

Original article

Increased expression of aquaporin 3 in atopic eczema

Background: Dry skin in atopic eczema depends on increased water loss. The mechanisms behind this are poorly understood. The aim of this work was to identify genes that may contribute to water loss in eczema.

Methods: Affymetrix DNA microarrays U133A were used to analyse gene expression in skin biopsies from 10 patients with atopic eczema and 10 healthy controls.

Results: DNA microarray analysis showed up-regulation of 262 genes and down-regulation of 129 genes in atopic eczema. The known functions of these genes were analysed using Gene Ontology to identify genes that could contribute to increased water loss. This led to identification of aquaporin 3 (*AQP3*), which has a key role in hydrating healthy epidermis. Increased expression of *AQP3* was found in eczema compared with healthy skin. This was confirmed with real-time polymerase chain reaction ($P < 0.001$). In healthy skin, epidermal *AQP3* immunoreactivity was weak and mainly found in the stratum basale. A gradient was formed with decreasing *AQP3* staining in the lower layers of the stratum spinosum. By contrast, in acute and chronic atopic eczema strong *AQP3* staining was found in both the stratum basale and the stratum spinosum.

Conclusions: Aquaporin 3 is the predominant aquaporin in human skin. Increased expression and altered cellular distribution of *AQP3* is found in eczema and this may contribute to water loss.

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Atopic eczema (AE) is one of the most common inflammatory skin diseases. The cumulative prevalence is 10–20% in children and 1–3% in adults (1). There is substantial clinical variation, but dry skin, pruritus and erythematous lesions are prominent features (2). In line with the Th1/Th2 hypothesis, increased release of cytokines from type 2 T helper cells as well as eosinophilia and increased IgE has been shown in AE (2). However, many other cells and mediators have also been implicated in the pathogenesis (2). DNA microarray studies have implicated a large number of genes in eczema and other allergic disorders (3–7). While many of these genes encoded inflammatory mediators, there were also genes associated with noninflammatory pathways suggesting that mechanisms other than those directly involved in the immune system may contribute to the pathogenesis (8).

In AE, pruritic and erythematous papules are consistent with inflammation. However, another common sign, i.e. dry skin, may indicate involvement of other pathogenic mechanisms. Dryness is frequently seen in both lesional and nonlesional skin, and causes considerable morbidity (9). Dry skin may also contribute to the pathophysiology of AE. The epidermis is a highly organized multi-layered epithelium where, water and solute transport has a critical

role for skin barrier function. In AE, dry skin is associated with barrier dysfunction, which leads to increased antigen absorption and cutaneous hyper-reactivity (10). Dry skin is thought to depend on increased trans-epidermal water loss. This may, in turn, depend on decreased content of water-retaining molecules such as, ceramides in the stratum corneum (SC) (2). The fluid is thought to come from the dermis, but the mechanisms by which the fluid enters and is transported through the epidermis have, however, not been elucidated (10).

The aim of this study was to identify genes that may be associated with increased water loss in AE. For this purpose skin biopsies from patients with AE and healthy controls were analysed using DNA microarrays.

Methods

Subjects

Patients with AE who fulfilled the criteria of Williams et al. (11) were studied. None of the patients had received local or systemic treatment with glucocorticoids or tacrolimus (FK506) within 2 weeks. Healthy controls with normal levels of IgE were included in the study. The controls were all symptom-free, had no history of AE or any other

atopic or chronic disease. Both patients and controls were of Caucasian origin. Skin biopsies from 10 patients and 10 controls were analysed with DNA microarrays and real-time polymerase chain reaction (PCR). The median (range) age of the patients was 40.3 years (21–50) of whom eight were females. The median age of the controls was 43 years (25–50). The group of controls was matched against the patients with respect to gender and age. In addition, skin biopsies from five patients and two controls were taken for immunohistochemistry. The median age of these patients was 31 years (21–54). The severity of the eczema was measured by a dermatologist (A.B.) using Severity Scoring of Atopic Dermatitis (SCORAD) system that gives a combined score of symptoms between 0 and 103, i.e. regarding eczema spread over body, cutaneous dryness, pruritus and insomnia (12). Total serum IgE in patients and controls was determined by a competitive radioimmunoassay (Pharmacia & Upjohn Diagnostics AB., Uppsala, Sweden). Two punch biopsies (4 mm diameter) were taken under local anaesthesia from lesional skin from each patient and matching skin location from each control. The biopsies were snap-frozen in liquid nitrogen and stored at -70°C until RNA preparation. Informed consent was obtained from all subjects involved in the study. The study was approved by the Ethics Committee of the Medical Faculty of Göteborg University.

Preparation of cRNA and DNA microarray analysis

The biopsies were homogenized for 60 s in Heidolph Diox 900 (Heidolph, Kelheim, Germany). RNA was isolated using the acid guanidinium thiocyanate, phenol–chloroform protocol and purified using RNeasy kit (Qiagen, Hilden, Germany). Quality of RNA samples was verified by agarose gel electrophoresis and by checking A_{260}/A_{280} ratio. Samples from 10 patients and 10 controls were analysed using DNA microarrays measuring the expression of 22 283 genes and variants according to the manufacturer's protocol (HuGe U133A GeneChip; Affymetrix, Santa Clara, CA, USA). The hybridization and analysis were performed according to the Minimum Information About a Microarray Experiment guideline and the raw array data are available from Gene Expression Omnibus (<http://www.ncbi.nlm.nih.gov/geo>).

Microarray data analysis

Signal values and arrays were background corrected and normalized using robust multi-array average and quantile–quantile normalization, respectively (13, 14). These methods are available in the R software (<http://www.bioconductor.org/>). A method described by Pan (15) was used to identify differentially expressed genes using 2000 permutations. This analysis was performed in Matlab (The MathWorks, Natick, MA, USA). In addition, the Affymetrix MAS 5.0 with default settings was used to get the detection call, i.e. to determine if the signal of a probe set is present, marginal or absent.

Identification of genes involved in water transport

The controlled gene function vocabulary in Gene Ontology (<http://www.geneontology.org/>) was used to identify differentially expressed genes involved in water transport. The ontology 'molecular function' was searched for subordinate ontologies that contained the term water. This identified the gene ontology term 'water channel activity' (GO: 0015250).

The differentially expressed genes were searched to find genes that matched this term. In case-differentially expressed genes from the microarray study were not included among the human genes in Gene Ontology, the corresponding murine genes were studied.

Tissue expression analysis

The expression levels of aquaporins were assessed in different cells and tissues using the SymAtlas dataset (<http://symatlas.gnf.org/SymAtlas/>, 16). This dataset describes gene expression levels in 79 different cells and tissues that have been analysed with duplicate DNA microarrays (HuGe U133A GeneChip). Tissue expression profiles were calculated as average signal values in each tissue duplicate. To allow comparison between tissues, each tissue specific profile was normalized by dividing with its average value. In addition, our own dataset was included among the tissue profiles after averaging the values of cases and controls in the same way as for the SymAtlas values.

Quantitative Real-Time PCR

Expression of the *AQP1* and *AQP3* genes were analysed using quantitative real-time PCR. The gene 'ribosomal protein, large, P0' (*RPLP0*) was selected as a reference gene after having evaluated the coefficient of variation of the microarray signal values for the commonly used reference genes *ACTB*, *GAPD*, *PPIA*, *RPLP0* and *RPLP1*.

Oligonucleotide primers and dual-labelled probes were purchased as predeveloped Assay-on-Demands (*AQP1*: Hs00166067, *AQP3*: Hs00185020_m1 and *RPLP0*: Hs99999902_m1) from Applied Biosystems (Nordic Regional HQ, Stockholm, Sweden). Total RNA was reverse transcribed to cDNA with TaqMan Reverse Transcription Reagents (Applied Biosystems) with random hexamers according to manufacturer's instruction at the thermal conditions 25°C for 5 min, 48°C for 30 min and finally 95°C for 5 min and used as template in PCR.

A standard curve, in the range of 0.625–40 ng of total RNA, was created from serial dilution of pooled skin cDNA. The same standard curve was used for all gene assays. Template amplification and detection was performed in ABI Prism 7700 Sequence Detection System (Applied Biosystems) in 30 μl volumes. All samples and standards were analysed in triplicates. The thermal conditions comprised initial denaturation at 95°C for 10 min and 40 cycles of denaturation at 95°C for 15 s, and annealing and elongation at 60°C for 1 min. The reference gene was used for normalization of the same samples. The statistical analysis was performed using Mann–Whitney test. A *P*-value smaller than or equal to 0.05 was considered significant.

Immunohistochemistry

The tissue distribution of AQP3 protein was investigated by immunohistochemistry on punch biopsy samples (4 mm) from lesional and nonlesional skin of patients with AE ($n = 5$) and healthy control subjects ($n = 2$). Deparaffinized formaline-fixed tissue sections (4 μm) were mounted on charged slides and subjected to heat-induced antigen retrieval in Tris/EDTA pH9 using a microwave oven for 10 min. Immunostaining was accomplished by an automated staining machine (DAKO Techmate 500 PLUS; DakoCytomation, Norden A/S, Glostrup, Denmark). The primary antibody used was AQP3 antibody in dilution 1 : 1000 (goat polyclonal antibody raised against a peptide mapping at the carboxy terminus of human AQP3; Santa Cruz Biotechnology, Santa Cruz, CA, USA) applied for 25 min. The secondary antibody was a biotinylated rabbit-antigoat IgG antibody from the same company (Santa Cruz Biotechnology). The result was visualized using a HRP rabbit/mouse LSAB-kit from DakoCytomation (Glostrup, Denmark) using diaminobenzidine as chromogen. All sections were examined and photographed by using standard bright-field optics.

Results

Skin biopsies were collected from a group of 10 AE patients, all of whom had high IgE levels, and a matched group of 10 healthy nonallergic controls. The median (range) IgE level in the patients was 1400 (150–14400) kU/l and 24.5 kU/l (8–65) kU/l in the controls. The median SCORAD index in the patients was 42.6 (20.6–60).

Gene expression in individual skin biopsies from all patients and all controls were examined with a total of 20 DNA microarrays. The statistical analysis identified 262 genes as up-regulated and 129 genes as down-regulated in lesional eczema skin. The known functions of these genes were examined using the Gene Ontology Database to find genes involved in water transport. This led to identification of *AQP3* that was up-regulated in the skin biopsies from the patients. This gene belongs to the aquaporin family that consists of the only proteins that can facilitate water diffusion through cellular membranes. Out of 10 different *AQP* measured by the DNA microarray analysis, *AQP3* was the most highly expressed in the skin biopsies, indicating the potential importance of *AQP3* in skin hydration (Fig. 1). Moreover, only *AQP3* was differentially expressed in AE compared with healthy controls. The DNA microarray used in this study contained three probe sets representing the *AQP3* gene, all of which were up-regulated. Of the remaining analysed aquaporins only *AQP1* showed detectable expression in the skin biopsies, but did not differ between patients and controls. Real-time PCR analysis verified that *AQP3* was up-regulated in eczema samples, while no difference in *AQP1* expression was found. Expression of *AQP1* and *AQP3* are presented as ratios of *AQP/RPLP0* signals in Fig. 2. The ratio for *AQP1* was 1.04 ± 0.27 in patients and 0.87 ± 0.24 in controls ($P = 0.38$). The corresponding values for *AQP3* were 1.3 ± 0.42 and 0.4 ± 0.06 ($P < 0.001$). This is a 3.25 fold-increase in *AQP3* expression in patients, which is

of a similar magnitude as the fold-increase found in the DNA microarray analysis.

To estimate the relevance of *AQP3* in skin tissue the expression levels of *AQP3* in skin were compared with other human tissues. For this purpose the SymAtlas Database was used (<http://symatlas.gnf.org/SymAtlas/>, 16). This database comprises 79 different tissues and cell types analysed with the same type of DNA microarray Gene Chip as in this study. Healthy skin had the second highest expression level of *AQP3* of all tissues included in the SymAtlas dataset, while expression of *AQP3* in eczema was the highest of all tissues (Fig. 3). By contrast, a low expression of *AQP1* was found in skin compared with other tissues and cell types (data not shown).

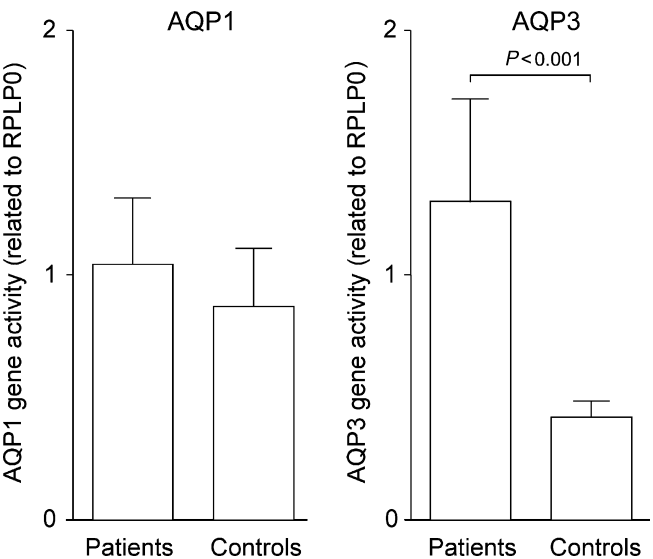


Figure 2. Real-time polymerase chain reaction analysis of expression aquaporin (*AQP*) 1 and 3 in skin biopsies from 10 patients with atopic eczema and 10 healthy controls. Values are presented as the mean $\pm$ sem *AQP*/ 'ribosomal protein, large, P0' (*RPLP0*) ratios. *** $P < 0.001$ (Mann–Whitney *U* test).

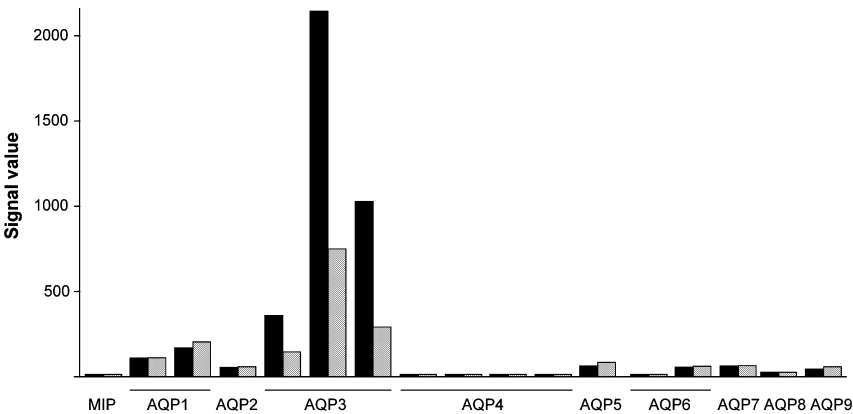


Figure 1. DNA microarray analysis of expression levels of 10 different aquaporins in skin biopsies from patients with atopic eczema (closed bars) and healthy controls (open bars). Each of the aquaporins 1, 3, 4 and 6 are represented by multiple probesets in the DNA microarray analysis. *AQP*, aquaporin; *MIP*, major intrinsic protein (also known as *AQP0*).

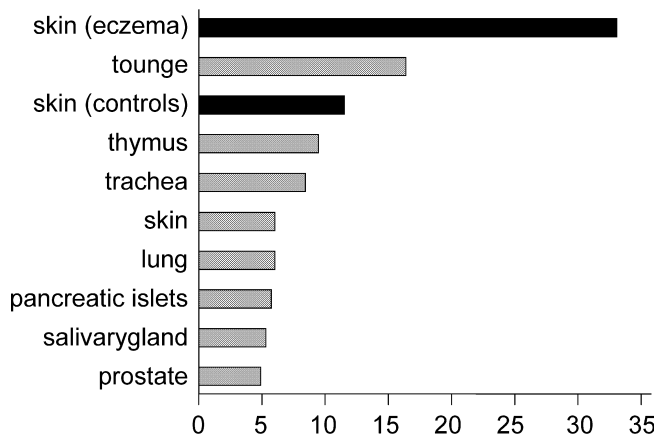


Figure 3. Tissue distribution of aquaporin 3 (*AQP3*) gene expression based on DNA microarray analysis of 81 different tissue samples. The figure shows the 10 tissues with the highest *AQP3* expression. Expression values are for skin is from this study and the remaining values from the SymAtlas database.

Immunohistochemical staining shows abnormal AQP3 staining in AE

Aquaporin 3 was detectable in the epidermis of healthy skin as well as in lesional and nonlesional skin of patients with AE (Fig. 4A–C). No AQP3 immunoreactivity was found in the dermis, except in sweat glands. In healthy skin, epidermal AQP3 staining was weak and mainly found in the stratum basale. A gradient was formed with decreasing AQP3 staining in the upper layers of the stratum spinosum. By contrast, in lesional skin from patients with AE, strong AQP3 staining was found in both the stratum basale and the stratum spinosum (Fig. 4C). In nonlesional AE skin, AQP3 immunoreactivity was found both in the stratum basale and the stratum spinosum but the staining was weaker than in lesional AE (Fig. 4B). Both in healthy controls and AE, AQP3 staining was mainly found in the cytoplasm in the stratum basale and in cell membranes in the stratum spinosum.

Discussion

Research during the last decades has shown the involvement of many different mediators in the pathogenesis of atopic eczema (AE) (2). DNA microarray studies of AE, allergic rhinitis and asthma have implicated several inflammatory genes (3–7). It is, however, also possible that altered expression of noninflammatory genes may be involved. A cardinal feature of AE is dry skin, which is thought to depend on increased trans-epidermal water loss (2). Dry skin is not only a significant cause of morbidity, but may also contribute to the pathogenesis of AE. This is because dryness is associated with impaired barrier function that leads to increased antigen absorption and cutaneous hyper-reactivity (2). Increased water loss is enhanced by abnormal lipid composition, with

both reduction in the amount of ceramides and altered distribution between the different ceramide types in the SC. Also the level of cholesterol is increased (17). However, the mechanisms that explain increased entry and transport of water through the epidermis are not clearly defined. The aim of this study was to identify genes that may contribute to increased water loss in AE. For this purpose, skin biopsies from patients with AE and healthy controls were analysed with DNA microarray. Only patients with AE who fulfilled the criteria of Williams et al.(11) were included in this study.

Using the public database Gene Ontology to examine the functions of differentially expressed genes aquaporin (*AQP*) 3 was identified. Aquaporin 3 belongs to a family of genes that encodes water-permeable channels. After the discovery of AQP1 in 1993, AQP have been described in epithelial cells in the skin, kidney, gastrointestinal and respiratory tracts. Their key role in water transport in red blood cells and the kidney has been clearly established (18). The functions of AQP in skin have been less thoroughly investigated, perhaps because water movements in the skin have been considered less important. There are 11 known human AQP, each of which has a unique tissue distribution. The DNA microarray analysis measured expression of 10 of these genes. *Aquaporin 3* was the predominant *AQP* in the skin. Moreover, a comparison with 79 other tissues showed that the expression of *AQP3* was particularly high in skin. By contrast, low expression of *AQP1* was found in skin compared with other tissues. This indicates that *AQP3* may have an important role in skin water transport. This is supported by experimental work by Sougrat et al.(19) showing that inhibition of AQP3 activity significantly decreased epidermal water flux. By contrast, higher expression of AQP3 in embryonal than in adult rat skin has been linked to increased transepidermal water loss (20). Immunohistochemical investigation of healthy skin has shown that AQP3 is expressed in the epidermis, but not in the loose tissue of the dermis in which water can diffuse more freely. In the healthy epidermis AQP3 is mainly found in the stratum basale with decreasing expression towards the stratum granulosum. Thus, a gradient is formed. No AQP3 is found in the SC. The AQP3 gradient may lead to a corresponding water gradient in the epidermis, with a sharp decrease of water content in the SC (19). In this way, water loss is prevented at the same time as the living layers of the epidermis are optimally hydrated. This isolates SC from the living tissue lower in the epidermis and contributes to skin barrier function. The AQP3-induced water gradient may be strengthened by another gradient formed by pH that also decreases in the SC (19); as AQP3 is inhibited by low pH the water transporting capacity of AQP3 is decreased in upper layers of the epidermis (19).

The DNA microarray analysis showed that mRNA expression of *AQP3* was about three times higher in AE

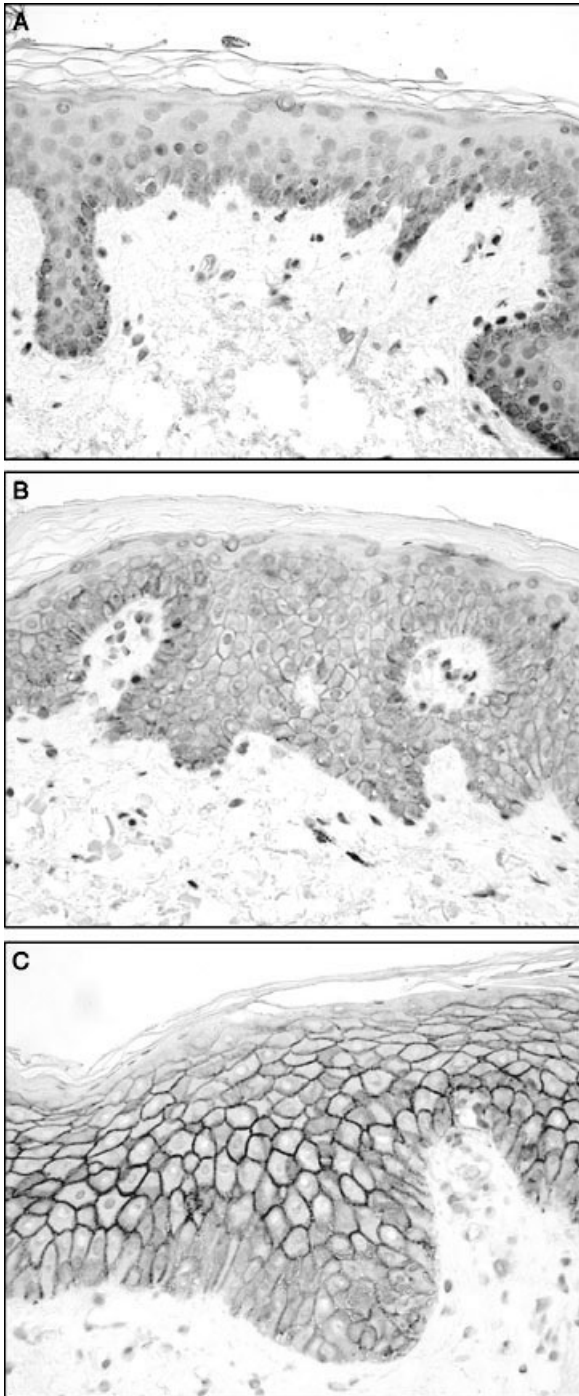


Figure 4. Aquaporin 3 (AQP3) staining is found in the epidermis of healthy controls (A), and nonlesional (B), as well as lesional atopic eczema (AE) skin (C). No staining is seen in the dermis. There is a weak AQP3 immunoreactivity in healthy skin, mainly in the stratum basale. A gradient with decreasing AQP3 staining towards the upper layers of the stratum spinosum is found (A). In lesional AE skin (C) AQP3 staining is increased compared with controls. Aquaporin 3 is found in both the stratum basale and the stratum spinosum, and no gradient is evident. A similar AQP3 distribution is seen in nonlesional AE skin, but the staining is weaker than in lesional AE.

than in healthy skin. This was confirmed with real-time PCR. In agreement with work by Sougrat et al. (19) immunohistochemical investigation of healthy skin showed staining for AQP3 in the epidermis. This staining was mainly found in the basal layers of the epidermis providing optimal hydration of the living layers of the epidermis as discussed above. In contrast to healthy skin increased immunoreactivity for AQP3 was found in the epidermis from patients with AE. Moreover, the increased AQP3 staining was found in both the stratum basale and the stratum spinosum. The staining was more pronounced in the stratum spinosum. Thus, the AQP3 gradient with decreasing staining found in healthy controls was absent. This may lead to increased water transport to the SC in AE. As the SC in patients with AE has reduced water-holding capacity this may contribute to increased loss of water and dry skin in AE.

Taken together, these findings support the hypothesis that increased expression of *AQP3* may lead to increased water loss in AE. While AQP3 staining was most pronounced in lesional skin of AE, staining was also found in both the stratum basale and the stratum spinosum of nonlesional skin. This is consistent with previous studies showing that nonlesional skin in AE can exhibit increased trans-epidermal water loss (21). It is also of note that pH is higher in AE than in healthy skin (22). As low pH inhibits AQP3 water permeability high pH in AE may further increase water loss. This is in agreement with the clinical observation that decreasing skin pH with low pH soaps ameliorates skin dryness.

Despite the importance of AQP for water transport little is known about their role in AE. Other relevant, but unresolved questions for future research are how *AQP3* expression relates to different phases of AE. For example, does AQP3 increase in acute AE, which is associated with marked oedema? Or does it decrease following treatment with glucocorticoids, tacrolimus or emollients? It would also be of interest to examine if AQP3 expression differs during different age periods, for example during senescence which is associated with dry skin. There are a few reports about AQP3 in skin diseases other than AE. Increased expression of *AQP3* has been described in erythema toxicum neonatorum, a common inflammatory skin disease in neonates (23). Aquaporin 3 and other AQP have been implicated in pulmonary inflammation and edema (24, 25). In this context it is noteworthy that expression of *AQP1* is regulated by glucocorticoids (26), *AQP3* by p73 (27) and *AQP5* by tumour necrosis factor- α (28). These findings indicate that AQP-mediated water transport may be an integral part of inflammatory processes. As AQP3 is the main AQP in skin, further studies are warranted to assess the role of AQP3 in AE.

In summary, AQP3 is the predominant AQP in human skin. Increased expression of *AQP3* was found in skin biopsies from patients with AE. Immunohistochemical investigation showed that, in contrast to healthy skin, strong AQP3 staining was found in both the stratum

basale and the stratum spinosum. This may contribute to increased water loss and dry skin in AE.

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