Free and Bioavailable 25-Hydroxyvitamin D Concentrations are Associated With Disease Activity in Pediatric Patients With Newly Diagnosed Treatment Naïve Ulcerative Colitis

Cary G. Sauer, MD, MSc,[*] Matthew S. Loop, PhD,[†] Suresh Venkateswaran, MD,[*] Vin Tangpricha, MD,[*] Thomas R. Ziegler, MD,[*] Ashish Dhawan, MD,[‡] Courtney McCall, BS,[§] Erin Bonkowski, BS,[§] David R. Mack, MD,[¶] Brendan Boyle, MD,[**] Anne M. Griffiths, MD,[††] Neal S. Leleiko, MD,[‡‡] David J. Keljo, MD,[§§] James Markowitz, MD,[¶¶] Susan S. Baker, MD,[***] Joel Rosh, MD,[†††] Robert N. Baldassano, MD,[‡‡‡] Sonia Davis, DrPH,[†] Shiven Patel,[*] Jessie Wang, MS,[†] Alison Marquis, MStat,[†] Krista L. Spada, BS,[§§§] Subra Kugathasan, MD,[*] Thomas Walters, MD,[††] Jeffrey S. Hyams, MD[§§§] and Lee A. Denson, MD[§]

Background: Vitamin D regulates intestinal epithelial and immune functions, and vitamin D receptor deficiency increases the severity of murine colitis. Bioavailable 25-hydroxyvitamin D (25(OH)D) is available to target tissues and may be a driver of immune function. The aim is to evaluate the relationship of bioavailable 25(OH)D to the clinical expression of treatment naive pediatric ulcerative colitis (UC).

Methods: The PROTECT (Predicting Response to Standardized Pediatric Colitis Therapy) study enrolled children ≤17 years newly diagnosed with UC. Free and total 25(OH)D were directly measured and 25(OH)D fractions were compared with disease activity measures.

Results: Data were available on 388 subjects, mean age 12.7 years, 49% female, 84% with extensive/pancolitis. The median (IQR) total 25(OH)D concentration was 28.5 (23.9, 34.8) ng/mL, and 57% of subjects demonstrated insufficient vitamin D status (25(OH)D < 30 ng/mL). We found no evidence of association between total 25(OH)D and disease activity. Regression models adjusted for age, sex, race, and ethnicity demonstrated that an increase from 25th to 75th percentile for bioavailable and free 25(OH)D were associated with a mean (95th CI) decrease in the Pediatric Ulcerative Colitis Activity Index (PUCAI) of -8.7 (-13.7, -3.6) and -3.1 (-5.0, -1.2), respectively. No associations were detected between 25(OH)D fractions and fecal calprotectin or Mayo endoscopy score.

Conclusions: Vitamin D insufficiency is highly prevalent in children with newly diagnosed UC. We found associations of free and bioavailable, but not total 25(OH)D, with PUCAI. Bioavailable vitamin D may contribute to UC pathophysiology and clinical activity.

Key Words: pediatric, inflammatory bowel disease, ulcerative colitis, vitamin D

What is Known/What is New

What is Known

- Vitamin D deficiency is common in pediatric UC patients.
- Vitamin D regulates intestinal epithelial and immune functions.
- Vitamin D receptor deletion in mice exacerbates experimental colitis.

What is New

- Free and bioavailable 25(OH) vitamin D but not total 25(OH) D are associated with disease activity as defined by the Pediatric Ulcerative Colitis Activity Index (PUCAI) in treatment naive pediatric UC patients.
- Bioavailable 25(OH)D may play a role in the pathogenesis of pediatric UC.

INTRODUCTION

Crohn's disease (CD) and ulcerative colitis (UC) are relapsing and remitting disorders of the gastrointestinal tract

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*Emory University, Atlanta, GA, USA; †Collaborative Studies Coordinating Center, University of North Carolina, Chapel Hill, NC, USA; ‡Cooper University Children's Regional Hospital; §Cincinnati Children's Hospital Medical Center, Cincinnati, OH, USA;¶Children's Hospital of East Ontario, Ottawa, Ontario, Canada; **Nationwide Children's Hospital Connecticut Children's Medical Center, Hartford, CT, USA; ††Hospital For Sick Children, Toronto, Canada; ‡‡Hasbro Children's Hospital, Providence, RI, USA; §§Children's Hospital of Pittsburgh, Pittsburgh, PA, USA; ¶¶Cohen Children's Medical Center Of New York, New Hyde Park, NY, USA; ***Women & Children's Hospital of Buffalo WCHOB, Buffalo, NY, USA; †††Goryeb Children's Hospital - Atlantic Health, Morristown, NJ, USA; ‡‡‡The Children's Hospital of Philadelphia, Philadelphia, PA, USA; §§§Connecticut Children's Medical Center, Hartford, CT, USA

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Address correspondence to: Cary G. Sauer, MD, MSc, Associate Professor, Department of Pediatrics, Emory University School of Medicine, Training Program Director, Emory Pediatric GI Fellowship, Endoscopy Director, Children's Healthcare of Atlanta, 2015 Uppergate Dr. NE, Suite 250, Atlanta, GA 30322. E-mail: csauer@emory.edu Tel: 404-712-2160.

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characterized by acute and chronic inflammation leading to mucosal injury.[1] Current evidence suggests that a complex interplay between the intestinal microbiota, intestinal epithelia, and innate and adaptive immune systems drives disease pathogenesis.[2] Emerging data over the past decade have suggested that vitamin D plays a significant role in both epithelial and immune system dysregulation in this setting, leading to practice guidelines for supplementation.[3–5] These studies have been limited by measurement of only total serum 25-hydroxvitamin D (25(OH)D), which reflects total body stores, but may not adequately define the bioavailable pool exerting beneficial effects in the gut.[6] For example, recent studies have demonstrated a stronger association between bioavailable 25(OH)D than total serum 25(OH)D for markers of bone mineral metabolism.[7] Whether this would also be the case for measures of disease activity in UC was not known.

Vitamin D has an established role in bone health and calcium homeostasis. However, both intestinal epithelial cells (IEC) and a wide variety of immune cells also possess the vitamin D receptor (VDR), suggesting a role for vitamin D in regulation of IECs and the immune system. The VDR has been identified on multiple types of immune cells including T-helper cells (CD4+), cytotoxic T-cells (CD8+), B cells, neutrophils, dendritic cells, and macrophages.[8] Thus, there is a growing body of literature that suggests vitamin D has a role not only in bone health and calcium homeostasis, but also a significant role in immune system function.

A recent meta-analysis demonstrated that adult-onset IBD patients were more than twice as likely to be vitamin D deficient as healthy controls.[9] A recent pediatric study demonstrated IBD patients with an elevated sedimentation rate had a significantly lower 25(OH)D compared to controls.[10] With one exception, IBD studies to date have evaluated only total 25(OH)D.[11] However, only a small portion (5%–10%) of total 25(OH)D is bioavailable and thus free to diffuse into cells to bind the VDR, with the rest bound to vitamin D-binding protein (VDBP).[6] Bioavailable vitamin D includes the free 25(OH)D and albumin-bound vitamin D fractions. It is this bioavailable vitamin D that is available to target tissues and is more likely to play a significant role in IEC and immune system regulation. Prior efforts to define bioavailable vitamin D have been limited by indirect methods that involved measurement of vitamin D-binding protein (VDBP) and likely overestimated the bioavailable vitamin D pool. With the recent availability of a method to directly measure free vitamin D and thereby precisely calculate bioavailable vitamin D, we sought to understand the relationship between free and bioavailable vitamin D and disease activity in a large pediatric UC inception cohort. The PROTECT Study: Predicting Response to Standardized Pediatric Colitis Therapy was initiated in 2012 to systematically examine the response of children and adolescents newly diagnosed with UC to standardized treatment regimens. During this prospective study, a large inception patient cohort was rigorously phenotyped clinically and endoscopically. We now report the relationship between free and bioavailable vitamin D and disease activity in this large pediatric UC inception cohort.

METHODS

PROTECT Study

The PROTECT enrolled 428 children with UC between the ages of 4 and 17 at diagnosis, before therapy, at 26 participating sites in North America. The study was approved by the Institutional Review Boards at each of the sites, and informed consent was obtained for each of the participants, with assent obtained for those age 11 and older. Demographic, clinical, laboratory, and endoscopic findings at diagnosis were recorded and only patients with a confirmed diagnosis of UC were included. Inclusion criteria included clinical symptoms (abdominal pain, loose stools, and rectal bleeding), a negative stool infectious workup, and endoscopic/histologic confirmation of UC based on established criteria with disease extending beyond the rectum. The current analysis included 388 participants with complete data for free, bioavailable, and total vitamin D fractions.

Determination of Vitamin D Fractions

Plasma collected at diagnosis before therapy was used to measure free 25(OH)D, total 25(OH)D, and albumin to determine vitamin D status. Total 25(OH)D was measured using the IDS-iSYS 25-Hydroxyvitamin D automated chemiluminescense immunoassay (Gaithersburg, MD) in a laboratory participating in the Vitamin D External Quality Assessment Scheme (DEQAS) and the NIST/NIH Vitamin D Quality Assurance Program (VitDQAP). The plasma free 25(OH)D was assayed by ELISA (DIAsource ImmunoAssays, Louvain-la-Neuve, Belgium)[12]

Albumin was measured at a central laboratory by ELISA per manufacturer's instructions (Cell Biolabs, Inc., San Diego, CA) for participants with no available clinical value (40 patients).

Bioavailable vitamin D ([Bio D]) was calculated from measured free vitamin D and measured albumin using the following formula.[13]

$$[\text{Bio D}] = [\text{DFree}] + [\text{DAlb}]$$

$$[\text{DAlb}] = \text{concentration of albumin-bound 25-hydroxyvitamin D}$$

$$[\text{DFree}] = \text{concentration of free (unbound) 25-hydroxyvitamin D}$$

$$[\text{DAlb}] = \text{Kalb} \cdot [\text{Total Serum Albumin}] \cdot [\text{DFree}]$$

$$K_{alb} = \text{affinity constant between 25-hydroxyvitamin D and albumin} = 6 \times 10^5\ M^{-1}$$

Fecal calprotectin was performed centrally by ELISA per manufacturer's instructions (Buhlmann Laboratories AG, Schönenbuch, Switzerland) from stool samples collected before colonoscopy cleanout or ≥2 days after colonoscopy but not more than 3 days after initial UC treatment The assay has a sensitivity of 10 mcg/gm with a standard range of 30 mcg/gm to 1800 mcg/gm. Samples above the standard range underwent serial dilution. The intraassay precision is 2.7% to 8.1%, and the interassay precision is 6.6% to 14.5%.

Statistical Analysis

We calculated summary statistics of 25(OH)D fractions (total, bioavailable, and free) in the total sample and tested for pairwise associations between the vitamin D fractions using the Spearman correlation test. We calculated summary statistics of demographics, clinical and laboratory measures, and disease activity (PUCAI and Mayo endoscopy subscore as determined by the site clinical investigator) in the total sample and by clinical cutpoints of total vitamin D (deficient was <20 ng/mL, insufficient was 20 to <30 ng/mL, and sufficient was ≥30 ng/mL) and by tertiles of bioavailable and free 25(OH) D. We conducted univariate tests of association between participant baseline characteristics and these vitamin D categories using ANOVA for Gaussian-distributed variables, the Kruskal-Wallis test for skewed continuous variables, the mean score chi-square test for nonordered categorical (ie, nominal) variables, and the nonzero correlation test for ordinal variables.

We used linear regression to fit models for the unadjusted relationship between PUCAI and $\log_{10}$ of the vitamin D fractions. Nonlinear relationships between PUCAI and 25(OH) D fractions were tested for using restricted cubic splines with 5 knots, with F-tests for the nonlinear model terms.[14] If the hypothesis tests for the nonlinear model terms were not statistically significant at the alpha = 0.05 level, then the nonlinear terms were dropped from the model. We fit additional models for each fraction of 25(OH)D that were adjusted for age, sex, race, and Hispanic ethnicity as potential confounders. As sensitivity analyses, unadjusted and adjusted models were also fit for: (1) the relationship between Mayo endoscopy subscore (severe/moderate vs mild) and 25(OH)D fraction using logistic regression; and (2) the relationship between fecal calprotectin and 25(OH) D fraction using linear regression. We reported mean differences with 95% confidence intervals for the difference between the 1st and 3rd quartile of the 25(OH)D fraction when modeling PUCAI and fecal calprotectin and odds ratios with 95% confidence intervals when modeling Mayo endoscopy subscore.

RESULTS

Demographic Characteristics of the Cohort

A total of 388 subjects with complete data for 25(OH) D fractions were included in the analysis. All subjects were enrolled at initial diagnosis of UC with clinical and laboratory measures obtained before therapy. The mean age of diagnosis was 12.7 years with 49% female. The sample was predominantly white (81%) but included Asians (5%) and blacks (7%) and 9% of the participants self-identified as Hispanic (Table 1).

Clinical and Laboratory Characteristics

The median (IQR) PUCAI for the patient population was 50 (35,65) with 129 (33%) patients demonstrating severely active colitis defined by a PUCAI ≥65, and 167 (43%) patients demonstrating moderately active disease defined by a PUCAI between 35 and 60. The Mayo endoscopy subscore similarly demonstrated endoscopically severe Mayo 3 disease in 132 (34%) patients and moderate Mayo 2 disease in 206 (53%) patients. A total of 325 (84%) patients presented with extensive or pancolitis disease location (Table 1). The median hemoglobin was 11.6 g/dL, the median erythrocyte sedimentation rate (ESR) was 24 mm/hour, and the median albumin was 3.8 g/dL. C-reactive protein (CRP) was greater than 2X the upper limit of normal in 88 (30%) patients. The median fecal calprotectin was 2286 mcg/g, with 176 of the 222 subjects (79%) having baseline stool calprotectin >1000 mcg/g.

Vitamin D Status of Participants

The median (IQR) total 25(OH)D concentration was 28.5 (23.9,34.8) ng/mL with 166 (43%) patients demonstrating vitamin D sufficiency defined by a level greater than or equal to 30 ng/mL. A total of 181 patients (47%) demonstrated vitamin D insufficiency defined as a level between 20 and 30 ng/mL and a total of 41 patients (11%) demonstrated vitamin D deficiency defined as a level below 20 ng/mL. The median (IQR) free vitamin D concentration was 4.1 (2.9, 6.0 pg/mL), and the median (IQR) bioavailable vitamin D concentration was 1.3 (0.8, 1.9) ng/mL. We then tested for associations between these vitamin D fractions. We observed a moderate correlation between bioavailable 25(OH)D and total 25(OH)D (Spearman correlation of 0.46, $P < 0.01$) and between free 25(OH)D and total 25(OH)D (Spearman correlation of 0.49, $P < 0.01$) (Fig. 1A and B). As expected, we confirmed a strong correlation between free and bioavailable 25(OH)D (Spearman r = 0.93, $P < 0.01$, Fig. 1C). Further examination demonstrates a strong correlation between free and bioavailable 25(OH)D within different categories of total vitamin D (Supplemental Figure 1). Collectively, these data demonstrated that bioavailable vitamin D represented approximately 4.7% (3.1%, 6.5%) of the total vitamin D pool within a newly diagnosed pediatric UC cohort, with a moderate correlation with total vitamin D levels.

Association between Total Vitamin D and Bioavailable/free Vitamin D

We evaluated the linearity between bioavailable 25(OH) D and total vitamin D and the linearity between free 25(OH) D and total vitamin D. The relationship between bioavailable 25(OH)D and total 25(OH)D is positive until approximately 40 ng/mL of total 25(OH)D at which point there appears to be no relationship, with similar findings for the relationship between free 25(OH)D and total 25(OH)D. This data suggest that as total 25(OH)D increases, free and bioavailable 25(OH) D also increase, up to a total 25(OH) D level of 40 ng/mL, at which any further increases in total 25(OH)D result in no additional increase in free or bioavailable 25(OH)D (Fig. 2).

TABLE 1: Clinical and Demographic Characteristics by Total Vitamin D Clinical Cut Points

	Total Vitamin D (ng/mL)				
Variable[a]	Total Sample (n = 388)	<20 ng/mL (n = 41)	20–<30 ng/mL (n = 181)	≥30 ng/mL (n = 166)	P-value[d]
Age, years[b]	12.7 (3.3)	13.7 (2.4)	13.0 (3.2)	12.2 (3.5)	0.009
Race					
Asian	18 (5%)	3 (7%)	14 (8%)	1 (1%)	0.001
Black	26 (7%)	5 (12%)	15 (8%)	6 (4%)	0.01
Other	28 (7%)	3 (7%)	15 (8%)	10 (6%)	0.29
White	316 (81%)	30 (73%)	137 (76%)	149 (90%)	
Females	191 (49%)	15 (37%)	97 (54%)	79 (48%)	0.92
Hispanic	34 (9%)	4 (10%)	18 (10%)	12 (7%)	0.40
Weight z-score[b]	-0.1 (1.1)	-0.3 (1.4)	-0.1 (1.1)	-0.0 (1.1)	0.36
Disease Location					
Proctosigmoiditis	23 (6%)	1 (2%)	19 (10%)	3 (2%)	0.04
Left-sided colitis	40 (10%)	7 (17%)	18 (10%)	15 (9%)	
Extensive / Pancolitis / Unassessable	325 (84%)	33 (80%)	144 (80%)	148 (89%)	
Albumin (g/dL)[c]	3.8 (3.2, 4.2)	3.8 (3.3, 4.3)	3.7 (3.0, 4.2)	3.9 (3.4, 4.2)	0.35
Hemoglobin (g/dL)[c]	11.6 (10.3, 12.9)	11.7 (9.0, 13.0)	11.9 (10.5, 13.0)	11.3 (9.9, 12.7)	0.17
ESR (mm/hr)[c]	24.0 (12.0, 41.0)	22.0 (12.5, 34.5)	23.0 (11.0, 44.0)	28.5 (14.0, 42.0)	0.24
CRP or hsCRP (mg/dL)					
>ULN	129 (45%)	15 (42%)	63 (46%)	51 (44%)	0.99
>2 × ULN	88 (30%)	6 (17%)	45 (33%)	37 (32%)	0.28
Fecal calprotectin (mcg/g)[c]	2285.9 (1171.7, 3906.7)	1891.1 (1157.1, 4181.1)	1798.6 (1071.0, 3849.9)	2633.8 (1388.5, 4008.8)	0.39
PUCAI[c]	50.0 (35.0, 65.0)	50.0 (30.0, 65.0)	45.0 (30.0, 65.0)	50.0 (40.0, 65.0)	0.50
Mild (10–34)	92 (24%)	11 (27%)	49 (27%)	32 (19%)	0.43
Moderate (35–64)	167 (43%)	17 (41%)	70 (39%)	80 (48%)	
Severe (≥65)	129 (33%)	13 (32%)	62 (34%)	54 (33%)	
Mayo endoscopy subscore					
Mild (1)[e]	50 (13%)	3 (7%)	26 (14%)	21 (13%)	0.82
Moderate (2)	206 (53%)	22 (54%)	96 (53%)	88 (53%)	
Severe (3)	132 (34%)	16 (39%)	59 (33%)	57 (34%)	

[a]Values are n (%) unless otherwise specified; [b]mean (standard deviation); [c]median (quartile 1, quartile 3); [d]P-value for ANOVA when mean (standard deviation) is presented, Kruskal-Wallis when median (quartile 1, quartile 3) is presented, mean score chi-square test for nominal row variables, and nonzero correlation test for ordinal row variables. For race variable, p-value is for that racial group vs whites.
[e]One participant with no disease evident was included in the mild group.

Association of Total Vitamin D and Demographic/Clinical Characteristics

Patients were grouped by total 25(OH)D into 3 categories based on current clinical guidelines (sufficient, insufficient, and deficient).[3] Patients with vitamin D deficiency/insufficiency were older at diagnosis than those with vitamin D sufficiency. The distribution of total vitamin D categories also varied with disease location, with extensive/pancolitis participants occurring more frequently in those with sufficient total vitamin D. We did not find statistically significant evidence of associations between categories of total vitamin D and albumin, hemoglobin, ESR, CRP, fecal calprotectin, PUCAI, or Mayo subscore (Table 1).

Association of Free and Bioavailable Vitamin D Fractions and Demographic/Clinical Characteristics

There are no standard clinical cut points for bioavailable 25(OH)D. Patients were therefore grouped according to tertiles of bioavailable 25(OH)D. As with total 25(OH)D, the oldest age-of-diagnosis was observed within the lowest tertile

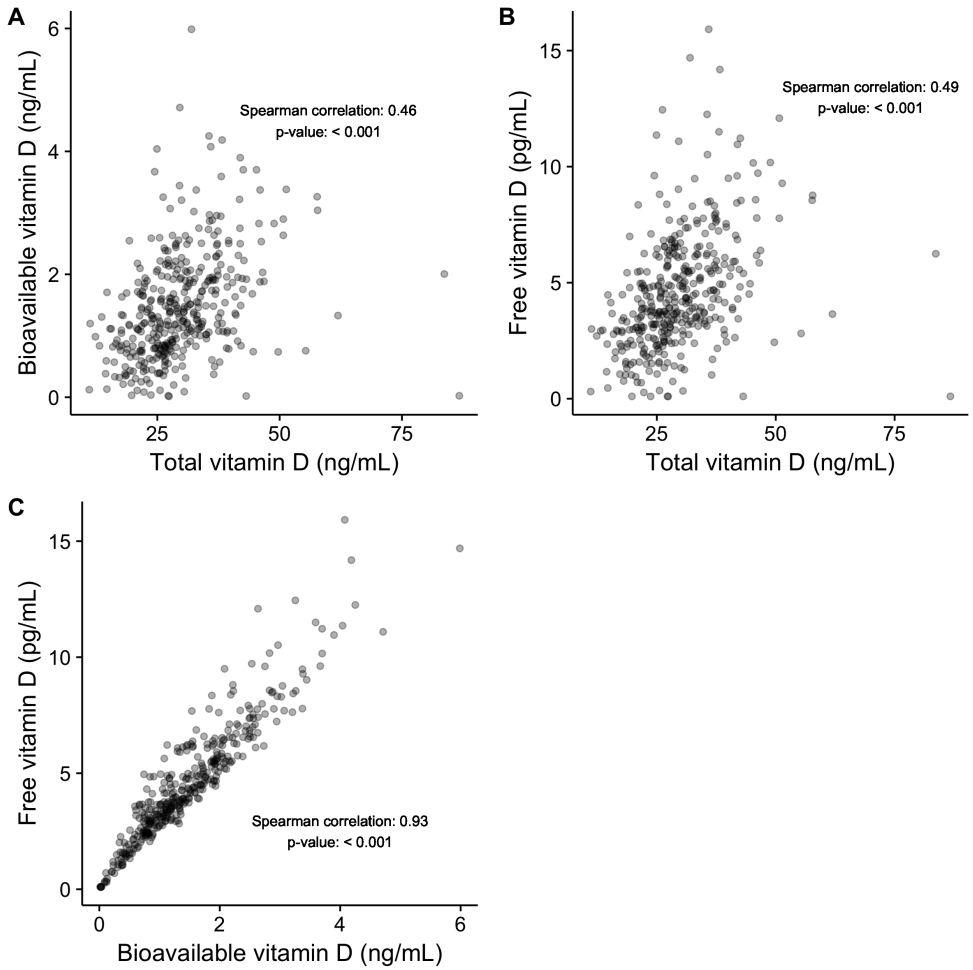

FIGURE 1. Spearman correlations between vitamin D fractions. A, Demonstrates moderate correlation between bioavailable and total 25(OH)D (Spearman correlation of 0.46, $P < 0.01$, n = 388). B, Demonstrates moderate correlation between free 25(OH)D and total 25(OH)D (Spearman correlation of 0.49, $P < 0.01$, n = 388). C, Demonstrates strong correlation between free and bioavailable 25(OH)D (Spearman r = 0.93, $P < 0.01$, n = 388).

of bioavailable vitamin D (Table 2). Similarly, the distribution of bioavailable 25(OH)D differed between whites and blacks, with blacks more likely to be in the lowest tertile (≤1.02 ng/mL) of bioavailable 25(OH)D. Similar associations with age and race were observed when considering tertiles of free 25(OH)D (Supplemental Table 1). The distribution of bioavailable 25(OH)D also varied with disease location, but in this case participants with extensive/pancolitis were more common in the lowest tertile of bioavailable 25(OH)D. In terms of the laboratory parameters, albumin and hemoglobin were lower, and the inflammatory markers ESR and CRP were higher, for participants within the lowest tertile of bioavailable 25(OH)D. A similar result was observed for free 25(OH)D, with participants with lower albumin and hemoglobin in the lowest tertile of free 25(OH)D (Supplemental Table 1). There was no statistically significant evidence that fecal calprotectin or the Mayo endoscopy subscore varied across the groups for either free or bioavailable 25(OH)D. However, consistent with the laboratory parameters, the clinical measure of disease activity, PUCAI, was increased in participants within the first or second tertiles of either free or bioavailable 25(OH)D, compared to participants in the third tertile (Table 2 and Supplemental Table 1).

Associations between Vitamin D Fractions and Disease Activity

Linear regression model results for the associations between vitamin D fractions and PUCAI are presented in Table 3. Bioavailable vitamin D was modeled using restricted cubic splines with 5 knots, whereas total 25(OH)D and free 25(OH)D were modeled using only linear terms, due to the lack of significance of the nonlinear terms for these fractions in preliminary models. All model results were reported for a change from the 1st quartile to the 3rd quartile of the vitamin D

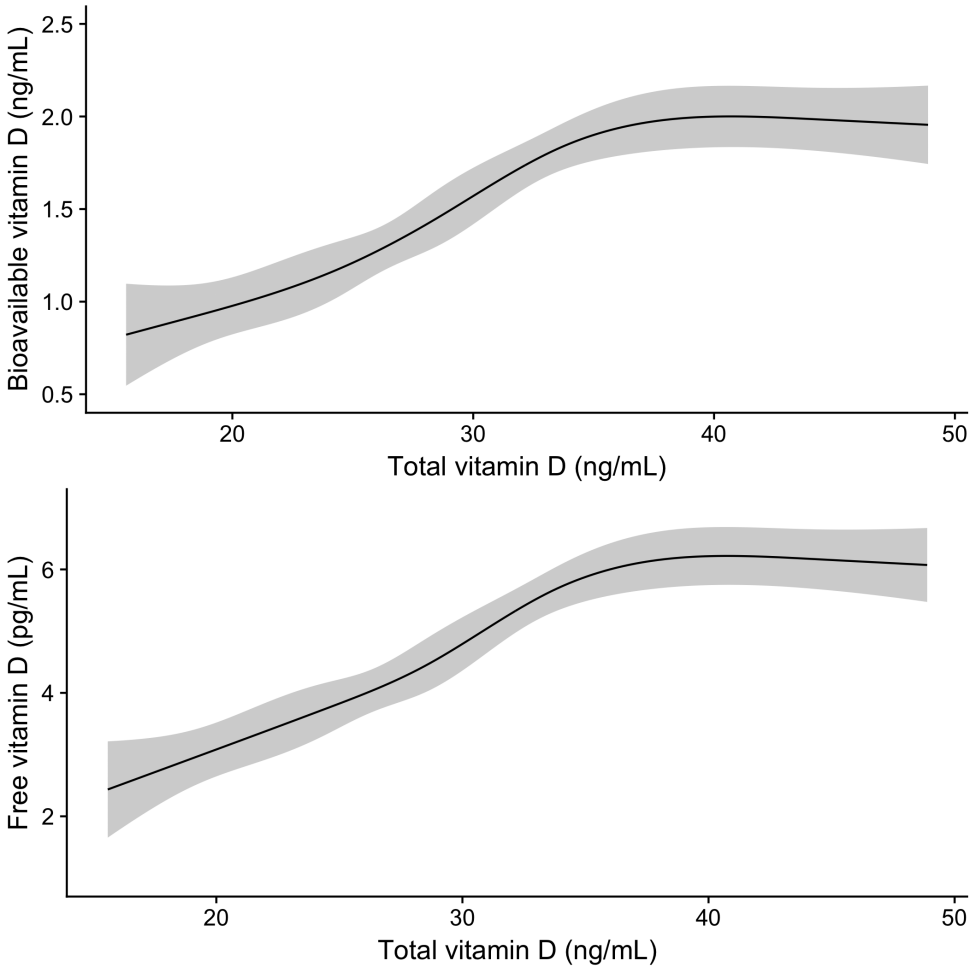

FIGURE 2. Relationship between total vitamin D and bioavailable/free vitamin D. Results demonstrate a positive relationship between total 25(OH) D and both free and bioavailable 25(OH)D until the total 25(OH)D reaches approximately 40 ng/ml.

fraction, due to the different measurement scales (ie, ng/mL vs pg/mL). In models adjusted for age, race, sex, and Hispanic ethnicity, a change from 23.9 ng/mL to 34.8 ng/mL in total 25(OH)D was not statistically significantly associated with a change in PUCAI (mean difference [95% confidence interval]: 0.8 [-1.8, 3.5]). However, in models adjusted for the same potential confounders, a change in bioavailable 25(OH)D from 0.84 ng/mL to 1.94 ng/mL was associated with a mean difference in PUCAI of -8.7 (-13.7, -3.6) points, indicating a statistically significant inverse relationship. Finally, a change in free 25(OH)D from 2.93 pg/mL to 5.96 pg/mL was statistically significantly associated with a mean difference in PUCAI of -3.1 (-5.0, -1.2) points. Predicted mean PUCAI and 95% confidence bands from these models are displayed in Figure 3, demonstrating a statistically significant decrease in PUCAI with increasing free and bioavailable 25(OH)D.

As sensitivity analyses, we also investigated the relationships between these vitamin D fractions and Mayo endoscopy subscore (moderate/severe vs mild; Supplemental Table 2) and fecal calprotectin (Supplemental Table 3). Only the linear terms for the vitamin D fractions were included in the models as the nonlinear terms were not found to be statistically significant. No vitamin D fractions, either in unadjusted or adjusted models, were significantly associated with Mayo endoscopy subscore or fecal calprotectin. However, the results for this exploratory sensitivity analysis for fecal calprotectin should be interpreted with caution due to extreme nonGaussian distributions of the model residuals that was not resolved via transformations of the model outcome, and the large fraction of participants (43%) missing a fecal calprotectin measurement.. Collectively, these analyses suggested a statistically significant association between increased free and bioavailable 25(OH)D and decreased clinical disease activity in a large treatment naive pediatric UC cohort.

DISCUSSION

To our knowledge we have conducted the first analysis of the relationship between bioavailable 25(OH)D and measures

TABLE 2: Clinical and Demographic Characteristics by Bioavailable Vitamin D Tertiles

		Tertile of Bioavailable Vitamin D (ng/mL)			
Variable[a]	**Total Sample (n = 388)**	**Tertile 1 (n = 130) ≤1.02 ng/mL**	**Tertile 2 (n = 129) >1.02–1.72 ng/mL**	**Tertile 3 (n = 129) >1.72 ng/mL**	**P-value[d]**
Age, years[b]	12.7 (3.3)	13.5 (2.9)	12.7 (3.3)	11.9 (3.6)	<0.001
Race					
Asian	18 (5%)	7 (5%)	6 (5%)	5 (4%)	0.43
Black	26 (7%)	18 (14%)	5 (4%)	3 (2%)	<0.001
Other	28 (7%)	9 (7%)	8 (6%)	11 (9%)	0.85
White	316 (81%)	96 (74%)	110 (85%)	110 (85%)	
Females	191 (49%)	69 (53%)	67 (52%)	55 (43%)	0.09
Hispanic	34 (9%)	13 (10%)	11 (9%)	10 (8%)	0.52
Weight z-score[b]	-0.1 (1.1)	-0.2 (1.2)	-0.0 (1.2)	0.0 (0.9)	0.14
Disease Location					
Proctosigmoiditis	23 (6%)	2 (2%)	10 (8%)	11 (9%)	0.01
Left-sided colitis	40 (10%)	10 (8%)	13 (10%)	17 (13%)	
Extensive / Pancolitis / Unassessable	325 (84%)	118 (91%)	106 (82%)	101 (78%)	
Albumin (g/dL)[c]	3.8 (3.2, 4.2)	3.4 (2.8, 3.8)	3.9 (3.4, 4.2)	4.1 (3.7, 4.4)	<0.001
Hemoglobin (g/dL)[c]	11.6 (10.3, 12.9)	10.8 (9.4, 12.3)	11.8 (10.5, 12.8)	12.1 (10.8, 13.3)	<0.001
ESR (mm/hr)[c]	24.0 (12.0, 41.0)	29.0 (15.0, 51.0)	26.5 (10.5, 44.0)	19.5 (10.0, 31.0)	0.001
CRP or hsCRP (mg/dL)					
>ULN	129 (45%)	56 (58%)	46 (47%)	27 (29%)	<0.001
>2 × ULN	88 (30%)	42 (43%)	28 (29%)	18 (19%)	<0.001
Fecal calprotectin (mcg/g)[c]	2285.9 (1171.7, 3906.7)	2132.6 (1087.2, 4021.6)	3332.9 (1380.8, 4181.1)	1921.2 (1150.5, 3540.5)	0.14
PUCAI^	50.0 (35.0, 65.0)	57.5 (40.0, 75.0)	50.0 (30.0, 65.0)	45.0 (30.0, 60.0)	<0.001
Mild (10–34)	92 (24%)	19 (15%)	33 (26%)	40 (31%)	<0.001
Moderate (35–64)	167 (43%)	51 (39%)	53 (41%)	63 (49%)	
Severe (≥65)	129 (33%)	60 (46%)	43 (33%)	26 (20%)	
Mayo endoscopy subscore					
Mild (1)[e]	50 (13%)	12 (9%)	18 (14%)	20 (16%)	0.05
Moderate (2)	206 (53%)	65 (50%)	72 (56%)	69 (53%)	
Severe (3)	132 (34%)	53 (41%)	39 (30%)	40 (31%)	

[a]Values are n (%) unless otherwise specified; [b]mean (standard deviation); [c]median (quartile 1, quartile 3); [d]P-value for ANOVA when mean (standard deviation) is presented, Kruskal-Wallis when median (quartile 1, quartile 3) is presented, mean score chi-square test for nominal row variables, and nonzero correlation test for ordinal row variables. For race variable, p-value is for that racial group vs whites.
[e]One participant with no disease evident was included in the mild group.

of clinical and endoscopic disease activity in a large treatment naive pediatric UC inception cohort. It is likely that biologic effects of 25(OH)D on intestinal epithelial and immune cells in UC are mediated by the free and albumin-bound fractions, which together constitute bioavailable 25(OH)D. The recent availability of a method to directly measure free vitamin D afforded the opportunity to test this, whereas avoiding the confounding introduced by prior approaches that relied on estimates of human serum carrier proteins (VDBP) abundance and binding affinity. We found that lower levels of both free and bioavailable 25(OH)D, but not total 25(OH)D, were inversely correlated with higher levels of clinical disease activity as measured by PUCAI. The association of bioavailable 25(OH)D with disease activity supports this hypothesis and provides a basis for future exploration into the wide-ranging effects of bioavailable vitamin D on the immune system and disease activity.

Fifty-seven percent of enrolled subjects were vitamin D deficient or insufficient. Pappa et al reported similar findings with 63.6% of 143 UC patients exhibiting a vitamin D concentration less than 32 ng/mL, 19.6% exhibiting a vitamin D concentration less than 20 ng/mL, and a higher likelihood of vitamin D deficiency in UC patients compared to CD

TABLE 3: Linear Regression Models of First Tertile vs Third Tertile of Vitamin D Fractions and Associated Mean Differences in PUCAI Score

Vitamin D fraction	Sample Size Used	Model[b]	Difference in mean PUCAI Score (95% Confidence Interval)[a]	P-value
Total vitamin D	388	Unadjusted	0.9 (-1.7, 3.4)	0.50
	384	Adjusted	0.8 (-1.8, 3.5)	0.54
Bioavailable vitamin D	388	Unadjusted	-8.4 (-13.3, -3.5)	<0.001
	384	Adjusted	-8.7 (-13.7, -3.6)	<0.001
Free vitamin D	388	Unadjusted	-2.9 (-4.7, -1.0)	0.002
	384	Adjusted	-3.1 (-5.0, -1.2)	0.001

[a]For total vitamin D, change is from 23.9 ng/mL to 34.8 ng/mL; for bioavailable vitamin D, change is from 0.84 ng/mL to 1.94 ng/mL; and for free vitamin D, change is from 2.93 pg/mL to 5.96 pg/mL
[b]Adjusted model includes age, sex, race, and Hispanic ethnicity.

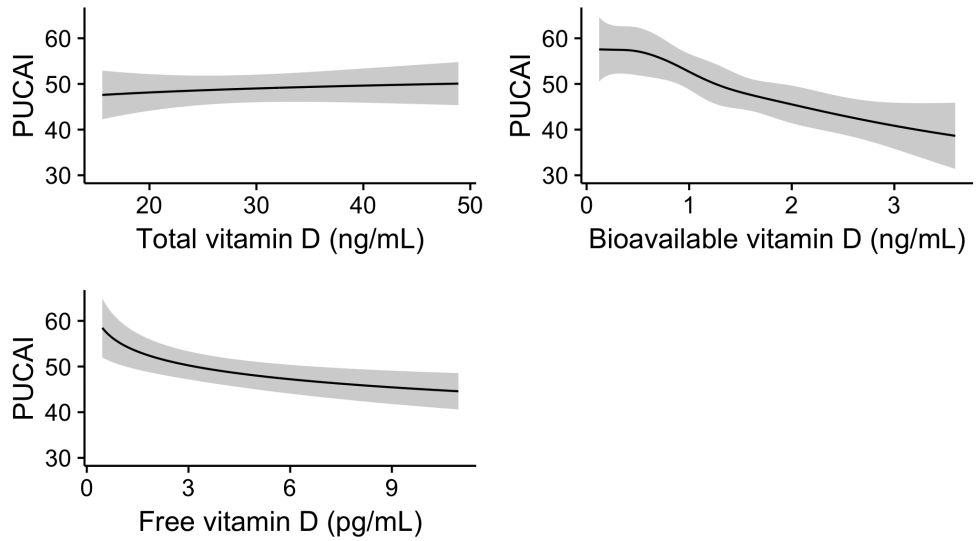

FIGURE 3. Predicted mean PUCAI with 95% confidence bands versus observed vitamin D fraction. Results are from linear regression models of $\log_{10}$ of vitamin D fraction, adjusted for age, race, sex, and Hispanic ethnicity (n = 384). Log_{10} bioavailable vitamin D was modeled as a restricted cubic spline with 5 knots. Figure 2 demonstrates no association between PUCAI and total 25(OH)D, an association between bioavailable 25(OH)D and PUCAI, and an association between free 25(OH)D and PUCAI.

patients.[15,16] In another cross-sectional study, vitamin D deficiency was detected in 62% of IBD patients.[17] Veit et al reported 18 UC patients of whom 83% had a total vitamin D less than 30 ng/mL and 50% less than 20 ng/mL.[10] In an inception cohort of 21 patients with UC, El-Matary et al reported a mean level of total 25(OH)D of 22.8 ng/mL.[18] Our study is therefore consistent with these prior reports documenting a high prevalence of total 25(OH)D insufficiency and deficiency in UC.

We did not find any evidence that total 25(OH)D concentration was related to classic laboratory or clinical measures of disease activity including albumin, hemoglobin, CRP/ESR, or PUCAI. Multiple cross-sectional pediatric studies have demonstrated an association of total vitamin D to ESR, but not albumin.[10,15,16,19] Our study did not show this association between vitamin D and ESR, although it should be noted that our study population is an inception cohort with high median ESR whereas previous studies were cross-sectional studies with much lower inflammatory markers. A small, inception UC cohort of 21 patients demonstrated no association between total vitamin D and PUCAI or albumin as does our study, but the limited sample size of that cohort likely led to underpowered tests of association between total vitamin D and laboratory and clinical measures of disease activity.[18]

Although we found no association between plasma total 25(OH)D and disease activity, bioavailable 25(OH)D was univariately associated with classic measures of

disease activity including albumin, hemoglobin, ESR, CRP, and PUCAI. Bioavailable 25(OH)D includes free vitamin D and albumin-bound vitamin D, thus, one would expect an association between bioavailable 25(OH)D and albumin, as was demonstrated in our study as well as others. Only 1 previous study has evaluated bioavailable 25(OH)D compared to laboratory measures of disease activity in a small cohort of adult CD and UC patients on therapy.[11] A potential limitation of that study was that free 25(OH)D was not directly measured, and so bioavailable 25(OH)D was calculated from a less precise measure of VDBP using a monoclonal assay.[20] Their study was not able to identify a correlation between bioavailable 25(OH)D and CRP, but did find evidence for a correlation with both albumin and platelets. Interestingly, bioavailable 25(OH)D was inversely correlated with clinical disease activity in UC defined by the Simple Clinical Colitis Activity Index, similar to our study.[11]

In our descriptive analyses, we found no statistically significant associations between bioavailable vitamin D and objective measures of mucosal inflammation including Mayo subscore and fecal calprotectin. Meckel et al demonstrated an association between total 25(OH)D and Mayo endoscopy score,[21] and Garg et al that total, free, and bioavailable 25(OH) D were inversely correlated with fecal calprotectin.[11] In both of these studies a vast majority of patients were in clinical and/or endoscopic remission. In a study of CD fecal calprotectin was associated with low serum 25(OH)D in patients in remission but not in patients with active disease.[22] Our findings are thus similar to this small study of active CD and suggest further exploration is necessary in a group with a more diverse range of disease activity.

We suggest that the reason free and bioavailable vitamin D did not predict mucosal disease as measured by Mayo score or calprotectin is due primarily to the low frequency of mild endoscopic severity (13%) in this cohort, and potentially interreader variability in the Mayo scoring system for endoscopic severity that has been previously published.[23] We observed a trend towards an increased frequency of severe endoscopic disease, and decreased frequency of mild endoscopic disease, in participants in the lowest tertile for bioavailable vitamin D (Table 2, $P = 0.05$). However, this relationship was not significant when testing for a difference in the frequency of mild versus moderate-to-severe endoscopic disease between the 1st and 3rd tertiles of bioavailable vitamin D in the adjusted logistic regression analysis (Suppl. Table 2). Likewise, regardingfecal calprotectin, our population demonstrates severe inflammation with a median fecal calprotectin over 2000 mcg/g, recognizing that there is less power for discrimination in our population. We will have the opportunity to test these relationships in future studies of mucosal healing at the week 52 time point with a more wide-range of endoscopic activity and fecal calprotectin measurements.

We demonstrated excellent correlation between bioavailable and free 25(OH)D. This finding is expected since bioavailable vitamin D includes free 25(OH)D in addition to albumin-bound vitamin D. We demonstrated only low or moderate correlation between total vitamin D and bioavailable vitamin D, which was expected given that on average only 4.7% of the total vitamin D was bioavailable. A majority of total vitamin D is bound to VDBP, and VDBP is highly variable, being predominantly driven by 2 well-known genetic polymorphisms.[13] It is not known whether VDBP production or binding affinity is altered in active inflammation or if VDBP is lost in the stool similar to albumin and other proteins. If so, this could have a profound effect upon the bioavailable vitamin D pool, which would vary significantly in inflamed and noninflamed states.

A particular strength of our study is that it is the first to directly measure free 25(OH)D, rather than calculate it from total vitamin D and VDBP. This is an important distinction, given limitations of current VDBP assays and the fact that the concentration and measurement of VDBP can be vastly different based on race and 2 well-known genetic polymorphisms.[13,20,24,25] Garg et al reported an adult UC cohort with mild disease with mean total 25(OH)D of 28 ng/mL similar to our population and mean bioavailable 25(OH)D of 3.6 ng/mL, which is significantly higher than our population of 1.3 ng/ml. It is likely that differences in direct measurement versus calculation are responsible for this dissimilarity, which would in turn be expected to have a profound effect upon tests for associations with measures of disease activity.[11] Additional strengths of our study include a large cohort of newly diagnosed and treatment naive patients. Finally, we provided both clinical and laboratory measures of activity and modeled the relationships between vitamin D fractions and disease activity when needed. Our study was limited by the inability to infer causal effects of bioavailable vitamin D on colitis activity. Such an inference would require additional randomized controlled trials. We also had a relatively small number of nonwhite participants. In addition, given that this is a study of newly diagnosed pediatric UC patients, our sample included primarily moderate-to-severe clinical and mucosal disease activity, which does not represent a typical patient population on therapy with greater variation in mucosal inflammation and disease activity. Finally, neither did we collect dietary information on vitamin D intake nor do we have healthy controls data.

Taken together, we suggest that our finding support the hypothesis that bioavailable 25(OH) D may exert an effect directly on the immune system. This finding supports current work in mouse models evaluating the effects of vitamin D on the immune system. In mice, vitamin D has been demonstrated to favor a Th2 differentiation of the immune system increasing anti-inflammatory cytokines.[26] In addition, Vitamin D has been shown to inhibit dendritic cell maturation, reducing proinflammatory cytokines.[27] Thus, vitamin D deficiency has been associated with decreased anti-inflammatory cytokines and increased proinflammatory cytokines, leading to the hypothesis that low vitamin D may be associated with an increased inflammatory

state. In addition to regulation of the inflammatory state, vitamin D also may have a role in gut epithelial wound healing and permeability, because VDR deficient mice have increased susceptibility to chemically induced inflammation[28,29] Altogether, these mechanistic studies in transgenic mice have established a direct role for vitamin D in regulating critical epithelial and immune functions involved in IBD pathophysiology.

In summary, we confirmed the high prevalence of vitamin D deficiency and insufficiency in pediatric UC at diagnosis. Bioavailable but not total 25(OH)D was statistically significantly associated with clinical symptoms (PUCAI) in regression models, and we found similar univariate relationships between these vitamin D fractions and laboratory measures of disease activity in descriptive analyses. We suggest that there was sufficient evidence that bioavailable vitamin D is likely involved in disease activity. Additional studies are needed to further explore the mechanisms linking bioavailable vitamin D to UC pathophysiology.

SUPPLEMENTARY DATA

Supplementary data are available at *Inflammatory Bowel Diseases* online.

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