

## Vitamin D is Associated with $\alpha 4\beta 7+$ Immunophenotypes and Predicts Vedolizumab Therapy Failure in Patients with Inflammatory Bowel Disease

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## ABSTRACT

**Background and Aims:** Vitamin D downregulates the *in vitro* expression of the gut-tropic integrin  $\alpha 4\beta 7$  on immune cells. The clinical relevance of this finding in patients with inflammatory bowel disease (IBD) is unclear. We tested the hypothesis that vitamin D is associated with  $\alpha 4\beta 7$  immunophenotypes and risk of vedolizumab (anti-  $\alpha 4\beta 7$ ) failure in IBD.

**Methods:** We performed single-cell immunophenotyping of peripheral and intestinal immune cells using mass cytometry (CyTOF) in vedolizumab-naïve patients with IBD (N=48). We analyzed whole-genome mucosal gene expression (GSE73661) from GEMINI I and GEMINI long-term safety (LTS) to determine the association between vitamin D receptor (VDR) and integrin alpha-4 (ITGA4) and beta-7 (ITGB7) genes. We estimated the odds of vedolizumab failure with low pre-treatment vitamin D in a combined retrospective and prospective IBD cohort (N= 252) with logistic regression.

**Results:** Immunophenotyping revealed that higher 25(OH)D was associated with decreased  $\alpha 4\beta 7^+$  peripheral blood mononuclear cells (R = -0.400, P < 0.01) and  $\alpha 4\beta 7^+$  intestinal leukocytes (R = -0.538, P= 0.03). Serum 25(OH)D was inversely associated with  $\alpha 4\beta 7^+$  peripheral B cells and natural killer (NK) cells and  $\alpha 4\beta 7^+$  intestinal B cells, NK cells, monocytes, and macrophages. Mucosal expression of VDR was inversely associated with ITGA4 and ITGB7 expression. In multivariate analysis, 25(OH)D < 25 ng/mL was associated with increased vedolizumab primary non-response during induction (OR 26.10, 95% CI

14.30-48.90, P<0.001) and failure at 1-year follow-up (OR 6.10, 95% CI 3.06-12.17, P<0.001).

**Conclusions:** Low serum 25(OH)D is associated with  $\alpha 4\beta 7+$  immunophenotypes and predicts future vedolizumab failure in patients with IBD.

**Key Words:** Vitamin D, integrins, vedolizumab, inflammatory bowel disease, clinical outcomes

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## INTRODUCTION:

The integrin  $\alpha 4\beta 7$  expressed on gut-tropic immune cells binds to mucosal addressin cell adhesion molecule 1 (MAdCAM-1) and facilitates leukocyte trafficking to the gut leading to intestinal inflammation [1]. Vedolizumab, a humanized monoclonal antibody to  $\alpha 4\beta 7$ , is approved for the treatment of inflammatory bowel disease (IBD) [2,3] and is thought to selectively inhibit gut leukocyte trafficking. Only about 40% of IBD patients achieve clinical remission with vedolizumab and a significant proportion of patients lose response over time [4,5]. The mechanisms underlying vedolizumab failure are unclear. Studies identifying modifiable risk factors predicting response to vedolizumab therapy are needed to elucidate mechanisms of treatment failure, enhance the precision of patient selection for therapy, and ultimately optimize the therapeutic efficacy of vedolizumab in patients with IBD.

Vitamin D status is a well-recognized risk factor implicated in inflammatory bowel disease [6]. Genetic polymorphisms in the vitamin D receptor (VDR) have been linked to disease susceptibility to ulcerative colitis and Crohn's disease [7,8]. Low vitamin D levels in patients with inflammatory bowel disease has been associated with active inflammation, disease severity, poor quality of life, and adverse clinical outcomes [9,10]. Prior mechanistic studies have suggested that the protective associations of higher vitamin D levels in patients with IBD are in part mediated through anti-inflammatory cytokine profiles [11] and antimicrobial peptides [12]. Prior observations have also suggested that pre-treatment vitamin D levels may impact response to therapy. For example, a higher pre-treatment vitamin D level in patients with IBD was associated with increased odds of primary response to anti-TNF therapy [13]. Whether pre-treatment vitamin D levels are associated with response to vedolizumab therapy in patients with IBD has not been previously explored.

The imprinting of the gut-tropic integrin  $\alpha 4\beta 7$  on leukocytes is regulated by gut lamina propria-derived CD103+ retinoic acid-metabolizing dendritic cells in mesenteric lymph nodes [14]. Interestingly, vitamin D has been identified as a regulator of integrin  $\alpha 4\beta 7$  expression

on immune cells *in vitro*. In one study [15], in the presence of retinoic acid, the active form of vitamin D (1,25(OH)<sub>2</sub>D<sub>3</sub>) downregulated  $\alpha$ 4 $\beta$ 7 expression on naïve T cells in a dose-dependent manner. In another study [16], addition of 1,25(OH)<sub>2</sub>D<sub>3</sub> to retinoic acid and IL-2 inhibited the *in vitro* production of proinflammatory cytokines and integrin  $\alpha$ 4 $\beta$ 7 expression on innate lymphoid cells (ILCs). Whether the effects of vitamin D on integrin  $\alpha$ 4 $\beta$ 7 expression on immune cells can be recapitulated *in vivo* and is clinically relevant in IBD are unclear. Specifically, it is unknown whether vitamin D levels correlate with  $\alpha$ 4 $\beta$ 7 expression on peripheral and intestinal immune cells in patients with IBD.

Given that low vitamin D is associated with increased inflammation and poor clinical outcomes in patients with IBD, vitamin D can downregulate the *in vitro* expression of  $\alpha$ 4 $\beta$ 7 on immune cells, and that  $\alpha$ 4 $\beta$ 7 immune cell expression in IBD patients may predict vedolizumab response [17], we hypothesized that low 25(OH)D is associated with increased gut-tropic  $\alpha$ 4 $\beta$ 7<sup>+</sup> immunophenotypes and predicts future vedolizumab therapy failure in patients with IBD. To test this hypothesis, we performed single-cell immunophenotyping of peripheral and intestinal immune cells in vedolizumab-naïve IBD patients, analyzed the association of mucosal gene expression of VDR and vitamin D metabolism enzymes with integrin gene expression, and performed retrospective and prospective cohort analyses in IBD patients treated with vedolizumab to estimate the odds of future vedolizumab failure with low pre-treatment serum 25(OH)D.

## **METHODS:**

### **Single-Cell Immunophenotyping of Peripheral and Intestinal Immune Cells in IBD Patients Using Mass Cytometry (CyTOF)**

Blood and intestinal biopsies were obtained from IBD patients (blood from 48 patients of which 12 patients had biopsies) enrolled in the Stanford IBD Registry and biobank (IRB 28427). Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood using the Ficoll-Paque method as previously described [18]. Intestinal leukocytes were isolated from intestinal biopsies using enzymatic treatment [19]. PBMCs and intestinal leukocytes were cryopreserved at -80°C until analysis. Immunophenotyping of PBMC and intestinal leukocytes was performed using mass cytometry (CyTOF) at the Stanford Human Immune Monitoring Center according to our previously published protocols [20]. All antibody conjugates were validated for accurate detection of their respective antigens and to ensure minimal isotope spillover by us, the Stanford Human Immune Monitoring Center, and/or in the literature using flow cytometry with antibody clones and mass cytometry with antibody–metal conjugates. Beads (Fluidigm, cat. #201078) were spiked into each sample for subsequent normalization using the Helios instrument software, and no cell stimulation or barcoding were used. Bead normalized sample files were obtained from the Helios instrument using on-board software. FlowJo was used for cleaning up files, concatenating files, and calculating manual gates and statistics. Doublets were carefully gated out in all samples. Cytobank was used to perform viSNE, CITRUS, and Spade analyses. viSNE analyses were run on live human single cells concatenated from individual samples by group.  $\alpha 4\beta 7^{\text{hi}}$  cells were identified, and subsequently canonical cell populations were gated from this subset as previously described. viSNE [21] was run on live human single cells concatenated from individual samples by clinical group. In total, 15,000 events were randomly subsampled from

each file of samples concatenated by clinical group, and clustering based on 16 canonical lineage antigens (CD3, CD4, CD8, CD11b, CD11c, CD14, CD16, CD19, CD20, CD25, CD27, CD45RO, CD56, CD123, CD127, and HLA-DR) was run on all concatenated files in parallel for blood or tissue samples using a random seed, 1,000 iterations, perplexity of 30, and theta of 0.5 in each of the two runs. We generated tSNE plots to display  $\alpha 4\beta 7$  cell surface protein expression gradients among immune cell subsets (CD4 T cells, CD8 T cells, T Regulatory Cells, B cells, natural killer (NK) cells, monocytes, macrophages, and innate lymphoid cells) as outlined in a previous study by our group [20]. We subsequently plotted serum 25(OH)D with percentage of  $\alpha 4\beta 7^+$  cells from PBMCs, intestinal leukocytes, and individual immune subsets and calculated Pearson's correlation coefficients for normally distributed datasets and Spearman's correlation coefficients for non-normally distributed datasets. In our CyTOF correlation analyses, we adjusted for potential confounders such as age, sex, ethnicity, active endoscopic inflammation (defined as a Mayo score  $\geq 2$  in UC and a simple endoscopic score (CD-SES)  $> 6$  in CD patients), and medications using multivariate regression. Finally, we analyzed expression of other leukocyte trafficking markers (CCR9, CCR1 and GPR15) on immune cell subsets and correlated these immunophenotypes with serum 25(OH)D.

### **Mucosal Gene Expression Analysis of Vitamin D Receptor (VDR), Vitamin D Metabolism Enzymes, and Integrin Gene Expression**

We used the NCBI Gene Expression Omnibus (GEO) database to download GSE73661 for correlation analyses between the Vitamin D pathway and integrin alpha-4 (ITGA4) and beta-7 (ITGB7) genes. GSE73661 was derived from baseline colon biopsies in 41 ulcerative colitis patients with active disease who were enrolled in two phase III vedolizumab clinical trials (GEMINI I and GEMINI long-term safety ((LTS)) [22]. Pearson correlations were computed after gene expression values were log<sub>2</sub>-normalized and scaled



using `rcorr` from the `Hmisc` package in R. P-values were adjusted for multiple comparisons using the Benjamini-Hochberg method. All correlation analyses were performed and visualized using `ggplot2` and R 3.6.3.

### **Retrospective and Prospective Cohort Analyses of IBD Patients Treated with Vedolizumab**

To test the hypothesis that low serum 25(OH)D is associated with increased risk of vedolizumab failure in patients with IBD, we performed a combined cohort analysis using two independent IBD cohorts (retrospective and prospective cohorts). Our study was approved by the Stanford University Institutional Review Board (IRB) under protocol 28427. For both cohorts, we included patients who were 18 years or older with inflammatory bowel disease (ulcerative colitis or Crohn's disease), who were previously vedolizumab-naïve and were started on vedolizumab therapy as part of standard of care determined by an IBD specialist based on symptoms (ulcerative colitis disease activity index (UCDAI) of total score  $> 2$  with all individual categories  $> 1$ , Harvey Bradshaw Index (HBI)  $> 7$ ), fecal calprotectin  $> 250 \mu\text{g/g}$ , or active endoscopic inflammation (if repeat endoscopy performed) defined as Mayo score  $\geq 2$ , Crohn's disease simple endoscopic score (CD-SES)  $> 6$ ), had available pre-treatment serum 25(OH)D measured prior to vedolizumab initiation (no more than two weeks before vedolizumab), and had response rates measured at induction (week 14) and maintenance therapy at 1-year follow-up. Patients without IBD, less than 18 years of age, without vedolizumab treatment, or who did not have available serum 25(OH)D to vedolizumab initiation were excluded. Our exposure was low pre-treatment serum 25(OH)D level (measured before of starting vedolizumab) defined as  $< 25 \text{ ng/mL}$  (lower limit of normal at Stanford lab). Our primary outcome was vedolizumab failure at 1-year follow-up which included patients who were both primary non-responders and patients who responded during induction but later failed during maintenance therapy at 1-year follow-up. Primary

non-response was defined by any of the following at or before week 14 of induction: ongoing symptoms (UCDAI total score  $> 2$  with all individual categories  $>1$ , HBI  $>7$ ), fecal calprotectin  $> 250 \mu\text{g/g}$ , or active endoscopic inflammation (if repeat endoscopy performed) defined as Mayo score  $\geq 2$ , Crohn's disease simple endoscopic score (CD-SES)  $> 6$ . Vedolizumab failure at 1-year was defined by UCDAI  $> 2$  with all individual categories  $>1$ , HBI (HBI)  $>7$ ), fecal calprotectin  $> 250 \mu\text{g/g}$ , or active endoscopic inflammation defined as Mayo score  $\geq 2$ , Crohn's disease simple endoscopic score (CD-SES)  $> 6$  at any time during induction therapy (up to week 14) or maintenance therapy up to 1-year follow-up. Our retrospective cohort was derived from the Stanford Research Repository (STARR) database. Patients were screened by ICD code (Crohn's disease ICD code K50.xx and ulcerative colitis ICD code K51.xx) and vedolizumab use and serum 25(OH)D was confirmed by chart review. For our prospective cohort, vedolizumab naïve patients who were started on vedolizumab as part of standard of care and had pre-treatment serum 25(OH)D levels checked were enrolled in our IBD registry and longitudinally followed and assessed for primary non-response at week 14 and vedolizumab failure during 1-year follow-up after vedolizumab initiation. For both cohorts, we collected baseline clinical data including patient demographics (age, sex, ethnicity), IBD subtype and characteristics per the Montreal classification, body mass index (BMI), smoking and alcohol use status, laboratory values (pre-treatment serum 25(OH)D, CRP, albumin, creatinine, fecal calprotectin), baseline endoscopic inflammation (Mayo endoscopic score for ulcerative colitis, and simple endoscopic score (SES-CD) for Crohn's disease), and medications (current steroids, current mesalamine use, current 6-mercaptopurine use/azathioprine, current methotrexate, prior anti-TNF failure, prior ustekinumab, prior tofacitinib, current opioid use, current NSAID use, and current vitamin D supplementation (not including multivitamin)). We defined baseline active endoscopic inflammation as a Mayo endoscopic score  $\geq 2$  for ulcerative colitis patients and a simple

endoscopic score (SES-CD)  $> 6$  for Crohn's disease. We recorded the rates of total vedolizumab failure at 1-year follow-up, primary non-response at week 14, and time to vedolizumab failure. Since this was an observational study, patients with low serum 25(OH)D received vitamin D supplementation as part of standard of care. Based on two previously developed and validated scoring tools for predicting response to vedolizumab therapy in patients with ulcerative colitis [23] and Crohn's disease [24], we included IBD disease duration  $\geq 2$  years, prior anti-TNF failure, baseline active endoscopic inflammation, baseline albumin level, prior bowel surgery, prior fistulizing disease, and baseline C-reactive protein (CRP) as co-variables in our logistic regression models for vedolizumab response.

### **Statistical and Sensitivity Analyses**

The rate of primary and secondary outcomes, predictive value of clinical variables on primary and secondary outcomes, odds ratio (OR) with its 95% confidence interval (CI), and P-values were calculated using Statistics/Data Analysis (Stata/IC 15.1 for Windows, College Station, TX). Dichotomous variables were analyzed for outcomes using the chi-squared test or the Fisher's exact test where appropriate, and continuous variables were analyzed using Student's T-tests if normally distributed, or the Wilcoxon signed-rank test for non-normal data. Correction for multiple testing was performed using the Bonferroni correction.

For our multivariate analyses, model building was based on forward stepwise logistic regression, with a P-value of 0.05 required for entry, and known predictors were also included. We tested the significance of a mediation effect using the Sobel, Aroian, and Goodman tests, which assess for interactions or statistically significant mediator variables for our primary outcome of vedolizumab failure. We performed time-to-event analyses to compare rates (hazard ratios) of primary non-response at week 14 and vedolizumab failure at 1-year follow-up between IBD patients with a pre-treatment serum 25(OH)D  $< 25$  ng/mL versus 25(OH)D  $\geq 25$  ng/mL. Time-to-event analyses were performed using GraphPad Prism

(version 8.3; GraphPad Software, Inc., La Jolla, CA). To test the robustness of our findings, we performed several sensitivity analyses. Our first sensitivity analysis involved using different serum 25(OH)D thresholds (from < 10 to < 60 ng/mL with increments of 5 ng/mL) to define a low vitamin status in our multivariate model for the outcome of vedolizumab failure. In our second sensitivity analysis, we performed our multivariate model separately in patients from retrospective versus prospective cohorts. Third, we performed our multivariate model separately in patients with ulcerative colitis versus Crohn's disease by combining patients from both retrospective and prospective datasets. Fourth, we analyzed our multivariate model according to endoscopic inflammation severity (moderate/severe versus quiescent/mild) using patients from both retrospective and prospective cohorts. Finally, we compared our results using vitamin D to predict vedolizumab failure with previously published vedolizumab prediction and clinical decision support tool (CDST) scores as previously described for ulcerative colitis [23] and Crohn's disease [24]. In these previously published CDST, low scores corresponded to decreased risk of vedolizumab response, whereas higher scores corresponded to higher probability of vedolizumab response.

## RESULTS:

### **Single-Cell Immunophenotyping of Peripheral and Intestinal Immune Cells in IBD Patients Using Mass Cytometry (CyTOF) Reveals an Inverse Correlation Between 25(OH)D and $\alpha 4\beta 7$ + Immunophenotypes**

The baseline clinical characteristics of the 48 vedolizumab-naïve IBD patients are summarized in Supplementary Table 1. The single cell distribution of  $\alpha 4\beta 7$  cell surface protein expression on various immune subsets in patients with IBD is summarized in Figure 1. Our multivariate correlation analysis is summarized in Supplementary Table 2. After adjusting for potential confounders such as age, sex, ethnicity, active endoscopic

inflammation, and medications, serum 25(OH)D was inversely associated with  $\alpha 4\beta 7+$  peripheral blood mononuclear cells (PBMC) ( $R = -0.400$ ,  $P < 0.01$ ) and  $\alpha 4\beta 7+$  intestinal leukocytes ( $R = -0.538$ ,  $P = 0.03$ ). Mayo endoscopic scores ( $R = -0.324$ ,  $P = 0.82$ ) and simple endoscopic scores ( $R = 0.038$ ,  $P = 0.90$ ) did not statistically correlate with percentage of  $\alpha 4\beta 7+$  PBMCs in patients with IBD (Supplementary Figure 1). In cross-sectional analysis (Supplementary Figure 2), serum 25 (OH)D was not associated with total peripheral CD4 T cells, CD8 T cells, T regulatory cells, innate lymphoid cells, NK cells, B cells, or monocytes. Serum 25(OH)D was inversely associated with total intestinal CD8 T cells ( $R = -0.573$ ,  $P = 0.02$ ) and intestinal macrophages ( $R = -0.555$ ,  $P = 0.03$ ), but not associated with total intestinal CD4 T cells, T regulatory cells, innate lymphoid cells, NK cells, B cells, or monocytes. In subgroup analysis, serum 25(OH)D was inversely associated with  $\alpha 4\beta 7+$  PBMCs in both patients with ulcerative colitis ( $R = -0.575$ ,  $P < 0.01$ ) and Crohn's disease ( $R = -0.472$ ,  $P < 0.05$ ). In analyses focused on specific immune subsets (Figure 2), higher serum 25(OH)D was associated with decreased peripheral  $\alpha 4\beta 7+$  B cells ( $R = -0.387$ ,  $P = 0.02$ ) and peripheral  $\alpha 4\beta 7+$  NK cells ( $R = -0.390$ ,  $P = 0.02$ ) and decreased intestinal  $\alpha 4\beta 7+$  B cells ( $R = -0.588$ ,  $P = 0.02$ ), intestinal  $\alpha 4\beta 7+$  NK cells ( $R = -0.565$ ,  $P = 0.02$ ), intestinal  $\alpha 4\beta 7+$  monocytes ( $R = -0.739$ ,  $P < 0.001$ ), and intestinal  $\alpha 4\beta 7+$  macrophages ( $R = -0.633$ ,  $P < 0.01$ ). Immunophenotyping (Supplementary Figure 3) based on other leukocyte trafficking markers (CCR9, CCR1, and GPR15) did not reveal associations between serum 25(OH)D and PBMCs or intestinal leukocytes.

## **Vitamin D Receptor (VDR) and Vitamin D Inactivating Enzyme CYP24A1 Gene Expression Are Associated with ITGA4 and ITGB7 Mucosal Expression in Patients with IBD**

Whole genome mucosal gene expression analysis (GSE73661) from baseline colon biopsies in 41 ulcerative colitis patients with active disease who were enrolled in two phase III vedolizumab clinical trials (GEMINI I and GEMINI LTS) [22] is summarized in Supplementary Table 3. In gene expression correlation analyses (Figure 3), vitamin D receptor (VDR) expression was inversely associated with expression of integrin subunits  $\alpha 4$  (ITGA4,  $R=-0.712$ ,  $FDR=5.33 \times 10^{-6}$ ) and  $\beta 7$  (ITGB7,  $R=-0.441$ ,  $FDR=0.03$ ). Conversely, gene expression of the vitamin D inactivating enzyme vitamin D3 24-hydroxylase (CYP24A1) was positively associated with gene expression of  $\alpha 4$  (ITGA4,  $R=0.434$ ,  $FDR=0.03$ ) and  $\beta 7$  (ITGB7,  $R=0.397$ ,  $FDR=0.04$ ). Gene expression of vitamin D activating enzymes (CYP27A1 and CYP27B1) was not associated with ITGA4 or ITGB7 gene expression. VDR expression was inversely associated with CCR1 expression ( $R=-0.562$ ,  $FDR<0.01$ ), whereas CYP24A1 was positively associated with CCR1 expression ( $R=0.418$ ,  $FDR=0.03$ ). Expression of VDR and vitamin D metabolism enzymes (CYP27A1, CYP27B1 and CYP24A1) were not associated with leukocyte trafficking markers CCR9 or GPR15.

## Low Pre-treatment Serum 25(OH)D is Associated with Increased Odds of Vedolizumab Failure at 1-Year Follow-up in Patients with IBD

The baseline clinical characteristics of all IBD patients included (combined retrospective and prospective cohorts) are summarized in **Table 1. Supplementary Table 4** summarizes rates of vedolizumab primary non-response during induction, vedolizumab failure at 1-year follow-up, rates of IBD-surgery, and medications after vedolizumab failure. A total of 73 patients (30%) failed vedolizumab at 1-year follow-up of whom 45 patients failed during induction therapy (primary non-responders). Among patients who failed vedolizumab at 1-year follow-up, 22 patients (30.1%) underwent surgery, 42 patients (57.6%) were on steroids (38 were continued on steroids, 4 were started on new steroids), 26 (36.5%) were switched to ustekinumab, 15 (20.5%) were switched to an Anti-TNF agent, and 6 (8.2%) were switched to Tofacitinib. **Table 2** summarizes our univariate and multivariate logistic regression analysis for clinical predictors of vedolizumab failure at 1-year follow-up for all IBD patients. In univariate logistic regression, 25(OH)D < 25 ng/mL (OR 6.77, 95% CI 3.50- 13.10, P <0.001), active endoscopic inflammation (OR 3.43, 95% CI 1.83- 6.43, P<0.001), and prior anti-TNF therapy (OR 2.31, 95% CI 1.15- 4.63, P=0.019) were associated with increased odds of vedolizumab failure at 1-year follow-up. In multivariate analysis, 25(OH)D < 25 ng/mL (OR 6.10, 95% CI 3.06- 12.17, P <0.001) and active endoscopic inflammation (OR 2.98, 95% CI 1.50- 5.93, P=0.002) were independently associated with vedolizumab failure. **Figure 4A** summarizes the time-to-event analyses comparing rates of vedolizumab failure at 1-year follow-up among IBD patients with a pre-treatment serum 25(OH)D < 25 ng/mL versus 25(OH)D ≥ 25 ng/mL. A pre-treatment serum 25(OH)D < 25 ng/mL was associated with greater rates (shorter time to event) of vedolizumab failure (HR 5.18, 95% CI 3.35- 8.01, P<0.001).

## Low Pre-treatment Serum 25(OH)D is Associated with Increased Odds of Vedolizumab Primary Non-Response During Induction Therapy in Patients with IBD

**Table 3** summarizes our univariate and multivariate logistic regression analysis for clinical predictors of vedolizumab primary non-response during induction therapy (14 weeks after vedolizumab initiation) in IBD patients. In univariate logistic regression, 25(OH)D < 25 ng/mL (OR 26.87, 95% CI 10.60- 58.35, P <0.001), active endoscopic inflammation (OR 3.34, 95% CI 1.55- 7.17, P=0.002), prior bowel surgery (OR 7.88, 95% CI 3.13- 19.80, P<0.001), and prior anti-TNF therapy (OR 2.48, 95% CI 1.04- 5.91, P=0.04) were associated with increased odds of vedolizumab primary non-response at week 14. In multivariate analysis, 25(OH)D < 25 ng/mL (OR 26.10, 95% CI 14.30 – 48.90, P <0.001) and prior bowel surgery (OR 9.45, 95% CI 4.62 – 28.87, P<0.001) were independently associated with vedolizumab primary non-response, whereas vitamin D supplementation use prior to vedolizumab initiation was independently associated with decreased risk (OR 0.36, 95% CI 0.14- 0.95, P=0.039) of vedolizumab primary non-response. **Figure 4B** summarizes the time-to-event analyses comparing rates of vedolizumab primary non-response at week 14 among IBD patients with a pre-treatment serum 25(OH)D < 25 ng/mL versus 25(OH)D ≥ 25 ng/mL. A pre-treatment serum 25(OH)D < 25 ng/mL was associated with greater rates (shorter time to event) of primary non-response (HR 9.57, 95% CI 6.40- 14.30, P<0.001).



## Sensitivity Analyses

In our sensitivity analyses evaluating different serum 25(OH)D thresholds to define a low vitamin D status (**Supplementary Table 5**), serum 25(OH)D was significantly associated with vedolizumab failure starting at 25(OH)D < 15 ng/mL extending to < 40 ng/mL in our combined cohorts. Lower 25(OH)D thresholds were associated with larger effect sizes (odds ratios). In our sensitivity analyses (**Supplementary Table 6**) comparing our results based on type of cohort (retrospective versus prospective), low vitamin D remained an independent predictor of vedolizumab failure at 1-year follow-up in both retrospective (OR 4.45, 95% CI 1.82-10.84, 95% CI, P=0.001) and prospective cohorts (OR 15.58, 95% CI 3.93-61.75, 95% CI, P <0.001). When we compared our results based on IBD subtype (**Supplementary Table 7**), the effect size of this association of low vitamin D with vedolizumab failure was greater in ulcerative colitis (OR 15.15, 95% CI 4.85-47.35, 95% CI, P <0.001) compared to Crohn's disease (OR 3.25, 95% CI 1.31-8.09, P =0.011). Given that baseline endoscopic inflammation was also associated with increased risk of future vedolizumab failure, we performed sensitivity analyses (**Supplementary Table 8**) separating patients with moderate/severe versus quiescent/mild endoscopic inflammation. Low pre-treatment serum 25(OH)D was associated with increased risk of vedolizumab failure in IBD patients with moderate/severe (OR 4.41, 95% CI 1.94-9.99, P<0.001) and quiescent/mild endoscopic inflammation (OR 11.56, 95% CI 3.27-40.91, P<0.001). We performed formal mediation analyses using the Sobel, Aroian, and Goodman tests to assess for interactions (mediator variables) with 25(OH)D and our primary outcome of vedolizumab failure. Our formal mediation analyses are summarized in **Supplementary Table 9**. None of the co-variables were found to have statistically significant (P<0.05) mediator effects on serum 25(OH)D and risk of vedolizumab failure by Sobel, Aroian, and Goodman tests. **Supplementary Table 10** summarizes our analysis comparing our vitamin D vedolizumab

clinical outcomes with previously published [23,24] vedolizumab prediction and clinical decision support tool (CDST) models. In these CDST models, a lower score corresponded with lower probability of vedolizumab response whereas a higher score was associated with higher probability of vedolizumab response. In our cohorts, patients with ulcerative colitis in the low CDST score group (< 26) had a greater proportion of patients with low vitamin D (27.4% vs 14.3%, P <0.05), vedolizumab primary non-response (20.5% vs 7.1%, P<0.01), and vedolizumab failure at 1-year (34.2% vs 7.1%, P <0.01) compared to the high CDST group (>32). Likewise, patients with Crohn's disease in the low CDST score group (< 13) had greater proportion of patients with low vitamin D (47.8% vs 14.8%, P <0.001), vedolizumab primary non-response (39.1% vs 11.1%, P<0.01), and vedolizumab failure at 1-year (69.6% vs 14.8%, P <0.001) compared to high CDST (>19). Taken together, using vitamin D levels to risk stratify patients to predict probability of vedolizumab failure correlated well with these prior CDST models.

## DISCUSSION:

In this multi-cohort study, we demonstrate novel associations between serum 25(OH)D and  $\alpha 4\beta 7$  cell surface expression on PBMCs and intestinal leukocytes and an association between VDR and vitamin D-inactivating enzyme CYP24A1 with ITGA4 and ITGB7 mucosal gene expression. Our findings also identify pre-treatment serum 25(OH)D as a novel biomarker and a potential modifiable risk factor in predicting response to vedolizumab therapy.

Since low vitamin D predicted vedolizumab failure in patients with IBD, we were interested in exploring which  $\alpha 4\beta 7^+$  immune cell subsets were inversely associated with vitamin D. Our data demonstrated that innate immune cells (monocytes, macrophages, NK cells) and B cells were inversely associated with vitamin D. Although our study did not evaluate whether and how these vitamin D-associated  $\alpha 4\beta 7^+$  innate immune cells and B cells

were mediating the risk of future vedolizumab failure, more recent studies have suggested that innate immune cells [25,26,27] and B cells [27] may predict the therapeutic efficacy of vedolizumab. In a prospective study of IBD patients treated with vedolizumab by Zeissig et al. [25], a decrease in M1/M2 macrophage ratio and macrophage-associated gene signatures were associated with response to vedolizumab. Furthermore, in a study by Verstockt et al. [27], M1 macrophages were enriched in vedolizumab non-responders, whereas naïve B cells were enriched in responders. Extending beyond the known  $\alpha 4\beta 7$  role on gut T cell trafficking and understanding the function of  $\alpha 4\beta 7^+$  innate immune cells and B cells in the pathogenesis of IBD may reveal insights into the mechanisms of anti-integrin therapy failure.

Our finding that low pre-treatment 25(OH)D is associated with future vedolizumab failure in patients with IBD may have several explanations. First, vitamin D may negatively regulate  $\alpha 4\beta 7$  expression in patients with IBD. By reducing the frequency of  $\alpha 4\beta 7^+$  immune cells able to migrate to the gut, higher serum 25(OH)D levels may work synergistically with vedolizumab to inhibit immune cell trafficking to the gastrointestinal tract and hence reduce gastrointestinal inflammation. This association with vitamin D may be specific to  $\alpha 4\beta 7$ , as there was no association with the other leukocyte trafficking markers CCR9, CCR1 and GPR15. Although the exact mechanisms of how vitamin D regulates  $\alpha 4\beta 7$  immunophenotypes are unclear and beyond the scope of this study, the biological plausibility of this relationship is supported by prior studies. Vitamin D has been shown to decrease the maturation and activation of dendritic cells, the master regulators of  $\alpha 4\beta 7$  imprinting on immune cells [28]. Decreased dendritic cell maturation may in turn result in attenuated  $\alpha 4\beta 7$  imprinting. Another potential mechanism is that vitamin D may reduce levels of IL-7, which has been shown to induce expression and activation of  $\alpha 4\beta 7$ , promoting immune cell homing to the intestinal mucosa [29]. Vitamin D supplementation has been shown to previously reduce serum IL-7 levels [30]. Second, vitamin D may mediate the risk of vedolizumab

failure through  $\alpha 4\beta 7$ -independent mechanisms. A prior study by Ananthkrishnan et al [31] demonstrated that gut microbiome function predicts response to anti-integrin therapy in IBD. Both the vitamin D receptor and vitamin D have been shown to be major factors in shaping the gut microbiome. The vitamin D receptor plays a role in regulation of gut microbiota composition [32] and in promoting healthy microbial metabolites [33]. Likewise, vitamin D has been shown to modulate the gut microbiota in IBD [34,35]. Vitamin D may mediate the risk of vedolizumab failure through regulation of the gut microbiome. Third, an alternative explanation is that IBD patients with low pre-treatment serum 25(OH)D had more severe baseline disease and thus were more likely to fail vedolizumab therapy. While our analysis did confirm that baseline active endoscopic inflammation was an independent predictor of vedolizumab failure, we adjusted for this co-variate in our multivariate models and sensitivity analyses and serum 25(OH)D remained an independent predictor of vedolizumab failure.

Our study has several strengths. First, our findings are novel. To our knowledge, this is the first study to demonstrate an association between 25(OH)D and  $\alpha 4\beta 7$  immunophenotypes in patients with IBD and to highlight a role of pre-treatment 25(OH)D in predicting vedolizumab response. While other groups have shown that vitamin D regulates *in vitro*  $\alpha 4\beta 7$  expression, our study directly translates these findings to patients with IBD. Second, we applied innovative techniques such as deep immunophenotyping with mass cytometry and whole-genome mucosal gene expression analyses to evaluate the association of vitamin D and VDR with  $\alpha 4\beta 7$  expression in patients with IBD. Third, our findings are both scientifically and clinically impactful. Our study highlighted the need to better understand vitamin D regulation of gut trafficking molecules on innate immune cells and B cells and the functional consequences of these immune cell subsets in IBD, which represent conceptual advances beyond the field's focus on T cell biology. Furthermore, our study suggests that pre-treatment 25(OH)D could be used as a novel biomarker to better risk stratify

patients with IBD who would most benefit from vedolizumab therapy. Given that serum 25(OH)D is an easily modifiable risk factor through diet and vitamin D supplementation, our study also raises the possibility of therapeutic interventions related to vitamin D. Well-powered randomized controlled trials evaluating the clinical benefit of concomitant vitamin D supplementation with vedolizumab induction therapy in patients with IBD are warranted.

Our study also has several limitations that warrant attention. First, our study was observational and may not establish causation or account for residual unmeasured confounders. However, our study met several Bradford Hill criteria [36] to support a plausible causal relationship [37]. First, the effect sizes for risk of vedolizumab failure were strong, and even stronger for primary non-response. Second, the relationship between low serum 25(OH)D and future vedolizumab failure was reproducible in an independent prospective verification cohort. Third, the association with 25(OH)D was specific to  $\alpha 4\beta 7$  and not other leukocyte-trafficking markers. Fourth, there was temporality in our association, as the exposure of low pre-treatment 25(OH)D preceded the outcome of future vedolizumab failure. Fifth, we demonstrated a dose-response relationship. Lower 25(OH)D thresholds were associated with much larger effect sizes for the outcome of vedolizumab failure. Finally, we previously discussed several potential mechanisms to support biological plausibility of vitamin D in regulating  $\alpha 4\beta 7$  immunophenotypes and affecting the risk of vedolizumab failure. A second limitation is that our clinical study was conducted at a single center, which limits the generalizability of our findings. However, as a tertiary referral center for a large northern California population, our patients are ethnically diverse and representative of the US population. Finally, due to study design, we were unable to determine the mechanisms of how low vitamin D leads to vedolizumab failure and how vitamin D alterations of  $\alpha 4\beta 7$  immunophenotypes mediate this risk. Future mechanistic studies and prospective validation of our CyTOF findings are warranted.

In conclusion, we demonstrate that serum 25(OH)D is inversely associated with  $\alpha 4\beta 7$  expression on PBMCs and intestinal leukocytes, VDR is inversely associated with mucosal gene expression of ITGA4 and ITGB7, and that low serum 25(OH)D is associated with increased risk of future vedolizumab failure among patients with IBD. Future studies are warranted to elucidate the exact mechanisms of these associations and to determine whether therapeutic interventions with vitamin D could be used to mitigate the increased risk of vedolizumab failure associated with low pre-treatment vitamin D levels.

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**Conflict of Interest:** None

**Author Contribution:** JG and AH planned and designed the study; SJSR and YH performed mass cytometry; LB performed the whole-genome gene expression analyses; JG and SJSR performed the CyTOF and vitamin D analyses, JG, SL, TB, AP, and AS performed the retrospective chart review for the discovery cohort, JG, TB, AP, and AS processed the prospective data from the Stanford IBD Registry; JG performed clinical study statistical and sensitivity analyses; JG drafted the manuscript; AH and SRS provided critical review of the manuscript; all authors interpreted the results and contributed to critical review of the manuscript; JG had full access to the study data and takes responsibility for the integrity of the data and accuracy of the analysis.

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**Figure 1.** Single-cell immunophenotyping by mass cytometry (CyTOF) reveals A)  $\alpha 4\beta 7$  immunophenotypes in patients with Crohn's disease, B)  $\alpha 4\beta 7$  immunophenotypes in patients with ulcerative colitis, C)  $\alpha 4\beta 7$  expression on peripheral and intestinal immune cells in patients with IBD, and D)  $\alpha 4\beta 7$  expression on peripheral blood mononuclear cells (PBMC) in patients with Crohn's disease and ulcerative colitis.

**Figure 2.** Single-cell immunophenotyping by mass cytometry (CyTOF) of A) peripheral immune cells (CD4 T cells, CD8 T cells, T Regulatory cells, B cells, and ILCs), NK cells, and monocytes) and B) intestinal immune cells (CD4 T cells, CD8 T cells, T Regulatory cells, B cells, NK cells, monocytes, macrophages, and ILCs).

**Figure 3.** Association of gene expression of gut tropic integrins ITGA4 (A) and ITGB7 (B) with vitamin D receptor (VDR) and vitamin D metabolism enzymes (CYP24A1, CYP27A1 and CYP27B1). Pearson correlations were done using log<sub>2</sub>-normalized scaled values for each gene and adjusted for multiple testing using the Benjamini-Hochberg method.

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**Abbreviations:** IBD, inflammatory bowel disease; 25(OH)D, vitamin D; VDR, vitamin D receptor; CYP24A1, gene encoding 24-hydroxylase; CYP27A1, gene encoding sterol 27-hydroxylase; CYP27B1, gene encoding 1-alpha-hydroxylase; UC, ulcerative colitis; CD, Crohn's disease; CyTOF, cytometry by time of flight; PBMC, peripheral blood mononuclear cells; NK cell, natural killer cell; ILC, innate lymphoid cell; ITGA4, gene encoding  $\alpha 4$ ; ITGB7, gene encoding  $\beta 7$ ; OR, odds ratio; HR, hazard ratio; CI, confidence interval; ROC, receiver operating characteristic; AUC, area under the curve; FDR, false discovery rate; 5-ASA, mesalamine; 6-MP/AZA, 6-mercaptopurine/azathioprine; anti-TNF, anti-tumor necrosis factor; IRB, institutional review board.

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**Table 1.** Baseline Clinical Characteristics of Inflammatory Bowel Disease (IBD) Patients Initiating Vedolizumab Therapy

Clinical Variables	All IBD Patients N= 232	Vedolizumab Responders N= 159	Vedolizumab Non-Responders N= 73	P-Value
<b>Age, years (mean ± SD)</b>	41.2 (±16.8)	40.9 (±16.6)	41.5 (± 17.4)	0.800
<b>Sex</b>				
Male , no. (%)	116 (50.0)	70 (44.0)	29 (39.7)	0.130
Female, no. (%)	116 (50.0)	89 (56.0)	44 (60.3)	
<b>Race</b>				
White, no. (%)	172 (74.1)	120 (75.5)	52 (71.2)	0.802
Hispanic, no. (%)	21 (9.1)	13 (8.1)	8 (11.0)	0.984
Black , no. (%)	5 (2.2)	3 (1.9)	1 (1.4)	0.963
Asian , no. (%)	28 (12.1)	20 (12.6)	8 (11.0)	0.912
Pacific Islander , no. (%)	1 (0.01)	1 (0.01)	0 (0)	0.320
Unknown , no. (%)	5 (2.2)	5 (2.2)	0 (0)	0.442
<b>Ulcerative Colitis</b>				
Total, no. (%)	122 (52.6)	84 (52.8)	39 (53.4)	0.636
E1 , no. (%)	4 (1.7)	3 (1.9)	1 (1.4)	0.963
E2 , no. (%)	42 (18.1)	28 (17.6)	14( 19.2)	0.953
E3 , no. (%)	76 (32.8)	54 (34.0)	22 (30.1)	0.500
<b>Crohn's Disease</b>				
Total, no. (%)	111 (47.8)	75 (47.2)	36 (49.3)	0.894
L1 , no. (%)	13 (5.6)	11 (6.9)	2 (2.8)	0.200
L2 , no. (%)	15 (6.5)	9 (5.7)	6 (8.2)	0.648
L3 , no. (%)	83 (35.8)	51 (32.1)	32 (43.8)	0.281
L4 , no. (%)	0 (0)	0 (0)	0 (0)	---
Perianal disease	50 (21.6)	32( 20.1)	18 (24.7)	0.471
B1 , no. (%)	20 (8.6)	15 (9.4)	5 (6.8)	0.656
B2 , no. (%)	35 (15.1)	23 (14.5)	12 (16.4)	0.968
B3 , no. (%)	74 (31.9)	40 (25.2)	34 (46.6)	0.181
<b>BMI, kg/m<sup>2</sup> (mean ± SD)</b>	25.0 (± 6.2)	24.7 (± 5.7)	25.7 (± 7.1)	0.844
<b>Current Smoker, no. (%)</b>	4 (1.7)	1 (0.6)	3 (4.1)	0.090
<b>Current Alcohol Use, no. (%)</b>	95 (40.9)	66 (41.5)	29 (39.7)	0.492
<b>Pretreatment 25(OH)D, ng/mL (mean ± SD)</b>	33.7 (± 13.7)	36.5 (± 11.7)	28.4 (±15.6)	<0.001
25(OH)D < 25 ng/mL, no. (%)	66 (28.4)	25(15.7)	41 (56.2)	
25(OH)D ≥ 25 ng/mL, no. (%)	166 (65.9)	134 (84.3)	32 (43.8)	
<b>Creatinine, mg/dL (mean ± SD)</b>	0.81 (±0.3)	0.84 (± 0.4)	0.75 (± 0.2)	0.470
<b>Albumin, g/L (mean ± SD)</b>	3.5 (± 0.5)	3.6 (± 0.5)	3.4 (± 0.6)	0.960
<b>C-reactive Protein, mg/dL (mean ± SD)</b>	3.66 (± 6.9)	3.30 (± 5.7)	4.3 (± 8.7)	0.783
<b>Fecal Calprotectin, µg/g (mean ± SD)</b>	665.5 (± 653.9)	551.9 (± 549.0)	892.9 (±785.7)	0.985
<b>Baseline Endoscopic Inflammation*</b>				
Quiescent/Mild, no. (%)	112 (48.3)	94 (59.1)	18 (24.7)	<0.001
Moderate/Severe, no. (%)	120 (51.7)	65( 40.9)	55 (75.3)	<0.001
<b>IBD Medication History</b>				
Current Steroids, no. (%)	103 (44.4)	65 (40.9)	38 (52.1)	0.387
Current 5-ASA, no. (%)	63 (27.2)	42 (26.4)	21 (28.8)	0.993
Current 6MP/Azathioprine, no. (%)	30 (12.9)	19 (11.9)	11 (15.1)	0.973
Current Methotrexate, no. (%)	21 (9.1)	15 (9.4)	6 (8.2)	0.906
Prior Anti- TNF Failure, no. (%)	167 (72.0)	107 (67.3)	60 (82.3)	0.017
Prior Ustekinumab, no. (%)	0 (0)	0 (0)	0 (0)	---
Prior Tofacitinib, no. (%)	0 (0)	0 (0)	0 (0)	---
Current Opioids, no. (%)	19 (8.2)	13 (8.2)	6 (8.2)	0.890
Current NSAIDs, no. (%)	34 (14.7)	20 (12.6)	14 (19.2)	0.562
Current Vitamin D Supplementation, no. (%)	127 (54.7)	87 (54.7)	40 (54.8)	0.277

\*Endoscopic Inflammation: Quiescent (Mayo =0, CD-SES =0-2), Mild (Mayo =1, CD-SES 3-6) Moderate (Mayo =2, CD-SES= 7-15), Severe (Mayo =3, CD-SES >15)

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**Table 2.** Univariate and Multivariate Predictors of Vedolizumab Therapy Failure in Patients with IBD at 1-Year Follow-up

Clinical Variables	All IBD Patients, N= 252					
	Univariate			Multivariate		
	Odds Ratio	95% CI	P-Value	Odds Ratio	95% CI	P-Value
IBD Disease Duration ≥ 2 Years	0.79	0.41 - 1.53	0.483			
Fistulizing Disease	1.82	0.69 - 4.79	0.184			
Low Serum 25(OH)D	6.77	3.50 - 13.10	<0.001	6.10	3.06 - 12.17	<0.001
Albumin (g/L)	0.65	0.33 - 1.04	0.068			
C-reactive Protein (mg/dL)	1.02	0.97 - 1.07	0.394			
Active Endoscopic Inflammation	3.43	1.83 - 6.43	<0.001	2.98	1.50 - 5.93	0.002
Prior Bowel Surgery	2.58	0.97 - 7.61	0.175			
Prior Anti-TNF Failure	2.31	1.15 - 4.63	0.019	1.94	0.90 - 4.20	0.092
Current Vitamin D Supplementation	0.73	0.41 - 1.29	0.278	0.65	0.34 - 1.20	0.205

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**Table 3.** Univariate and Multivariate Predictors of Vedolizumab Primary Non-Responders (Vedolizumab Failure During Induction)

Clinical Variables	All IBD Patients, N= 252					
	Univariate			Multivariate		
	Odds Ratio	95% CI	P-Value	Odds Ratio	95% CI	P-Value
IBD Disease Duration ≥ 2 Years	0.77	0.22 - 1.12	0.080			
Fistulizing Disease	1.97	0.85 - 4.56	0.112			
Low Serum 25(OH)D	24.87	10.60 - 58.35	<0.001	26.10	14.30 - 48.90	<0.001
Albumin (g/L)	0.56	0.30 - 1.05	0.071			
C-reactive Protein (mg/dL)	0.98	0.92 - 1.05	0.604			
Active Endoscopic Inflammation	3.34	1.55 - 7.17	0.002	2.51	0.87 - 7.29	0.090
Prior Bowel Surgery	7.88	3.13 - 19.80	<0.001	9.45	4.62 - 28.87	<0.001
Prior Anti-TNF Failure	2.48	1.04 - 5.91	0.041	1.48	0.50 - 4.41	0.482
Current Vitamin D Supplementation	0.64	0.33 - 1.25	0.192	0.36	0.14 - 0.95	0.039

ACC



**FIGURE 1**

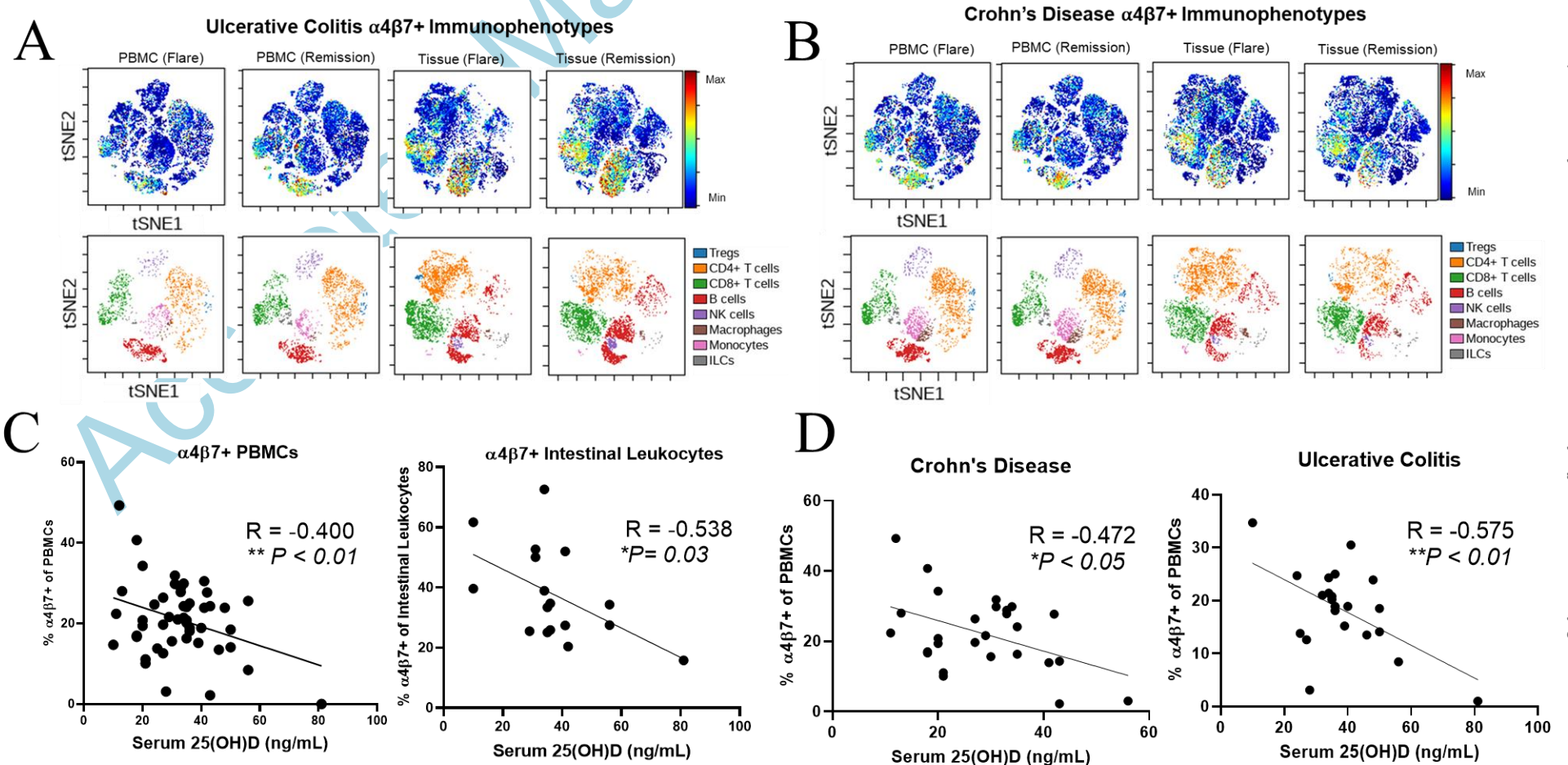




FIGURE 2

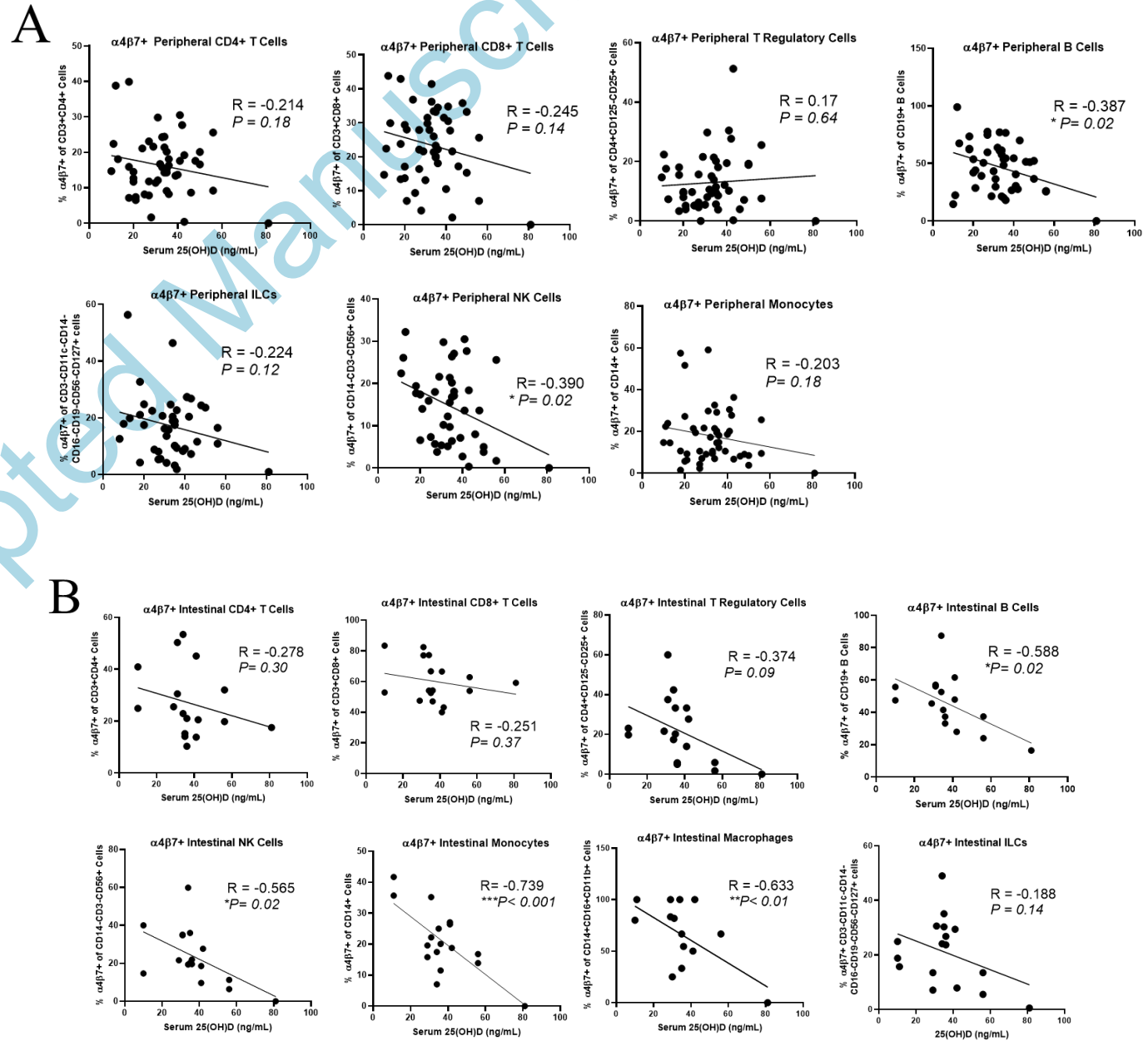
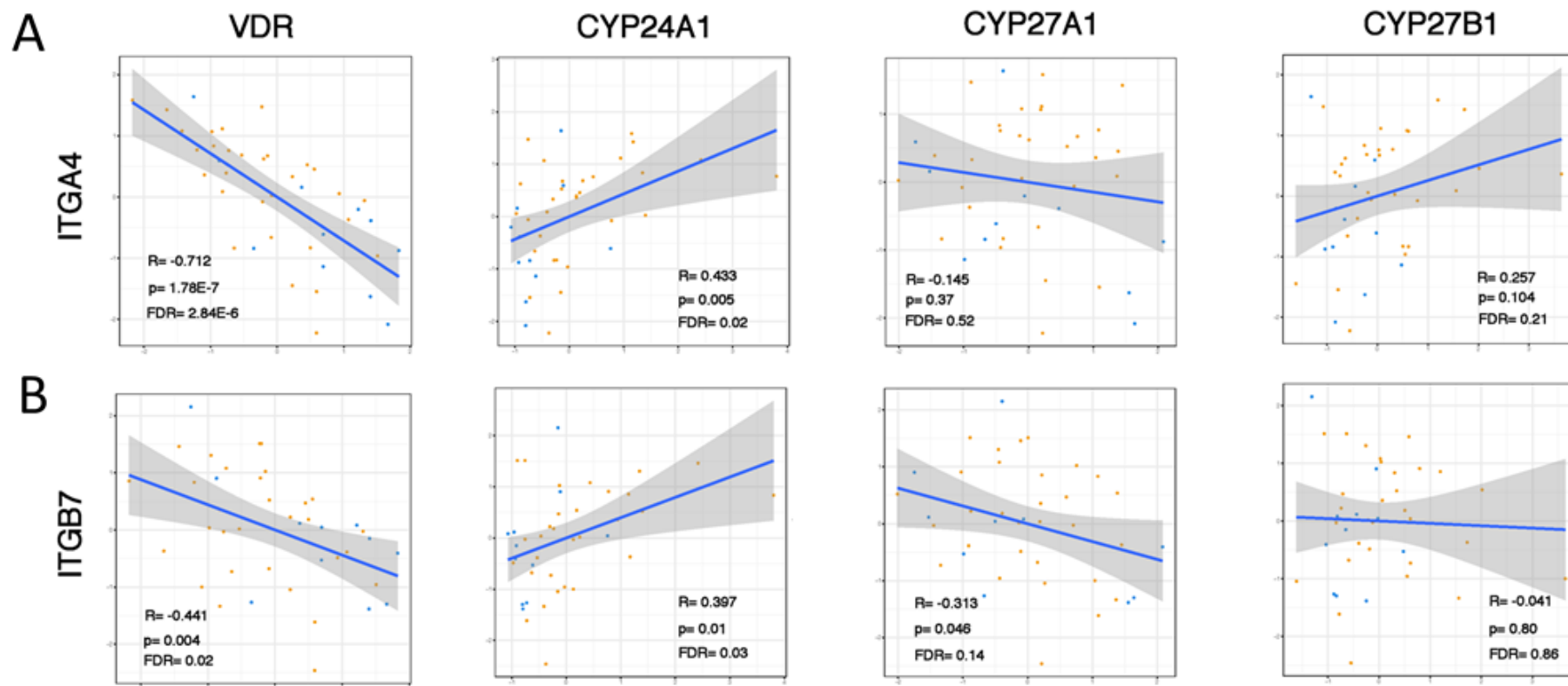


FIGURE 3



**FIGURE 4**

