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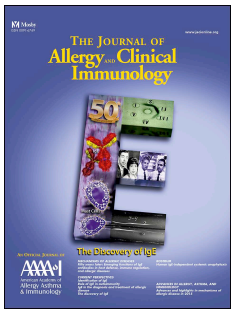

Vitamin D and antimicrobial peptide levels in patients with Atopic Dermatitis (AD) and Atopic Dermatitis complicated by Eczema Herpeticum (ADEH): A Pilot Study

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Vitamin D and antimicrobial peptide levels in patients with Atopic Dermatitis (AD) and Atopic Dermatitis complicated by Eczema Herpeticum (ADEH): A Pilot Study

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Key words: atopic dermatitis, eczema herpeticum, vitamin D, cathelicidin, LL-37, antimicrobial peptide, children/pediatric

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Capsule summary:

In this study, Vitamin D supplementation results in improved clinical severity of atopic dermatitis and increased skin surface LL-37 levels, analyzed by a novel, non-invasive method. Vitamin D supplementation could be a therapeutic option in AD.

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To the Editor:

Atopic dermatitis (AD) is a relapsing condition prone to infections such as Herpes Simplex virus (HSV), resulting in AD with Eczema Herpeticum (ADEH), (a more severe clinical manifestation).[1] Recent medical research has implicated Vitamin D (VD) in AD. [2, 3] It appears essential for skin barrier structure, increasing pro-filaggrin and lipid lamella production.[4] Of interest is the effect of VD on the antimicrobial peptide LL-37 expression,[3] which demonstrates significant anti-viral activity against HSV, and immune modifying characteristics. [5]

Modern research has demonstrated low LL-37 levels in AD and ADEH patients,[6] increasing after VD supplementation.[3, 7] VD deficiency has been inversely correlated with AD severity.[8] Furthermore, a few randomized controlled trials found AD improvement with VD supplementation.[7, 9-12] Despite these major advances, the extent of VD deficiency in AD is unknown.

We conducted a clinical service evaluation at the Sheffield Children's Hospital Dermatology Department to firstly determine the level of VD deficiency in AD children and establish its association with disease severity. Secondly we aimed to establish the effect of VD supplementation on AD, using LL-37 levels as a prognostic marker.

Following approval by the Sheffield Children's Hospital (CA309), AD children were screened for VD deficiency during three summer months. 25 (OH) VD levels were classified as: >75 nmol/L = sufficient, < 75 = insufficient (50-75 nmol/L = suboptimal, <50 nmol/L = deficient).[13] AD children with insufficient 25(OH) VD levels were then assessed clinically on a subsequent visit using SCORAD by a single dermatologist. POEM scores were also determined. LL-37 levels were quantified

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from superficial samples of stratum corneum using novel method described in the supplementary material. This group was supplemented for two months depending upon the level of deficiency and age (cholecalciferol 6000 IU daily in ages 1-12 years; 10,000 IU daily for ages 12-18) for 2 months, as recommended by the British National Formulary. Sub-optimal levels were corrected with over-the-counter (OTC) preparations containing 100% RDA of VD. On the third visit all levels were re-checked and clinical severity reassessed. Patients continued all other topical and oral medications. If in need of new oral treatment, the patient was not included in the final analysis.

Ninety children between the ages of 1 and 18 (mean age 9) attended the dermatology clinic during the period of this clinical audit and underwent screening for VD (Demographics in supplement, TableS1). The majority of patients were receiving topical immunotherapy; eleven were on oral immuno-suppressants for more than one year prior to the study. Baseline 25(OH)VD levels revealed 57 % patients with VD deficiency, and a further 26% with sub-optimal levels, totaling 83% with insufficient VD levels.

ADEH patients comprised 51% of the sample population. Baseline 25(OH) VD levels were significantly lower in ADEH (37± 20 nmol/l) than AD patients (61± 28 nmol/l, p <0.001, two sample t test, Figure 1A). Only two ADEH patients had normal 25(OH) VD levels.

After screening, 18 patients were lost to follow up (Figure S1); 10 patients excluded due to commencement of oral therapy. Consequently, a total of 47 patients were analyzed: 12 AD and 35 ADEH. Patients with normal VD levels were not followed up as part of this audit.

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Using SCORAD, patients were classified into: mild <25, moderate 25-50 and severe >50,[8] and showed a significant difference in 25(OH)VD levels (Figure 1B, means 31±17, 40±15, and 57±21 respectively, p = 0.02, one way ANOVA). Bonferonni's post-test showed a significant difference between mild and severe scores (p = 0.01). A significant inverse relationship was found between 25(OH) VD and SCORAD (p = 0.01, Pearson's r = - 0.36). LL-37 levels were also significantly different between the groups; with the most severe AD patients displaying the lowest levels (Figure 1C, p =0.018, one way ANOVA). Bonferroni's post-test revealed a significant relationship between both mild and moderate (p =0.04), and mild and severe groups (p= 0.01). ADEH children had lower LL-37 than AD children (n=35, mean score 0.4 ±0.5 μg/g; n=12, mean score 0.5 ±0.6 μg/g respectively; p=0.46). Moreover 25(OH) VD levels were found to correlate with LL-37 levels (Figure 1D, r =0.3, p=0.02).

Following a 2-month period of VD supplementation SCORAD and POEM improved significantly with a mean reduction of 42% and 47% respectively (p < 0.001, Figure 2a and b, paired t test). This improvement in severity was accompanied by a significant increase in LL-37 levels (lesional and non-lesional) by 4-fold (therapeutic or OTC) (p = 0.0004, Figure 2c, d and e, two sample t test). The severity of AD was significantly correlated with LL-37 (r= -0.32, p =0.01), suggesting a causal relationship.

VD deficiency is now recognized as a worldwide problem. Recently 35-40 % of healthy UK [14] and US children [15] were VD deficient. A study in Kuwait showed 57% of AD children with less than 50nmol/l.[16] Our study shows VD levels significantly lower in children with moderate and severe AD compared to mild AD, similar to recent studies.[8] Children with ADEH also displayed significantly lower VD levels than those with AD.

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LL-37 levels are up-regulated in wound injury to participate in re-epithelialization.[4] Previous studies have reported low LL-37 levels in AD[3], with further reductions in ADEH.[6, 17] This was echoed here, but not statistically significant, possibly due to the smaller sample size of AD patients (AD =12 vs. ADEH= 35).

VD supplementation has previously been shown to increase lesional and non-lesional LL-37 levels in skin biopsies of AD patients.[2, 3] Moreover RCTs have reported reduced AD severity with VD supplementation.[7, 9-12] In our study, two months VD supplementation significantly improved AD and ADEH severity; LL-37 levels also increased significantly within the stratum corneum. Therefore VD deficiency could lead to a decrease in LL-37, resulting in reduced antimicrobial defense and increased disease severity with secondary infections.

As VD itself has been reported to influence lipid lamellae formation,[4] it could have contributed to improved AD in our cohort by improving permeability barrier function. The discovery of increased *VDR* polymorphisms in AD patients in comparison to healthy controls, suggests an important role of VD in the pathogenesis of AD.[18]

This was a practice evaluation study designed to emulate regular clinical practice not a randomized controlled trial. All medications continued with no constraints. Another limitation of this study is the lack of clinical scores in the AD patients with normal 25(OH) VD levels, to allow for unknown factors that could contribute to clinical improvement. In addition, our study could be underpowered to detect differences due to the sample size. Nevertheless, the significant results of VD supplementation in this study, renders that possibility unlikely.

In conclusion, VD deficiency is common, and could lead to decreased LL-37 levels and increased severity of AD and ADEH. We developed a novel, non-invasive

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method for quantifying LL-37 that simplifies collection, permitting analysis of larger and younger populations. Monitoring of this peptide may be a useful prognostic clinical marker. Further research and larger samples are necessary to fully examine the relationship between VD and LL-37.

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(See supplement for remaining references)

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Figure Legends:

Figure 1: Pre supplementation analysis. **(A)** Baseline VD level comparison. ADEH children (black dots, n=45) with lower VD levels than AD (gray squares, n=45). Levels 50-75 nmol/l (dotted lines) = suboptimal, < 50 nmol/l = deficient. **(B)** AD patients (47) classified into: severe AD (n=13), moderate AD (n= 30), and mild AD (n=4) showed significantly different VD levels **(C)** Baseline LL-37 levels stratified according to SCORAD were significantly different **(D)** VD and LL-37 correlation (*p* = 0.01).

Figure 2: Post-supplementation analysis (n=47)**. (A)** SCORAD reduced 42.3% post-supplementation. **(B)** POEM showed significant reduction (46.6%). **(C)** LL-37 levels increased significantly from mean = 2 ± 0.7 Log10 (LL-37pg/g) to 2.8 ± 0.8 Log10(LL-37pg/g). **(D)** Lesional LL-37 increased from mean = 2.3± 0.7 Log10LL-37pg/g to mean= 3 ± 0.7 Log10(LL-37pg/g). **(E)** Non lesional LL-37 increased from mean = 2.3 ± 0.6 Log10(LL-37pg/g) to 2.7 ± 0.8 Log10(LL-37pg/g).

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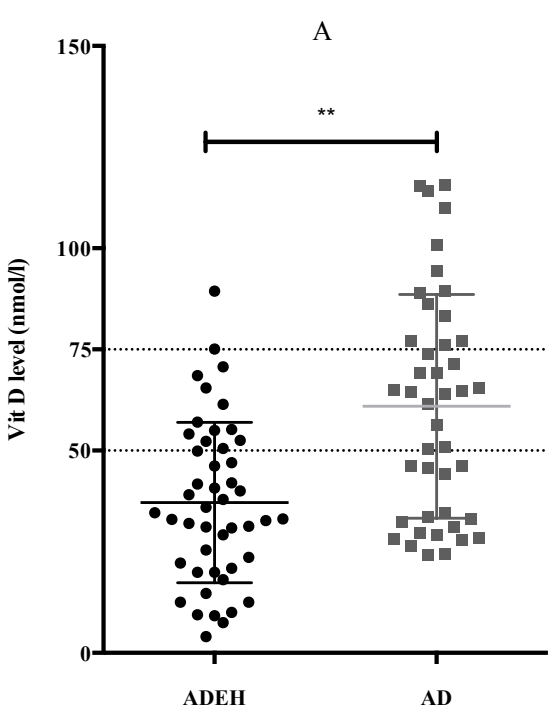
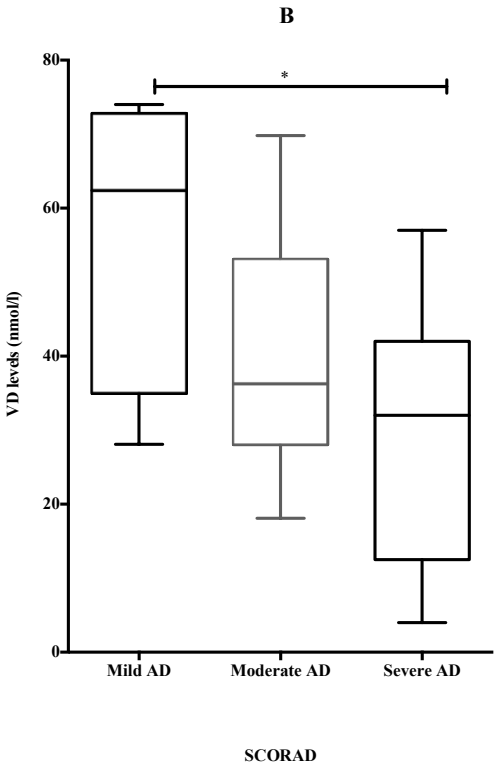
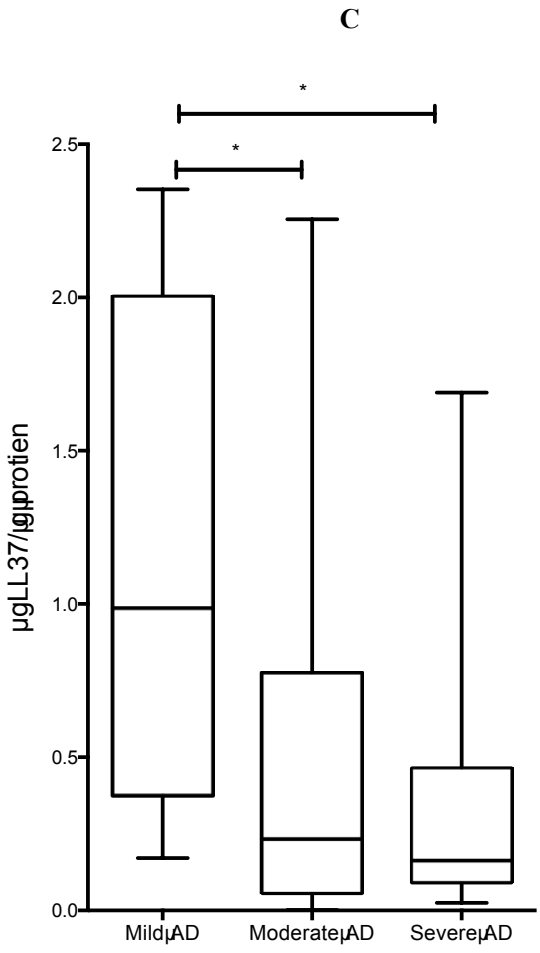
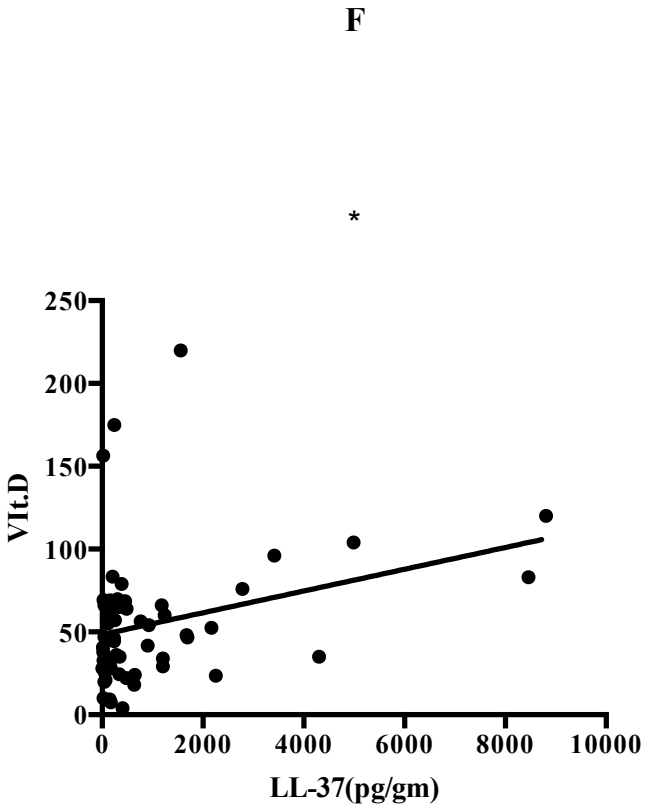
Demographics of the AD children

Characteristics	Initial AD children screened (n=90)	AD children included in study (n=47)
Age	mean age=9 (1-18)	Mean age=11(1-18)
Gender:		
Females	47 (52%)	21 (45%)
males	43 (48%)	26 (55%)
Ethnicity:		
Caucasian	57(63%)	25 (53%)
Asian	24 (27%)	14 (30%)
Chinese	3 (4%)	3 (6%)
African	2 (2%)	1 (2%)
mixed	4 (4%)	4 (9%)
Clinical classification:		
AD	45 (50%)	12 (26%)
ADEH	45 (50%)	35 (74%)
Healthy 25(OH)VD levels	15 (17 %)	
Unhealthy 25(OH)VD levels:	75 (83%)	47
- Sub optimal 25(OH)VD (50-75nmol/l)	23 (26 %)	12 (25%)
- Deficient 25(OH) VD (<50nmol/l)	35 (39%)	23 (50%)
- Severely deficient VD (<25nmol/l)	17 (18%)	12 (25%)


A
**
Vit D level (nmol/l)
150
100
75
50
0
ADEH
AD


B
*
VD levels (nmol/l)
80
60
40
20
0
Mild AD
Moderate AD
Severe AD
SCORAD


C
*
*
µgLL37/µgprotien
2.5
2.0
1.5
1.0
0.5
0.0
MildµAD
ModerateµAD
SevereµAD
SCORAD


F
*
VIt.D
250
200
150
100
50
0
0
2000
4000
6000
8000
10000
LL-37(pg/gm)

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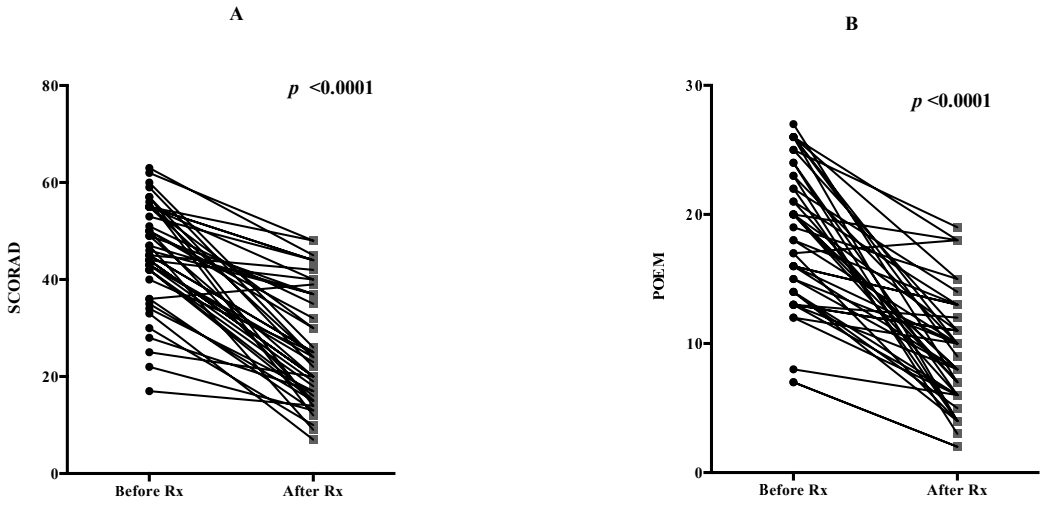
A
p <0.0001
SCORAD
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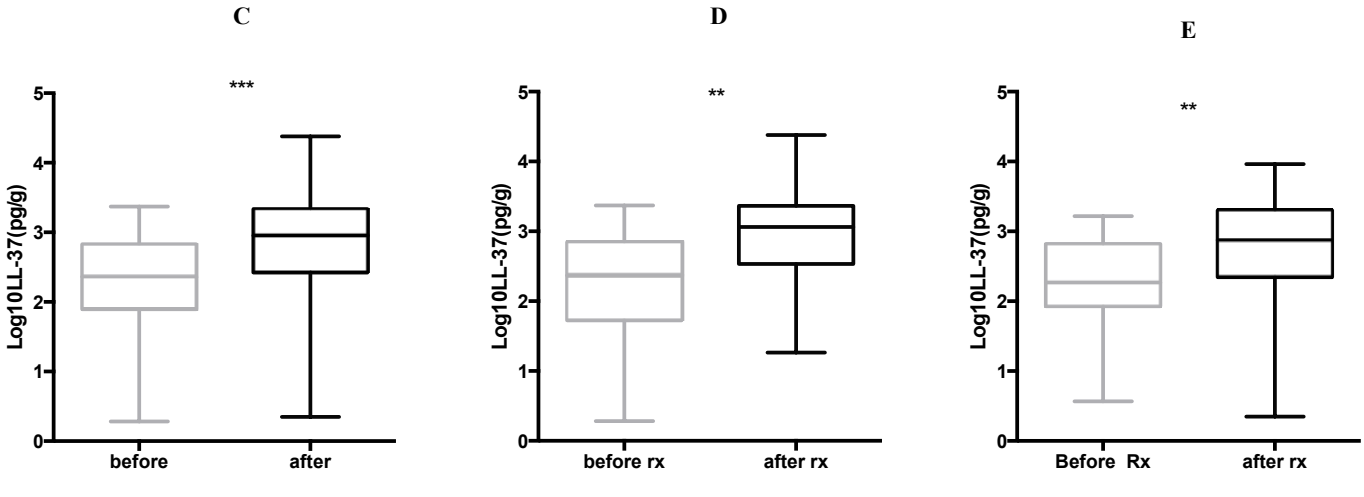
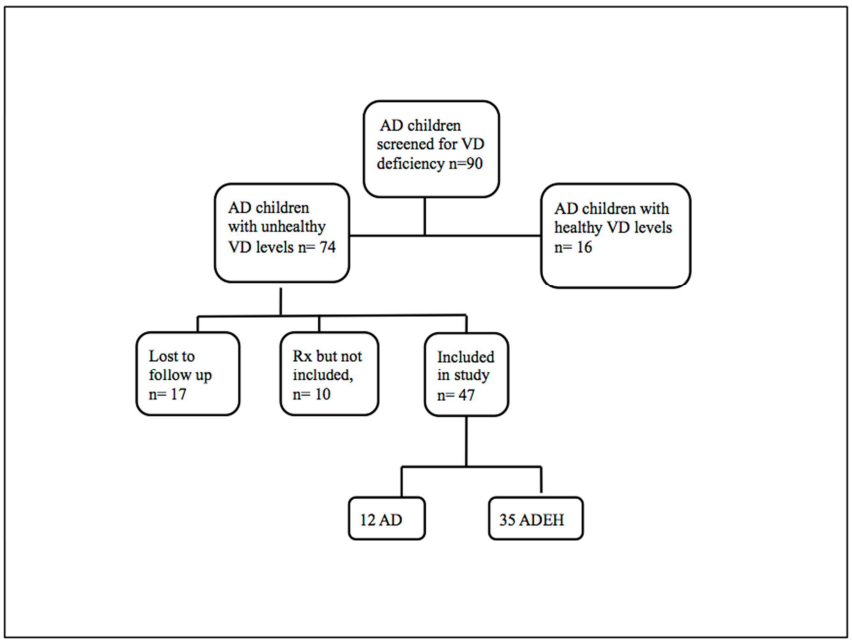
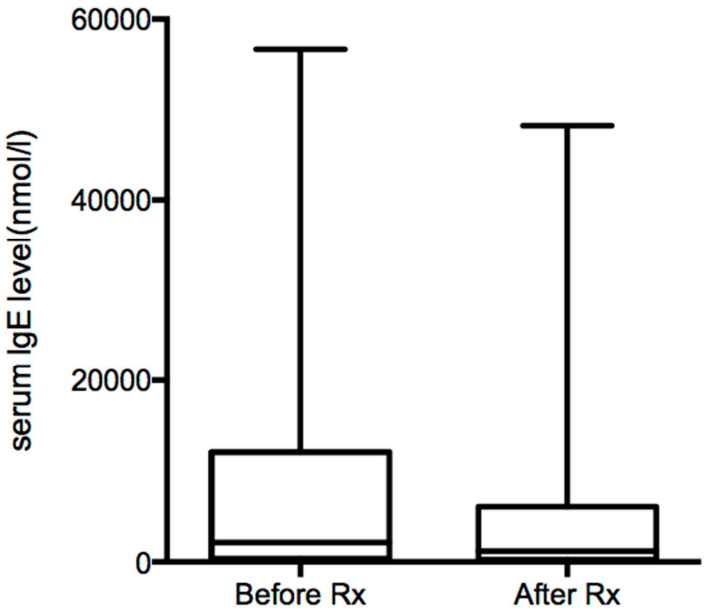
60
40
20
0
Before Rx
After Rx
B
p <0.0001
POEM
30
20
10
0
Before Rx
After Rx


C
***
Log10LL-37(pg/g)
5
4
3
2
1
0
before
after
D
**
Log10LL-37(pg/g)
before rx
after rx
E
**
Log10LL-37(pg/g)
Before Rx
after rx


AD children screened for VD deficiency n=90
AD children with unhealthy VD levels n= 74
AD children with healthy VD levels n= 16
Lost to follow up n= 17
Rx but not included, n= 10
Included in study n= 47
12 AD
35 ADEH



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Supplementary Information

Materials and Methods

Sample collection and quantification of LL-37

Skin cells were collected using non-invasive cytology brushes (Cytotak brushes, Medical Wire Co., UK) from lesional and non-lesional sites of the VD deficient patients. Three brushes were gently brushed against the skin surface, placed in 1.5 ml tube, and frozen at – 80°C. The samples were extracted in 800µl buffer (10 mM disodium phosphate pH 7.4, 0.2% sodium dodecyl sulphate, 0.5% propylene glycol) containing protease inhibitors (Complete mini, Roche, Germany) for 30 minutes with sonication (Ultrawave Ltd. UK). The LL-37 levels were determined using the Human LL-37 ELISA kit (Hycult, The Netherlands), and expressed as a proportion of total protein, determined by Bicinchoninic Acid (BCA) analysis (Sigma-Aldrich, UK).

Statistical Analysis

GraphPad Prism (UK) was used for statistical analysis. AD and ADEH groups were analyzed by two sided two-sample t-test. Scatter plot of AD vs ADEH shows mean and standard deviation (SD). One-way analysis of variance (ANOVA) was used to compare VD levels and LL-37 levels stratified by clinical severity. Post-hoc comparisons were analyzed with Bonferroni's multiple comparisons test. Boxplot graphs show midline to represent median; boxes represent the 25^{th} and 75^{th} quartile, and whiskers represent the minimum to maximum values. Pearson's analysis was used for correlations between LL-37, VD levels, and SCORAD. SCORAD and POEM measurements collected before and after VD supplementation were analyzed by paired t-test. LL37 levels collected before and after were analyzed by two-sample t test. Level of significance was set at 0.05. All values were expressed as mean ± standard deviation.

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TableS1: Demographics of sample population. n=90 the total amount of children screened initially. n=47 the amount of children entered into the practice evaluation study, supplemented and clinically evaluated.

Figure S1: Flowchart of study. 90 AD children screened for VD deficiency and classified into AD and ADEH groups on initial consultation. Of these 90, 15 were found to have normal VD levels, 75 had low VD levels. Of the 75 AD children, 18 lost to follow up and 10 excluded. Therefore 47 children were included in the practice evaluation study. These 47 children were clinically assessed and supplemented.

Figure S2: IgE level did not show significant change from mean score 7010 ± 2370 nmol/l to 7824 ± 3221nmol/l (p =0.93, unpaired t-test).

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