentration was between 0.1 ng/ml to up to 100 ng/ml of Cathelicidin. The unknown concentration was derived by plotting absorbance of samples against known concentrations of standards. All standard curve has R2 greater than 0.90.

2.2.6 Estimation of Chemerin peptide by ELISA

Chemerin levels in plasma samples were determined using a commercially available sandwich ELISA kit (Glory Bioscience, USA, cat #11406). The inter-assay coefficient of variation was less than 10%, and the within-assay coefficient of variation was less than 5%. The sensitivity of the ELISA assay was 0.5–10 ng/ml, and the midrange of the assay was 5 ng/ml. The least detectable concentration of human Chemerin was 0.5 ng/ml.

2.3 Statistical Methods

Data was analyzed by statistical software of SPSS 19 and Microsoft Excel. Mann Whitney U test and Median test was applied for comparison of median difference in two groups. Friedman two way test was applied for comparison of ranks between two analytes. ROC analysis was carried out for identification of discriminatory marker between TB and EC groups. A p value <0.05 was considered a significant between groups.

3. RESULTS AND DISCUSSION

3.1 Characteristics of the Study Groups

TB group comprised of (54%) of male and (45%) female. The mean age of TB and EC groups were 25.136±8.697yrs (range 16-50yrs), and 31.647±5.219yrs (24-46yrs) respectively. BCG scar was present in 27% of TB patients and up to 90% in healthy controls. Overall incidence of HIV in TB cases is 0.96% in Pakistan [1]. TB patients were not screened for HIV positivity.

3.2 Relationship of circulating level of Cathelicidin, Chemerin and Vitamin D in TB and EC Group

The criteria for plasma Vitamin D [25(OH)D3] deficiency is based on international convention (reference plasma levels) (deficient = <20 ng/ml;

insufficient =21-29 ng/ml; sufficient >30 ng/ml) [4]. A majority (71%; N= 28/39) of study participants had deficient Vitamin D levels [<20 ng/ml of plasma 25(OH) D3].

Fig. 2 shows the comparison of Chemerin (Fig. 2a), Cathelicidin (Fig. 2b) Vitamin D (Fig. 2c) and TNF α (Fig. 2d) between TB and EC groups in box plots. In TB group, the level of Chemerin was significantly lower compared to controls (p=0.02). The average weight of TB patients was 41.22±3.77kg, and that of EC was 63.83±12.31 kg (T test; p=0.00004). The lower level of Chemerin in TB group is presumably due to lower body weight or less adipocytes.

We next compared the Cathelicidin and TNF α among TB and EC group, the level of Cathelicidin and TNF α are presumably higher in TB patients, although the medians are not significantly different. Cathelicidin and TNF α showed similar trends in box plots which were later confirmed by scatter plot that showed a linear relationship (Fig. 4). There was no difference in Vitamin D level between TB and EC group.

In order to determine the pairwise comparison of biomarkers, we performed rank order analysis of whole study group to observe the trend of markers across the board. Table 1 shows the distribution of Cathelicidin, Chemerin and Vitamin D using rank order test. The average rank for Cathelicidin was 2.49 followed by 1.95 in Chemerin and 1.57 in Vitamin D. The difference between average ranks of Cathelicidin and Chemerin are significantly different (p=0.04). Similarly the average ranks of Vitamin D and Cathelicidin are also significantly different (p<0.0001). However, no such relationship was observed between Vitamin D and Chemerin. We also observed a higher average rank for TNF α cytokine (4.00) compared to other variables (p<0.0001).

We next compared Cathelicidin and Chemerin levels in TB and EC groups. For this analysis, groups were divided in two categories of >median and \leq median values of Cathelicidin (Fig 3A) and Chemerin (Fig 3B). The median value of Cathelicidin was 42.74 (IQR: 32.120-78.650) in total study subjects. Fourteen of twenty two (14/22) (63.6%) TB patients had Cathelicidin level greater than 42.74 ng/ml compared to 5/17 (29.4%) of EC group. For \leq median category, 12/17(70%) of EC had less than 42.72 ng/ml of Cathelicidin compared to 8/22 (36%) in TB (Median test; p=0.03).

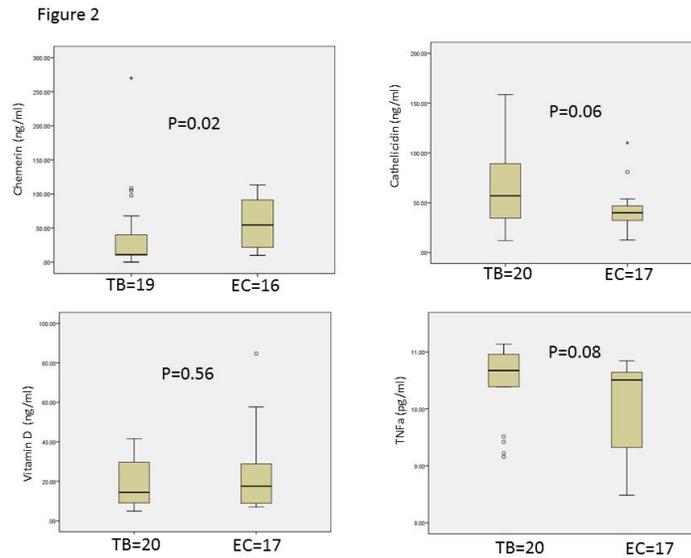


Fig. 2. Distribution of antimicrobial peptide (cathelicidin), adipokine (chemerin), vitamin D and TNFα in TB and EC groups

Box plot shows the serum level of Chemerin(A), Cathelicidin(B), 25(OH)D3 (C), and TNFα (D) in TB and EC groups. The results are shown as median, 25th and 75th percentiles. Asterisks and circles are shown as outliers. The estimation of Chemerin, Cathelicidin and vitamin D in blood was carried out by commercial sandwich ELISA kits. Vitamin D was estimated by inhibition ELISA technique where as Cathelicidin and Chemerin was done by regular ELISA. TNFα detection was carried out by in-house Sandwich ELISA using pairs of monoclonal antibodies. Mann Whitney U test was applied for comparison of significant difference in two groups. P<0.05 was considered significant

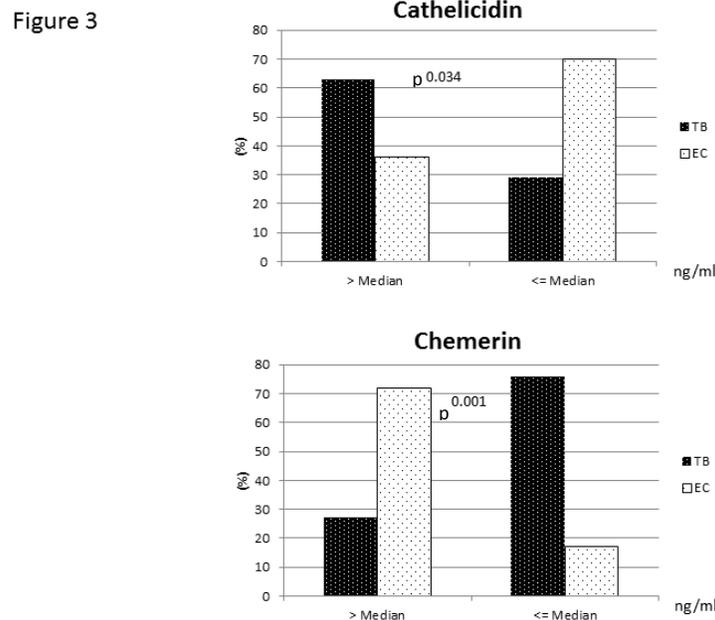


Fig. 3. Elevated level of Cathelicidin but not Chemerin in TB patients

The bar graph shows the proportion of TB and EC group in two categories of >median and < median of Cathelicidin (A) and Chemerin (B). X-axis shows the median categories of Cathelicidin and chemerinin ng/ml and Y-axis shows the % in each category. All details of experiments are same as of Fig. 2. Median test was applied to test the proportion of subjects in two categories

Table 1. Pairwise comparison of vitamin D, Cathelicidin, Chemerin and TNF α in study subjects (n=39)

Relationship between variables	Average rank	# Significance
i. Vitamin D- Chemerin	1.57-1.95	0.311
ii. Vitamin D- Cathelicidin	1.57-2.49	<0.0001
iii. Vitamin D-TNF α -	1.47-4.00	<0.0001
iv. Chemerin- Cathelicidin	1.95-2.49	0.040
v. Chemerin-TNF α	1.95-4.00	<0.0001
vi. Cathelicidin - TNF α	2.49-4.00	<0.0001

*Friedman's two-way analysis of variance by ranks

The median value of Chemerin was 17.24 (IQR: 10.13-54.34). TB group had 6/22(27%) of cases above the median value compared to 13/17 (76%) of EC. For \leq median category, 16/22 (72%) of TB had less than 17.24 ng/ml of Chemerin compared to only 3/17 (18%) in EC group (Median test, $p=0.001$). Median test for Vitamin D was not significant as it was observed that both groups were equally deficient for Vitamin D. These results show non homogenous distribution of two peptides in this study group.

3.3 Correlation of Peptides (Cathelicidin and Chemerin) with Vitamin D and TNF α

Fig. 4 shows the correlation of Vitamin D and Cathelicidin (A), Chemerin and Cathelicidin (B), TNF α and Cathelicidin (C) and TNF α and Chemerin (D). There was a negative correlation between Vitamin D and Cathelicidin ($\rho=0.189$, $p=0.248$) and Vitamin D and Chemerin ($\rho=0.132$, $p=0.430$). Chemerin and Cathelicidin also showed no correlation ($\rho=0.023$, $p=0.894$). However, the numbers are fewer to draw a definitive conclusion about this relationship. TNF α showed a trend of positive correlation with Cathelicidin ($\rho=0.367$, $p=0.033$) and negative correlation with Chemerin ($\rho=0.191$, $p=0.287$) and Vitamin D ($\rho=0.230$, $p=0.185$). The trend of positive correlation between TNF α and Cathelicidin indicates simultaneous regulation of both markers in inflammatory state.

3.4 ROC Analysis for Biomarker of TB

In order to assess the discriminatory power of markers in TB vs. EC groups, we carried out ROC analysis (Fig. 5). In ROC analysis, Cathelicidin was identified as most discriminatory marker with AUC of 0.780 ($p=0.007$), followed by TNF α (AUC of 0.673 ($p=0.097$)). Both Vitamin D and Chemerin had no discriminatory power. This further confirms no relationship of Cathelicidin

and Chemerin in TB disease. High level of Cathelicidin in TB patient was also shown in Fig. 2 with marginal significance compared to EC group ($p=0.06$). Cathelicidin is also emerging as a marker of acute inflammation in TB patients, which was also confirmed by ROC analysis (Fig. 5).

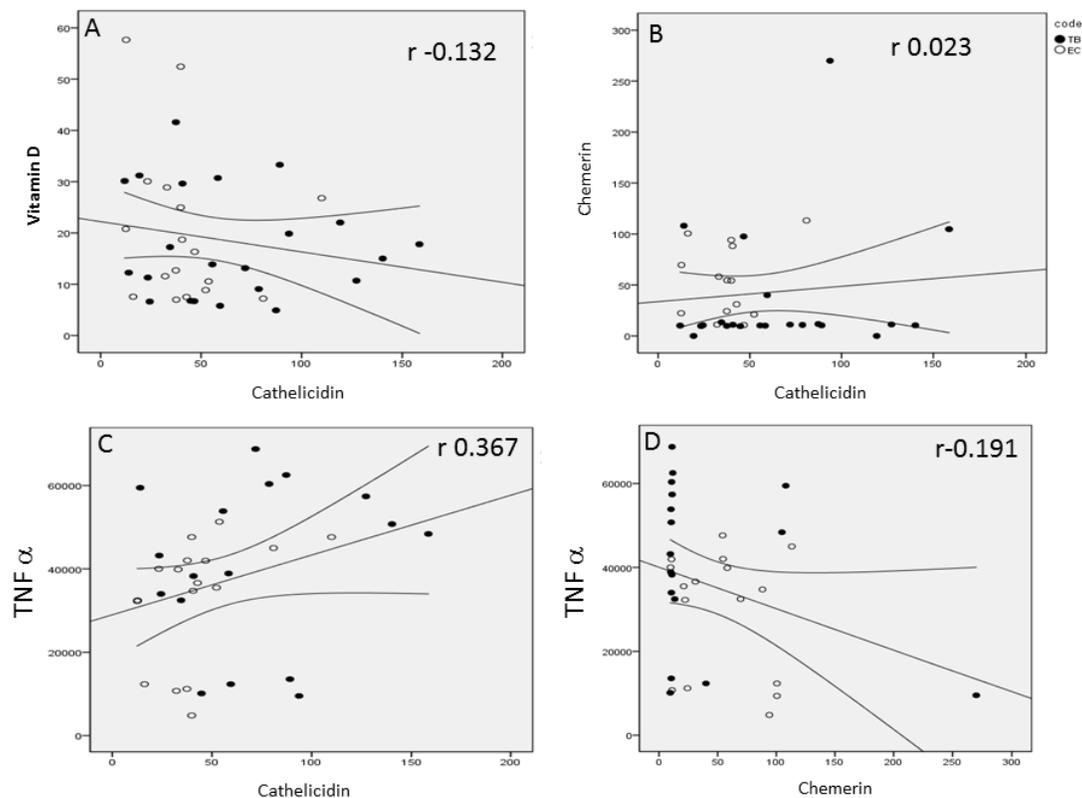
To confirm an anti-inflammatory role of Vitamin D, we also performed whole blood stimulation assay with active form of Vitamin D [1,25-(OH) $_2$ D $_3$]. For this analysis, study group was divided in to three categories of Vitamin D and effect of stimulation of active form of Vitamin D was observed on IFN γ and IL10 cytokines. The results of whole blood assay showed an up regulation of IL-10 but not IFN γ in Vitamin D deficient group (Wilcoxon; $p=0.04$) (suppl. Fig. 1).

4. DISCUSSION

The current study was carried out to delineate the relationship of two chemotactic and antimicrobial peptides in TB and EC groups with similar level of Vitamin D deficiency.

Chemerin and Cathelicidin have structural homology at N terminus region of hCAP18 [25]. Both peptides are potent chemoattractant and possess antimicrobial activity against microbes [26]. The antibacterial activity was particularly exhibited by truncated peptides, Chem S 157 (truncated bioactive Chemerin lacking 6 amino acids) and S 155 against *E. coli* and *K. pneumoniae* [26]. These peptides also possess chemoattractant property for CMKLR1+ blood cells. Chemerin has been identified as a marker of pre-diabetic stage [27], whereas Cathelicidin as a marker of acute inflammation and probable biomarker of TB [28]. Vitamin D deficiency is prevalent worldwide including Pakistan [2]. National Nutrition Survey of Pakistan 2011, reported a widespread deficiency of Vitamin D in

Figure 4

**Fig. 4. Correlation of biomarkers**

Correlations of biomarkers are shown in scatter plot. A) Vitamin D vs. Cathelicidin. B) Chemerin vs. Cathelicidin. C) TNF α vs. Cathelicidin. D) TNF α vs. Chemerin. Straight lines indicate linear relationship between biomarkers and curve lines are 95% CI among points lie close to linear line. The open and filled circles indicate EC and TB groups respectively

both pregnant and non-pregnant women [29]. This deficiency was prevalent in both urban (87.9%) and rural (84.2%) women of child bearing age. Low serum Vitamin D was also correlated with Cathelicidin in sepsis patients [8] and TB patients with smear positivity but not with over all vitamin D status [7].

The increasing evidence of Vitamin D deficiency in both TB and diabetes has prompted us to investigate the actual relationship of markers of TB and Diabetes in context of Vitamin D. To our knowledge, this is the first study in Pakistani subjects delineating intricate relationship of Cathelicidin level with Chemerin in context of Vitamin D deficiency. The induction of Cathelicidin is dependent on sufficient level of active form of Vitamin D, which is also important for anti-mycobacterial activity of macrophages. However, our results showed no linear

relationship between circulating inactive Vitamin D [25(OH) D3] and Cathelicidin peptide (Cathelicidin) (Fig. 4). This was also confirmed by ROC analysis where Cathelicidin showed up as a single marker to discriminate TB group from healthy controls with 80% sensitivity and 40% specificity. Cathelicidin is also emerging as a new biomarker of TB disease [28]. A nonlinear relationship of Cathelicidin with Vitamin D was also reported in TB patients, where high level of Cathelicidin was associated with high AFB positivity or disease severity [7]. Similar finding was reported by Iacob et al. [30] in HCV infected patients. The other reports had conflicting results about correlation of Cathelicidin and Vitamin D in healthy controls [31,32]. Dixon et al reported positive correlation between Cathelicidin and Vitamin D using <32 ng/ml cutoff in healthy controls. In contrast to this, another report showed a positive correlation with Vitamin D, and

Figure 5

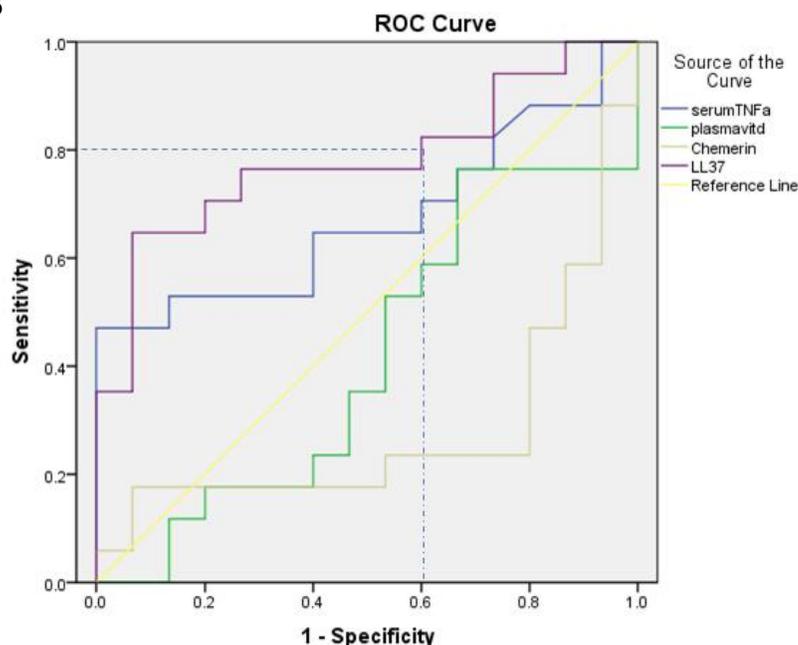


Fig. 5. ROC analysis of biomarkers

Receiver Operating Characteristic (ROC) analysis was performed on serum Chemerin (brown), TNF α (blue) and vitamin D (green): TB ($n=20$) was compared using control ($n=17$) as non-disease state. The coordinate line is shown in yellow. The dotted line shows the arbitrary cutoff for sensitivity and specificity of Cathelicidin

a trend of age dependent decrease in Cathelicidin. To confirm the role of Cathelicidin as a marker of inflammation, we also looked at TNF α in both study groups (TB and EC), which showed a direct linear relationship with Cathelicidin (Fig. 4D).

Due to the ubiquitous expression of Chemerin in inflammatory and metabolic pathway, its regulation is broadly classified as agonists for nuclear receptors, and immuno-modulators for chronic inflammation. The pathway of Chemerin release is meant to be regulated by Vitamin D receptor (VDR) [33]. An increase in expression of Chemerin mRNA was observed when adipocytes were treated with Vitamin D [34]. Furthermore, high level of circulating immune mediator such as Chemerin and VCAM are highly suggestive of inflammatory response in obese children with concurrent low vitamin D level [35]. Although our results did not show such regulation of Chemerin and Vitamin D in our TB group, as both groups were deficient with Vitamin D but distribution of Chemerin was not homogenous in these groups. This relationship of Chemerin and Vitamin D holds true in case of inflammation of adipose

tissue and fibroblast [34]. Based on our results, we speculate that inflammatory response of Chemerin is primarily due to inflammation in adipose tissue which differs from chronic inflammation in TB patients as level of Chemerin are significantly lower in lean TB group.

In this study, we also studied the effect of exogenous addition of the active form of Vitamin D [1,25-(OH) $_2$ D $_3$] in whole blood on the secretion of IFN γ and IL-10. Exogenous active form of Vitamin D stimulates anti-inflammatory cytokine (IL-10) and down regulates pro-inflammatory cytokine (IFN γ).

Since there was no difference in Vitamin D level, therefore TB and EC group were pooled for invitro experiment. In our *in vitro* experiments, we found an anti-inflammatory effect of 1,25-(OH) $_2$ D $_3$, that enhances the secretion of IL-10, while having a negative or no effect on IFN γ secretion. Bioactive Vitamin D[1,25-(OH) $_2$ D $_3$] has immunosuppressive effect on various pro-inflammatory cytokines (IFN γ TNF α & IL12) [22,36], and exerts its regulatory effect on T cells via inhibition of DCs maturation [37].

The major limitation of our study was in accessibility of TB patient with co-morbid of Diabetes, which is critical to draw conclusion about role of Chemerin in TB with DM. The results of this study highlight the role of Cathelicidin as biomarker of TB which is also supported by concomitant increase in TNF α in TB patient but not with Vitamin D and Chemerin. Vitamin D deficiency is prevalent in Pakistani population. Both TB patients and EC are deficient with circulating level of Vitamin D. This is interesting to investigate what factors in addition to Vitamin D increase susceptibility to TB infection, such as genetic, environmental and nutritional status.

5. CONCLUSION

Our results show a trend of nonlinear relationship of Cathelicidin and Chemerin in TB patients and healthy controls (EC) without comorbidity of DM. However, a linear relationship of Cathelicidin was observed with TNF α but not with Chemerin and Vitamin D. Cathelicidin was also identified as most discriminatory marker of inflammation in TB followed by TNF α . It is also important to study the relationship of these two peptides in TB patients with or without DM to further dissect the role of Chemerin and Cathelicidin in TB disease, and whether or not these markers can be used for screening TB with DM.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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