

Cigarette smoke exposure is associated with vitamin D3 deficiencies in patients with chronic rhinosinusitis

Jennifer K. Mulligan, PhD,^{a,b,c} Whitney Nagel, BS,^a Brendan P. O'Connell, MD,^a Jennifer Wentzel, BS,^a Carl Atkinson, PhD,^d and Rodney J. Schlosser, MD^{a,c} Charleston, SC

Background: Cigarette smoke (CS) plays a role in the exacerbation of chronic rhinosinusitis (CRS); however, the mechanism for this is unknown. We hypothesize that CS impairs human sinonasal epithelial cell (HSNEC) conversion of 25(OH) D3 (25VD3) to 1,25-dihydroxyvitamin D3 (1,25VD3) and, furthermore, that supplementation with 1,25VD3 will reverse smoke-induced inflammatory responses by HSNECs.

Objective: We sought to determine the effect of CS on vitamin D3 (VD3) levels, conversion, and regulation of CS-induced inflammation in control subjects and patients with CRS.

Methods: Blood and sinus tissue explants were collected at the time of surgery from control subjects, patients with chronic rhinosinusitis without nasal polyps, and patients with chronic sinusitis with nasal polyps (CRSwNP). Expression of VD3 metabolizing enzymes were measured by using RT-PCR.

Primary HSNECs were cultured from tissue explants. 25VD3 with and without cigarette smoke extract (CSE) was used to examine conversion of 25VD3 to 1,25VD3, as well as HSNEC production of proinflammatory cytokines.

Results: CS exposure was associated with reduced circulating and sinonasal 25VD3 levels in all groups compared with those seen in CS-naïve, disease-matched counterparts. CS exposure decreased expression of *CYP27B1* and was especially pronounced in patients with CRSwNP. CSE impairs control HSNEC conversion of 25VD3. HSNECs from patients with CRSwNP also demonstrate an intrinsic reduction in conversion of 25VD3 to 1,25VD3. Exogenous 1,25VD3 reduces CSE-induced cytokine production by HSNECs.

Conclusions: Exposure to CS is associated with reduced 25VD3 levels and an impaired ability of HSNECs to convert 25VD3 to 1,25VD3. Addition of 1,25VD3 reduces the proinflammatory effects of CS on HSNECs. Impaired VD3 conversion by CS

exposure represents a novel mechanism through which CS induces its proinflammatory effects. (*J Allergy Clin Immunol* 2014;■■■:■■■-■■■.)

Key words: Vitamin D, secondhand smoke, sinusitis, epithelial cell

Chronic rhinosinusitis (CRS) is a health condition characterized by diffuse inflammation of the sinonasal mucosa and is composed of a variety of clinical phenotypes. Patients with chronic rhinosinusitis without nasal polyps (CRSsNP) have increased levels of T_H1 and T_H2 mediators, resulting in a mixed neutrophilic and eosinophilic infiltrate.¹⁻³ Chronic rhinosinusitis with nasal polyps (CRSwNP) is the most difficult form of the disease to treat and displays a T_H2-skewed immune phenotype similar to that of asthmatic patients, including increased mast cell and eosinophil numbers.^{4,5} Although the exact cause of the inflammation associated with CRS is unknown, it is thought to be the result of numerous interactions between environmental factors and intrinsic alterations in the host immune system.

Active smoking and environmental tobacco smoke (ETS) exposure have been shown to cause or exacerbate numerous diseases of the upper and lower airways, including CRS.⁶⁻⁹ Exposure to ETS during childhood and adulthood has been identified as a risk factor for the development of a number of respiratory diseases, including CRS.¹⁰⁻¹³ In the upper airway, cigarette smoke (CS) has been shown to induce nasal irritation and congestion, as well as increased nasal obstruction. Lastly, *in vitro* studies have shown that cigarette smoke extract (CSE)-treated human sinonasal epithelial cells (HSNECs) produce a robust inflammatory response demonstrated by a significant increase in proinflammatory cytokine levels.^{14,15} Although CS-induced respiratory inflammation is becoming more widely accepted, the mechanism through which CS exacerbates CRS is unclear.

Vitamin D3 (VD3) is a potent steroid hormone involved in the regulation of calcium homeostasis; antibacterial products, such as cathelicidin; and inflammatory cell processes. VD3 regulates a variety of cell types, including epithelial cells, dendritic cells (DCs), monocytes, macrophages, and T cells, and has been shown to promote immune tolerance in a number of contexts.¹⁶⁻²⁰ For example, it has been demonstrated that 1 α ,25-dihydroxyvitamin D3 (1,25VD3) diminishes DC stimulation of T_H1/T_H2 differentiation by blocking monocyte to DC differentiation and maturation^{21,22} and also recruits regulatory T cells, which produce the anti-inflammatory cytokine IL-10.^{23,24}

VD3 synthesis begins in the skin, where pro-VD3 is converted to pre-VD3. After binding to vitamin D binding protein, it is transported to the liver and converted to 25-hydroxycholecalciferol (25VD3).²⁵ Originally it was thought that conversion of 25VD3 to the active metabolite, 1,25VD3, by 1 α -hydroxylase occurred in the kidneys.²⁶ However, a number

From the Departments of ^aOtolaryngology-Head & Neck Surgery, ^bPediatrics, and ^dMicrobiology and Immunology, Medical University of South Carolina, and ^cthe Ralph H. Johnson VA Medical Center.

Supported by grants from the Flight Attendant Medical Research Institute (092401 to J.K.M. and 113039 to R.J.S.), the National Institutes of Health (R01HL091944 to C.A.), and a grant from the Department of Veterans Affairs.

Disclosure of potential conflict of interest: J. K. Mulligan has received research support from the Flight Attendant Medical Research Institute, the US Department of Veterans Affairs, and the National Institutes of Health. R. J. Schlosser has received research support from the Flight Attendant Medical Research Institute, the US Department of Veterans Affairs, NeilMed, ArthroCare, Intersect, and Optinose; has consultant arrangements with BrainLAB, Olympus, and United Allergy; has received payment for lectures from NeilMed; and has a patent pending for vitamin D use in respiratory tract disorders. The rest of the authors declare that they have no relevant conflicts of interest.

Received for publication May 1, 2013; revised January 10, 2014; accepted for publication January 22, 2014.

Corresponding author: Jennifer K. Mulligan, PhD, 135 Rutledge Ave, MSC 550, Charleston, SC 29425. E-mail: konopa@musc.edu.

0091-6749/\$36.00

© 2014 American Academy of Allergy, Asthma & Immunology

<http://dx.doi.org/10.1016/j.jaci.2014.01.039>

Abbreviations used

BEGM:	Basal epithelial cell growth medium
CRS:	Chronic rhinosinusitis
CRSsNP:	Chronic rhinosinusitis without nasal polyps
CRSwNP:	Chronic rhinosinusitis with nasal polyps
CS:	Cigarette smoke
CSE:	Cigarette smoke extract
C _t :	Cycle threshold
DC:	Dendritic cell
ETS:	Environmental tobacco smoke
HSNEC:	Human sinonasal epithelial cell
VD3:	Vitamin D3
1,25VD3:	1 α ,25-Dihydroxyvitamin D3
25VD3:	25-Hydroxycholecalciferol
VDR:	Vitamin D receptor

of reports have shown that other peripheral tissues, including respiratory epithelial cells, contain 1 α -hydroxylase²⁷ and can serve as a source of 1,25VD3.

Increasing evidence suggests that VD3 plays an important role in respiratory health. For instance, higher 25VD3 levels were associated with reduced likelihood for being hospitalized for asthma-related complications and reduced use of anti-inflammatory medications.²⁸ Serum 25VD3 levels have been shown to be inversely associated with the occurrence of upper respiratory tract infections, and this association was even stronger in those with asthma or chronic obstructive pulmonary disease.²⁹ In the upper airway we have previously reported that patients with CRSwNP have systemic 25VD3 deficiencies that are independent of age, race, sex, and asthma. Additionally, VD3 deficiency was associated with more severe bone erosion and increased DC infiltrates in patients with CRSwNP.^{30,31} However, what is still unidentified is whether intrinsic changes in local VD3 regulation are associated with CRS or whether extrinsic insults, such as CS, alter VD3 regulation. In these studies, we explored the relationship between CS exposure and regulation of VD3 metabolism in patient with CRS. As we report here, CS exposure is associated with reduced circulating and tissue levels of 25VD3 and impaired VD3 conversion of 25VD3 to 1,25VD3. Furthermore, addition of exogenous 1,25VD3 reverses smoke-induced inflammation by HSNECs.

METHODS**Patients**

The Institutional Review Board at the Medical University of South Carolina approved these studies, and written informed consent was obtained for all patients. Blood, hair, and sinus tissue were taken at the time of endoscopic sinus surgery. Inclusion criteria include patients with CRSsNP and those with CRSwNP who met the diagnostic criteria outlined by the European Position Paper on Rhinosinusitis and Nasal Polyps 2012.³² Control subjects were undergoing surgery for cerebrospinal fluid leak repair or to remove non-hormone-secreting pituitary tumors. Disease severity was assessed by using the Lund-Mackay radiologic staging system³³ for patients with available computed tomographic scans. Scores were assigned by 2 blinded graders and then averaged to yield a mean score for each patient.

Exclusion criteria include use of oral steroids or immunomodulatory agents within the preceding 30 days; use of vitamin D supplements other than standard multivitamins; other immunologic (eg, rheumatoid arthritis, immunodeficiency, cystic fibrosis, ciliary dyskinesia, and malabsorption), renal, gastrointestinal, endocrine, or skeletal disorders; and pregnancy.

Smoking status determination

CS-exposed patients included active smokers and those with ETS exposure. Self-reported smoking status was obtained at the time of surgery and recorded in the preoperative medical records. Hair samples were analyzed for nicotine at the Wellington Hospital Laboratory in Newton, New Zealand, by using methods previously described.³⁴ One centimeter of hair provides a mean exposure over the previous month and is less vulnerable to day-to-day fluctuations in environmental exposures than other measures, such as urinary or serum cotinine. Patients were classified as (1) smoke naive (ie, those without ETS exposure and hair nicotine <2 ng/mg); (2) ETS exposed (ie, those with a hair nicotine level of 2–30 ng/mg); or (3) active smokers (ie, those with hair nicotine levels of >30 ng/mg, those who admitted to actively smoking, or both).³⁵ Admitted former smokers were excluded.

Tissue processing and HSNEC line establishment

Sinus tissue explants were returned immediately to the laboratory for processing on ice. Samples to be used for RT-PCR and tissue homogenate analysis were snap-frozen and stored in RNAlater at -80°C until use. Tissue lysates were prepared, as described previously.³⁶ The total protein concentration of each sample was determined by using a bicinchoninic acid assay (Thermo Scientific, Waltham, Mass).

Samples used to generate primary HSNEC lines were processed, as previously described.³⁷ Briefly, tissue explants were minced with a scalpel and then digested in Pronase (Sigma-Aldrich, St Louis, Mo) for 90 minutes at 37°C . Cell suspensions were then passed over a $70\text{-}\mu\text{m}$ cell strainer and washed in PBS. Cells were plated for 2 hours on standard tissue culture-treated plates to remove contaminating fibroblasts that adhere faster than HSNECs and then cultured with basal epithelial cell growth medium (BEGM). Cells were expanded for 5 to 7 days on collagen IV-coated 75-cm^2 dishes. Subconfluent HSNECs were trypsinized and reseeded on human collagen type IV-coated, 6-well tissue-culture dishes. Cell purity was confirmed by means of flow cytometry with positive immunostaining for the epithelial cell marker MUC1 (>95%) and negative immunostaining (<2%) for the fibroblast marker FSP. All cells were used at passage 2.

Blood processing

Peripheral blood was collected at the time of intravenous line placement and processed, as previously described.³⁰ Briefly, blood was collected in vacutainers containing K2EDTA. Blood was centrifuged at 500g, and then plasma was collected and stored at -80°C .

RNA isolation and RT-PCR analysis

An RNeasy Plus Kit (Qiagen, Hilden, Germany) was used to isolate RNA and eliminate genomic DNA. All tissue was processed according to the manufacturer's instructions. RT-PCR analysis was conducted by using the RT² Profiler Array System (SABiosciences, Valencia, Calif) for the following genes: *GC* (VD3 binding protein), *CYP2R1* (vitamin D 25-hydroxylase), vitamin D receptor (*VDR*), and *CYP27B1* (1- α hydroxylase). Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used for data normalization. Execution of the array was conducted with a Mastercycler ep realplex PCR system (Eppendorf AG, Hauppauge, NY). For RT-PCR analysis, ΔC_t values from 5 control subjects were averaged together and then used to calculate $\Delta\Delta C_t$ values for individual noncontrol patients. Fold change was calculated from $2^{(-\Delta\Delta C_t)}$. Fold change values of less than 1 were then calculated by using the negative inverse and reported as fold downregulation.

Measurement of VD3 levels

Plasma and tissue 25VD3 and 1,25VD3 levels were measured by using an enzyme immunoassay (Immunodiagnostic Systems, Fountain Hills, Ariz), according to the manufacturer's instructions. 25VD3 insufficiency was defined as less than 32 ng/mL.^{38–40} Assay detection limits were 2.4 to 200 pg/mL and 5 to 152 ng/mL for 1,25VD3 and 25VD3, respectively.

CYP27B1 analysis in tissue explants and HSNECs by means of immunostaining

Single-cell suspension of sinonasal tissue explants was prepared, as previously described.⁴¹ FcγII/III receptors were blocked with Human FC Block (eBioscience, San Diego, Calif) and 10% BSA for 5 minutes. HSNECs were then incubated with the Fixation/Permeabilization kit from eBioscience, blocked again with Human FC Block, and stained with the following antibodies at the concentrations recommended by the manufacturer: anti-CYP27B1 Antibody (Abgent, San Diego, Calif) and F(ab')₂ Donkey Anti-Rabbit IgG PE (eBioscience). The cells were analyzed with a Guava 8HT flow and analyzed with FCS Express 4.0. Dead cells (7-aminoactinomycin D positive) were excluded from the final data analysis.

HSNEC treatments

HSNECs were treated with vehicle (BEGM) or indicated doses of 25VD3 or 1,25VD3 diluted in BEGM. 25VD3 dosing was based on previous reports for conversion of bronchial epithelial cells.²⁷ CSE was generated by bubbling the CS from two 3R4F research-grade cigarettes through 25 mL of BEGM with a smoking apparatus.⁴² CSE was passed through a 0.22-μm filter and then diluted in BEGM to a final concentration of 10% similar to previously described results.⁴³ For HSNEC treatments with CSE, CSE was added to media containing 25VD3 or 1,25VD3 and immediately added to cells and allowed to incubate concurrently for 24 hours. Twenty-four hours after initial treatment, supernatants were collected and immediately assessed for the presence of 1,25VD3. Protein levels for IL-6, IL-8 (BD Biosciences, San Jose, Calif), and CCL20 (R&D Systems, Minneapolis, Minn) were measured by using ELISA. All doses of CSE and 1,25VD3 used were confirmed not to induce cell death, as determined by using the MTS Cell Viability Assay (Promega, Madison, Wis).

Statistical analysis

Statistical analysis was conducted with GraphPad Prism 6.0 software (GraphPad Software, La Jolla, Calif). A D'Agostino and Pearson omnibus test was used to determine whether data sets were normally distributed. For data in which all groups were normally distributed, a 1-way ANOVA with the *post hoc* unpaired Student *t* test was used. For analysis in which all data sets were not normally distributed (including RT-PCR analysis), a Mann-Whitney test was used to determine statistically significant differences between indicated groups.

Spearman correlation analysis was used to determine whether a significant correlation existed between patient-matched tissue and plasma 25VD3 levels.

RESULTS

CS exposure is associated with decreased circulating and sinonasal tissue 25VD3 levels

To determine the effect of CS on systemic VD3 levels, we measured plasma 25VD3 levels in smoke-naïve and CS-exposed control subjects, patients with CRSsNP, and patients with CRSwNP (demographics are summarized in Table I). Consistent with our previous reports,^{30,31} patients with CRSwNP were VD3 deficient (Fig 1, A) and had reduced plasma 25VD3 levels compared with those in control subjects ($P = .0001$) or patients with CRSsNP ($P < .0001$). Exposure to CS was associated with decreased 25VD3 levels when comparing smoke-naïve with CS-exposed patients in all 3 diagnostic groups. There was no significant difference in 25VD3 levels between ETS-exposed versus active smokers in any of the diagnostic groups (data not shown).

We next examined sinonasal tissue lysates for 25VD3 levels to determine whether systemic measures of 25VD3 were reflective of local tissue levels. Similar to plasma results, we observed

CRSwNP lysates had reduced 25VD3 levels compared with those seen in control subjects ($P = .0145$) and patients with CRSsNP ($P = .0381$; Fig 1, B). In all groups examined, tissue 25VD3 levels were reduced in those with CS exposure compared with their smoke-naïve, cohort-matched counterparts. Lastly, a positive correlation was observed between plasma and tissue 25VD3 levels (Fig 1, C). Together, these results demonstrate that CS exposure is associated with reduced circulating and tissue 25VD3 levels in all patient groups and that CS exposure exacerbates existing 25VD3 deficiencies in patients with CRSwNP.

CS exposure is associated with downregulation of select VD3 genes in the sinus mucosa

Next, we examined the effect of CS exposure on sinonasal expression of a number of VD3 regulatory genes using RT-PCR. *GC*, *CYP2R*, and *VDR* expression did not yield any changes greater than 2-fold when examining the effect of either disease or CS exposure (data not shown). However, *CYP27B1* demonstrated differences in gene expression based on both disease status and CS exposure.

CYP27B1 is the gene that codes for 25-hydroxyvitamin D3 1-α-hydroxylase, which is responsible for the conversion of 25VD3 to 1,25VD3. When examining the effect of disease state, smoke-naïve patients with CRSsNP had a 2.0-fold downregulation in *CYP27B1* expression compared to control. Smoke-naïve patients with CRSwNP had an even greater 4.583-fold downregulation of *CYP27B1* expression, which was significantly lower than that seen in patients with CRSsNP ($P = .04$, Fig 2). When examining the effect of CS exposure, this was associated with downregulation of *CYP27B1* expression in all diagnostic groups. The control group with CS exposure and the patients with CRSsNP with CS exposure were associated with downregulation of *CYP27B1* expression of -3.63 -fold and -3.87 -fold, respectively. In patients with CRSwNP, CS exposure was associated with a downregulation of *CYP27B1* expression by -9.83 -fold. This expression was greater than that seen in smoke-naïve patients with CRSwNP ($P = .025$). *Ex vivo* analysis of *CYP27B1* sinonasal tissue explant protein levels also demonstrated that *CYP27B1* expression was downregulated in patients with CRSwNP compared with that seen in control subjects ($P = .02$) or patients with CRSsNP ($P = .009$; see Fig E1, A, in this article's Online Repository at www.jacionline.org). Furthermore, a significant downregulation of epithelial cell-specific *CYP27B1* expression was observed in sinonasal epithelial cells from patients with CRSwNP compared with those from control subjects ($P = .02$) or patients with CRSsNP ($P = .03$; see Fig E1, B).

Epithelial cell conversion of 25VD3 to 1,25VD3 is reduced in patients with CRSwNP and in the presence of CS

Next, we investigated the influence of CS on HSNEC metabolism of 25VD3. Primary HSNECs from smoke-naïve control subjects were treated with varying doses of 25VD3 with or without 10% CSE for 24 hours. In cells treated with vehicle control (BEGM) or 10% CSE alone, less than 1.5 pg/mL 1,25VD3 was detected (Fig 3, A). A dose-dependent increase in 1,25VD3 levels was measured in supernatants from cells treated with

TABLE I. Demographics for patients shown in Fig 1

	No.	25VD3 (ng/mL)	Race	Sex (% male)	Age (y)	Computed tomographic score	ETS (%); active (%)
Plasma 25VD3							
Control group	12	46.6 ± 16.18	W, 8; AA, 3; H, 0; O, 1	67	48 ± 19	2 ± 3	0; 0
CRSsNP group	14	47.55 ± 17.93	C, 13; AA, 0; H, 1; O, 0	36	49 ± 20	5 ± 4	0; 0
CRSsNP group	30	27.53 ± 12.04	C, 21; AA, 6; H, 0; O, 3	70	38 ± 17	16 ± 6	0; 0
Control-CS group	9	25.32 ± 20.99	C, 4; AA, 5; H, 0; O, 0	33	44 ± 13	1 ± 1	22; 78
CRSsNP-CS group	26	27.54 ± 14.52	C, 25; AA, 1; H, 0; O, 0	54	38 ± 17	6 ± 4	46; 54
CRSsNP-CS group	15	18.93 ± 9.16	C, 7; AA, 8; H, 0; O, 0	67	39 ± 23	10 ± 4	53; 47
Lysate 25VD3							
Control group	7	7.13 ± 2.58	C, 5; AA, 1; H, 0; O, 1	71	47 ± 20	NA	0; 0
CRSsNP group	8	6.69 ± 2.60	C, 8; AA, 0; H, 0; O, 0	25	60 ± 11	3 ± 2	0; 0
CRSsNP group	14	4.80 ± 1.44	C, 9; AA, 5; H, 0; O, 0	79	41 ± 22	17 ± 9	0; 0
Control-CS group	4	2.03 ± 0.66	C, 3; AA, 1; H, 0; O, 0	50	45 ± 20	0 ± 0	50; 50
CRSsNP-CS group	10	2.43 ± 2.29	C, 10; AA, 0; H, 0; O, 0	50	48 ± 15	9 ± 4	60; 40
CRSsNP-CS group	15	2.36 ± 1.05	C, 6; AA, 9; H, 0; O, 0	60	34 ± 22	11 ± 5	47; 53

Values are averages ± SDs.

AA, African American; CS, cigarette smoke exposure (active + ETS); ETS, environmental tobacco smoke exposure; H, Hispanic; NA, not applicable; O, other; W, white.

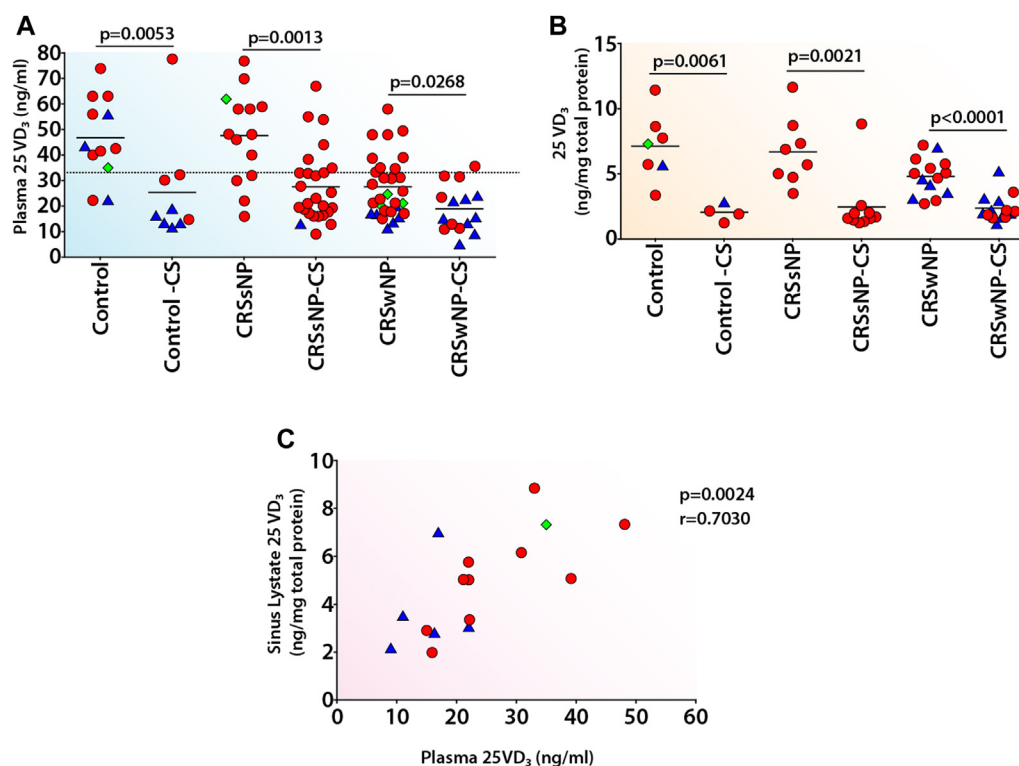


FIG 1. Circulating and tissue levels of 25VD3 are reduced with CS exposure. **A** and **B**, CS exposure is associated with lower 25VD3 levels in plasma (Fig 1, **A**) and sinonasal tissues (Fig 1, **B**). Statistics shown are for the Mann-Whitney test between indicated diagnostic groups. **C**, Pearson correlation analysis with patient-matched plasma and sinonasal tissue 25VD3 levels. ○, White; ▲, African American; ♦, other.

25VD3 ($P < .05$ for each dose vs control). Addition of CSE reduced detectable levels of 1,25VD3, suggesting that CSE impaired HSNEC conversion of 25VD3 to 1,25VD3.

Given the disease-specific variations in *CYP27B1* in the absence of CS exposure (Fig 2), we then investigated the ability of HSNECs from various CRS groups to convert VD3 (Fig 3, **B**). All patient groups demonstrated conversion of 25VD3 to 1,25VD3 ($P < .007$ for each diseased-matched comparison). However, HSNECs from patients with CRSsNP demonstrated an impaired ability to convert 25VD3 and had

significantly lower 1,25VD3 levels compared with those seen in control subjects ($P = .02$) or patients with CRSsNP ($P = .03$), demonstrating intrinsic disease-specific impairment of 25VD3 metabolism. Exposure of HSNECs from different patient cohorts to CSE resulted in significantly reduced detection of 1,25VD3 in all 3 patient groups compared with that seen in vehicle-treated samples. Further analysis of HSNEC *CYP27B1* expression supported this observation, demonstrating reduced expression of *CYP27B1* after CSE exposure (Fig 4).

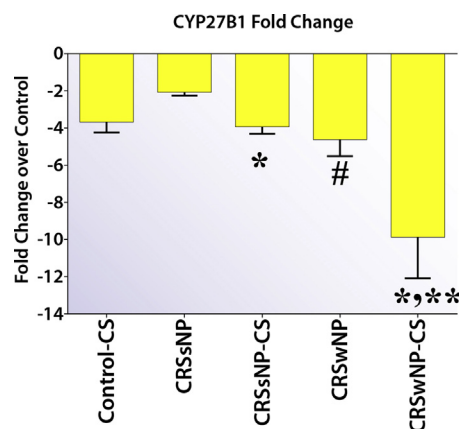


FIG 2. Sinonasal tissue expression of *CYP27B1* and CS exposure. RT-PCR analysis of sinonasal mucosa for *CYP27B1* was performed. * $P < .05$ versus smoke-naïve, disease-matched subjects. # $P < .05$ versus the CRSsNP group. ** $P < .05$ versus the control-CS or CRSsNP-CS groups. Control group, $n = 5$; CRSsNP group, $n = 6$; CRSwNP group, $n = 6$; Control-CS group, $n = 5$; CRSsNP-CS group, $n = 8$; CRSwNP-CS group, $n = 10$.

An alternative mechanism that was explored was whether CSE was directly degrading 25VD3 or 1,25VD3. To test this, 50 pg/mL of either 1,25VD3 or 25VD3 was added to FBS-supplemented BEGM with or without 10% CSE. Both 25VD3 and 1,25VD3 components were measured by using ELISA, and there was no significant difference between samples with or without CSE (data not shown). This supports that the mechanism of smoke-impaired local 25VD3 metabolism is the result of failed HSNEC conversion and not by degradation of 25VD3 or 1,25VD3 directly by CSE.

1,25VD3 blocks CSE-induced proinflammatory cytokine production by HSNECs

Given our findings that smoke impairs 25VD3 conversion to 1,25VD3, we tested the ability of exogenous 1,25VD3 to inhibit CSE-induced cytokine production by HSNECs. Control HSNECs were treated concurrently with either CSE, 50 pg/mL 1,25VD3, or a combination. As shown in Fig 5, CSE induced a significant increase in IL-6, IL-8, and CCL20 production versus that seen in control subjects ($P < .01$ for each analyte). Exogenous 1,25VD3 inhibited CSE-induced changes in all measured analytes compared with CSE-treated control subjects ($P < .008$ vs CSE treatment). Collectively, these results suggest that addition of exogenous 1,25VD3 can reduce the proinflammatory effects of CSE on HSNECs.

DISCUSSION

In this study we examined the effect of CS on systemic 25VD3 levels and its local metabolism in control subjects and patients with CRS. Previously, we reported that patients with CRSwNP were systemically VD3 deficient, as determined by using ELISA and liquid chromatography/tandem mass spectrometry^{30,31}; comparison of these results found no statistically significant difference between data obtained with either method. Here we determined that CS exposure exacerbates local and systemic VD3 deficiencies in patients with CRSwNP. These effects were not limited to patients with CRSwNP because control subjects

and patients with CRSsNP who were CS exposed had reduced 25VD3 levels systemically and locally as well. We also examined the effect of CS on sinonasal tissue expression of a number of VD3 regulatory genes. Although CRSwNP was associated with a downregulation of *CYP27B1* expression even in smoke-naïve patients, CS exposure was associated with an increased downregulation of *CYP27B1* gene expression in all diagnostic groups. A significant portion of our focus centered around the effect of CSE on HSNEC regulation of VD3. Both CRSwNP and exposure to CSE were found to result in reduced conversion of VD3 by HSNECs. Lastly, addition of 1,25VD3 to HSNECs was able to rescue CSE-induced inflammation.

CS has been shown to have a potent effect on airway epithelial cell functions. A number of reports have shown that CSE induces the production of a large number of proinflammatory factors, including complement components IL-6, IL-8, and a broad range of chemokines.^{15,43-46} Although the inflammatory effects of smoke are well known, as is the anti-inflammatory nature of VD3, the relationship between these 2 in patients with CRS has been largely unreported. Therefore we explored the ability of exogenous 1,25VD3 to overcome inflammation induced by CSE. Here we report the novel finding that CS exposure profoundly affects 25VD3 conversion to 1,25VD3 and that exogenous 1,25VD3 can block CSE-induced inflammation.

A small but increasing number of reports suggest that a relationship might exist between VD3 levels and CS. In a study of more than 9000 subjects, 25VD3 deficiency was a risk factor for the development of tobacco-related cancers.⁴⁷ This report is consistent with earlier data from the Normative Aging Study showing that VD3 sufficiency is protective against lung damage associated with smoking.⁴⁸ Murine studies have shown a similar pattern, with VDR knockout mice prematurely having emphysema,⁴⁹ suggesting that VD3 might have a protective effect against the inflammatory effects of smoke. In a study of 550 perimenopausal women, active smoking was associated with lower serum 25VD3 and 1,25VD3 levels compared with those in nonsmoking control subjects.⁵⁰ Our results build on these findings, demonstrating that exposure to ETS and active smoking are associated with reductions in systemic and tissue 25VD3 levels in both control subjects and patients with CRS compared with levels in their smoke-naïve counterparts. A further decrease in 25VD3 circulating levels was noted in ETS-exposed patients with CRSwNP, who are already systemically deficient. However, it is still unclear what is causing this exacerbation of 25VD3 deficiency in patients with CRSwNP or in smoke-exposed subjects.

Local analysis of *CYP27B1*, which codes for 25-hydroxylase, did not show any differences based on smoke exposure or disease state. Given that most 25VD3 is produced in the liver, lack of changes in *CYP27B1* expression in the sinus mucosa is not entirely surprising. We were also unable to detect any significant local changes in *VDR* gene expression based on disease state or smoke exposure. This observation is in contrast with other reports in murine models. Agrawal et al⁵¹ reported that VDR expression was reduced by inflammation or VD3 deficiency in a murine allergic model. Based on this study, it would be anticipated that VD3-deficient patients with CRSwNP would have reduced local *VDR* expression; however, no differences were observed in this human study.

One item of focus was the effect of disease state and smoke exposure on *CYP27B1* expression. Patients with CRSwNP had

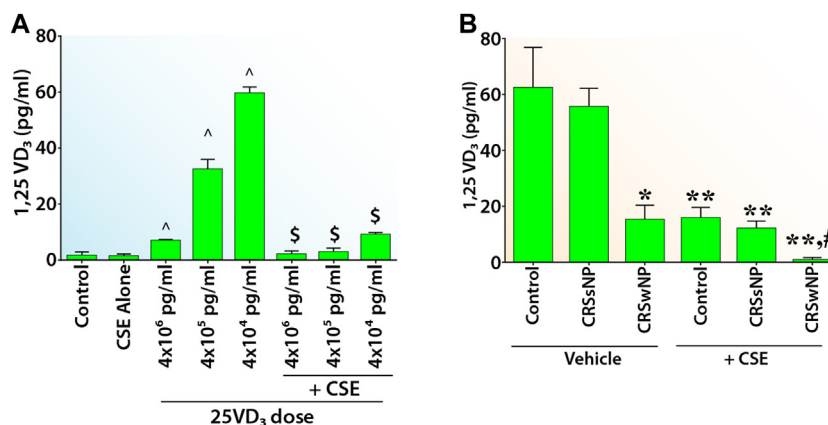


FIG 3. HSNEC VD₃ conversion is impaired in patients with CRSwNP and by CSE. Data shown are means $\pm$ SEMs. **A**, Control HSNEC cultures treated with indicated 25VD₃ dose plus 10% CSE. $^{\wedge}P < .05$ versus control or CSE-alone groups. $^{\$}P < .001$ versus the dose-matched, non-CS-treated group. $N = 3$ control patients. **B**, HSNECs treated with 4×10^4 pg/mL 25VD₃ to determine disease-specific conversion and effect of CSE. $^*P < .05$ versus the control group. $^{**}P < .05$ versus each disease-matched, vehicle control subject. $^{\#}P < .05$ versus control + CSE or CRSsNP + CSE. Control subjects and patients with CRSsNP, $n = 3$; patients with CRSwNP, $n = 4$.

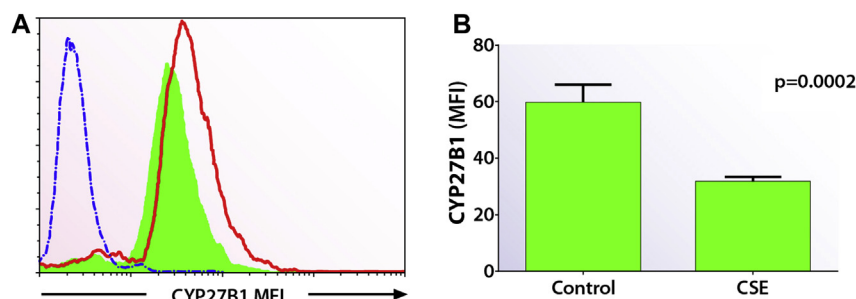


FIG 4. CSE exposure downregulates HSNEC *CYP27B1* expression. Intracellular staining and flow cytometric analysis of *CYP27B1* expression 12 hours after treatment with media (control) or 10% CSE is shown. **A**, Representative histograms of isotype (blue dashed line), control-treated (red solid line), or CSE-treated (filled green area) groups. **B**, Mean $\pm$ SD *CYP27B1* expression. $N = 3$ control subjects. Statistics shown are unpaired t tests. *MFI*, Mean fluorescence intensity.

nearly 5-fold downregulation of this gene, whereas patients with CRSsNP had a more modest reduction. Exposure to CS in patients with CRSwNP had a pronounced effect, with a nearly 10-fold downregulation in *CYP27B1* expression. Results were similar yet less dramatic in patients with CRSsNP exposed to CS. In addition to the effect of smoke, local 25VD₃ metabolism is affected by disease type. HSNECs from control subjects and patients with CRSsNP are equally able to convert 25VD₃ to 1,25VD₃. The dosages and amounts of 1,25VD₃ yielded were similar to those reported with normal human bronchial epithelial cells.²⁷ 25VD₃ conversion by HSNECs from patients with CRSwNP was significantly impaired compared with that seen in control subjects or patients with CRSsNP. The inability to find any 1,25VD₃ in CSE-treated CRSwNP supernatants is consistent with *CYP27B1* sinus tissue results that demonstrated a more robust effect of CS on patients with CRSwNP than control subjects or patients with CRSsNP. These findings suggest a possible mechanism in which the effect of CS exposure is greater on those with underlying intrinsic airway diseases when compared with that seen in otherwise healthy subjects.

VD₃ has been shown to have anti-inflammatory properties that protect against a number of inflammatory mediators. For

example, 25VD₃ reduced bronchial epithelial cell production of IL-6 and several chemokines in response to polyinosinic-polycytidylic acid stimulation.⁵² 1,25VD₃ has also been shown to reduce IL-6 and IL-8 production by corneal epithelial cells in response to *Pseudomonas aeruginosa*.⁵³ Recently, Uh et al⁵⁴ showed that CS inhibited VDR translocation in bronchial epithelial cells. Some VDR function appears to be retained despite the presence of CSE because we did observe that addition of 1,25VD₃ was able to reduce CSE-induced cytokine production, suggesting that VDR binding and signaling remain functional. The amount of 1,25VD₃ used (50 pg/mL) is within the range found in the plasma of healthy subjects. Based on our analysis of smoke impairment of VD₃ conversion, normal levels of 25VD₃ might not be sufficient to overcome the smoke-induced downregulation of *CYP27B1*. However, one limitation of our studies is the use of a submerged and not an air-liquid interface system in the culturing and treatment of sinonasal epithelial cells. Additional studies are needed to determine what effect oral administration of 25VD₃ might have in reducing CS-induced airway inflammation. Impairment of VD₃ signaling represents a novel mechanism through which CS can exacerbate respiratory diseases.

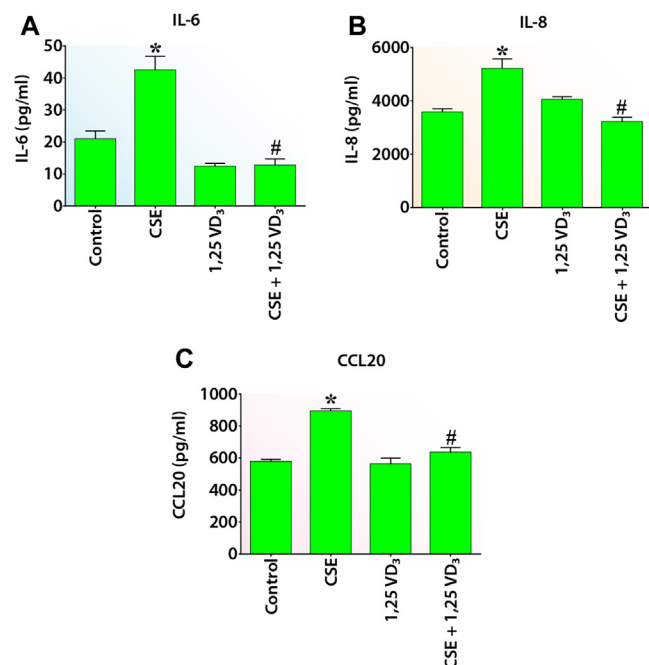


FIG 5. 1,25VD₃ abrogates smoke-induced cytokine production by HSNECs. HSNECs were cultured with vehicle or 10% CSE in the presence or absence of 50 pg/mL 1,25VD₃. Conditioned media were assessed for IL-6 (A), IL-8 (B), or CCL20 (C). Data shown are means $\pm$ SEMs ($n = 3$ control patients for each trial). * $P < .01$ versus the control group. # $P < .008$ versus 10% CSE treatment.

We thank Emy Yarborough for her technical support.

Key messages

- Smoke exposure is associated with reduced circulating and tissue 25VD₃ levels in control subjects and patients with CRS.
- CS impairs conversion of 25VD₃ to 1,25VD₃.
- Exogenous 1,25VD₃ can block smoke-induced inflammation by sinonasal epithelial cells.

REFERENCES

- Kern RC, Conley DB, Walsh W, Chandra R, Kato A, Tripathi-Peters A, et al. Perspectives on the etiology of chronic rhinosinusitis: an immune barrier hypothesis. *Am J Rhinol* 2008;22:549-59.
- Zeile TV, Claeys S, Gevaert P, Maele GV, Holtappels G, Cauwenberge PV, et al. Differentiation of chronic sinus diseases by measurement of inflammatory mediators. *Allergy* 2006;61:1280-9.
- Cao PP, Li HB, Wang BF, Wang SB, You XJ, Cui YH, et al. Distinct immunopathologic characteristics of various types of chronic rhinosinusitis in adult Chinese. *J Allergy Clin Immunol* 2009;124:478-84, e1-2.
- Carney AS, Tan LW, Adams D, Varelias A, Ooi EH, Wormald PJ. Th2 immunological inflammation in allergic fungal sinusitis, nonallergic eosinophilic fungal sinusitis, and chronic rhinosinusitis. *Am J Rhinol* 2006;20:145-9.
- Kim YM, Munoz A, Hwang PH, Nadeau KC. Migration of regulatory T cells toward airway epithelial cells is impaired in chronic rhinosinusitis with nasal polyposis. *Clin Immunol* 2010;137:111-21.
- Hastan D, Fokkens WJ, Bachert C, Newson RB, Bislimovska J, Bockelbrink A, et al. Chronic rhinosinusitis in Europe—an underestimated disease. A GA(2)LEN study. *Allergy* 2011;66:1216-23.
- Besaratinia A, Pfeifer GP. Second-hand smoke and human lung cancer. *Lancet Oncol* 2008;9:657-66.
- Lesmes GR, Donofrio KH. Passive smoking: the medical and economic issues. *Am J Med* 1992;93:38S-42S.
- Percival J, Queally B. Second-hand smoke: how damaging is it to health? *Nurs Times* 2005;101:50-2.
- Martin-Pujol A, Fernandez E, Schiaffino A, Moncada A, Ariza C, Blanch C, et al. Tobacco smoking, exposure to second-hand smoke, and asthma and wheezing in schoolchildren: a cross-sectional study. *Acta Paediatr* 2013;102:e305-9.
- Higgins TS, Reh DD. Environmental pollutants and allergic rhinitis. *Curr Opin Otolaryngol Head Neck Surg* 2012;20:209-14.
- Treyster Z, Gitterman B. Second hand smoke exposure in children: environmental factors, physiological effects, and interventions within pediatrics. *Rev Environ Health* 2011;26:187-95.
- Reh DD, Lin SY, Clipp SL, Irani L, Alberg AJ, Navas-Acien A. Secondhand tobacco smoke exposure and chronic rhinosinusitis: a population-based case-control study. *Am J Rhinol Allergy* 2009;23:562-7.
- Schlosser RJ, Mulligan RM, Casey SE, Varela JC, Harvey RJ, Atkinson C. Alterations in gene expression of complement components in chronic rhinosinusitis. *Am J Rhinol Allergy* 2010;24:21-5.
- Davis KS, Casey SE, Mulligan JK, Mulligan RM, Schlosser RJ, Atkinson C. Murine complement deficiency ameliorates acute cigarette smoke-induced nasal damage. *Otolaryngol Head Neck Surg* 2010;143:152-8.
- McNally P, Coughlan C, Bergsson G, Doyle M, Taggart C, Adorini L, et al. Vitamin D receptor agonists inhibit pro-inflammatory cytokine production from the respiratory epithelium in cystic fibrosis. *J Cyst Fibros* 2011;10:428-34.
- Chirayath MV, Gajdzik L, Hulla W, Graf J, Cross HS, Peterlik M. Vitamin D increases tight-junction conductance and paracellular Ca²⁺ transport in Caco-2 cell cultures. *Am J Physiol Gastrointest Liver Physiol* 1998;274:G389-96.
- Hewison M. Vitamin D and immune function: an overview. *Proc Nutr Soc* 2012; 71:50-61.
- Takeda M, Yamashita T, Sasaki N, Nakajima K, Kita T, Shinohara M, et al. Oral administration of an active form of vitamin D₃ (calcitriol) decreases atherosclerosis in mice by inducing regulatory T cells and immature dendritic cells with tolerogenic functions. *Arterioscler Thromb Vasc Biol* 2010;30:2495-503.
- Penna G, Amuchastegui S, Giarratana N, Daniel KC, Vulcano M, Sozzani S, et al. 1,25-Dihydroxyvitamin D₃ selectively modulates tolerogenic properties in myeloid but not plasmacytoid dendritic cells. *J Immunol* 2007;178:145-53.
- Adorini L, Giarratana N, Penna G. Pharmacological induction of tolerogenic dendritic cells and regulatory T cells. *Semin Immunol* 2004;16:127-34.
- Hackstein H, Thomson AW. Dendritic cells: emerging pharmacological targets of immunosuppressive drugs. *Nat Rev Immunol* 2004;4:24-34.
- Litonjua AA, Weiss ST. Is vitamin D deficiency to blame for the asthma epidemic? *J Allergy Clin Immunol* 2007;120:1031-5.
- Weiss ST, Litonjua AA. Childhood asthma is a fat-soluble vitamin deficiency disease. *Clin Exp Allergy* 2008;38:385-7.
- Cox G, Gaudie J, Jordana M. Bronchial epithelial cell-derived cytokines (G-CSF and GM-CSF) promote the survival of peripheral blood neutrophils in vitro. *Am J Respir Cell Mol Biol* 1992;7:507-13.
- Deeb KK, Trump DL, Johnson CS. Vitamin D signalling pathways in cancer: potential for anticancer therapeutics. *Nat Rev Cancer* 2007;7:684-700.
- Hansdotir S, Monick MM, Hinde SL, Lohan N, Look DC, Hunninghake GW. Respiratory epithelial cells convert inactive vitamin D to its active form: potential effects on host defense. *J Immunol* 2008;181:7090-9.
- Brehm JM, Celedon JC, Soto-Quiros ME, Avila L, Hunninghake GM, Forno E, et al. Serum vitamin D levels and markers of severity of childhood asthma in Costa Rica. *Am J Respir Crit Care Med* 2009;179:765-71.
- Ginde AA, Mansbach JM, Camargo CA Jr. Association between serum 25-hydroxyvitamin D level and upper respiratory tract infection in the Third National Health and Nutrition Examination Survey. *Arch Intern Med* 2009;169:384-90.
- Mulligan JK, White DR, Wang EW, Sansoni SR, Moses H, Yawn RJ, et al. Vitamin D₃ deficiency increases sinus mucosa dendritic cells in pediatric chronic rhinosinusitis with nasal polyps. *Otolaryngol Head Neck Surg* 2012;147:773-81.
- Mulligan JK, Bleier BS, O'Connell B, Mulligan RM, Wagner C, Schlosser RJ. Vitamin D₃ correlates inversely with systemic dendritic cell numbers and bone erosion in chronic rhinosinusitis with nasal polyps and allergic fungal rhinosinusitis. *Clin Exp Immunol* 2011;164:312-20.
- Fokkens WJ, Lund VJ, Mullol J, Bachert C, Alobid I, Baroody F, et al. EPOS 2012: European position paper on rhinosinusitis and nasal polyps 2012. A summary for otorhinolaryngologists. *Rhinology* 2012;50:1-12.
- Lund VJ, Kennedy DW. Quantification for staging sinusitis. The Staging and Therapy Group. *Ann Otol Rhinol Laryngol Suppl* 1995;167:17-21.
- Mahoney GN, Al-Delaimy W. Measurement of nicotine in hair by reversed-phase high-performance liquid chromatography with electrochemical detection. *J Chromatogr B Biomed Sci Appl* 2001;753:179-87.
- Chetiyanukornkul T, Toriba A, Kizu R, Kimura K, Hayakawa K. Hair analysis of nicotine and cotinine for evaluating tobacco smoke exposure by liquid chromatography-mass spectrometry. *Biomed Chromatogr* 2004;18:655-61.

36. Ayers CM, Schlosser RJ, O'Connell BP, Atkinson C, Mulligan RM, Casey SE, et al. Increased presence of dendritic cells and dendritic cell chemokines in the sinus mucosa of chronic rhinosinusitis with nasal polyps and allergic fungal rhinosinusitis. *Int Forum Allergy Rhinol* 2011;1:296-302.
37. Mulligan JK, Mulligan RM, Atkinson C, Schlosser RJ. Human sinonasal epithelial cells direct dendritic cell function and T-cell T helper 1/T helper 2 skewing following *Aspergillus* exposure. *Int Forum Allergy Rhinol* 2011;1: 268-74.
38. Binkley N, Ramamurthy R, Krueger D. Low vitamin D status: definition, prevalence, consequences, and correction. *Endocrinol Metab Clin North Am* 2012;39:287-301.
39. Ahmed W, Khan N, Glueck CJ, Pandey S, Wang P, Goldenberg N, et al. Low serum 25 (OH) vitamin D levels (<32 ng/mL) are associated with reversible myositis-myalgia in statin-treated patients. *Transl Res* 2009;153:11-6.
40. Heaney RP. Vitamin D in health and disease. *Clin J Am Soc Nephrol* 2008;3: 1535-41.
41. Psaltis AJ, Schlosser RJ, Yawn JR, Henriquez O, Mulligan JK. Characterization of B-cell subpopulations in patients with chronic rhinosinusitis. *Int Forum Allergy Rhinol* 2013;3:621-9.
42. Hautamaki RD, Kobayashi DK, Senior RM, Shapiro SD. Requirement for macrophage elastase for cigarette smoke-induced emphysema in mice. *Science* 1997;277:2002-4.
43. Mulligan RM, Atkinson C, Vertegel AA, Reukov V, Schlosser RJ. Cigarette smoke extract stimulates interleukin-8 production in human airway epithelium and is attenuated by superoxide dismutase in vitro. *Am J Rhinol Allergy* 2009; 23:e1-4.
44. Damia Ade D, Gimeno JC, Ferrer MJ, Fabregas ML, Folch PA, Paya JM. A study of the effect of proinflammatory cytokines on the epithelial cells of smokers, with or without COPD. *Arch Bronconeumol* 2011;47:447-53.
45. Yamin M, Holbrook EH, Gray ST, Harold R, Busaba N, Sridhar A, et al. Cigarette smoke combined with Toll-like receptor 3 signaling triggers exaggerated epithelial regulated upon activation, normal T-cell expressed and secreted/CCL5 expression in chronic rhinosinusitis. *J Allergy Clin Immunol* 2008;122:1145-53.e3.
46. Hudy MH, Traves SL, Wiehler S, Proud D. Cigarette smoke modulates rhinovirus-induced airway epithelial cell chemokine production. *Eur Respir J* 2010;35:1256-63.
47. Afzal S, Bojesen SE, Nordestgaard BG. Low plasma 25-Hydroxyvitamin D and risk of tobacco-related cancer. *Clin Chem* 2013;59:771-80.
48. Lange NE, Sparrow D, Vokonas P, Litonjua AA. Vitamin d deficiency, smoking, and lung function in the normative aging study. *Am J Respir Crit Care Med* 2012; 186:616-21.
49. Sundar IK, Hwang JW, Wu S, Sun J, Rahman I. Deletion of vitamin D receptor leads to premature emphysema/COPD by increased matrix metalloproteinases and lymphoid aggregates formation. *Biochem Biophys Res Commun* 2011;406: 127-33.
50. Brot C, Jorgensen NR, Sorensen OH. The influence of smoking on vitamin D status and calcium metabolism. *Eur J Clin Nutr* 1999;53:920-6.
51. Agrawal T, Gupta GK, Agrawal DK. Vitamin D deficiency decreases the expression of VDR and prohibitin in the lungs of mice with allergic airway inflammation. *Exp Mol Pathol* 2012;93:74-81.
52. Schrumph JA, van Sterkenburg MAJA, Verhoosel RM, Zuyderduyn S, Hiemstra PS. Interleukin 13 exposure enhances vitamin D-mediated expression of the human cathelicidin antimicrobial peptide 18/LL-37 in bronchial epithelial cells. *Infect Immun* 2012;80:4485-94.
53. Xue ML, Zhu H, Thakur A, Willcox M. 1 alpha,25-Dihydroxyvitamin D3 inhibits pro-inflammatory cytokine and chemokine expression in human corneal epithelial cells colonized with *Pseudomonas aeruginosa*. *Immunol Cell Biol* 2002;80: 340-5.
54. Uh ST, Koo SM, Kim YK, Kim KU, Park SW, Jang AS, et al. Inhibition of vitamin d receptor translocation by cigarette smoking extracts. *Tuberc Respir Dis (Seoul)* 2012;73:258-65.

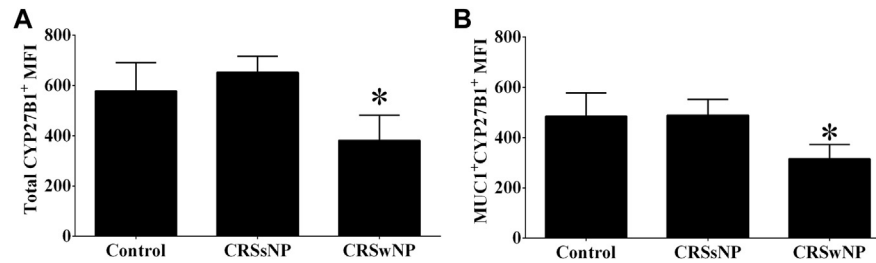


FIG E1. *Ex vivo* analysis of sinus tissue explants by means of intracellular flow cytometry demonstrates general downregulation of CYP27B1 protein in all cells from sinus explants (**A**), as well as epithelial cell-specific CYP27B1 expression (**B**). Data shown are mean fluorescence intensity (MFI) $\pm$ SEM. * $P < .05$ versus control or CRSsNP. Control group, $n = 6$; CRSsNP group, $n = 4$; CRSwNP group, $n = 4$.