

Vitamin D₃ replacement enhances antigen-specific immunity in older adults

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Abbreviations:

25(OH)D	- 25-hydroxyvitamin D
CBA	– cytometric bead array
CRP	- C reactive protein
p38-MAPK	- p38 mitogen-activated protein kinase
Tregs	- T regulatory cells
T _{RM}	- resident-memory T cells
VZV	- Varicella Zoster Virus

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Abstract:

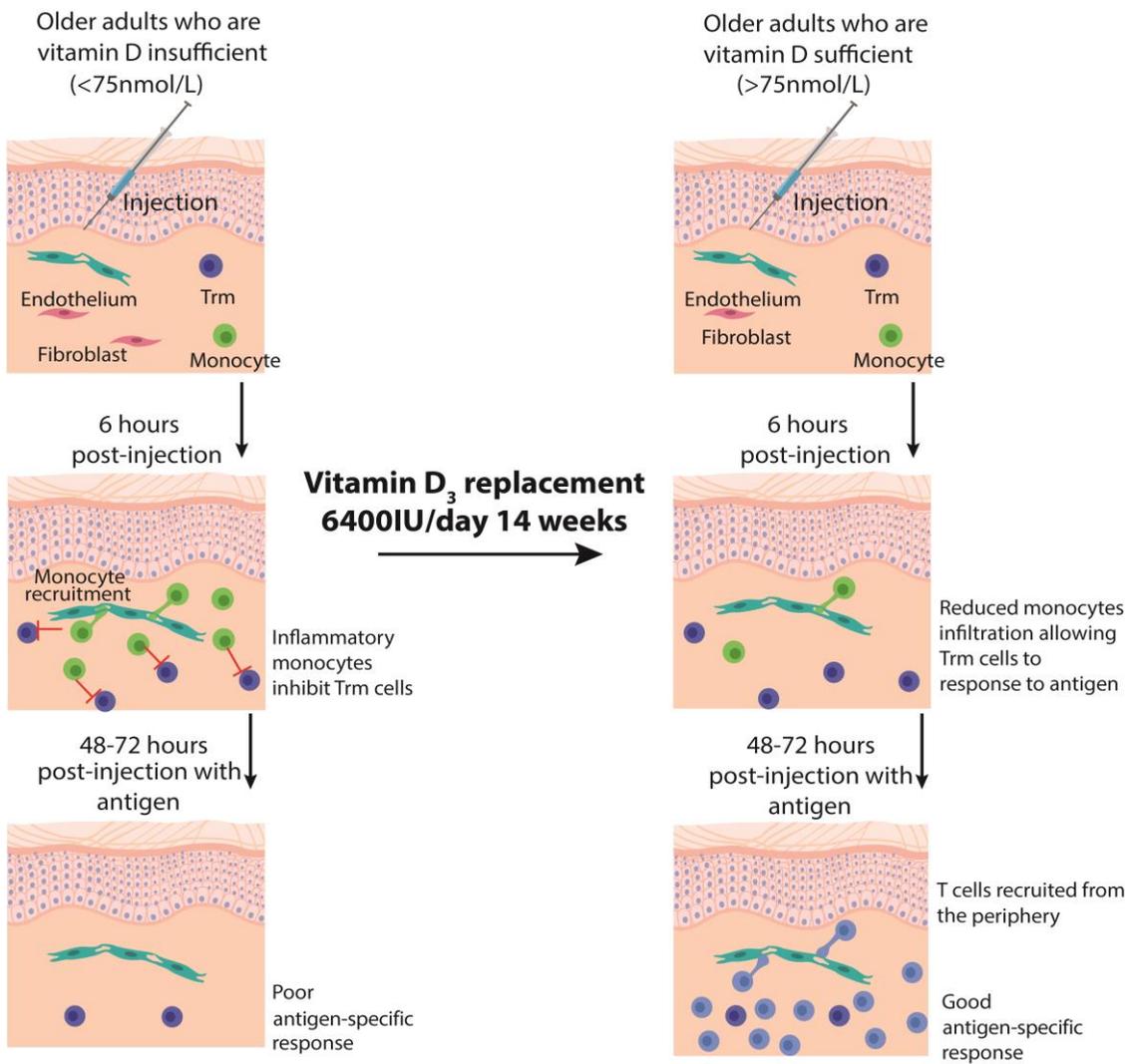
Ageing is associated with increased number of infections, decreased vaccine efficacy and increased systemic inflammation termed inflammageing. These changes are reflected by reduced recall responses to varicella zoster virus (VZV) challenge in the skin of older adults. Vitamin D increases immunoregulatory mechanisms and has the potential to inhibit inflammageing. Since vitamin D deficiency is more common in the old and has been associated with frailty and increased inflammation. Therefore we investigated the use of vitamin D₃ replacement to enhance cutaneous antigen-specific immunity in older adults (≥65 years).

We showed that that older adults had reduced VZV-specific cutaneous immune response and increased non-specific inflammation as compared to young. Increased non-specific inflammation observed in the skin of older adults negatively correlated with vitamin D sufficiency. Therefore, vitamin D₃ replacement was investigated to determine if it could improve VZV-specific cutaneous immune responses in older adults. Vitamin D insufficient older adults (n=18) were administered 6400IU of vitamin D₃/day orally for 14 weeks. Antigen-specific immunity to VZV was assessed using transcriptional analysis of skin biopsies collected from challenged injection sites pre- and post-vitamin D₃ replacement. We showed that vitamin D₃ supplementation significantly increased the response to cutaneous VZV antigen challenge in older adults. This enhancement was associated with a reduction in inflammatory monocyte infiltration with a concomitant enhancement of T cell recruitment to the site of antigen challenge in the skin.

In conclusion vitamin D₃ replacement can boost antigen-specific immunity in older adults with sub-optimal vitamin D status.

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Graphical Abstract



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Introduction:

Immunity decreases during ageing as demonstrated by the increased susceptibility to bacterial and viral infections, re-activation of latent infections such as varicella zoster virus (VZV), decreased vaccine efficacy and increased incidence of cancer (1-3). In addition, there is an increase in low grade systemic inflammation in older humans termed inflammageing. This is characterised by high serum levels of the inflammatory cytokines IL-6, IL-1 β , TNF α and C reactive protein (CRP) (4), and is a strong predictor for frailty and mortality (5, 6). Inflammageing is also believed to contribute to reduced antigen-specific immunity that is observed with older age (≥ 65 years) (7, 8).

Antigen-specific cutaneous recall responses are reduced in healthy old as compared to young individuals (8-11). We have shown that intradermal injections of air, saline or antigen into the skin of older adults are associated with induction of an early non-specific inflammation which directly contributes to reduced secondary cutaneous immunity (12). We proposed that this non-specific inflammation is driven by senescent fibroblasts recruiting inflammatory monocytes that secrete PGE₂ and directly inhibit antigen-specific immunity (12). Blockade of inflammation using the anti-inflammatory drug Losmapimod (a specific p38 MAP kinase inhibitor) can restore antigen-specific immunity in older adults via inhibiting the non-specific inflammation in the skin (8, 12).

Vitamin D has key immunomodulatory properties including increasing the abundance of regulatory T cells (Tregs) (13-15), reducing inflammatory cytokine production by T cells and monocytes (16, 17) as well as increasing antimicrobial peptide production (18). Vitamin D insufficiency, as determined by serum 25-hydroxyvitamin D (25[OH]D) levels < 75 nmol/L, is more common in the older adult (> 65 years) population, particularly in those who are frail and who have elevated inflammatory markers (19-21). Therefore, vitamin D insufficiency may exacerbate inflammageing and non-specific inflammation observed in older adults.

As vitamin D insufficiency is associated with ageing and inflammation, we initiated a clinical study using vitamin D replacement in older adults with sub-optimal vitamin D status to assess if vitamin D₃ replacement improves secondary cutaneous immunity. Older adults with vitamin D insufficiency (25(OH)D < 75 nmol/L), were orally administered 6400IU of vitamin D₃ per day for 14 weeks. Antigen-specific immunity was assessed by measuring the clinical response to VZV challenge and by transcriptional analysis of skin biopsies collected pre- and post-vitamin D₃ replacement. We show that vitamin D₃ replacement can significantly improve VZV-specific cutaneous immunity in older adults. Vitamin D therefore has the potential to be used as a cheap, safe and effective therapy to enhance antigen-specific immunity in the skin of elderly humans.

Materials and Methods:

Study approval:

This study was approved by the NHS Queen Square Research Ethics Committee (reference 17/SC/0196) and by the UCL Research Ethics Committee. All participants provided written informed consent and study procedures were performed in accordance with the principles of the declaration of Helsinki. We were advised by the UK's Medicines and Healthcare products Regulatory Agency (MHRA) that the study was not classified as a Clinical Trial of an Investigational Medicinal Product (IMP) as defined by the EU Directive 2001/20/EC. As this experimental medicine study was designed to test a hypothesis in humans *in vivo* and not to determine the therapeutic outcome or efficacy of vitamin D₃ for patient benefit.

Study participants

For the study involving young (<40 years) and old (≥65 years) adults (Figure 1) we recruited healthy individuals of white European ethnicity. We excluded individuals with co-morbidities that are associated with significant internal organ or immune dysfunction including heart failure, severe chronic obstructive pulmonary disease (COPD), diabetes mellitus and rheumatoid arthritis, and individuals receiving immunosuppressive treatment (e.g. oral glucocorticoids, methotrexate, azathioprine and cyclosporin) for autoimmune or chronic inflammatory diseases.

For the study involving vitamin D₃ (Figures 2-4), healthy older adults were recruited to take part through local GP surgeries. When individuals expressed an interest in the study they were screened and recruited according to the inclusion and exclusion criteria see Supplementary Table 1. We recruited 18 healthy older individuals, VZV skin test and saline injection were performed and biopsies were collected at 6 and 48 hours. Subsequently, individuals were given 6400IU of vitamin D₃ per day for 14 weeks orally. After vitamin D₃ supplementation the participants repeated the same VZV skin test and skin biopsies were collected as before (see Figure 3A). Serum CRP levels were measured using a Roche cobas high sensitive immunoturbidimetric assay and 25(OH)D concentrations were measured with a Roche cobas electrochemiluminescence immunoassay (ECLIA).

Skin tests

VZV antigen (BIKEN, The Research Foundation for Microbial Diseases of Osaka University, Japan) or 0.9% saline solution were injected intradermally into sun unexposed skin of the medial proximal volar forearm as per manufacturer's instructions. Induration, palpability and the change in erythema from baseline were measured and scored on day 2 or 3 as validated and described previously(11). A clinical score (range 0-10) based on the summation of these parameters was then calculated.

RNAseq analysis of skin biopsies:

Three separate 3 mm punch biopsies were collected from each volunteer: one from normal (un-injected) skin, one from the saline injection site at 6 hours post-injection and one from the VZV injection site at 48 or 72 hours post-injection. Biopsies were immediately stabilised in RNAlater for

cryostorage. Total RNA was extracted from bulk tissue homogenates using RNeasy Mini Kit (Qiagen) as previously described (8). Library preparation for RNAseq was performed using the Kappa Hyperprep kit (Roche Diagnostics) and sequencing was performed by the Pathogens Genomic Unit (UCL) on the Illumina Nextseq 500 (Illumina) using the NextSeq 500/550 High Output 75 cycle kit (Illumina) according to manufacturers' instructions, resulting in a median of 22.7 million (range 1.4-38.6 million; IQR 20.8-24.4 million) 41 bp paired-end reads per sample.

Module analysis: RNAseq data were mapped to the reference transcriptome (Ensembl Human GRCh38 release 99) using Kallisto (22). The transcript-level output counts and transcripts per million (TPM) values were summed on gene level and annotated with Ensembl gene ID, gene name, and gene biotype using the R/Bioconductor packages tximport and BioMart (23, 24). Downstream analyses were restricted to gene biotypes with selected BioMart annotations (protein coding, IG_C_gene, IG_D_gene, IG_J_gene, IG_V_gene, TR_C_gene, TR_D_gene, TR_J_gene, TR_V_gene), resulting in 23,402 Ensembl gene IDs.

Heatmap and individual gene analysis: Reads were aligned to Genome Reference Consortium Human Build 38 (GRCh38) using Hisat2(25). Samtools was used to select for reads with paired mates. Transcript assembly was carried out using StringTie (26), with gene-level Fragments per Kilobase of transcript per Million mapped read (FPKM) generated using Ballgown(27). Statistical comparisons were made on gene count estimates generated by StringTie. Genes with low expression or short transcript lengths (<200 nucleotides for the longest transcript) were removed. The count matrix was normalised using the TMM method in edgeR (version 3.22.5)(28), followed by contrast fit with voom in limma (version 3.36.5)(29), treating the subject ID as a blocking variable. Genes with an adjusted p-value of less than 0.05 and expression change of greater than 2-fold up or down, were considered to be statistically significant.

Microarray data

Data from previous microarray experiments were utilized in this study (8). Following robust multi-array average (RMA) normalization with the R/Bioconductor package affy (30), only unique gene name annotations were retained, selecting the probe ID with highest average expression across all samples.

Transcriptional modules

The gene expression modules for T cells and monocytes have been described (31, 32) and validated previously (33). The VZV-specific model was generated by the mean expression of genes in a transcriptional module comprising differential gene expression in biopsies from the site of VZV-injection in young adults as compared to normal (unmanipulated) skin. The saline-specific module was represented by the mean expression of genes in a transcriptional module comprising differentially gene expression in biopsies from the site of saline-injection in old individuals as compared to normal skin. In each case, differentially expressed genes with false discovery rate (FDR) <0.05 and log₂ fold difference ≥1 were identified using DeSeq2 and SARTools (34) for RNAseq data, and Mann-Whitney tests in MultiExperiment Viewer v4.9 (<http://www.tm4.org/mev.html>) for microarray data, based on false discovery rate (FDR) <0.05 and log₂ fold difference ≥1. Gene module

scores were subsequently calculated as mean expression of the constituent gene names in each module. For RNAseq data, log₂-transformed TPM values were used, following the addition of a pseudocount of 0.001 to enable log₂ transformation. Where duplicate gene names were present in the RNAseq data, the highest log₂ transcript per million (TPM) value was used for each sample.

Reactome pathway enrichment among module genes was analysed with the XGR R package (35). For visualization purposes, 20 pathway groups were identified by hierarchical clustering of Jaccard indices to quantify similarity between the gene composition of each pathway. For each group, the pathway with the largest total number of genes was then selected to provide a representative annotation.

Serum cytokine measurements

Cytokine concentration in serum was assessed by cytometric bead array (CBA; BD Biosciences) according to the manufacturer's protocol. Samples were analysed using a BD Verse flow cytometer (BD Biosciences). The lower limit of detection for each analyte were 1.5pg/mL.

Statistics

Statistical analysis was performed using GraphPad Prism version 8.00 (GraphPad Software, San Diego, California, USA). Data was assessed for normality and the subsequent appropriate statistical test was performed as indicated in the legend of each figure.

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Results:

Low serum 25-hydroxyvitamin D concentrations correlate with inflammatory response to saline

We have shown previously that older adults exhibit an early non-specific inflammatory response to intradermal injection which is associated with a reduced delayed-type hypersensitivity responses to the VZV skin test (9). We sought to extend these findings by performing modular bioinformatic analysis, as validated previously (33). We intradermally-challenged healthy young (<40 years) and old (≥ 65 years) individuals with VZV antigen (in individuals who had pre-existing VZV immunity) for donor characteristics see Table 1). The site of challenge in the skin was biopsied 72 hours later and RNAseq or microarray analysis was performed and compared to normal, unmanipulated, skin (Figure 1A). In line with our previous studies (8, 12), 6 hour saline injection was used as a control for non-specific (needle-injury) responses.

We derived transcriptional modules (signatures) to quantify the VZV-specific cutaneous immune response (Supplementary Figure 1A and Supplementary Table 2). As expected, the expression of genes within the VZV-specific module was increased in young and old adults after skin challenge (8). However, the magnitude of the secondary response to VZV antigen was significantly lower in older individuals compared to the young individuals (Figure 1B). In a previous study, we have observed that there was a significant accumulation of T cells at the site of VZV antigen challenge in young subjects which was greatly reduced in older adults (36). To identify if T cells are as important for a VZV response, expression of a previously generated T cell-specific gene module was used (33). We observed that following injection with VZV there was a significant increase in expression of the T cell-specific module (Figure 1C). Since the magnitude of expression of the T cell-specific module correlates directly with the number of T cells present (33), this suggested that there was an increase in T cell numbers in antigen-injected skin as compared to normal skin. Indeed, our analysis showed that the expression level of the genes in the T cell-specific module correlated directly with the magnitude of the VZV clinical score (Supplementary Figure 2).

We previously showed that a monocyte-driven inflammatory response to injection is responsible for the impaired T cell response to VZV in the skin of older individuals (8, 12). We therefore created a gene module associated with non-specific saline injection based upon gene expression in 6 hour saline injected old skin. This saline-specific module was enriched in genes and pathways associated with the innate immune system and interleukin signalling (Supplementary Figure 1B, Supplementary Table 3). We confirmed that there was a significant induction of an inflammatory response in saline-injected older skin that was not observed in the young (Figure 1D). Consistent with our previous observation we found that enrichment of a monocyte specific module was significantly greater in saline injection sites of older compared to younger individuals (Figure 1E). Expression of the monocyte-specific module was also increased in the skin of older adults 6 hours after injection with VZV antigen (Figure 1F), confirming the non-specific recruitment of monocytes to the tissue damage caused by needle injection rather than specific to saline (12).

Next, in order to evaluate the potential role of vitamin D in inflammaging, we sought to understand if vitamin D insufficiency was associated with the exaggerated non-specific monocytic inflammatory response to saline injection we found in older individuals. In keeping with this, we found that there was a significant negative correlation between serum 25(OH)D concentrations and both the

expression of the saline induced transcriptional module and the monocyte module in older adults (Figure 1G and H).

Therefore this data suggests that vitamin D insufficiency is associated with increased non-specific inflammation in the skin of older adults.

Vitamin D₃ supplementation significantly improved cutaneous secondary immune response in older adults.

We hypothesised that if vitamin D insufficiency may be causally related to inflammaging, and in turn mechanistically linked to attenuation of antigen specific recall responses, then vitamin D₃ supplementation may rescue age-related diminution of recall responses. We tested this hypothesis by evaluating immune responses before and after of vitamin D replacement (6400IU of vitamin D₃ per day orally for 14 weeks) among older adults (median age 69 years; 6 males and 12 females), with low concentrations of serum 25(OH)D (median 43nmol [22.9-68.3nmol/L]) (Figure 2A). We utilised 6400IU/day in order to maximise our chances of elevating circulating 25(OH)D levels into high physiological range, without risking toxicity by exceeding the Tolerable Upper Intake Level (UL) of 10,000 IU/day (37). All older adults had a significant increase in their serum 25(OH)D concentrations after vitamin D replacement (Figure 2B) confirming compliance with the vitamin D supplementation regime. We observed a significant increase in VZV clinical scores after vitamin D supplementation (Figures 2C and D) using an ordinal scale clinical score (11). The increase in VZV clinical score was not due to repeated exposure of antigen, as we have shown previously that repeated exposure to VZV antigen over the same time frame as used in this study, does not increase VZV clinical score (8).

We further stratified the participants into three groups based on the magnitude of their clinical response following vitamin D₃ supplementation: non-improvers, who did not have an improvement in clinical score; mild-improvers, clinical score improved by 1; improvers, those who had an improvement in their clinical score of ≥ 2 (Figure 2D). Analysis of the characteristics of each of these groups revealed that there were no significant differences in their ages, serum 25(OH)D or CRP concentrations at baseline (Table 2) or after vitamin D supplementation. There was, however, an increased proportion of females in the improvers when compared to the other two groups (Table 2).

These data suggest that vitamin D replacement can significantly enhance antigen-specific immunity during ageing.

Vitamin D₃ supplementation decreased non-specific monocyte-driven inflammation

Following 14 weeks of vitamin D₃ replacement, there was no significant impact on circulating inflammatory cytokine or CRP concentrations (Table 3). This suggested that the beneficial anti-inflammatory effect of vitamin D₃ is specific to the site of antigen challenge in the skin. Next, we evaluated the effect of vitamin D₃ supplementation on the non-specific inflammatory response to saline injection. 3mm skin biopsies were collected from normal and saline-injected skin (6 hours post-injection) pre- and post-vitamin D₃ replacement. As observed previously (8, 12), there was a large proinflammatory response to saline injection in older adults which was characterised by

increased expression of monocyte chemoattractants and cytokines such as *CCL2*, *CCL8* and *IL1B*. The expression of the inflammatory genes was reduced after vitamin D₃ supplementation (Figure 3A). Focusing on the eight most upregulated genes in response to saline prior to vitamin D₃ replacement, we observed that, after supplementation these genes were no longer statistically significantly upregulated as compared to normal skin (Figure 3B). Consistent with these findings, we found that expression of both the saline-induced and monocyte transcriptional modules were significantly decreased after vitamin D₃ supplementation (Figures 3C and D), suggesting that vitamin D₃ supplementation can reduce the non-specific inflammation and the associated inflammatory monocyte recruitment which was associated with needle challenge in older adults.

Vitamin D supplementation enhances T cell accumulation in the skin after antigen challenge

We have previously shown that inflammatory monocytes recruited to the skin of older adults in response to needle challenge blocks antigen-specific T cell responses and that inhibiting monocyte infiltration can improve cutaneous immunity (12). We wanted to investigate whether vitamin D₃ supplementation could also reverse inflammatory monocytes recruitment and thus the attenuated T cell responses to VZV antigen in older adult skin. Specifically, we wanted to determine whether the decrease in monocyte infiltration following vitamin D₃ supplementation leads to an enhancement of T cell accumulation at the site of antigen challenge. To assess this, gene expression in VZV-injected skin (48 hours after injection) was compared by RNAseq analysis pre- and post-vitamin D₃ supplementation and no significant differential overall gene expression was observed (Figure 4A). We reasoned that the heterogeneity of the effect of vitamin D₃ supplementation meant that our sample size was underpowered to detect statistically consistent differences in the whole group, particularly in view of the multiple testing penalty for gene-wide analysis. Therefore, we focused our analysis on VZV-induced and T cell transcriptional modules after stratifying participants by the vitamin D₃ associated improvement in their VZV clinical score, into those who were non-improvers or mild-improvers (clinical score change ≤ 1) as compared to improvers (clinical score change > 1). We found that improvers had a significant increase in the expression of the VZV-specific module after vitamin D₃ supplementation as compared to those who were mild/ non-improvers (Figure 4B). In addition, the T cell-specific module was significantly increased in VZV injected skin in the improvers but not in the mild/non-improvers (Figure 4C). Interestingly, individuals whose VZV clinical score increased by ≥ 2 had a higher expression of the T cell module in response to VZV prior to vitamin D₃ supplementation.

Collectively, our data is consistent with a mechanistic model in which vitamin D status may enhance antigen-specific immunity by reducing non-specific monocyte driven inflammation and enhancing T cell mediated recall responses.

Discussion:

In this study we confirmed that antigen-specific cutaneous immune responses were reduced in the skin of older adults (≥ 65 years) when compared to young (< 40 years). In agreement with our previous work the reduced secondary cutaneous response was associated with an increased monocyte-derived non-specific inflammatory response to needle-challenge in the older adults. As vitamin D has a role in controlling inflammation, we investigated whether vitamin D insufficiency correlated with the increased inflammatory response that occurs in the skin after needle challenge. There was increased non-specific inflammation in response to injection (determined by increased expression of genes in the saline response module) in individuals that were most vitamin D deficient. Furthermore, we demonstrated that vitamin D₃ supplementation in older adults (6400 IU vitamin D₃ per day for 14 weeks) significantly improved cutaneous secondary immune responses to VZV antigen. Our transcriptional analyses suggested that this increase in cutaneous immunity was associated with decreased early monocyte-driven inflammation and subsequent increased recruitment of T cells to the site of antigen-challenge.

In this paper we confirm using bioinformatic modular analysis, our earlier observation that an early (6 hours) monocyte-driven non-specific inflammatory response is observed in older adults but not in the young (8, 12). This non-specific inflammatory response is associated with worse antigen-specific cutaneous immunity, as characterised by reduced T cells present in VZV injected skin. Vitamin D₃ replacement significantly reduced monocyte gene signatures in saline injected skin and increased T cell signatures in those individuals who had an improvement in their clinical score. This data proposes that vitamin D₃ supplementation inhibits monocyte recruitment to injected skin of older people and therefore limits monocyte-driven suppression of T resident memory (T_{RM}) cells at the site of antigen challenge. It is interesting to note that the T cell signature only increases in VZV injected skin of individuals that had an improvement in their clinical score even though the non-specific inflammatory response is reduced in the majority of participants after vitamin D₃ replacement. One reason for this might be that the T cell response is only increased in those individuals who have a more measurable cell response to antigen prior to vitamin D₃ supplementation.

Older adults have increased risk of mortality from primary infections such as influenza virus and the SARS-CoV-2 coronavirus, and have an increased risk of reactivation of persistent virus infections such as VZV leading to shingles (1, 38, 39). We have previously observed that older adults have reduced recall responses to antigens such as VZV or candida, resulting in a reduced recruitment of T cells and dendritic cells at the site of antigen challenge (8). This defect in immunity is not due to alterations in circulating antigen-specific cells but is a consequence of inflammatory defects in the skin environment (9, 36). In this study we confirm that there is decreased recall responses in the skin of older adults as compared to young. The defect in the skin of older adults may be applicable to other tissue sites such as the lung and warrants further investigation.

Vitamin D insufficiency is increased in the older adult population (20) and is considered to be due in part to decreased outdoor activity and aging-related alterations in vitamin D metabolism (40). In addition, vitamin D insufficiency in older adults is associated with frailty and increased systemic inflammation (19, 21). Previous studies have shown that vitamin D₃ supplementation in older adults with chronic inflammatory diseases such as osteoarthritis and heart failure significantly decreases the levels inflammatory mediators such as TNF α in the circulation (41, 42). In contrast to these

earlier studies, we did not observe significant decreases in circulating inflammatory mediators after vitamin D₃ supplementation in the healthy volunteers with no overt inflammatory disease, consistent with data in an independent study of healthy older adults (43). We did however observe that vitamin D₃ supplementation was associated with a significant decrease in the non-specific inflammatory response to needle challenge in the skin.

Vitamin D has a plethora of effects on the immune system. Indeed, it is known that vitamin D enhances the number and function of Foxp3⁺ and IL-10⁺ Tregs (13-15), and thus Tregs could directly reduce non-specific inflammation observed in the skin after needle challenge. Another important function of vitamin D is that it enhances T cell receptor (TCR) signalling, as it increases expression of PLC γ and facilitates activation of T cells in response to antigen (44), suggesting an additional means by which vitamin D₃ supplementation could be mediating the effects described in this study.

There were limitations to this study including the study size, gender distribution and ethnic origin of the donors. Although this study had a higher proportion of female donors, we have previously observed that there is no significant difference in non-specific inflammatory response with between males and females (12). Our initial investigations in young and old individuals were carried out on people of diverse backgrounds and found no obvious difference between different racial groups (8, 9, 36). However, this study was designed to be only carried out on caucasians to exclude any potential effects of ethnic backgrounds. Further studies should now be performed to determine the impact of ethnicity, using our data on caucasians as a reference point. As our study was an experimental study to establish mechanisms, rather than confirm the efficacy of vitamin D₃, it will be important to do a larger study to assess the impact of vitamin D₃ replacement on cutaneous immunity.

Another important health challenge within older populations is the reduction in vaccine efficacy with increasing age when compared to younger adults (3). It has been proposed that inflammation has a detrimental effect on the functioning immune system and vaccine responses (7). Therefore there is a drive to develop therapies which can block inflammation to enhance vaccine responses. One such therapy that has been shown to improve influenza vaccine efficacy in older adults is the use of a TORC1 inhibitor. Inhibition of the mTOR pathway significantly enhances the immune response to vaccination and by doing so reduces influenza infections (45, 46). We have also demonstrated that cutaneous immunity can be enhanced by a four day course of oral treatment with p38-MAPKinase inhibitor Losmapimod (8, 12). However, the use of either inhibitor could potentially result in undesirable side effects, especially when used in the long-term. In contrast, the use of vitamin D supplementation is safe, cheap and readily available. Our data suggest that if used as part of a public health initiative targeting older adults, this has the potential to significantly improve the health-span by improving antigen-specific immunity and increasing vaccine efficacy.

Vitamin D insufficiency has also been linked with worse clinical outcomes in the current COVID-19 pandemic (47). In addition, older people are more at risk of increase morbidity and mortality from infection with the Sars-CoV-2 coronavirus (48). Vitamin D is known to be important for respiratory health through the increasing production of antimicrobial peptides (such as cathelicidin) and reducing inflammation (17, 18, 49). Therefore, vitamin D₃ supplementation could be considered as a straightforward, cheap and safe means to help improve immunity to SARS-CoV-2 infection.

Collectively, our data show that vitamin D₃ supplementation could be a simple, cheap and readily available therapy that could enhance antigen-specific immunity in older adults.

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Data availability statement: RNAseq data relating to the young vs old comparison (Figure 1) that support the findings of this data have been deposited on ArrayExpress accession number E-MTAB-9789. RNAseq data relating to the vitamin D₃ replacement study that support the findings of this study have been deposited in NCBI Gene Expression Omnibus, <https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE156212>

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Author contribution: ESC designed and performed experiments and wrote the manuscript. MVS was involved in the overall design of the study and wrote the manuscript. GP, ET and HT performed the experiments. BBS, CTT, NM and TCF performed the bioinformatic analysis of the RNA-seq samples. MHR was the clinical lead for the study and was involved with scientific discussions. MN was involved in the experimental design and editing the manuscript. ARM was involved in study design, provision of clinical advice during the study and editing of the manuscript. ANA was involved in the overall design of the study, initiated and coordinated the collaborative interaction between the different research groups, interpreted the data, contributed writing and edited the manuscript.

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Figure legends:**Figure 1: Decreased cutaneous immunity with age correlates with vitamin D insufficiency.**

A, study schematic, Young (white) and Old (grey) individuals were injected with either antigen or saline and biopsies were collected at specified time-points and RNAseq or Microarray analysis was performed. Samples were compared to Normal (unmanipulated; - [young n=5 and old n=32]) skin. **B**, antigen-specific gene module was generated and **C**, T cell-specific gene module in VZV injected skin (72 hours post-injection; young n=6 and young n=9). **D**, saline-specific gene module and **E**, monocyte-specific gene module in saline injected skin (6 hours post-injection; young n=9 and old n=37). **F**, monocyte-specific gene module in VZV injected skin (6 hours post-injection; young n=6 and young n=9). **G**, saline-specific module and **H**, monocyte-specific module in saline-injected skin from old donors was correlated with serum 25(OH)D concentrations (nmol/L). B-F were analysed with an unpaired t test and G and H were analysed by a Pearson correlation test. ** = $p < 0.01$; *** = $p < 0.001$; **** $p < 0.0001$.

Figure 2: Vitamin D₃ supplementation significantly improves VZV-specific cutaneous immunity.

A, Clinical study schematic. **B**, Serum 25(OH)D concentrations and **C**, and **D**, VZV clinical scores in older adults pre- and post-supplementation (n=18). B and C were analysed with a paired t test. *** = $p < 0.001$; **** $p < 0.0001$.

Figure 3: Vitamin D₃ supplementation is associated with reduced inflammatory monocyte recruitment in response to saline.

A, RNAseq analysis of 3mm biopsies collected from normal and saline-injected skin (6 hours post-injection) pre- and post-vitamin D₃ supplementation. The top 30 genes upregulated in saline injected skin as compared to normal skin before pre-Vitamin D₃ and **B**, dot plots of top 8 upregulated saline-associated genes pre-vitamin D₃. **C**, saline specific module and **D**, monocyte-specific module in saline injected skin pre- and post-vitamin D₃ supplementation (n=17). B, analysed by C and D, analysed with a Wilcoxon-matched paired test. * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$; **** = $p < 0.0001$.

Figure 4: Vitamin D₃ supplementation increases the accumulation of T cells at the site of VZV challenge.

A, RNAseq analysis of 3mm biopsies collected from normal and VZV-injected skin (48 hours post-injection) pre- and post-vitamin D. The top 30 genes upregulated in VZV injected skin as compared to normal skin before post-Vitamin D₃ (n=16). **B**, VZV-specific module and **C**, T cell specific module in VZV injected skin pre- and post-vitamin D₃ supplementation separated based upon improvement in VZV score change of <2 (white; n=9) and change ≥2 (red; n=7). Paired data was analysed using a Wilcoxon-matched paired test and unpaired data with Mann Whitney test . * = p<0.05; ** p<0.01.

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Tables:

	Normal		VZV		Saline	
Characteristic	Young	Old	Young	Old	Young	Old
Age	19.0 (18-23)	69.0 (65-82)	25.5 (20-27)	74.0 (66-83)	19.0 (18-23)	69.0 (65-82)
Gender	3 Male 2 Female	14 Males 18 Female	5 Male 1 Female	2 Male 7 Female	5 Male 4 Female	17 Male 20 Female
Serum 25(OH)D (nmol/L)	49 (29-88)	50.5 (25-103)	No data	No data	54.0 (35-88) [#]	52.5 (26-108)
Number of donors	5	32	6	9	9	37

Table 1: Donor characteristics of young and old donors

Data shown as median \pm 10-90 Percentile. # = 3 donors had no serum 25(OH)D measurements. Normal = unmanipulated biopsied skin; VZV = varicella zoster virus.

Donor Characteristic	Non-improver (NI)	Mild-improver (MI)	Improver (I)	Significant?
Age	70 (65-82)	73 (68-81)	69 (65-69)	ns
Gender	3 Male ; 4 Females	2 Male; 2 Female	1 Male ; 6 Females	
VZV clinical score at baseline	1	0.5	3	*** NI vs I
CRP at baseline (mg/L)	0.8 (0.3-24.3)	0.7 (0.3-24.3)	0.8 (0.4-2.6)	ns
Serum 25(OH)D at baseline (nmol/L)	40.0 (23-68)	53.0 (37-65)	42.0 (22-71))	ns
Serum 25(OH)D after Vitamin D3 supplementation (nmol/L)	89.0 (47-102)	103.5 (87-118)	78.0 (50.0-136.0)	ns
Change in Clinical Score	0	1	2	*** NI vs I
Number of donors	7	4	7	

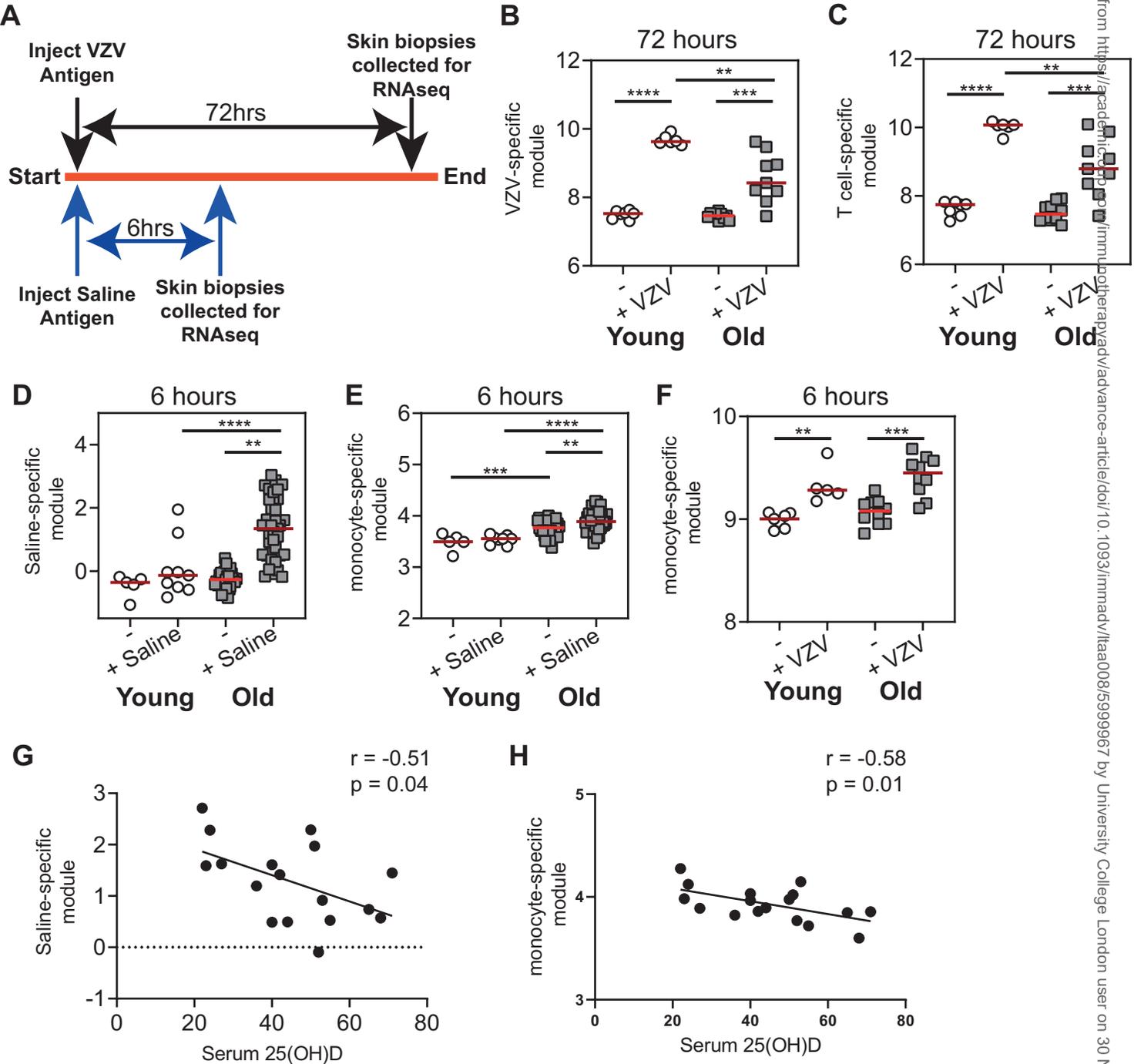
Table 2: Donor characteristics

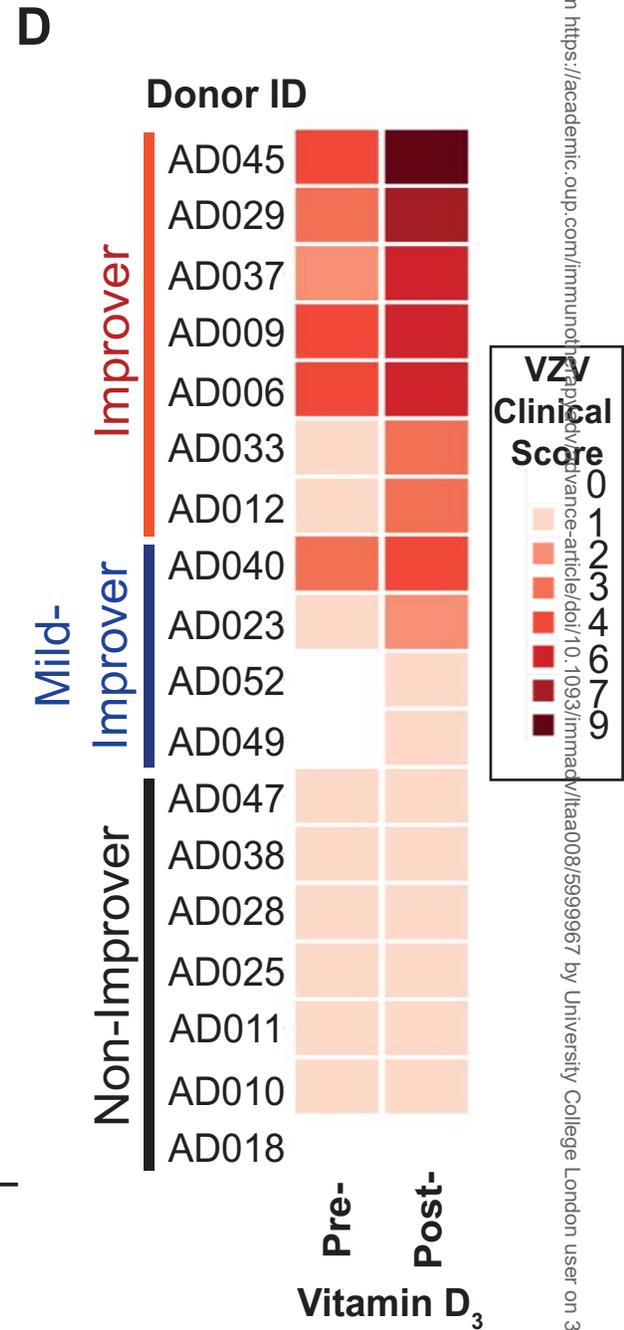
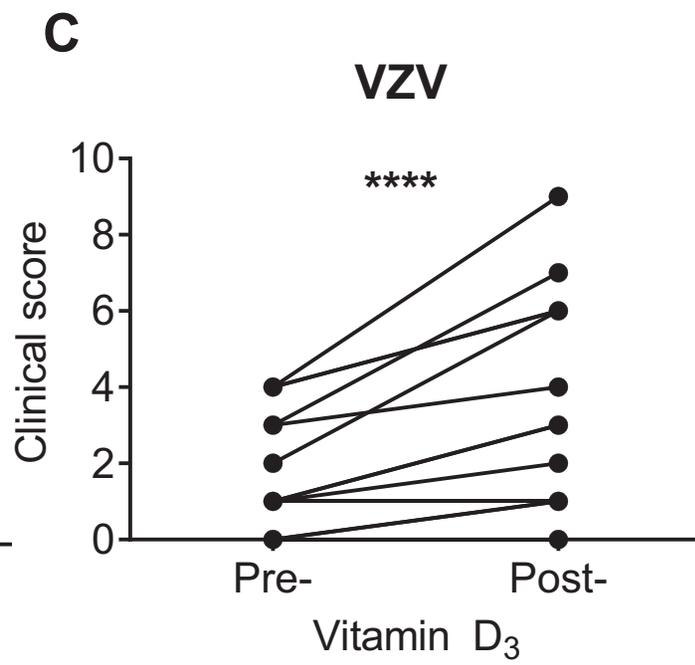
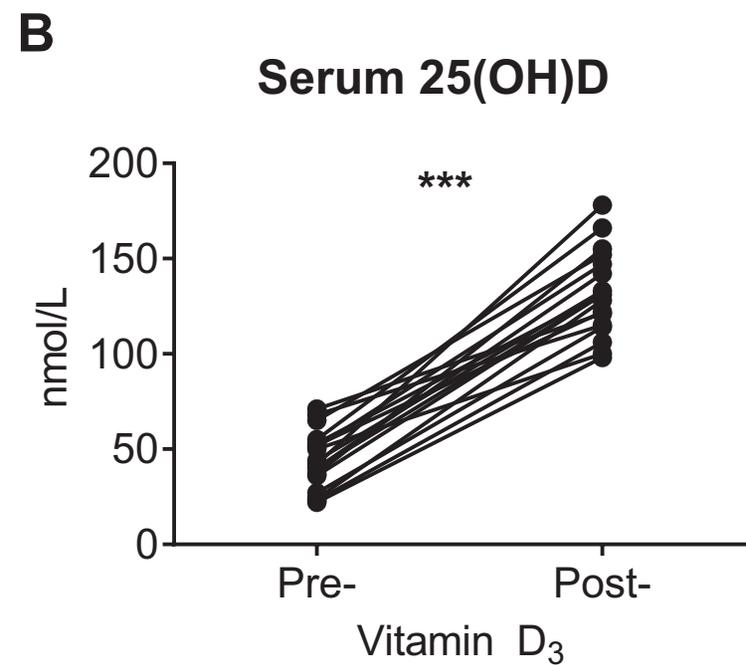
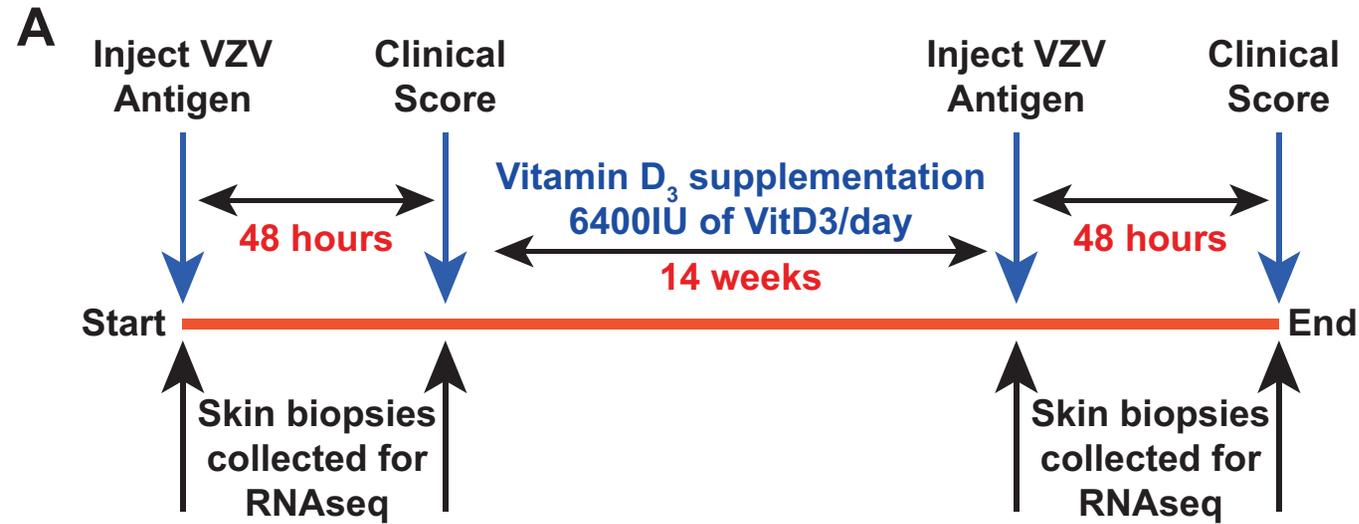
Non-improvers VZV clinical score change of 0, mild improvers VZV clinical score change of 1 and improvers VZV clinical score change of >1 after vitamin D₃ supplementation. Data shown as median ± 10-90 Percentile. Data analysed by Kruskal-Wallis test. *** = p<0.001; ns = non-significant; VZV = varicella zoster virus.

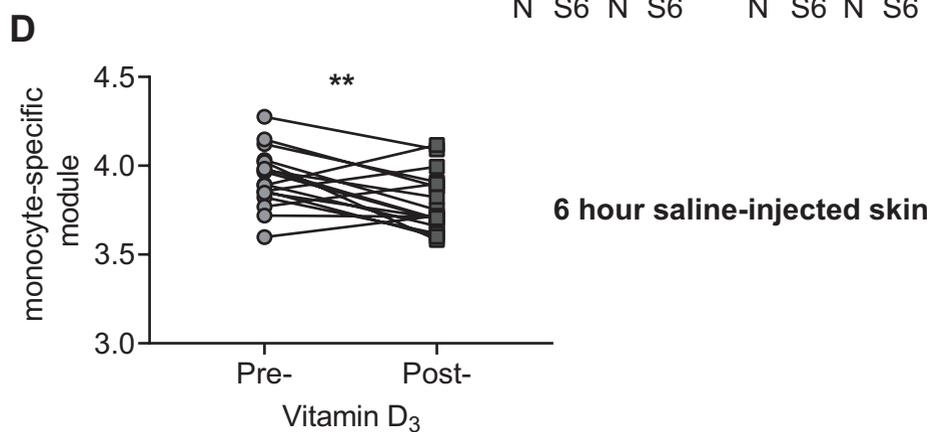
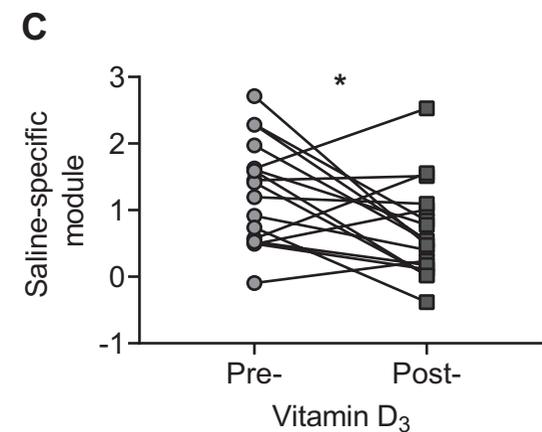
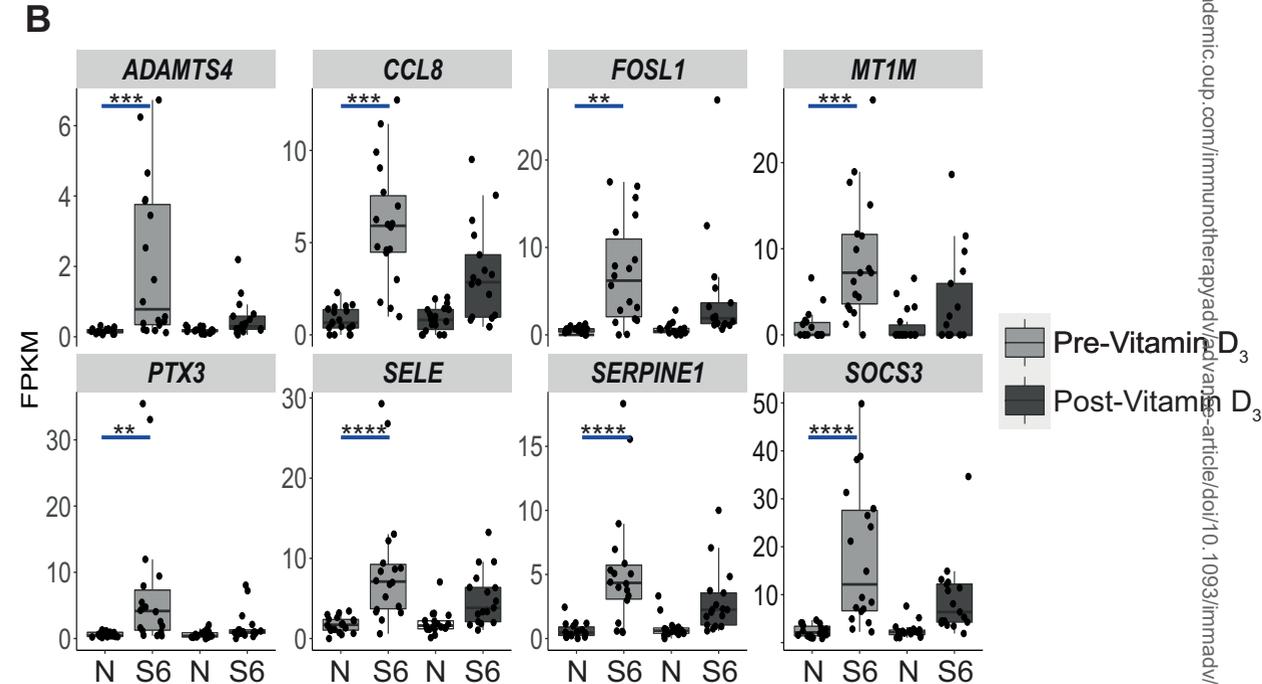
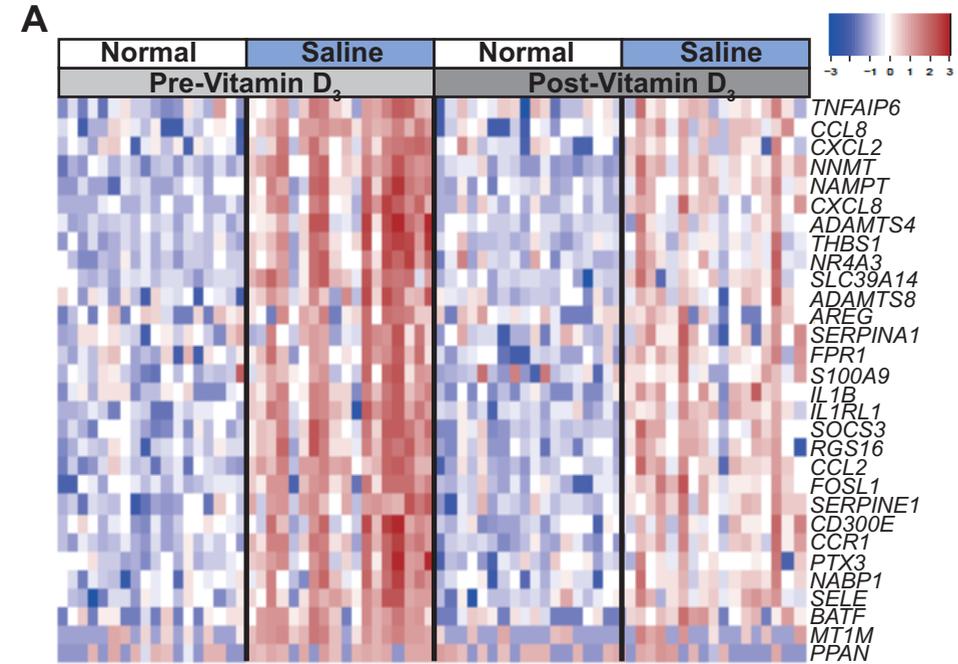
Cytokine	Pre-vitamin D ₃	Post-vitamin D ₃	P value
CCL2	12.1pg/ml (8.47-15.7)	11.6pg/ml (8.67-14.6)	0.99
IL-1β	0.17pg/ml (0.00-0.40)	0.15pg/ml (0.00-0.30)	0.84
IL-6	0.60pg/ml (0.28-0.91)	0.54pg/ml (0.22-0.85)	0.58
IL-8	6.58pg/ml (4.36-8.82)	16.0pg/ml (4.01-27.9)	0.14
IFNα	9.68pg/ml (0.94-18.4)	8.97pg/ml (0.00-18.4)	0.48
TNFα	0.41pg/ml (0.01-0.80)	0.60pg/ml (0.00-1.25)	0.67
CRP	2.42 mg/L (0.00-5.16)	1.98 mg/L (0.87-3.10)	0.73

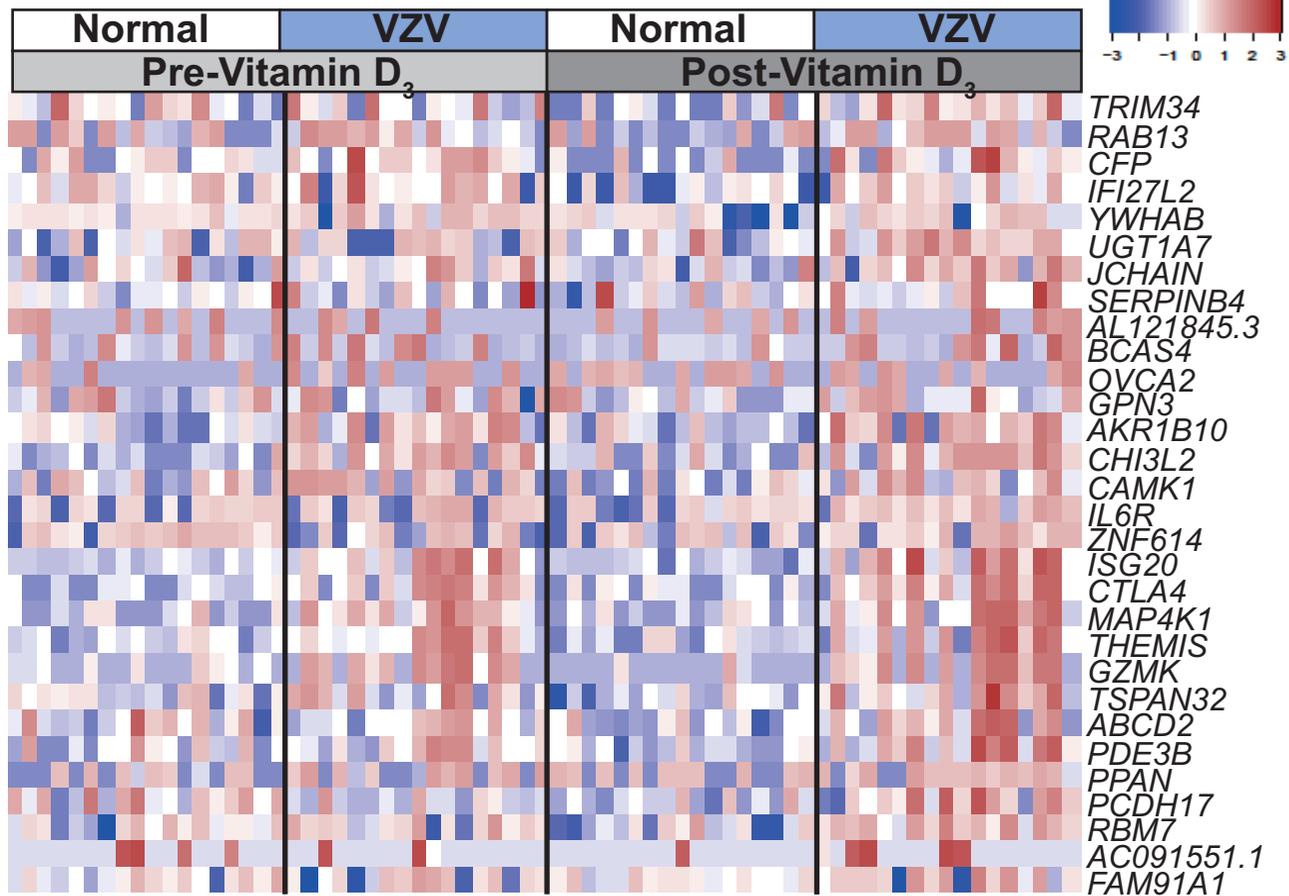
Table 3: Serum inflammatory cytokines pre- and post-vitamin D₃ supplementation

Serum samples were collected pre- and post-vitamin D₃ supplementation (n=18). Cytokine concentrations were assessed by cytometric bead array. Data shown as mean \pm 95%CI. Data analysed by paired t test.

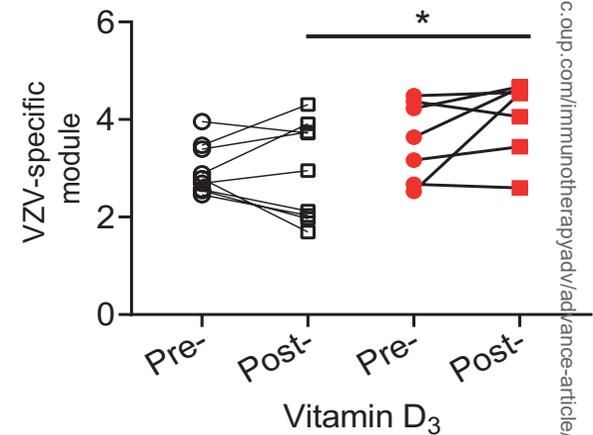






A

48 hour VZV-injected skin

B**C**