The Effects of Vitamin D on Keratoconus Progression

NICOLÒ BARTOLOMEO, MATTEO PEDERZOLLI, SILVIA PALOMBELLA, PHILIPPE FONTEYNE, GIUSEPPE SUANNO, GIANLUCA TILARO, STEFANO DE PRETIS, FRANCESCA BORGO, FEDERICO BERTUZZI, CARLOTTA SENNI, MASSIMO DE MICHELI, FRANCESCO BANDELLO, AND GIULIO FERRARI

• PURPOSE: **The aim of this study was to assess whether vitamin D (Vit D) supplementation affects local disease progression, as well as systemic inflammation, collagen degradation, and oxidative stress in adolescents affected by keratoconus (KC) and Vit D deficiency.**

• DESIGN: **Prospective, interventional single-center study.**

• SUBJECTS: **Forty patients (age range, 12.2-19.9) presenting with both KC and Vit D insufficiency (<30 ng/mL) were included in the study.**

• METHODS: **Vit D was prescribed for 6 months as per standard of care. Follow-up visits were scheduled for 12 months. Each visit included the measurement of best spectacle-corrected visual acuity, maximal keratometry (Kmax), and thinnest corneal thickness. Blood samples were collected at month 0 and month 6 to measure Vit D levels and systemic biomarkers of inflammation, collagen degradation, and oxidative stress by ELISA or real-time polymerase chain reaction; full RNA sequencing was performed on 20 patients at month 0 and month 6.**

• MAIN OUTCOME MEASURES: **The primary outcome of the study was the percentage of patients with a Kmax progression less than 1 diopter (D) throughout the entire study (ie, stable patients).**

• RESULTS: **Overall, 65% of patients remained stable (75% of eyes) after 12 months. Specifically, best spectacle-corrected visual acuity, Kmax, and thinnest corneal thickness rates remained stable during the 12-month observational period. ELISA performed on blood plasma showed that Vit D upregulated the expression of Vit D binding protein. QPCR performed on peripheral leukocytes showed an increase in the expression of VDR and CD14 with no changes in the principal enzymes involved in Vit D activation/deactivation. ELISA and qPCR showed the modulation of collagen degradation and collagen crosslinking. Subgroup analysis with RNA sequencing showed differential response to Vit D treatment. Responder patients showed downregulation in inflammatory and platelet activation pathways, and upregulation of proteoglycan metabolism/biosynthesis enrichment.**

• CONCLUSIONS: **Our findings support the hypothesis that Vit D supplementation can affect KC progression in adolescent patients with Vit D insufficiency possibly through the modulation of systemic inflammation, inhibition of collagen degradation, and promotion of proteoglycan synthesis. Our results strongly suggest that KC may be the ocular manifestation of a systemic disorder. (Am J Ophthalmol 2025;276: 235–251. © 2025 Published by Elsevier Inc.)**

AJO.com Supplemental Material available at AJO.com.
Accepted for publication April 7, 2025.

From the Eye Repair Unit, Division of Neuroscience (N.B., S.P., P.F., G.S., G.T., and G.F.), IRCCS San Raffaele Scientific Institute, Milan, Italy; Ophthalmology Unit (M.P., F.B., C.S., F.B., and G.F.), IRCCS San Raffaele Scientific Institute, Milan, Italy; Vita-Salute San Raffaele University (G.S., F.B., and G.F.), Milan, Italy; Center for Omics Sciences (S.d.P. and F.B.), IRCCS San Raffaele Scientific Institute, Milan, Italy; Ospedale Maggiore di Lodi (M.D.M.), Ophthalmology Unit, Lodi, Italy

Inquiries to Giulio Ferrari, Via Olgettina, 60 20132 Milano, Italy.; e-mail: ferrari.giulio@hsr.it

INTRODUCTION

Keratoconus (KC) is the most common primary corneal ectasia and results in progressive corneal thinning, irregular astigmatism, and decreased visual acuity.[1,2] Global prevalence varies depending on the country of reference, ranging from 120 up to 4.790/100.000 inhabitants.[3] Although the exact etiological factors and the mechanisms that regulate its progression are not well defined, KC is indicated as a multifactorial degenerative disease primarily caused by corneal proteins structural impairment, increased proteinases and augmented oxidative stress.[4] The disease traditionally presents itself within the second decade of life and is generally more aggressive in younger patients.[5,6] KC is generally managed according to its severity and evidence of progression; initial cases are treated with spectacles or contact lenses. If progression occurs or is expected, corneal cross-linking (CXL) may be employed to increase biomechanical stability and rigidity of the cornea.

Collagen degradation or dislocation of collagen lamellae has been described in KC corneas, and reactive oxygen species are increased in KC patients both locally and systemically.[7-9] It has also been suggested that KC might be associated with systemic inflammation associated with immune-mediated diseases.[10]

0002-9394/$36.00
https://doi.org/10.1016/j.ajo.2025.04.009
© 2025 Published by Elsevier Inc.

Vitamin D (Vit D) is a fat-soluble prohormone with pleiotropic actions in the human body, including effects on the immune system.[11] It has been previously shown that KC patients have lower serum Vit D levels compared to healthy controls.[11-13] In this vein, we previously showed that Vit D supplementation—a noninvasive and inexpensive treatment-inhibits collagenolysis and shows potential for arresting progression of the disease in a clinical trial involving 20 patients.[14]

This study aims to test whether those preliminary clinical observations were reproducible in a larger sample. Moreover, we analyzed the complete mRNA expression in peripheral blood mononuclear cells (PBMC) using RNA sequencing (RNAseq) in a subset of patients, to search for a mechanism driving the clinical effect we observed.

MATERIALS AND METHODS

- **STUDY DESIGN:**

Patient enrollment

This prospective pilot study was conducted at the Cornea and Ocular Surface Unit of the San Raffaele Scientific Institute, Milan, Italy. The study was carried out in accordance with the tenets of the Declaration of Helsinki, and it was approved by the Institutional Review Board/Ethics Committee (Comitato Etico Istituto Scientifico Ospedale San Raffaele). Written informed consent was obtained from the patients at enrollment. A schematic representation of the study design is shown in Figure 1.

Briefly, after the screening visit (month 0 [M0]), patients were followed up for 12 months. Follow-up visits were scheduled at 2, 4, 6, 9, and 12 months from M0. A full ophthalmic exam was performed at each visit, which included measures of best spectacle-corrected visual acuity (BSCVA) and corneal tomography and topography. Maximal keratometry (Kmax), thinnest corneal thickness (TCT), and epithelial and stromal minimal thickness were evaluated. At each visit, patients were reminded not to rub. The examination was always performed at the same time of the day (14-15 hours). Additionally, blood samples were collected at the screening visit, and at months 4, 6, and 12.

Patients presenting KC and Vit D insufficiency, that is serum levels less than 30 ng/mL were included in the study.[15] Exclusion criteria were the following: prior surgical procedures of the cornea, including CXL; diagnosis of end-stage KC defined as corneal thickness less than 300 μm and/or extensive apical leucoma and/or corneal hydrops; diagnosis of active keratitis/conjunctivitis. The diagnosis of KC was confirmed by corneal tomography/topography (MS-39 AS-OCT; CSO). At the screening visit, medical history was collected, including the presence of allergies and rubbing habits. All patients were instructed not to rub regardless of having the habit or not. A full ophthalmic exam was performed, including measurement of (1) BSCVA, (2) uncorrected visual acuity (3) Kmax, and (4) epithelial and stromal minimal thickness and TCT by corneal tomography and topography. Patients were treated as per standard of care in case of Vit D insufficiency with oral cholecalciferol supplementation (50,000 IU once a week for the first 3 months). Maintenance treatment was then continued with 50,000 IU once a month up to month 6 (M6).

When CXL was performed during the study, the treated eye (s) was/were not further considered for the analysis. However, if the eye had worsened more than 1 D before CXL, it was included among worsened eyes; while if the eye had worsened less than 1 D before the procedure, it was excluded from further analysis.

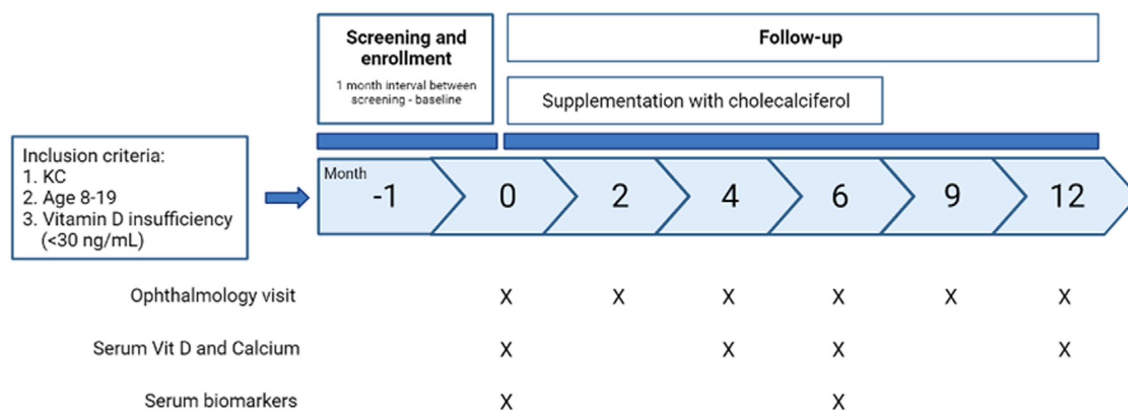

FIGURE 1. Schematic representation of the study design. Patients diagnosed with KC and presenting Vit D insufficiency (<30 ng/mL) were enrolled. They were followed up for 12 months. Full ophthalmic visits were scheduled at months 0, 2, 4, 6, 9, and 12. Serum Vit D and calcium levels were evaluated at months 0, 4, 6, 12. In addition, peripheral blood samples were collected to analyze other serum biomarkers at months 0 and 6.

Primary outcome

The primary outcome measure was defined as the percentage of patients with Kmax progression of less than 1 D throughout the 12-month follow-up time.

Secondary outcomes

Secondary outcome measures were changes in BSCVA, Kmax, and TCT, as well as Vit D and calcium serum levels, during the 12-month follow-up time.

Clinical parameters

BSCVA, recorded in Snellen equivalents during visits, was converted into logMAR scale values "Counting finger" was converted to 2.0, and "hand motion" was converted to 3.0 logMAR values.[16,17] BSCVA rate was defined as the difference between BSCVA at a certain month of follow-up and BSCVA at enrolment, divided by the number of months that intercurred between the two-time points, eg, BSCVA rate at M6 = (M6 logMAR—M0 logMAR)/6.

Kmax, TCT, epithelial thickness, and stromal thickness were measured using the MS-39 AS-OCT corneal tomographer/topographer. Kmax rate and TCT rate were calculated in a similar manner as the BSCVA rate.

Thus, an increase in each rate was associated with KC-related worsening of the index parameter: increased BSCVA rates meant that BSCVA had decreased over time, increased Kmax rates meant that Kmax had increased, and increased TCT rates meant that TCT had reduced.

• **SYSTEMIC BIOMARKER ASSESSMENT AND PBMC ISOLATION:** Blood samples were collected at M0, M4, M6, and M12 to monitor serum 25-OH Vit D and calcium levels through standardized methods: electrochemiluminescence immunoassay[18] and the *o*-cresolphthalein complexone method,[19] respectively, using a Cobas C 800 autoanalyzer (Roche). At M0 and M6, PBMCs and plasma samples were isolated using a density gradient centrifugation based on Ficoll (Lymphoprep; Stemcell Technologies). After collection, the PBMC was stored at –20°C until further analysis.

• **ENZYME-LINKED IMMUNOSORBENT ASSAY:** Plasma samples collected at M0 and M6 were centrifuged at 1000*g* for 15 minutes at 4°C to remove debris.

Supernatants were separated, diluted following the manufacturer's instructions, and immediately assessed. Highly sensitive ELISA kits were employed following the manufacturer's instructions for the determination of matrix metalloproteinase-9 (MMP9) (EH0238; FineTest), tissue metalloproteinase inhibitor-1 (EH0294; FineTest), Vit D binding protein (VDBP) (ab108853, Abcam), and procollagen type 1 C-terminal propeptide (PICP) (EH0957; FineTest). A 1:2 dilution was used for each marker.

• **REAL-TIME POLYMERASE CHAIN REACTION:** PBMC samples collected at M0 and M6 were used. Total RNA extraction, DNAse treatment, retrotranscription, and real-time polymerase chain reaction were performed as described elsewhere.[20] The following genes were evaluated by the TaqMan Gene Expression Assays (Applied Biosystems): lysyl oxidase (LOX, Hs00942480_m1), Vit D receptor (VDR, Hs01045843_m1), cytochrome P450 2R1 (CYP2R1, Hs01379776_m1), cytochrome P450 27A1 (CYP27A1, Hs01017992_g1), cytochrome P450 24A1 (CYP24A1, Hs00167999_m1), cluster of differentiation 14 (CD14, Hs02621496_s1), procollagen-lysine, 2-oxoglutarate 5-dioxygenase 2 (PLOD2, Hs01118190_m1), as well as glyceraldehyde 3-phosphate dehydrogenase (GAPDH, Mm99999915_g1) as a reference gene. Results are shown as a relative expression with the $\Delta\Delta$CT method.

• **RNA SEQUENCING:** RNA was isolated from PBMC as described in the previous section. Five hundred picograms RNA was used from each sample as template for cDNA generation using the Takara Smart-Seq v4 Ultra Low Input RNA kit (Takara Bio USA) following the manufacturer's instructions, with 15 cycles of cDNA amplification. Smart-Seq cDNA was assessed for quality on High Sensitivity D5000 Screen Tape Assay on the 4200 TapeStation System. Starting from 500 cDNA picograms, libraries were prepared through a DNA library prep Illumina kit (Illumina), following the manufacturer's instructions. Final libraries were quantified (High Sensitivity D5000 Screen Tape Assay) and pooled at 0, 8 nM concentration, then sequenced 1 × 100 bp on the Illumina NovaSeq 6000 platform. One sample failed due to insufficient RNA concentration.

Bioinformatic analysis

FASTQ sequencing reads were adaptor-trimmed and quality-filtered with Trimmomatic,[21] prior to mapping to the hg38 human reference genome (https://www.gencodegenes.org/human/) with STAR.[22] Gene counts were obtained using featureCounts[23] and Genecode basic annotation v31. Downstream gene expression analysis has been done using the R/Bioconductor framework.[24] Sequencing coverage was considered inadequate for 3 samples, which were removed from the analysis. Only genes with a mean of at least 10 read counts per sample were considered for further analyses. Following this criterion, 12,891 protein-coding and 6675 noncoding genes were identified, and for the sake of this analysis, only protein-coding genes were considered.

Principal component analysis

Principal component analysis (PCA) was performed on the 1000 coding genes with the highest amount of variability (ie, explanatory power) in our dataset. Based on this data, the two main dimensions, dimension 1 (26.2% variance) and 2 (12.4% variance) were subsequently taken into consideration, collectively explaining 38.6% of our dataset variance.

UMAP visualization and unbiased clustering analysis
The top 9 components of the PCA, collectively explaining 78.8% of our dataset variance, have been used to compute the 6 nearest neighbors for each sample and generate the Uniform Manifold Approximation and Projection (UMAP) plot via the *umap* R package and the unbiased clustering using the Leiden algorithm (resolution 0.8) via the *igraph* R package.

Differential gene expression analysis
Differential gene expression (DGE) analysis was performed in R (version 4.1.1) using DESeq2.[25] Adjusted *P* values were obtained based on the Benjamini-Hochberg method. Genes with a false discovery rate <10% and absolute log2FoldChange >0.25 were considered significant. Differentially expressed genes were subjected to Gene Ontology analysis using the R/Bioconductor package clusterProfiler.[26]

• **CELL TYPE DECONVOLUTION:** Deconvolution of the RNA-seq samples was performed to estimate the contribution of individual PBMCs in defining the bulk transcriptomic profiles of each analyzed sample. In particular, CIBERSORTx[27] and a single-cell reference of the human blood obtained from the Tabula Sapiens consortium[28] were used to run the "Impute cell fraction" functionality.

Variant calling
Variant calling analysis was performed following GATK guidelines for RNA-seq.[29] In particular, STAR's two-pass mode alignment was performed to improve alignments around novel splice junctions, duplicate reads were identified, RNA alignments at intronic positions were converted to DNA conventions (SplitNCigarReads), and systematic errors in the base quality scores were detected and corrected with the BaseRecalibrator tool. Finally, HaplotypeCaller was used to call variants toward the reference genome. Variants were then hard filtered using VariantFiltration to exclude likely false-positive ones. Only variants passing filter were kept in the downstream analysis.

• **STATISTICS:**

Sample size
Published data suggest that KC progression of at least 1 D at the apex occurs in more than 80% of cases.[30] We considered a reduction of KC progression to 50% after 12 months to be clinically significant in the Vit D—supplemented group as opposed to 80% in the untreated population. Hence, considering an alpha error of 0.05 and beta error of 0.2, with 0.8 power, 32 patients were necessary. Considering a potential loss at follow-up of 20%, we enrolled 40 patients.

Analysis
For the primary endpoint, we considered each patient separately. For all the other analyses, we considered each eye from the same patient separately. Data were expressed as standard error of the mean. The statistical significance of the differences between the two groups for continuous variables was assessed using the Wilcoxon signed-rank test. For categorical variables, Fisher's exact test was employed. A two-sided $P \leq .05$ was considered statistically significant. RStudio software (RStudio, PBC) and Prism 10.0 (GraphPad Software) were used for the statistical calculations.

RESULTS

• **BASELINE CHARACTERISTICS:** We recruited 40 patients (mean age, 16.6 ± 1.9; range, 12.2-19.9 years) presenting Vit D insufficiency (30 ng/mL) and diagnosed with KC in at least one eye. Ten eyes were excluded from further analysis, as the patients had previously undergone corneal CXL (Figure 2). Most of the patients were males (87.5%). In addition, 32.5% of patients reported allergies. The patient's demographics are summarized in Table 1.

• **CLINICAL PROGRESSION AFTER VIT D SUPPLEMENTATION:** Table 2 summarizes the values of Kmax, TCT, USCVA, and BSCVA at the beginning of the study and 12 months later. All parameters did not significantly change during the 12-month follow-up. Clinical data expressed as rates did not vary significantly throughout the study, indicating the stabilization of KC progression (Figure 3).

Corneal pachymetry analysis of epithelial and stromal thickness showed no significant differences in all variables 12 months after study initiation (Table 3).

• **CHANGES IN PLASMA VIT D LEVELS AND METABOLISM:** Serum Vit D levels statistically increased up to month 4 (49.9 ng/mL) and decreased thereafter, although they remained above or equal to 30 ng/mL (reference value for sufficiency)[15] up to month 12 (Figure 4, A). Calcium levels remained within the normal range (2.10-2.60 mmol/L) throughout the study, suggesting that the dosage regimen is safe (M4, 2.4683 ± 0.0760 mmol/L; M6, 2.4808 ± 0.0866 mmol/L; M12, 2.4543 ± 0.0791 mmol/L).

After 6 months of treatment, patients showed a statistically significant increase of the levels of VDBP, the principal transporter of Vit D in the plasma ($P = .0049$), followed by a significant increased expression of VDR ($P < .0001$), and CD14 ($P < .0001$), the principal and most rapidly upregulated target gene of VDR.[31] Vit D metabolism-related genes, instead, remained stable across the study ($P = .3945$, $P = .0967$, and $P = .4809$, respectively) (Figure 4).

• **FULL RNASEQ ANALYSIS OF DIFFERENTIALLY REGULATED GENES AFTER VIT D SUPPLEMENTATION:** The 10 most topographically responder patients and the 10 most worsened patients were chosen and used for full RNA seq analysis.

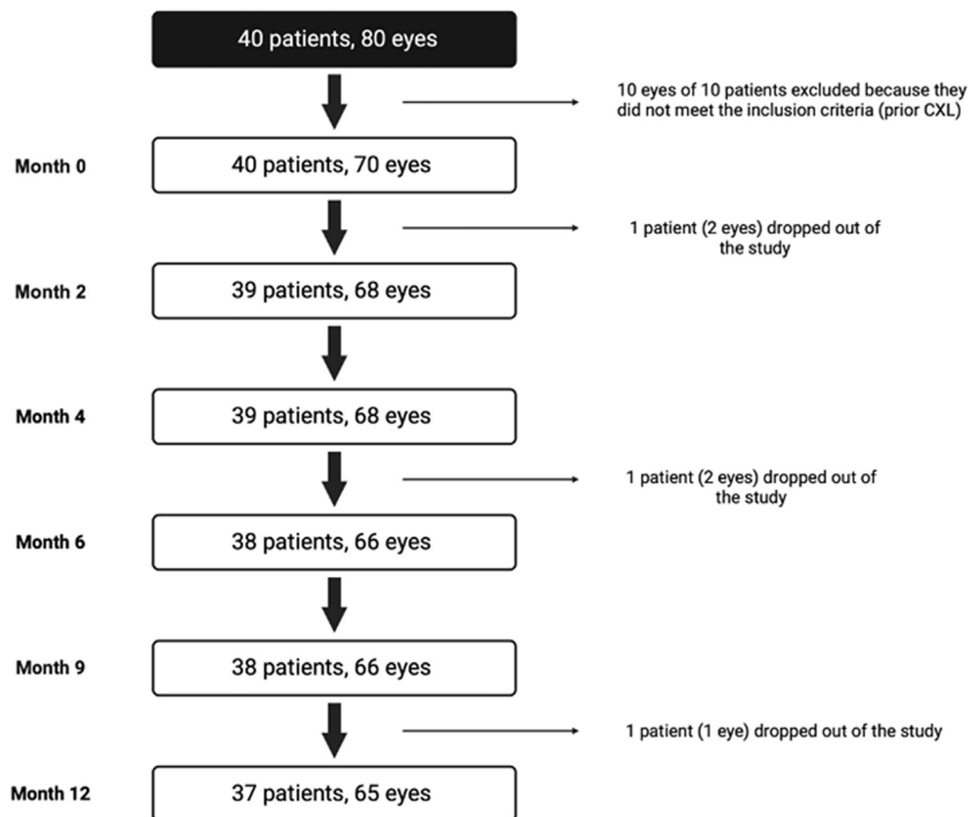

FIGURE 2. Study flow chart. Forty patients (80 eyes) were enrolled. From these, 70 eyes were used for the analysis because CXL had already been performed in 10 eyes. Patients were followed for up to 12 months. Five eyes (from three different patients) underwent CXL during the study and were excluded from the final analysis.

TABLE 1. Demographic Characteristics of the Study Population

Parameter		KC-Vit D Patients M0	KC-Vit D Patients M12
Patients, *n*		40	37
Gender, *n*			
	Male	35	32
	Female	5	5
Age (y), mean (range)		16.6 (12.2-19.9)	17.5 (13.2-20.9)
Allergies, *n* (%)		13 (32.5%)	11 (29.73%)

TABLE 2. Clinical Parameters Before and After Vit D Supplementation

Parameter	KC-Vit D Patients M0	KC-Vit D Patients M12	*P* Values$_{0-12}$
Kmax (D), mean ± SD	51.5 ± 6.6	52.1 ± 6.9	.6141
TCT (μM), mean ± SD	494.1 ± 32.2	492.2 ± 34.0	.8532
UCVA (logMAR), mean ± SD	0.23 ± 0.20	0.24 ± 0.26	.6800
BSCVA (logMAR), mean ± SD	0.11 ± 0.14	0.09 ± 0.16	.292

Comparison of clinical parameters (Kmax, TCT, UCVA, BSCVA) at M0 and M12.

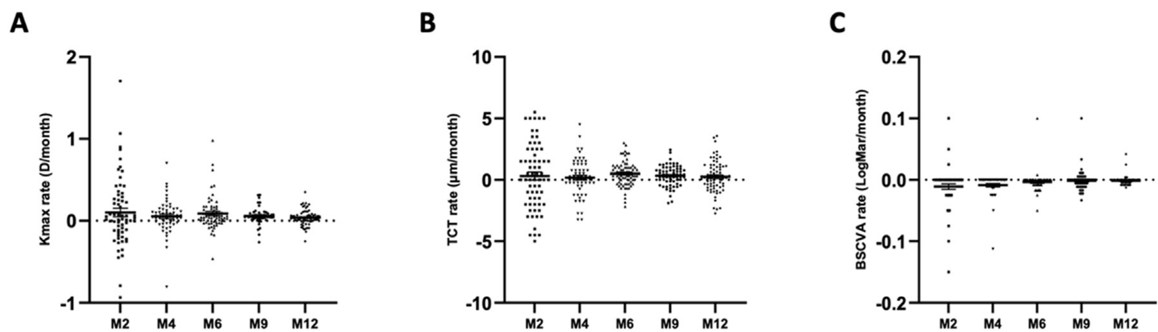

FIGURE 3. Vit D supplementation arrests KC progression. (A-C) Quantification of Kmax rate, TCT rate, and BSCVA rate indicating KC stability over time. Results are expressed in terms of rates (how parameters changed at each time point compared to M0). Sample size is indicated in the supplementary material.

TABLE 3. Epithelial and Stromal Thickness Remains Stable Following Vit D Supplementation

Variables	KC—Vit D		
	M0 ($N = 39$)	M12 ($N = 32$)	*P* Value M0-12
Epithelium			
3 mm (µm)	50.34 ± 6.13 (32-63)	50.88 ± 6.27 (32-64)	.5262
6 mm (µm)			
Superior	52.74 ± 4.42 (45-64)	54.39 ± 5.13 (44-65)	.08077
Inferior	54.90 ± 4.70 (46-72)	55.27 ± 4.95 (45-68)	.6632
Nasal	54.98 ± 4.90 (47-68)	55.88 ± 5.95 (46-72)	.526
Temporal	52.40 ± 4.06 (44-63)	52.82 ± 4.37 (44-62)	.6075
8 mm (µm)			
Superior	48.74 ± 4.66 (41-62)	47.70 ± 4.43 (40-59)	.4021
Inferior	51.47 ± 4.27 (41-63)	51.57 ± 3.98 (42-58)	.6248
Nasal	55.37 ± 4.72 (47-69)	54.30 ± 4.97 (46-69)	.2553
Temporal	52.91 ± 4.05 (44-61)	52.25 ± 4.75 (43-61)	.4019
Stroma			
3 mm (µm)	457.81 ± 27.56 (374-516)	454.82 ± 29.11 (371-520)	.567
6 mm (µm)			
Superior	528.68 ± 29.05 (472-599)	528.96 ± 26.86 (473-588)	.9459
Inferior	501.74 ± 26.36 (448-577)	503.88 ± 27.60 (449-584)	.8506
Nasal	511.43 ± 29.29 (454-578)	512.93 ± 28.16 (464-571)	.7555
Temporal	484.00 ± 27.26 (424-588)	482.98 ± 25.32 (421-545)	.984
8 mm (µm)			
Superior	607.31 ± 39.96 (497-680)	607.32 ± 34.07 (532-678)	.9508
Inferior	577.54 ± 38.20 (512-672)	580.73 ± 37.86 (511-667)	.5945
Nasal	576.69 ± 33.43 (529-651)	577.95 ± 56.40 (426-856)	.94
Temporal	531.81 ± 28.32 (485-607)	534.71 ± 26.56 (479-598)	.6075

Comparison of epithelial and stromal thickness between KC patients at M0 and M12.

Following RNAseq analysis, responders were characterized by a noteworthy change in the gene expression profile compared to nonresponders (Figure 5). To explain this difference, we visualized our transcriptomic results by means of a dimensionality reduction strategy, namely PCA (Supplemental Data, Figure 1), that represented 37% of the overall variability in the first two dimensions.

This analysis shows that responders have a coherent systemic response to Vit D (arrow pointing toward the same direction in the PCA space) resulting in appreciable DGE, while nonresponders do not collectively show a change in gene expression due to incoherent response profiles interfering with one another and resulting in an overall neutral expression. These results suggest that responders have a homogenous systemic response profile to Vit D supplementation.

• **VDR POLYMORPHISM ANALYSIS:** We then wondered whether the presence of nonresponders could be explained by different frequency of VDR polymorphisms in differ-

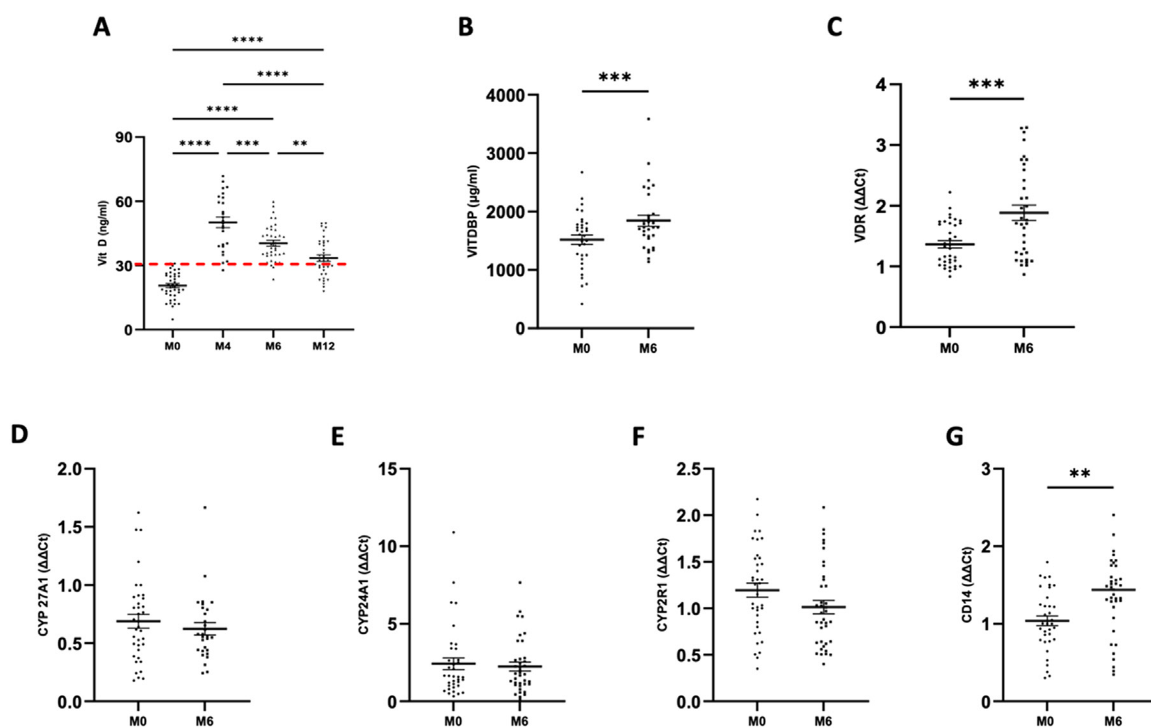

FIGURE 4. Vit D treatment increases circulating Vit D levels and the expression of VDBP, VDR, CD14. No change in the expression of genes associated with Vit D metabolism was observed. (A) Quantification of Vit D levels at four time points. (B) Vit D binding protein expression after treatment by ELISA. (C) Quantification of vitamin D receptor expression by PCR. (D-F) Evaluation of cytochromes involved in vitamin D metabolism by qPCR. (G) CD14, a vitamin D target gene, is increased after treatment. Sample size is indicated in the supplementary material.

ent patient cohorts. However, the frequency of VDR polymorphisms did not differ between the general population and either responders or nonresponders (Figure 6). Data on general population were obtained from the 1000 Genomes Project Consortium.[32]

• **SYSTEMIC INFLAMMATION AND PLATELET ACTIVATION:** A blood cell panel was performed to investigate the systemic effect of Vit D. Upregulated genes were associated with PBMC cell function processes, and were compatible with the shift from acquired immunity toward natural immunity known to be promoted by Vit D.[33] Interestingly, pathway enrichment showed that the main genes that were downregulated in responders pertained to platelet activation pathways associated with a residual platelet fraction found in PBMC samples (Figure 7).

To rule out the possibility of procedural errors causing selective platelet contamination, we reviewed preparation batches. All preparations were processed with the same methods and reagents. Two preparations were done on the same day, while all other preparations were conducted on separate days.

Subsequently, estimation of cell type abundances was performed using a single-cell-based deconvolution method.

The analysis was performed using CIBERSORTx[27] (a tool that provides an estimation of the abundances of member cell types in a mixed cell population, using gene expression data). Single-cell expression references were taken from the Tabula Sapiens consortium portal.[28]

Absolute quantification of platelet content in the different samples highlighted a general decrease of platelet content after Vit D treatment (Figure 8, A). The homogenous pattern of estimated platelet count variation before and after supplementation was not compatible with casual differences in platelet fraction. Therefore, we interpreted this data as a numerical decrease in the platelet fraction associated with PBMC sample preparation, and/or a decrease of RNA transcripts in platelets.

Interestingly, a decrease in platelet number and platelet-lymphocyte ratio, a well-known inflammatory index[34] was found after treatment (Figure 8, B and C), which was more evident in responders (Figure 8, D). These data further corroborate the hypothesis that Vit D supplementation reduces systemic inflammation in KC patients.

• **UNBIASED CLUSTERING OF RNA-SEQ SAMPLES REVEALS TRANSCRIPTIONAL PROFILES ASSOCIATED WITH PLATELET DECREASE IN RESPONSIVE PATIENTS:** To further characterize the transcriptional response to Vit D treatment, we performed an unbiased clustering analysis based on the neighborhood of our samples in the transcriptional space. In addition, we exploited UMAP to better visualize

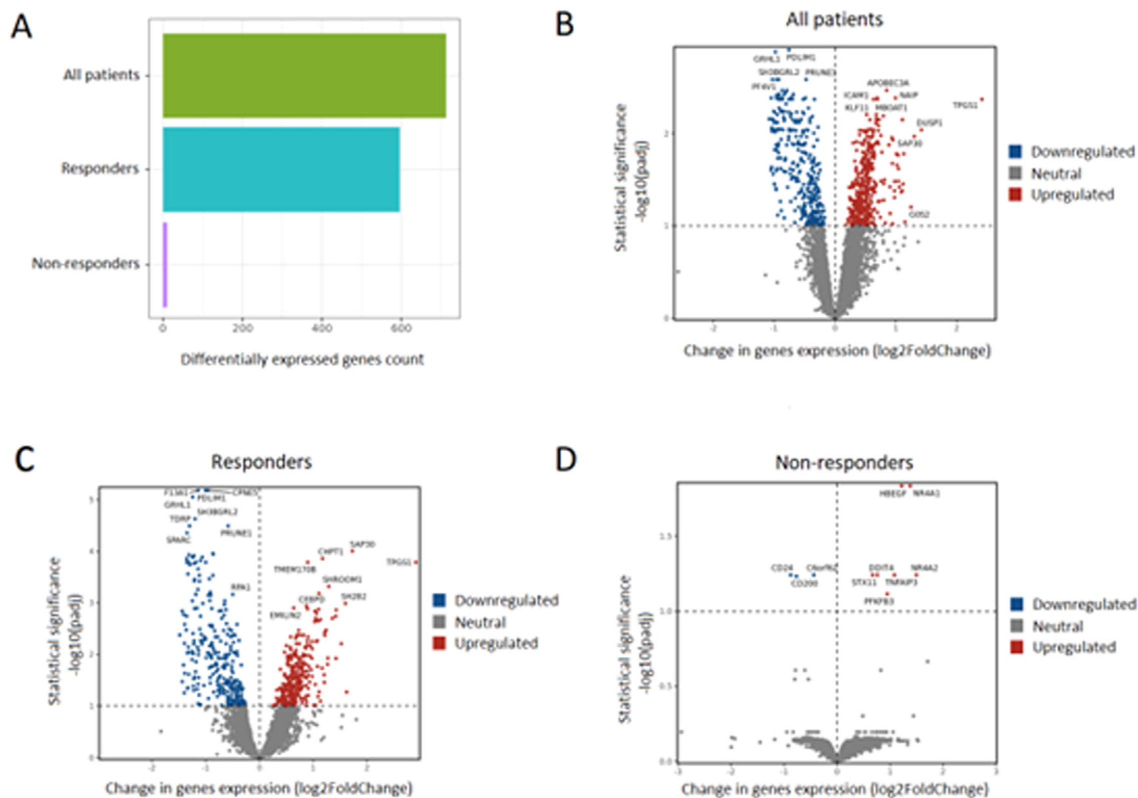

FIGURE 5. Vit D supplementation induces a characteristic pattern of differentially expressed genes (DEG) that is observed in responders but is absent in nonresponders. (A) Bar plot of total number of differentially expressed genes (DEGs) for three groups: all patients, responders to treatment and nonresponders, highlighting a different RNA expression in response to treatment (note that the "all patients" bar is not the algebraic sum of the two subgroups, as the same amount of upregulation/downregulation of the same gene in a population averages out as neutral expression); (B-D) Volcano plots showing significant (above the horizontal line) gene regulation in the total RNA-seq population (B), in responders (C) and nonresponders (D) (M0 $n = 20$; M6 $n = 20$).

the complex transcriptional space (see methods). UMAP embedding and clustering analysis of our dataset revealed 4 transcriptional clusters with different representation in terms of clinical groups (Figure 9, A). Clusters 1 and 2 were represented mainly by untreated patients. Conversely, clusters 3 and 4 were represented mainly by treated patients (Figure 9, B, left panel). Further stratification of patients by their responses to Vit D revealed that, among clusters associated with the treatment, cluster 3 was mainly associated to nonresponder patients, while cluster 4 was exclusive for responders (Figure 9, B, right panel). Clusters were also associated with Vit D and platelets levels, with a progression from low Vit D levels and high platelets in cluster 1 to high Vit D levels and low platelets in cluster 4 (Figure 9, C). This data supports the evidence that the Vit D levels, platelet count can be associated to responders and nonresponders by means of their transcriptional profiles. To characterize transcriptional differences among the clusters, we performed DGE analysis of each cluster against all the others. This analysis confirmed that cluster 1, which is peculiar of untreated patients, is associated with platelets activation. Cluster 3, peculiar of treated nonresponsive patients, is associated with the modulation of immune system mediated by interleukins (Figure 9, D). Cluster 4, peculiar of treated responsive patients, is associated with the expression of different HLA genes and other genes related to the antigen processing (Figure 9, D). Overall, this analysis supports that Vit D could exert different transcriptional changes in responders and nonresponder patients, involving different immune system response.

- **COLLAGEN AND EXTRACELLULAR MATRIX TURNOVER:** MMP9 was significantly reduced after treatment ($P < .01$) (Figure 10, A). A significant increase of MMP inhibitor was also observed ($P = .0274$) (Figure 10, B).

The gene expression of procollagen type I was significantly decreased ($P < .0001$) (Figure 10, C).

Processes involved in keratan sulfate (a proteoglycan that participates in corneal extracellular matrix composition) metabolic and biosynthetic processes were enriched in responders (Figure 11). The analysis could not be carried out in nonresponders as DEG expression was insufficient.

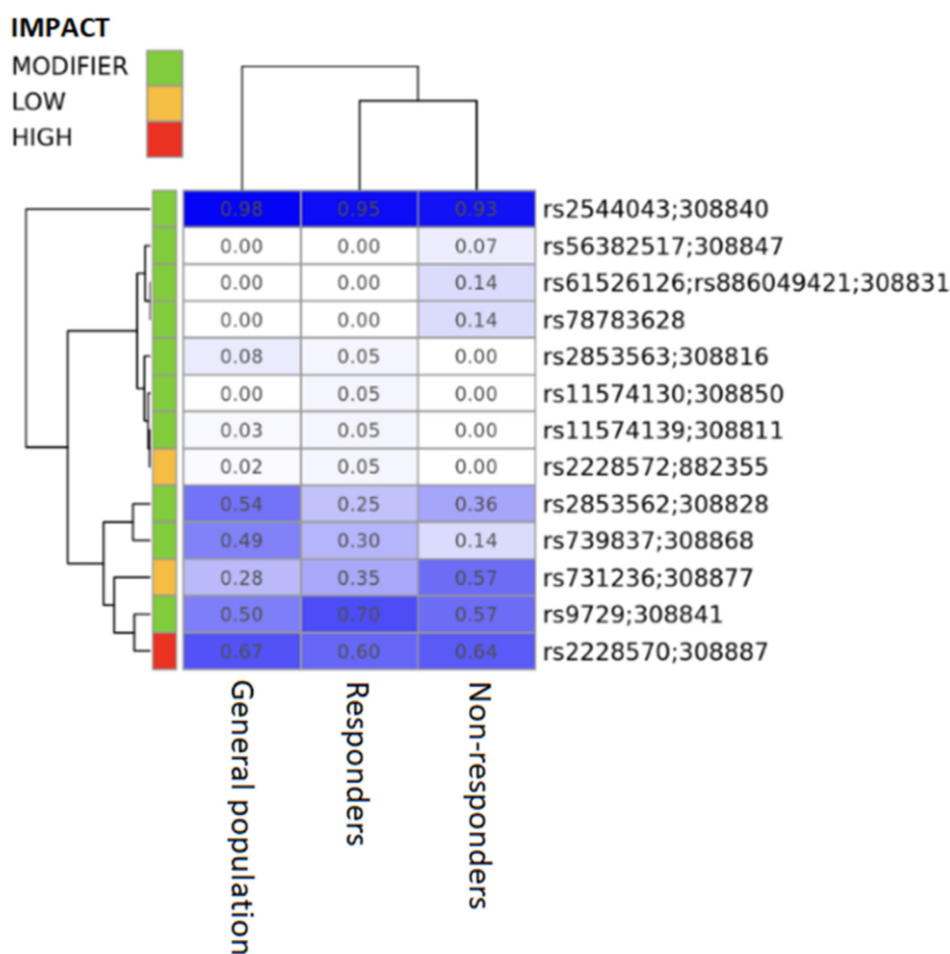

FIGURE 6. The frequency of VDR polymorphisms does not differ between responders, nonresponders, and the general population, as shown by RNA-seq polymorphism analysis (M0 $n = 20$; M6 $n = 20$).

DISCUSSION

KC has been traditionally considered a noninflammatory corneal degeneration, characterized by progressive changes in corneal structure and organization of collagen. The concept that KC may be considered the ocular manifestation of a systemic disease is supported by several clinical findings, including substantial KC comorbidities,[4] exceedingly high rates of KC associated with certain systemic genetic disorders,[4] and epidemiology studies.[12,35] Interestingly, most, if not all, of these disparate disorders share a high prevalence of Vit D deficiency. Interestingly, Vit D insufficiency is also common in children and adolescent patients, possibly because of reduced sunlight exposure[36] although other factors could contribute (eg, obesity).[37-45]

In 1937, Blackberg and Knapp[46] reported the results of their experiments on Vit D-deprived dogs and rats, describing a link with the development of KC. Two years later, Knapp reported KC improvement (measured on casts of eyes before and after treatment) upon administering Vit D supplementation.[46] However, technologies available at the time do not allow to draw definitive conclusions, nor they provide a potential mechanism for this clinical observation.

Recently, a renewed interest for the function of Vit D in corneal homeostasis and KC pathogenesis has arisen.[47] A meta-analysis by Gupta et al has drawn the conclusion that Vit D levels are significantly lower in KC patients compared to the general population. Quite notably, Vit D correlated with the aggressiveness of the disease: lower serum levels were associated with progressive disease.[11,12,14,48-50] However, the molecular mechanisms behind this interaction were still unclear. We previously showed that Vit D supplementation is associated with reduced markers of systemic collagenolysis and may promote KC stability. In this study, we expanded the treated cohort and provided full RNAseq analysis of a patient subset. Our data corroborate those from our previous paper as 65% of patients (72% of eyes) remained stable for 1 year, which is remarkable considering that adolescent patients generally show much higher rates of progression.[14]

FIGURE 7. **Vit D modulates platelet function and immunity by downregulating platelet-related processes and upregulating selected immune-related processes. The figure shows the most downregulated and upregulated processes after supplementation in the RNA seq population.**

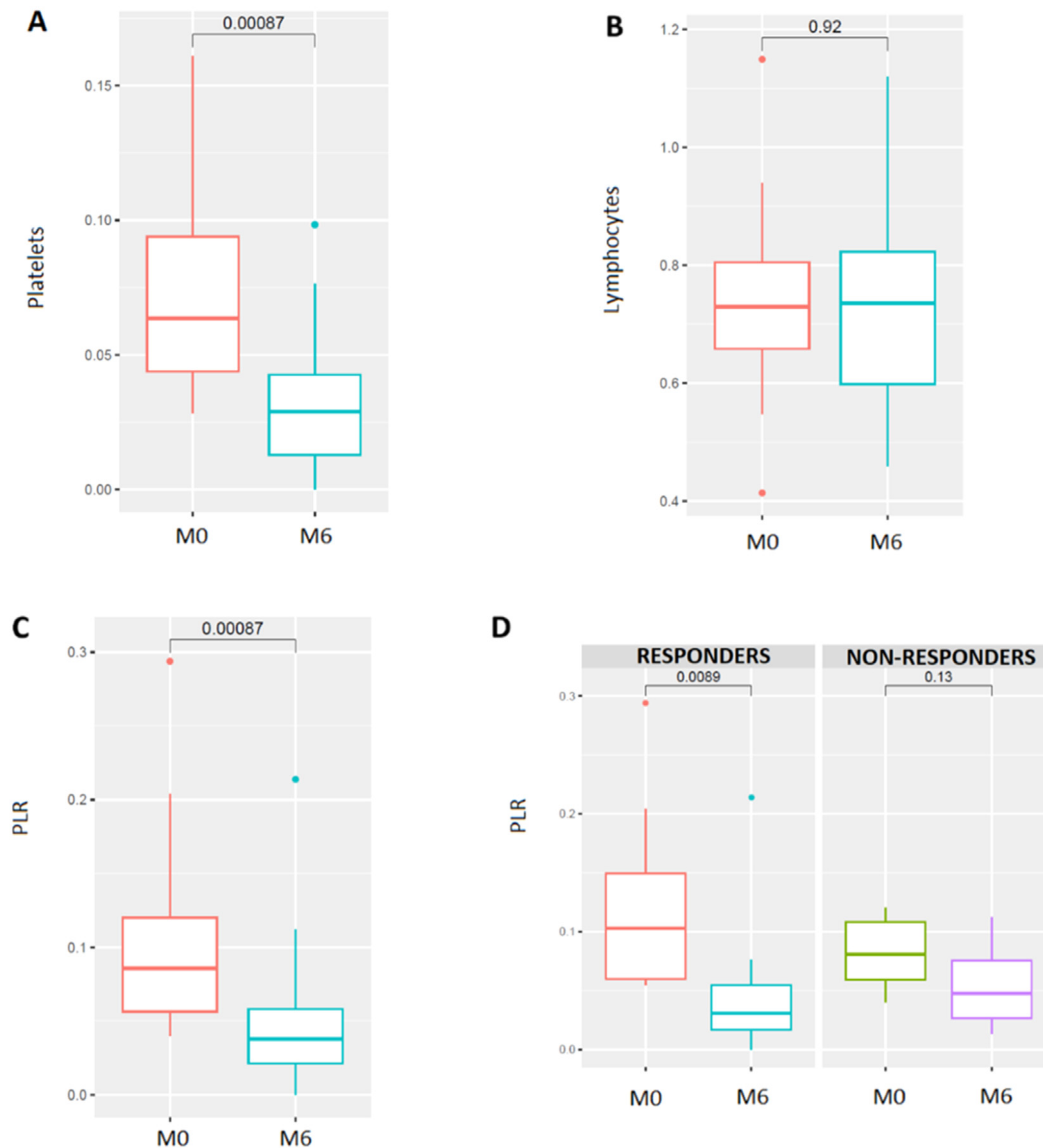

FIGURE 8. Platelet/Lymphocyte ratio decreases after Vit. D supplementation, more evidently in responders. (A) Platelet-related gene expression decreases in the RNA-seq population after treatment; (B) lymphocyte-related genes remain stable after treatment; (C) PLR decreases after treatment in the total population, more evidently in responders (D). All graphs are represented as box plots (M0 *n* = 20; M6 *n* = 20).

• **CLINICAL FINDINGS:** Our findings support that KC is a partially systemic disease and that Vit D is a key factor for the biomechanical stability of the cornea. Indeed, we have shown that KC remained stable (Kmax change < 1 D) in 24 patients (65%) and 49 eyes (75%) over 12 months following Vit D supplementation. Although in this study we did not have a control group, data from the literature clearly shows that in a similar age range, KC progression occurs in 80%-up to 90% of cases in children.[51] We believe that the effects of Vit D show promise for the stabilization of the disease. Interestingly, TCT, UCVA, and BSCVA also remained stable at month 12 as well as epithelial and stromal thickness. In our study, only 25% of KC eyes worsened during the 12 months of follow-up. These data confirm our initial observations in a smaller subgroup of patients.[14]

• **BIOLOGICAL MECHANISM (S) EXPLAINING CLINICAL DATA:** To find a biological mechanism that could explain our clinical data, we first analyzed Vit D metabolism extensively. Serum Vit D levels after enrollment were significantly increased for the following 12 months and remained

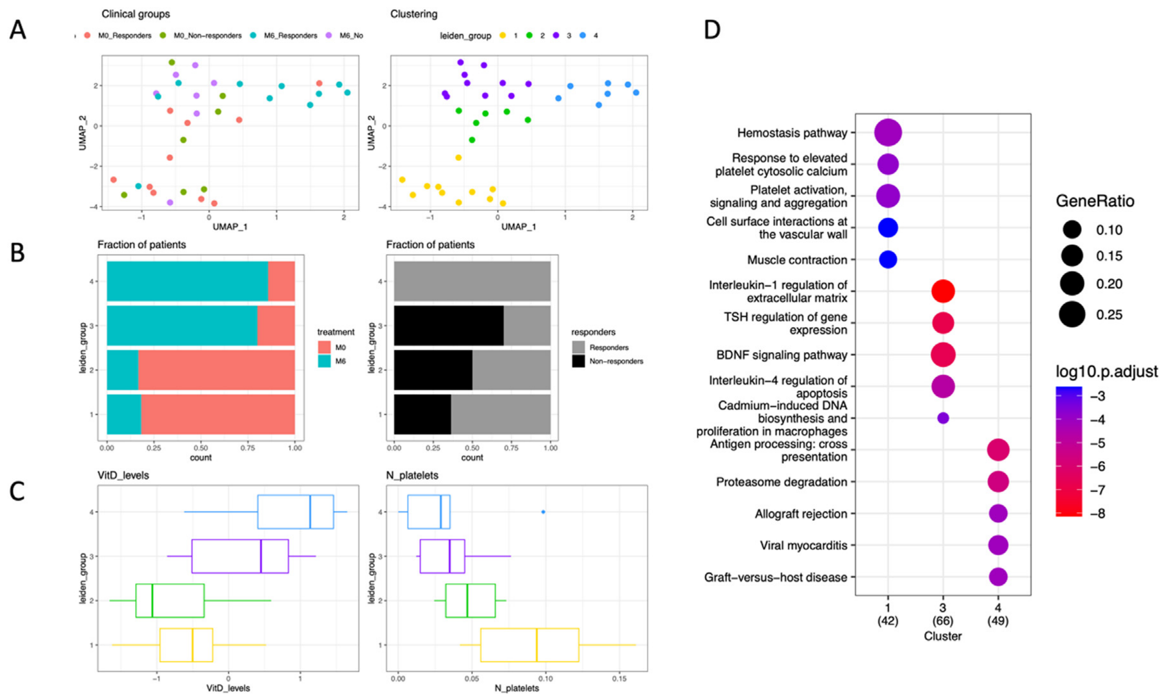

FIGURE 9. **UMAP plots demonstrating cell clustering changes before and after vitamin D supplementation. (A) UMAP representation of the RNA-seq dataset, colored by clinical group (left) or Leiden transcriptional cluster (right); (B) bar plots representing the fraction of treated and untreated patients (left), or responders and nonresponders (right), within each Leiden transcriptional cluster. (C) Boxplots representing the vitamin D (left) or platelets estimated abundance (right) within each Leiden transcriptional cluster. (D) Top 5 BioPlanet pathways enriched among the DEGs of each Leiden transcriptional cluster compared to others.**

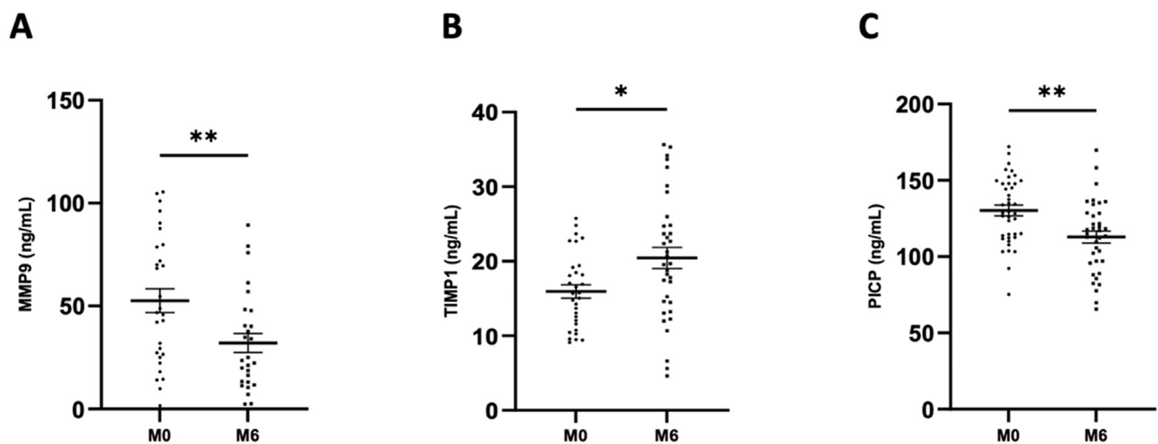

FIGURE 10. **Vit D treatment reduced collagen turnover. Quantification of matrix metalloproteinase-9 (A), metallopeptidase inhibitor (B), procollagen type I synthesis (C). All measurements are performed by means of ELISA assays.**

above or equal to 30 ng/mL, which is the reference value for sufficiency.[15] After Vit D supplementation, the levels of VDBP rose, increasing Vit D transport to its inducible receptor, whose expression was eventually upregulated. All genomic and biological actions of the active form of Vit D are mediated by the transcription factor VDR, which is the only protein that binds 1α, 25 (OH) $_2D_3$ effectively at subnanomolar concentrations.[52,53] These data indicate adequate treatment compliance and appreciable activation of systemic pathways related to Vit D.

Interestingly, however, the systemic biological response to Vit D supplementation was not uniform in our popu-

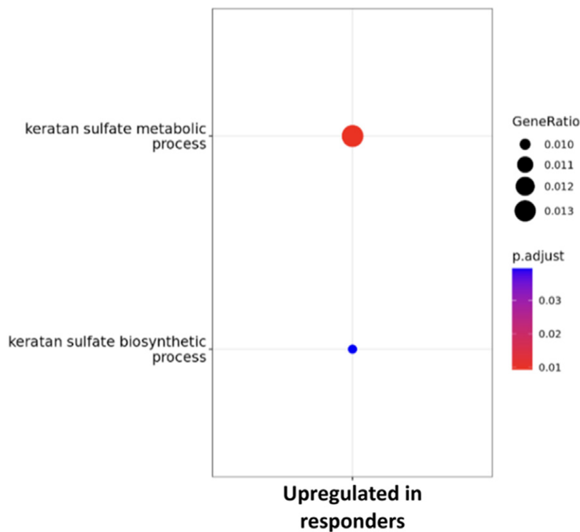

FIGURE 11. Keratan sulfate metabolism-related genes are increased in responder patients. The DEG analysis could not be carried out in nonresponders due to the limited differential gene expression (as seen in Figure 5, D). Genes involved in keratan sulfate metabolic processes: CHST2/SLC35D2/GNS/ST3GAL2. Genes involved in keratan sulfate biosynthetic process: CHST2/SLC35D2/ST3GAL2.

lation. A notable factor of differentiation between clinical responders and nonresponders was RNA seq coherence in treatment response. Responders displayed a coherent response to the treatment allowing the identification of significantly regulated genes and mechanisms, whereas nonresponders showed lack of total DEG changes. It should be noted that this observation does not imply that there was no transcriptional response to supplementation in nonresponders, but rather an incoherent response resulting in reciprocal annihilation of changes in single genes (as found with PCA analysis, see Supplemental Data).

The difference in expression between the two groups suggests that responders have a functional response to Vit D supplementation, while nonresponders may have patterns of systemic response that are different from each other, with variable degrees of dysfunctionality. Other predisposing factors not analyzed in this study may have worsened the clinical course for this subgroup.

Because VDR polymorphisms can influence systemic response to Vit D, we performed a variant analysis on our population, which showed no difference in the prevalence of any single variant between responders or nonresponders, or the general population. Therefore, our analysis supports that variant distribution does not play a role in differential Vit D response, both systemic and in terms of protection against KC evolution. Vit D exerts several VDR-independent biological activities. Indeed, it can directly/indirectly modulate mitochondrial function and metabolism and have cell-protective properties.[54]

As expected, Vit D supplementation affected the systemic inflammatory framework. While the etiopathology of KC remains unknown, evidence accumulating in recent

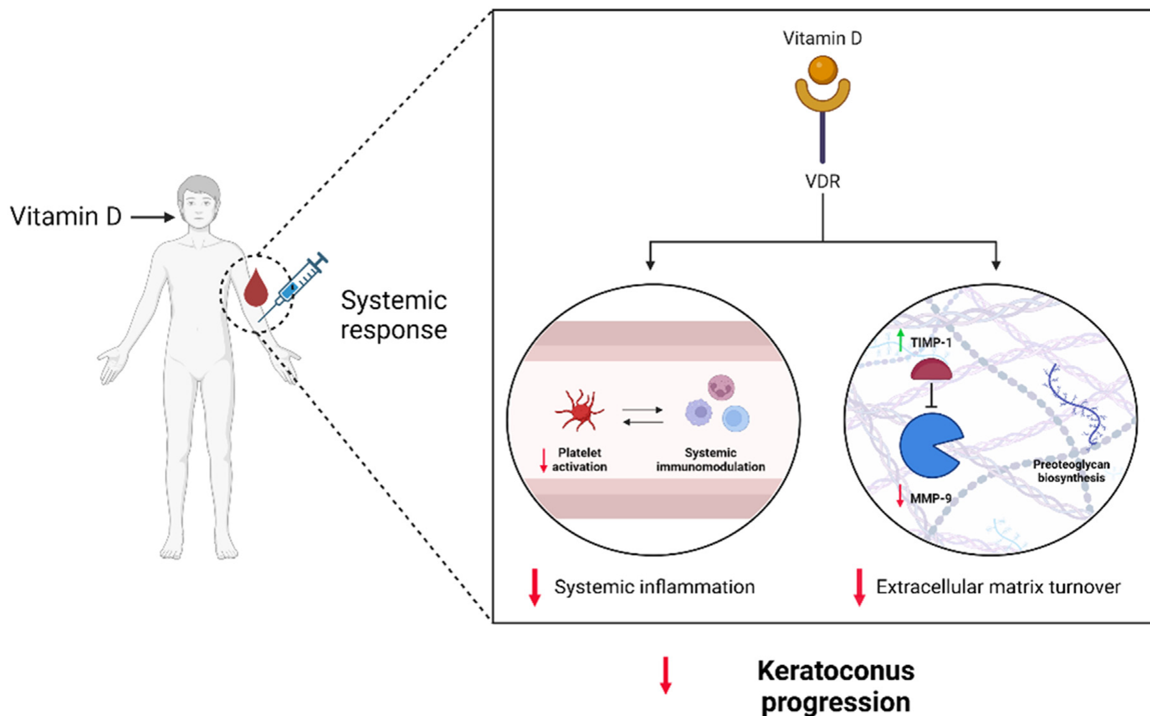

FIGURE 12. Hypothesis of the biological mechanisms leading to stabilization of KC progression.

years suggests that inflammation may be involved in KC progression.[55,56] For instance, it has been proposed that atopy (probably through the effect of eye rubbing) is a predictor of KC progression.[57] Systemic inflammatory indexes, including platelet-lymphocyte ratio, have been found to be higher in patients with KC than in healthy controls.[58]

A possible association with thyroid gland disease has also been found[59] although there is no unanimous consensus on this matter.[60] It is known that inflammatory cells produce metalloproteinases which lead to collagen degradation. Indeed, MMPs can be induced by proinflammatory cytokines, including IL-1, IL-6, and TNF.[61,62]

Our observation that the expression of soluble CD14 is increased after Vit D supplementation corroborate these findings because it has been shown that it reduces the expression of proinflammatory cytokine Interleukin 8.[63]

Platelet-activation processes were upregulated in the study population following supplementation. Batch review revealed that the presence of a small fraction of platelets was systematic and not influenced by procedural errors. Interestingly, an inverse correlation between Vit D and platelets has been reported: Vit D deficiency is associated with increased platelet count.[64-66]

VDR is present in platelets, and VDR knockout mice have increased thrombogenicity.[67] In platelets, VDR has a nontranscriptional role (as they lack a nucleus), but it plays a direct role in calcium-dependent activation.[68] In conclusion, our data shows a down-regulation of platelet-related transcript at M6 which could reflect actual changes in platelet count and/or platelet activation (as it has been suggested before).[69]

Inflammation and platelet activation are deeply associated,[70,71] and platelet count is significantly associated with platelet activity.[72] Interestingly, it has been reported that reduced platelet activation also inhibits the activation of latent MMPs into active MMPs through prostoglandin E2, and tissue-type plasminogen activator,[73,74] which could explain our observation of reduced MMP9 expression and collagenolysis in Vit D supplemented patients. Finally, Vit D inhibited systemic collagenolysis-as demonstrated by reduced MMP9 and increased tissue metalloproteinase inhibitor-1 expression- and promoted preoteoglycan biosynthesis. It should be noted that the cornea is composed by large amounts of collagen and that the orientation and stability of collagen lamellae is maintained by a highly organized proteoglycan architecture.[75] Therefore, our observations strongly point to a therapeutic role of Vit D supplementation in KC, because it is well-known that KC corneas undergo progressive disruption of collagen fibers,[8,76] and also profound alterations of proteoglycan composition, specifically keratocan.[77] Moreover, our data suggest that Vit D supplementation inhibits systemic profibrotic pathways. Specifically, our observation that Vit D regulates collagen turnover is corroborated by its additional role in the metabolism of copper, a key cofactor for collagen CXL, which we reported before in the setting of KC.[14] Vit D may also play a direct role in cofactor availability for CXL reactions, as we previously observed.[14]

To further dig into our transcriptomic analysis, we performed unsupervised clustering analysis and UMAP visualization. The unbiased clusters found with our approach largely resembled both the treatment and the clinical features of our samples, with clusters represented by untreated patients and others by responsive or nonresponsive samples. This analysis supported the use of transcriptomics both to evaluate the response to Vit D treatment and to identify differences in the treatment effects between the responsive and the unresponsive population. Moreover, the comparison between clusters associated with responsive and nonresponsive treated samples highlighted a different activation of the immune processes following Vit D administration, supporting the idea of a specific role of the immune system in the progression of the disease.

We acknowledge that our study has some limitations. First, a randomized clinical trial will be needed to definitively confirm our results. Second, additional studies, conducted on larger cohorts, will also be helpful to validate biomarkers and RNAseq to reduce confounding factors linked to interindividual variability. Third, platelet contamination may have led to systemic bias in RNAseq analysis. However, the consistency of trends from M0 to M6 among the whole patient population makes this occurrence highly unlikely, and strongly suggest that such changes are not casual. In any case, addition of a complete blood count test will have to be implemented in future studies.

In conclusion, we suggest that KC is the local manifestation of a systemic disease, and we hypothesize that systemic inflammation, platelet activation, and collagen degradation play a primary role in this process. Eye rubbing, recurrent allergic/vernal conjunctivitis are well-known factors associated with KC progression and could well be local actors of this systemic proinflammatory condition. As such, the exploration and introduction of a systemic treatment may be beneficial. Here, we have shown that (1) Vit D may reduce the progression rate of KC in pediatric patients with Vit D insufficiency, and this clinical observation is associated with (2) reduced markers of systemic inflammation, platelet count and/or activation, and extracellular collagen matrix degradation.

The modulation of systemic inflammation (both directly and through decreased platelet activation/numbers) and the increased extracellular matrix stability which we measured in the blood is parallelled by clinical measures of substantial KC stability (Figure 12). In fact, we found specific patterns of differential gene regulation between responders and nonresponders. Interestingly, we found a significant reduction in platelet/lymphocyte ratio after treatment only in responders. These transcriptomic observations suggest that systemic response and KC progression in our population tended to occur together, pointing to a role of Vit D as a modifier of the clinical course of KC in responders.

Importantly, our findings also suggest that platelet/lymphocyte ratio is a minimally invasive, inexpensive marker of KC stability during Vit D supplementation.

CREDIT AUTHORSHIP CONTRIBUTION STATEMENT

Nicolò Bartolomeo: Writing – original draft, Investigation, Formal analysis, Data curation. **Matteo Pederzolli:** Writing – original draft. **Silvia Palombella:** Methodology, Investigation. **Philippe Fonteyne:** Methodology, Data curation, Conceptualization. **Giuseppe Suanno:** Methodology, Investigation. **Gianluca Tilaro:** Formal analysis. **Stefano de Pretis:** Formal analysis, Data curation. **Francesca Borgo:** Formal analysis, Data curation. **Federico Bertuzzi:** Visualization, Data curation. **Carlotta Senni:** Visualization, Data curation. **Massimo De Micheli:** Visualization, Validation. **Francesco Bandello:** Visualization, Validation, Resources. **Giulio Ferrari:** Validation, Supervision, Funding acquisition, Conceptualization.

Financial Disclosures: The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Giulio Ferrari reports financial support was provided by Associazione Italiana CHEratoconici. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this article.
Acknowledgments: The Authors would like to thank Associazione Italiana CHEratoconici for their precious support, and Luana Sorrenti and Arianna Zennato for their help.

REFERENCES

1. Gomes JAP, Tan D, Rapuano CJ, et al. Global consensus on keratoconus and ectatic diseases. *Cornea*. 2015;34(4):359–369. doi:10.1097/ICO.0000000000000408.
2. Santodomingo-Rubido J, Carracedo G, Suzaki A, Villa-Collar C, Vincent SJ, Wolffsohn JS. Keratoconus: An updated review. *Contact Lens and Anterior Eye*. 2022;45(3):101559. doi:10.1016/J.CLAE.2021.101559.
3. Gomes JAP, Rodrigues PF, Lamazales LL. Keratoconus epidemiology: A review. *Saudi Journal of Ophthalmology*. 2022;36(1):3. doi:10.4103/SJOPT.SJOPT_204_21.
4. Ferrari G, Rama P. The keratoconus enigma: A review with emphasis on pathogenesis. *Ocul Surf*. 2020;18(3):363–373. doi:10.1016/J.JTOS.2020.03.006.
5. Jiménez JLO, Jurado JCG, Rodriguez FJB, Laborda DS. Keratoconus: age of onset and natural history. *Optom Vis Sci*. 1997;74(3):147–151. doi:10.1097/00006324-199703000-00025.
6. Kankariya VP, Kymionis GD, Diakonis VF, Yoo SH. Management of pediatric keratoconus - Evolving role of corneal collagen cross-linking: An update. *Indian J Ophthalmol*. 2013;61(8):435–440. doi:10.4103/0301-4738.116070.
7. Kenney MC, Brown DJ. The cascade hypothesis of keratoconus. *Cont Lens Anterior Eye*. 2003;26(3):139–146. doi:10.1016/S1367-0484(03)00022-5.
8. Mathew JH, Goosey JD, Söderberg PG, Bergmanson JPG. Lamellar changes in the keratoconic cornea. *Acta Ophthalmol*. 2015;93(8):767–773. doi:10.1111/AOS.12811.
9. Chwa M, Atilano SR, Reddy V, Jordan N, Kim DW, Kenney MC. Increased stress-induced generation of reactive oxygen species and apoptosis in human keratoconus fibroblasts. *Invest Ophthalmol Vis Sci*. 2006;47(5):1902–1910. doi:10.1167/IOVS.05-0828.
10. Claessens JLJ, Godefrooij DA, Vink G, Frank LE, Wisse RPL. Nationwide epidemiological approach to identify associations between keratoconus and immune-mediated diseases. *Br J Ophthalmol*. 2022;106(10):1350–1354. doi:10.1136/BJOPHTHALMOL-2021-318804.
11. Gupta P, Pathak M, Thakur B, Fogla R, Agarwal A, Ram J. Association of keratoconus with serum levels of 25-hydroxyvitamin D and antioxidant trace elements: A systematic review and meta-analysis. *Indian J Ophthalmol*. 2022;70(8):2818–2824. doi:10.4103/IJO.IJO_3216_21.
12. Zarei-Ghanavati S, Yahaghi B, Hassanzadeh S, Mobarhan MG, Hakimi HR, Eghbali P. Serum 25-hydroxyvitamin D, selenium, zinc and copper in patients with keratoconus. *J Curr Ophthalmol*. 2020;32(1):26–31. doi:10.1016/J.JOCO.2019.06.003.
13. Akkaya S, Ulusoy DM. Serum Vitamin D Levels in Patients with Keratoconus. *Ocul Immunol Inflamm*. 2020;28(3):348–353. doi:10.1080/09273948.2019.1604002.
14. Vitar RML, Fonteyne P, Knutsson KA, et al. Vitamin D Supplementation Impacts Systemic Biomarkers of Collagen Degradation and Copper Metabolism in Patients With Keratoconus. *Transl Vis Sci Technol*. 2022;11(12). doi:10.1167/TVST.11.12.16.
15. Holick MF. Vitamin D deficiency. *N Engl J Med*. 2007;357(3):266–281. doi:10.1056/NEJMRA070553.
16. Lasagni Vitar RM, Triolo G, Fonteyne P, et al. Epidemiology of Corneal Neovascularization and Its Impact on Visual Acuity and Sensitivity: A 14-Year Retrospective Study. *Front Med (Lausanne)*. 2021;8:733538. doi:10.3389/FMED.2021.733538/FULL.
17. Holladay JT. Proper method for calculating average visual acuity. *J Refract Surg*. 1997;13(4):388–391. doi:10.3928/1081-597X-19970701-16.
18. Arneson WL, Arneson DL. Current Methods for Routine Clinical Laboratory Testing of Vitamin D Levels. *Lab Med*. 2013;44(1):e38–e42. doi:10.1309/LMONQZQ27TIN7XFS.
19. Connerty H V, Briggs AR. Determination of serum calcium by means of orthocresolphthalein complexone. *Am J Clin Pathol*. 1966;45(3):290–296. doi:10.1093/AJCP/45.3.290.
20. Vitar RML, Barbariga M, Fonteyne P, Bignami F, Rama P, Ferrari G. Modulating Ocular Surface Pain Through Neurokinin-1 Receptor Blockade. *Invest Ophthalmol Vis Sci*. 2021;62(3):26. doi:10.1167/IOVS.62.3.26.
21. Bolger AM, Lohse M, Usadel B. Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics*.

2014;30(15):2114–2120. doi:10.1093/BIOINFORMATICS/BTU170.
22. Dobin A, Davis CA, Schlesinger F, et al. STAR: ultrafast universal RNA-seq aligner. *Bioinformatics*. 2013;29(1):15–21. doi:10.1093/BIOINFORMATICS/BTS635.
23. Liao Y, Smyth GK, Shi W. featureCounts: an efficient general purpose program for assigning sequence reads to genomic features. *Bioinformatics*. 2014;30(7):923–930. doi:10.1093/BIOINFORMATICS/BTT656.
24. Huber W, Carey VJ, Gentleman R, et al. Orchestrating high-throughput genomic analysis with Bioconductor. *Nature Methods*. 2015;12(2):115–121 *2015 12:2*. doi:10.1038/nmeth.3252.
25. Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol*. 2014;15(12):1–21. doi:10.1186/S13059-014-0550-8/FIGURES/9.
26. Wu T, Hu E, Xu S, et al. clusterProfiler 4.0: A universal enrichment tool for interpreting omics data. *Innovation*. 2021;2(3). doi:10.1016/J.XINN.2021.100141/ATTACHMENT/19F0A0E0-397E-4A0C-8995-02608B6642C2/MMC2.PDF.
27. Newman AM, Steen CB, Liu CL, et al. Determining cell type abundance and expression from bulk tissues with digital cytometry. *Nature Biotechnology*. 2019;37(7):773–782 *2019 37:7*. doi:10.1038/s41587-019-0114-2.
28. Jones RC, Karkanias J, Krasnow MA, et al. The Tabula Sapiens: A multiple-organ, single-cell transcriptomic atlas of humans. *Science (1979)*. 2022;376(6594). doi:10.1126/SCIENCE.ABL4896/SUPPL_FILE/SCIENCE.ABL4896_MDAR_REPRODUCIBILITY_CHECKLIST.PDF.
29. Brouard JS, Bissonnette N. Variant Calling from RNA-seq Data Using the GATK Joint Genotyping Workflow. *Methods in Molecular Biology*. 2022;2493:205–233. doi:10.1007/978-1-0716-2293-3_13.
30. Chatzis N, Hafezi F. Progression of keratoconus and efficacy of corneal collagen cross-linking in children and adolescents. *Journal of Refractive Surgery*. 2012;28(11):753–758. doi:10.3928/1081597X-20121011-01.
31. Koivisto O, Hanel A, Carlberg C. Key Vitamin D Target Genes with Functions in the Immune System. *Nutrients*. 2020;12(4):1140. doi:10.3390/NU12041140.
32. Auton A, Abecasis GR, Altshuler DM, et al. A global reference for human genetic variation. *Nature*. 2015;526(7571):68. doi:10.1038/NATURE15393.
33. Giannini S, Giusti A, Minisola S, et al. The Immunologic Profile of Vitamin D and Its Role in Different Immune-Mediated Diseases: An Expert Opinion. *Nutrients*. 2022;14(3):473. doi:10.3390/NU14030473/S1.
34. Balta S, Ozturk C. The platelet-lymphocyte ratio: A simple, inexpensive and rapid prognostic marker for cardiovascular events. *Platelets*. 2015;26(7):680–681. doi:10.3109/09537104.2014.979340.
35. Pearson AR, Soneji B, Sarvananthan N, Sanford-Smith JH. Does ethnic origin influence the incidence or severity of keratoconus? *Eye (Lond)*. 2000;14(Pt 4):625–628 4. doi:10.1038/EYE.2000.154.
36. Gordon-Shaag A, Millodot M, Shneor E, Liu Y. The Genetic and Environmental Factors for Keratoconus. *Biomed Res Int*. 2015;2015. doi:10.1155/2015/795738.
37. Tang YG, Picornell Y, Su X, Li X, Yang H, Rabinowitz YS. Three VSX1 gene mutations, L159M, R166W, and H244R, are not associated with keratoconus. *Cornea*. 2008;27(2):189–192. doi:10.1097/ICO.0B013E31815A50E7.
38. Barbaro V, Di Iorio E, Ferrari S, et al. Expression of VSX1 in human corneal keratocytes during differentiation into myofibroblasts in response to wound healing. *Invest Ophthalmol Vis Sci*. 2006;47(12):5243–5250. doi:10.1167/IOVS.06-0185.
39. Aldave AJ, Yellore VS, Salem AK, et al. No VSX1 Gene Mutations Associated with Keratoconus. *Invest Ophthalmol Vis Sci*. 2006;47(7):2820–2822. doi:10.1167/IOVS.05-1530.
40. Štabuc-Šilih M, Stražišar M, Hawlina M, Glavač D. Absence of pathogenic mutations in VSX1 and SOD1 genes in patients with keratoconus. *Cornea*. 2010;29(2):172–176. doi:10.1097/ICO.0B013E3181AEBF7A.
41. Liskova P, Ebenezer ND, Hysi P, et al. Molecular analysis of the VSX1 gene in familial keratoconus. *Mol Vis*. 2007;13:1887 Accessed October 6, 2024. /pmc/articles/PMC5466152/.
42. Stabuc-Silih M, Ravnik-Glavac M, Glavac D, Hawlina M, Strazisar M. Polymorphisms in COL4A3 and COL4A4 genes associated with keratoconus. *Mol Vis*. 2009;15:2848 Accessed October 6, 2024. /pmc/articles/PMC2796875/.
43. Kokolakis NS, Gazouli M, Chatziralli IP, et al. Polymorphism analysis of COL4A3 and COL4A4 genes in Greek patients with keratoconus. *Ophthalmic Genet*. 2014;35(4):226–228. doi:10.3109/13816810.2014.946055.
44. Lu Y, Vitart V, Burdon KP, et al. Genome-wide association analyses identify multiple loci associated with central corneal thickness and keratoconus. *Nat Genet*. 2013;45(2):155–163. doi:10.1038/NG.2506.
45. Hughes AE, Bradley DT, Campbell M, et al. Mutation altering the miR-184 seed region causes familial keratoconus with cataract. *Am J Hum Genet*. 2011;89(5):628–633. doi:10.1016/J.AJHG.2011.09.014.
46. Blackberg SN, Knapp AA. The influence of the vitamin-D-Calcium-phosphorus complex in the production of ocular pathology. 1. A histological study of the changes in the fibrous tunic. *Am J Ophthalmol*. 1937;20(4):405–407. doi:10.1016/S0002-9394(37)91477-6.
47. Patel D V, Ku JYF, Johnson R, McGhee CNJ. Laser scanning in vivo confocal microscopy and quantitative aesthesiometry reveal decreased corneal innervation and sensation in keratoconus. *Eye (Lond)*. 2009;23(3):586–592. doi:10.1038/EYE.2008.52.
48. Gorimanipalli B, Shetty R, Sethu S, Khamar P. Vitamin D and eye: Current evidence and practice guidelines. *Indian J Ophthalmol*. 2023;71(4):1127–1134. doi:10.4103/IJO.IJO_3174_22.
49. Akkaya S, Ulusoy DM. Serum Vitamin D Levels in Patients with Keratoconus. *Ocul Immunol Inflamm*. 2020;28(3):348–353. doi:10.1080/09273948.2019.1604002.
50. Aslan MG, Findik H, Okutucu M, et al. Serum 25-Hydroxy Vitamin D, Vitamin B12, and Folic Acid Levels in Progressive and Nonprogressive Keratoconus. *Cornea*. 2021;40(3):334–341. doi:10.1097/ICO.0000000000002475.
51. Anitha V, Vanathi M, Raghavan A, Rajaraman R, Ravindran M, Tandon R. Pediatric keratoconus - Current perspectives and clinical challenges. *Indian J Ophthalmol*. 2021;69(2):214. doi:10.4103/IJO.IJO_1263_20.
52. Haussler MR, Haussler CA, Jurutka PW, et al. The vitamin D hormone and its nuclear receptor: molecular actions and dis-

ease states. *Journal of Endocrinology*. 1997;154(3_Suppl):S57–S73. doi:10.1677/JOE.0.154S057.
53. Lin R, White JH. The pleiotropic actions of vitamin D. *BioEssays*. 2004;26(1):21–28. doi:10.1002/BIES.10368.
54. Żmijewski MA. Nongenomic Activities of Vitamin D. *Nutrients*. 2022;14(23). doi:10.3390/NU14235104.
55. Lema I, Durán JA. Inflammatory molecules in the tears of patients with keratoconus. *Ophthalmology*. 2005;112(4):654–659. doi:10.1016/J.OPHTHA.2004.11.050.
56. Jun AS, Cope L, Speck C, et al. Subnormal Cytokine Profile in the Tear Fluid of Keratoconus Patients. *PLoS One*. 2011;6(1):e16437. doi:10.1371/JOURNAL.PONE.0016437.
57. Antunes-Foschini R, Doná H, de Mello PHS, et al. Natural history and predictors for progression in pediatric keratoconus. *Scientific Reports*. 2023;13(1):1–10 *2023 13:1*. doi:10.1038/s41598-023-32176-5.
58. Elbeyli A, Kurtul B. Systemic immune-inflammation index, neutrophil-to-lymphocyte ratio, and platelet-to-lymphocyte ratio levels are associated with keratoconus. *Indian J Ophthalmol*. 2021;69(7):1725. doi:10.4103/IJO.IJO_3011_20.
59. El-Massry A, Doheim MF, Iqbal M, et al. Association between keratoconus and thyroid gland dysfunction: A cross-sectional case-control study. *Journal of Refractive Surgery*. 2020;36(4):253–257. doi:10.3928/1081597X-20200226-03.
60. AlHawari HH, Khader YS, AlHawari HH, et al. Autoimmune Thyroid Disease and Keratoconus: Is There an Association? *Int J Endocrinol*. 2018;2018(1):7907512. doi:10.1155/2018/7907512.
61. Manicone AM, McGuire JK. Matrix Metalloproteinases as Modulators of Inflammation. *Semin Cell Dev Biol*. 2007;19(1):34. doi:10.1016/J.SEMCDB.2007.07.003.
62. Kossakowska AE, Edwards DR, Prusinkiewicz C, et al. Interleukin-6 Regulation of Matrix Metalloproteinase (MMP-2 and MMP-9) and Tissue Inhibitor of Metalloproteinase (TIMP-1) Expression in Malignant Non-Hodgkin's Lymphomas. *Blood*. 1999;94(6):2080–2089. doi:10.1182/BLOOD.V94.6.2080.
63. Hidaka M, Wakabayashi I, Takeda Y, Fukuzawa K. Vitamin D3 derivatives increase soluble CD14 release through ERK1/2 activation and decrease IL-8 production in intestinal epithelial cells. *Eur J Pharmacol*. 2013;721(1-3):305–312. doi:10.1016/J.EJPHAR.2013.09.014.
64. Park YC, Kim J, Seo MS, Hong SW, Cho ES, Kim JK. Inverse relationship between vitamin D levels and platelet indices in Korean adults. *Hematology*. 2017;22(10):623–629. doi:10.1080/10245332.2017.1318334.
65. Alanli R, Küçükay MB, Yalçin KS. Relationship between vitamin D levels and platelet count: A retrospective study. *Gulhane Medical Journal*. 2020;62(3):174–178. doi:10.4274/GULHANE.GALENOS.2020.762.
66. Kucukay MB, Alanli R. Vitamin D Replacement Effect on Platelet Counts. *J Coll Physicians Surg Pak*. 2021;31(9):1064–1068. doi:10.29271/JCPSP.2021.09.1064.
67. Aihara KI, Azuma H, Akaike M, et al. Disruption of nuclear vitamin D receptor gene causes enhanced thrombogenicity in mice. *J Biol Chem*. 2004;279(34):35798–35802. doi:10.1074/JBC.M404865200.
68. D'Amelio P, Cristofaro MA, De Vivo E, et al. Platelet vitamin D receptor is reduced in osteoporotic patients. *Panminerva Med*. 2016;54(3):225–231. Accessed October 6, 2024. https://iris.unito.it/handle/2318/121424 .
69. Mitchell AJ, Gray WD, Hayek SS, et al. Platelets confound the measurement of extracellular miRNA in archived plasma. *Scientific Reports*. 2016;6(1):1–11 *2016 6:1*. doi:10.1038/srep32651.
70. Stokes KY, Granger DN. Platelets: a critical link between inflammation and microvascular dysfunction. *J Physiol*. 2012;590(Pt 5):1023. doi:10.1113/JPHYSIOL.2011.225417.
71. Chen Y, Zhong H, Zhao Y, Luo X, Gao W. Role of platelet biomarkers in inflammatory response. *Biomark Res*. 2020;8(1):1–7. doi:10.1186/S40364-020-00207-2/FIGURES/1.
72. Würtz M, Hvas AM, Kristensen SD, Grove EL. Platelet aggregation is dependent on platelet count in patients with coronary artery disease. *Thromb Res*. 2012;129(1):56–61. doi:10.1016/J.THROMRES.2011.08.019.
73. Yen JH, Kocieda VP, Jing H, Ganea D. Prostaglandin E2 Induces Matrix Metalloproteinase 9 Expression in Dendritic Cells through Two Independent Signaling Pathways Leading to Activator Protein 1 (AP-1) Activation. *Journal of Biological Chemistry*. 2011;286(45):38913–38923. doi:10.1074/JBC.M111.252932.
74. Hu K, Yang J, Tanaka S, Gonias SL, Mars WM, Liu Y. Tissue-type plasminogen activator acts as a cytokine that triggers intracellular signal transduction and induces matrix metalloproteinase-9 gene expression. *Journal of Biological Chemistry*. 2006;281(4):2120–2127. doi:10.1074/JBC.M504988200.
75. Puri S, Coulson-Thomas YM, Gesteira TF, Coulson-Thomas VJ. Distribution and Function of Glycosaminoglycans and Proteoglycans in the Development, Homeostasis and Pathology of the Ocular Surface. *Front Cell Dev Biol*. 2020;8:557884. doi:10.3389/FCELL.2020.00731/BIBTEX.
76. Meek KM, Tuft SJ, Huang Y, et al. Changes in Collagen Orientation and Distribution in Keratoconus Corneas. *Invest Ophthalmol Vis Sci*. 2005;46(6):1948–1956. doi:10.1167/IOVS.04-1253.
77. García B, García-Suárez O, Merayo-Lloves J, et al. Differential Expression of Proteoglycans by Corneal Stromal Cells in Keratoconus. *Invest Ophthalmol Vis Sci*. 2016;57(6):2618–2628. doi:10.1167/IOVS.15-16692.