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***THE EFFECT OF VITAMIN D
SUPPLEMENTATION ON ATOPIC
DERMATITIS***

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To my husband; my white knight; my hero

*Without his love and support, especially during these two years, I would
not have come so far.*

*To my kids, Tamara, Roosha and Habi, for always making my world a
wonderful, happy mess.*

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ABSTRACT

Atopic Dermatitis (AD) is recognized as one of the major worldwide health concerns. Despite being first described in the nineteenth century by Besnier, management options continue to be limited and are primarily palliative. AD patients are susceptible to many infections, such as the Herpes simplex virus (HSV), resulting in a more serious clinical subgroup of patients with AD complicated by Eczema Herpeticum (ADEH).

Supplementation with vitamin D (VD) has shown positive effects on the clinical outcome of AD in previous randomized controlled trials and clinical studies which could be due, in part, to the up regulation the antimicrobial peptide LL-37, which has known antimicrobial, immune-modulatory and wound healing effects.

Objective: We sought to determine the level of VD deficiency in AD and ADEH patients in a pediatric dermatology clinic, to investigate whether there was a difference in baseline VD levels between AD and ADEH, to examine skin surface LL-37 levels in both and to establish whether VD supplementation would result in clinical improvement.

Methods: A practice evaluation study was performed at the Sheffield Children's Hospital. All AD patients were screened for VD deficiency. Those that were found deficient were assessed using SCORAD and POEM. Skin surface cells were collected from lesional and non-lesional sites using a novel non-invasive technique for LL-37 evaluation. In addition, serum IgE levels and serum calcium levels were also

checked. Supplementation was then commenced for two months after which clinical severity was reassessed and all levels were rechecked.

Results: Ninety children (mean age 9) were screened for VD deficiency; 83 % had low VD levels. VD levels were significantly lower in ADEH children than AD children ($p < 0.0001$). Baseline SCORAD was also significantly higher in ADEH patients ($p < 0.0001$). Skin surface LL-37 levels were reduced in ADEH patients in comparison to AD patients ($p = 0.46$), and were significantly reduced in patients with severe AD (SCORAD >50) in comparison to patients with moderate (SCORAD 25-50) or mild AD (SCORAD <25 ; $p=0.018$). Two months of VD supplementation resulted in significant improvement of SCORAD ($p <0.0001$) and POEM ($p < 0.0001$) in both groups; LL-37 levels also increased significantly ($p = 0.0004$).

Conclusions: VD deficiency was found to be common in AD children, and VD supplementation reduced ADEH and AD severity in children. Part of the explanation is through increased LL-37 production.

Clinical implications: All AD patients should be screened for VD deficiency and supplemented accordingly, which has become routine clinical practice at the Sheffield Children's Hospital.

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LIST OF ABBREVIATIONS:

AD= Atopic Dermatitis

ADEH= Atopic Dermatitis complicated by Eczema Herpeticum

HSV= Herpes Simplex Virus

VD= Vitamin D

EASI= Eczema Area and Severity Index

SCORAD= SCORing of AD

POEM= Patient Oriented Eczema Measure

K= Keratin

SC= Stratum Corneum

KLK= Kallikrien

SPINK-5= Serine Protease Inhibitor Kazal-5

LEKTI= Lympho-Epithelial Kazal-Type Inhibitor

AMP= Anti Microbial Peptide

hCAP-18= human Cationic Antimicrobial Peptide (inactive precursor)

S. aureus= *Staphylococcus aureus*

LPS= Lipopolysaccharides

TNF= Tumour Necrosis Factor

TLR= Toll-like Receptor

PAMP= Pathogen Associated Molecular Patterns

Tregs= Regulatory T lymphocytes

TGF= Transforming Growth Factor

DC= Dendritic cells

VD₂= Vitamin D₂ or ergocalciferol

VD₃= Vitamin D₃, or cholecalciferol

DBP= Vitamin D binding protein

VDR= Vitamin D Receptor

VDRE= Vitamin D Response Element

CaR= Calcium Receptor

CAMP= Cathelicidin Anti Microbial Peptide

OTC= Over The Counter

RCT= Randomized controlled Trials

RDA= Recommended Daily Allowance

Chapter One: Introduction

I. Chapter one: Introduction

1.1. Atopic Dermatitis

Atopic dermatitis (AD) is a common, persistent inflammatory disease of the skin with a prevalence of 9-20% worldwide (ISAAC 1998). In Kuwait it affects 31% of children (Nanda, Al-Hasawi et al. 1999). AD is characterized by dry, extremely pruritic skin, which causes the person to scratch. This leads to the appearance of erythematous scaly patches, excoriations and lichenification. AD severity can range from a few xerotic patches on the skin, to involvement of the whole body causing intense psychological distress. It is also typically very fluctuant in its course, rendering the diagnosis uncertain in some cases (Williams 2000).

The location of the lesions in AD depends upon the age of the patient (Remitz and Reitamo 2008). In infants, it affects predominantly the cheeks, wrists and extensor surfaces of the arms and legs. In older children, it is mainly seen on the ante-cubital and popliteal fossae, as well as the nape of the neck. Peri-oral and peri-ocular involvement is also known to occur. Food allergies are found to be important triggers during infancy, whereas as the child gets older, other factors such as climate and stress become more important (Remitz and Reitamo 2008).

In 1980, Hanifin and Rajka introduced a series of criteria for the diagnosis of AD (Hanifin and Rajka 1980). These consisted of four major criteria and over twenty minor ones. The list, however, contained invasive tests such as serum IgE levels and skin prick tests, and was therefore too complex to use on a daily basis.

In 1994, a refined list was agreed upon (Williams, Burney et al. 1996), which proved to be easier for clinicians and epidemiological studies (Box 1).

In order to qualify as a case of Atopic Dermatitis, a child must have:

An itchy skin condition

Plus three or more of the following:

1. Onset below the age of two
2. History of flexural involvement
3. History of dry skin
4. Personal history of other atopic diseases or family history of first degree relative

Box 1. The refined Hanifin and Rajka diagnostic criteria for Atopic Dermatitis.

Over the years, many have suggested different diagnostic criteria and scoring systems for AD (Ricci, Dondi et al. 2009). There are now more than twenty different scoring systems; the Nottingham Eczema Severity Score, Eczema Area and Severity Index (EASI), Severity Scoring of Atopic Dermatitis index (SCORAD), and Patient- oriented Eczema Measure (POEM) are the most recognized (Williams and Grindlay 2010).

A recent study examined all these scoring systems, with the conclusion that only SCORAD, EASI and POEM are the only systems that have been tested sufficiently for use (Schmitt, Langan et al. 2007).

1.II. Atopic Dermatitis complicated by Eczema Herpeticum (ADEH)

AD patients are particularly prone to many types of infections. One of the most prominent of these infections is the Herpes Simplex virus (HSV). This results in a subgroup of AD patients with Eczema Herpeticum (ADEH) presenting a more severe clinical manifestation (Beck, Boguniewicz et al. 2009), and could be more challenging to diagnose and treat.

ADEH was first described in the 19th century by Moritz Kaposi (Kaposi 1887). It presents as a monomorphic, vesiculo-papular eruption commonly affecting the head, trunk and neck (Wollenberg, Wetzel et al. 2003). It can vary in severity from generalized body dissemination to few vesicular clusters, rendering the diagnosis difficult in some patients (Frisch and Siegfried 2011). Complications of ADEH include kerato-conjunctivitis, viremia and meningitis (Wollenberg, Wetzel et al. 2003), and historically was associated with a 75 % mortality rate before use of antiviral treatment (Sanderson, Brueton et al. 1987).

1.II Pathogenesis of AD: “Genes vs. Environment”

Genetic predisposition is undeniably an important factor for the development of AD. It seems to be responsible for 25-50% of one's predisposition to develop AD, dependant upon whether single or multiple family members are involved (Williams 2000). Indeed, some studies have shown that AD shows a monozygotic concordance rate of 77% (Schultz Larsen 1993), and as high as 84.6% (Yilmaz-Demirdag, Prather et al. 2010). Dizygotic twins also show a strong concordance of 62.5% (Yilmaz-Demirdag, Prather et al. 2010). Individuals were found to have a seven times increased risk of developing AD if a monozygotic sibling was affected; three times increase if dizygotic (Thomsen, Ulrik et al. 2007).

Having a positive family history for atopy has always been used to identify high-risk individuals (Hanifin and Rajka 1980; Williams, Burney et al. 1996). Maternal atopy seems to be the strongest influence (20-26%), and atopy in a single first degree relative was found responsible for 76% of those developing AD at two years of age (Arshad, Kurukulaarachy et al. 2008). Sandini and co-workers (2011) found a 1.75 increased odds ratio of developing AD at the age of two in a child with both parents having atopy compared with children with only one parent affected. The discovery of a link between variants of the filaggrin gene, encoding the structural protein of the skin, and the development of AD certainly emphasizes the importance of genetics (Palmer, Irvine et al. 2006). Research has shown that loss of function mutations in the filaggrin gene is present in up to 50% of all AD patients, depending upon clinical severity (Osawa, Akiyama et al. 2011).

The prevalence of AD, however, has increased meteorically within the past five decades. Studies show that the cumulative incidence of AD in children born before the year 1960 is less than three percent, a further increase to 4-8% for children born between 1960-1970, rising even more to over 12% for children born afterwards (Williams 2000). The ISAAC study identified the increase in atopy to be consistent worldwide, varying from 0.3% to 20.5% (ISAAC 1998). In Kuwait, AD has an incidence of 31% (Nanda, Al-Hasawi et al. 1999). Since genetics cannot have altered in those years to such an extent, scientists have turned to the environmental factors in an attempt to explain this increase.

Brunello Wuthrich first recognized and classified AD into two subtypes: extrinsic and intrinsic AD (Wuthrich, Cozzio et al. 2007). Extrinsic AD was described as having elevated levels of total IgE, positive levels of specific IgE to common aero and food allergens, positive skin prick tests to these allergens, and respiratory involvement. Intrinsic AD was defined as having total IgE levels less than 200 kU/l, negative specific IgE and skin prick tests, and no asthma. This set off the eternal debate between allergists, scientists, and clinicians whether to regard these subtypes as two separate entities, or simply two phases within the AD spectrum (Bieber 2010).

The latter was the conclusion reached by Folster-Holst *et al* in Germany (Folster-Holst, Pape et al. 2006). Out of over 200 patients with AD, only 18 patients were found to have 'intrinsic' AD. After a median follow up of 7.5 years, four of those patients also developed respiratory symptoms, although IgE levels did not rise above 200 kU/l.

The World Allergy Organization recently defined atopy as being only in association with increased IgE levels (Johansson, Bieber et al. 2004), thus they defined the extrinsic type to be “true AD”, and the intrinsic as “non-atopic AD” (Bieber 2010). In the small proportion that never progress to “true AD”, however, it is reasonable to believe that there is also an abnormality in the skin barrier that contributed to the manifestation of AD (Cork, Robinson et al. 2006). This is reinforced by the finding that uninvolved skin in all AD patients, both true and non-atopic, is still relatively thinner (White, Jenkinson et al. 1987) than healthy people. In addition, the skin barrier still displays abnormal properties even after five years of having clear skin (Elias 2008).

I.III The Epidermal Barrier

I.III.I Epidermal differentiation

Epidermal thickness ranges from as thin as 54 μm in the eyelids, to 637 μm thick in the soles of the feet (Kusuma, Vuthoori et al. 2010). The postauricular and popliteal fossae have also been found to be thin (Lee and Hwang 2002; Cork, Danby et al. 2008). Notably, these areas, with a naturally thin epidermis, are commonly affected in AD patients.

The keratinocyte is the major cell in the epidermis, and it undergoes major changes during differentiation (Candi, Schmidt et al. 2005) (Figure I.1). This process is tightly regulated by various factors, the most important of them being the calcium gradient, discussed later in greater detail (Bikle, Oda et al. 2004).

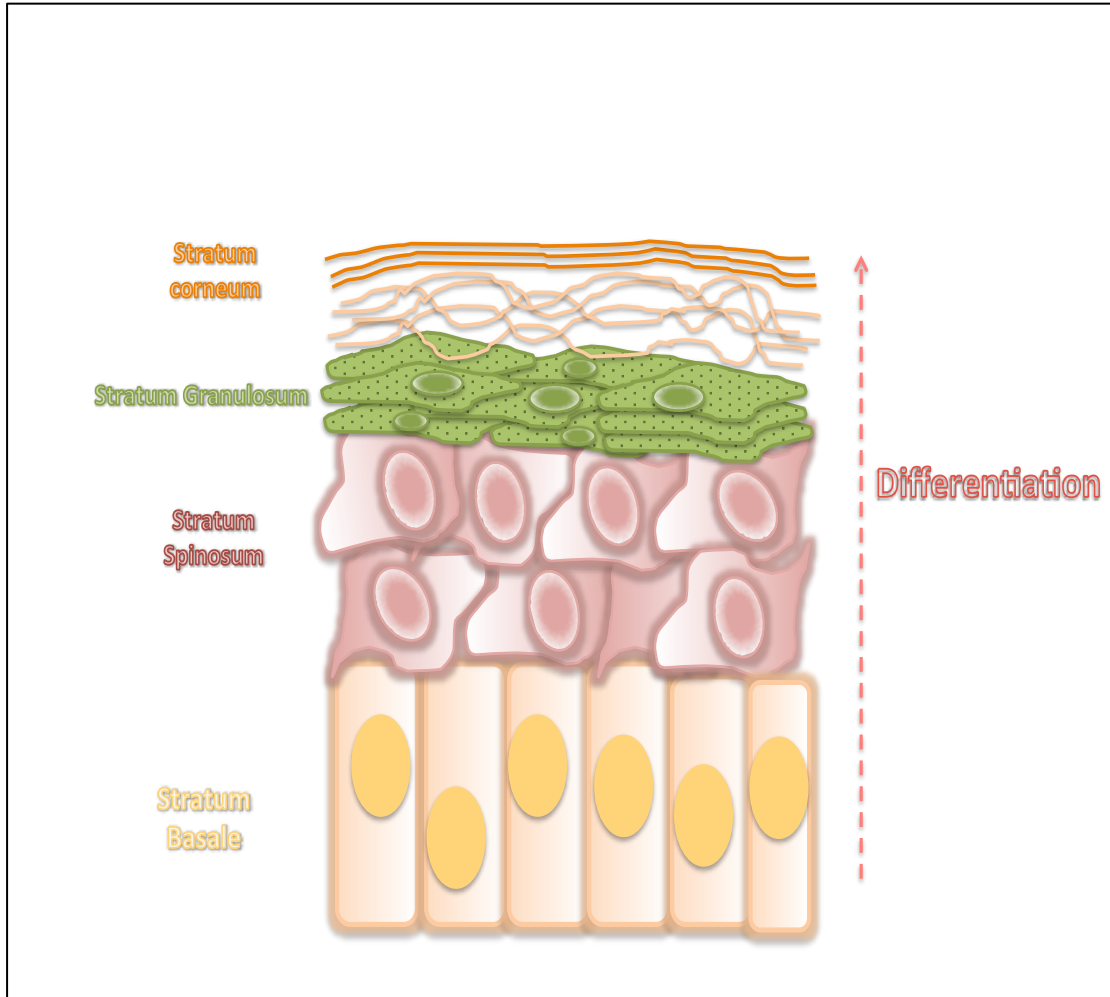


Figure I. 1: Layers of the Epidermis

As it migrates from the basal germinal layer (stratum basale), the keratinocyte changes from columnar and nuclear, gradually becoming polygonal and granular at the stratum granulosum level. (Candi, Schmidt et al. 2005). Eventually it extrudes the nucleus, and at the stratum corneum (SC), the keratinocyte is anucleate and flat, becoming a “corneocyte” (Rito and Pineiro-Maceira 2009).

Keratins 5 and 14 are the main structural proteins in the keratinocytes at the basal layer, and are organized into microfilaments. They are involved in forming the cytoskeleton of the cell, along with microtubules and microfilaments (actin) (Candi, Schmidt et al. 2005).

As the keratinocyte differentiates, K5 and 14 are replaced by K1 and 10 at the stratum spinosum (Strelkov, Herrmann et al. 2003). The structural protein involucrin is also synthesized at this level. At the stratum granulosum, loricrin and trichohyalin, along with the filaggrin precursor, profilaggrin are all synthesized (Bikle 2012). Profilaggrin is subsequently cleaved to liberate filaggrin molecules (Chatterjea, Resing et al. 2011), which then aggregates the keratin fibers into tight bundles at the stratum corneum (SC) level, changing the shape of the keratinocyte cell into a flat “corneocyte” (Kalinin, Marekov et al. 2001).

In addition, the plasma membrane of the keratinocytes becomes reinforced at the SC level with the afore-mentioned structural proteins, involucrin, trichohyalin and loricrin, which are then cross-linked together with filaggrin, by enzymes called transglutaminases. This results in the formation of a tough, water impermeable case around the cell, referred to as the cornified envelope. This cornified envelope acts as a scaffold for the formation of an outer lipid envelope (Ishida-Yamamoto 1998; Candi, Schmidt et al. 2005).

Cell-cell adhesion in the epidermis

There are several types of junctions found in the many layers of the epidermis (Figure 1.2): tight junctions, adherens junctions/desmosomes and gap junctions (Candi, Schmidt et al. 2005). Two types of cell junctions in the epidermis are found to be responsible mainly for cell cohesion: adherens junctions and desmosomes.

Adherens junctions mainly connect the actin filaments of adjacent cells together (Proksch, Brandner et al. 2008). E-cadherin, an important protein involved in the composition of adherens junctions, has shown reduced expression in spongiotic lesions, which is characteristic of AD lesions (Ohtani, Memezawa et al. 2009). Desmosomes are involved in connecting the keratin filaments of neighboring cells, and will be talked about in more detail later.

Tight junctions are cell-cell junctions formed of trans membranous proteins such as claudins and occludins (Proksch, Brandner et al. 2008). They possess many functions; they are involved in controlling the para-cellular pathway of various molecules and inflammatory cells, known as the barrier function. In addition, they function in preventing intramembranous diffusion of contents between the apical and basal sides of the cell membrane, referred to as the fence function. They are also involved in cell proliferation and differentiation, and are seen to be synthesized before the SC during epidermal regeneration (Brandner 2009). The expression of tight junctions, specifically claudin-1, were found to be significantly reduced in AD patients (De Benedetto, Rafaels et al. 2011).

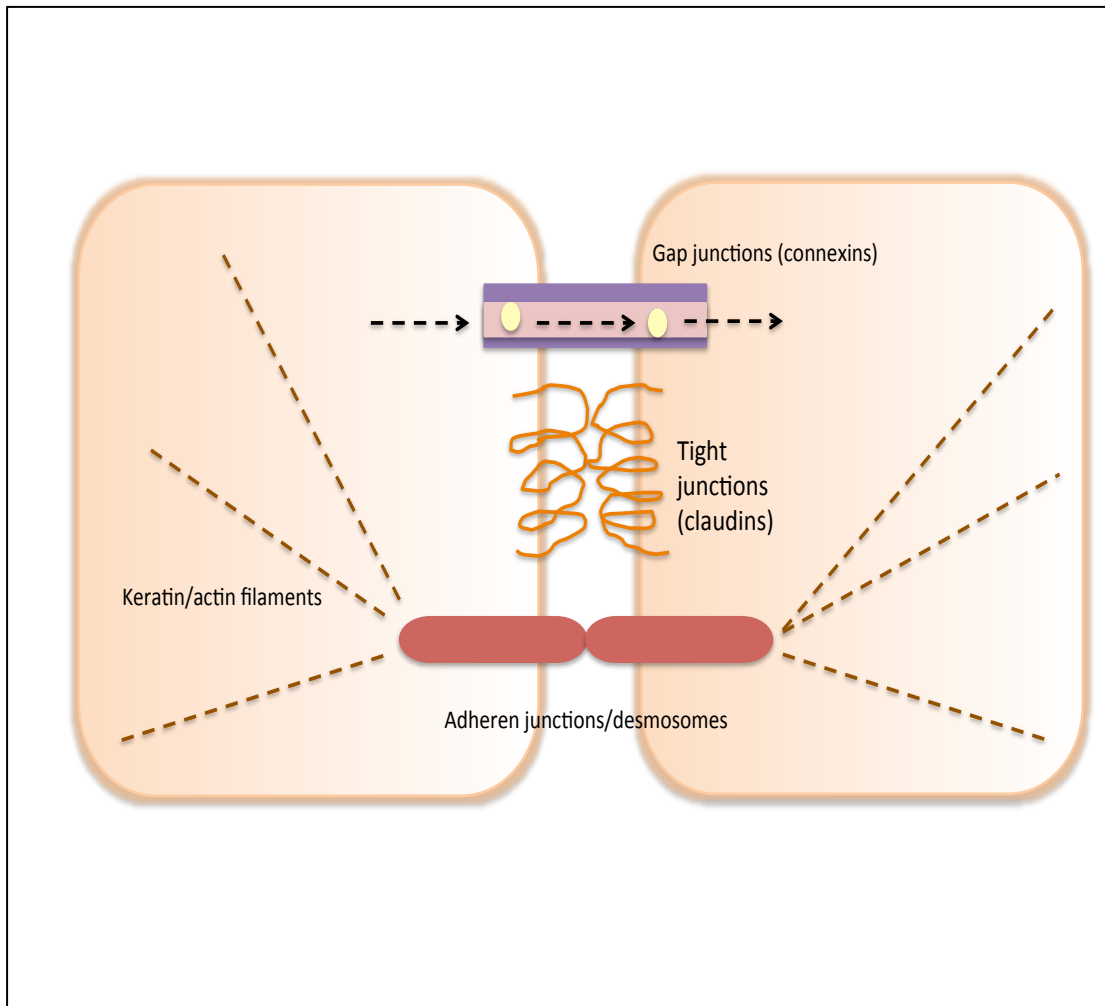


Figure I. 2: Schematic diagram of the different junctions within the epidermis.

Several junctions contribute to epidermal cohesion and stability. Gap junctions function as channels allowing intercellular exchange. Adherens junctions and desmosomes are mainly responsible for intercellular linkage. Tight junctions, have many functions. They allow paracellular passage of molecules, separation of the cell membrane layers, and are involved in epidermal regeneration.

Gap junctions are mainly involved in intercellular transport of molecules, such as calcium and magnesium, between adjacent cells (Proksch, Brandner et al. 2008), and are formed from the trans membranous protein molecules called connexins. Thus they are believed to have a major regulatory role in cell differentiation (Jensen and Proksch 2009). Interestingly, AD patients have demonstrated increased expression of connexin-26 (De Benedetto, Yoshida et al. 2012), which is associated with delayed skin barrier recovery in similar models (Djalilian, McGaughey et al. 2006).

I.III.II The epidermal barrier

The epidermal barrier is at the lower part of the SC, acting as a shield from various environmental influences (Cork, Danby et al. 2008). It serves as a protector from chemical and physical forces (Proksch, Brandner et al. 2008). It also functions as an antimicrobial barrier against infections (Elias, Hatano et al. 2008). Furthermore, it regulates water diffusion from the skin into the environment, which is known as trans epidermal water loss (TEWL)(Elias 2005).

The “brick and mortar” analogy has become very popular (Figure I.3). In this analogy, the corneocytes are the building blocks or “bricks”, and the “mortar” is the lipid sheets in between called the lipid lamellae (Elias 1983). Others have broadened the comparison, likening the specialized junctions that bind the corneocytes together, corneodesmosomes, to iron rods passing through the bricks (Cork, Robinson et al. 2006).

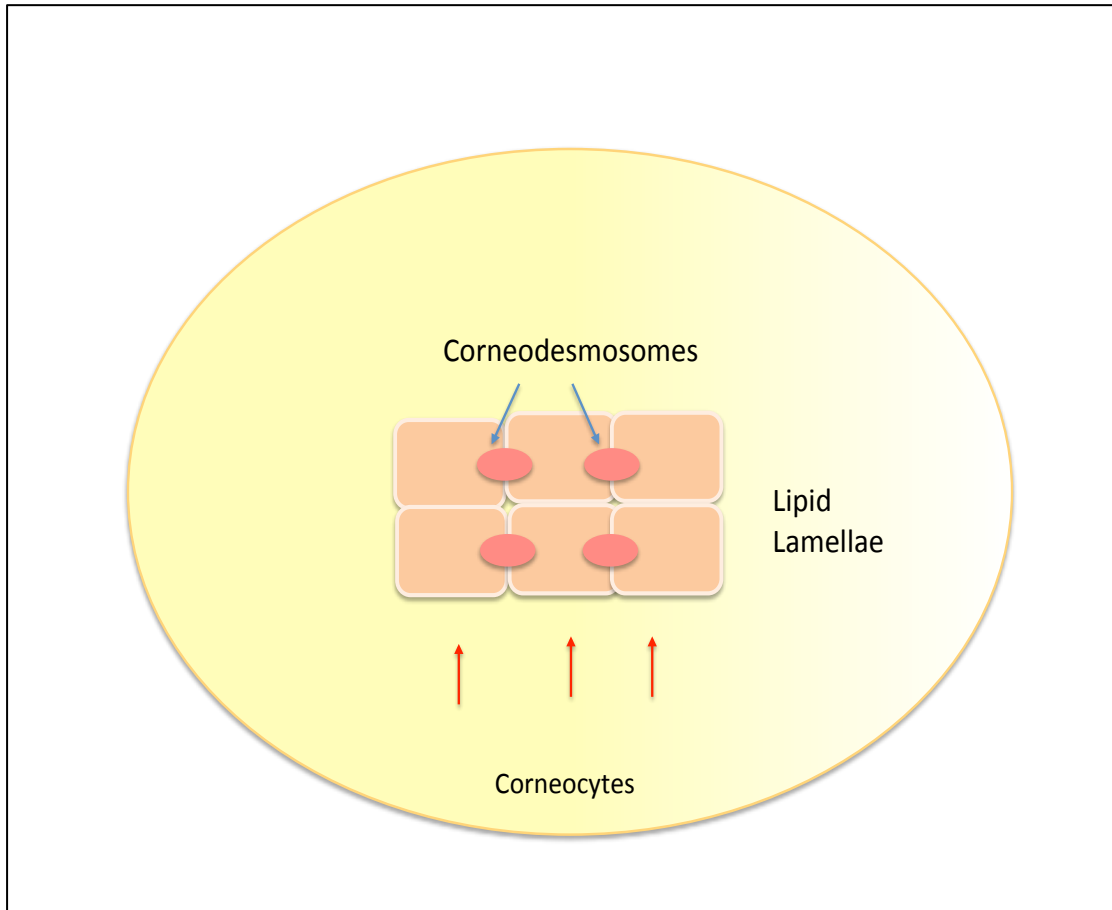


Figure I. 3: Structure of the skin barrier.

The skin barrier is formed of corneocytes that are bound together with corneodesmosomes to form the cornified envelope (Ishida-Yamamoto 1998). The lipid lamella is a lipid rich substance encasing these corneocytes (Elias 1983).

A. Filaggrin “reinforcing the brick”

Filaggrin can be considered one of the key elements in the formation of the cornified envelope. It was identified in 1977, and was termed ‘filaggrin’ for filament aggregating protein (Dale, Lonsdale-Eccles et al. 1980). It is derived from profilaggrin, which is a histidine rich polypeptide forming the keratohyalin granules located in the cytoplasm of cells in the stratum granulosum (O'Regan and Irvine 2008). At the uppermost layer of the granular layer, profilaggrin is cleaved to form filaggrin in a process involving the protease caspase 14 (Kamata, Taniguchi et al. 2009; Hoste, Kemperman et al. 2011).

It then proceeds, in the lowermost two layers of the SC, to aggregate the keratin bundles, at the end of which it disintegrates into hydrophilic amino acids. These amino acids then become incorporated into what is called Natural Moisturizing Factor (NMF). NMF is important for water retention in the corneocytes, preserving moisture and giving the corneocytes flexibility (Cork, Danby et al. 2009). It also aids in modulation of the SC pH, important for regulating protease activity and synthesis of the lipid lamellae (Rippke, Schreiner et al. 2004; Hachem, Roelandt et al. 2010).

Several structural defects of epidermal differentiation have been found in AD, the most prominent of them affecting filaggrin (Palmer, Irvine et al. 2006). Filaggrin mutations were first identified in Ichthyosis Vulgaris, a disease characterized primarily of dry, flaky skin and hyperlinearity of the palms and soles with seasonal variation (Dereure 2007).

Now, it is found in 15-50% of AD patients, and is associated with AD of higher severity (Irvine, McLean et al. 2011).

Involucrin, in addition to being a structural component of the cornified envelope, is important for the attachment of lipids to the outer surface of the corneocyte cell membrane, enabling formation of the lipid envelope (Nemes, Marekov et al. 1999). Research has also demonstrated reduced expression of involucrin in AD (Jensen, Folster-Holst et al. 2004).

B. Lipid lamellae “The mortar”

The lipid lamellae is an arrangement of lipids into lamellar sheets, which surround the corneocytes, filling the extracellular space (Feingold 2007). It is composed of approximately 50% ceramides, 25% cholesterol and 15% free fatty acids. These lipids are derived from both exogenous sources, and secretory granules in the keratinocytes coined lamellar bodies (Elias 2008).

Lamellar bodies are first seen in the stratum spinosum (Fartasch, Bassukas et al. 1992). They then cluster and fuse with the apical cell membranes in the stratum granulosum and excrete their contents (Figure 1.4), which are a mixture of phospholipids, sphingolipids (glucosylceramides, sphingomyelin) and cholesterol (Elias and Schmuth 2009). Furthermore, they secrete various lipid processing enzymes, such as β -glucocerebrosidase, acidic sphingomyelinase, and secretory phospholipase A₂ (Menon, Grayson et al. 1988). These enzymes help metabolize the lipid precursors into the lipids that constitute the lipid lamellae (Fartasch 2004).

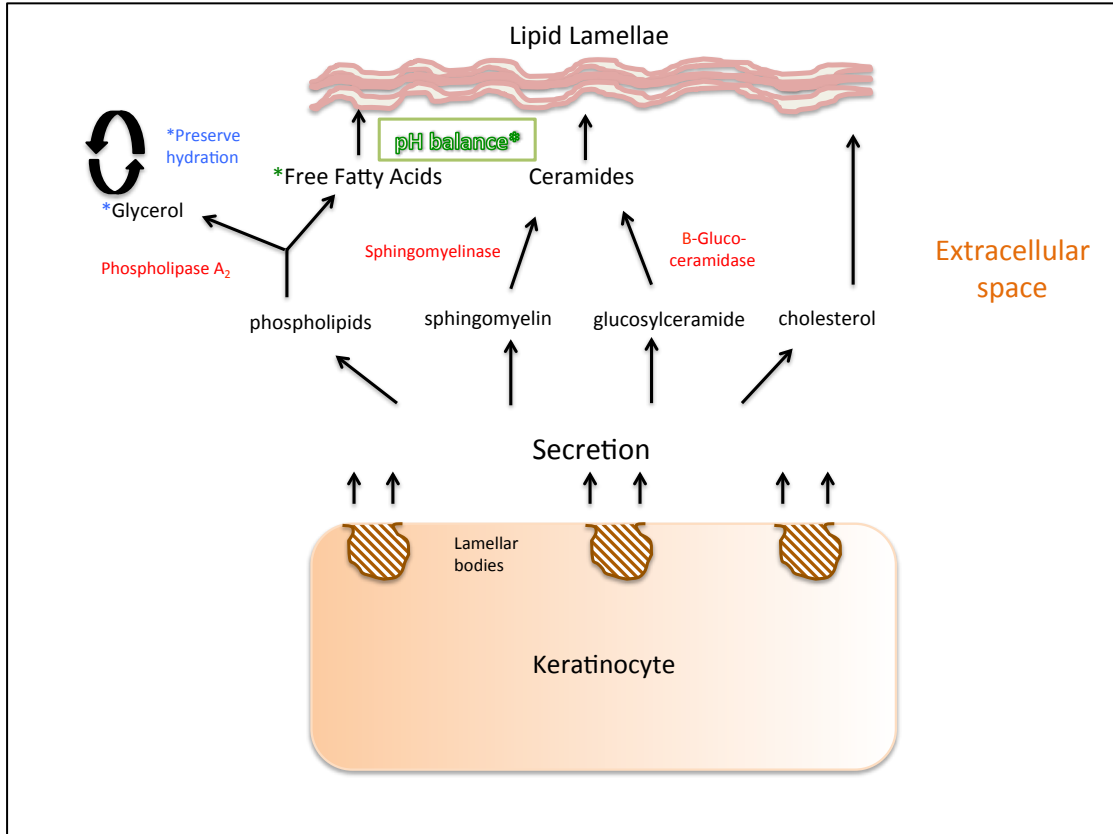


Figure I. 4: Lipid lamellae formation.

Lamellar bodies fuse with the cell membrane, extruding a mixed content of lipid precursors and their lipid processing enzymes. The lipids are then processed by the relevant enzymes, and the end products incorporated into the lipid lamellae. The end products are also involved in other functions; glycerol aids in hydration of the stratum corneum, free fatty acids help in pH maintenance.

Research has shown reduced ceramide levels in the lipid lamellae of AD patients in comparison with healthy controls (Kawashima, Morita et al. 1990; Imokawa, Abe et al. 1991; Hara, Higuchi et al. 2000). The reduction in ceramide levels was thought to be due to abnormal expression of enzymes, such as glucosylceramide deacylase (Ishibashi, Arikawa et al. 2003), sphingomyelin acylase (Murata, Ogata et al. 1996), and sphingomyelin deacylase (Hara, Higuchi et al. 2000).

Studies have shown sphingomyelin deacylase, in particular, to have five times higher activity in AD lesional skin and approximately three times higher in non-lesional AD skin in comparison to healthy controls. In addition, atypical forms of ceramides have also been found in AD (Bleck, Abeck et al. 1999).

Furthermore, many aberrations have been discovered in lamellar bodies as well. Fartasch and colleagues found that in atopic skin, lamellar bodies have somewhat dysfunctional lipid extrusion (Fartasch, Bassukas et al. 1992). In addition, there was a reduction in the number of lamellar bodies delivered and fused with the cell membrane in AD patients in comparison with controls.

C. Corneodesmosomes “the iron rods”

Corneodesmosomes, mentioned earlier, are specialized junctions found only at the SC level (Serre, Mils et al. 1991). They are formed from desmosomes, which are junctions composed of proteins such as desmoglein-1, desmocollin, and plakoglobin and plakophilin (Candi, Schmidt et al. 2005).

These then become reinforced with a protein expressed in the upper granular layers of the epidermis, corneodesmosin (Serre, Mils et al. 1991). Together, they form one of the main structures responsible for corneocyte cohesion, the corneodesmosome.

Proteases, such as kallikriens (KLK), are also secreted by lamellar bodies and act by cleaving the corneodesmosomes (Cork, Danby et al. 2008). This leads to separation of the corneocytes, and shedding (Cork, Danby et al. 2008). Other corneocytes, advancing from below, already differentiated, replace these cells (Egelrud 2000). Hence the stratum corneum is continually renewed but maintained at a constant thickness in a process called desquamation (Egelrud 2000).

Eight different types of KLKs are present in skin tissue (Komatsu, Tsai et al. 2006). They are secreted as latent precursors, after which they are activated by either auto-activation, or other KLKs (Yousef and Diamandis 2001). There have been reports linking a variant of *KLK7* gene, encoding KLK7, to an increased risk of AD (Vasilopoulos, Cork et al. 2004). The *KLK7* gene has been found to be over expressed (Saaf, Tengvall-Linder et al. 2008), and KLK7 activity to be increased in AD patients, resulting in increased SC breakdown (Vasilopoulos, Cork et al. 2004; Nemoto-Hasebe, Akiyama et al. 2009).

Protease inhibitors are equally important in controlling the desquamation process (Egelrud 2000). In disorders such as Netherton syndrome, which is characterized by generalized scaly erythroderma and atopic manifestations (Bitoun, Chavanas et al. 2002), mutations have been found in the *serine protease inhibitor Kazal-5 (SPINK-5)* gene, encoding for the protease inhibitor SPINK-5, previously known as *Lympho-epithelial Kazal-type 5 serine protease inhibitor (LEKTI)* (Chavanas, Bodemer et al. 2000).

This could lead to the inactivity of SPINK-5, and therefore over activity of the protease KLK7, leading to skin barrier breakdown (Chavanas, Bodemer et al. 2000; Richard, Sprecher et al. 2000; Kato, Fukai et al. 2003). The similarities in the clinical presentation of Netherton syndrome and AD have led researchers to focus on SPINK-5 in association with AD (Ishida-Yamamoto, Igawa et al. 2011). This led to the discovery of *SPINK-5* polymorphisms in AD patients (Kato, Fukai et al. 2003; Nishio, Noguchi et al. 2003), as well as reduced activity of SPINK-5 protein (Roedl, Traidl-Hoffmann et al. 2009). Both protease and protease inhibitor activity are pH dependent (Schade and Marchionini 1928). A summary of the defects identified in the skin barrier can be found in Figure I.5 and Table 1.

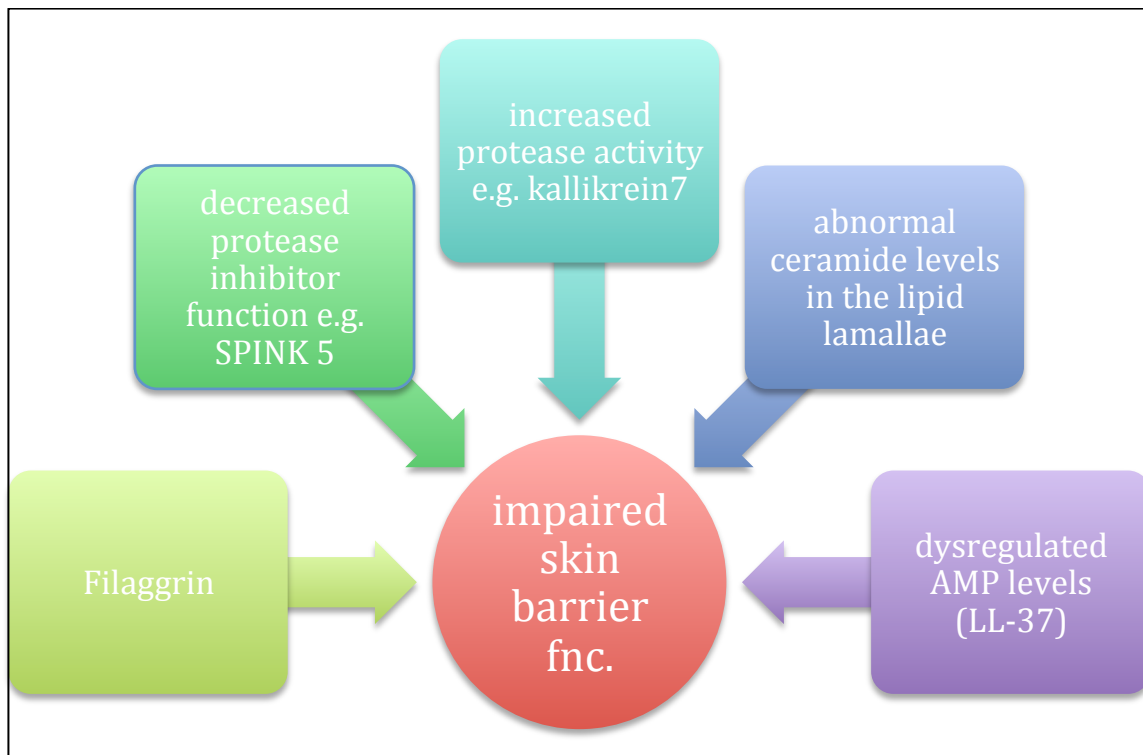


Figure I. 5: Defects identified in the skin barrier.

The structural protein, filaggrin, is abnormal due to mutations in the filaggrin encoding gene (Palmer, Irvine et al. 2006). This leads to fragile corneocytes and decreased NMF. Protease inhibitors have also been found to have reduced activity (Vasilopoulos, Cork et al. 2004); there also are abnormalities in protease activity (Saaf, Tengvall-Linder et al. 2008). Antimicrobial peptides (AMP) and ceramide levels have been found to be abnormal in AD. All these can lead to breakdown of the epidermal barrier, and the manifestation of AD.

Component	Function	Defect associated with AD
Structural components - Filaggrin - Involucrin	Stability, NMF, pH balance	Decreased expression (Palmer <i>et al</i> , 2006) (Jensen <i>et al</i> , 2004)
Cell-cell adhesion - Tight junctions (claudins) - Gap junctions (connexins) - Corneo-desmosomes	Stability, intercellular regulation	Decreased expression of claudin (De Benedetto <i>et al</i> , 2011) Increased expression of connexin26 (De Benedetto <i>et al</i> , 2012) Decreased expression of desmoglein-1 (De Benedetto <i>et al</i> , 2008)
Lipids - Lamellar bodies - Sphingolipids (ceramides)	Water repellence, Hydration, pH balance	Dysfunctional lipid extrusion (Fartasch <i>et al</i> , 1992) Decreased ceramide levels (Hara <i>et al</i> , 2000)
Regulatory enzymes - Proteases (KLKs) - Protease inhibitors	Regulation of the desquamation process	Overexpression of proteases (Saafi <i>et al</i> , 2008) Increased activity (Vasilopoulos <i>et al</i> , 2004) Reduced expression (Kato <i>et al</i> , 2003)

Table 1. The different components involved in epidermal differentiation, and the defects discovered in association with AD.

I.III.III The antimicrobial barrier

One of the main functions of the epidermal barrier is antimicrobial defense. This is achieved by both maintenance of skin surface pH (Korting, Lukacs et al. 1992), and through expression of antimicrobial peptides (AMP) (Lai and Gallo 2009).

A. Skin Surface pH:

It was Schade and Marchionini whom first described the skin surface as being acidic and most favorable for skin barrier function (1928). Firstly, proteases function under neutral pH (7.0). As the pH decreases, so does the activity of the proteases (Rippke, Schreiner et al. 2004), thereby maintaining stratum corneum cohesion (Figure I.6). This finding was demonstrated on hairless mice (Hachem 2003; Fluhr, Mao-Qiang et al. 2004). Secondly, low pH aids the formation and organization of the lipid lamellae (Bouwstra, Gooris et al. 1998; Pilgram, Vissers et al. 2001). This is because the afore-mentioned lipid processing enzymes have acidic optimum (Elias, Williams et al. 2002). Finally, an acidic environment is important for discouraging colonization of pathogenic bacteria, such as *Staphylococcus aureus* (*S. aureus*) and *propionibacteria* (Korting, Lukacs et al. 1992).

As mentioned previously, filaggrin mutations can result in disruption of the NMF, resulting in increased pH. In addition, disturbed lipid processing can also lead to elevated pH. Protease activity is elevated as a consequence, leading to cleavage of the corneodesmosomes (Rippke, Schreiner et al. 2002).

This in turn results in disintegration of the corneocytes, and barrier breakdown (Rippke, Schreiner et al. 2002). Elevated pH can also cause swelling of the corneocytes and modification of the lipid structure (Bouwstra, Gooris et al. 1998). A recent study demonstrated a clear association between AD patients with filaggrin mutations and elevation of skin surface pH (Jungersted, Hogh et al. 2010)

Several studies have found skin pH to be higher than the normal range in AD patients (Seidenari and Giusti 1995; Eberlein-Konig, Schafer et al. 2000; Knor, Meholjic-Fetahovic et al. 2011). Eberlein-Konig and colleagues examined over 300 primary school children for eczema and skin dryness, and found increased pH of non-lesional skin in the AD children (pH 5.32) in comparison with controls (pH 5.12)(Eberlein-Konig, Schafer et al. 2000). Another more recent study demonstrated lesional, peri-lesional and non-lesional sites in AD patients had significantly higher pH values than healthy subjects (Knor, Meholjic-Fetahovic et al. 2011) Thus increased skin pH is now a recognized factor in AD development.

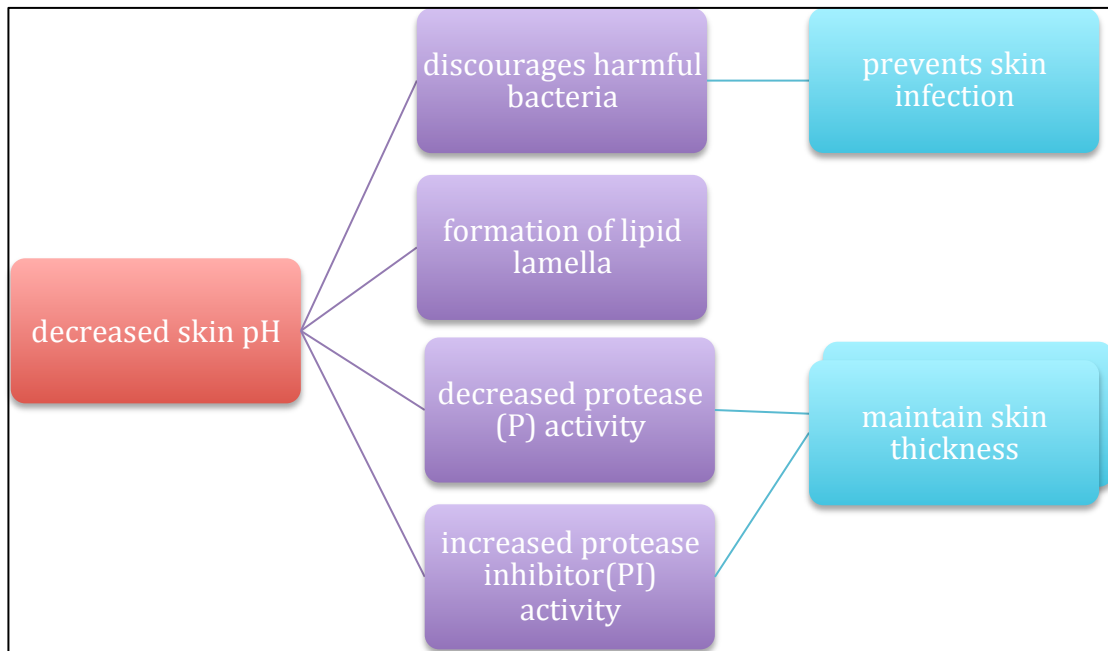


Figure I. 6: Importance of skin surface pH.

Skin pH has an important regulatory effect on the desquamation process. It increases protease inhibitor activity and decreases protease activity, therefore maintaining a healthy skin thickness (Rippke, Schreiner et al. 2004). Secondly, it aids lipid lamella formation (Bouwstra, Gooris et al. 1998). It also discourages the colonization of harmful bacteria, reducing skin infection (Korting, Lukacs et al. 1992).

B. Antimicrobial peptides

AMPs are a group of individually configured peptides with a capability to kill pathogens (Braff, Bardan et al. 2005). In addition, they stimulate and modify several aspects of the innate and adaptive immune system (Schauber and Gallo 2008). Many cells in the body, such as sebocytes (Lee, Yamasaki et al. 2008), neutrophils, and keratinocytes (Braff, Di Nardo et al. 2005), express these important proteins. In the past twenty years more than 1800 different AMPs have been identified (Wang and Wang 2003), and can be classified according to their structure into three groups: peptides with α -helix structures, peptides with β -sheet structures and peptides with loop structures (Lai and Gallo 2009). Although they all predominantly inhibit microbial growth, their immunological functions are diverse and quite specific to each group (Guani-Guerra, Santos-Mendoza et al. 2010).

Approximately twenty different peptides have been discovered in the skin with antimicrobial activity (Schauber and Gallo 2008). Some, such as the melanocyte stimulating hormone, have a biological function as well (Braff and Gallo 2006). The two main AMP groups, however, are the defensins and cathelicidins (Beard, Bearden et al. 2011). Cathelicidins, in particular, are well documented for their inhibitory effects against HSV (Gordon, Romanowski et al. 2004; Leung, Wollenberg et al. 2006; Ogawa, Kawamura et al. 2010).

Cathelicidins

Cathelicidins are AMPs characterized by an N-terminal cathelin domain and a C-terminus with antimicrobial activity (Bals and Wilson 2003). In the epidermis, they are stored as inactive precursors (h-CAP18) within the lamellar bodies in keratinocytes (Braff, Di Nardo et al. 2005). They are then extruded at the upper level of the stratum granulosum, and liberated as the active form LL-37 by extracellular cleavage with KLK 5 and 7 (Morizane, Yamasaki et al. 2010).

Although there are several different types of cathelicidins identified in bovine and porcine tissue (Zanetti, Gennaro et al. 1995), only one type (h-CAP18) has been identified so far in humans (Cowland, Johnsen et al. 1995). LL-37, specifically, has been found in abundance in several tissues of the body such as lung airway fluid (Agerberth, Grunewald et al. 1999), plasma (Sorensen, Cowland et al. 1997), and the epithelial cells of the epididymis (Malm, Sorensen et al. 2000).

LL-37 shows interaction with the inner and outer membranes of Gram-negative bacteria, due to the presence of lipopolysaccharides (LPS). This is generally through one of three methods (Figure I.7). Method one is the barrel stave technique in which they form a trans membrane pore in the target cell. Method two involves aggregation of several peptides to form several pores, leading to the leakage of the intracellular substances. Method three involves encapsulating the target pathogen in a carpet like manner, leading to the collapse of the membrane (Bals and Wilson 2003).

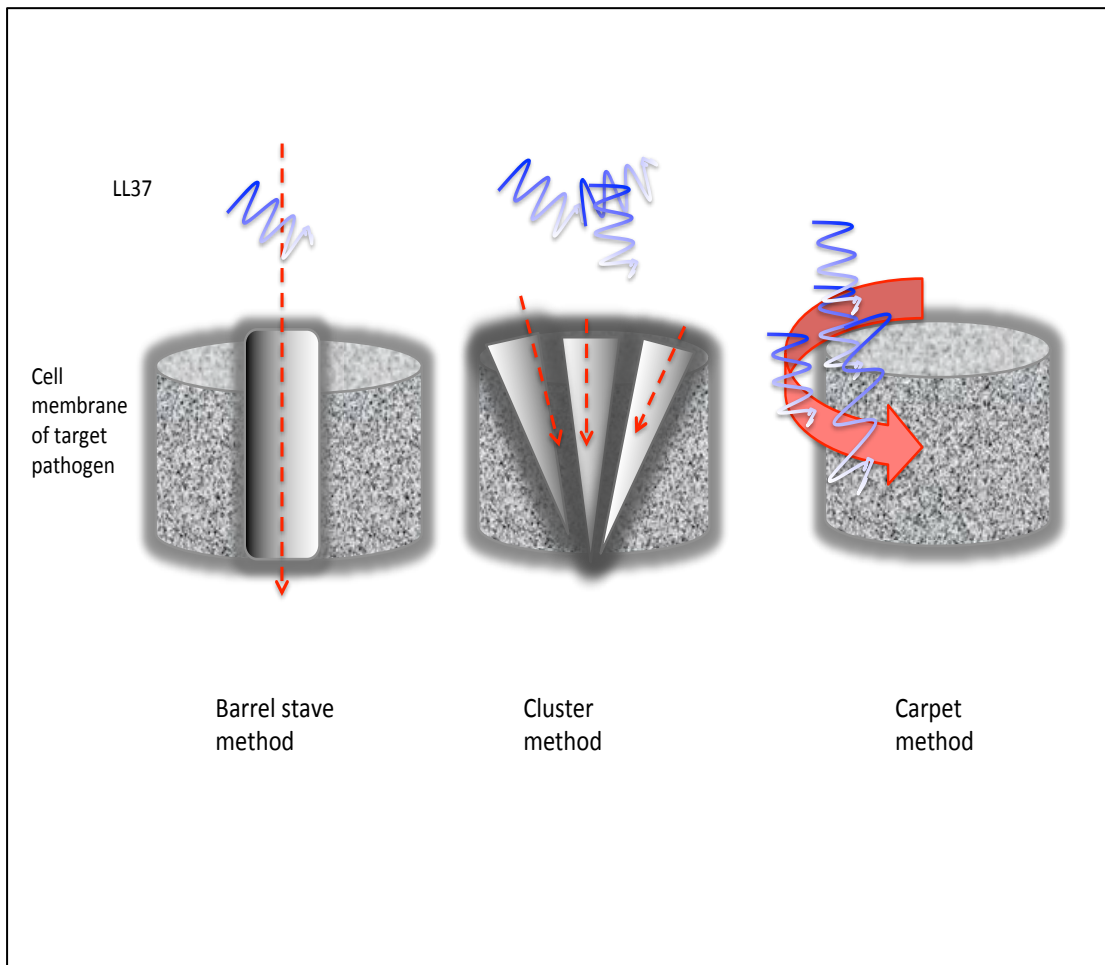


Figure I. 7: AMP mechanism of actions.

AMPs antimicrobial effects are brought about by either forming a trans-membranous pore (Barrel Stave method), the clustering of several peptides together to form small pores through which intracellular substances seep through (clustering method), or by enclosing the pathogen membrane (carpet method) (Bals and Wilson 2003).

Gram-positive bacteria are also susceptible, however the mechanisms have yet to be understood (Nelson, Hultenby et al. 2009). LL-37 has also been shown to have the capability to bind and inactivate bacterial endotoxin (Hirata, Kirikae et al. 1998; Kirikae, Hirata et al. 1998).

Despite this, many bacteria have developed resistance against the effects of AMP. *Shigella species* have been shown to release plasmid DNA which inhibits the expression of LL-37 (Lai and Gallo 2009). *S. aureus* can release proteases to inactivate LL-37 (Sieprawska-Lupa, Mydel et al. 2004), as well as modulate the LPS surface membrane, reducing their recognizing properties (Peschel and Collins 2001).

LL-37 has also shown significant anti viral activity (Figure I.8). *In vivo* and *In vitro* studies show inhibited replication of HSV (Howell, Wollenberg et al. 2006), the Influenza virus (Barlow, Svoboda et al. 2011), the Vaccinia virus (Howell, Jones et al. 2004) and HIV (Wang, Watson et al. 2008) when exposed to cathelicidins. LL-37 also displays anti-fungal activity (Lopez-Garcia, Lee et al. 2005; Tsai, Yang et al. 2011).

LL-37 has been found to exhibit immuno-modulatory functions as well. They are directly chemotactic to neutrophils, monocytes and T cells via G protein coupled formyl peptide receptor-like 1 (FPRL-1) (Yang, Chertov et al. 2001). It also displays indirect chemotactic properties by inducing secretion of the inhibitory cytokine IL-10 (Yu, Mookherjee et al. 2007).

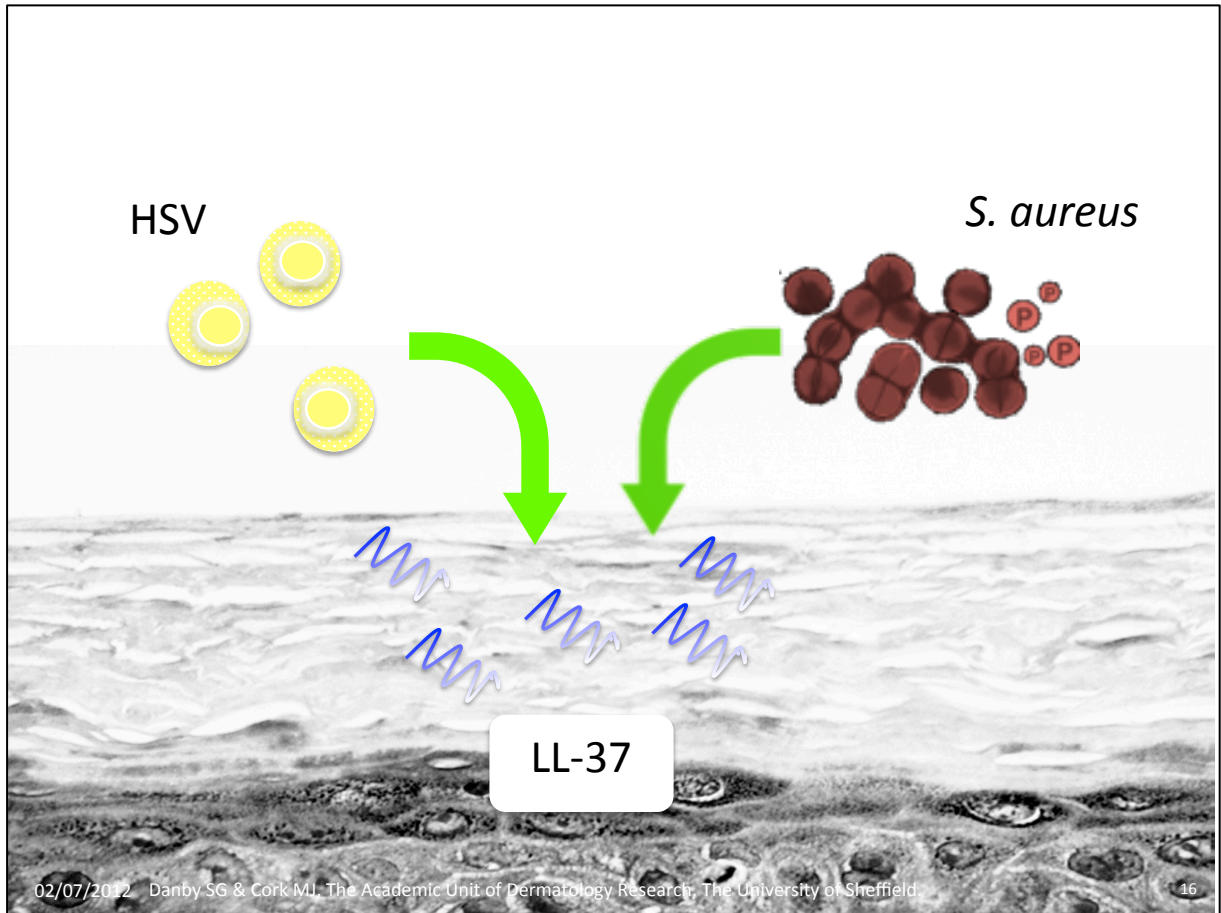


Figure I. 8: The functions of LL-37 in the skin.

Keratinocytes express LL-37 in response to infections. LL-37 displays inhibitory action on various pathogens, such as HSV and *S. aureus*, which are the main causative factors of secondarily infected AD.

In addition, LL-37 was found to inhibit maturation of dendritic cells (DC) (Kandler, Shaykhiev et al. 2006), and the release of TNF α from monocytes (Mookherjee, Brown et al. 2006). Therefore it is possible that it has a regulatory effect on inflammatory responses.

Furthermore, LL-37 appears to be important for wound repair. It has shown to promote keratinocyte migration to aid re-epithelialization (Tokumaru, Sayama et al. 2005). It also apparently stimulates endothelial cell proliferation to promote angiogenesis (Koczulla, von Degenfeld et al. 2003). It directly acts on dermal fibroblasts, inhibiting collagen release and fibrosis of wounds, promoting healthy wound development (Park, Cho et al. 2008).

Cathelicidin levels in AD patients

As stated before, in normal wounds injury leads to up-regulation of LL-37. This is in order for LL-37 to participate in re-epithelialization (Braff, Bardan et al. 2005). Malbris and colleagues, however, found decreased LL-37 expression in the wounds of 14 AD patients (Mallbris, Carlen et al. 2009). Howell and co-workers performed a study to determine LL-37 levels in the lesional skin of AD patients (Howell, Wollenberg et al. 2006).

Patients were stratified into AD, and ADEH. Ten AD patients were biopsied, in addition to ten ADEH patients. It was found that not only are LL-37 levels decreased in AD patients with HSV, but that their levels were significantly less ($p < 0.05$) than those in uncomplicated AD patients.

I.IV. Pathophysiology of AD

AD is identified as being the first step in the 'atopic march', progressing to asthma and allergic rhinitis (Cantani 1999). This atopic march occurs early; over 50% of AD children will develop asthma or allergies before the age of three (Boguniewicz and Leung 2011). This is due to a primary skin barrier defect, accompanied with an adaptive immunity Th₁-Th₂ paradigm (Zheng, Yu et al. 2011).

The disrupted skin barrier function in AD leads to permeation of allergens, irritants and pathogens (Figure 1.9). The allergens are then captured and processed by Langerhans cells, which are the main local antigen presenting cells. In AD, Langerhans cells are found to express higher levels of the high affinity IgE receptor, FcεRI (Boguniewicz and Leung 2010). This unusual phenotype could lead to the heightened responsiveness of the local T cells to outside allergens at normal concentrations, inducing a state of hyper reactivity (Leung 1999). AD lesions also have increased levels of T cells of the CLA⁺ type, which are the homing antigen/allergen-reactive memory cells (Leung, Boguniewicz et al. 2004). This could also mean a low threshold for skin activation.

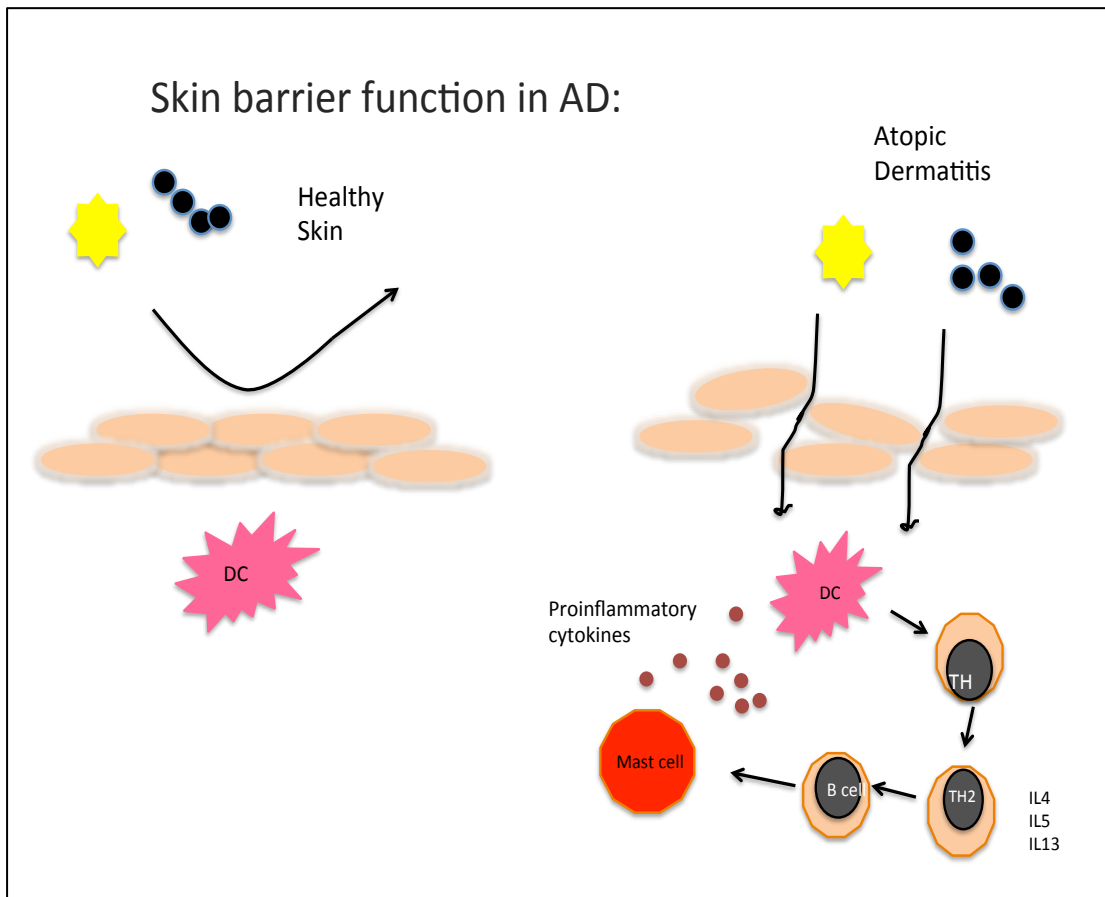


Figure I. 9: Pathogenesis of AD.

In a healthy individual, the skin barrier is intact and able to prevent permeation of allergens and pathogens (left). In a person with a compromised skin barrier, pathogens and allergens are able to penetrate through the skin, inducing the activation of DCs. This then leads to antigen presentation to the Th cells, inducing a Th₁- Th₂ switch. This then leads to formation of IgE cells, which diffuse into the blood stream and mount a systemic Th₂ response.

Monocytes in AD also exhibit the FcεRI receptor, as well as an increased production of prostaglandin E₂ (Bologna, Jorizzo et al. 2003). This leads to increased secretion of IL-10, inhibiting Th₁ cell activity and encouraging a Th₂ predominant infiltrate composed of IL-4, IL-5, and IL-13. This encourages further switching to the IgE antibody class (Galli, Tsai et al. 2008).

Interestingly, IL-4 and IL-13 both have shown to down regulate expression of filaggrin (Howell, Kim et al. 2007), loricrin and involucrin (Kim, Leung et al. 2008), thereby leading to reduced cornified envelope formation and decreased skin barrier integrity.

Consequently, IgE diffuses systemically via blood and lymphatics, creating a Th₂ predominant immune system. Subsequent exposure to allergens leads to mast cell degranulation and release of histamine and leukotrienes (Galli, Tsai et al. 2008).

The persistent inflammation seen in AD is due to the ongoing inflammation associated with tissue damage (Dimeloe, Nanzer et al. 2010). In chronic or more severe AD, Th₁ cells are also thought to contribute to ongoing inflammation, possibly through ongoing secretion of the immunomodulatory cytokine interferon γ (IFNγ) (Grewe, Bruijnzeel-Koomen et al. 1998; Dokmeci and Herrick 2008)

The Th₁₇ subset is relatively new and predominantly secrete IL-17 and IL-22 (Barnes 2011). It is also believed to induce AMP production (Peric, Koglin et al. 2009). Its role in AD, however, is under much debate and has yet to be determined fully.

Although some studies have reported diminished IL-17 levels in AD patients (Hayashida, Uchi et al. 2011), others have found high levels (Toda, Leung et al. 2003) and some have found normal levels (Nogralles, Suarez-Farinas et al. 2010). Some research has suggested that Th₂ counteracts Th₁₇, preventing AMP induction (Eyerich, Pennino et al. 2009).

T regulatory cells

In healthy individuals, regulatory T cells (TRegs) aid in the down regulation of the inflammatory response. They can be classified into two subgroups: naturally occurring TRegs (nTRegs) produced in the thymus, and induced TRegs (iTRegs), in response to infection and cancer (McGuirk, Higgins et al. 2010). The nTRegs express forkhead-winged helix transcription factor, forkhead box P₃ (Fox P₃), which are apparently important for TReg cell function (Sakaguchi 2000). Not only do nTRegs release inhibitory cytokines, but they also directly block DC maturation, and as a consequence suppress their ability to activate T effector cells (Onishi, Fehervari et al. 2008).

The iTRegs, which can either be FoxP₃⁺ or FoxP₃⁻, are thought to be induced peripherally and act by secreting inhibitory cytokines such as IL-10, IL-35 and transforming growth factor (TGF-β) (Marie, Letterio et al. 2005; Collison, Workman et al. 2007).

IL-10 has shown many anti-allergic properties, such as mast cell and eosinophil, inhibition. It also acts on B cells, stimulating a switch to IgG4 production, an antibody that was found to inhibit IgE mediated histamine release (Till, Francis et al. 2004). TGF- β also acts by inhibiting Th₁ and Th₂ inflammation (Veldhoen, Uyttenhove et al. 2008).

In AD, TReg functions have been found to be impaired (Hawrylowicz 2005). In addition, studies have found decreased levels of TGF- β and Fox P₃⁺ TRegs cells in the lung cells of asthmatic patients when compared with healthy controls (Luo, Liu et al. 2006).

This is especially evident in the disorder called immune dysregulation polyendocrinopathy X-linked (IPEX), which is characterized by autoimmunity and marked atopy in the form of AD, food allergies and high IgE levels (Li, Samanta et al. 2007). Asthma patients were found to have lesser levels than healthy controls of TRegs in broncho alveolar lavage (Hartl, Koller et al. 2007). The peripheral blood of people suffering from hay fever were also found to contain decreased levels TRegs in comparison with controls (Ling, Smith et al. 2004). Some have also identified specific FoxP₃ gene polymorphisms associated with allergic rhinitis (Zhang, Zhang et al. 2009).

I.V. Vitamin D and AD

The role of vitamin D (VD) in bone mineralization has been recognized and confirmed for several decades (Anderson, Turner et al. 2012). Recently, however, the extra skeletal importance of VD has increasingly become recognized (Bartley 2010). Recent studies have associated VD deficiency with an increased risk of malignancy (Pilz, Tomaschitz et al. 2009), cardiovascular disease (Shirts, Howard et al. 2012), and autoimmune disorders (Andreoli, Piantoni et al. 2012). VD is involved in various processes in the body, and some have even argued its essential role in evolution (Bikle 2011). In addition, it appears to have significant immunomodulating effects in both the innate and adaptive immune systems (Hewison 2010; Peelen, Knippenberg et al. 2011).

VD has recently emerged as potential therapeutic option for AD as well (Sidbury, Sullivan et al. 2008; Javanbakht, Keshavarz et al. 2011; Amestejani, Salehi et al. 2012). Recent research has implicated a role for VD supplementation in AD (Hata, Jackson et al. 2008; Hata, Kotol et al. 2008), due to its various effects on the skin barrier (Braga, Hodiwalla et al. 1995), innate immunity and adaptive immunity (Baeke, Takiishi et al. 2010), all of which will be dealt with in this section.

I.V.I. VD Sources

VD is acquired from two major sources: diet and sunlight, which is responsible for more than 90 % of an individual's supply (Box 2) (Pearce and Cheetham 2010). Fair skinned people require 20-30 minutes of sunlight three times a week in order to achieve optimum VD levels (Pearce and Cheetham 2010). The elderly, and individuals with darker skin will need approximately twice as much to achieve healthy levels (Working Group of the, New Zealand et al. 2005). Two main forms of VD exist: VD₃ (cholecalciferol), which is formed in the skin and can also be obtained through the diet from animal sources, and VD₂ (ergocalciferol), obtained from fungi (Baeke, Takiishi et al. 2010).

Sources of VD

1. Ultraviolet light
2. Oily fish: mackerel, salmon, sardines, tuna (smoked=160 IU; raw=1600IU/100 gm).
3. Fish oils: cod liver oil
4. Mushrooms (very minimal amounts).
5. Eggs: specifically the egg yolk (20 IU per yolk).
6. Fortified milk and fortified cereals (160 IU-320/100gm).

Box 2. Sources of VD.

I.V.II. Clinical manifestation of VD deficiency

VD deficiency is generally thought to present as skeletal abnormalities, with an increase in fractures, and in severe cases, bowing of legs and knock knees, manifesting as rickets (Mughal 2011). It can, however, present a subtler picture, with increased irritability, a history of multiple infections and poor growth (Beck-Nielsen, Jensen et al. 2009).

In adults, VD deficiency can manifest as general pain and muscle weakness (Pearce and Cheetham 2010). Research has also linked VD deficiency to chronic low back pain (Lewis 2005; Atherton, Berry et al. 2009; Straube, Moore et al. 2009).

I.V.III. VD and the Skin barrier

VD₃ is a hormone produced from thermal cleavage of 7-dehydrocholesterol in the skin after ultraviolet B exposure (Schwalfenberg 2011). It then binds to the VD binding protein (DBP) and undergoes systemic transportation (Baeke, Takiishi et al. 2010). It is thereafter hydroxylated into the hormonally active form 1,25 dihydroxyvitamin D₃ twice; in the liver by the enzyme 25 hydroxylase (CYP27A1), and in the kidney by 1 α hydroxylase (CYP27B1) (Dombrowski, Peric et al. 2010) (Figure I.10). Dietary VD, both VD₂ and VD₃, also undergo metabolic activation along the same pathway (Baeke, Takiishi et al. 2010). Keratinocytes in the stratum basale, however, contain both enzymes and therefore can activate VD independently (Bikle, Pillai et al. 1989). This active form of VD₃ then binds to the intracellular VD receptor (VDR), regulating the expression of up to 900 genes (Wang, Tavera-Mendoza et al. 2005).

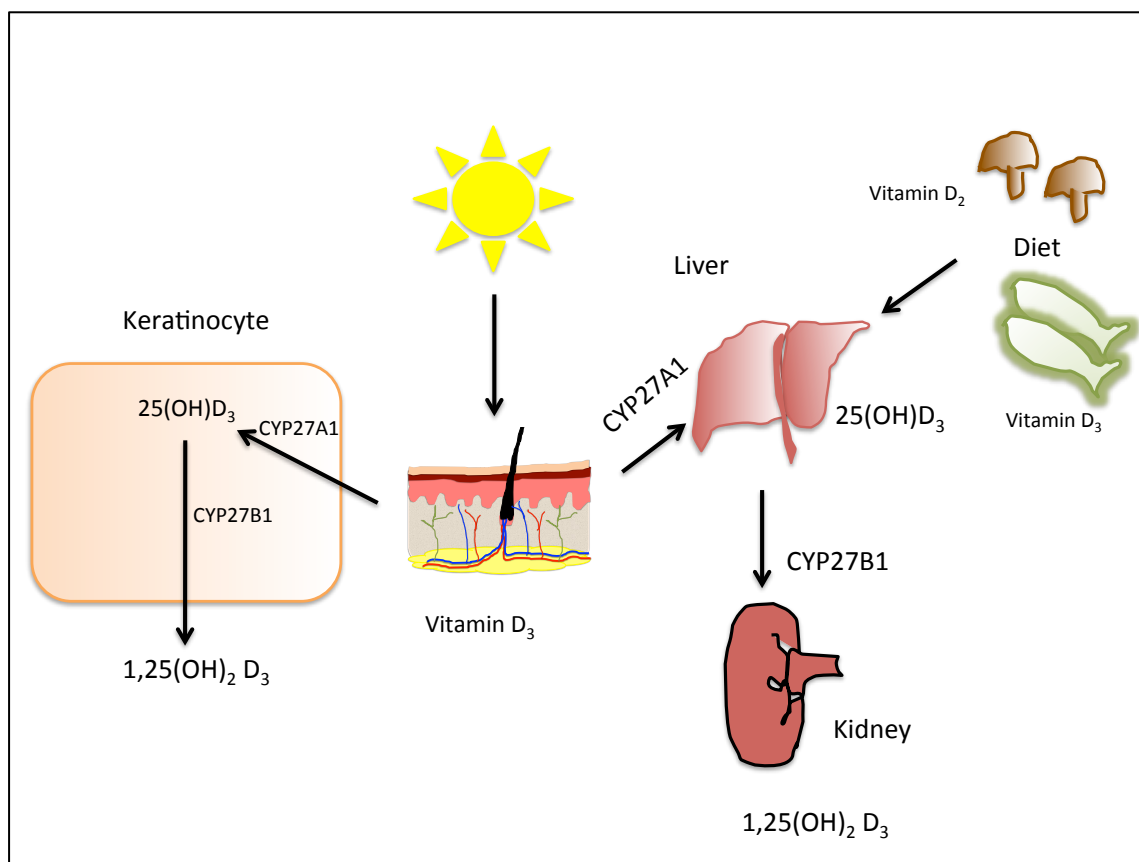


Figure I. 10: VD Production.

Ultraviolet light is absorbed through the skin, leading to thermal cleavage of 7 dehydrocholesterol into VD₃. VD₃ then binds to DBP, and is transported to the liver. Ingested VD₂ and VD₃ are also transported to liver. In the liver, they are hydroxylated by 25 hydroxylase (CYP27A1) into 25 hydroxyvitamin D₃. They are then transported to the kidney to undergo further processing by 1 α hydroxylase (CYP27B1) into the active 1,25 dihydroxyvitamin D₃. The keratinocyte, however, contains both enzymes and is able to activate VD₃ independently.

VD₃ is essential for epidermal barrier integrity. It affects epidermal differentiation in two ways: through its effects on the calcium gradient in the epidermis, and by direct regulation via VDR (Bikle 2012). The calcium gradient is essential for epidermal differentiation, and is characterized by an increase in calcium concentration as the keratinocyte advances towards the stratum granulosum (Figure I.11) (Bikle, Oda et al. 2004). The first enzyme involved in hydroxylation of VD, 25 hydroxylase, has been shown to have higher activity at low calcium concentrations, which corroborates the importance of the lower calcium levels found in the stratum basale (Pillai, Bikle et al. 1988).

Increased extracellular calcium leads to increased intracellular calcium concentration via activation of the calcium-sensing trans membranous receptor (CaR) in keratinocytes (Tu, Chang et al. 2001). This receptor is also induced by VD₃, rendering the keratinocytes more sensitive to calcium activation (Bikle 2012). This increase in intracellular calcium then triggers the redistribution of several calcium regulated proteins, causing them to rearrange next to the cell membrane, resulting in the formation of intercellular connections, the most prominent of them being E-cadherin (Braga, Hovalva et al. 1995). *In vitro* studies have also shown redistribution of desmoplakin and plakoglobulins to form desmosomes in response to the increased intracellular calcium level (Watt, Matthey et al. 1984). Desmogleins and desmocollins also rigidify in response to increased intracellular calcium, aiding in desmosomal formation (Brooke, Nitoiu et al. 2012).

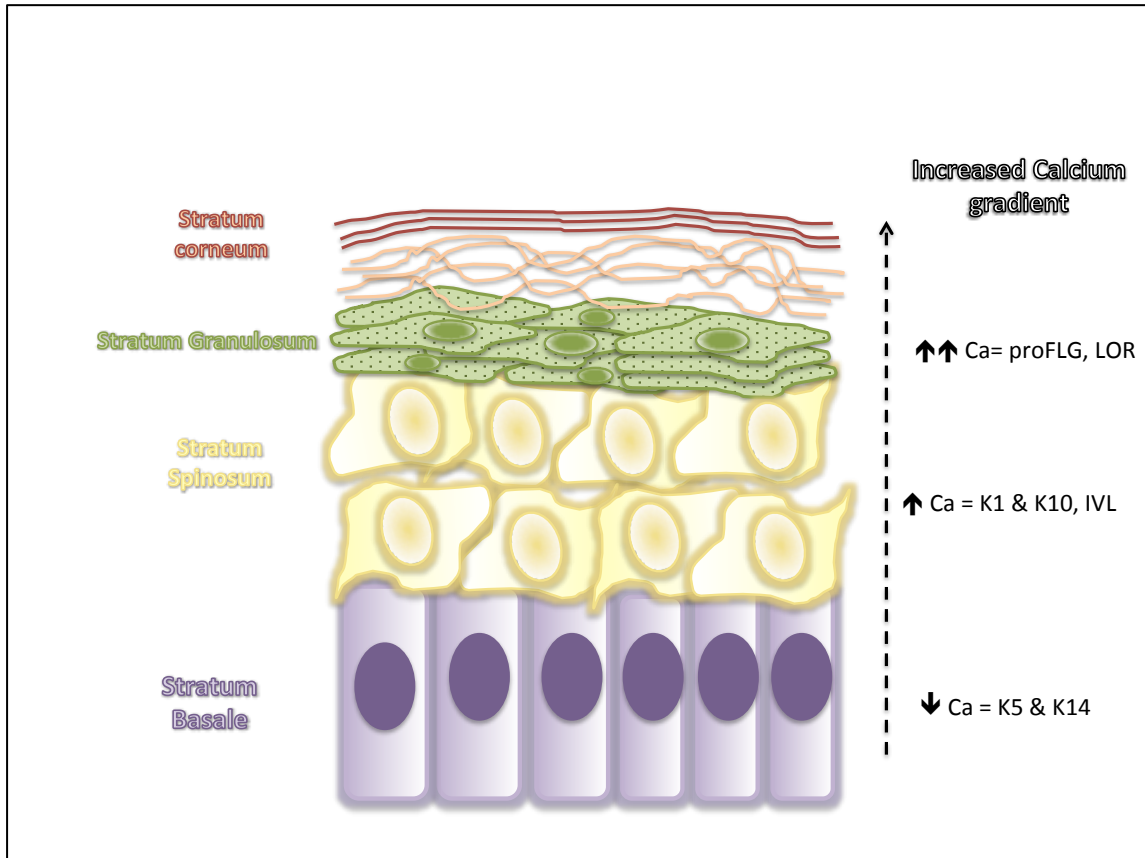


Figure I. 11: Calcium gradient in the epidermis.

The calcium gradient is fundamental to epidermal differentiation. At the level of the stratum basale, the low calcium level is elemental to 25 hydroxylase activity. It is also important for production of K5 and 14. The increase in intracellular calcium upwards towards the stratum corneum triggers the switch to production of K1, K10 and involucrin (IVL) at the level of the stratum spinosum. Further increase in the calcium concentration triggers production of profilaggrin (proFLG) and Loricrin (Lor).

Increased calcium levels along the gradient also induce keratinocytes to switch from producing Keratins 5 and 14 to K1 and K10 (Yuspa, Kilkenny et al. 1989). This, shortly after, is followed by an increased production of profilaggrin, involucrin and loricrin, all of which are essential for cornified envelope formation (Hohl, Lichti et al. 1991), and is evident within two days of extracellular calcium increase (Hennings, Steinert et al. 1981).

VDR, specifically, is reportedly essential in inducing expression of the genes that encode the proteins required for gap junctions (Clairmont, Tessmann et al. 1996), adherens junctions (Gniadecki, Gajkowska et al. 1997; Lopes, Carvalho et al. 2012) and tight junctions (Chirayath 1998; Kong, Zhang et al. 2008). Furthermore, VD_3 has also been shown to directly induce expression of both involucrin and transglutaminases via VDR (Su, Bikle et al. 1994; Bikle, Oda et al. 2004).

In vivo studies in mice with mutated VDR or 1α hydroxylase have shown decreased involucrin and profilaggrin in addition to loss of keratohyalin granules (Xie, Komuves et al. 2002). The 1α hydroxylase null mice also demonstrated an abnormal calcium gradient in the epidermis, and delayed skin barrier recovery (Bikle, Chang et al. 2004).

In addition, the VDR has been shown to affect sphingolipid production, specific to the epidermis (Oda, Uchida et al. 2009). Silencing of VDR in mice resulted in decreased sphingolipid production, decreased expression of the enzyme ceramide glucosyl transferase, and defects in lamellar body formation.

The effect of VD₃ on LL-37

Of particular interest is the effect of VD₃ on the expression of cathelicidins (Beard, Bearden et al. 2011). Interestingly enough, only the gene encoding for human cathelicidin, *CAMP* (cathelicidin anti microbial peptide), contains the VD response element (VDRE) (Gombart, Saito et al. 2009). An important study revealed the presence of VDRE mediated cathelicidin production in human keratinocytes, monocytes and neutrophils (Wang, Nestel et al. 2004). These cells were directly stimulated with VD₃, and were found to increase cathelicidin secretion via induction of *CAMP* gene expression.

Yuk et al found that VD₃ induces autophagy of monocytes and destruction of *Mycobacterium tuberculosis* through cathelicidin induction (Yuk, Shin et al. 2009). Similarly, inhibition of VD₃ induced *CAMP* production lead to an increased mycobacterial growth (Liu, Stenger et al. 2007). Liu and colleagues further demonstrated that LL-37 expression was absent in people with VD deficiency, and that this could be corrected by VD supplementation (Liu, Stenger et al. 2006; Modlin, Liu et al. 2006).

Keratinocytes treated with VD₃ were found to increase expression of KLK 5, 7 and LL-37 (Morizane, Yamasaki et al. 2010). This could lead to the increased activation of LL-37, thereby enhancing the antimicrobial response; a function that could prove valuable in ADEH patients with decreased LL-37.

I.V.IV VD and the Innate Immune system

VD₃ has shown significant roles in all aspects of the immune system (Figure I.12). This was realized upon discovery of the VDR in almost all the immune cells (Provvedini, Tsoukas et al. 1983; Takahashi, Nakayama et al. 2002). In the innate immune system, VD₃ has been demonstrated to directly induce expression of genes encoding the pattern-recognition receptors known as toll-like receptors (TLRs). TLRs are expressed in the antigen presenting monocytes and macrophages (McInturff, Modlin et al. 2005), as well as in keratinocytes (Lew, Sim et al. 2009).

The activation of TLRs results in several outcomes. Firstly, TLRs increase the chemotactic and phagocytic activity of monocytes and macrophages, which are considered to be the first line of defense against infection (Baeke, Korf et al. 2010). Secondly, TLRs can detect pathogen-associated molecular patterns (PAMPs) (Takeda and Akira 2005), such as LPS from Gram-negative bacteria and lipoteichoic acid from Gram-positive bacteria (Beutler 2004). Finally, TLR activation triggers the production and release of AMPs (Liu, Stenger et al. 2006), thereby aiding in anti microbial defense.

VD₃, however, has also been found to inhibit expression of TLR-2 and TLR-3 on human monocytes at 72 hours *in vitro*, leading to a reduction in the level of the pro-inflammatory cytokine TNF α (Scherberich, Kellermeyer et al. 2005; Sadeghi, Wessner et al. 2006).

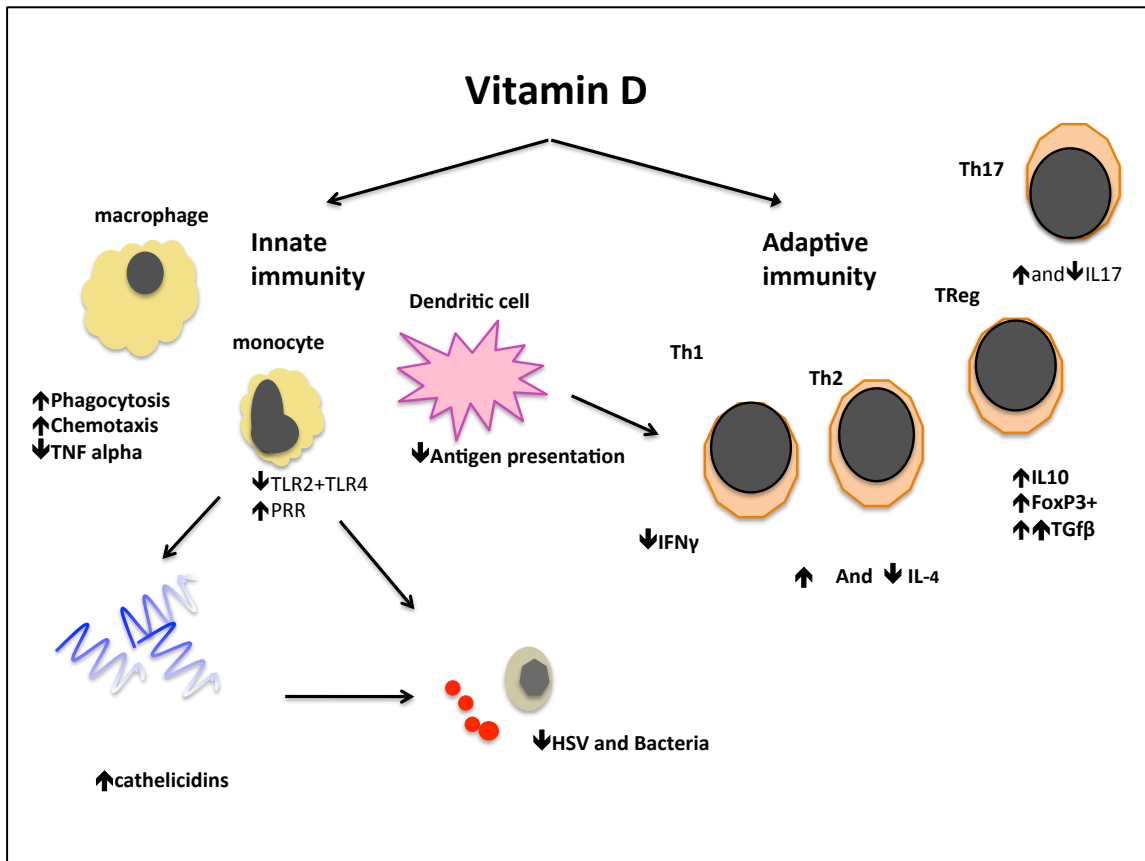


Figure I. 12: The effect of VD₃ on the immune system.

VD₃ has various effects on the immune system. In the innate immune system, it has been shown to increase chemo taxis and phagocytic activity of monocytes, macrophages and DCs, but to decrease antigen presentation of DC. It also up regulates expression of cathelicidins (LL-37).

In adaptive immunity, it decreases Th₁ predominant IFN γ secretion; increase expression of FoxP₃⁺ Treg cells and IL-10 secretion. On Th₂ cells, it seems to have both inhibitory and stimulatory functions, apparently depending upon VD₃ concentration.

This was explained to be a negative feedback mechanism, preventing disproportionate TLR activation and inflammation at the late stages of infection (Baeke, Takiishi et al. 2010).

Antigen presenting DCs are affected by VD_3 as well (Baeke, Takiishi et al. 2010). These cells essentially link the innate and adaptive immune systems together. DCs capture, process and present antigens to the T lymphocytes (Baeke, Takiishi et al. 2010). They also have been found to express VDR, and their antigen presentation seems to be inhibited when adding VD_3 *in vitro* (Penna and Adorini 2000). This is thought to prevent over stimulation of T cells; a conclusion supported by the lower graft rejection rate of mice treated with VD_3 (Adorini, Penna et al. 2001; Gregori, Casorati et al. 2001).

I.V.V. VD and the Adaptive Immune System

Historically, there was controversy surrounding VD and its effect in allergy, due to the negative effects found in mice (Li, Hener et al. 2006). It has increasingly become evident, however, that VD has a positive outcome on AD (Sidbury, Sullivan et al. 2008; Javanbakht, Keshavarz et al. 2011; Peroni, Piacentini et al. 2011).

The role of VD_3 in the adaptive immune system is still under scrutiny. It has been shown to inhibit production of $IFN\gamma$, the principal Th_1 cytokine (Mahon, Wittke et al. 2003), however it has been shown both to promote (Boonstra, Barrat et al.

2001) and inhibit (Staeva-Vieira and Freedman 2002) expression of Th₂ cells. It could be that different concentrations of VD₃ could explain the differing effects on Th₂ differentiation. High concentrations of VD₃ have been found to induce Th₂ secretory cytokines *in vitro*, whereas low concentrations were suppressive (Dimeloe, Nanzer et al. 2010). Hyponnen et al (2009) in a recent study found that excessively high (<135nmol/L) and very low (<25nmol/) VD levels were associated with high serum IgE levels in British adults, which is consistent with the above *in vitro* results.

On Th₁₇, considered to induce AMP production as mentioned previously, studies have also produced contradicting results. VD₃ has been shown to increase (Peelen, Knippenberg et al. 2011) and decrease IL-17 (Palmer, Lee et al. 2011). Therefore, more research is needed in this area.

Again, there have been conflicting results with regards to VD₃ effects on the inflammatory regulators, the TReg cells, another population found to have impaired function in AD (Hawrylowicz 2005). In mice, VD₃ was found to either reduce TReg levels (Chang, Cha et al. 2010) or have no effect (Mayne, Spanier et al. 2011). Human studies on the other hand found that VD₃ stimulates the production of IL-10 producing TReg cells, important for Th₂ response regulation (Xystrakis, Kusumakar et al. 2006). This is also the effect of corticosteroids, which are extensively used in the treatment of atopy (Dimeloe, Nanzer et al. 2010).

A positive correlation has been found between VD₃ and FoxP₃₊ TReg levels (Hamzaoui, Dhifallah et al. 2010) and VD₃ levels and FoxP₃₊ TReg function (Smolders, Thewissen et al. 2009) in patients with autoimmune disorders. Therefore VD₃ seems to have positive effects on TRegs.

I.V. Aims of this study

Previous studies have found reduced levels of the human cathelicidin LL-37 in AD and ADEH patients in skin biopsies from lesional skin (Mallbris, Carlen et al. 2009; Hata, Kotol et al. 2010). VD supplementation was also reported to increase expression of LL-37 (Frohman, Agerberth et al. 1997; Hata, Jackson et al. 2008). In addition, small, randomized controlled trials (RCTs) have found an improvement in the severity of AD with VD supplementation (Sidbury, Sullivan et al. 2008; Javanbakht, Keshavarz et al. 2011; Amestajani, Salehi et al. 2012).

Therefore, to date, there have been twelve studies examining the effect of VD status on AD severity (Giannetti, Ricci et al. 2011; Peroni, Piacentini et al. 2011; Shim, Kim et al. 2012), the relationship between VD and LL-37 (Howell, Wollenberg et al. 2006; Leung, Wollenberg et al. 2006; Mallbris, Carlen et al. 2009) and the effect of VD supplementation on both AD and LL-37 levels (Hata, Jackson et al. 2008; Hata, Kotol et al. 2008; Sidbury, Sullivan et al. 2008; Stahle, Mallbris et al. 2008; Javanbakht, Keshavarz et al. 2011; Amestajani, Salehi et al. 2012).

Together, all previous research suggests that VD supplementation is of benefit in AD; this is likely to be greater in ADEH, and altered LL-37 expression is a possible mechanism for this beneficial effect. . A major limitation of these studies was the need to perform skin biopsies to measure anti microbial peptide levels.

This meant that studies were small and none had been performed in pediatric patients with AD.

Therefore, this study was designed with the following aims:

- To determine the extent of VD deficiency in pediatric AD patients
- To investigate whether there was a difference in VD status between AD and ADEH patients.
- To examine lesional and non-lesional LL-37 levels in AD and ADEH patients at the skin surface, by a novel, non-invasive technique.
- To determine IgE levels and serum calcium levels
- To establish whether VD supplementation would improve the clinical severity of ADEH patients in particular, as well as AD patients, when no other variable is changed in clinical practice.
- To examine the change in LL-37, IgE and serum calcium levels postsupplementation.

Chapter Two: Materials and Methods

II. Chapter Two: Materials and Methods

II.1. Study design:

A practice evaluation study was performed in the Dermatology department of the Sheffield Children's Hospital. Ninety children were diagnosed with AD based on the UK Working Party's diagnostic criteria upon routine attendance to the dermatology clinic (Williams, Burney et al. 1996) . These children were then stratified further to either AD or ADEH. ADEH patients included were those that had been diagnosed clinically as having herpetic lesions by a dermatologist and treated for at least six months with antiviral medications. Both groups were screened for VD deficiency. Total IgE and serum calcium levels were also checked.

Patients with VD deficiency were then assessed clinically on a follow up visit by means of the SCORAD index by a single dermatologist, and the patient-oriented eczema measure (POEM) scores were also determined. In addition, samples of skin surface cells were collected using a novel non-invasive technique for evaluation of LL-37 levels from lesional and non-lesional sites of the VD deficient AD patients. Quantification of LL-37 in superficial skin samples was performed as explained below.

Taking the International Osteoporosis Foundation's (IOF) recommendations (Dawson-Hughes, Mithal et al. 2010) , levels >75 nmol/L were defined as sufficient VD levels. Levels <50 nmol/L were deemed deficient; and supplementation commenced.

Levels of 50-75 nmol/L were considered suboptimal and corrected with over the counter (OTC) multivitamin preparations containing the 100% RDA of VD. Those that were considered deficient were supplemented depending upon the level of deficiency and age (6000 IU of cholecalciferol daily in ages 1-12 years; 10,000 IU daily for 12-18 years of age) according to the British National Formulary (British Medical Association and Royal Pharmaceutical Society of Great Britain 2011) for two months.

Patients continued on the same topical and oral medications. No treatment was changed, or withheld during this period, and patients were treated accordingly as usual clinical practice. In the event that a patient needed to commence new treatment, such as immune-suppressants or anti viral medication, VD supplementation was commenced, however the patient was not included in the final analysis. Therefore newly diagnosed ADEH patients were not included in the final analysis.

On the follow up visit after two months of supplementation, SCORAD was reassessed by the same dermatologist and POEM was repeated. Serum VD levels, total IgE level and serum calcium levels were rechecked. In addition, samples of skin surface cells were collected again from lesional and non-lesional sites for reassessment of LL-37 levels.

The study was approved by the Sheffield Children's Hospital ethics committee.

II.II Evaluation of clinical severity

II.II.I SCORAD

SCORAD is a system that incorporates three components: extent of involvement, intensity and subjective systems as indicators of quality of life (European Task Force On Atopic 1993). AD patients in this study were evaluated on these three components in the dermatology clinic.

Extent of area involved is evaluated based on the rule of nine, originally proposed for assessment of burns (Berkow 1924). The lesions included in the assessment are only inflammatory patches of eczema and not general dryness, and are directly plotted onto a printed figure in the evaluation sheet.

The intensity items are composed of six criteria: erythema, edema/papulation, oozing/crusting, excoriations, lichenification and dryness. For evaluation of intensity, for each item an area is chosen on the patient that is regarded representative of all the lesions in the patient. Therefore, the worst area affected should be excluded (European Task Force On Atopic 1993).

Dryness should be scored on general condition, not on acute lesions or lichenified areas. Each item is graded on a score of 0 to 3 (0=absent, 1=mild, 2=moderate, 3=severe). The subjective symptoms, pruritis and sleep loss, are determined by the patient and/or parents, according to last three days and nights. This is scored on a scale 0-10; 10 being the worst the patient has ever experienced. The results are then computed using the online computer software www.adserver.sante.univ-nantes.fr/SCORAD.

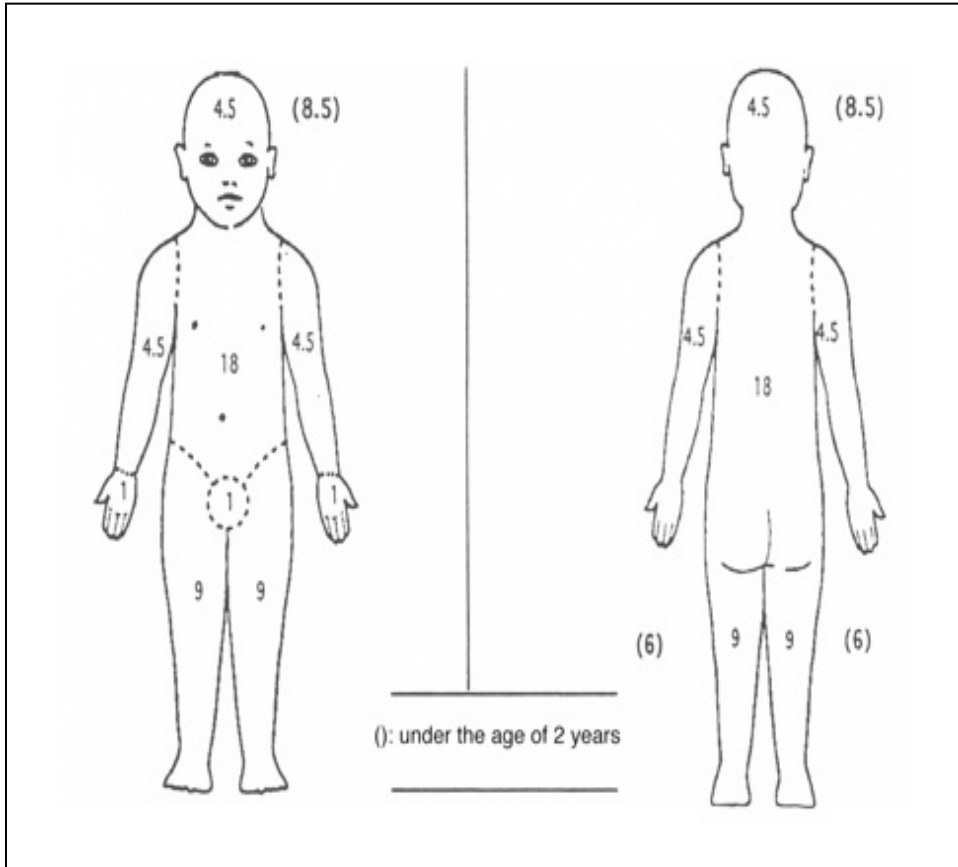


Figure II. 1: SCORAD; extent of area involved in AD.

Rule of nine is applied to evaluate the extent of AD in a patient. In this method, both the front and back of patient is plotted. In patients over two years of age, the head accounts for 4.5%; the trunk for 18 % of body surface area on each side; the upper limbs for 9 %, each of the lower limbs for 9%. Each hand accounts for 1 %; the groin accounts for 1 % as well.

The figures in parentheses are for children less than 2 years of age, where the head accounts for 8.5%, and the posterior aspect of the lower limbs for 6% each.

II.II.III The Patient Oriented Eczema Measure (POEM)

POEM is a measure developed for the self-assessment and monitoring of AD (Charman, Venn et al. 2004). It is important due to the fact it provides insight into the patient's perspective on their illness and their experience. It mainly examines symptom frequency rather than overall severity, which is important for assessing relapses and remissions, as well as general quality of life.

AD patients completed a questionnaire consisting of seven questions (Figure II.2), addressing issues such as itchiness, dryness, sleep loss, bleeding and infection signs such as weeping and oozing. All answers were allocated a score from which a total score, maximum 28, is determined.

Patient-Oriented Eczema Measure

Please circle one response for each of the seven questions below. Young children should complete the questionnaire with the help of their parents. Please leave blank any questions you feel unable to answer.

1. Over the last week, on how many days has your/your child's skin been itchy because of the eczema?

No Days 1-2 Days 3-4 Days 5-6 Days Every Day

2. Over the last week, on how many nights has your/your child's sleep been disturbed because of the eczema?

No Days 1-2 Days 3-4 Days 5-6 Days Every Day

3. Over the last week, on how many days has your/your child's skin been bleeding because of the eczema?

No Days 1-2 Days 3-4 Days 5-6 Days Every Day

4. Over the last week, on how many days has your/your child's skin been weeping or oozing clear fluid because of the eczema?

No Days 1-2 Days 3-4 Days 5-6 Days Every Day

5. Over the last week, on how many days has your/your child's skin been cracked because of the eczema?

No Days 1-2 Days 3-4 Days 5-6 Days Every Day

6. Over the last week, on how many days has your/your child's skin been flaking off because of the eczema?

No Days 1-2 Days 3-4 Days 5-6 Days Every Day

7. Over the last week, on how many days has your/your child's skin felt dry or rough because of the eczema?

No Days 1-2 Days 3-4 Days 5-6 Days Every Day

Total Score (maximum 28) _____

Figure 1. The patient-oriented eczema measure. Responses are scored as follows: 0, no days; 1, 1 to 2 days; 2, 3 to 4 days; 3, 5 to 6 days; and 4, every day.

Figure II. 2: POEM.

The patient and/or parents answered the above seven questions, after which the total score was calculated (Charman, Venn et al. 2004).

II.III. Evaluation of Cathelicidin levels (LL-37) in the stratum corneum

II.III.I Collection of superficial skin samples

Sterile nylon brushes (Cytotak Transwab brushes, Medical Wire and Equip. LTD, UK) were used to collect surface cells of the stratum corneum. Brushes were gently brushed against the skin surface, then placed in 1.5 ml micro tubes (Sarstedt, Aktiengesellschaft and Co., Germany) and placed in a - 80 freezer until analysis (Forma Scientific, Inc. USA).

II.III.II Analysis of LL-37 levels in superficial skin samples

Enzyme-Linked Immuno-Sorbent Assay (ELISA), is a popular type of analytic biochemistry assay used to detect the presence of an antigen in a liquid sample (Ueda 2007). Detecting antigens in a sample is through the following steps: The specific antigens, presumed to be in the sample adhere to a surface. Then an antibody, specific to these antigens, is placed over the surface to allow antigen-antibody binding. This specific antibody is connected to an enzyme. The enzyme then reacts with a substrate present in the last solution added, and this reaction produces a color change in the substrate, which is then read by a spectrophotometer.

To prepare the samples, an extraction buffer (10 mM disodium phosphate/0.2% sodium dodecyl sulphate/ 0.5% propylene glycol) at pH 7.4 was prepared (Hendrix, Miller et al. 2007).

Protease inhibitors (Complete mini, Roche, Germany) were then added (1 tablet per 10 ml of extraction buffer) to prevent undue proteolysis of LL-37. These tablets contain the following enzymes: Pancreas-extract 0.02 mg/ml, Pronase 0.005 mg/ml, Thermolysin 0.0005 mg/ml, Chymotrypsin 0.003mg/ml and Papain 0.33 mg/ml.

To each micro tube of sample, each containing three brushes, 800 μ L of the prepared extraction buffer was added. The samples were then sonicated for 30 minutes in a sonication water bath (Ultrawave ltd., UK). The brushes were removed from the micro tubes and discarded; 275 μ L of each sample was placed in duplicate wells in a 96 multi-well plate, pre-coated in the LL-37 capture antibody provided in the ELISA kit (Human LL-37 ELISA kit, Hycult biotech, The Netherlands). Standards of LL-37 of known concentrations, prepared according to the manufactures guidelines, were also loaded on to the plate (Table 2). The plate was then incubated for one hour at room temperature. The plate was washed four times with wash/dilution buffer supplied in the kit. Boiotynylated tracer antibody was added (100 μ L to each well), and the plate was incubated again for one hour. The wash procedure was then repeated as before, after which 100 μ L of streptavidin-peroxidase was added to each well and incubated for an hour.

Concentration of Standards (ng/ml)	Volume of standard	Volume of Wash/dilution Buffer
100	150 μ l	230 μ l
33	125 μ l	250 μ l
11	125 μ l	250 μ l
3.7	125 μ l	250 μ l
1.2	125 μ l	250 μ l
0.4	125 μ l	250 μ l
0.1	125 μ l	250 μ l
0	0	250 μ l

Table 2: Concentration of Standards used in the Human LL-37 Elisa kit.

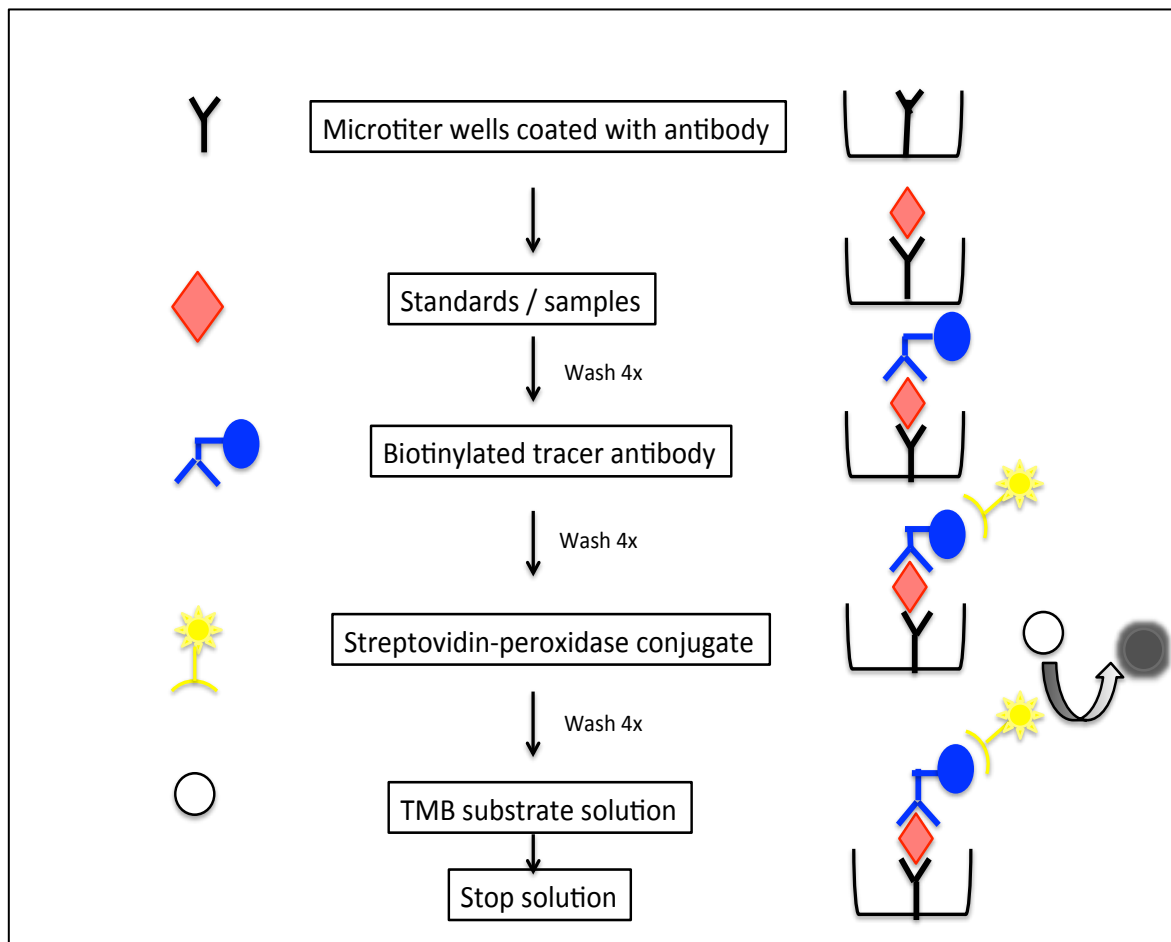


Figure II. 3: Overview of LL-37 ELISA Protocol.

Micro well plates precoated with LL-37 binding antibodies are supplied. Samples and standards are loaded and incubated for one hour at room temperature (20-25°C). Biotinylated tracer antibody is then loaded after wash procedure, which will bind to the captured LL-37. After another hour incubation period, the streptavidin-peroxidase conjugate, added will then bind to the biotinylated tracer antibody. After incubation and wash procedure, the streptavidin- peroxidase will then react with the substrate added, tetra methyl bezidine. This reaction is then stopped after 30-40 minutes, and the absorbance is read by a spectrophotometer.

Tetra methyl benzidine substrate, supplied in the kit, was added after repeating the wash procedure above, and plate was incubated for 30-40 minutes. The stop solution oxalic acid was added to each well to stop the reaction, and the plate was read using a Spectramax ME5 plate reader (Molecular devices, LLC, USA) at a wavelength of 450 nm.

The concentration of LL-37 in each sample was extrapolated from a standard curve generated with the absorbance readings from the standards included on each assay plate. These readings, in addition to the total protein readings obtained from the BCA assay below, were used to determine the amount of LL 37 per gram of protein.

II.III.III The Bicinchoninic Acid (BCA) analysis

The Bicinchoninic Acid (BCA) assay is a method used to determine the amount of protein present in a solution, based on Copper reduction from Cu^{2+} to Cu^{1+} . The amount of Cu^{2+} reduced is proportional to the amount of protein present in the solution.

The standards for the BCA assay were prepared from a BSA 1mg/ml stock solution supplied in the BCA kit (Sigma-Aldrich, UK) and were prepared at the following concentrations:

Concentrations of Standards ($\mu\text{l}/\text{ml}$)	Volume of BSA	Volume of de ionized H₂O
30	300 μl	9.7 ml
20	200 μl	9.8 ml
15	150 μl	9.85 ml
10	100 μl	9.90 ml
5	50 μl	9.95 ml
2	20 μl	9.98 ml
1	10 μl	9.99 ml
0	0	10.00 ml

Table 3: Concentrations of Standards used in the BCA assay

Samples were loaded into the micro well plates in duplicates; a two-fold stepwise dilution was then performed. The plate was then incubated at 60 °C for two hours, and then read with a spectrophotometer, at a wavelength of 562 nm. The total protein concentration is proportional to the degree of change.

The absorbance readings of the standards were then plotted against the concentrations of standards, and analyzed by linear regression using Microsoft Excel to obtain a linear calibration curve. The absorbencies of the samples were then extrapolated.

II.IV. Statistical Analyses:

Statistical analysis was performed using Prism 5 (Graph Pad Prism Software, Inc., a Jolla, USA). SCORAD and POEM results were analyzed by two tailed paired students t-test. LL-37, IgE and serum calcium results were analyzed using an unpaired students t-test. One-way analysis of variance (ANOVA) was used to analyze data stratified according to SCORAD. Post hoc comparisons were done with Bonferroni's multiple comparisons test. Level of significance was set at $p < 0.05$. All values were expressed as mean \pm standard error of the mean (SEM).

Chapter Three: Results

III. Chapter Three: Results

III.I. Patient demographics:

Ninety children with a mean age of 9 (ages 1-18) were screened, of which 52% were male and 48% female. The demographics were as follows: 63% Caucasian, 27% Asian descent, 4% of mixed race, 4% Chinese and 2% of African descent (Table 2). Fifty percent of the patients screened were classified as ADEH and were either under treatment or had been treated previously with anti-viral medication.

Eleven of the patients were on immunosuppressant treatment for at least a year prior to the study. The majority of the children were on a combination of emollients, topical corticosteroids and topical calcineurin inhibitors; only two patients were just on emollient therapy.

Of the 90 AD children, only fifteen had healthy VD levels (Table 4). Fifty seven percent of the patients were found to be VD deficient, 26% were found to have sub-optimal levels, making it a total of 83% with unhealthy VD levels (<75 nmol/l). Further stratification according to ethnic background is in Table 5. When grouping the children into Caucasian and non-Caucasian subgroups, the incidence of VD deficiency was 61.5% and 38.5% respectively.

Characteristic	N (%)
Gender:	
Females	47 (52%)
Males	43 (48%)
Ethnicity:	
Caucasian	57(63%)
Asian	24 (27%)
Chinese	3 (4%)
African	2 (2%)
Mixed	4 (4%)
Clinical classification:	
AD	45 (50%)
ADEH	45 (50%)
Healthy VD levels	15 (17 %)
Unhealthy VD levels:	75 (83%)
- Sub optimal VD (50-75nmol/l)	23 (26 %)
- Deficient VD (<50nmol/l)	35 (39%)
- Severely deficient VD (<25nmol/l)	17 (18%)

Table 4: Demographics of the AD patients screened in the clinic.

Ethnic group	Severely Deficient (<25nmol/l) N=17	Deficient (25-50nmol/l) N= 35	Sub optimal (50-75nmol/l) N= 23	Healthy (>75nmol/l) N=15
Caucasian	8 (47%)	24 (68%)	16 (70%)	12 (80%)
Asian	7 (41%)	7 (20 %)	3 (13%)	3 (20%)
Chinese	1 (6 %)	1 (3 %)	1 (4 %)	0
African	0	0	3 (13 %)	0
Mixed origin	1 (6%)	3 (9 %)	0	0

Table 5: Classification of VD status according to ethnic group in the population screened.

After screening, eighteen patients were lost to follow up (Figure III.1). Another ten patients either immediately started supplementation through their GP or local dermatologist, or needed either antivirals or immunosuppressant treatment, and therefore could not be included. Consequently, a total of 47 patients were included in the practice evaluation study. The demographics of this cohort were as follows: 53% Caucasian, 30% Asian, 9 % mixed race, 6% Chinese and 2% of African origin (Table 6). Of those 47 patients, twelve were AD; thirty-five patients were diagnosed to have ADEH. Stratification of VD status according to ethnic background in this group is in Table 7.

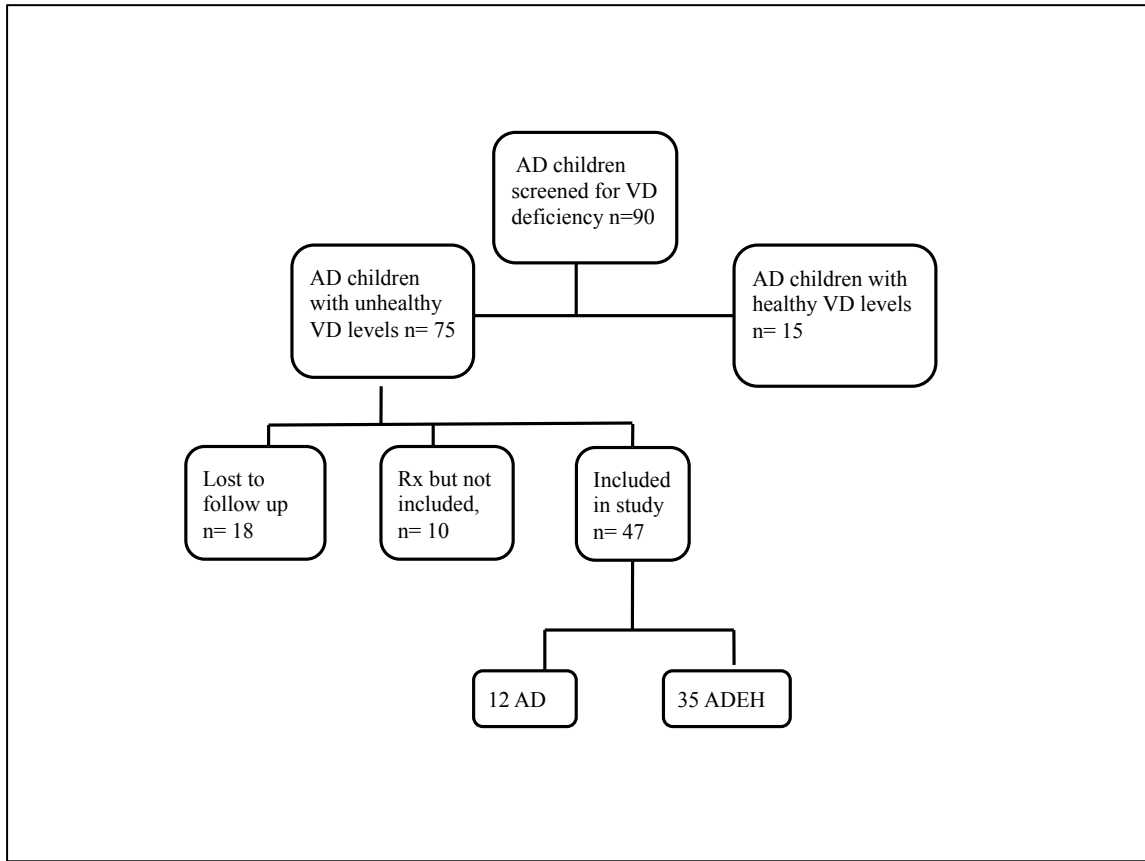


Figure III. 1: Flowchart of study.

Ninety children with AD were screened for VD deficiency: 15 were found to have normal VD levels, 75 had low VD levels. Of the 75 AD children, 18 were lost to follow up and 10 commenced VD supplementation via GP or local dermatologist. Therefore 47 children were included in the practice evaluation study.

Characteristic	N (%)
Gender: Females Males	21 (45%) 26 (55%)
Ethnicity: Caucasian Asian Chinese African Mixed	25 (53%) 14 (30%) 3 (6%) 1 (2%) 4 (9%)
Clinical classification: AD ADEH	12 (26%) 35 (74%)
Unhealthy VD levels: - Sub optimal VD (50-75nmol/l) - Deficient VD (<50nmol/l) - Severely deficient VD (<25nmol/l)	47 12 (25%) 23 (50%) 12 (25%)

Table 6: Demographics of the AD patients included in the practice evaluation study.

Ethnic group	Severely Deficient (<25nmol/l) N=12	Deficient (25-50nmol/l) N= 23	Sub optimal (50-75nmol/l) N= 12
Caucasian	4 (33%)	13 (57%)	8 (67%)
Asian	6 (50%)	6 (26%)	2 (17%)
Chinese	1 (8%)	1 (4%)	1 (8%)
African	0	0	1 (8%)
Mixed origin	1 (8%)	3 (13%)	0

Table 7: Classification of VD status according to ethnic group in the AD patients included in the practice evaluation study.

III.II. Pre-supplementation assessment:

In the original population screened, only two of the 45 ADEH patients had sufficient VD levels by current standards. ADEH patients were found to have significantly lower VD levels than AD patients ($p < 0.0001$, mean ADEH score= 37.27 ± 2.95 , mean AD score= 60.81 ± 4.12 , unpaired t-test, Figure III.2).

Using SCORAD, the 47 remaining patients included in the study were classified into three groups based on severity: mild < 25 , moderate 25-50 and severe > 50 , as described in previous studies, which will be discussed in detail in the discussion chapter. VD levels were found to be significantly different between the groups by one-way ANOVA (Figure III.3, $p = 0.016$, means 30.6 ± 4.5 , 40.4 ± 2.72 , and 56.7 ± 10.31 respectively). Bonferroni's post-test revealed a statistically significant difference in VD levels between children with severe and mild AD. VD levels and SCORAD also showed a significant inverse correlation ($p = 0.014$, Pearson's correlation coefficient $r = -0.4$, Figure III.4).

SCORAD was found to be significantly lower in AD children when compared with those of ADEH children ($p < 0.0001$, mean AD score= 34.75 ± 3.09 , mean ADEH score= 49.17 ± 1.28 , unpaired t-test, Figure III.5). POEM was also found to differ significantly between AD and ADEH groups ($p = 0.0076$, mean scores= 14.92 ± 1.39 and 19.79 ± 0.91 respectively, unpaired t-test, Figure III.6).

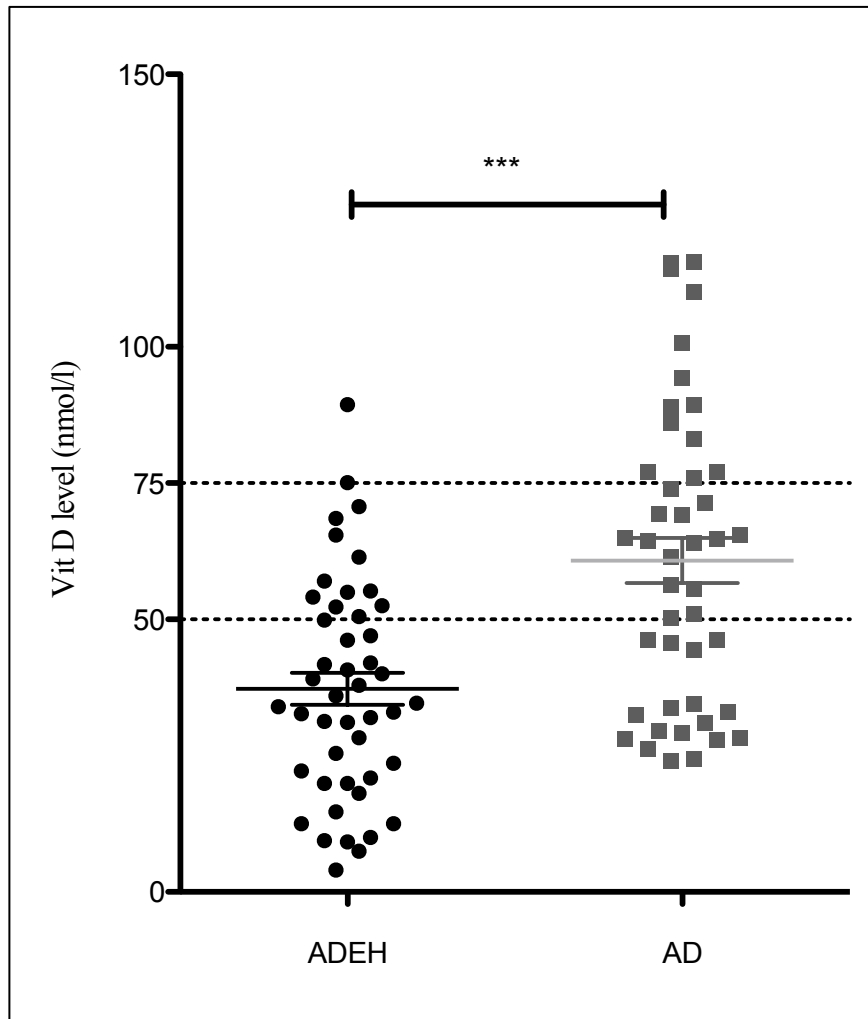


Figure III. 2: VD levels in AD and ADEH children.

Ninety children screened for VD deficiency revealed ADEH patients (black dots, mean score= 37.27 ± 2.95 , $n=45$) to have significantly lower VD levels than AD patients (gray squares, mean score= 60.81 ± 4.12 , $n=45$; $p < 0.0001$, unpaired t-test). Levels between 50 and 75 nmol/l (dotted lines) were considered suboptimal, levels below 50 nmol/l were considered deficient.

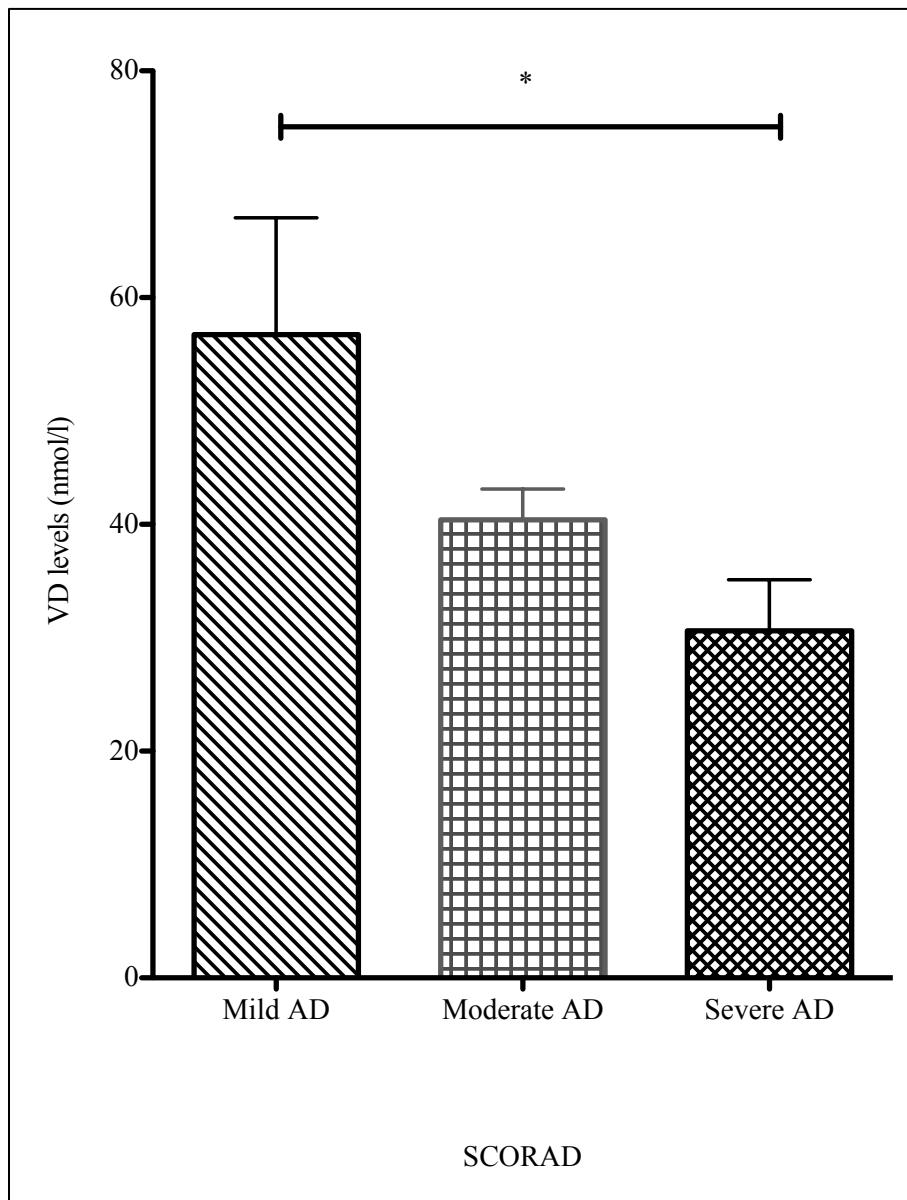


Figure III. 3: Classification of patients based on SCORAD.

AD patients (47) were classified into three groups: severe AD (>50, n=13), moderate AD (25-50, n= 30), and mild AD (<25, n=4). VD levels were significantly different ($p = 0.016$, mean severe AD= 56.7 ± 10.31 , mean moderate score= 40.4 ± 2.72 , mean mild score= 30.6 ± 4.5 ; one way ANOVA). Post-test with Bonferonni's multiple comparisons showed a significant difference between mild and severe scores.

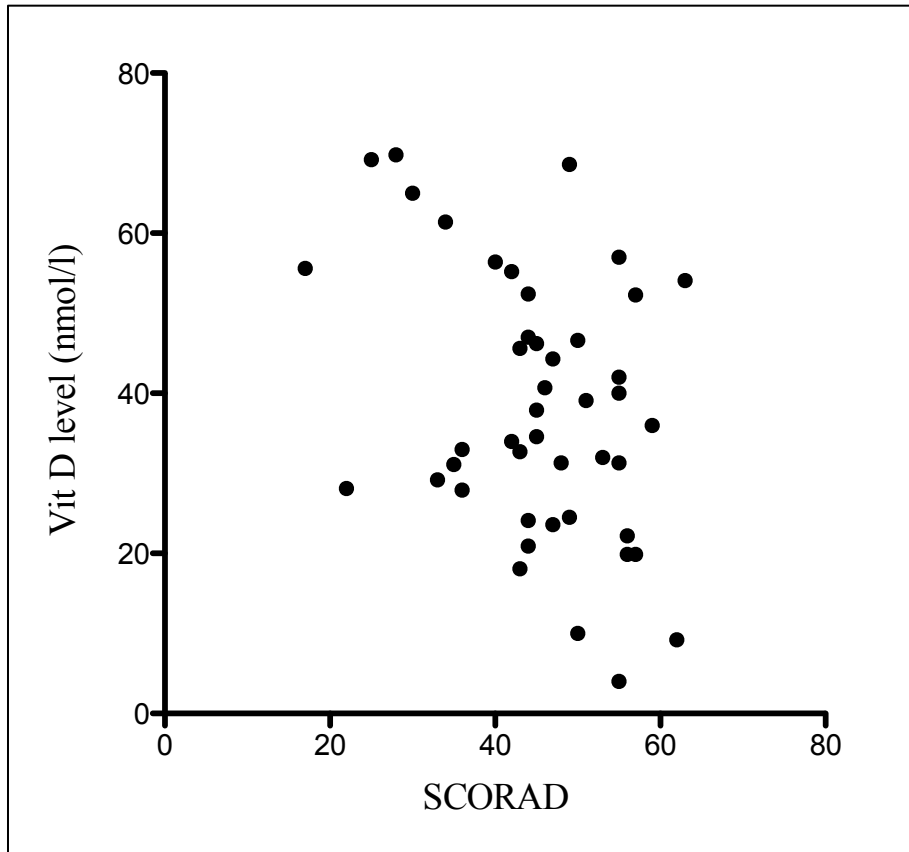


Figure III. 4: Correlation of SCORAD and VD levels.

Correlation of VD levels and SCORAD also showed a significant inverse relationship ($p = 0.014$, Pearson's correlation coefficient $r = -0.4$, $n = 47$).

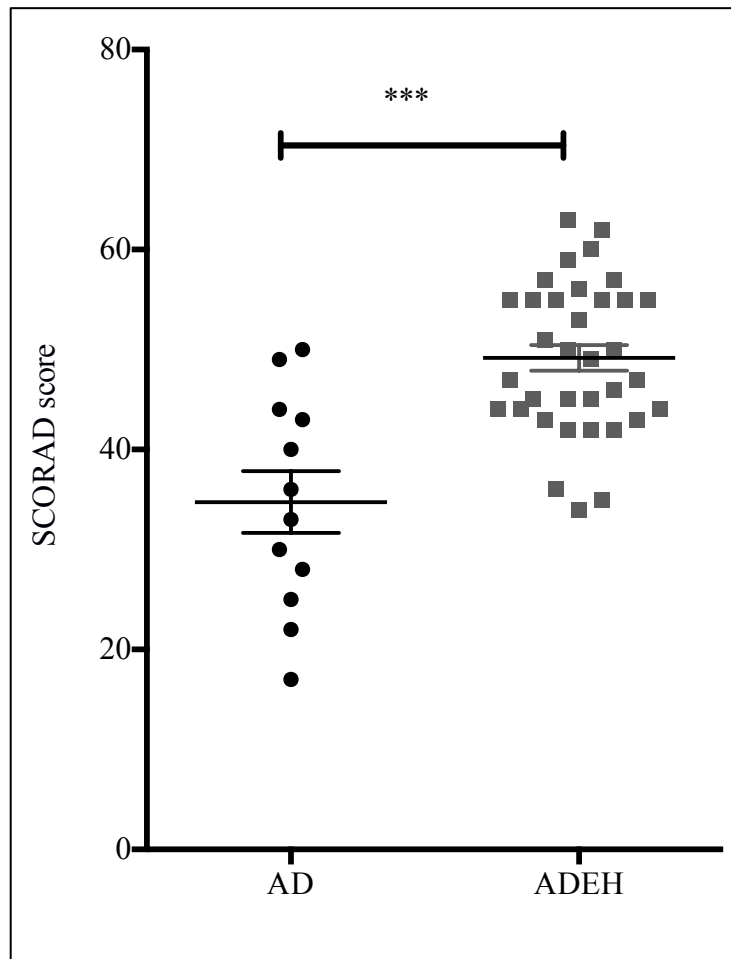


Figure III. 5: SCORAD in AD and ADEH.

There was a significant difference in baseline SCORAD between 12 AD children (black dots, mean score = 49.17 ± 1.28) and 35 ADEH children (grey squares, mean score = 34.75 ± 3.09) ($p < 0.0001$, unpaired t-test).

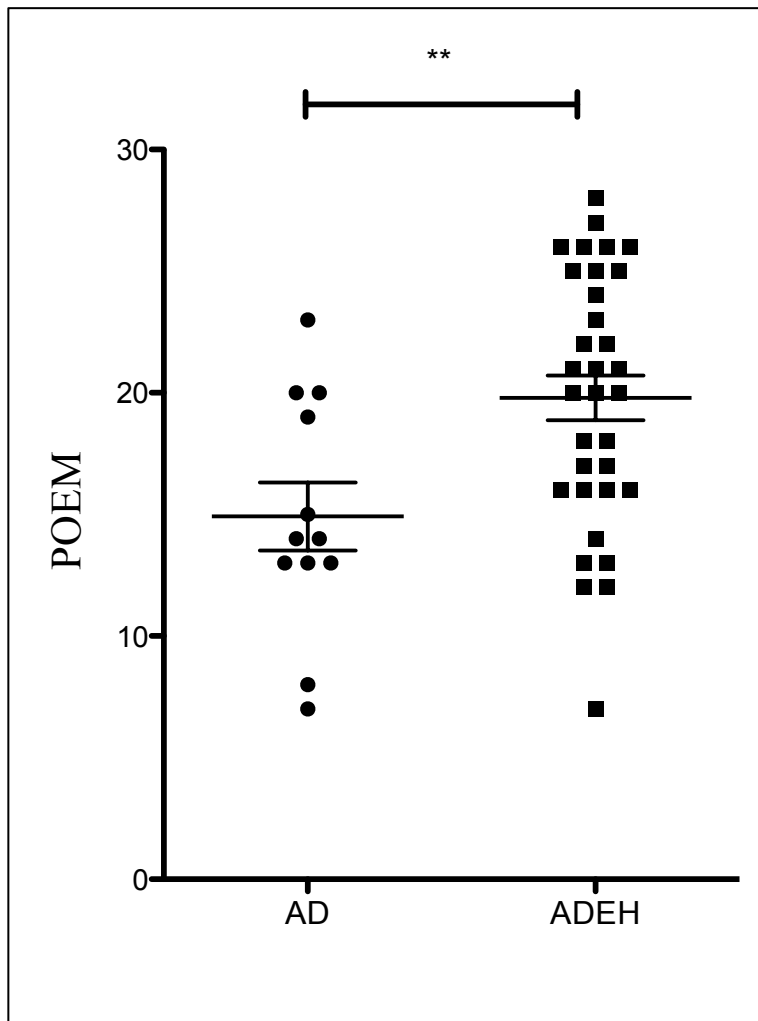


Figure III. 6: POEM in AD and ADEH.

POEM was found to differ significantly between AD (mean score= 14.92 ± 1.39 , $n=12$) and ADEH groups (mean score= 19.79 ± 0.91 , $n=35$; $p= 0.0076$, unpaired t-test).

The baseline LL-37 levels measured were also stratified, similarly to the VD levels, according to SCORAD. Analysis by one-way ANOVA showed a significant difference in LL-37 levels between mild, moderate and severe SCORAD ($1.15 \pm 0.39 \mu\text{g/g}$, $0.47 \pm 0.09 \mu\text{g/g}$ and $0.34 \pm 0.09 \mu\text{g/g}$ respectively, $p = 0.018$, Figure III.7). Bonferroni's post-test revealed a significant relationship between both mild and moderate, and mild and severe groups. Although there was an observed trend for lesional and non-lesional LL-37 levels to decrease with increasing SCORAD severity, this did not reach statistical significance ($p = 0.32$ and $p = 0.31$ respectively, Figure III.8).

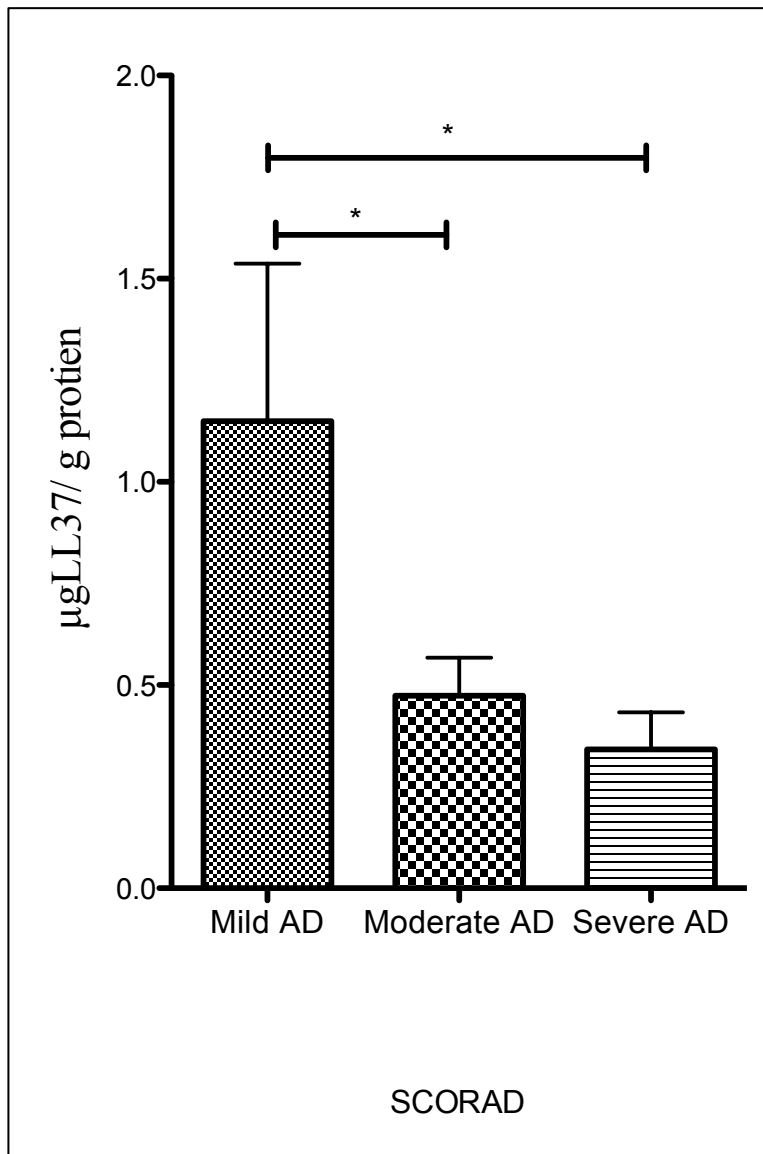


Figure III. 7: LL-37 levels according to SCORAD.

LL-37 levels were stratified according to SCORAD severity into mild, moderate and severe. Analysis by one-way ANOVA showed a significant difference in LL-37 levels between mild, moderate and severe SCORAD ($1.15 \pm 0.39 \mu\text{g/g}$, $0.47 \pm 0.09 \mu\text{g/g}$ and $0.34 \pm 0.09 \mu\text{g/g}$ respectively, $p = 0.018$). Bonferroni's post-test revealed a significant relationship between both mild and moderate, and mild and severe groups ($p < 0.05$).

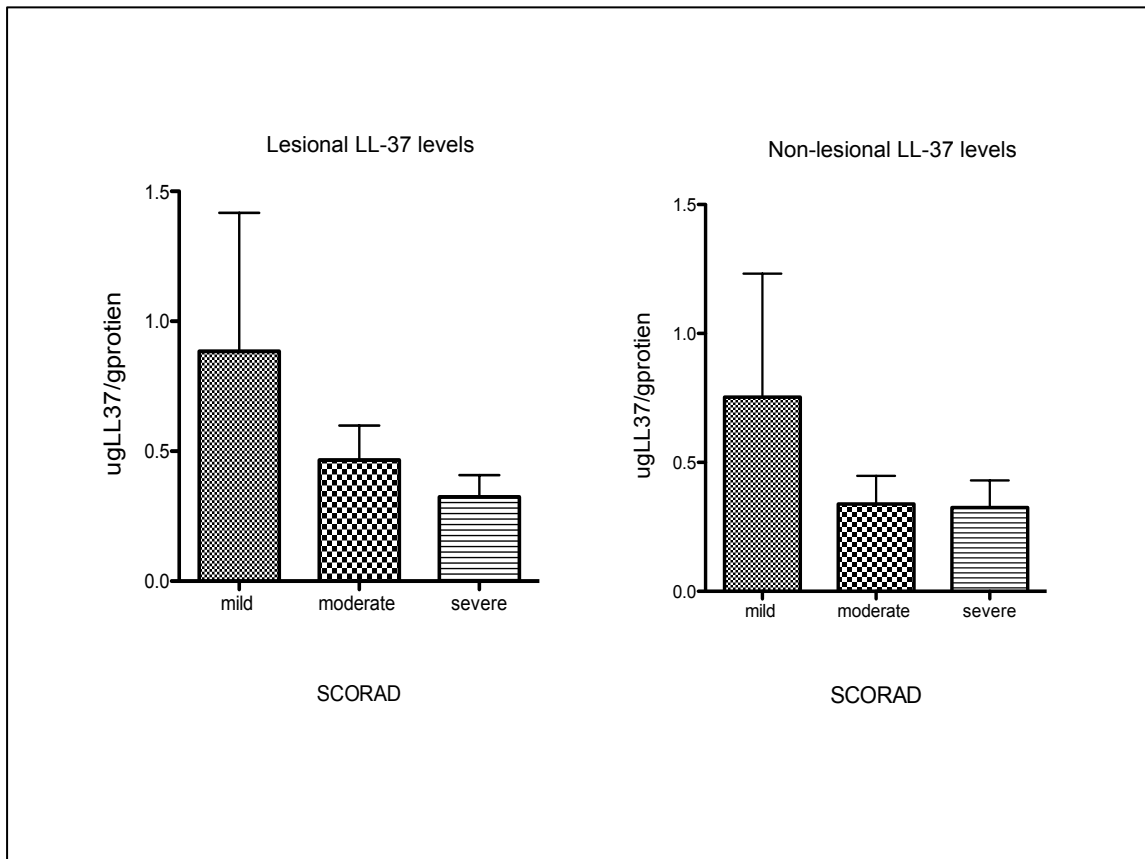


Figure III. 8: Lesional and non-lesional LL-37 levels vs SCORAD.

Left graph: Upon stratifying lesional LL-37 levels according to SCORAD, no significant difference was detected between the mean score of mild, moderate and severe SCORAD groups ($0.82 \pm 0.5 \mu\text{g/g}$, $0.47 \pm 0.13 \mu\text{g/g}$, $0.32 \pm 0.08 \mu\text{g/g}$ respectively; $p = 0.32$; one way ANOVA).

Right graph: non-lesional LL-37 levels did not show significant difference between SCORAD groups as well (mild = $0.75 \pm 0.48 \mu\text{g/g}$, moderate = $0.34 \pm 0.108 \mu\text{g/g}$, severe = $0.32 \pm 0.105 \mu\text{g/g}$; $p = 0.31$; one way ANOVA).

The baseline LL-37 levels measured in ADEH patients (n=35, mean score= $0.414 \pm 0.08 \mu\text{g/g}$) were lower than the levels in AD patients (n=12, mean score= $0.526 \pm 0.13 \mu\text{g/g}$), however it did not reach significance ($p= 0.46$, unpaired t-test, Figure III.9). Further stratification of LL-37 within each group into those taken from lesional and non-lesional sites did not show significance (Figure III.10). Lesional LL-37 levels measured in the ADEH patients (mean levels= $0.538 \pm 0.17 \mu\text{g/g}$) were lower than those in the AD patients (mean levels= $0.66 \pm 0.21 \mu\text{g/g}$), however this did not reach significance ($p =0.66$; unpaired t-test). Non-lesional LL-37 levels were also not significantly different between ADEH and AD groups (mean levels are $0.39 \pm 0.107 \mu\text{g/g}$ and $0.35 \pm 0.125 \mu\text{g/g}$ respectively, $p =0.86$, unpaired t-test).

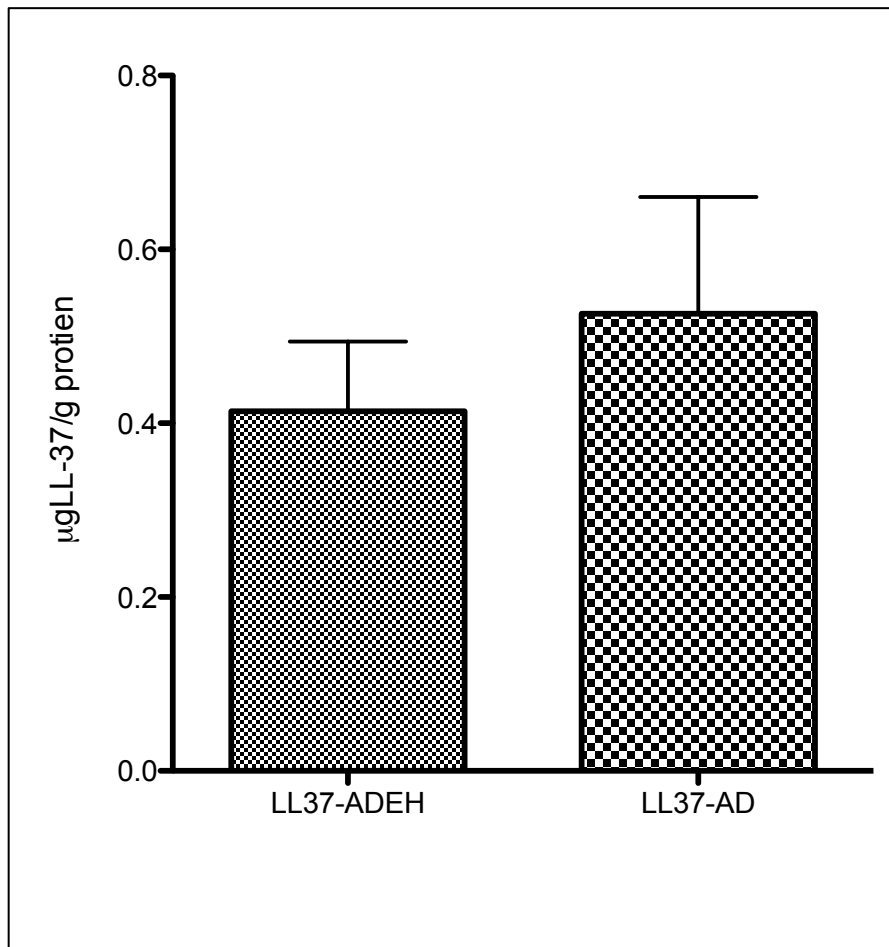


Figure III. 9: LL-37 levels in AD and ADEH patients.

The LL-37 levels measured in the lesional and non-lesional skin of ADEH patients (n=35, mean score= 0.414 ± 0.08 µg/g) were lower than the levels in AD patients (n=12, mean score= 0.526 ± 0.13 µg/g), however it did not reach significance ($p= 0.46$, unpaired t-test).

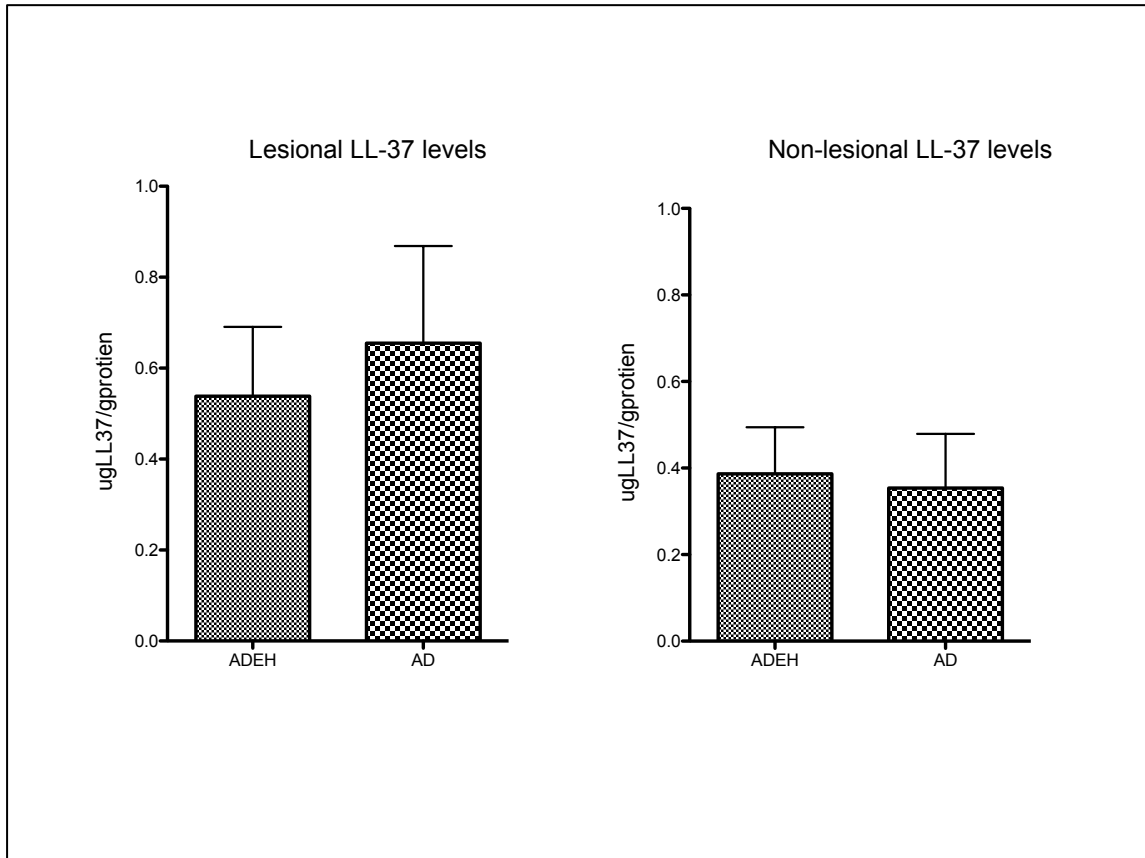


Figure III. 10: Lesional and Non-lesional LL-37 levels in AD and ADEH.

Left graph: Lesional LL-37 levels measured in the ADEH patients (mean levels= $0.538 \pm 0.17 \mu\text{g/g}$) were lower than those in the AD patients (mean levels= $0.66 \pm 0.21 \mu\text{g/g}$), however this did not reach significance ($p = 0.66$; unpaired t test).

Right graph: Non-lesional LL-37 levels were not significantly different between ADEH and AD groups (mean levels are $0.39 \pm 0.107 \mu\text{g/g}$ and $0.35 \pm 0.125 \mu\text{g/g}$ respectively, $p = 0.86$, unpaired t-test). LL-37 levels in lesional skin were increased in AD and ADEH in comparison with non-lesional LL-37 levels.

III.III. Post-supplementation assessment:

After two months of VD supplementation, VD levels showed a significant two-fold increase from mean level 37.99 ± 2.8 to level 84.93 ± 11.93 nmol/l, however remained within normal levels ($p < 0.0001$, unpaired t-test, Figure III.11).

Similarly, SCORAD scores also showed significant improvement with a mean reduction of 42.3% ($p < 0.0001$, paired t-test, Figure III.12). Stratification of patients into ADEH and AD also revealed significant reduction in SCORAD ($p < 0.0001$, ADEH from mean score 48.67 ± 1.4 to 27.78 ± 1.9 ; AD from mean score 35.69 ± 2.9 to 20.46 ± 2.68 ; Figure III.13, paired t-test).

Upon stratifying patients into two groups: patients who received the therapeutic VD supplements (concentrated cholecalciferol), and patients on OTC, both groups displayed significant reduction of SCORAD severity after two months (Figure III.14). The group receiving OTC supplements had a 40.7% reduction in SCORAD from mean score 38.8 ± 3.79 to 23.9 ± 3.23 ($p = 0.0006$, paired t-test); the group receiving cholecalciferol showed a 42.2% reduction from mean score 46.53 ± 1.55 to 27.14 ± 1.94 ($p < 0.0001$, paired t-test).

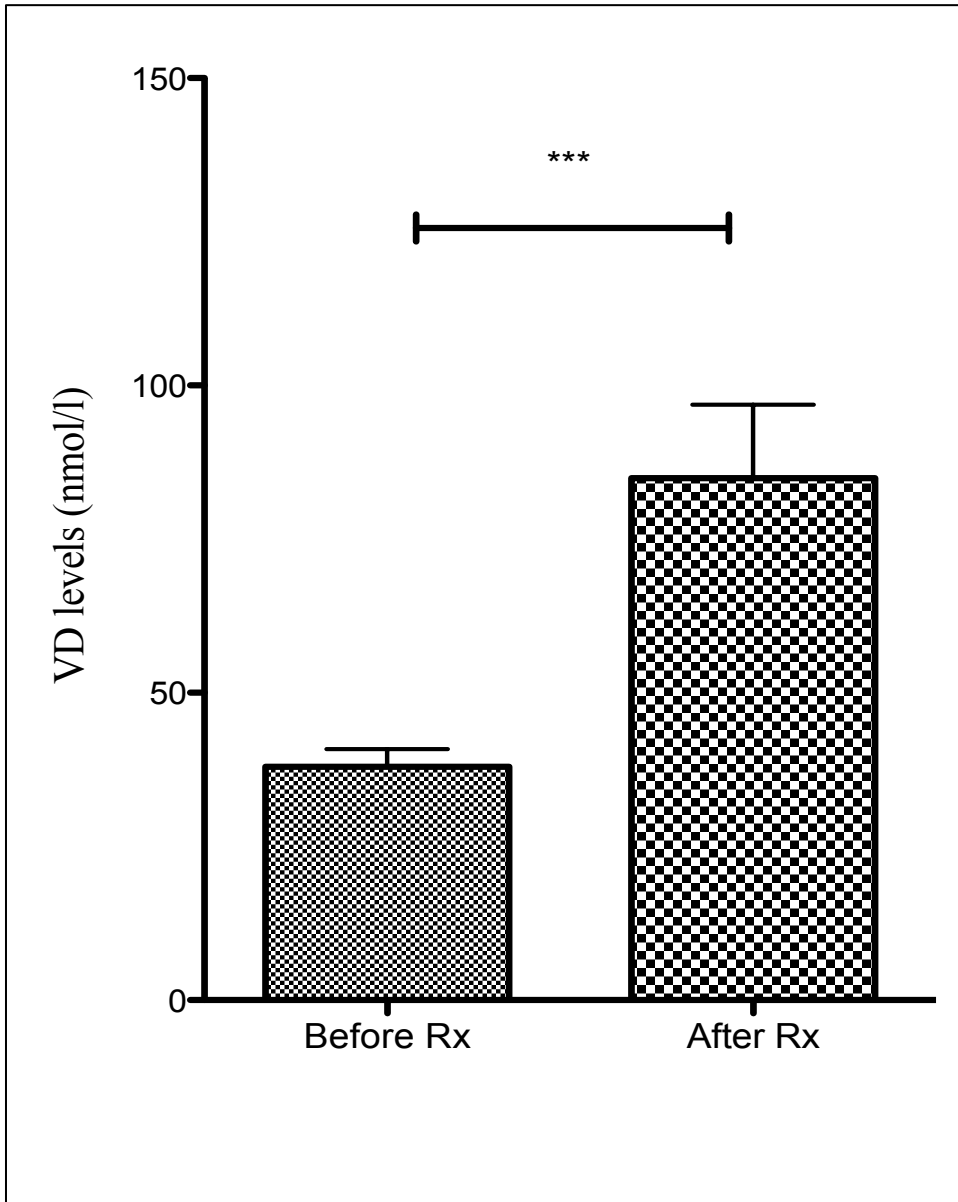


Figure III. 11: Comparison of VD levels.

After two months of VD supplementation, VD levels showed a significant increase from mean level 37.99 ± 2.8 to level 84.93 ± 11.93 nmol/l, however remained within normal levels ($p < 0.0001$, unpaired t-test).

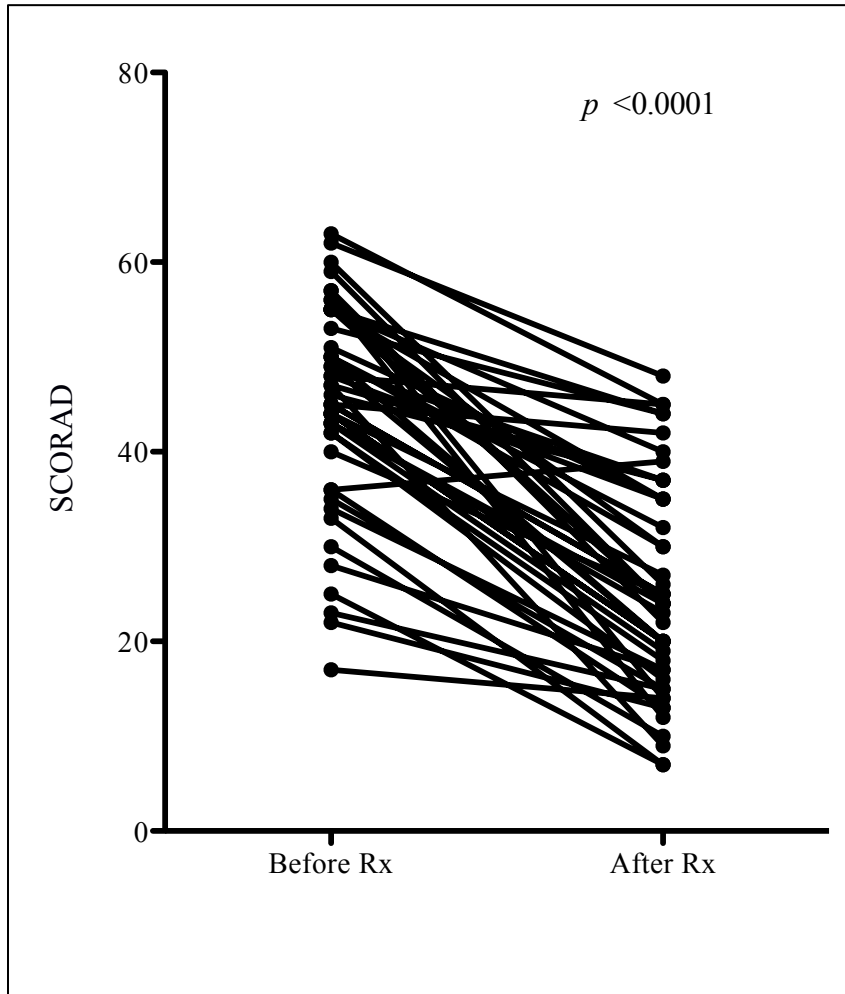


Figure III. 12: SCORAD after two months of VD supplements.

SCORAD in 47 AD patients showed significant reduction from mean score 45.5 ± 1.53 to 25.8 ± 1.7 (paired t-test) after two months of VD supplementation (mean reduction= 42.3%).

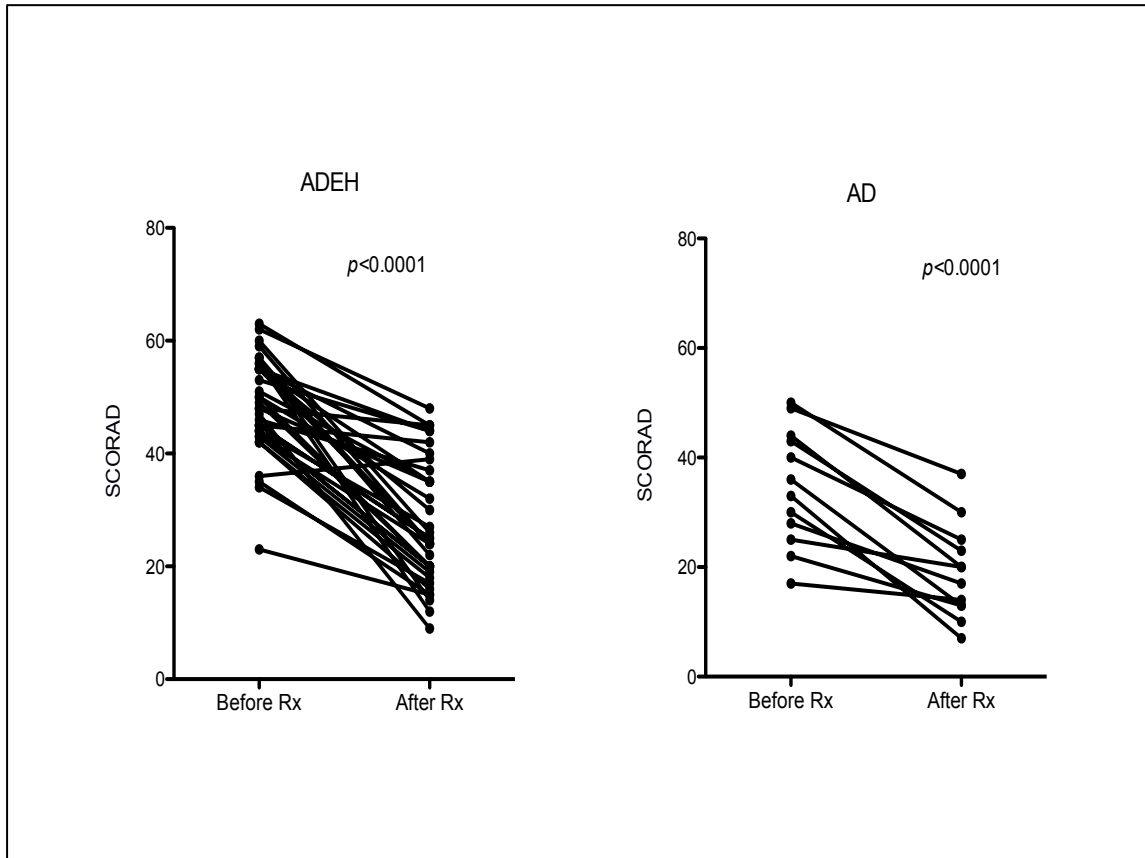


Figure III. 13: SCORAD in AD and ADEH patients.

ADEH patients (left graph) showed a significant reduction in SCORAD after two months of VD supplementation (from mean score 48.67 ± 1.4 to 27.78 ± 1.9 ; paired t-test). AD patients (right graph) also showed a significant reduction in SCORAD (from mean score 35.69 ± 2.9 to 20.46 ± 2.68 ; paired t-test).

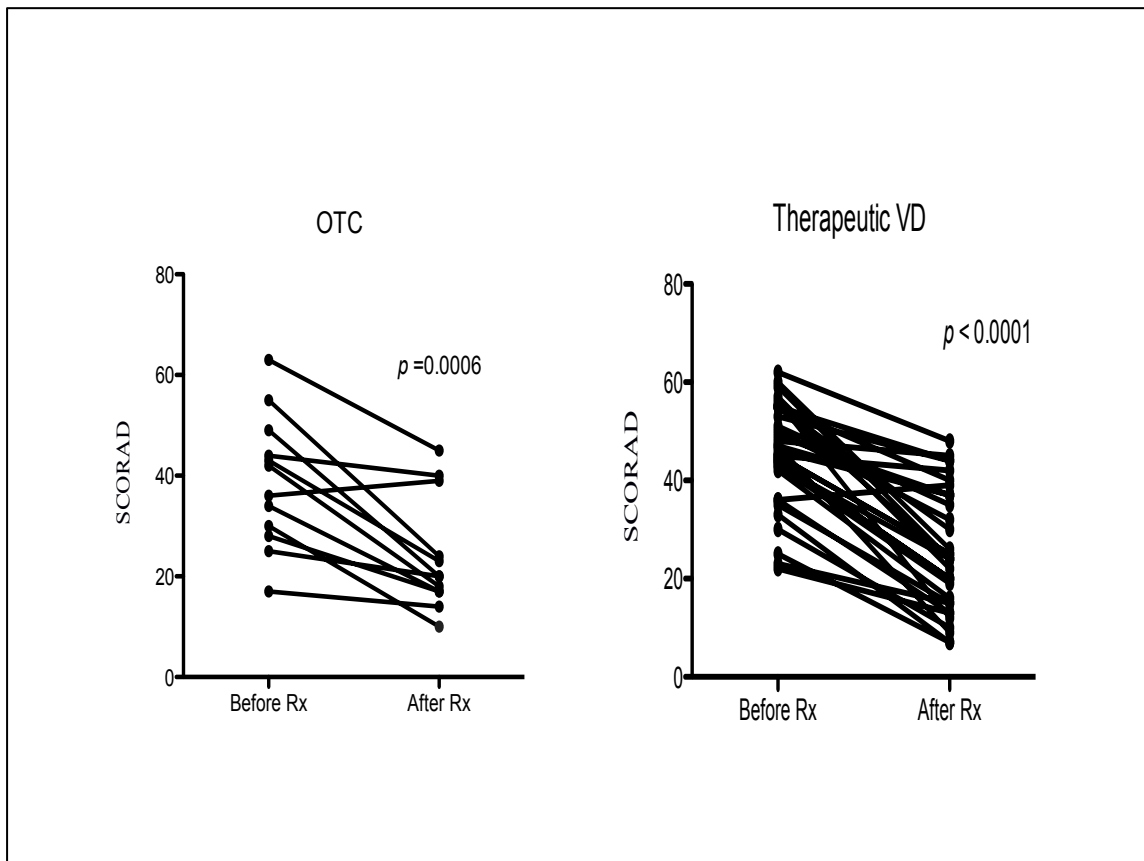


Figure III. 14: Stratification of SCORAD based upon type of VD supplement.

Stratification of patients into those treated with OTC VD supplements (left graph, n= 12) and those whom received the therapeutic dose (right graph, n= 35) revealed both groups to have significantly reduced SCORAD scores after two months of supplementation (OTC=40.7% SCORAD reduction from mean score 38.8 ± 3.79 to 23.9 ± 3.23 ; Cholecalciferol= 42.2% reduction from mean score 46.53 ± 1.55 to 27.14 ± 1.94 ; paired t-test).

Comparison of reduction rates in both groups showed no significant difference ($p=0.8$, unpaired t-test, Figure III.15). POEM scores also showed a significant reduction of 46.6% (from mean score 18.3 ± 0.8 to 9.2 ± 0.7 , $p < 0.0001$, paired t-test, Figure III.16), indicating a reduction in AD severity from the patient's perspective. Similarly, stratifying POEM into AD and ADEH also showed significant reduction in the scores (Figure III.17, AD mean score 14.92 ± 1.4 to 7.67 ± 1.11 , $p = 0.0009$, paired t-test; ADEH mean score 19.73 ± 0.92 to 10.52 ± 0.83 , $p < 0.0001$, paired t-test).

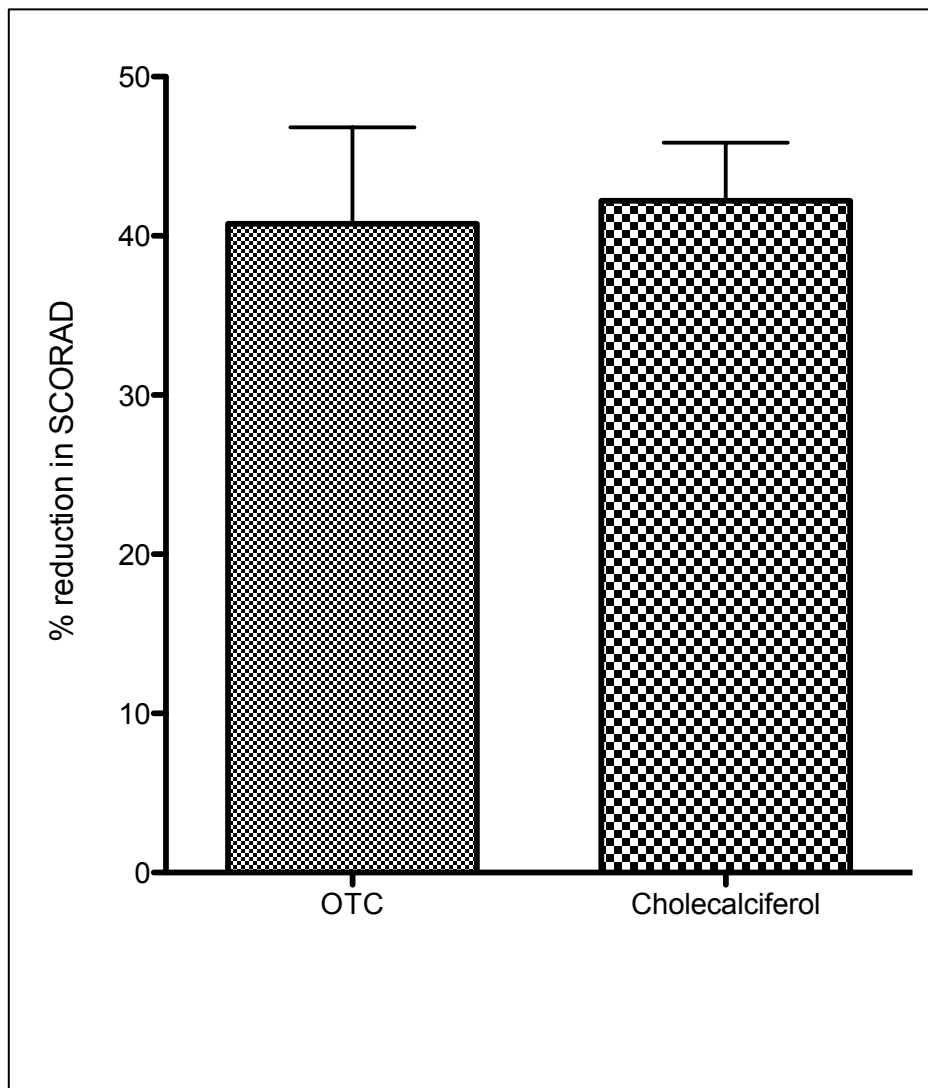


Figure III. 15: The percentage of SCORAD reduction in OTC and Cholecalciferol groups.

Comparison of individual reduction rates within the OTC group (mean reduction= 40.7 ± 6.02) and the Cholecalciferol group (mean reduction= 42.2 ± 3.65) did not show statistical significance ($p=0.82$, unpaired t-test).

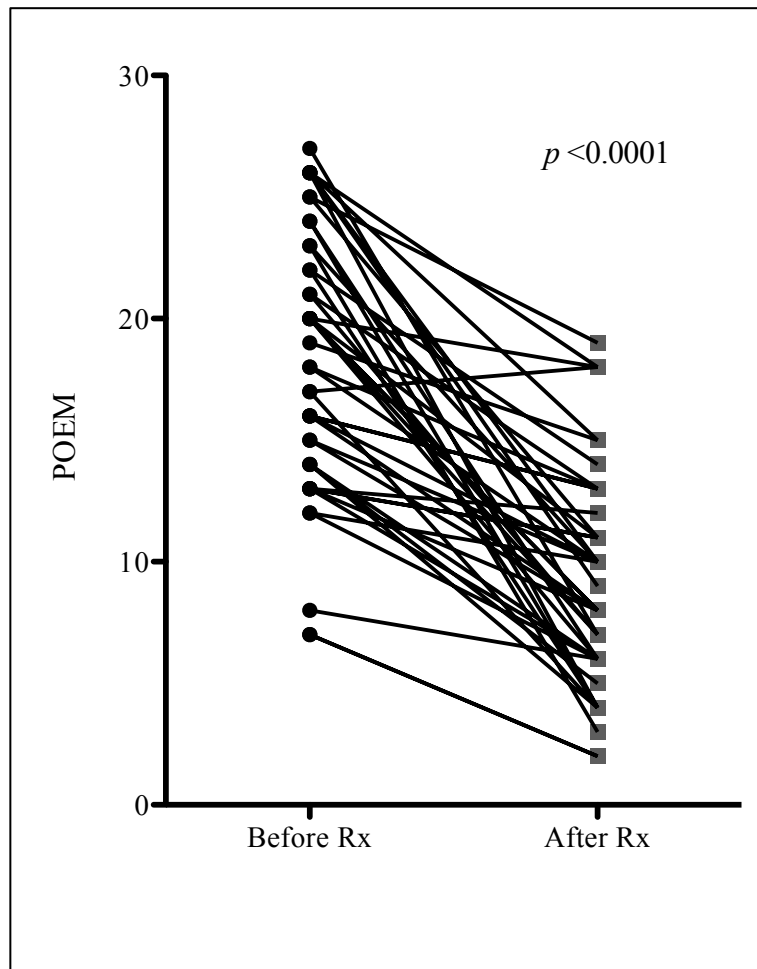


Figure III. 16: POEM scores after two months of VD supplements.

POEM scores in 47 AD patients showed a significant reduction from mean score 18.3 ± 0.8 to 9.2 ± 0.7 (mean reduction=46.6%) after two months of supplementation (paired t-test).

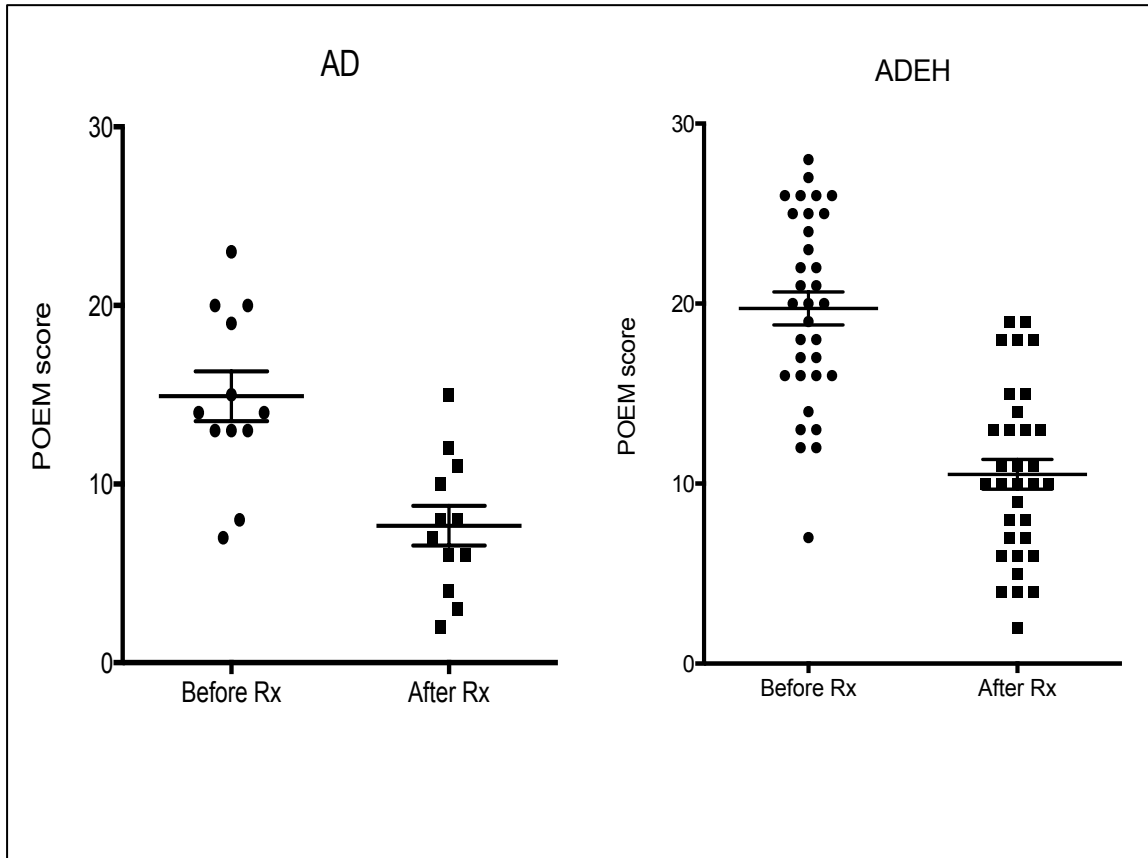


Figure III.17: POEM scores in AD and ADEH patients.

POEM in AD patients showed a significant reduction after two months of VD supplementation (from mean score 14.92 ± 1.4 to 7.67 ± 1.11 , $p = 0.0009$, paired t-test). ADEH patients also showed a significant reduction in the POEM scores (from mean score 19.73 ± 0.92 to 10.52 ± 0.83 , $p < 0.0001$, paired t-test).

LL-37 levels showed a significant four fold increase after two months supplementation ($p = 0.0004$, unpaired t-test, Figure III.18). Both lesional and non-lesional LL-37 showed a significant increase post supplementation (Figure III.19).

Serum calcium levels also showed a significant increase ($p = 0.0037$, unpaired t-test, Figure III.20), which differed from IgE levels, which did not show significant change ($p = 0.84$).

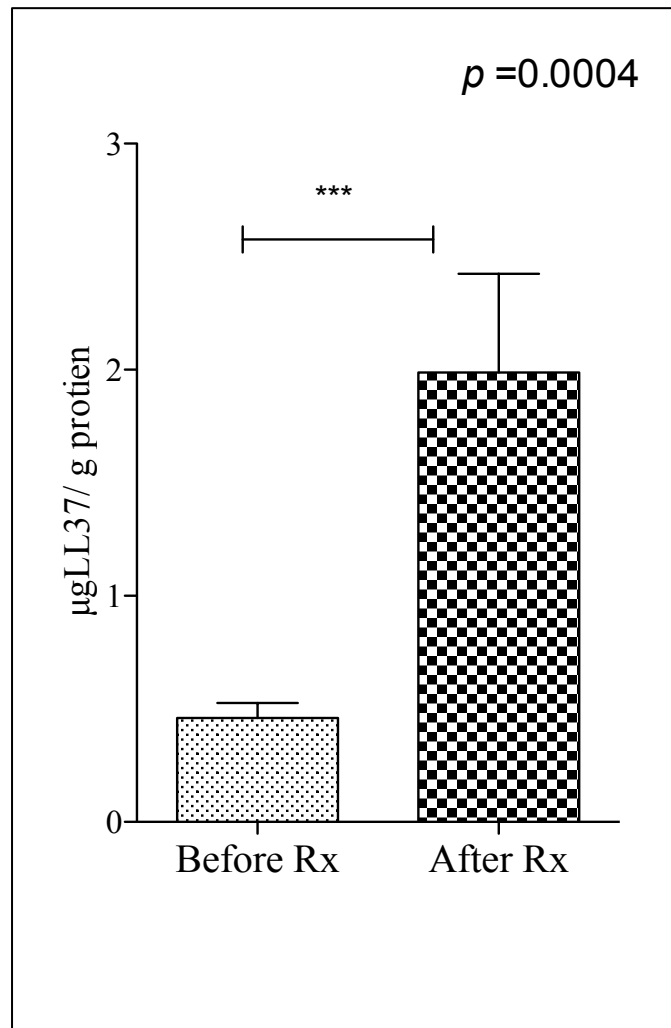


Figure III. 18: LL-37 levels in AD patients.

After two months of supplementation with VD, LL-37 levels increased significantly from mean LL-37 level= $0.46 \pm 0.06 \mu\text{g/g}$ to $1.98 \pm 0.44 \mu\text{g/g}$ ($p= 0.0004$, unpaired t-test).

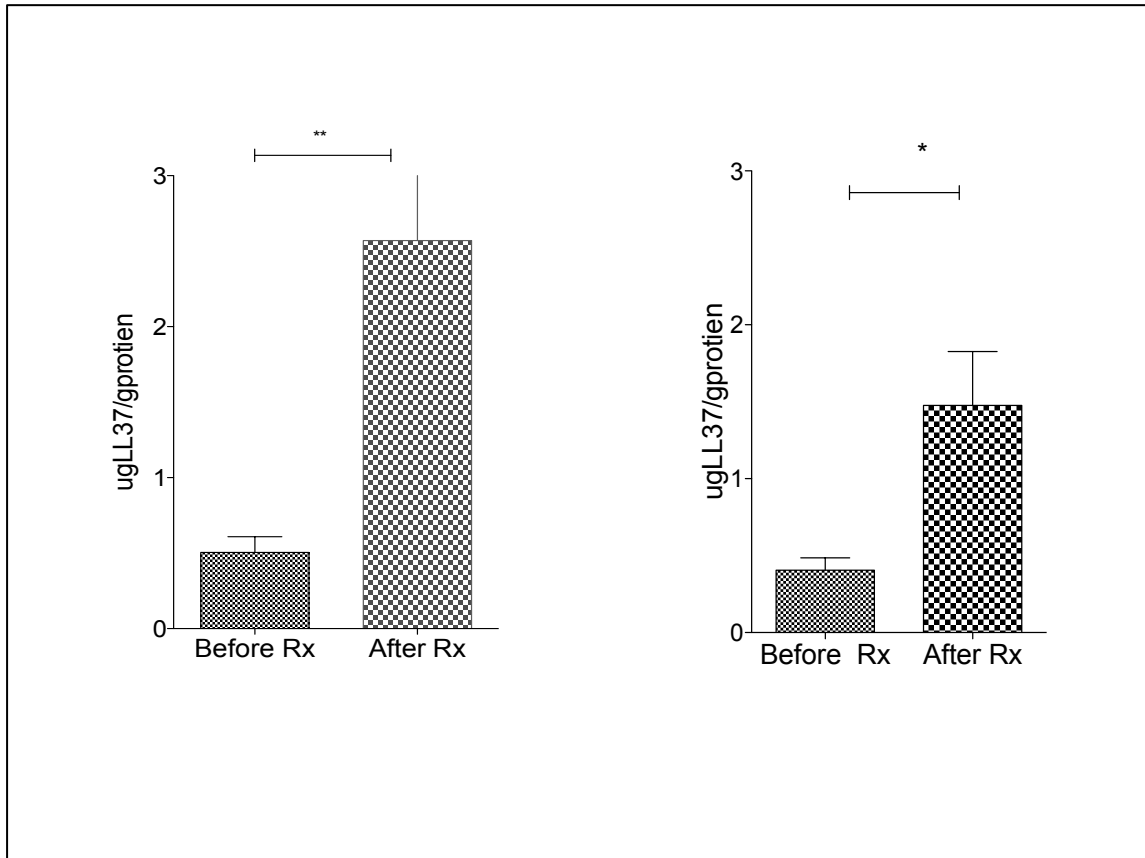


Figure III. 19: Lesional and non-lesional LL-37 levels in AD

Left graph: LL-37 sampled at lesional sites showed a significant increase from mean LL-37 level $0.504 \pm 0.104 \mu\text{g/g}$ to mean level $2.57 \pm 0.82 \mu\text{g/g}$ ($p = 0.0072$). Right graph: LL-37 sampled from non-lesional sites also showed a significant increase from mean level $0.405 \pm 0.08 \mu\text{g/g}$ to $1.47 \pm 0.35 \mu\text{g/g}$ ($p = 0.046$, unpaired t-test).

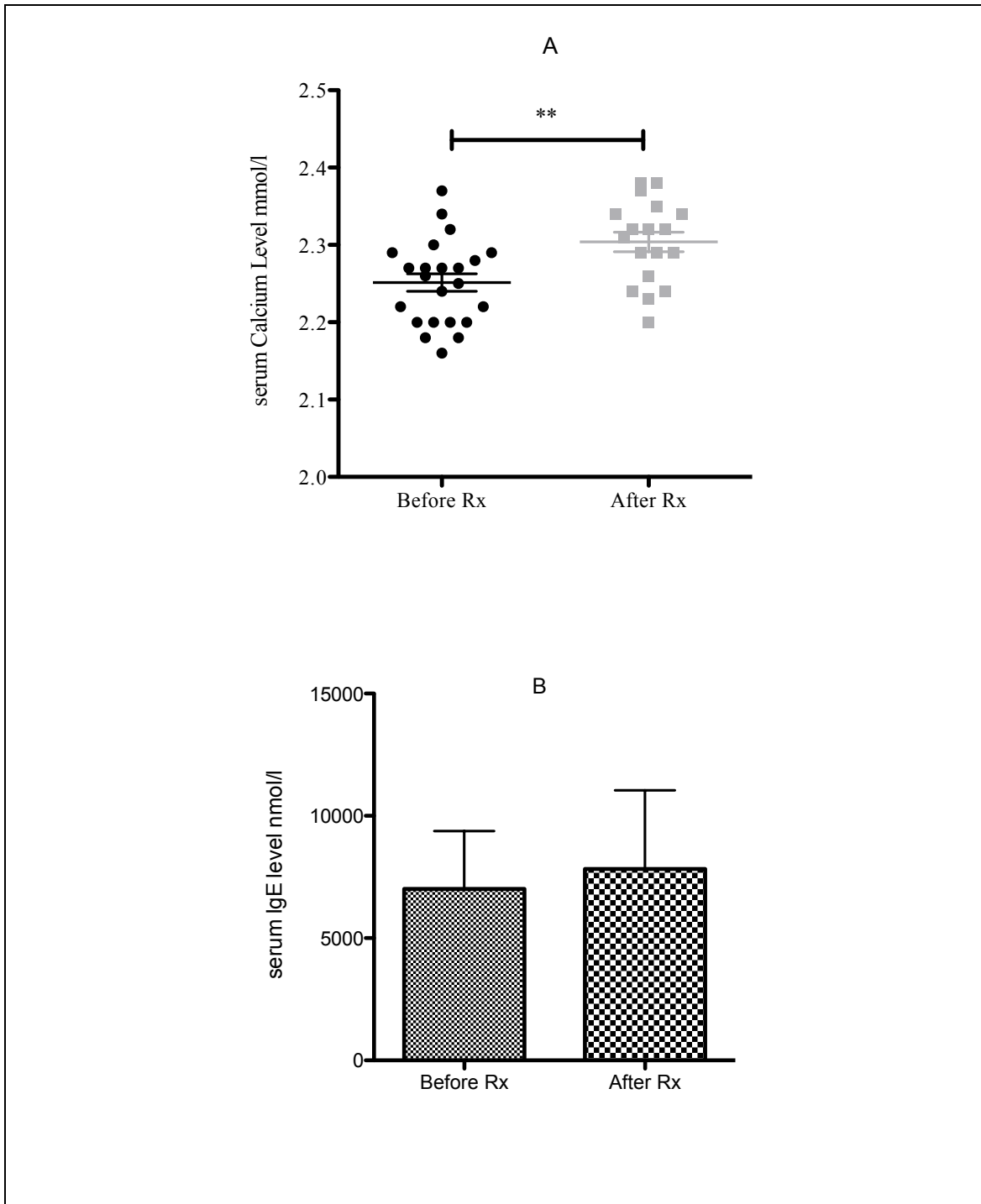


Figure III. 20: Serum calcium and IgE levels.

Top graph: serum calcium also showed a significant increase from mean level 2.25 ± 0.011 mmol/l to 2.3 ± 0.013 mmol/l ($p = 0.0037$, unpaired t-test). Bottom graph: IgE level did not show significant change from mean score 7010 ± 2370 nmol/l to 7824 ± 3221 nmol/l ($p = 0.84$, unpaired t-test).

Chapter Four: Discussion

IV. Chapter Four: Discussion

IV.1. Defining VD deficiency

VD deficiency is now recognized to be a worldwide problem (Ford, Graham et al. 2006; Hypponen and Power 2007). Approximately 50 % of the adult British population have insufficient levels of VD (< 50nmol/l) (Hypponen and Power 2007). A further 16% are severely deficient during winter and spring months (Ford, Graham et al. 2006). In children less than five years of age, the incidence of symptomatic VD deficiency (identified by either radiographic evidence of rickets, or by diagnosis of hypocalcemic convulsions) was found to be 7.5 per 100, 000 (Callaghan, Moy et al. 2006). Another recent study in Scotland found an annual incidence of 23 cases of symptomatic VD deficiency in children from 2002-2007, and an increase to 42 cases in 2008 (Ahmed, Franey et al. 2011).

In Kuwait, 40% of mothers and 60% of neonates were found to be deficient on the day of delivery (Molla, Al Badawi et al. 2005). Seventy five percent of young Kuwaiti women were found to consume less than two thirds of the recommended daily allowance (RDA) of VD (Alshawi 1992).

Studies in the US found the prevalence of VD deficiency (< 75 nmol/l) to equal 40% in 365 healthy infants and toddlers (Gordon, Feldman et al. 2008); 42% (< 50 nmol/l) in 307 healthy adolescents (Gordon, DePeter et al. 2004). A study on elderly women in Europe found VD deficiency (<30 nmol/l) to be extremely high in Italy, Spain and Greece, ranging from 80-92% (van der Wielen, de Groot et al.

1995).

The increasing awareness of the value of VD has led to controversy over the definition of VD deficiency. Recently the Institute of Medicine (IOM) defined VD sufficient at 50nmol/L, and recommended a daily intake of 600 IU/day for ages 1-70. A higher intake was recommended for the elderly (800 IU/day). (Ross, Manson et al. 2011). These levels, however, were estimated sufficient with respect to bone requirements only. Evidence for extra skeletal requirements was deemed insufficient and inconclusive, and as such the IOM encouraged more research in this area.

The IOM recommendations have already been challenged by many (Bischoff-Ferrari, H and W 2011; Hollis and Wagner 2011; Schwalfenberg and Whiting 2011), declaring these levels too low for optimal bone health. Indeed, trials have determined that levels < 60 nmol/l lead to a 19% increase in fracture falls (Bischoff-Ferrari, Dawson-Hughes et al. 2009). Levels less than 75 nmol/l were associated with an increased risk in hip fractures (Bischoff-Ferrari 2009).

Furthermore, arguments have also been made against the insufficiency of this level to prevent extra skeletal VD deficiency effects (Holick 2011). A meta-analysis of 18 RCTs studying the effect of VD supplementation on mortality concluded that VD supplements appeared to reduce the risk of mortality (Autier and Gandini 2007). Levels less than 44.5 nmol/l have been associated with 26% increased risk of mortality (Melamed, Michos et al. 2008). An RCT on 334 children found that VD supplementation to levels greater than 75 nmol/l were associated with a reduction in influenza A incidence in school children (Urashima, Segawa et al. 2010). Another

RCT showed VD levels over 75 nmol/l could improve vascular stiffness in adolescents (Dong, Stallmann-Jorgensen et al. 2010).

De Boer and colleagues in a recent study evaluated the relationship between serum VD levels and the risk of hip fractures, myocardial infarctions, cancer and mortality (de Boer, Levin et al. 2012). The conclusion reached was that levels of approximately 50 nmol/l are associated with an increased risk for multiple disease outcomes. Hence the International Osteoporosis Foundation (IOF) recommended a level of 75nmol/L (Dawson-Hughes, Mithal et al. 2010), as did the Endocrine Society Clinical Practice Guideline (Holick, Binkley et al. 2011).

Taking all this into account, only patients with levels > 75nmol/l were considered to have a healthy VD status, levels <50 nmol/l were considered deficient. Therefore 83% percent of AD children in this study were found to have unhealthy VD levels, compared to 40% in healthy children: 57% were deficient, a finding similar to a previous RCT on AD children (Javanbakht, Keshavarz et al. 2011). However, if adjusting the cut off level at 50 nmol/l in accordance with IOM recommendations, 57% of the AD patients would still be considered higher than levels found in healthy individuals (Gordon, DePeter et al. 2004).

IV.II. The Selection of the appropriate VD formulation

VD is present in many formulations; the two main formulations used for VD deficiency are VD₂ and VD₃ (Baeke, Takiishi et al. 2010). Other formulations, such as 1 α hydroxycholecalciferol and 1, 25 dihydroxycholecalciferol also exist,

but are mainly used in severe renal impairment (British Medical Association and Royal Pharmaceutical Society of Great Britain 2011).

Clinical studies comparing the efficacy of VD₂ supplements to VD₃ have yielded conflicting results. Some studies have found VD₂ to be as efficient as VD₃ in raising and preserving serum VD level (Holick, Biancuzzo et al. 2008), whereas most found VD₃ to be more effective (Armas, Hollis et al. 2004; Glendenning, Chew et al. 2009; Heaney, Recker et al. 2011). This could be due to the fact that VD₂ is known to have a more rapid metabolism and quicker clearance than VD₃ (Brown, Ritter et al. 2004). Research has also shown VD₂ to have less affinity in binding to DBP (Hollis 1984) and VDR (Houghton and Vieth 2006), as well as a less rate of hydroxylation by 25-hydroxylase in the liver (Holmberg, Berlin et al. 1986). A recent RCT showed VD₂ supplemented individuals to have a reduction in their serum VD levels during the winter months, whereas VD₃ supplemented individuals maintained a stable VD level throughout the six months of supplementation (Logan, Gray et al. 2013). Similarly, a meta-analysis of RCTs comparing VD₂ and VD₃ efficacy has recommended the use of cholecalciferol supplements for VD deficiency (Tripkovic, Lambert et al. 2012), and is the supplement used in this study.

IV.III. The Genetics and Demographics of VD deficiency

Pigmented skin is considered a risk factor for the development of VD deficiency (Pearce and Cheetham 2010). Several studies have found higher levels of VD deficiency in non-white populations (Looker, Dawson-Hughes et al. 2002;

Callaghan, Moy et al. 2006). The current recommendations on the use of sunscreens, hats and clothing in the sun, however, has now placed the white population at comparable risk for VD deficiency (Pearce and Cheetham 2010).

In our study population, both Caucasian and non-Caucasian groups had a similar incidence of VD deficiency. This could be due to either our sample size, or to rigorous sun protection.

VD deficiency is known to run in families, highlighting a genetic link (Berry and Hypponen 2011). This observation was also noted in our study. Research has identified polymorphisms in the *VDR* encoding gene, as well as the 1α hydroxylase encoding gene in asthma patients (Raby, Lazarus et al. 2004; Bossé, Lemire et al. 2009), and in alopecia (Malloy and Feldman 2011). A recent case control study investigated the presence of *VDR* gene polymorphisms in 265 AD patients, and found an increase in a specific *VDR* polymorphism in severe AD in comparison to the healthy control group (Heine, Hofer et al. 2013) . Therefore *VDR* could be a factor in AD development and control, through the effect of VD on skin barrier integrity and the immune system.

IV.IV. VD deficiency and AD

The SCORAD index was recently identified to be the most widely used in scoring system in RCTs on AD patients (Jones and Charman 2011). To simplify interpretation of AD clinical severity, some have recommended to classify AD

according to SCORAD into three categories:

mild (<25), moderate (25-50), and severe AD (>50) (Oranje, Glazenburg et al. 2007).

Using this system, VD levels have been found to be significantly higher in children with milder disease compared to patients with moderate and severe disease (Peroni, Piacentini et al. 2011), which is confirmed in our study. SCORAD and VD levels were also significantly correlated in our study, similar to previous studies (Giannetti, Ricci et al. 2011; Peroni, Piacentini et al. 2011; Shim, Kim et al. 2012).

In a study performed on patients with ages ranging from 1 to 80 years (mean age 21), ADEH patients were shown to have a higher and more severe clinical scoring (Beck, Boguniewicz et al. 2009), a finding that was echoed here. In addition, the ADEH children in our study were found to have significantly lower VD levels than AD children.

Research has identified LL-37 to possess significant antiviral activity (Leung, Wollenberg et al. 2006). The addition of LL-37 to keratinocytes infected with HSV led to significant killing and inhibition of viral replication. Furthermore, LL-37 levels, analyzed by PCR and verified by immune-staining techniques in skin biopsies, have been found to be deficient in AD lesional skin biopsies, and reduced further in ADEH lesional skin (Howell, Wollenberg et al. 2006; Leung, Wollenberg et al. 2006; Hata, Kotol et al. 2010). Howell *et al* showed a significant reduction of LL-37 levels in the lesional skin biopsies of 10 ADEH patients in comparison with 10 AD patients (Howell, Wollenberg et al. 2006).

This was also found in another study, however no difference was found in LL-37 expression in non-lesional sites (Hata, Kotol et al. 2010). In our present study, ADEH patients exhibited reduced expression of baseline LL-37 levels (lesional and non-lesional) in comparison with AD patients (not statistically significant). This could be due to the fact that the AD group was much smaller than the ADEH group (12 vs. 35).

LL-37 levels from non-lesional sites did not show a significant difference between AD and ADEH patients. A possible explanation could be that ADEH patients could have normal basal LL-37 levels, but a deficiency in up regulation of LL-37 in response to injury/eczema lesions (Stahle, Mallbris et al. 2008).

Historically, LL-37 levels taken from blood and saliva have not shown a difference in AD patients, or have not correlated with skin LL-37 levels, which could mean that the reduction of LL-37 observed in ADEH in lesional skin are skin specific (Hata, Udall et al. 2007). A recent publication however, presented otherwise (Kanda, Hau et al. 2012); twenty seven AD patients presented decreased circulating LL-37 compared to healthy donors, however the levels did not correlate with SCORAD.

Interestingly, another recent study found circulating LL-37 to increase with increased AD severity (Leung, Ching et al. 2012), however the levels did not significantly differ from the healthy controls. Possible explanations for the different results could be that circulating LL-37 might not be an accurate indicator, as the previous studies mentioned before have shown low LL-37 levels in lesional

skin only (Stahle, Mallbris et al. 2008; Hata, Kotel et al. 2010). Therefore it could be that only LL-37 analyzed from skin samples reflects accurate antimicrobial status of the skin.

In our study, LL-37 levels (both lesional and non-lesional) showed a significant difference when stratifying them according to SCORAD, showing a decrease with increased AD severity.

When further differentiating the LL-37 samples into those taken from lesional and non-lesional sites of our patients, no significant difference was revealed, although there was an observed trend of decreasing LL-37 levels with decreased clinical severity in lesional and non-lesional skin.

Treatment with VD topically has been found to increase LL-37 expression in lesional AD sites (Stahle, Mallbris et al. 2008). VD supplementation for 21 days at 4000 IU daily has shown increased LL-37 expression in both lesional and non-lesional biopsies (Hata, Jackson et al. 2008; Hata, Kotel et al. 2008). Two months of VD supplementation significantly improved the clinical scoring in both our AD and ADEH children. Furthermore, POEM and SCORAD showed similar reduction rates in the clinical severity in our children (42.3 % vs 46.6 %), reflecting an agreement between the two clinical tools, and indicating strong concordance between physicians assessment and patients perception of disease severity.

The LL-37 levels also increased significantly after VD treatment. This increase may account, at least in part, for the observed clinical improvement, as LL-37 has been known to not only act as an antimicrobial agent, but as an anti inflammatory (Kandler, Shaykhiev et al. 2006), exhibiting chemotactic properties and

inducing the production of inhibitory cytokines (Lai and Gallo 2009). In addition, it promotes healthy wound development (Tokumaru, Sayama et al. 2005; Park 2008).

The observed clinical improvement in both groups, AD and ADEH, could also be due to the involvement of VD in other processes in the skin barrier, such as increased production of structural proteins, such as filaggrin and involucrin (Bikle, Chang et al. 2004), increased production of sphingolipids (Oda, Uchida et al. 2009), and its effect on the calcium gradient and epidermal differentiation. Therefore VD supplementation could possibly increase cornified envelope formation, and maintain skin barrier structure and function.

It could also be due to the aforementioned effects of VD on both the innate and adaptive immune systems; VD has been shown to inhibit DC antigen presentation to T helper cells, thereby preventing over stimulation of T cells and reduction of inflammation (Baeke, Takiishi et al. 2010).

It also seems to affect the TReg cell population (Dimeloe, Nanzer et al. 2010). Therefore VD could have a regulatory effect on the immune system, and decrease inflammation.

VD toxicity is always a fear of most clinicians, as reports of toxicity are rare but could become more frequent as more people are alerted to the beneficial effects of VD supplementation. Research does show however, that only levels > 250 nmol/l can cause hyper-calcemia of more than 3.5 mmol/l leading to toxicity symptoms such as fever, vomiting, weight loss and loss of appetite (Root, Diamond et al. 1996).

The cases that have been reported were due to either depot injections of VD containing 300,000 IU without a previous history of VD deficiency (Sezer, Guran et al. 2012), or manufacturing errors of OTC supplements leading to an ingestion of up to 970,000 IU daily dose of VD (Araki, Holick et al. 2011).

In addition, previous trials done with 12-16 weeks of VD supplementation at doses of 400 IU daily found their subjects still within suboptimal range (50-75nmol/l) after the trial (Stellinga-Boelen, Wieggersma et al. 2007; Dong, Stallmann-Jorgensen et al. 2010).

Long-term supplementation with high doses of VD (2000 IU/d) was tolerated well with no incidence of VD toxicity, and VD levels remained in the normal range (90-145 nmol/l) (Maalouf, Nabulsi et al. 2008).

After two months of VD supplementation in our study, VD levels also showed a significant increase, however remained within normal levels. In addition, although serum calcium levels showed a significant increase, the mean level still remained within normal range.

Another point to consider is the optimal VD levels needed for various health outcomes. Research has shown that levels < 100 nmol/l are associated with a reduction of pancreatic responsiveness to insulin (Chiu, Chu et al. 2004). Levels < 80 nmol/l were associated with an increase in colorectal cancer risk (Gorham, Garland et al. 2007), as well as an increased risk of periodontal disease (Dietrich, Joshipura et al. 2004).

A meta-analysis conducted on multiple health outcomes recommended the optimal level of VD to be in the range of 90-100nmol/l (Bischoff-Ferrari, Giovannucci et al. 2006). To date, however, no research has been done on the optimal levels for AD.

The role of VD in atopic disorders, since they are primarily Th₂ driven (Zheng, Yu et al. 2011), has produced conflicting results, showing, as mentioned previously, both induction (Boonstra, Barrat et al. 2001) and suppression (Staeva-Vieira and Freedman 2002) of the Th₂ cell population *in vitro*. Hyponnen *et al* (2009) in a recent study found atopic patients with excessively high (>135nmol/L) and very low VD levels (<25nmol/) were associated with high serum IgE levels.

Our study does not show a significant change in total serum IgE levels after two months of treatment with VD, which could mean that supplementation of people with low VD would not dramatically change, but improve, the allergic state of AD or ADEH patients by returning VD to healthy levels.

As it was a practice evaluation study all medications, oral and topical, continued as normal with no constraints, which is a limitation in our study. Another drawback, for the same reason, is that the AD patients with normal VD levels were not followed for clinical scoring as well, to allow for unknown factors, which could be contributing to the clinical improvement of the VD deficient AD patients. Nevertheless, the extremely significant results of VD supplementation in this study, renders that possibility unlikely.

In conclusion, this study has examined AD patients in a pediatric clinic, and determined VD deficiency to be a common problem in AD children. Our study is the first to determine ADEH children to have significantly lower VD levels than AD children.

VD supplementation for two months showed a significant reduction in the clinical severity of ADEH children, both by clinical evaluation using SCORAD, and by patient and/or parent self assessment using POEM. This was also true for AD children.

A two fold increase in VD levels was associated with a four fold increase in LL-37 levels in both lesional and non-lesional SC layers of the skin. This, therefore, could be one of the mechanisms in the improvement of AD and ADEH.

In addition, we introduced a novel, non-invasive method for LL-37 measurement, which simplifies collection of samples as well as increases compliance. Treatments such as VD are a cost effective and simple therapeutic option in AD patients, and could possibly lessen the use of systemic immunosuppressive agents. It has become our practice to check VD levels in all AD patients.

V. Future work:

This thesis has answered several questions, however has raised many more.

Several areas could be explored further such as:

- An audit to determine the true prevalence of VD deficiency in AD children, with a sample size of at least 250 patients.
- A cross sectional study to determine the prevalence of VD deficiency in healthy children in the UK.
- A genetic epidemiological study of our VD deficient AD children to determine the presence of VDR polymorphisms, as well as the presence of other polymorphisms in the VD processing enzymes.
- A clinical study to evaluate the effect of VD supplementation on skin barrier integrity and function such as TEWL and hydration.

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VII. Appendix: Vitamin D/SCORAD sheet

Vitamin D supplementation in AD

Date:

Patient number:

Gender:

Ethnic group:

Skin type:

Topical treatments used:

Systemic treatments used:

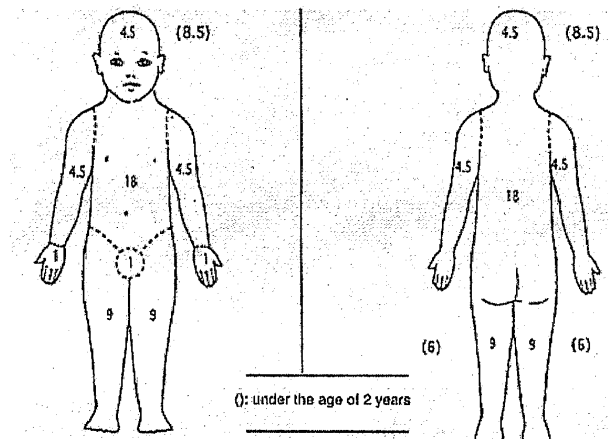
Vit D level:

IgE level:

Serum Calcium Level:

SCORAD:

A. Extent:



B. Intensity

Criteria	Intensity
Erythema	
Edema/papulation	
Oozing/Crusting	
Excoriation	
Lichenification	
Dryness	

(0=absence, 1=mild, 2=moderate, 3=severe)

C. Subjective symptoms for the last three days or nights:

Pruritis (1-10)

Sleep loss (1-10)

LL-37:

Sites sampled

