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Altered cord serum 25-hydroxyvitamin D signalling and placental inflammation is associated with pre-term birth

Running title: Vitamin D alters placental inflammation in PTB

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Abstract

Problem: Vitamin D is well-known for having anti-inflammatory and immunomodulatory properties. Impaired maternal Vitamin D status has been known to increase the risk of adverse pregnancy outcomes like pre-term birth. The present study aims to evaluate the impact of fetal cord serum 25-hydroxyvitamin D mediated signalling in mediating inflammatory responses in placenta during pre-term birth.

Methods of Study: For the above purpose, cord serum 25(OH) D were measured in term (n=20) and pre term (n=20) born babies using ELISA. Vitamin D downstream signalling has also been checked in placenta (VDR, CYP27B1, Cathelicidin LL37) along with expression of inflammatory markers (S100A8, HMGB1, TLR2, pNF-kappaB) using Western blotting and immunohistochemistry. Pearson correlation model was used to do correlation study.

Results: Compared with term born babies (59.31+3.476), decline in cord serum 25(OH) D levels is observed in pre-term born babies (22.26+1.083, p=<0.0001) that showed strong positive correlation with gestational age (r=0.9368***), and birth weight (r=0.9559***). On the other hand, Vitamin D signalling markers were found to be downregulated and inflammatory markers were upregulated in placental tissue of pre-term born babies.

Conclusion: Thus, our study demonstrated that insufficient cord 25(OH) D levels may disturb the homeostasis of inflammation in placenta. Altered cord serum 25(OH) D mediated anti-inflammatory signalling may be acting as trigger signals in modulating inflammatory responses in placenta and eliciting premature activation of spontaneous labor in pre-term birth.

Keywords: Vitamin D, pregnancy, placental inflammation, pre-term birth, anti-inflammatory

Abbreviations: PTB, pre-term birth; 25(OH)D, 25 hydroxyvitamin D; S100A8, S100 Calcium binding protein A8; HMGB1, High mobility group box 1; TLR2, toll like receptor 2; p-NF-kB phosphorylated Nuclear factor Kappa light-chain-enhancer of activate B cells; VDR, vitamin D receptor, CYP27B1, Cytochrome P450 family 27 subfamily B member1.

Introduction

A healthy and successful pregnancy needs a balanced co-ordination between pro-inflammatory and anti-inflammatory response of the immune system at maternal-fetal interface. If the balance gets skewed towards pro-inflammatory state due to any of the known or unknown factors, pre-mature breakdown of the uterine quiescence occurs which in turn leads to onset of labor or commonly called pre term birth.

Among the globally reported cases of all pregnancies, 8-10% of them are incidences of pre-term birth. Of that 8-10%, 40% of the pre-term labor are infection induced [1,2]. That is why it is of primary importance to address the infection vis-à-vis inflammation. During pregnancy, at the time of infection, innate immune system gets activated, recognizes the common repeating patterns present on most of the microorganisms as danger signals [3–6]. These molecular patterns known as DAMPs (Damage associated molecular patterns) or PAMPs (Pathogen associated molecular patterns) gives us an alarming signal. Pattern recognition receptors (PRRs) like 'toll like receptors' (TLRs) recognize these molecular signature patterns and elicit an inflammatory response via activation of central

inflammatory transcription factor NF-kappaB with infiltration of different immune cells at site and prevents the spreading of infection[7,8]. Untimely, exaggerated and dysregulated inflammatory response is a characterized cause of pre-term delivery. Vitamin D plays a pivotal role in modulating these responses. The association of Vitamin D deficiency and insufficiency with adverse pregnancy outcomes is globally reported[9–12] . The relationship of Vitamin D and inflammation resembles hen and egg conundrum; whether inflammation reduces Vitamin D concentration or Vitamin D dampens inflammation still remains a puzzle. However, recent literature and in-vitro studies have shown Vitamin D as anti-inflammatory in nature, regulating the activity of regulatory T cells and TH17 cells [13–17] . In cancer and other immunological disorders, Vitamin D has been extensively known to inhibit the expression and function of central transcription factor NF-κB [18,19] . Vitamin D has the potential to skew more aggressive cell mediated pro-inflammatory state to more tolerant anti-inflammatory state during pregnancy[20] .

Enough evidences suggest that deficiency or insufficiency of maternal serum Vitamin D lead to the incidence of pre-term birth[21–23] . Therefore, aforementioned observations instigated us to study if any alterations in Vitamin D mediated signals originating from fetal side modulate the maternal immune system in triggering the pre mature activation of labor as the information till date is fragmented and inconclusive, thus bestowing a major gap between two pathways. Therefore, we intend to deep dive directly into maternal-fetal interface by analyzing cord serum 25(OH) D and drawing its co-relation with placental inflammation.

For the scope of present study, we intend to quantify the evident role played by cord serum 25(OH) D in facilitating normal term delivery and its immunomodulatory response as an inflammation inhibiting hormone during placental inflammation. For this, we quantified cord serum 25(OH) D concentrations in pre- term born and term born babies; performed expression analysis of DAMPs (S100A8, HMGB1), PRRs (TLR2), to check the status of placental inflammation in both the groups, a well-known inflammatory transcription factor: p-NF-kappaB p-65 to analyze any correlation with cord serum 25 (OH) D concentrations. Hence, to observe the impact of inflammation on placenta, we have also analyzed the histological changes in placenta of PTB and term babies. To further investigate the significance of anti-inflammatory role of Vitamin D, we assayed the expression of Vitamin D mediated signaling molecules (Vitamin D receptor, 1α hydroxylase enzyme) and its anti-microbial

action via Cathelicidin-LL37, an anti-microbial peptide involved in host defense mechanism. The results clearly indicates that pre-term born babies had insufficient level of cord serum 25(OH)D concentration as compared to term born babies and is found to be negatively co-related with pNF-kappaB p-65, a central inflammatory transcription factor. Our study revealed an up-regulation of inflammatory genes with down-regulation of Vitamin D signaling molecules. By investigating the changes in expression profile and its co-relation with altered vitamin D levels we could provide new insights in understanding the pathophysiology of the pre-term birth.

2. Materials and Methods

2.1. Study population

The institutional human ethical committee of Institute of Science, Banaras Hindu University approved this study. The patients were counseled, and then signed the written consent form (Ref. no: F.Sc./Ethical Committee/2014-2015). They were ensured for full and strict confidentiality of their information. Study enrolled two groups: The case group comprised neonates who got delivered prematurely from mother undergoing spontaneous labor (27+0 to 36 weeks of gestation, n= 20) and controls comprised neonates who got delivered at full term of pregnancy (>37 weeks of gestation). Both controls and cases group sample was recruited from Department of Obstetrics and Gynaecology at Sir Sunderlal Hospital, Banaras Hindu University. The subjects with any known medical conditions (fetal distress syndrome, fetal anomalies, severe respiratory distress syndrome, immune deficiency disorder or any previous pregnancy complications) were excluded from the study. The samples for the present study were recruited evenly throughout the year. Therefore, exposure to sunlight for controls and cases would have been equal. Both groups were not on any Vitamin D supplementation. Cord blood and placental tissue was collected immediately after delivery and brought instantly to the lab. Cord blood was then centrifuged at 4ºC and serum was stored at -20ºC until analysis. The conditions for placental sampling were uniform for both cases and control group. Fresh tissue was collected immediately after normal vaginal delivery at pre-term and term delivery from the labor room. A small specimen of placental tissue (1cm x 1cm x 1cm) was collected macroscopically from the normal areas excluding any sign of calcification or infarction. The tissue was washed in ice cold 1XPBS to remove any blood remains. One part of dissected tissue were chopped in small pieces and kept in RNA later

solution (Thermo Scientific) for RNA isolation, one part was stored at -80ºC for protein isolation and one part was fixed in Bouin's solution for histological studies.

Pre term born babies are termed as cases and term born babies are termed as controls in the manuscript.

Reagents and antibodies:

Total 25(OH) Vitamin D Enzyme Immunoassay was purchased from Epitope Diagnostics, Inc. (KT-815). For Western blotting and immunohistochemistry, sheep anti human S100A8 mAb (catalog no AF 4570) were obtained from R&D, rabbit anti human HMGB1 (Catalog no 3935S) was purchased from CST, mouse anti human TLR2 (ab24192) were obtained from Abcam, rat anti human VDR mAb (MAI-710, clone9A7) was obtained from Thermo Scientific, sheep anti human Cathelicidin (Catalog no AF7497) were obtained from R&D and anti human Beta actin HRP was purchased from Sigma (Catalog no A3854).

2.2 ELISA of 25(OH) D in human cord blood

Serum concentration of human cord 25 (OH) D (Vitamin D) in controls and cases were measured using ELISA kit (Epitope Diagnostics, Inc Cat no. KT-815, Lot no. VDK002) according to manufactures instructions. According to Endocrine society of India (2011), Vitamin D <10ng/ml is considered severe deficient, 10-20ng/ml is deficient, 20-30ng/ml falls under insufficient category and 30-100ng/ml range is for optimum concentration of Vitamin D in the body. In short, 25µl of calibrators, controls and test samples were added in each microwells followed by addition of Vitamin D analogue and incubating the plate at room temperature for 60 min on ELISA plate shaker at 170 or 450 rpm. The plate was washed 5 times with wash solution and proceeded with addition of 100µl Biotinylated Vitamin D analogue at room temperature for 30 min. The plate was washed again and 20 min incubation with 100ul Streptavidin-HRP was done at room temperature. The wells were again washed 5 times till all the contents were aspirated. Then 100 ul tetramethylbenzidinechromagen solutions were added and plate was incubated for 20 min at room temperature in static position.

Finally, 100µl Stop solution was added and mixed gently. The absorbance was captured at 450 nm using a microplate reader. The assay was carried out in triplicate.

2.3 Histology analysis

Histology analysis was performed in chorionic villi tissue samples using standard protocol. Briefly, tissue collected from the patients was fixed in Bouin's solution for 24 hours at room temperature followed by washing with 70% alcohol twice and dehydration in ascending alcohol concentrations. The tissue was embedded in paraffin blocks and 6µ sections were cut to get mounted on poly-L-lysine coated slides. These fine sections on the slide were deparaffined using xylene, rehydrated in descending alcohol concentrations followed by routine histopathology specific hematoxylin and eosin staining. The slides were observed under light microscope for any histological changes occurring in placental tissue samples of pre-term birth.

2.4 Quantitative Real-Time PCR

Total RNA was isolated from placental tissue samples using Trizol method, followed by DNase I (NEB) treatment. The concentration of isolated RNAs was determined using Nanodrop 2000 (Thermo Fisher Scientific, Waltham, MA, USA). The synthesis of complementary DNA (cDNA) strands was done as per the manufacturer's protocol (Applied Biosystems). Briefly for 15 µL total reaction volume, 7.5 µL SYBER Green PCR Master mix (Applied Biosystems), 0.25 µL each of forward and reverse primers(10pM/µl) and 2 µL cDNA was used in PCR using ABI 7500 instrument. Each reaction was performed in triplicates. PCR was done with an initial incubation at 50°C for 2 minutes, followed by 10 minutes of denaturation at 95°C and 40 cycles at 95°C for 15 seconds, 60°C for 1 minute, and 72°C for 40 seconds (VDR forward primer, 5′TCCTCCTGCTCAGATCACTG3′; VDR reverse primer, 5′AGGGTCACAGAA GGGTCATC3′ and CYP27B1 forward primer, 5' CACCCGACACGGAGACCTT 3'; CYP27B1 reverse primer, 5' TCAACAGCGTGGACACAAACA 3'). Finally, the expression of these genes was normalized to the messenger RNA (m-RNA) levels of a housekeeping gene, Beta-actin (Gene ID: 60). Gene expression changes between the case and the control groups were estimated according to the relative quantitation method by calculating ΔΔCT. Statistical significance ($P<0.05$) was determined by Student's t test using GraphPad Prism5 software.

2.5 Western Blotting

The placental tissue samples were homogenized in 1x cell lysis buffer prepared from 10x cell lysis buffer (CST 0.01M Tris pH 7.6, 0.001M EDTA pH=8.0, 0.1M NaCl, 1 µg/ml aprotinin, 100µg/ml PMSF). Tissue was homogenized using 150µl of 1x cell lysis buffer to 100mg of tissue, followed by centrifugation at 1200 rpm for 30 minutes at 4 degree Celsius. Supernatant was collected and estimated using Bradford assay. Equal amount of proteins (50 µg) as determined by Bradford's method was loaded on to 15% SDS-PAGE for low molecular weight proteins (S100A8, Cathelicidin); 10% SDS-PAGE for HMGB1, VDR, pNF-κB and 8% SDS-PAGE for TLR2. After overnight transfer of proteins on polyvinylidene fluoride membrane (Millipore India Pvt. Ltd.), membranes were blocked for 1.5 hour with 5% fat-free dry milk solution in Tris-buffered saline (TBS; Tris 50mM (pH 7.5), NaCl 150mM, 0.02% Tween 20) and incubated with primary antibodies (detail in supplementary Table1) for overnight at 4 degree. After incubation with primary antibody, membranes were washed two times with TBST for 10 min. After this, membranes were incubated with horseradish peroxidase tagged secondary antibody (at a dilution recommended for each antibody) for 2 h. Finally, the blot was washed three times with TBST and developed with enhanced Chemiluminescence (ECL) detection system (Bio-Rad, USA). Blot for each protein was repeated for three times. The densitometric analysis of the blots was performed by scanning and quantifying the bands for density value by using computer assisted image analysis (Image J 1,38x, NIH, USA). The densitometric data were presented as the mean of the integrated density value ± SEM. The bands obtained from western blot were normalized to β-actin (Sigma Aldrich, St. Louis, MO, USA).

2.6 Immunohistochemistry

Immunohistochemistry was performed in paraffin embedded tissue sections having thickness of 6 µm. In brief, sections were deparaffinized by dipping in xylene overnight and were rehydrated with successive changes of 100%, 90%, 70% and 50% ethanol and distilled water. Antigen retrieval was done by dipping the slides in boiling citrate buffer (pH-6) for 10 minutes. After hydration and antigen retrieval, endogenous peroxidase blocking was done using Bloxall (SP-6000, Vector Laboratories, Inc.) for 30 min. The sections were then blocked with blocking serum for 1 h, followed by incubation with the primary antibody (S100A8, 1:50; HMGB1, 1:50; TLR2, 1:50; VDR, 1:50) over night at 4^0C

temperature. The sections were then washed with 1x TBS and incubated with the horseradish peroxidase tagged secondary antibody for 1.5 h at room temperature. After secondary antibody incubation, sections were washed again and incubated with DAB chromogen substrate (DAB Peroxidase substrate kit, Catalogue no. SK4100) for 2-3 minutes. After this, sections were dehydrated using ascending alcohol changes followed by mounting with DPX. Slides were analyzed under a light microscope (Nikon, Tokyo, Japan) and photographed.

2.7 Statistical analysis:

Data collected were analyzed using Student's t –tests by GraphPad Prism Software, Inc. La Jolla, CA, USA. Data are presented as the mean ± SD for continuous variables and percentage for categorical variables. Pearson's correlation was used for correlating the studied parameters. The data were considered significant if* $p< 0.01$, **$p<0.001$ and ***$p<0.0001$.

3. Results

No significant difference is observed between the mean age, BMI (body mass index) of mothers whose cord blood and tissue samples were collected. Moreover, gravidity has no significant association with the occurrence of pre-term birth. However, a significant difference between gestational age ($p=0.0016$) and birth weight of the newborn ($p<0.0001$) is seen between cases and controls as shown in **Table 1**.

3.1 Changes in cord blood serum 25(OH) Vitamin D levels in pre-term birth as compared to term birth.

Cord serum 25(OH) D concentration of controls and cases were measured. Pre-term born babies showed significant decrease and were insufficient (22.26+ 1.083) in 25(OH) D levels as compared to term born babies (59.31+3.476) ($p<0.0001$) as shown in **Figure 1(a).** To further explore whether gestational age has any role to play in affecting the concentration of cord serum 25(OH) D levels and if altered levels of 25(OH) D levels affects the weight of newborn babies, we did correlation analysis and interestingly found that cord serum 25(OH) D concentration is positively correlated with both age

of gestation (r=0.9368, p<0.0001) **Fig 1 (b)** and weight of the newborn babies (r=0.9559, p<0.0001) **Figure 1(c).**

3.2 Changes in the histology of PTB cases and term controls

We did histological examination of twenty placental tissue collected from mothers undergoing preterm (27 to 36 weeks gestation) and term deliveries (37to 39 weeks gestation). Tissues in both the groups were selected based on the age of gestation. Histopathological changes were observed in placental tissue of cases, having discontinuous syncytiotrophoblast layer and distorted cytotrophoblast cells. Villous tissue lacked proper vascularization, promoted vacuolization and condensed fibrous stroma fills the space. Highly vascularized placental tissue with continuous syncytiotrophoblast cell layer and presence of oval cytotrophoblast cells is observed as the gestation time reaches term. The change in placental histology can be because of increased placental inflammation in PTB cases. **(Figure 2(a).**

3.3 Real-Time Reverse Transcription–PCR Analysis

The expression of Vitamin D receptor and 1α hydroxylase genes were checked at transcript level (n=20) using quantitative real time PCR (qPCR). Livak method (Livak and Schmittgn 2001) was used to determine relative quantification[24]. Beta actin gene was used for normalization of expression levels and relative to the calibrator (ΔCT) of comparison groups. ΔΔCT and the relative fold change of VDR and 1α hydroxylase gene was calculated. A significant decrease in expression of VDR transcript is seen in PTB cases as compared to term controls, shown in **Figure 2(b**), but we could not find significant difference in the expression of 1α hydroxylase gene, shown in **Figure 2(c**).

3.4 Changes in the expression of inflammatory markers and Vitamin D signalling cascade in PTB cases and term controls

We did expression analysis for S100A8, HMGB1, TLR2, pNF-KB, VDR and Cathelicidin proteins to assess the alterations in their expression at protein level. S100A8 level was overexpressed in pre-term cases when compared to the control (**Figure 3a**). At histology level, inflammatory protein, S100A8 expression was mostly seen in syncytiotrophoblast cells in our control group but its expression was predominantly localized in syncytiotrophoblast cells and endothelium of fetal capillaries **(Figure 3b**). The overexpression of another inflammatory protein HMGB1 was seen in pre-term cases (Figure 3a). Histological study revealed the same, showing more immunogenic reaction in syncytiotrophoblast

cells as compared to controls (**Figure 3b**). The expression of their receptor, TLR2 showed overexpression in pre-term cases as compared to term controls (**Figure 3a**). The intensity of TLR2 expression was predominant in syncytiotrophoblast cells and endothelial cells of fetal capillaries as compared to the control group (**Figure 3b**). Overexpression of pNF-κB, a well-known transcription factor involved in inflammation is seen in placental tissue of pre-term cases with low expression in term controls (**Figure 3a**).

The expression study of Vitamin D signalling protein was validated using immunoblotting and immunohistochemistry. The low expression level of Vitamin D receptor protein was observed in pre-term placental tissues showing good correlation with RNA level (**Figure 2b and 2d**). At histology level, it showed positive reaction in syncytiotrophoblast cells but intense immunostaining was seen in both cytotrophoblast and syncytiotrophoblast cells of term placental tissues (**Figure 2f**). We also looked at the expression of one of the VDR response element genes; Cathelicidin, an antimicrobial peptide which is known to lower the extent of bacterial or viral infection. The expression of cathelicidin was significantly reduced in placental tissue of pre-term cases as compared to controls **(Figure 2e**).

4. Discussion

To the best of our knowledge, this is the first study evaluating the potential impact of altered cord serum 25 (OH) D concentrations on exaggerated placental inflammation during pre-term birth. The key finding of our study is: cord serum 25 (OH) D concentration is found to be positively correlated with gestational age and birth weight but negatively correlated with central inflammatory transcription factor pNF-KB. Elevated expression profiling of inflammatory marker proteins (S100A8, HMGB1, and TLR2) and downregulated expression of Vitamin D signalling molecules (VDR, Cathelicidin-LL37) confirmed exaggerated placental inflammation in pre-term birth. Till date, only maternal Vitamin D insufficiency or deficiency is reported to have adverse pregnancy outcome. This is the first study correlating insufficient cord serum 25 (OH) D concentrations and its altered anti-inflammatory signalling with enhanced placental inflammation during PTB.

The establishment and maintenance of successful pregnancy requires a balanced coordination of proinflammatory and anti-inflammatory state within feto-placental unit. Optimal inflammatory setting is prerequisite for efficacious implantation in the first trimester and during parturition in the

third trimester of pregnancy. Previous studies and animal models have well established that infection and inflammation is a major risk factor in PTB [25–27].

Directly or indirectly, maternal infection invades and promotes placental inflammation via secretion of cytokines and chemicals released by fetal immune cells at fetal maternal interface. Any type of enhanced and untimely inflammatory reactions during pregnancy may lead to high risk pregnancy cases like pre-term birth[1]. Reports till date have shown the activation of Damage associated molecular patterns (HMGB1, S100 family proteins), various TLRs during maternal intra-amniotic and intrauterine infection but less is talked about the activation of these molecules in eliciting a response in aberrant placental inflammation[28,29] and therefore we selected these molecules for expression analysis to investigate their involvement in placental inflammation. The involvement of a mammalian calcium binding protein S100A8 (Calgranulin A) and High mobility group box protein 1 (HMGB1) during development and progression of several inflammatory diseases is well known [30,31]. In terms of pregnancy, various transcriptomics and proteomics studies in different gestational tissues and fluids have reported that the expression of S100A8 and HMGB1 are elevated and thus act a potential biomarker in complications like preeclampsia, pregnancy loss and pre-term birth [29,32,33]. Earlier reports from our lab showed an altered expression of S100A8 in endometrial decidual tissue samples of recurrent pregnancy loss cases [34]. These endogenous proteins are known to elicit pro-inflammatory response via NF-kappaB, a central inflammatory transcription factor [35]. No studies till date showed the functional relevance of S100A8 and HMGB1 at maternal fetal interface during pre-term birth and if Vitamin D, being anti-inflammatory in nature has any significance in regulating the activation of NF-kappaB signalling.

Our results clearly showed elevated expression of S100A8, HMGB1, TLR2 and p-NF-KB in placental tissue of PTB cases as compared to term controls. In the present study, we observed an increase in the magnitude of inflammation in PTB cases which were in concordance with previous studies, where mutations in TLRs and intra amniotic administration of HMGB1, induced spontaneous pre-term labor [28,36]. Inflammation is thus conjectured to prompt a suit of changes in placental tissue breaking uterine quiescence and initiating the labor. Though the association of Vitamin D with inflammation always remained controversial, but a wealth of data is available reporting Vitamin D as immunomodulatory

and anti-inflammatory molecule which modulate both innate and adaptive immunity of an individual[14].

Regarding Vitamin D status, we observed significant decrease in the mean level of 25(OH) D concentrations in our cases as compared to controls. Considering Vitamin D as immunomodulatory in nature, decreased concentration in PTB cases implicate disturbed innate and adaptive immunity of newborn. The involvement of cord 25(OH) D in regulating placental inflammation during PTB is reported for the first time, where the expression of pNF-KappaB was found to be negatively correlated with cord serum 25 (OH) D concentrations (r= -0.9174**). Therefore, our present study distinctively indicates that decreased level of cord serum 25(OH) D levels might be involved in exaggerating placental inflammation.

The prior knowledge regarding placental expression of VDR on immune cells and their reduced expression in decidual tissue during spontaneous abortion gives an impression of having its immunological role at fetal maternal interface [37,38]. In infection like tuberculosis, Vitamin D enhances the expression of host defensive peptide, cathelicidin for limiting the growth of mycobacterium that causes tuberculosis [39–41]. In agreement of the aforementioned studies, we found a decline in the expression of VDR and antimicrobial peptide Cathelicidin, LL-37 in placental tissue of PTB cases, with no significant changes in the expression of 1α hydroxylase gene that converts pro-hormone into its active form. Thus, low expression of VDR and cathelicidin in our study may be involved in proliferation of microbes invading genital tract or intrauterine compartment contributing to enhanced placental inflammation.

To further confirm, whether altered inflammatory and Vitamin D signalling in PTB affect the histological architecture of placenta, we did histological analysis of placental tissue from both the groups and observed histopathological alterations in placental tissue of PTB cases when compared to term controls. Healthy and highly vascularized villus is seen in full term placental tissue with smooth layer of syncytiotrophoblast layer while distorted and degenerative placental tissue having fibrous stroma and reduced vascularization is seen in PTB cases. As pre term birth is somewhere pathologically associated with disturbed maternal-fetal immune interactions, placenta may be an important and interesting target to focus on.

In conclusion, the present study for the first time summarizes the correlation of cord serum 25(OH) D with central inflammatory transcription factor, p-NF-kappaB. These findings suggests that decrease in 25(OH) D concentration may lead to increase in p-NF-kB expression and support the established cause of infection and resulting placental inflammation in pre-term birth. Inflammatory marker proteins (S100A8, HMGB1 and TLR2) act via NF-kB pathway and activate pro-inflammatory cytokines. Therefore increased expression of these proteins and decreased expression of VDR and antimicrobial peptide, Cathelicidin and poor vascularization of placenta will all together set a state of unfavorable inflammatory milieu which may lead to initiation of premature labor.

The present study states that low cord serum Vitamin D level enhances placental inflammation which might be initiating the pre-mature activation of labor. However, controversial theories still exist to contemplate over the question 'Is low Vitamin D level a cause of, or consequence of inflammation?' Considerable evidences support the anti-inflammatory nature of Vitamin D [42,43] and simultaneously several other studies in Ageing and inflammatory health disorders believe that low Vitamin D levels is a consequence of chronic inflammation [44–46]. Therefore, further longitudinal studies are needed to validate our observational study and discover better relationship between placental inflammation and Vitamin D levels in pre-term birth. Functional studies are also needed to further confirm the interplay of two pathways and establish Vitamin D as anti-inflammatory in pre-term birth. Given that the percentage of pre-term birth rate is increasing, clinical interventions are needed that could decrease the magnitude of inflammation and lessen the burden of disease.

Author contributions: S.B performed and analysed all the experiments and also contributed in writing the manuscript. P.V and R.V contributed in writing the manuscript. S.G and S.R helped in providing human samples and doing clinical evaluation of patients. K.S designed and coordinated the study design and critically reviewed the manuscript. All authors approved the final version of manuscript.

References:

1. Agrawal V, Hirsch E. Intrauterine infection and preterm labor. In: Seminars in Fetal and Neonatal Medicine. Elsevier; 2012. p. 12–9.
2. Kemp MW. Preterm birth, intrauterine infection, and fetal inflammation. Front Immunol. 2014;5:574.
3. Boyle AK, Rinaldi SF, Norman JE, Stock SJ. Preterm birth: Inflammation, fetal injury and treatment strategies. J Reprod Immunol. 2017;119:62–6.
4. Cappelletti M, Della Bella S, Ferrazzi E, Mavilio D, Divanovic S. Inflammation and preterm birth. J Leukoc Biol. 2016;99(1):67–78.
5. Burdet J, Paula Dominguez Rubio A, Ines Salazar A, Laura Ribeiro M, Ibarra C, Maria Franchi A. Inflammation, infection and preterm birth. Curr Pharm Des. 2014;20(29):4741–8.
6. Romero R, Espinoza J, Gonçalves LF, Kusanovic JP, Friel L, Hassan S. The role of inflammation and infection in preterm birth. In: Seminars in reproductive medicine. Copyright© 2007 by Thieme Publishers, Inc., 333 Seventh Avenue, New York, NY …; 2007. p. 21–39.
7. Piccinini AM, Midwood KS. DAMPening inflammation by modulating TLR signalling. Mediators Inflamm. 2010;2010.
8. Romero R, Chaiworapongsa T, Alpay Savasan Z, Xu Y, Hussein Y, Dong Z, et al. Damage-associated molecular patterns (DAMPs) in preterm labor with intact membranes and preterm PROM: a study of the alarmin HMGB1. J Matern Neonatal Med. 2011;24(12):1444–55.
9. Barrett H, McElduff A. Vitamin D and pregnancy: an old problem revisited. Best Pract Res Clin Endocrinol Metab. 2010;24(4):527–39.
10. Websky K Von, Abdallah A, Reichetzeder C, Tsuprykov O. Journal of Steroid Biochemistry and Molecular Biology Impact of vitamin D on pregnancy-related disorders and on o ff spring outcome. 2018;180(August 2017):51–64.
11. Heyden EL, Wimalawansa SJ. Vitamin D: Effects on human reproduction, pregnancy, and fetal well-being. J Steroid Biochem Mol Biol. 2018;180:41–50.
12. Shah BA, Padbury JF, Anderson MP, Holick MF, Szyld E, Gordon CM. Vitamin D and associated perinatal – neonatal outcomes among extremely low-birth-weight infants. 2018;25.
13. Ji J, Zhai H, Zhou H, Song S, Mor G, Liao A. The Role and Mechanism of Vitamin

D-Mediated Regulation of Treg/Th17 Balance in Recurrent Pregnancy Loss. Am J Reprod Immunol. 2019;e13112.

14. Zahran AM, Zharan KM, Hetta HF. Significant correlation between regulatory T cells and vitamin D status in term and preterm labor. J Reprod Immunol. 2018;129:15–22.
15. Sassi F, Tamone C, D'Amelio P. Vitamin D: nutrient, hormone, and immunomodulator. Nutrients. 2018;10(11):1656.
16. Cantorna M, Snyder L, Lin Y-D, Yang L. Vitamin D and 1, 25 (OH) 2D regulation of T cells. Nutrients. 2015;7(4):3011–21.
17. Ji J, Muyayalo KP, Zhang Y, Hu X, Liao A. Immunological function of vitamin D during human pregnancy. Am J Reprod Immunol. 2017;78(2):e12716.
18. Stio M, Martinesi M, Bruni S, Treves C, Mathieu C, Verstuyf A, et al. The Vitamin D analogue TX 527 blocks NF-κB activation in peripheral blood mononuclear cells of patients with Crohn's disease. J Steroid Biochem Mol Biol. 2007;103(1):51–60.
19. Yu X-P, Bellido T, Manolagas SC. Down-regulation of NF-kappa B protein levels in activated human lymphocytes by 1, 25-dihydroxyvitamin D3. Proc Natl Acad Sci. 1995;92(24):10990–4.
20. Urry Z, Chambers ES, Xystrakis E, Dimeloe S, Richards DF, Gabryšová L, et al. The role of 1α, 25-dihydroxyvitamin D 3 and cytokines in the promotion of distinct F oxp3+ and IL-10+ CD 4+ T cells. Eur J Immunol. 2012;42(10):2697–708.
21. Bodnar LM, Klebanoff MA, Gernand AD, Platt RW, Parks WT, Catov JM, et al. Maternal vitamin D status and spontaneous preterm birth by placental histology in the US Collaborative Perinatal Project. Am J Epidemiol. 2013;179(2):168–76.
22. Zhou S, Tao Y, Huang K, Zhu B, Tao F. Vitamin D and risk of preterm birth: Up-to-date meta-analysis of randomized controlled trials and observational studies. J Obstet Gynaecol Res. 2017;43(2):247–56.
23. Chen Y-H, Fu L, Hao J-H, Wang H, Zhang C, Tao F-B, et al. Influent factors of gestational vitamin D deficiency and its relation to an increased risk of preterm delivery in Chinese population. Sci Rep. 2018;8(1):3608.
24. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2− ΔΔCT method. methods. 2001;25(4):402–8.

25. Houser BL. Decidual Macrophages and Their Roles at the Maternal-Fetal Interface. 2012;85(1):105–18.
26. Challis JR, Lockwood CJ, Myatt L, Norman JE, Strauss III JF, Petraglia F. Inflammation and pregnancy. Reprod Sci. 2009;16(2):206–15.
27. PrabhuDas M, Bonney E, Caron K, Dey S, Erlebacher A, Fazleabas A, et al. Immune mechanisms at the maternal-fetal interface: perspectives and challenges. Nat Immunol. 2015;16(4):328.
28. Gomez-lopez N, Romero R, Plazyo O. Intra-amniotic Administration of HMGB1 Induces Spontaneous Preterm Labor and Birth. 2017;75(1):3–7.
29. Baumbusch MA, Buhimschi CS, Oliver EA, Zhao G, Thung S, Rood K, et al. High Mobility Group-Box 1 (HMGB1) levels are increased in amniotic fluid of women with intra-amniotic inflammation-determined preterm birth, and the source may be the damaged fetal membranes. Cytokine. 2016;81:82–7.
30. Bae J-S. Role of high mobility group box 1 in inflammatory disease: focus on sepsis. Arch Pharm Res. 2012;35(9):1511–23.
31. Wang S, Song R, Wang Z, Jing Z, Wang S, Ma J. S100A8/A9 in Inflammation. Front Immunol. 2018;9:1298.
32. Zhu L, Zhang Z, Zhang L, Shi Y, Qi J, Chang A, et al. HMGB1–RAGE signaling pathway in severe preeclampsia. Placenta. 2015;36(10):1148–52.
33. Shao J, Zhao M, Tong M, Wei J, Wise MR, Stone P, et al. Increased levels of HMGB1 in trophoblastic debris may contribute to preeclampsia. Reproduction. 2016;152(6):775–84.
34. Nair RR, Khanna A, Singh K. Role of inflammatory proteins S100A8 and S100A9 in pathophysiology of recurrent early pregnancy loss. Placenta. 2013;34(9):824–7.
35. Liu T, Zhang L, Joo D, Sun S-C. NF-κB signaling in inflammation. Signal Transduct Target Ther. 2017;2:17023.
36. Lorenz E, Mira JP, Frees KL, Schwartz DA. Relevance of mutations in the TLR4 receptor in patients with gram-negative septic shock. Arch Intern Med. 2002;162(9):1028–32.
37. Anagnostis P, Karras S, Goulis DG. Vitamin D in human reproduction: a narrative review. Int J Clin Pract. 2013;67(3):225–35.

38. Li N, Wu HM, Hang F, Zhang YS, Li MJ. Women with recurrent spontaneous abortion have decreased 25 (OH) vitamin D and VDR at the fetal-maternal interface. Brazilian J Med Biol Res. 2017;50(11).
39. Selvaraj P. Vitamin D, vitamin D receptor, and cathelicidin in the treatment of tuberculosis. In: Vitamins & Hormones. Elsevier; 2011. p. 307–25.
40. Gombart AF. The vitamin D–antimicrobial peptide pathway and its role in protection against infection. Future Microbiol. 2009;4(9):1151–65.
41. Khoo A-L, Chai LYA, Koenen HJPM, Oosting M, Steinmeyer A, Zuegel U, et al. Vitamin D3 down-regulates proinflammatory cytokine response to Mycobacterium tuberculosis through pattern recognition receptors while inducing protective cathelicidin production. Cytokine. 2011;55(2):294–300.
42. Holick MF, Chen TC. Vitamin D deficiency: a worldwide problem with health consequences. Am J Clin Nutr. 2008;87(4):1080S-1086S.
43. Ota K, Dambaeva S, Han A-R, Beaman K, Gilman-Sachs A, Kwak-Kim J. Vitamin D deficiency may be a risk factor for recurrent pregnancy losses by increasing cellular immunity and autoimmunity. Hum Reprod. 2013;29(2):208–19.
44. Noordam R, De Craen AJM, Pedram P, Maier AB, Mooijaart SP, Van Pelt J, et al. Levels of 25-hydroxyvitamin D in familial longevity: the Leiden Longevity Study. Cmaj. 2012;184(18):E963–8.
45. Autier P, Boniol M, Pizot C, Mullie P. Vitamin D status and ill health: a systematic review. lancet Diabetes Endocrinol. 2014;2(1):76–89.
46. Mangin M, Sinha R, Fincher K. Inflammation and vitamin D: the infection connection. Inflamm Res. 2014;63(10):803–19.

Figure legends:

Figure 1: (a) Bar graph showing significant decrease in the cord serum 25(OH) D concentration between healthy term (n=20) and PTB cases (n=20) ($p<0.0001$). **(b)** Correlation between cord serum 25(OH)D with the age of gestation in weeks and **(c)** Correlation between cord serum 25(OH) concentration with birth weight of the newborn. A significant positive correlation was found between

cord serum 25(OH)D and age of gestation ($r=0.9368$, $p<0.0001$). Also, a significant positive correlation was found between cord serum 25(OH) D and birth weight of newborn ($r=0.9559$, $p<0.0001$). r= Pearson coefficient value is significant when $p<0.05$.

Figure 2: (a) Light microscopic histopathological analysis of placental tissue of healthy term controls and PTB cases. Black arrow shows the morphological differences in cellular architecture (b) Bar graph of RT-PCR analysis showing the lower expression of Vitamin D receptor mRNA with no significant change in expression of CYP27B1 gene (c) in the placenta of PTB cases. Fold change was determined using ΔΔCT method of relative quantification. The graph was plotted using log (Relative average fold change i.e $2^{-\Delta\Delta CT}$). (d) Representative immunoblot for VDR and Cathelicidin-LL37 protein in placental tissue of PTB cases and term control. β-actin was used as loading control and band intensity were analyzed by densitometric analysis. b) Immunohistochemical localisation of VDR in placental tissue of controls and cases. Black arrows show the region of positive immunostaining. The resolution used to capture images is 40X.

Figure 3: (a) Representative immunoblot analysis of inflammatory marker proteins (S100A8, HMGB1, TLR2, p-NF-Kb) in placental tissue of control and PTB patients. β-actin was used as loading control and band intensity were analyzed by densitometric analysis. (b) Immunohistochemical localisation of inflammatory markers in term controls and PTB cases. Black arrows show the region of positive immunostaining.

Table 1. Demographic and clinical parameters of PTB cases and controls

Parameters	Controls (N=20)	Cases (N=20)	P value
Maternal			
a. Age	25.36 ± 0.7309	27.43 ± 1.529	0.199
b. Origin	North Indian	North Indian	-
b. Gestational Age	36.91 ± 1.812	30.58 ± 0.4476	**0.0016****
c. BMI	24.87 ± 0.566	23.55 ± 0.331	0.052
d. Gravidity **i) Primigravida** **ii) Multigravida**	 11(55%) 09(45%)	 14(70%) 06(30%)	 0.327
Neonatal			
a. 25(OH) D (ng/ml)	(59.31+3.476)	(22.26+ 1.083)	**< 0.0001**
b. Birth weight(g)	2371 ± 140.8	1078 ± 57.93	**<0.0001**

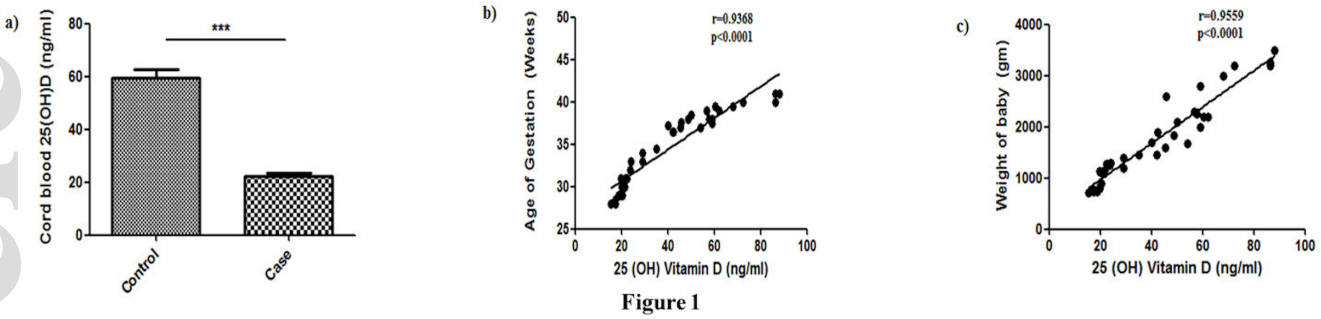

Figure 1

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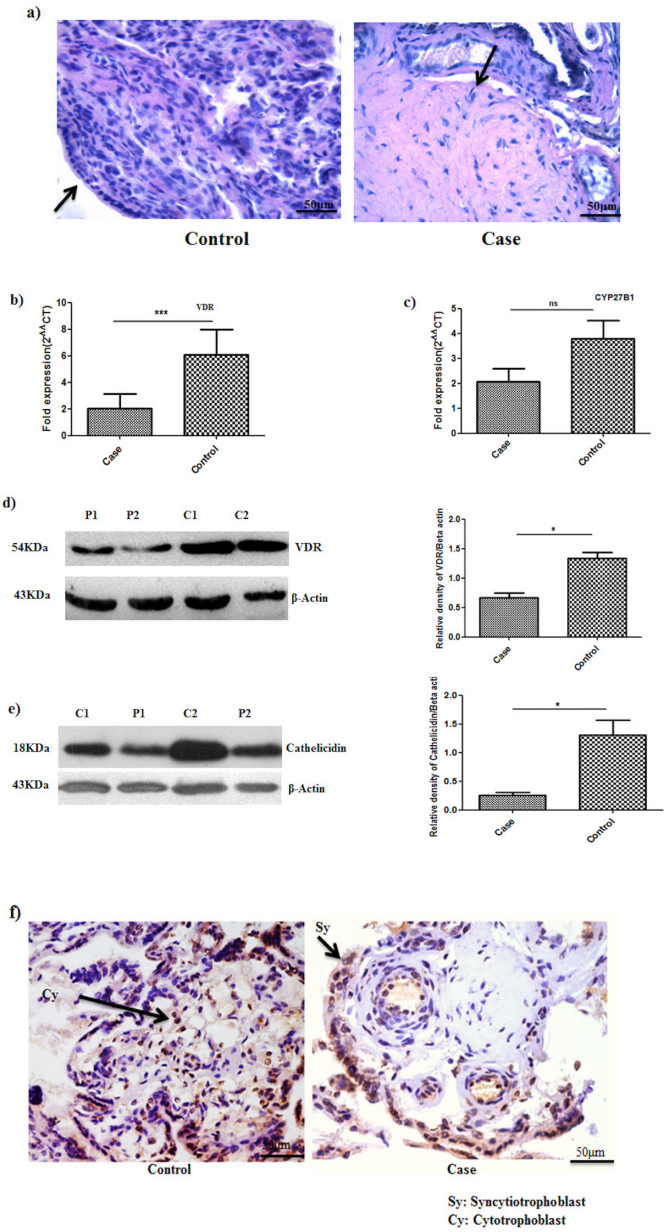

Figure 2

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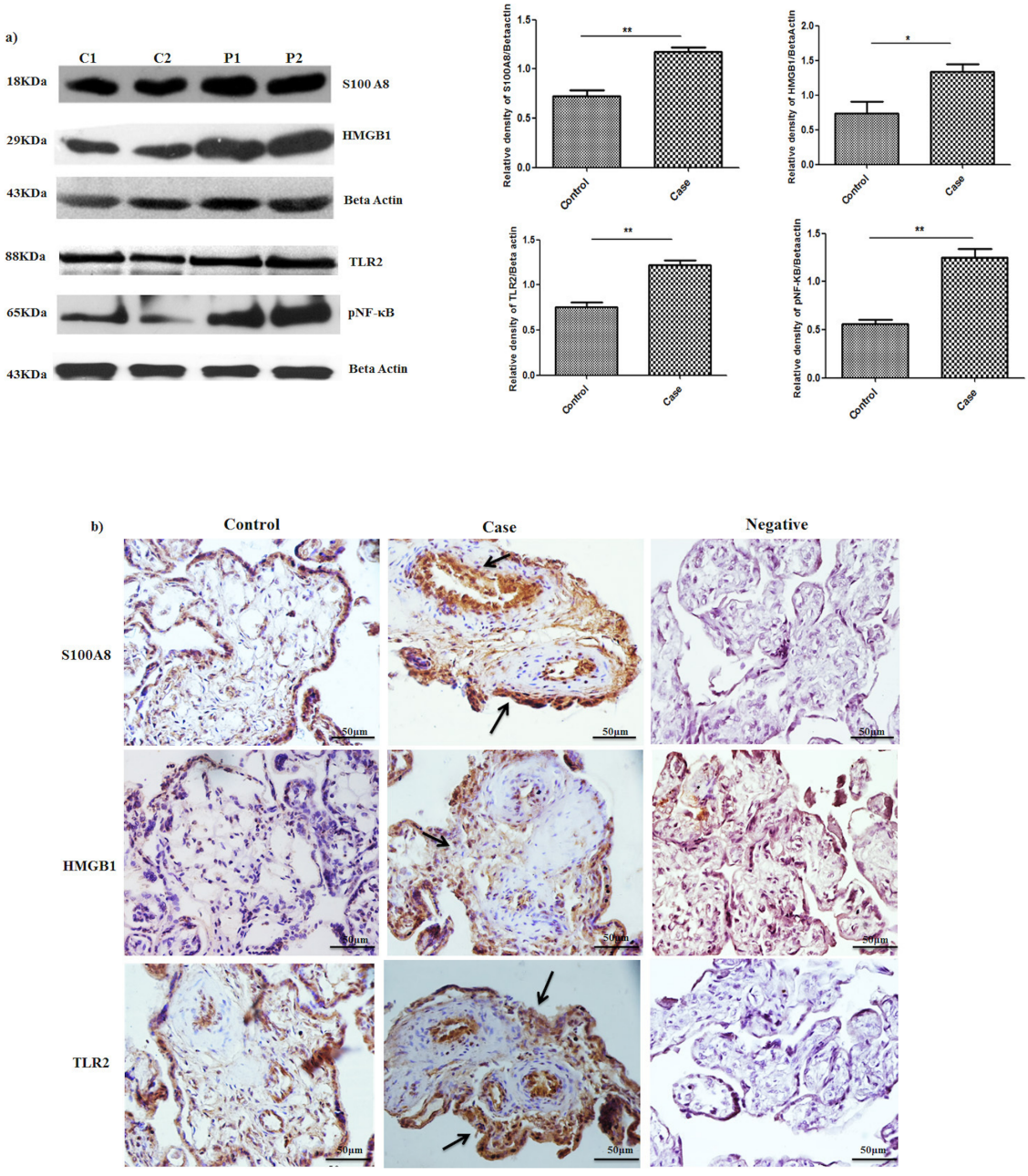

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