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Circulating cytokine concentrations are not altered by supplemental vitamin D in knee osteoarthritis: A pilot study

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HIGHLIGHTS

- The vitamin D-cytokine relationship was examined in knee osteoarthritis.
- Serum cytokines were higher with higher serum 25(OH)D concentrations before vitamin D supplementation at baseline.
- Serum cytokine concentrations were not altered by vitamin D supplementation.

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ABSTRACT

The purpose of this investigation was to identify if raising serum 25-hydroxyvitamin D (25(OH)D) through vitamin D supplementation modulates circulating cytokine concentrations in subjects with knee osteoarthritis (OA). This study consisted of a randomized, double-blind, placebo-controlled study design. Twenty-nine subjects with knee OA were randomly assigned to one of two oral-supplement groups: 1) placebo (PL; $n = 15$) or 2) vitamin D (VD; $n = 14$; 4000 IU/d, cholecalciferol). Supplements were taken daily for 84-d. Serum 25(OH)D and cytokine concentrations were measured in fasting blood samples obtained prior to (i.e., at Baseline (Bsl)), during, and following supplementation. At Bsl, circulating interleukin (IL)-10 and IL-12 concentrations were significantly (all $p < 0.05$) higher in subjects above (i.e., ≥ 26.3 ng/mL, $n = 14$) compared to below (i.e., < 26.3 ng/mL, $n = 15$) the median serum 25(OH)D concentration prior to supplementation. Following supplementation, serum 25(OH)D concentrations were significantly ($p < 0.05$) increased (~45%) in the VD group and circulating cytokine concentrations were not significantly different between groups (i.e., PL vs VD). Based on these findings, we conclude that higher serum 25(OH)D concentrations at baseline associate with higher serum IL-10 and IL-12 concentrations in subjects with knee OA. However, raising serum 25(OH)D concentrations with vitamin D supplementation did not perturb serum cytokine concentrations. ClinicalTrials.gov identifier: NCT04121533.

1. Introduction

Knee osteoarthritis (OA) is a degenerative joint condition and a leading contributor to the global burden of disease [1]. Estimates indicate that approximately 14 million people in the United States have symptomatic knee OA, and nearly half of those individuals are between 45 and 65 years of age [2]. Over the years, data have extended our knowledge regarding the early premise of knee OA being the sole consequence of "wear and tear" processes of articular cartilage and it is now recognized that knee OA arises, in part, as a consequence of

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cytokine-mediated cellular and signaling events [3–6].

Cytokines are pleiotropic proteins instrumental to the immune response, host defenses, and intra- and inter-cellular signaling [7]. Tumor necrosis factor (TNF)-α and interleukin (IL)-1β are pro-inflammatory cytokines that promote the catabolic and destructive events of knee OA in animal [3,4] and human studies [5]. These findings are corroborated by data illustrating chondrocytes as a site for pro-inflammatory cytokine production in knee OA [6], and that disease severity [8–10] and progression [10–12] associate with increasing TNF-α, IL-1β, and other cytokine concentrations in the circulation and transcriptional expression in peripheral blood leukocytes. Fortunately, IL-10 is an anti-inflammatory cytokine expressed in chondrocytes [13] and possesses chondroprotective properties by inhibiting pro-inflammatory cytokine production. While some factors are unavoidable or unpreventable, such as aging, trauma, and genetic predisposition, disrupting the cytokine network could alter OA development and progression.

Vitamin D has been found to be a potent cytokine modulator in various pathophysiological and physiological conditions [14–24]. Unfortunately, few studies identify the cytokine-modulating capacity of vitamin D in knee OA. Data from our lab indicates that vitamin D deficiency (i.e., serum 25(OH)D ≤ 20 ng/mL) is associated with muscular weakness (i.e., deficit in quadriceps strength of the involved leg), a predominant impairment in knee OA, but not with cytokine alterations in the blood [25]. More recently, vitamin D supplementation for 24 months was found to stabilize knee joint effusion during disease progression [26], but it was ineffective at modulating circulating cytokine concentrations in subjects with knee OA [27]. However, the monthly dose of supplemental vitamin D administered in aforementioned study was equivalent to approximately 1700 IU/d [27], and therefore, it is unknown if a higher dose of daily supplemental D perturbs circulating cytokine concentrations in subjects with knee OA.

The primary purpose of this investigation was to identify if raising serum 25(OH)D through vitamin D supplementation modulates circulating cytokine concentrations in subjects with knee OA. We hypothesized that raising serum 25(OH)D would decrease pro- and increase anti-inflammatory cytokines in subjects with knee OA. To test this hypothesis, circulating cytokine concentrations were compared between a placebo and vitamin D supplement groups in a randomized, double-blind study design. Placebo and vitamin D supplements were taken for 84-d. As a secondary objective, we also sought to identify if serum concentrations were different above and below the study specific median serum 25(OH)D concentration prior to supplementation at baseline.

2. Materials and methods

Modestly active (minimum of 30 min of continuous exercise or physical exertion 3 times per week during the previous year) subjects older than 18 but younger than 60 years of age were initially recruited and consented to study participation. Subjects with suspected knee OA were approached and recruited from The Orthopedic Specialty Hospital (Intermountain Healthcare, Murray, UT USA) by a clinical research coordinator. Recruitment and all data collection (see below) were performed at The Orthopedic Specialty Hospital. During recruitment, subjects were excluded from participation if: they had a recent (within 2 years) surgery on the involved or non-involved leg, had a history of metabolic bone disease, skeletal muscle pathology, cardiac or peripheral cardiovascular system abnormality, clotting disorder, coronary artery disease, peripheral vascular disease, stroke, cancer, high cholesterol or triglycerides, or high blood pressure. Potential subjects were also excluded from participation if they were using warfarin or other anti-coagulants prior to study enrollment, using cholesterol lowering medication, using corticosteroid medication, orlistat, phenobarbital, phenytoin, or thiazide, diagnosed with diabetes mellitus, impaired liver or kidney function, pregnant, using a daily dietary supplement or vitamin during the previous year, morbidly obese (body mass index > 40 kg/m^2), were smokers, planning on increasing or decreasing the amount of time spent in the sun or tanning bed, or traveling south of 37°N in latitude during study participation. Subjects were coincidentally not receiving any osteoarthritis specific treatments or medications upon recruitment and enrollment. Subjects were informed of and provided written and verbal consent to the protocol and procedures. This study was registered (NCT: 04121533) and the Central Region Institutional Review Board (number: 1023358) at Intermountain Healthcare (Salt Lake City, UT USA) approved this study. Data was collected between July 2011 and January 2013 in Salt Lake City, UT (40°N latitude). A preliminary analysis of some of the baseline data from this study has been previously published [28].

2.1. Eligibility screening

Following consent, each subject provided a fasting blood draw sample during eligibility screening. Subjects with hypo- (total calcium < 8.4 mg/dL) or hyper-calcemia (total calcium > 10.4 mg/dL), hypo- (parathyroid hormone < 12 pg/mL) or hyper-parathyroidism (parathyroid hormone > 72 pg/mL), elevated rheumatoid factor (> 15 IU/mL), or increased uric acid (females > 7.5 mg/dL; males > 8.5 mg/dL) were excluded from participation. Serum intact parathyroid hormone (iPTH; pg/mL) with calcium (mg/dL) concentrations were measured in each blood sample using an electrochemiluminescent immunoassay (ARUP Laboratories). Serum rheumatoid factor (IU/mL) was quantitated using an immunoturbidimetry assay and uric acid (mg/dL) was determined using a quantitative spectrophotometry assay (ARUP Laboratories) in the eligibility screening blood samples.

To confirm the presence of knee OA, potential subjects were further screened for unilateral knee pain, muscular weakness, and radiographic evidence of knee OA. Subjects were excluded from participation if: 1) the Western Ontario and McMaster Universities Osteoarthritis Index (WOMAC) [29] pain score was < 2 on any of the five questions in its subsection, 2) there was no evidence of muscular weakness (i.e., deficit in peak isokinetic-knee extension or flexion torque at 60°/sec) in the involved compared to the non-involved leg, and 3) a Kellgren-Lawrence grade < 2 was scored in the involved knee [30] as determined from X-ray images obtained on each knee in the anterior-posterior view at 45° of knee flexion. Thus, subjects with circulating total calcium, parathyroid hormone, rheumatoid factor and uric acid within clinically-based reference ranges, and with reported pain, muscular weakness, and radiographic evidence of OA in the involved knee were included in this study.

2.2. Study design and protocol

This study consisted of a randomized, double-blind, placebo controlled experimental design. After eligibility screening, twenty-nine subjects were randomly assigned to one of two oral-supplement groups: 1) placebo (PL, n = 15) or 2) vitamin D (VD, n = 14; cholecalciferol, 4000 IU/d per tablet). Supplements were taken daily for 84-days. USANA Health Sciences, Inc. (Salt Lake City, UT USA) generously donated and provided quality control analysis of the PL and VD supplements. Supplement tablets were identical in appearance, size, taste, and texture. Groups were permuted in random blocks of six as determined from computer-generated random numbers. Allocation was concealed throughout the study duration and secured in a separate location from study recruitment and data collection on a password protected computer. Subjects were instructed to take one tablet per day with a meal and to keep dietary habits consistent throughout data collection. All subjects were asked to refrain from using any other dietary or vitamin supplements during study participation. Subjects were also asked to refrain from using non-steroidal anti-inflammatory drugs, including aspirin, ibuprofen, and naproxen sodium, throughout the duration of the study. As an alternative, subjects were allowed to use

acetaminophen.

Subjects were also asked to refrain from any osteoarthritis specific treatments or medications during study participation; but if needed, to consult with the research team that included several orthopedic surgeons prior to use. No subjects requested the use of osteoarthritis treatments or medications during study participation. Subject characteristics, including gender, age, height, and body mass, were collected upon study enrollment. Height was recorded to the nearest 0.1 cm and body mass to the nearest 0.1 kg using standard clinical anthropometric assessment tools. Body mass index (kg/m^2) was calculated from body mass and height.

Each subject provided nine fasting (10–12 h) blood draw samples: baseline (Bsl, following screening and prior to supplementation), and 7-, 14-, 21-, 28-, 42-, 56-, 70-, and 84-days after starting supplementation. All blood draws were performed at The Orthopedic Specialty Hospital by a trained phlebotomist. Blood was drawn from the antecubital vein. Plasma and serum from gel Vacutainers were separated by centrifugation (Fisher Scientific, Centrific, Model 228, Pittsburgh, PA, USA) at 1380 *g* for 15 min within 20 min of sample collection. Plasma and serum from non-gel Vacutainers were separated by centrifugation (VWR International, Clinical 50 Centrifuge, Radnor, PA USA) at 1068 *g* for 10 min within 20 min of sample collection. After centrifugation, plasma and serum samples were aliquoted into several different cryotubes and stored at −80 °C until later analysis at the conclusion of the study.

A comprehensive metabolic panel was performed on Bsl plasma samples (ARUP Laboratories). Serum intact parathyroid hormone (iPTH; pg/mL) with calcium (mg/dL) concentrations were measured in each blood sample (i.e., Bsl through 84-d). Subjects completed a separate sun exposure questionnaire (i.e., < 10 min/d, 10–30 min/day, or > 30 min/day) for weekdays and weekends, and reported the daily use of sunscreen and the sun protection factor (SPF) of the sunscreen at Bsl, 28-d, 56-d, and 84-d.

2.3. Analytical procedures

2.3.1. Serum 25(OH)D and vitamin D binding proteins concentrations

Serum 25(OH)D concentrations (ng/mL) were measured in duplicate or triplicate (coefficient of variation (CV) = 6.24%) in each blood sample (Bsl through 84-d), as previously described [31]. In brief, analytes were separated on an Agilent (series 6460, Model G6460A, Santa Clara, CA USA) high performance-liquid chromatography system and detected on an Agilent tandem mass spectrometer using atmospheric pressure chemical ionization detection (350 °C gas temperature, 400 °C vaporizer). Serum $25(OH)D_2$ and $25(OH)D_3$ concentrations were determined relative to authentic standards and corrected for recovery of the $25(OH)D_3$ internal standard. Serum $25(OH)D_2$ (limit of detection = 2.0 ng/mL) was not detected in any of the subjects and serum $25(OH)D_3$ concentrations are therefore referred to as serum 25(OH)D concentrations hereafter. Subjects were classified as vitamin D deficient, insufficient, or sufficient if they had a serum 25(OH)D concentration ≤ 20, > 20 and < 30, or ≥ 30 ng/mL, respectively [32]. Serum 25(OH)D concentrations were measured at the conclusion of the study and not known during data collection. In Bsl blood samples, serum vitamin D binding protein (VDBP) concentrations (pg/mL) were assessed using the multiplex (EMD Millipore; Billerica, MA USA) technology of Luminex (MAGpix; Austin, TX USA) and albumin concentrations were measured at ARUP Laboratories (Salt Lake City, UT USA).

2.3.2. Serum cytokines, soluble cytokine receptors, and high-sensitivity C-reactive protein (hsCRP)

The primary outcomes of this study included serum cytokine concentrations. Serum cytokine (granulocyte-macrophage colony stimulating factor (GM-CSF), interferon (IFN)-γ, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12, IL-13, and TNF-α) concentrations (pg/mL) were determined using the multiplex technology of Luminex (MAGPix) with high sensitivity (EMD Millipore) at Bsl, 28-d, and 84-d. Serum soluble cytokine receptor (high-sensitivity sIL-1r1, sIL-1r2, sIL-4r, sIL-6r, sTNFr1, and sTNFr2; EMD Millipore) concentrations (pg/mL) were determined using the multiplex technology of Luminex (MAGPix) at Bsl. Serum hsCRP concentrations (mg/L) were quantitated using immunoturbidimetry assay (ARUP Laboratories) in each blood draw sample (i.e., Bsl through 84-d). Serum soluble cytokine receptor and hsCRP concentrations were secondary outcomes.

2.4. Statistical analyses

The analysis consisted of an intention to treat. Data were checked for normality prior to statistical analyses with a Shapiro-Wilk Test. Group differences at Bsl were assessed with a *t*-test or a Mann-Whitney U test. Statistical significance of categorical data were assessed with separate Pearson Chi-Square tests. Statistical significance of data were assessed with separate repeated measures analysis of variance (ANOVA) and followed by a Bonferroni correction on multiple pairwise comparisons when appropriate. Non-normally distributed data were assessed for significance with a Kruskal-Wallis one-way ANOVA and followed by a Dwass-Steel-Chritchlow-Fligner test for pairwise comparisons when appropriate. Age, disease severity, body mass index (BMI), and gender served as statistical covariates when comparing the cytokine concentrations between the VD and PL groups. Significance was set at $p < 0.05$ and all statistical analyses were performed with SYSTAT (version 13.1, Chicago, IL, USA). Data are presented as mean (SEM) unless noted otherwise.

3. Results

3.1. Baseline subject characteristics in the PL and VD groups

At Bsl, serum 25(OH)D concentrations were 27.5 (1.5) ng/mL for all subjects (n = 29, GROUP). Approximately 14% of the subjects possessed a serum 25(OH)D concentration deemed vitamin D deficient, while ~58% were insufficient and ~28% were sufficient (Table 1). GROUP characteristics, clinical chemistries, and Kellgren-Lawrence scores are provided in Table 1.

Following group separation, subject characteristics, comprehensive metabolic panel data, vitamin D binding proteins (i.e., vitamin D binding protein (VDBP) and albumin), Kellgren-Lawrence score, and vitamin D status classification (i.e., deficient, insufficient, and sufficient) were not significantly different between the PL and VD groups at Bsl (Table 1). Sun exposure, sunscreen use and SPF (Supplemental Table 1) were not significantly different between the PL and VD groups at Bsl.

3.2. Below compared to above the median serum 25(OH)D concentration at Bsl

Few subjects (see Table 1) displayed a serum 25(OH)D concentration ≤ 20 ng/mL prior to supplementation. Therefore, to assess the influence of a higher serum 25(OH)D concentration on circulating cytokines prior to supplementation, subjects were separated below (Below, < 26.3 ng/mL) and above (Above, ≥ 26.3 ng/mL) the median serum 25(OH)D concentration (see Fig. 1A). Subject characteristics, comprehensive metabolic panel data, and Kellgren-Lawrence scores were not significantly different in the Below compared to the Above group (Supplemental Table 2). Likewise, sun exposure, sunscreen use and SPF (Supplemental Table 3), and activity levels (*data not shown*) were not significantly different between the Below and Above groups. High-sensitivity C-reactive protein, most of the serum cytokines, and the majority of the soluble cytokine receptor concentrations were not significantly different between groups (Supplemental Table 4). Conversely, serum IL-10, IL-12, and the sIL-4r concentrations were

Table 1
Baseline subject characteristics, plasma metabolic panel, and serum vitamin D binding protein data.

	GROUP	PL	VD
n, f/m	29 (16/13)	15 (8/7)	14 (8/6)
age, y	48.9 (1.6)	46.8 (2.7)	51.1 (1.6)
height, cm	169 (2)	167 (2)	172 (3)
body mass, kg	92.3 (3.6)	87.7 (4.1)	97.3 (6.0)
BMI, kg/m^2	32.2 (1.1)	31.6 (1.4)	32.9 (1.7)
sodium, mmol/L	147 (1)	146 (2)	148 (2)
potassium, mmol/L	4.17 (0.08)	4.10 (0.10)	4.24 (0.13)
chloride, mmol/L	108 (1)	107 (2)	109 (2)
CO_2, mmol/L	21.0 (0.4)	21.0 (0.5)	21.0 (0.5)
anion gap, mmol/L	17.9 (0.3)	17.7 (0.4)	18.1 (0.3)
urea nitrogen, mg/dL	16.1 (0.7)	15.5 (1)	16.7 (0.9)
creatinine, mg/dL	0.88 (0.03)	0.90 (0.04)	0.88 (0.05)
glucose, mg/dL	107 (4)	107 (6)	108 (6)
alkaline phosphatase, U/L	75.1 (3.3)	73.0 (4.6)	77.4 (4.8)
AST, U/L	21.1 (1.4)	21.9 (2.3)	20.2 (1.6)
ALT, U/L	17.2 (2.5)	19.4 (4.1)	14.9 (2.7)
total protein, g/dL	7.19 (0.10)	7.27 (0.14)	7.09 (0.12)
total bilirubin, mg/dL	0.47 (0.05)	0.47 (0.08)	0.48 (0.06)
albumin, g/dL	4.33 (0.06)	4.31 (0.10)	4.36 (0.08)
VDBP, pg/mL	43.7 (2.7)	47.8 (4.2)	39.2 (3.0)
Kellgren-Lawrence score			
2, n	3	1	2
3, n	18	8	10
4, n	8	6	2
vitamin D status			
deficient, n	4	4	0
insufficient, n	17	8	9
sufficient, n	8	3	5

Data presented as mean (SEM) unless noted otherwise.
ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; GROUP, all subjects combined; VDBP, vitamin D binding protein.

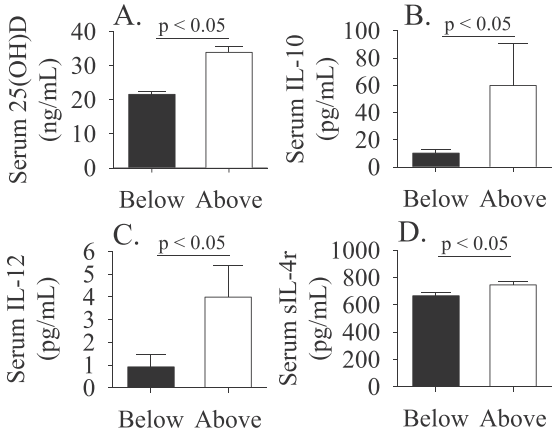

Fig. 1. Serum 25(OH)D, IL-10, IL-12, and sIL-4r concentrations Below and Above the median serum 25(OH)D concentration at Bsl. (**A**) Serum 25(OH)D (ng/mL), (**B**) IL-10 (pg/mL), (**C**) IL-12 (pg/mL), and (**D**) IL-4r (pg/mL) concentrations were significantly (bar (−)) increased in the Above (serum 25(OH)D ≥ 26.3 ng/mL) compared to the Below group (serum 25(OH)D < 26.3 ng/mL). Data presented as mean (SEM).

significantly higher in the Above compared to the Below group (Fig. 1B–D).

3.3. Serum 25(OH)D and iPTH with calcium following supplementation

Serum 25(OH)D concentrations were not significantly different between the PL and VD groups at Bsl (Fig. 2). Serum 25(OH)D concentrations were not significantly different in the PL group during supplementation. In the VD group, serum 25(OH)D concentrations progressively increased (~32%) to 28-d and plateaued thereafter during supplementation. At the conclusion of supplementation, serum 25(OH)D concentrations increased approximately 45% from Bsl and all subjects achieved a concentration deemed sufficient (i.e., ≥30 ng/mL; range, 32.0–57.9 ng/mL) in the VD group. In comparison to the PL group, serum 25(OH)D concentrations were significantly increased throughout the duration of supplementation (i.e., 7- to 84-d) in the VD group.

Plasma iPTH and calcium were not significantly different within or between the PL and VD groups (Table 2). Likewise, sun exposure (weekday and weekend) and daily sunscreen use were not significantly different between the PL and VD groups during study participation (see Supplemental Table 1).

3.4. Serum cytokines and hsCRP concentrations

Serum hsCRP (Table 2) and cytokine (Table 3) concentrations were not significantly different between groups prior to or following supplementation. It is noteworthy, however, that one outlier (i.e., more than a 1.5 interquartile range above the upper quartile) was present in the VD group Bsl through 84-d. Statistical results and assumptions were not significantly different with or without the inclusion of the outlier. Therefore, the outlier was included in the final analysis and presented herein.

4. Discussion

This investigation provides original research demonstrating that a higher serum 25(OH)D at baseline associates with higher circulating IL-10, IL-12, and sIL-4r concentrations in subjects with knee OA. Following supplemental vitamin D, however, circulating cytokine concentrations were not altered despite a significant increase in serum 25(OH)D. These discordant findings underscore the importance of interpretive caution when deciphering the relationship between vitamin D and cytokines, and illustrate that circulating cytokines in subjects with knee OA are not perturbed by supplemental vitamin D.

4.1. Higher circulating cytokine and soluble cytokine receptor concentrations with higher serum 25(OH)D prior to supplemental vitamin D at baseline

In other pathophysiological and non-pathophysiological conditions, IL-10 increases [14–20] and IL-12 remains unchanged [25,33,34] with an increase in serum 25(OH)D concentrations. However, studies identifying the impact of disparate serum 25(OH)D concentrations on circulating IL-10 and IL-12 in subjects with knee OA are limited. In general, data suggest that circulating IL-10 and IL-12 concentrations are not disturbed by contrasting serum 25(OH)D concentrations [25]. In our previous study [25], *The Endocrine Society*-based serum 25(OH)D concentration guidelines were implemented to demarcate vitamin D status group comparisons [32,35], while herein, subjects were separated above and below the study-specific median serum 25(OH)D concentration. Differences in the serum 25(OH)D concentration demarcating group comparisons could account for the contrasting IL-10 and IL-12 findings between studies, and therefore, underscore the necessity of establishing disease- and physiology system-specific vitamin D status criteria.

As previously mentioned, TNF-α and IL-1β are pro-inflammatory cytokines and mediators of knee OA [3,4]. In OA and other joint inflammatory conditions, IL-10 is locally produced in arthritic cartilage and synovial tissue [13,36,37]; and importantly, possesses chondroprotective properties by inhibiting pro-inflammatory cytokine production and mediated events [for review, see Refs. [38,39]]. In circulation, IL-10 concentrations are lower in OA than control subjects [37]

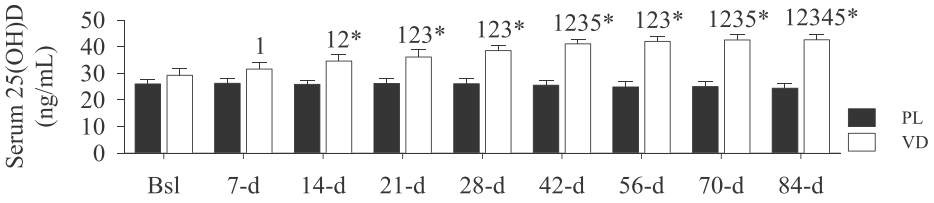

Fig. 2. Serum 25(OH)D concentrations (ng/mL). Serum 25(OH)D concentrations were significantly (treatment × time interaction, $p < 0.05$) different between the PL and VD groups. $^{1}p < 0.05$ vs Bsl; $^{2}p < 0.05$ vs 7-d; $^{3}p < 0.05$ vs 14-d; $^{4}p < 0.05$ vs 21-d; $^{5}p < 0.05$ vs 28-d; *$p < 0.05$ vs corresponding PL. Legend provided in the Figure. Data presented as mean (SEM).

Table 2
Plasma iPTH, calcium, and serum hsCRP data.

	PL	VD
iPTH, pg/mL		
Bsl	54.0 (6.7)	41.9 (3.4)
7-d	48.3 (4.7)	39.6 (2.7)
14-d	45.6 (4.4)	39.1 (3.6)
21-d	46.0 (4.5)	42.1 (4.0)
28-d	48.9 (4.8)	39.3 (4.1)
42-d	45.9 (4.5)	39.5 (3.4)
56-d	44.4 (4.4)	40.7 (3.2)
70-d	44.7 (4.8)	38.8 (3.0)
84-d	48.3 (4.5)	41.1 (3.0)
Calcium, mg/dL		
Bsl	9.34 (0.08)	9.41 (0.10)
7-d	9.43 (0.32)	9.46 (0.09)
14-d	9.41 (0.12)	9.41 (0.10)
21-d	9.40 (0.10)	9.36 (0.08)
28-d	9.37 (0.12)	9.46 (0.07)
42-d	9.38 (0.11)	9.52 (0.06)
56-d	9.46 (0.09)	9.34 (0.09)
70-d	9.43 (0.08)	9.34 (0.08)
84-d	9.42 (0.08)	9.40 (0.10)
hsCRP, mg/L		
Bsl	2.99 (0.81)	4.86 (1.63)
7-d	2.72 (0.85)	4.29 (1.07)
14-d	3.32 (0.79)	3.90 (0.96)
21-d	3.36 (1.04)	3.25 (0.78)
28-d	2.51 (0.77)	4.61 (1.12)
42-d	3.17 (1.02)	3.97 (1.06)
56-d	3.42 (1.06)	4.22 (0.94)
70-d	3.16 (1.19)	4.14 (1.08)
84-d	3.03 (0.95)	4.31 (0.86)

Data presented as mean (SEM).

and a decrease in *ex vivo* whole-blood IL-10 production increases the risk of OA [40]. Therefore, the higher circulating IL-10 concentration with a higher serum 25(OH)D could reflect a greater systemic anti-inflammatory cytokine status and protect against the catabolic events governed by pro-inflammatory cytokines.

In addition to IL-10, this study provides novel data that a higher serum 25(OH)D associates with a significantly higher serum sIL-4r concentration at Bsl. In knee OA, circulating sIL-4r concentrations increase [41], which could assist with protecting, stabilizing [42] and enhancing the activity [43] of another anti-inflammatory cytokine, IL-4 [44–46], as found in other experimental models. Although there is a gap in our knowledge regarding the vitamin D association with IL-4 receptors in knee OA, evidence indicates that 1,25-dihydroxyvitamin D (1,25(OH)D), the most biological active form of vitamin D, increases IL-4 receptor gene transcription in a murine osteoblast precursor cell line (i.e., MC3T3) [47]. Nevertheless, despite previous data revealing an inverse association between serum sIL-4r and physical dysfunction in knee OA [28], additional research is needed to identify if a higher sIL-4r concentration with higher serum 25(OH)D potentially minimizes patient-reported disability with knee OA.

IL-12 is a pro-inflammatory cytokine present in the synovial membrane during knee OA [48,49]. Data from experimental mice demonstrates that collagen-induced arthritis is less frequent and less severe with IL-12 (p40) deficiency [50], while the overexpression of IL-12 in the knee joint aggravates streptococcal cell wall-induced arthritis [51]. Thus, protecting against higher IL-12 concentrations could be advantageous in combating the deleterious events mediating OA. In culture, 1,25(OH)D decreases IL-12 mRNA expression in THP-1 cells [52] and protein secretion from dendritic [53] and peripheral blood mononuclear cells [54]. Serum 1,25(OH)D was not measured in the present investigation, but a previous report identifies a positive correlation between serum 25(OH)D and 1,25(OH)D concentrations [34], implying a higher 1,25(OH)D in the Above group. In support of this relationship, 1,25(OH)D concentrations increase following a bolus (i.e., 100,000 IU of cholecalciferol) of supplemental vitamin D and a corresponding increase in serum 25(OH)D in reportedly healthy adults [55]. Nevertheless, despite a higher serum 25(OH)D, circulating IL-12 concentrations were higher in the Above group. Clearly, resolution surrounding the vitamin D and IL-12 relationship in knee OA awaits future research, but based on available data, it is reasonable to assume that the higher circulating IL-12 concentration in the Above group could be detrimental to knee OA. Alternatively, the higher circulating IL-12 in the Above group could serve as a systemic biomarker reflective of localized cartilage damage and synovium inflammation [56] in knee OA and independent from serum 25(OH)D perturbations.

4.2. Supplemental vitamin D does not modulate circulating cytokines in knee OA

Contrasting with our hypothesis, supplemental vitamin D was ineffective at altering circulating cytokines despite an ~45% increase in serum 25(OH)D concentrations and all subjects achieving a sufficient vitamin D status following supplementation. This finding extends a previous report demonstrating that a lower dose of supplemental vitamin D (i.e., ~1700 IU/d compared to 4000 IU/d in the present study) does not modify serum cytokine concentrations in subjects with knee OA [27], and is in general agreement with other studies performed in overweight or obese [57–59], multiple sclerosis [60], and reportedly healthy subjects [31,55,61]. Conversely, supplemental vitamin D has been reported to govern circulating pro- and anti-inflammatory cytokine fluctuations in other disease [17,19–22] and non-disease conditions [18,23,24]. A potential explanation for the null findings herein could be that circulating cytokine concentrations are too low in knee OA to identify a response to vitamin D supplementation or that the cytokine response to supplemental vitamin D is localized. Along these lines, supplemental vitamin D was found to protect against the increase in knee joint effusion-synovitis volume [26]. Therefore, future research is needed to identify if supplemental vitamin D perturbs local (i.e., synovial fluid, synovial membrane, and chondrocytes) as opposed to circulating cytokine levels in knee OA. Another potential explanation is serum 25(OH)D concentrations prior to supplementation were not low enough to identify a circulating cytokine response to supplemental vitamin D [34]. In a previous report [27], and including those here (see Fig. 2), serum 25(OH)D concentrations were approximately 17.0–29.2 ng/mL prior to supplementation, suggesting studies investigating the role of supplemental vitamin D on circulating cytokines in knee OA consisted of subjects with a borderline deficient to insufficient vitamin D status.

In addition to those above, there are a few study limitations worthy of discussion. First, this study consisted of a rather small sample size. However, stringent inclusion and exclusion criteria were implemented in an attempt to minimize the potential influence of confounding variables and co-morbidities. Second, the duration of supplemental

Table 3
Serum cytokine concentrations.

	PL	VD		PL	VD
IL-1β, pg/mL			IL-10, pg/mL		
Bsl	0.40 (0.13)	0.93 (0.36)	Bsl	13.0 (4.5)	56.8 (30.9)
28-d	0.44 (0.16)	0.86 (0.34)	28-d	11.9 (3.4)	46.5 (22.8)
84-d	0.55 (0.21)	0.84 (0.34)	84-d	12.2 (3.6)	40.3 (18.7)
IL-2, pg/mL			IL-12, pg/mL		
Bsl	0.84 (0.29)	2.19 (0.93)	Bsl	2.90 (1.30)	1.87 (0.82)
28-d	0.98 (0.37)	2.49 (0.17)	28-d	3.11 (1.14)	1.52 (0.76)
84-d	1.18 (0.44)	2.17 (0.92)	84-d	3.24 (1.99)	1.12 (0.52)
IL-4, pg/mL			IL-13, pg/mL		
Bsl	20.5 (13.4)	56.6 (31.2)	Bsl	15.7 (5.4)	18.8 (7.9)
28-d	34.3 (17.7)	57.0 (31.8)	28-d	19.1 (6.6)	17.6 (8.1)
84-d	34.0 (18.5)	48.0 (24.9)	84-d	18.2 (6.6)	17.2 (7.6)
IL-5, pg/mL			GM-CSF, pg/mL		
Bsl	0.22 (0.14)	0.51 (0.26)	Bsl	1.41 (0.86)	4.36 (2.76)
28-d	0.33 (0.16)	0.45 (0.23)	28-d	1.91 (1.12)	3.74 (2.66)
84-d	0.36 (0.20)	0.42 (0.20)	84-d	2.37 (1.37)	2.96 (2.04)
IL-6, pg/mL			IFN-γ, pg/mL		
Bsl	1.55 (0.45)	1.49 (0.32)	Bsl	7.68 (3.83)	12.2 (5.5)
28-d	1.09 (0.20)	1.29 (0.37)	28-d	8.75 (4.31)	8.25 (4.13)
84-d	1.12 (0.25)	1.49 (0.36)	84-d	8.09 (4.58)	8.53 (4.21)
IL-7, pg/mL			TNF-α, pg/mL		
Bsl	3.18 (0.50)	3.77 (0.84)	Bsl	3.50 (0.60)	4.59 (0.72)
28-d	2.78 (0.52)	2.68 (0.46)	28-d	4.08 (0.64)	4.21 (0.72)
84-d	2.69 (0.58)	3.15 (0.64)	84-d	3.86 (0.80)	4.07 (0.61)
IL-8, pg/mL					
Bsl	5.02 (0.54)	6.97 (1.22)			
28-d	5.25 (0.57)	6.35 (0.84)			
84-d	5.17 (0.43)	6.58 (0.85)			

Date presented as mean (SEM).

vitamin D was 84-d. Although serum 25(OH)D concentrations rapidly increase and eventually plateau during the intervention, it is unknown if 84-d was long enough and if the dose of cholecalciferol was optimal to reveal an impact on circulating cytokines in knee OA. Third, additional research is needed to identify the potential impact of vitamin D obtained from dietary sources on circulating cytokine concentrations in subjects with knee OA. Finally, subject recruitment, enrollment, and data collection was not limited to season. Future research should consider the influence of seasonal fluctuations on serum 25(OH)D concentrations and potentially circulating cytokines.

5. Conclusion

Osteoarthritis is a heterogenous condition and product of a variety of factors. Of these factors, evidence continuously reveals diverse cytokines as potential regulators in OA development, progression, and disease-specific clinical outcomes (i.e., imaging, patient-reported, and performance-based). Here, we extend those findings by providing the first evidence indicating that a higher serum 25(OH)D concentration at baseline associates with higher circulating cytokine and soluble cytokine receptor concentrations in subjects with knee OA. Following supplemental vitamin D, circulating cytokine concentrations were not altered despite a significant increase in serum 25(OH)D. Based on these findings, we conclude that select circulating cytokines and a soluble cytokine receptor are elevated with a higher serum 25(OH)D concentration in subjects with knee OA and without supplemental vitamin D. However, raising serum 25(OH)D concentrations with vitamin D supplementation did not perturb serum cytokine concentrations compared to a placebo. We speculate that protecting against low serum 25(OH)D could be more influential on circulating cytokine concentrations as opposed to substantial serum 25(OH)D concentration increases following an arguably large daily dose (i.e., 4000 IU) of supplemental vitamin D in subjects with knee OA and with borderline vitamin D deficiency or insufficiency at baseline. Future research is clearly warranted to confirm our conclusion and proposed premise.

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