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ORIGINAL PAPER

In vivo studies of aquaporins 3 and 10 in human stratum corneum

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Abstract Aquaporins (AQPs) constitute one family of transmembrane proteins facilitating transport of water across cell membranes. Due to their specificity, AQPs have a broad spectrum of physiological functions, and for keratinocytes there are indications that these channel proteins are involved in cell migration and proliferation with consequences for the antimicrobial defense of the skin. AQP3 and AQP10 are aqua-glyceroporins, known to transport glycerol as well as water. AQP3 is the predominant AQP in human skin and has previously been demonstrated in the basal layer of epidermis in normal human skin, but not in stratum corneum (SC). AQP10 has not previously been identified in human skin. Previous studies have demonstrated the presence of AQP3 and AQP10 mRNA in keratinocytes. In this study, our aim was to investigate if these aquaporin proteins were actually present in human SC cells. This can be seen as a first step toward elucidating the possible functional role of AQP3 and AQP10 in SC hydration. Specifically we investigate the presence of AQP3 and AQP10 in vivo in human SC using "minimal-invasive" technique for obtaining SC samples. SC samples were obtained from six healthy volunteers. Western blotting and immunohistochemistry were used to demonstrate the presence of AQP3 as well as AQP10. The presence of AQP3 and AQP10 was verified by Western blotting, allowing for detection of proteins by specific antibodies. Applying immunohistochemistry, cell-like structures in the shape of corneocytes were identified in all samples by AQP3 and AQP10 antibodies. In conclusion, identification of AQP3 and AQP10 protein in SC in an in vivo model is new. Together with the new "minimal-invasive" method for SC collection presented, this opens for new possibilities to study the role of AQPs in relation to function of the skin barrier.

Keywords AQP3 · AQP10 · Stratum corneum · Minimal-invasive technique

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Introduction

The skin is the external barrier of the body, and an intact skin barrier is an important shield against chemical agents and microbes. The skin barrier is also involved in temperature regulation and water evaporation from the body. The outermost layer of the skin, stratum corneum (SC) is the limiting barrier to water loss by evaporation and should be kept well hydrated to avoid fissures and keep the barrier intact. A deteriorated SC is a central first step in the development of irritant contact dermatitis and is also considered an important part of the pathogenesis in atopic dermatitis.

Aquaporins (AQPs) constitute one of the several families of transmembrane proteins forming water channels and thus facilitating the transport of water across cell membranes [3, 15]. Thirteen AQP isoforms have been identified in human (AQP0–AQP12). AQP3, 7, 9 and 10 are aqua-glyceroporins, known to transport glycerol as well as water [4]. In the skin AQP3 in keratinocytes is involved in skin hydration; however, there are indications of other implications of AQP3 in this tissue. A recent study performed on human cell culture points to a possible effect of AQPs on the antimicrobial defense [6]. AQPs are also possibly involved in keratinocyte migration and proliferation [9], but probably not in keratinocyte differentiation [5, 10].

In healthy skin, epidermal AQP3 immune staining has been reported mainly from the stratum basale of the epidermis, indicating its expression in immature keratinocytes, with a gradient formed with decreasing AQP3 staining in the lower layers of the stratum spinosum. In acute and chronic atopic dermatitis, however, strong AQP3 staining was found in both the stratum basale and the stratum spinosum in human skin biopsies [12]. In skin diseases associated with elevated transepidermal water loss (TEWL) and reduced SC hydration, an up-regulation of expression of AQP3 has been shown [2]. In mice with significantly deteriorated skin barrier function (DHCR24−/− mice), AQP3 was expressed throughout the epidermis. The increased AQP3 expression in the epidermis of these mice was followed by an increased glycerol uptake and content [8]. Apart from AQP3, other AQPs have only sparsely been reported in human skin. mRNA for both AQP3 and AQP10 has been found in cell cultures from human keratinocytes [1], indicating the AQP10 protein as another possible player in relation to skin barrier function, but this finding has until now not been confirmed in in vivo studies.

Today, most knowledge on AQPs relies on studies performed on cell cultures or in mice, and only very few human studies have been published. In the present study, we aim (1) to demonstrate the presence of AQP3 and AQP10 protein in human SC and (2) to introduce a "minimal-invasive" model making it possible to study changes in AQP3 and AQP10 presence over time and in different physiological and pathological situations.

Materials and methods

The study was approved by the local ethical committee (H-4-2011-039) and performed after written informed consent from all volunteers. The Declaration of Helsinki protocols were followed.

A total of six healthy volunteers were examined (1 male and 5 females, median age 35 years, range 32–64), none had any major dermatological diseases, past or present.

Two types of skin samples were collected by marking two areas of 5 × 4 cm^2 on the volar forearm with a skin pencil marker. From one of these areas, SC samples for immunohistochemistry were collected using D-squame tapes (Cu-Derm Corporation), applying a pressurizer (Cu-Derm Corporation) on top of each tape for 10 s and then removed using a gloved hand. Tapes 1–3 were disregarded, and tape 4 was put into vials and kept at −80 °C until further analysis. From the other of the two marked skin areas on the forearm, SC samples for Western blotting were collected by scraping 20 times with a scalpel at a 90° angle using light pressure and scraping in one direction. The SC samples were put into vials and kept at −80 °C until further analysis.

Western blotting

SC samples were thawed on ice before being dissolved in 50 µl of 2× sodium dodecyl sulfate (SDS) sample buffer (Invitrogen, Nearum, Denmark) with 1:100 protease inhibitor cocktail (for use with mammalian cells and tissue, Sigma Aldrich, Broendby, Denmark) and 1:100 phenylmethane-sulfonyl fluoride solution (Sigma Aldrich, Broendby, Denmark). For AQP3 and AQP10 detection, skin samples were left for incubation for 5 h for AQP3 and 3 h for AQP10, with continuous inversion before loading on a 4–12 % tris–glycine gel (Invitrogen, Nearum, Denmark). Gels were run at 125 V on an Expedeon Dual Run and Blot system (Kem-En-Tec, Taastrup, Denmark) with a 1× SDS running buffer before blotting onto a polyvinylidene difluoride (PVDF) membrane at 30 V overnight at 4 °C. The membrane was developed using a chromogenic Western blot immunodetection kit, anti-rabbit, based on BCIP/NBT substrate for alkaline phosphatase (WesternBreeze, Invitrogen, Naerum, Denmark). We followed the protocol of the kit with minor exceptions. For AQP3 detection, the membrane was incubated with the primary antibody; anti-water channel aquaporin 3 antibody produced in rabbit (anti-AQP3) (Sigma Aldrich, Broendby, Denmark)

overnight before incubation with the secondary antibody overnight, followed by incubation with the chromogenic substrate overnight. For AQP10 detection, the membrane was incubated with the primary antibody; anti-water channel aquaporin 10 antibody produced in rabbit (anti-AQP10) (Sigma Aldrich, Broendby, Denmark) overnight before incubation with the secondary antibody for 3 h, followed by incubation with the chromogenic substrate overnight. The Western blot was dried before it was photographed with an 8 megapixels iPhone 4S camera (Apple, California).

To verify the specificity of the antibody of human AQP, controls of pure proteins were included in the blots. In the Western blot for AQP3, recombinant human AQP3 (0.1 μg) served as a positive control for the specific binding of the AQP3 antibody to AQP3 (Fig. 1, lane 2) and recombinant human AQP10 was included as a negative control; in the Western blot for AQP10, recombinant human AQP10 (0.02 μg) served as a positive control for the specific binding of the AQP10 antibody to AQP10 (Fig. 3, lane 2) and recombinant human AQP3 was included as a negative control.

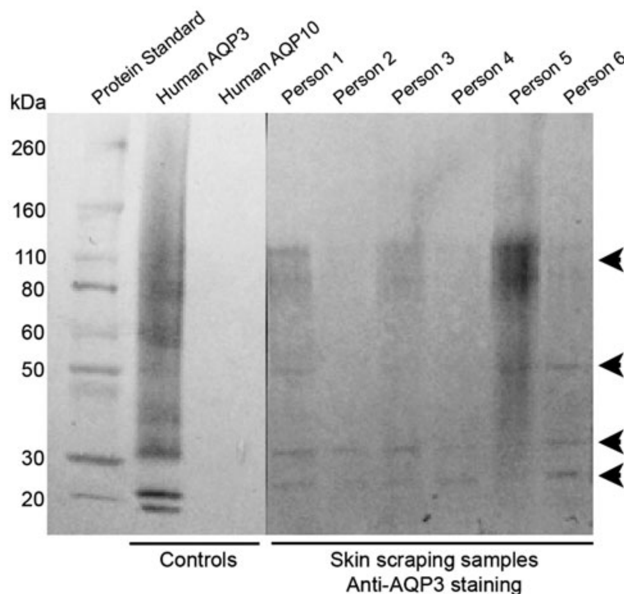

Fig. 1 AQP3-specific Western blot of skin scraped samples from test persons 1–6. Recombinant human AQP3 (0.1 μg) and human AQP10 (0.02 μg) were included as negative and positive controls, respectively. Staining of the positive control, human AQP3 and lack of staining of human AQP10 verify the specificity of the anti-AQP3 antibody toward AQP3. Bands corresponding to the monomeric (25 kDa), glycosylated monomeric (30 kDa) as well as the dimeric (50 kDa) forms of the protein are observed. Heavier bands of 80–120 kDa corresponding to higher order oligomers and non-dissolved SC as well as several unspecific bands are also observed. In conclusion, we identified AQP3 in skin samples for all six test persons

The sizes of the proteins detected on the Western blot were determined by comparing to the Novex sharp pre-stained protein ladder (Invitrogen, Naerum, Denmark).

Immunohistochemistry

Samples from three of the six test persons were analyzed. Tape-strips with SC skin samples were hydrated in 20 % ethanol in 1× phosphate buffered saline (PBS) solution for 24 h at 50 °C. The ethanol solution was removed and the tape-strips washed twice in a 1× PBS solution with 0.2 % bovine serum albumin and 0.1 % Triton X-100 (PBT solution) for 5 min with continuous inversion. The tape-strips were incubated in PBT solution with 5 % goat serum and primary antibody (1:50 dilution ratio) at room temperature overnight with continuous inversion. The primary antibodies were either anti-aquaporin 3 antibody (anti-AQP3) or anti-aquaporin 10 antibody (anti-AQP10), both produced in rabbit (Sigma Aldrich, Broendby, Denmark). Following incubation, the tape-strips were washed three times for 5 min in PBS before incubation with the secondary antibody (Alexa Fluor 546 goat anti-rabbit IgG) and dissolved in PBT solution (1:50 dilution ratio) with 5 % goat serum for 3 h with continuous inversion. The sample was visualized using a Zeiss AxioScope A1 microscope equipped with a Zeiss AxioCam MRc CCD color camera with a resolution of 1,388 × 1,040 pixels.

Results

AQP3

Identification of AQP3 in SC samples obtained by skin scraping and analyzed by Western blotting is shown in Fig. 1. Several bands were detected for all six samples, representing one individual person each (Fig. 1, lanes 4–9). Clearly defined bands are present representing proteins of the sizes ≈25, ≈30 and ≈50 kDa. Furthermore, diffuse bands spanning the sizes ≈80–120 kDa were present. Pure AQP3 showed clear staining, while pure AQP10, included as a negative control, showed no staining.

Immunohistochemistry on SC samples obtained by tape stripping was performed for the physiological localization of AQP3 in SC. Micrographs showing overlaid images recorded with optical transmission and fluorescence microscopy of the same sample are shown in Fig. 2. In all samples, cell-like structures in the shape of corneocytes were identified. The fluorescent parts show binding of the fluorescent probe, confirming the presence of AQP3. Negative controls omitting the incubation step with the primary antibody was performed, and no fluorescence was detected (results not shown).

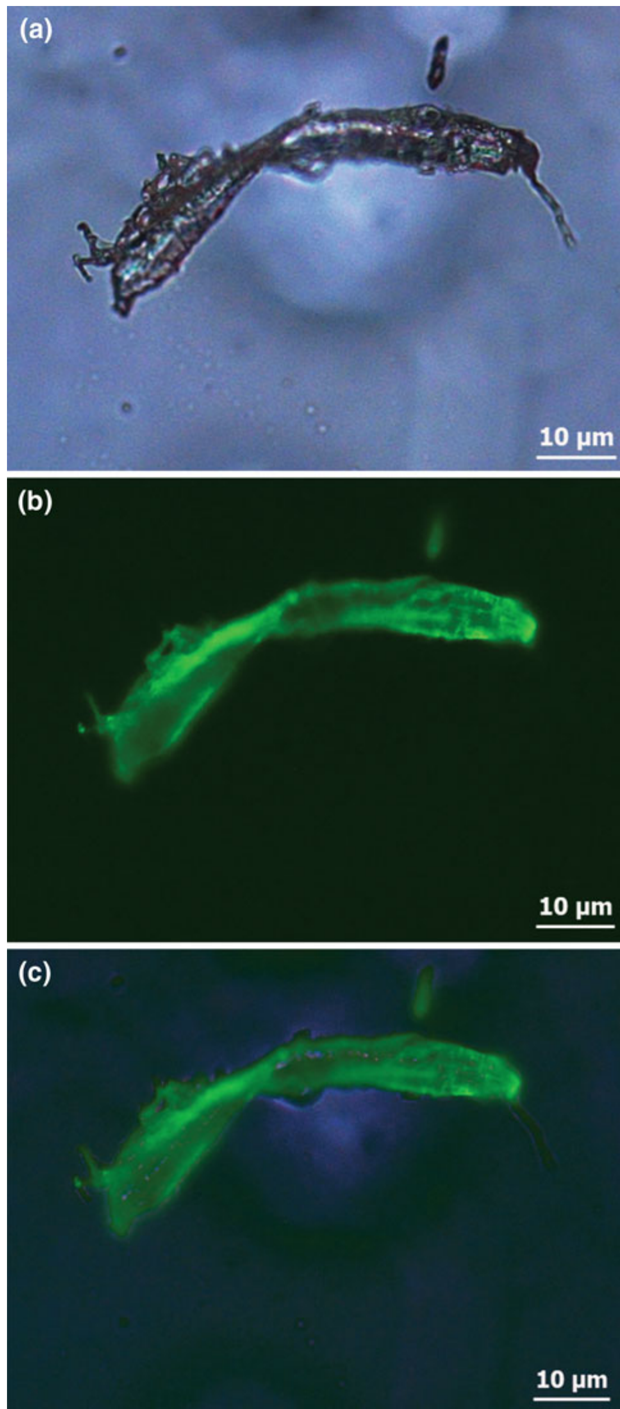

Fig. 2 Immunohistochemistry shows the presence of AQP3 in corneocytes in stratum corneum. Micrographs showing images recorded with optical transmission microscopy (**a**) and fluorescence microscopy (**b**), together with an overlay (**c**). The skin cells were stained using a specific primary antibody targeting AQP3 and a secondary fluorescence-tagged antibody. The strong fluorescent signals detected from the cells show that AQP3 is localized in the corneocytes

AQP10

Verification of AQP10 in SC samples obtained by skin scraping and analyzed by Western blotting is shown in Fig. 3. The blot was incubated with antibodies specifically targeting human AQP10.

Pure AQP10 was included as a positive control and shows as a clearly stained band with a size of ≈25 kDa, as expected for the AQP10 monomer, as well as a dimeric form of the protein at ≈50 kDa. Pure AQP3, included as a negative control, showed no protein staining at all. For the patient SC samples, a clear and well-defined band was found with a size of ≈50 kDa, corresponding to the dimer (Fig. 3). Furthermore, a band of about 60 kDa was present, as well as sharply stained bands with an apparent molecular weight of ≈80 and ≈110 kDa, and a diffuse band spanning the sizes ≈160–270 kDa were found (Fig. 3). No bands corresponding to the monomer for AQP10 were found in the patient samples.

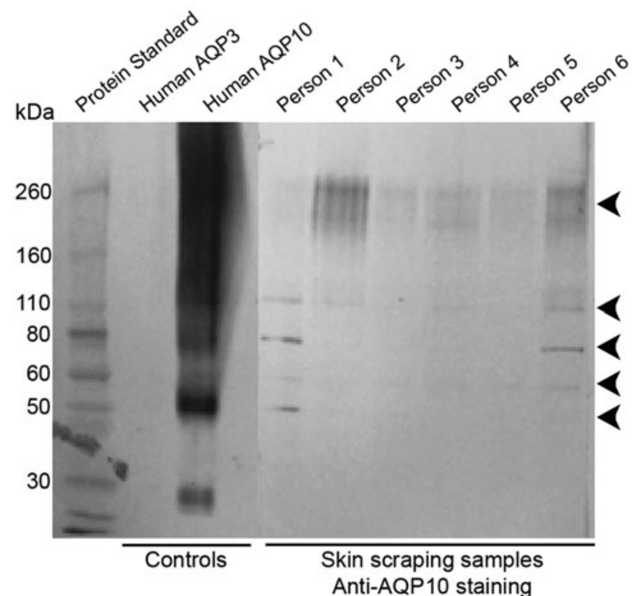

Fig. 3 AQP10-specific Western blot of skin scraped samples from test person 1–6. Recombinant human AQP3 (0.1 μg) and human AQP10 (0.02 μg) were included as negative and positive controls, respectively. Staining of the positive control, human AQP10 and lack of staining of human AQP3 verify the specificity of the anti-AQP10 antibody toward AQP10. In the SC samples, bands are observed for the dimer (≈50 kDa), for the trimer (≈80 kDa) as well as vague bands for the native tetramer (≈110 kDa). Bands of 60 kDa are observed as well which correspond to a glycosylated dimer. Heavier aggregates of 200–260 kDa are observed corresponding to SC that was left undissolved by the SDS treatment. In conclusion, we detected AQP10 in SC samples from all six test persons

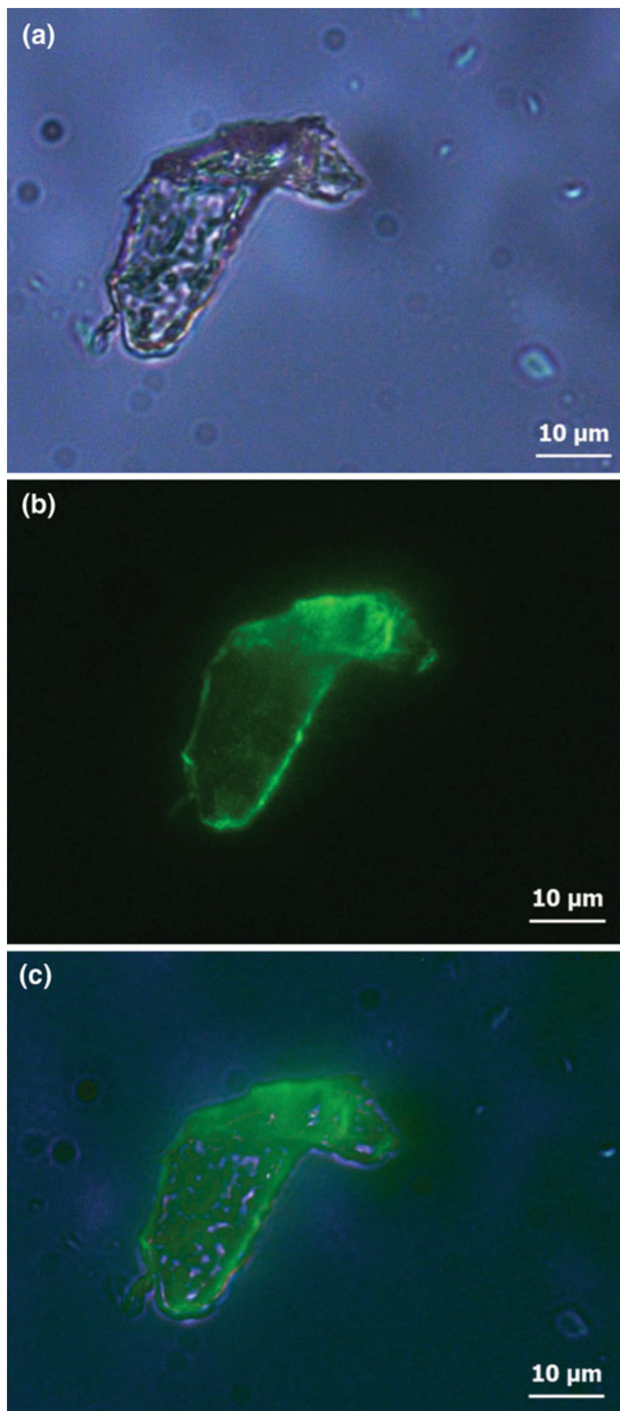

Fig. 4 Immunohistochemistry shows the presence of AQP10 in corneocytes in stratum corneum. Micrographs showing images recorded with optical transmission microscopy (**a**) and fluorescence microscopy (**b**), together with an overlay (**c**). The skin cells were stained using a specific primary antibody targeting AQP10 and a secondary fluorescence-tagged antibody. The strong fluorescent signals detected from the cells show that AQP10 is localized in the corneocytes

Immunohistochemistry on SC samples obtained by tape stripping was performed for validation of our findings of AQP10 in SC, and micrographs showing overlaid images recorded with optical transmission and fluorescence microscopy of the same sample are shown in Fig. 4. For all three test persons included, a uniform fluorescent signal from corneocyte-like structures could be detected confirming the presence of AQP10 in those cells (Fig. 4). Negative controls omitting the incubation step with the primary antibody were performed and no fluorescence was detected (results not shown).

Discussion

This study is the first to show the presence of AQP3 and AQP10 in human SC by Western blot analysis and confirmed by immunohistochemistry. The presence of AQPs in the SC indicates that they play a role in maintenance of hydration state of the SC and of skin barrier function, although their presence as a simple "spill over phenomenon" from the lower parts of the epidermis cannot be excluded. AQP3 has previously been reported in basal layer of the epidermis in normal skin [12], while the presence of AQP10 in human skin has not previously been reported. It has been suggested that AQP10 is present in human SC [1], but in vivo studies to confirm this have been lacking until now.

In the present study, AQPs were identified in SC samples obtained by a "minimal-invasive" tape stripping and skin scraping techniques. This method makes it possible to study in vivo changes in the AQP profile in human SC due to physiological and pathophysiological changes, and the perspective of this method is that the response to exogenous challenges and topical treatment regimes can be studied over time. The methods are minimally invasive and easy to handle, and produce enough material for the analysis.

With respect to the Western blot analysis of AQP3 (Fig. 1), the $\approx$25 kDa corresponds to the monomer size of hAQP3 of 26 kDa [14], while the $\approx$30 kDa band could possibly correspond to a glycosylated form of human AQP3, consistent with what has previously been described for AQP3 in the human epidermis [11, 14]. The $\approx$50 kDa band corresponds to the dimer of AQP3. With respect to the diffuse band spanning the sizes $\approx$80–120 kDa, we suggest that it represents both higher order oligomers as well as aggregates of skin that was not fully dissolved during the SDS treatment of the skin samples. With respect to the Western blot analysis of AQP10, a well-defined band

was found as a size of ≈50 kDa, corresponding to the dimer (Fig. 3). A band of ≈60 kDa was present, and we suggest that this corresponds to a glycosylated form of the dimer. Sharply stained bands with an apparent molecular weight of ≈80 and ≈110 kDa were observed as well, which we assign to represent higher order oligomerization and thus show the presence of trimers and tetramers. Furthermore, a diffuse band spanning the sizes ≈160–270 kDa was recognized (Fig. 3), which we assigned to aggregates of skin that was not fully dissolved during the SDS treatment of the skin samples. In contrast to the AQP3 findings, we do not observe bands corresponding to the monomer in the SC sample analysis for AQP10. However, as for AQP3, the tape-stripping samples show uniform fluorescent signal from the cells clearly demonstrating the uniform presence of AQP10 in human SC corneocytes (Fig. 4). The fact that the negative controls (pure AQP10 included in the Western blot for AQP3 and pure AQP3 included in the Western blot for AQP10, respectively) showed no protein staining indicates the specificity of the antibodies for respective AQP.

With respect to immunofluorescence for both AQP3 and AQP10, a fluorescent signal was detected uniformly over the cells, indicating not only that both AQPs are indeed present in human SC, but also that they are expressed uniformly in corneocytes, indicating homogeneity of AQPs occurrence in the cells.

Since the barrier function of the skin is located mainly in the SC, the finding of AQP3 and AQP10 in this layer makes it plausible that the role of AQP can be added to our overall understanding of factors influencing the skin barrier function, like filaggrin mutations, antimicrobial peptides, stratum corneum lipids [7, 13], and other players involved. A limitation of the present method is the lack of quantitative measure for the AQPs, which is an aim to be pursued in future studies. To fully understand the role of AQP3 and AQP10 in the skin barrier function, studies on their existence and regulation in different barrier affected diseases, like atopic dermatitis and psoriasis, as well as their response to different treatment regimes, need to be examined.

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Conflict of interest The authors state no conflict of interest.

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