



JID Open

Oral Vitamin D Rapidly Attenuates Inflammation from Sunburn: An Interventional Study

Jeffrey F. Scott¹, Lopa M. Das², Sayeeda Ahsanuddin², Yuqi Qiu³, Amy M. Binko², Zachary P. Traylor², Sara M. Debanne³, Kevin D. Cooper^{1,2}, Rebecca Boxer⁴ and Kurt Q. Lu^{1,2}

The diverse immunomodulatory effects of vitamin D are increasingly being recognized. However, the ability of oral vitamin D to modulate acute inflammation *in vivo* has not been established in humans. In a double-blinded, placebo-controlled interventional trial, 20 healthy adults were randomized to receive either placebo or a high dose of vitamin D₃ (cholecalciferol) one hour after experimental sunburn induced by an erythemogenic dose of UVR. Compared with placebo, participants receiving vitamin D₃ (200,000 international units) demonstrated reduced expression of proinflammatory mediators tumor necrosis factor- α ($P = 0.04$) and inducible nitric oxide synthase ($P = 0.02$) in skin biopsy specimens 48 hours after experimental sunburn. A blinded, unsupervised hierarchical clustering of participants based on global gene expression profiles revealed that participants with significantly higher serum vitamin D₃ levels after treatment ($P = 0.007$) demonstrated increased skin expression of the anti-inflammatory mediator arginase-1 ($P = 0.005$), and a sustained reduction in skin redness ($P = 0.02$), correlating with significant expression of genes related to skin barrier repair. In contrast, participants with lower serum vitamin D₃ levels had significant expression of proinflammatory genes. Together the data may have broad implications for the immunotherapeutic properties of vitamin D in skin homeostasis, and implicate arginase-1 upregulation as a previously unreported mechanism by which vitamin D exerts anti-inflammatory effects in humans.

Journal of Investigative Dermatology (2017) 137, 2078–2086; doi:10.1016/j.jid.2017.04.040

INTRODUCTION

Vitamin D is a ubiquitous fat-soluble hormone important in calcium homeostasis and bone metabolism (Jackson et al., 2006). The majority of vitamin D arises from *de novo* synthesis in the skin triggered by UVR, with smaller contributions from dietary sources (Bikle, 2011). Although considerable attention has been placed on vitamin D deficiency and optimizing supplementation strategies, appreciation for the diverse biological effects and long-term outcomes of vitamin D now include the modulation of immune responses, inflammatory disease, cardiovascular health, and carcinogenesis (Giovannucci et al., 2006; Martins et al., 2007; Rosen, 2011; Sanders et al., 2010; Wobke et al., 2014). However, there is a lack of evidence demonstrating that intervention with vitamin D is capable of

resolving acute inflammation in target tissues and organs in humans.

Keratinocytes and macrophages produce active vitamin D within the skin (Baeke et al., 2010; Bikle et al., 1986). Vitamin D has pleiotropic effects on the immune system, including the enhancement of antimicrobial responses, induction of autophagy, and suppression of proinflammatory mediators, including tumor necrosis factor- α (TNF- α) (Di Rosa et al., 2012; Liu et al., 2006; Zhang et al., 2012). We recently demonstrated that intervention with a single dose of vitamin D is capable of rapidly attenuating an inflammatory response in a mouse model of chemical induced skin injury through inhibition of inducible nitric oxide synthase (iNOS; or *NOS2* gene) and TNF- α (or *TNFA* gene) by activated macrophages (Au et al., 2015). Therefore, we designed a pilot, proof-of-principle interventional study in humans, modeled after a randomized, double-blinded, placebo-controlled clinical trial, to test the hypothesis that a single high dose of oral vitamin D₃ (cholecalciferol) would be capable of rapidly attenuating experimental sunburn induced by simulated solar radiation (SSR).

RESULTS

Dose-dependent response of high-dose oral vitamin D₃ and UV irradiation

Subjects randomized to receive various doses of vitamin D were divided into 4 categories (Figure 1a), whereas the overall study design constituting the control phase and an investigative phase (Figure 1b) required monitoring of subjects for a total of 6 weeks. The randomized treatment groups did not differ in their baseline characteristics (Table 1). No participant was taking supplemental

¹Department of Dermatology, University Hospitals Cleveland Medical Center, Cleveland, Ohio, USA; ²Department of Dermatology, Case Western Reserve University School of Medicine, Cleveland, Ohio, USA;

³Department of Epidemiology and Biostatistics, Case Western Reserve University, Cleveland, Ohio, USA; and ⁴Department of Medicine, Division of Geriatrics, University of Colorado Anschutz Medical Campus, Aurora, Colorado, USA

Correspondence: Kurt Q. Lu, Department of Dermatology, University Hospitals Cleveland Medicine Center, Lakeside 3500, 11100 Euclid Avenue, Cleveland, Ohio 44106, USA. E-mail: kql1@case.edu

Abbreviations: iNOS, inducible nitric oxide synthase; IU, international units; MED, minimal erythema dose; SSR, simulated solar radiation; TNF- α , tumor necrosis factor- α ; 25(OH)D₃, serum 25-hydroxyvitamin D₃

Received 7 February 2017; revised 14 March 2017; accepted 7 April 2017; accepted manuscript published online 30 May 2017

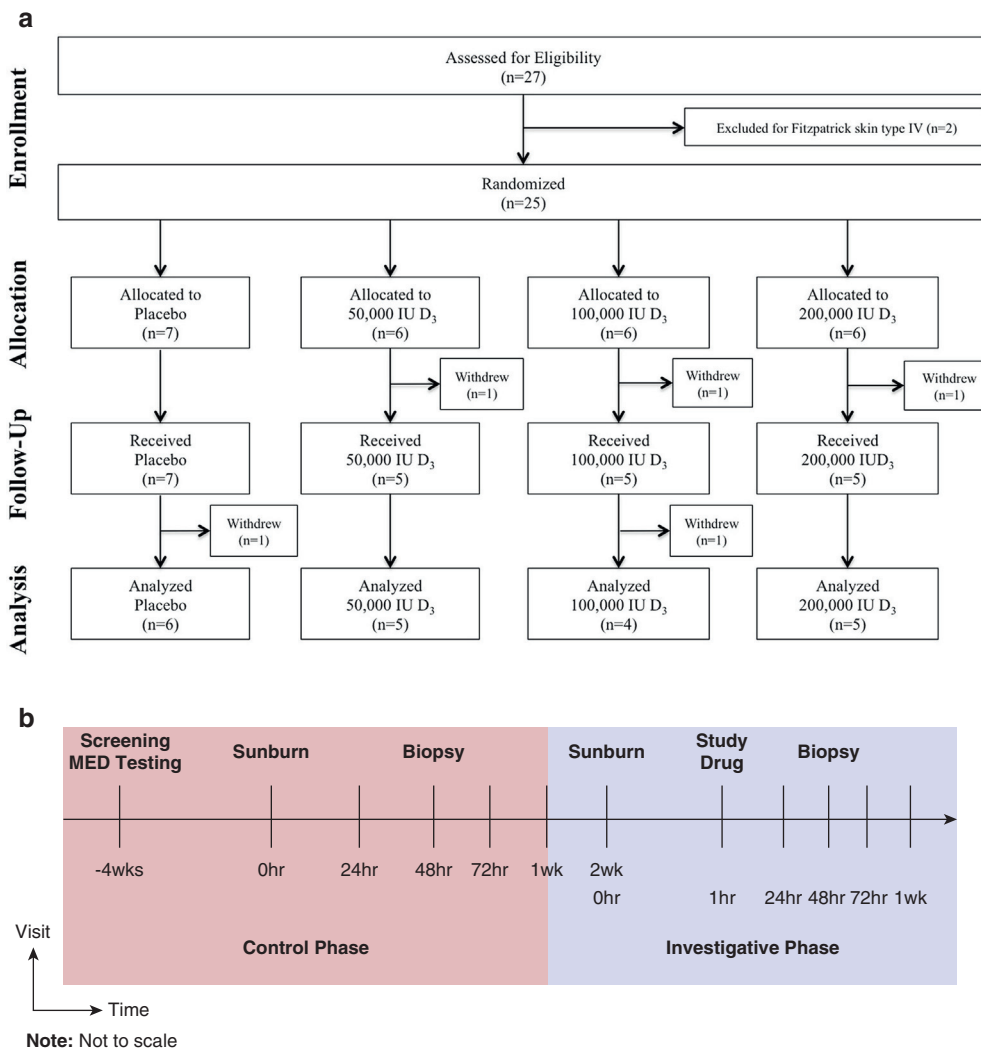


Figure 1. Study design and baseline characteristics. (a) The enrollment, allocation, follow-up, and analysis of participants. (b) A schematic of the parallel phase study design. Skin erythema and thickness were measured 24 hours, 48 hours, 72 hours, and 1 week after experimental sunburn induced by an erythemogenic dose of simulated solar radiation. Participants returned 2 weeks after conclusion of the control phase of the study for the investigative phase, and were subsequently randomized to receive the study drug as a postexposure treatment 1 hour after experimental sunburn on the contralateral arm. Punch biopsies for tissue TNF- α , iNOS, and microarray analyses were obtained 48 hours after experimental sunburn in both phases of the study. iNOS, inducible nitric oxide synthase; MED, minimal erythema dose; TNF- α , tumor necrosis factor- α .

vitamin D₃ before study initiation. Serum 25-hydroxyvitamin D₃ (25(OH)D₃), a marker of vitamin D₃ stores, increased after treatment in a vitamin D₃ dose-dependent fashion (Supplementary Figure S1a online). Similar trends were observed for the active form of vitamin D₃, 1,25(OH)₂D₃, as well as an inactive breakdown product, 24,25(OH)₂D₃ (Supplementary Figure S1b). No measured vitamin D₃ metabolite increased into a toxic range in any of the treatment groups throughout the study period. Furthermore, there were no instances of clinically significant hypercalcemia occurring in any of the treatment groups throughout the study period (Supplementary Figure S2 online).

Sunburn is a stereotypical inflammatory response induced by exposure to an erythemogenic dose of UVR. Sunburn is characterized clinically by redness, mediated by dermal vasodilation, and edema, mediated by increased vascular permeability and inflammatory cell infiltration (Clydesdale et al., 2001; Cooper et al., 1993; Ouhtit et al., 2000). Although skin redness peaks early after UVR exposure, skin thickness increases steadily for up to 2 weeks after irradiation (Clydesdale et al., 2001; Ouhtit et al., 2000). Compared with one minimal erythema dose (MED), exposure to 2MED increased skin erythema 24 hours and 48 hours after irradiation, and exposure to 3MED

increased skin thickness 72 hours and 1 week after irradiation ($P < 0.05$ for all) (Supplementary Figure S3a, S3b online). We observed saturation of skin redness after exposure to 3MED, limiting the ability to discern subtle differences among treatment groups with this high dose of UVR.

High-dose oral vitamin D₃ attenuates skin inflammation

Clinically, irradiated skin appeared red and swollen 48 hours after UVR exposure (Figure 2a). Irradiated skin also displayed histologic evidence of structural damage as compared with nonirradiated skin, including epidermal vesiculation and edema formation, which improved in a vitamin D₃ dose-dependent fashion (Figure 2b). Skin expression of TNF- α and iNOS was lower in participants receiving 200,000 international units (IU) D₃ as compared with placebo 48 hours after irradiation ($P = 0.04$ for TNF- α ; $P = 0.02$ for iNOS) (Figure 2c). With higher doses of vitamin D₃, there was a trend for decreased skin thickness after irradiation, which reached significance in the 100,000 IU D₃ group at both 72 hours ($P = 0.03$) and 1 week ($P = 0.02$) after irradiation (Supplementary Table S1 online). Comparison of global gene expression profiles among the treatment groups revealed that

Table 1. Baseline Characteristics of the Randomized Treatment Groups

	Placebo (n = 6)	50,000 IU D ₃ (n = 5)	100,000 IU D ₃ (n = 4)	200,000 IU D ₃ (n = 5)
Age, median (range)	24.0 (21–46)	35.0 (21–58)	36.5 (22–50)	27.0 (21–53)
Sex, N (%)				
Male	3 (50.0)	3 (60.0)	3 (75.0)	4 (80.0)
Female	3 (50.0)	2 (40.0)	1 (25.0)	1 (20.0)
BMI, median (range)	22.8 (16.1–26.8)	23.1 (21.3–31.9)	28.6 (22.6–33.1)	23.0 (21.7–41.6)
FST, N (%)				
I	0 (0.0)	0 (0.0)	0 (0.0)	1 (20.0)
II	2 (33.3)	3 (60.0)	2 (50.0)	3 (60.0)
III	4 (66.6)	2 (40.0)	2 (50.0)	1 (20.0)
Baseline vitamin D ₃ Metabolites, mean (95% CI)				
25(OH)D ₃ (ng/ml)	26.8 (23.3–30.4)	30.9 (21.1–40.6)	18.6 (10.8–26.4)	22.4 (15.4–29.4)
1,25(OH) ₂ D ₃ (pg/ml)	61.8 (47.1–76.6)	54.5 (50.3–58.8)	57.7 (46.1–69.4)	54.8 (42.4–67.2)
24,25(OH) ₂ D ₃ (ng/ml)	2.4 (1.8–3.0)	3.0 (1.5–4.5)	1.4 (0.9–1.8)	1.8 (0.9–2.6)

Abbreviations: BMI, body mass index (the weight in kilograms divided by the square of the height in meters); CI, confidence interval; D, vitamin D₃ (cholecalciferol); FST, Fitzpatrick skin type; IU, international units.

the 200,000 IU D₃ group had a distinct gene expression profile that was very different from the placebo group, and most closely related to the 100,000 IU D₃ group (Figure 2d).

Elevated serum levels of 25(OH)D₃ correlate with decreased skin redness

To further investigate a potential link between vitamin D₃ and gene expression, we analyzed the global gene expression profiles of all participants, blinded to their allocated treatment groups. The dendrogram resulting from this analysis produced two clusters of participants (Figure 3a), representing an unbiased, unsupervised hierarchical clustering of all individuals based on similarities in gene expression profiles.

Of note, *ARG* (arginase)-1, known to enhance tissue repair and inhibit inflammation through the utilization of iNOS precursors, was significantly downregulated in cluster 1 ($P = 0.016$) and upregulated in cluster 2 ($P = 0.046$) (Figure 3a) (Bronte and Zanovello, 2005). Increased *ARG1* gene expression observed in cluster 2 compared with cluster 1 was subsequently validated with quantitative real-time reverse transcriptase-PCR ($P = 0.005$) (Figure 3b). Additionally, confocal microscopy analysis of a representative participant from cluster 2 revealed increased expression of the arginase-1 protein localized to CD163+ macrophages after vitamin D₃ treatment (Figure 3c).

Unblinding of the participants' demographics and treatment group allocation revealed that the two clusters of participants did not differ in their baseline characteristics (Supplementary Table S2 online). However, cluster 1 contained all participants randomized to receive placebo, as well as a mixture of participants from the various vitamin D₃ treatment groups (Figure 3a). Cluster 2 predominately contained participants who had received higher doses of vitamin D₃, and notably none of the participants who had received placebo. Although the two clusters had similar baseline serum 25(OH)D₃ levels, participants in cluster 2 had significantly higher 25(OH)D₃ levels after treatment as compared with participants in clusters 1 ($P < 0.05$ for all time points) (Figure 4a). When subjects receiving placebo were excluded from this analysis, serum 25(OH)D₃ levels after treatment remained lower for participants in cluster 1 as compared

with participants in cluster 2 ($P < 0.05$ for 24 hours, 48 hours, and 1 week) (Figure 4b). We will now refer to participants from cluster 2 as vitamin D₃ responders and participants from cluster 1 as vitamin D₃ nonresponders.

As indicated above, body mass index, age, gender, and baseline 25(OH)D₃ stores had no effect on the serum response to oral vitamin D₃. Furthermore, along with higher serum 25(OH)D₃ levels, vitamin D₃ responders demonstrated a statistically significant sustained reduction in skin redness at all time points after irradiation as compared with vitamin D₃ nonresponders ($P < 0.05$ for all) (Figure 4c), and a trend for reduced skin thickness 1 week after irradiation ($P = 0.09$; data not shown).

Differential gene expression profiles characterize vitamin D₃ responders and vitamin D₃ nonresponders

Vitamin D₃ nonresponders displayed upregulation of various proinflammatory genes not observed in the gene expression profiles of vitamin D₃ responders, including matrix metalloproteinases (*MMP1*, *MMP3*), interleukin-1 alpha (*IL-1A*), and monocyte chemokines (*CCL2*) (Supplementary Figure S4a online). Likewise, canonical pathways related to leukocyte migration and IL-6 signaling were significantly activated in vitamin D₃ nonresponders, including TNF- α as a predicted upstream regulator ($P < 0.0001$ for all) (Supplementary Figure S4b). Conversely, vitamin D₃ nonresponders displayed a strikingly different gene expression profile, characterized by upregulation of genes implicated in skin barrier repair, including tissue transglutaminases (*TGM3*, *TGM5*), keratins (*KRT78*, *KRT80*), corneodesmosin (*CDSN*), and calmodulin-like 5 (*CALML5*) (Supplementary Figure S4a).

DISCUSSION

In this pilot, proof-of-principle human interventional study modeled after a randomized, double-blinded, placebo-controlled trial, we provide in vivo evidence that a single high dose of oral vitamin D₃ is capable of rapidly attenuating a local inflammatory response to UVR. Participants responding to high doses of vitamin D₃ demonstrated a sustained reduction in skin redness after experimental sunburn, as well as less epidermal structural damage, reduced

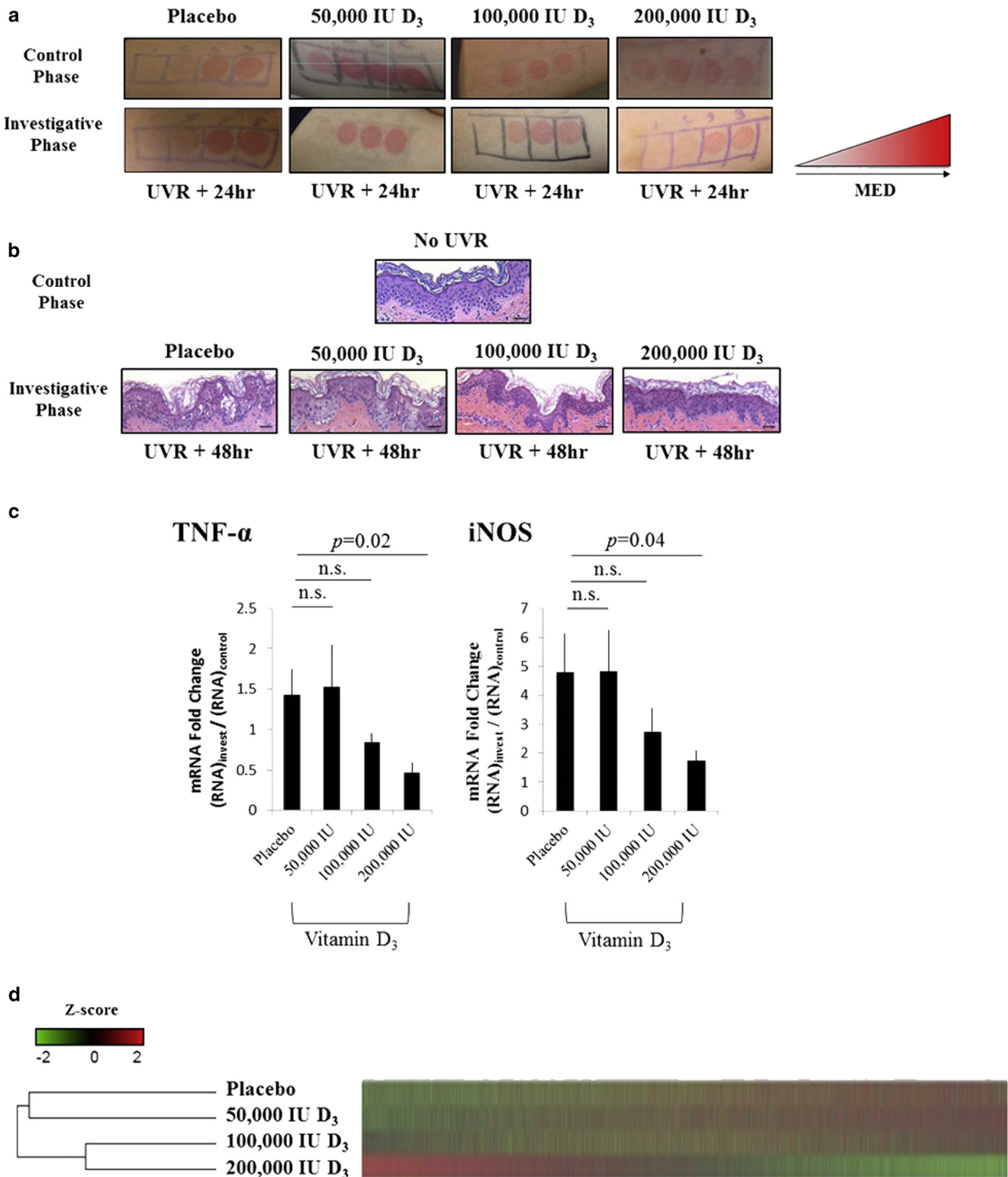


Figure 2. Primary outcomes of randomized treatment groups. (a) Representative clinical images of irradiation sites of participants in each treatment group in the control and investigative phases of the study. (b) Representative hematoxylin and eosin stained histological images obtained from punch biopsies from participants in each treatment group 48 hours after irradiation with 3MED. (c) The difference in TNF- α and iNOS mRNA expression obtained from punch biopsies between the investigative and control phases of the study [(RNA)_{invest}/(RNA)_{control}]. Bars represent the mean, and error bars represent the standard error of the mean for the placebo (n = 4), 50,000 IU D₃ (n = 5), 100,000 IU D₃ (n = 4), and 200,000 IU D₃ (n = 5) groups. Two participants were excluded from the placebo analysis given poor RNA sample quality. (d) A heat map depicting global gene expression averages for each treatment group, with dendrogram depicting the unbiased hierarchical clustering of treatment groups based on similarities in gene expression profiles. Red indicates increased gene expression and green indicates decreased gene expression, correlating to a row-wise z-score. Statistical comparisons are made between vitamin D3 treatment groups and the placebo group. Scale bar = 100 μ m. iNOS, inducible nitric oxide synthase; IU, international units; MED, minimal erythema dose; n.s., nonsignificant; TNF- α , tumor necrosis factor- α .

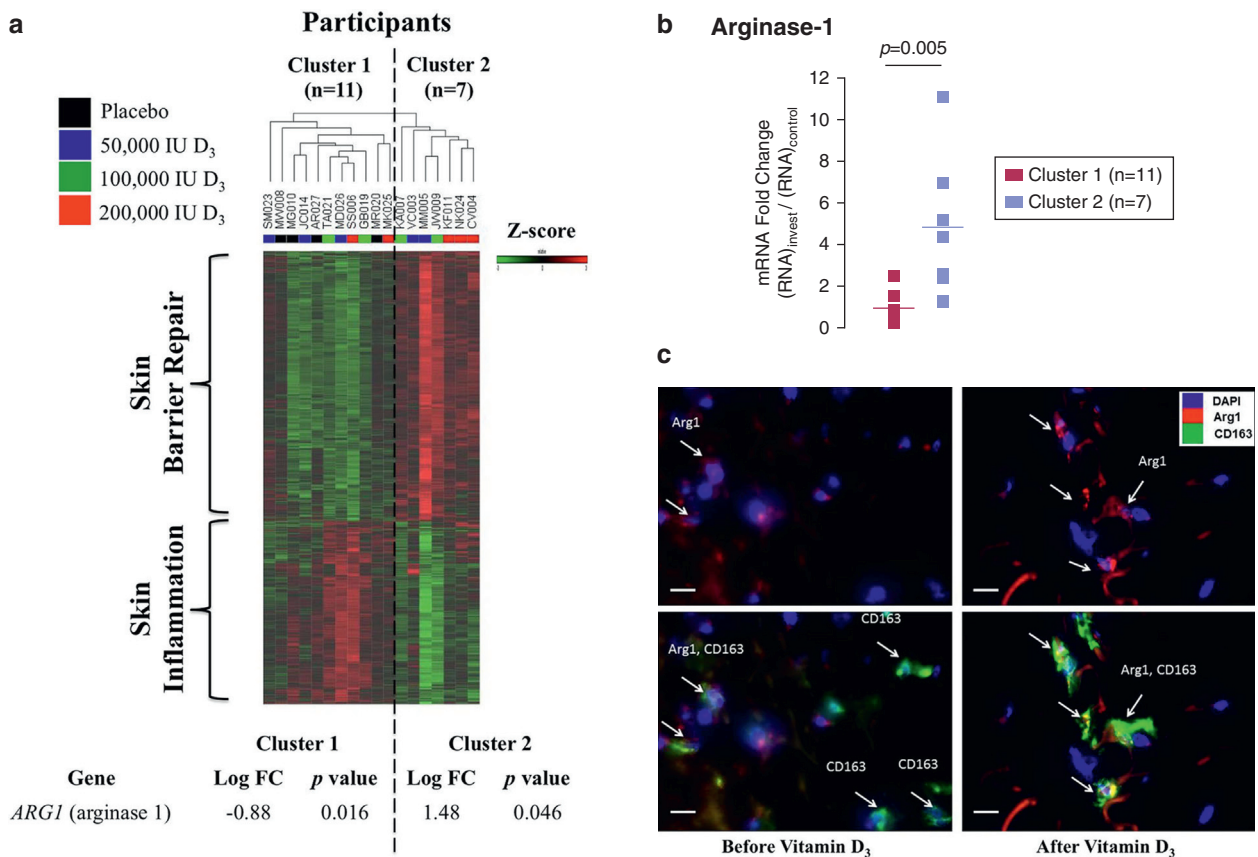


Figure 3. Unsupervised clustering of participants based on gene expression profiles. (a) A heat map depicting global gene expression profiles for individual participants, with the resulting dendrogram depicting the unsupervised hierarchical clustering of participants based only on similarities in gene expression. Red indicates increased gene expression and green indicates decreased gene expression, correlating to a row-wise z-score. Two unique clusters emerged from this unbiased and blinded analysis. Cluster 1 was characterized by downregulation of *ARG1*, and upregulation of genes involved in skin inflammation. Cluster 2 was characterized by upregulation of *ARG1* and genes involved in skin barrier repair. (b) The fold change difference in skin *ARG1* mRNA expression from punch biopsies obtained in the investigative and control phases of the study [(RNA_{48hr})_{invest}/(RNA_{48hr})_{control}]. Data points represent *ARG1* fold change differences for individual participants at each time point. Horizontal lines represent the mean for cluster 1 (n = 11) and cluster 2 (n = 7). (c) Immunofluorescently stained sections from a representative participant receiving 200,000 IU D₃ before and after vitamin D₃ intervention. Nuclear DNA is depicted in blue (DAPI), arginase-1 protein expression is depicted in red, and CD163, a marker of macrophages, is depicted in green. Arginase-1 protein levels are increased primarily within CD163+ macrophages after vitamin D₃ intervention. The white scale bar represents 20 μm. IU, international units; FC, fold change.

expression of proinflammatory markers in the skin, and a gene expression profile characterized by upregulation of skin barrier repair genes. This study also demonstrates that regardless of baseline serum vitamin D₃ levels, a single high dose of oral vitamin D₃ is safe, with serum vitamin D₃ and calcium concentrations remaining within a normal reference range (Rosen, 2011). The simplicity and safety of high-dose oral vitamin D₃ treatment, combined with its rapid and sustained therapeutic efficacy, suggest that these proof-of-concept findings may ultimately be translated to routine clinical use once larger studies are performed on diverse populations of subjects (Ilahi et al., 2008; Sanders et al., 2010).

Moreover, upregulation of arginase-1 is associated with the anti-inflammatory effects of vitamin D₃ in humans (Figure 5). Although arginase has been identified to be present at physiological levels in inflammatory skin diseases, tumors, and chronic wounds, to our knowledge the induction of arginase-1 expression by vitamin D₃ in human skin in vivo is previously unreported (Abd-El-Aleem et al., 2000; Bruch-Gerharz

et al., 2003; Gokmen et al., 2001). These findings suggest that arginase-1 may also be a clinically useful tissue biomarker for monitoring the immunomodulatory effects of vitamin D₃ in humans. Given the presence of a putative vitamin D₃ response element upstream of the *ARG1* promoter, future studies should be aimed at defining the mechanism by which vitamin D₃ treatment activates the arginase-1 pathway (Andrukhova et al., 2014).

Exploratory analyses suggest that the host's response to vitamin D₃ intervention plays a critical role in the modulation of inflammation. Participants segregated into two clusters based on similarities in global gene expression profiles, and these two clusters differed significantly in their serum vitamin D₃ levels after treatment. The pharmacokinetic properties of oral vitamin D₃ are complex, however, and an individual's serum response to oral vitamin D₃ depends on the dose of vitamin D₃, age, body mass index, baseline vitamin D₃ stores, and genetic polymorphisms (Didriksen et al., 2013; Ilahi et al., 2008). Large randomized, double-blinded, placebo-controlled trials will be required to elucidate factors

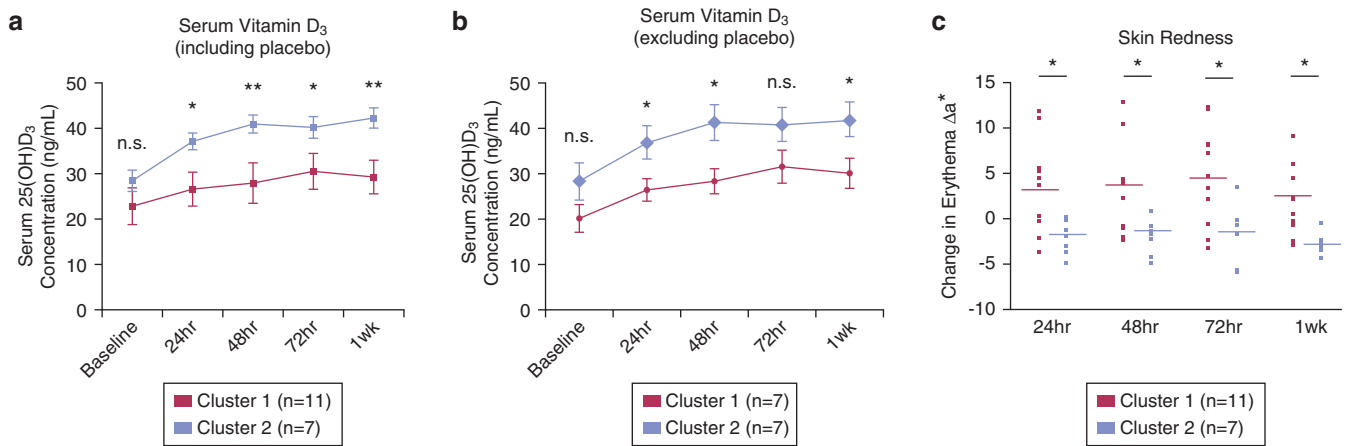


Figure 4. Serum vitamin D₃ and skin erythema after experimental sunburn in cluster 1 and cluster 2. (a) The serum 25(OH)D₃ levels over time after study drug administration for cluster 1 (n = 11) and cluster 2 (n = 7). (b) The serum 25(OH)D₃ levels over time after study drug administration for cluster 1 (n = 7) and cluster 2 (n = 7), excluding the participants from cluster 1 who received placebo (n = 4). Error bars represent the standard error of the mean for each group at each time point. (c) The change in erythema over time after experimental sunburn with 2MED for each cluster. Data points represent erythema values for individual participants at each time point. Horizontal lines represent the mean for cluster 1 (n = 11) and cluster 2 (n = 7) at each time point. Statistical comparisons are between cluster 1 and cluster 2 at each time point. **P* < 0.05; ***P* < 0.01. MED, minimal erythema dose.

responsible for the therapeutic variability of vitamin D₃ treatment within populations and to determine whether individuals with lower baseline vitamin D₃ levels require higher treatment doses to achieve immunomodulation.

Exposure to erythemogenic doses of UVR initiates an influx of inflammatory cells into the skin, generating a microenvironment rich in inflammatory mediators (Cooper et al., 1993; Ouhitt et al., 2000). Specifically, release of TNF- α by damaged keratinocytes and other inflammatory cells plays a crucial role in initiating and sustaining UVR-induced inflammation (Clydesdale et al., 2001; Di Rosa et al., 2012). Following an inflammatory insult, classically activated M1-polarized macrophages infiltrate the skin and produce iNOS as part of an oxidative burst in an evolutionarily conserved attempt to prevent infection (Bronte and Zanovello, 2005; Mills, 2012). However, excessive production of iNOS perpetuates tissue damage, retards the resolution of inflammation, and prevents tissue repair (Bronte and Zanovello, 2005; Mills, 2012). We and others have shown using murine models that vitamin D₃ inhibits the production of TNF- α , and is capable of attenuating skin inflammation by reducing macrophage-specific iNOS production (Au et al., 2015; Zhang et al., 2012).

In the presence of retinoic acid, vitamin D₃ induces the *in vitro* differentiation of monocytes into alternatively activated, M2-polarized CD163+ macrophages expressing arginase-1 (Di Rosa et al., 2012; Takahashi et al., 2014). Furthermore, it has been shown that exposure to acute UVR increases endogenous retinoids in the skin of mice (Gressel et al., 2015). Taken together, our results combined with these data suggest that a potential mechanism by which vitamin D₃ mediates resolution of experimental sunburn is via the upregulation of arginase-1 by endogenous repair molecules, leading to the selective induction of anti-inflammatory, M2-polarized CD163+ macrophages. Additionally, vitamin D₃ may have other protective mechanisms in skin, including reducing DNA damage and keratinocyte apoptosis after experimental sunburn, as was shown in mice

treated topically with the active form of vitamin D₃ immediately after exposure to UVR (Dixon et al., 2007).

It is likely that the generation of vitamin D₃ from cholesterol precursors in the skin after UVR evolved to perform vital homeostatic functions (Bikle, 2011). Moreover, skin-resident cells are capable of locally converting vitamin D₃ into its active form, which can then signal in an intracrine, autocrine, and paracrine fashion to exert diverse biological effects (Bikle, 2011; Di Rosa et al., 2012). It is worthwhile to conjecture that vitamin D₃ may provide an “endocrine barrier” within the skin, utilizing energy derived from sunlight to reduce inflammation, and promote wound healing, tissue repair, and an enhanced epidermal barrier. This would provide the host with additional protection against environmental insults by complementing the classically described brick and mortar mechanical, melanin pigment, and Langerhans cell immunologic barriers.

MATERIALS AND METHODS

Screening, randomization, and study design

The Institutional Review Board at University Hospitals Cleveland Medical Center approved this pilot study, which was conducted between March 2013 and February 2015. The study was modeled after a randomized, double-blinded, placebo-controlled trial. The trial is registered with clinicaltrials.gov (NCT02920502). Twenty-seven healthy adults 18 years and older were screened for eligibility and provided written informed consent (Figure 1a). A total of 25 participants were randomized to receive, in a double-blinded fashion, either placebo or a single oral dose of vitamin D₃ (cholecalciferol) at 50,000, 100,000, or 200,000 IU one hour after SSR exposure. UVR was administered as SSR emitted from a 1,000 W Xenon arc lamp (Newport, Stratford, CT), a full spectrum light source that closely resembles natural sunlight (Clydesdale et al., 2001).

This study was designed in a parallel fashion with exposure to SSR occurring on one arm without study drug administration (control phase), followed 2 weeks later by exposure to SSR on the contralateral arm with study drug administration (investigative phase) (Figure 1b). The MED to experimentally induce sunburn was

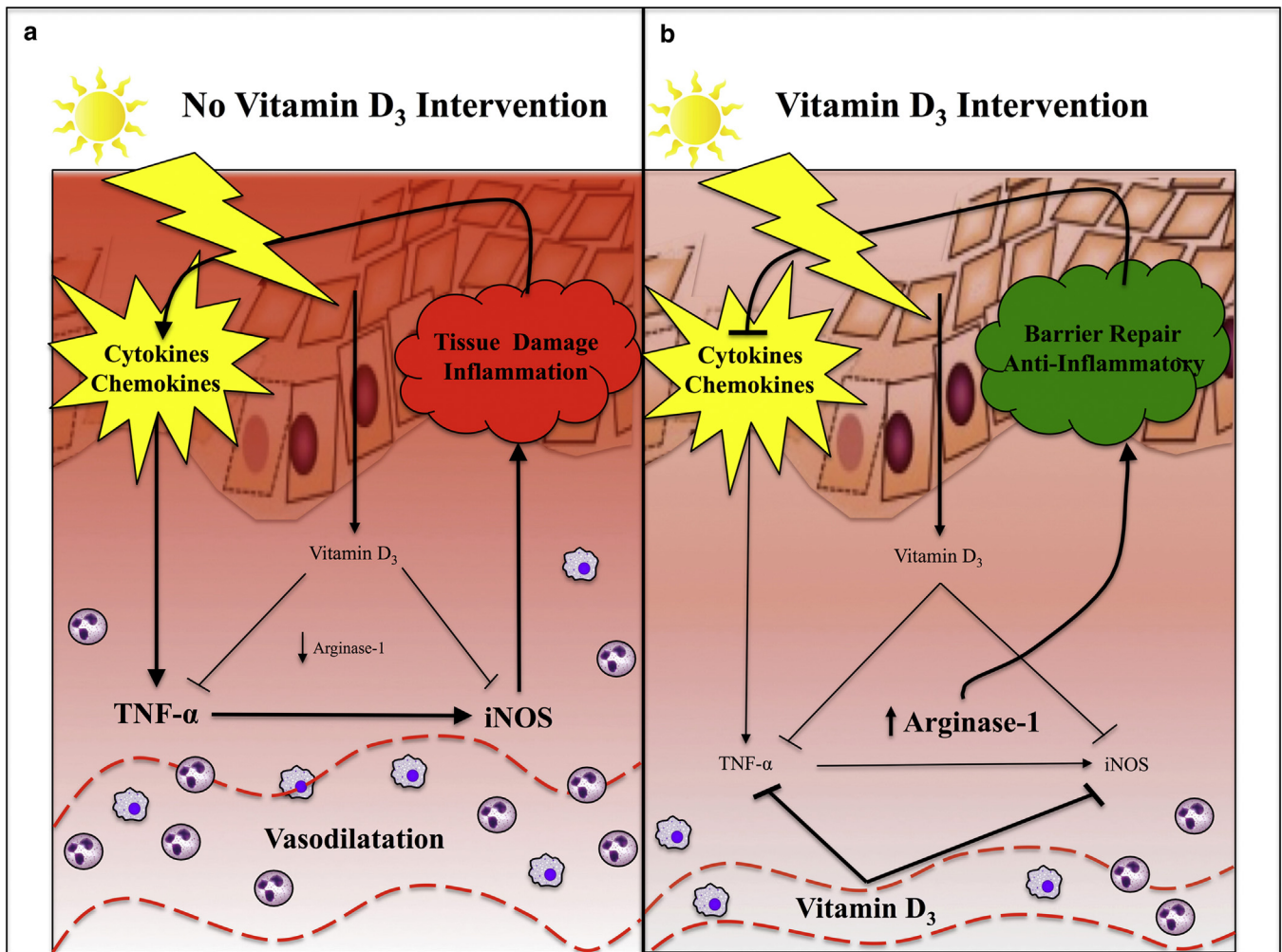


Figure 5. The effect of oral vitamin D₃ intervention on skin inflammation. (a) Levels of vitamin D₃ are at baseline levels in the absence of high-dose oral vitamin D₃ intervention. In this context, exposure to erythemogenic doses of UVR results in sunburn and the release of proinflammatory cytokines and chemokines in the skin, including TNF-α and iNOS, which further propagate tissue inflammation. Increased skin redness and thickness are mediated by vasodilation, an influx of inflammatory cells, and vascular congestion within the skin. The gene expression profile of skin at this time is characterized by increased expression of various proinflammatory genes. (b) Levels of vitamin D₃ rapidly rise within the serum after high-dose oral vitamin D₃ intervention. Arginase-1 is upregulated within the skin, and production of the proinflammatory mediators TNF-α and iNOS is attenuated after sunburn. Reduced skin erythema and thickness are observed clinically. The gene expression profile of skin at this time is characterized by increased expression of skin barrier genes, which help to repair the epidermal barrier and attenuate the inflammatory insult. iNOS, inducible nitric oxide synthase; TNF-α, tumor necrosis factor-α.

determined for each participant during the initial screening visit as previously described (Heckman et al., 2013). Participants were irradiated with one, two, and three times the MED on the sun-shielded, upper arm using plastic holed templates to ensure that adjacent skin was not exposed. A total of 20 participants completed both phases of the study and were included in the per-protocol analysis.

Quantification of vitamin D₃ metabolites and calcium

The concentrations of the vitamin D₃ metabolites 25(OH)D₃, 1,25(OH)₂D₃, and 24,25(OH)₂D₃, as well as total serum calcium, were measured from freshly frozen serum obtained during the screening visit, as well as 24 hours, 48 hours, 72 hours, and 1 week after receiving the study drug. Serum levels of 25(OH)D₃ (ng/ml) and 1,25(OH)₂D₃ (pg/ml) were measured by Liaison assay, and serum levels of 24,25(OH)₂D₃ (ng/ml) were measured by liquid chromatography-mass spectrometry (Heartland Assays, Ames, IA). Toxic serum 25(OH)D₃ levels were defined as those greater than

150 ng/ml (Holick, 2003). Total serum calcium was measured by the University Hospitals Cleveland Medical Center core laboratory (Cleveland, OH), and the normal reference range was considered 8.8–10.7 mg/dl.

Primary outcomes of randomized participants

Primary outcomes included noninvasive measurements of skin erythema and thickness 24 hours, 48 hours, 72 hours, and 1 week after irradiation, as well as tissue expression of TNF-α and iNOS 48 hours after irradiation. Skin erythema (redness) was quantified using a CR300 chromameter (Minolta, Ramsey, NJ). The difference in erythema (Δa^*) between irradiated and nonirradiated skin ($a^*_{\text{irrad}} - a^*_{\text{nonirrad}}$) was calculated for each time point after SSR exposure (Δa^*_{time}). The difference in Δa^*_{time} between the investigative and control phases of the study was calculated to determine the effect of the study drug on skin redness after SSR exposure [$(\Delta a^*_{\text{time}})_{\text{invest}} - (\Delta a^*_{\text{time}})_{\text{control}}$].

Skin thickness, an acute measure of edema, was quantified using a Mitutoyo 9 mm dial caliper (Northamptonshire, UK). Thickness measurements were repeated in triplicate and the mean was used for all calculations. The difference in thickness (Δth) between irradiated and nonirradiated skin ($th_{\text{irrad}} - th_{\text{nonirrad}}$) was calculated for each time point after SSR exposure (Δth_{time}). The difference in Δth_{time} between the investigative and control phases of the study was calculated to determine the effect of the study drug on skin thickness after UVR exposure [$(\Delta th_{\text{time}})_{\text{invest}} - (\Delta th_{\text{time}})_{\text{control}}$].

A 6-mm punch biopsy specimen was obtained from the 3MED site 48 hours after irradiation in both the control and investigative phases of the study. RNA was extracted from fresh frozen tissue using the RNeasy Lipid Mini Kit (Qiagen, Redwood City, CA), and tissue mRNA expression of TNF- α and iNOS was quantified as previously described (Au et al., 2015). A fold change representing the difference in RNA expression between the investigative and control phases of the study was calculated for TNF- α and iNOS [$(\text{RNA}_{48\text{hr}})_{\text{invest}} / (\text{RNA}_{48\text{hr}})_{\text{control}}$].

Unsupervised hierarchical clustering of participants

An exploratory analysis was performed utilizing clusters obtained from the unsupervised hierarchical clustering of individual participants based on similarities in their gene expression profiles, regardless of the allocated treatment group. A minimum of 3 μg of total RNA was submitted to the Gene Expression and Genotyping facility at Case Western Reserve University for microarray analysis (Cleveland, OH). Human Genome 2.0 ST arrays (Affymetrix, Santa Clara, CA) were the chosen platform to analyze transcriptomic changes incurred by treatment. For microarray analyses, normalized linear data post- (T_{interv}) and pre- (T_{control}) study drug intervention were averaged amongst the 18 participants. The microarray raw data for two participants was not interpretable given poor sample quality. A fold change representing the difference in RNA log expression between the investigative and control phases of the study was determined for each gene for each participant [$(\text{RNA}_{48\text{hr}})_{\text{invest}} - (\text{RNA}_{48\text{hr}})_{\text{control}}$]. Data analysis was performed using Rv3.2.2/Bioconductor (R Studio, Boston, MA). The oligo package was used to read, background correct, and normalize the raw sample data using the Robust Multiarray Average algorithm (Bolstad et al., 2003). The limma package was used to create a paired design matrix and conduct differential gene expression analysis based on a linear model fit and empirical Bayes methodology (Ritchie et al., 2015).

Heatmaps and dendrograms were generated utilizing 26,599 transcripts with unique gene names using the GENE-E software (<http://www.broadinstitute.org/cancer/software/GENE-E/index.html>, Accessed June 14, 2016). Hierarchical clustering was used to recursively merge samples based on pairwise distance, determined using the 1-Pearson correlation coefficient and average linkage methods. Ingenuity Pathway Analysis (Qiagen, www.qiagen.com/ingenuity) was used to determine statistically significant canonical pathways, predicted upstream regulators, and biological networks most likely affected by the set of genes differentially expressed for each group of participants.

Tissue ARG1 mRNA expression was quantified using quantitative real-time reverse transcriptase-PCR as described above. To analyze the expression of the arginase-1 protein in skin, freshly frozen biopsy samples embedded in optimal cutting temperature compound were cut into 8- μm sections and stained immunofluorescently as previously described (Au et al., 2015). CD163

was used as a marker for skin macrophages, and DAPI was used to stain nuclear DNA.

Statistical analysis

Given the pilot nature of the study design, power calculations were not performed. To control for type I error rate, hierarchical closed-testing procedures were utilized for analysis of primary outcomes. Unpaired *t*-tests were used to compare the means of groups with respect to changes in the primary outcomes at each time point. Fisher's exact test was used for intergroup comparisons of categorical data and for determining significance of the canonical pathways. The Benjamini-Hochberg correction was used to determine differentially expressed genes in the microarray analysis, adjusting for multiple comparisons (Benjamini and Hochberg, 1995). A two-sided *P*-value of 0.05 or less was considered to indicate statistical significance.

CONFLICT OF INTEREST

The authors state no conflict of interest.

ACKNOWLEDGMENTS

We would like to thank T.S. McCormick for critical discussions and reading of the manuscript; K. Honda for hematoxylin and eosin tissue slide analysis; and the Skin Disease Research Center at Case Western Reserve University for their recruitment of participants and execution of the study protocol. Grant support was provided by National Institute of Arthritis Musculoskeletal and Skin Diseases (NIAMS) (P30-AR039750) and National Institute of Health (U01-AR064144).

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at www.jidonline.org, and at <http://dx.doi.org/10.1016/j.jid.2017.04.040>.

REFERENCES

- Abd-El-Aleem SA, Ferguson MW, Appleton I, Kairsingh S, Jude EB, Jones K, et al. Expression of nitric oxide synthase isoforms and arginase in normal human skin and chronic venous leg ulcers. *J Pathol* 2000;191:434–42.
- Andrukhova O, Slavic S, Zeitz U, Riesen SC, Heppelmann MS, Ambrisko TD, et al. Vitamin D is a regulator of endothelial nitric oxide synthase and arterial stiffness in mice. *Mol Endocrinol* 2014;28:53–64.
- Au L, Meisch JP, Das LM, Binko AM, Boxer RS, Wen AM, et al. Suppression of hyperactive immune responses protects against nitrogen mustard injury. *J Invest Dermatol* 2015;135:2971–81.
- Baeke F, Takiishi T, Korff H, Gysemans C, Mathieu C, Vitamin D. modulator of the immune system. *Curr Opin Pharmacol* 2010;10:482–96.
- Benjamini Y, Hochberg Y. Controlling the false discovery rate: a practical and powerful approach to multiple testing. *J R Stat Soc Series B Stat Methodol* 1995;57:289–300.
- Bikle DD. Vitamin D metabolism and function in the skin. *Mol Cell Endocrinol* 2011;347:80–9.
- Bikle DD, Nemanic MK, Whitney JO, Elias PW. Neonatal human foreskin keratinocytes produce 1,25-dihydroxyvitamin D3. *Biochemistry* 1986;25:1545–8.
- Bolstad BM, Irizarry RA, Astrand M, Speed TP. A comparison of normalization methods for high density oligonucleotide array data based on variance and bias. *Bioinformatics* 2003;19:185–93.
- Bronte V, Zanovello P. Regulation of immune responses by L-arginine metabolism. *Nat Rev Immunol* 2005;5:641–54.
- Bruch-Gerharz D, Schnorr O, Suschek C, Beck KF, Pfeilschifter J, Ruzicka T, et al. Arginase 1 overexpression in psoriasis: limitation of inducible nitric oxide synthase activity as a molecular mechanism for keratinocyte hyperproliferation. *Am J Pathol* 2003;162:203–11.
- Clydesdale GJ, Dandie GW, Muller HK. Ultraviolet light induced injury: immunological and inflammatory effects. *Immunol Cell Biol* 2001;79:547–68.
- Cooper KD, Duraiswamy N, Hammerberg C, Allen E, Kimbrough-Green C, Dillon W, et al. Neutrophils, differentiated macrophages, and monocyte/

- macrophage antigen presenting cells infiltrate murine epidermis after UV injury. *J Invest Dermatol* 1993;101:155–63.
- Di Rosa M, Malaguarnera G, De Gregorio C, Palumbo M, Nunnari G, Malaguarnera L. Immuno-modulatory effects of vitamin D3 in human monocyte and macrophages. *Cell Immunol* 2012;280:36–43.
- Didriksen A, Grimnes G, Hutchinson MS, Kjaergaard M, Svartberg J, Joakimsen RM, et al. The serum 25-hydroxyvitamin D response to vitamin D supplementation is related to genetic factors, BMI, and baseline levels. *Eur J Endocrinol* 2013;169:559–67.
- Dixon KM, Deo SS, Norman AW, Bishop JE, Halliday GM, Reeve VE, et al. In vivo relevance for photoprotection by the vitamin D rapid response pathway. *J Steroid Biochem Mol Biol* 2007;103:451–6.
- Giovannucci E, Liu Y, Rimm EB, Hollis BW, Fuchs CS, Stampfer MJ, et al. Prospective study of predictors of vitamin D status and cancer incidence and mortality in men. *J Natl Cancer Inst* 2006;98:451–9.
- Gokmen SS, Aygit AC, Ayhan MS, Yorulmaz F, Gulen S. Significance of arginase and ornithine in malignant tumors of the human skin. *J Lab Clin Med* 2001;137:340–4.
- Gressel KL, Duncan FJ, Oberyzyzn TM, La Perle KM, Everts HB. Endogenous retinoic acid required to maintain the epidermis following ultraviolet light exposure in SKH-1 hairless mice. *Photochem Photobiol* 2015;91:901–8.
- Heckman CJ, Chandler R, Kloss JD, Benson A, Rooney D, Munshi T, et al. Minimal erythema dose (MED) testing. *J Vis Exp* 2013:e50175.
- Holick MF. Evolution and function of vitamin D. *Recent Results Cancer Res* 2003;164:3–28.
- Ilahi M, Armas LA, Heaney RP. Pharmacokinetics of a single, large dose of cholecalciferol. *Am J Clin Nutr* 2008;87:688–91.
- Jackson RD, LaCroix AZ, Gass M, Wallace RB, Robbins J, Lewis CE, et al. Calcium plus vitamin D supplementation and the risk of fractures. *N Engl J Med* 2006;354:669–83.
- Liu PT, Stenger S, Li H, Wenzel L, Tan BH, Krutzik SR, et al. Toll-like receptor triggering of a vitamin D-mediated human antimicrobial response. *Science* 2006;311:1770–3.
- Martins D, Wolf M, Pan D, Zadshir A, Tareen N, Thadhani R, et al. Prevalence of cardiovascular risk factors and the serum levels of 25-hydroxyvitamin D in the United States: data from the Third National Health and Nutrition Examination Survey. *Arch Intern Med* 2007;167:1159–65.
- Mills CD. M1 and M2 macrophages: oracles of health and disease. *Crit Rev Immunol* 2012;32:463–88.
- Ouhitit A, Muller HK, Davis DW, Ullrich SE, McConkey D, Ananthaswamy HN. Temporal events in skin injury and the early adaptive responses in ultraviolet-irradiated mouse skin. *Am J Pathol* 2000;156:201–7.
- Ritchie ME, Phipson B, Wu D, Hu Y, Law CW, Shi W, et al. limma powers differential expression analyses for RNA-sequencing and microarray studies. *Nucleic Acids Res* 2015;43:e47.
- Rosen CJ. Clinical practice. Vitamin D insufficiency. *N Engl J Med* 2011;364:248–54.
- Sanders KM, Stuart AL, Williamson EJ, Simpson JA, Kotowicz MA, Young D, et al. Annual high-dose oral vitamin D and falls and fractures in older women: a randomized controlled trial. *JAMA* 2010;303:1815–22.
- Takahashi H, Hata Y, Iriyama N, Hasegawa Y, Uchida H, Nakagawa M, et al. Induced differentiation of human myeloid leukemia cells into M2 macrophages by combined treatment with retinoic acid and 1 α ,25-dihydroxyvitamin D3. *PLoS One* 2014;9:e113722.
- Wobke TK, Sorg BL, Steinhilber D. Vitamin D in inflammatory diseases. *Front Physiol* 2014;5:244.
- Zhang Y, Leung DY, Richers BN, Liu Y, Remigio LK, Riches DW, et al. Vitamin D inhibits monocyte/macrophage proinflammatory cytokine production by targeting MAPK phosphatase-1. *J Immunol* 2012;188:2127–35.

Editor's Note

During the review of this article, questions arose concerning the post-enrollment trial registration date reported herein. The authors have explained this as follows:

“Our aim was to conduct a proof-of-concept study demonstrating that vitamin D can modulate acute inflammation in target tissues and, secondarily, to discover new skin repair genes *in vivo*. No established studies were available to inform on dosing to correlate with biologic activity in the skin. Therefore dose-finding was also incorporated in conceptualizing this study. To achieve rigor in the data and remove potential bias, the pilot study was modeled after a randomized double-blinded placebo-controlled trial design. The findings in this study and the results from exploratory analysis with gene array will help define parameters for primary and secondary outcomes in larger clinical trials. In preparation of the manuscript, we learned that medical journals have a broader definition of clinical trials than the FDA. Pre-registration for this pilot study was not done because our understanding was that non-regulated supplements such as Vitamin D did not constitute an applicable clinical trial. Since learning of the International Committee of Medical Journal Editors guidelines, the study was retrospectively registered and the data were made available.”

The JID requires preregistration of all clinical trials as part of its broad interest in assuring that all published data are free of bias of any kind. In this case, however, it is the Editor's judgment that sharing these results has priority and the investigators' misunderstanding of what constitutes a clinical trial should not preclude publication.

Mark C. Udey
Editor



This work is licensed under a Creative Commons Attribution 4.0 International License. To view a copy of this license, visit <http://creativecommons.org/licenses/by/4.0/>