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CONCISE COMMUNICATION

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Transient receptor potential vanilloid 3 expression is increased in non-lesional skin of atopic dermatitis patients

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Abstract

TRPV3 (transient receptor potential vanilloid 3) is a pro-inflammatory ion channel mostly expressed by keratinocytes of the human skin. Previous studies have shown that the expression of TRPV3 is markedly upregulated in the lesional epidermis of atopic dermatitis (AD) patients suggesting a potential pathogenetic role of the ion channel in the disease. In the current study, we aimed at defining the molecular and functional expression of TRPV3 in non-lesional skin of AD patients as previous studies implicated that healthy-appearing skin in AD is markedly distinct from normal skin with respect to terminal differentiation and certain immune function abnormalities. By using multiple, complementary immunolabelling and RT-qPCR technologies on full-thickness and epidermal shave biopsy samples from AD patients (lesional, non-lesional) and healthy volunteers, we provide the first evidence that the expression of TRPV3 is markedly upregulated in non-lesional human AD epidermis, similar to lesional AD samples. Of further importance, by using the patch-clamp method on cultured healthy and non-lesional AD keratinocytes, we also show that this upregulation is functional as determined by the significantly augmented TRPV3-specific ion current (induced by agonists) on cultured non-lesional AD keratinocytes when compared to healthy ones.

KEYWORDS
atopic dermatitis, inflammatory skin diseases, keratinocytes, TRPV3

1 | BACKGROUND

Members of the transient receptor potential (TRP) channel superfamily are a diverse set of cellular sensors capable of responding to a wide variety of chemical and physical stimuli. While most of these cation channels were first described on nerves, many have also been proven to be functionally expressed on epithelial cells and cells of the immune system.[1] TRP channels are also expressed in the skin, where they are important contributors to multiple homeostatic and inflammatory processes on keratinocytes,

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melanocytes, sebaceous glands, hair follicles and even some tumours.[2]

Of particular interest in the context of skin physiology is TRP vanilloid (TRPV) 3, since it was first described on (murine) keratinocytes, and not on neurons.[3] Perhaps the largest breakthrough regarding its function was the groundbreaking work that linked the hairless and pruritic dermatitis phenotype of DS-Nh mice with a Gly573Ser substitution in the channel.[4] This lead not only to hairlessness, but also spontaneous dermatitis and itch, as well as *Staphylococcus aureus* colonization, higher IgE and IL-4 serum levels, increased T-cell infiltration and hyperkeratosis in the affected mice.[5] Mutations in the TRPV3 channel have also been described in humans, where gain-of-function and even missense mutations in the gene have resulted in the development of Olmsted syndrome,[6-10] further highlighting its relevance in skin pathophysiology.[6]

TRPV3 activation has also been shown to result in the release of signalling molecules that induce inflammation and itch in neighbouring cells, such as prostaglandin E2, ATP, nerve growth factor and thymic stromal lymphopoietin.[5,11-13] Further, we have previously shown that TRPV3 channels are expressed on *human* epidermal keratinocytes, and that the activation of the channel by the non-specific agonist carvacrol causes an increase in calcium concentration, as well as decreasing the proliferation of these cells and inducing cell death. The opening of the channel was also followed by increased production of inflammatory mediators in a TRPV3-dependent manner via the activation of the NF-κB pathway.[14]

Of significant translational relevance, TRPV3 expression was found to be increased in lesional skin of atopic dermatitis (AD) patients as well as in pruritic post-burn lesions on the mRNA level.[13,15] Inhibition of TRPV3 by various compounds has been proven to be effective in ameliorating AD-like symptoms in animal models,[16-21] and the channel has also been linked to proteinase-activated receptor 2.[22,23] A more recent report has expanded on these results, by showing that TRPV3 expression is also increased on the protein level in AD lesions, and that heat-induced itch in AD patients is dependent on TRPV3. Further, keratinocytes isolated from lesional skin of AD patients showed increased sensitivity to heat in a TRPV3-dependent manner, which resulted in release of pruritogens.[24]

2 | QUESTIONS ADDRESSED

Interestingly, the above studies did not investigate in detail whether the expression and function of TRPV3 is altered in non-lesional skin of AD patients that is reportedly distinct from normal skin with respect to terminal differentiation and certain immune function abnormalities.[25,26] Therefore, in the current study, we aimed at assessing and comparing the expressional and functional characteristics of TRPV3 in non-lesional and lesional AD as well as in healthy specimens.

3 | EXPERIMENTAL DESIGN

Quantitative expressional (RT-qPCR, Western blotting, immunohistochemistry) analyses were performed on punch and shave biopsies from AD (non-lesional, lesional) patients and from healthy volunteers as well as on in vitro primary keratinocyte cultures (both AD and healthy). Further, functional (patch-clamp) studies were additionally carried out on cultured primary keratinocytes (for more information on the Methods, see Supplementary information).

4 | RESULTS

We first employed TRPV3 immunolabelling of skin biopsies ($n = 14$) obtained from non-lesional (NL) and lesional (L) skin of patients with AD as well as samples from healthy volunteers ($n = 14$) (for relevant data of the enrolled patients, please see Table S1). As expected from previous studies,[13,24,27] we found that the expression of TRPV3 protein in the epidermis was significantly increased in AD-L skin compared to healthy controls and, as a new data, to AD-NL samples (Figure 1A,B) (appropriate negative controls are shown in Figure S1). In addition, there was a tendency of increase of TRPV3 in AD-NL skin compared to that of the healthy controls which did not reach significance. The expression of TRPV3 was further validated by 3 independent monoclonal antibodies revealing similar quantitative and qualitative patterns (Figure S2).

We also had the chance to obtain epidermis-enriched shave biopsies from 3 patients (both NL and L regions) as well as from 3 healthy donors (for relevant data of the enrolled patients, please see Table S3). These shave biopsies were divided into 3 parts: two parts were immediately processed and subjected to Western blot and RT-qPCR analysis to quantitatively determine the TRPV3 expression at the protein and mRNA levels, respectively; whereas the third part was used to initiate in vitro epidermal keratinocyte cultures. These cultures were then further analysed for quantitative assessment of the molecular (Western blot, RT-qPCR) and functional (patch-clamp) expressions of TRPV3.

RT-qPCR analysis revealed that TRPV3 expression was significantly increased at the mRNA level in shave biopsies taken from AD-L skin compared to both healthy and AD-NL skin in all donors (Figure 1C shows the expression of TRPV3 on Donor 1 whereas data obtained from the other 2 donors are shown in Figure S3). Importantly, the mRNA expression of TRPV3 was also significantly higher in AD-NL shave biopsies compared to healthy controls in 2 donor comparisons whereas a non-significant tendency of increase was identified in the third case. Intriguingly, by Western blot, the protein level expression of TRPV3 was below the detection level in all healthy donors (Figure 1D presenting pooled Western blot densitometry analysis data of the 3 donors and Figure S4 presenting the original Western blots of all donor samples). In contrast, we were able to detect marked TRPV3 protein expression in all 3 diseased donor samples with a significantly higher expression in the AD-NL

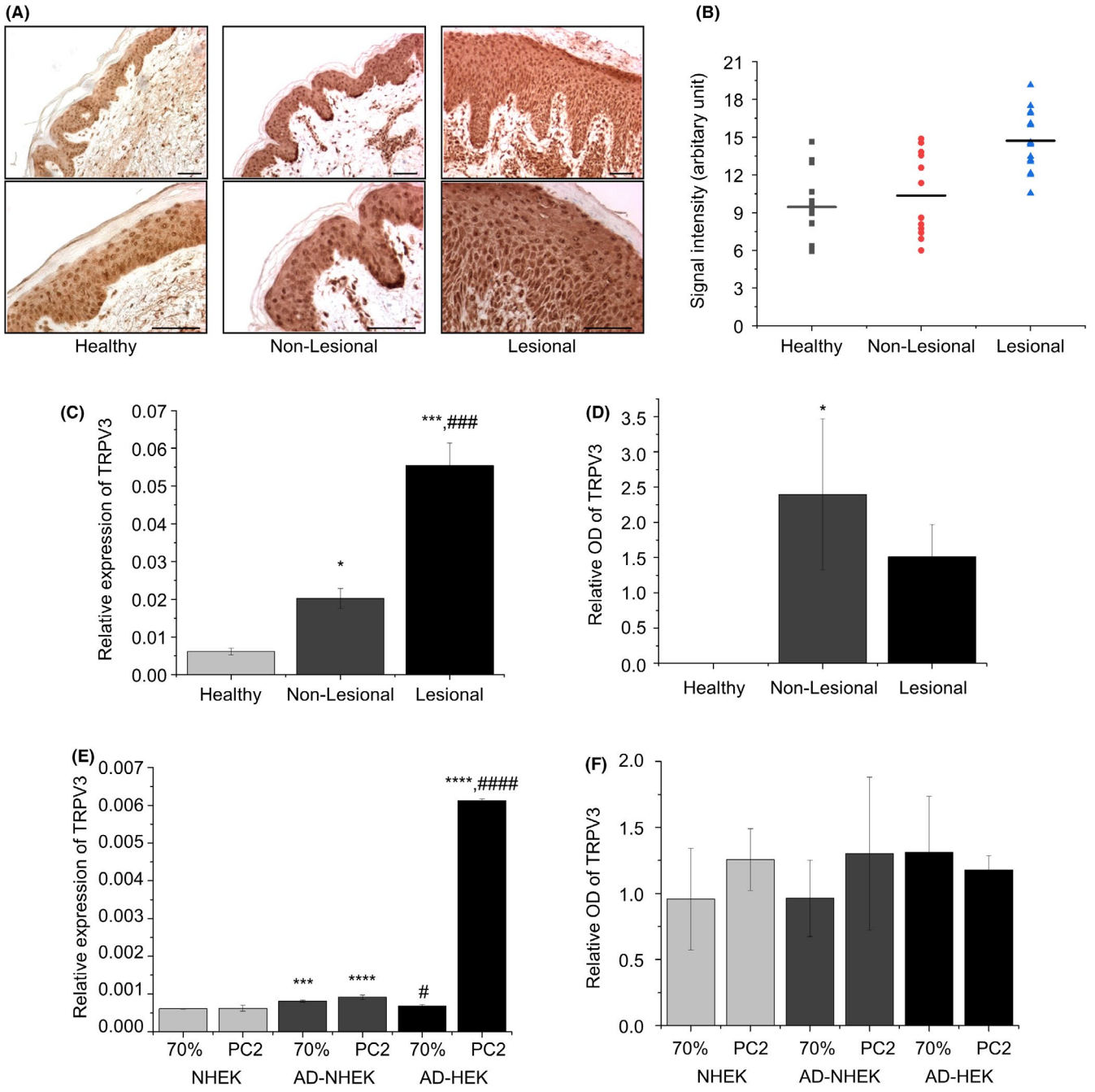

FIGURE 1 Expression of TRPV3 is significantly increased in AD patients. (A) TRPV3-specific immunoreactivity (IR), as shown by immunohistochemistry on representative healthy, non-lesional and lesional atopic dermatitis (AD) skin sections. Magnification 200x (top row) and 400x (bottom row). Scale bars = 50 μm. (B) Semiquantitative analysis of TRPV3 immunoreactivity on 4 histological sections per group. Results are expressed as mean ± *SD*. (C, E) TRPV3 mRNA expression in healthy human (Healthy) as well as non-lesional and lesional AD (Non-Lesional and Lesional) skin tissue, and cell lysates of keratinocytes isolated from healthy human (NHEK: normal human epidermal keratinocyte) and non-lesional and lesional AD (AD-NHEK: Non-Lesional human epidermal keratinocyte from atopic dermatitis patient and AD-HEK: Lesional human epidermal keratinocyte from atopic dermatitis patient, 70%: samples harvested at 70% confluence, PC2: samples harvested on day 2 after 100% confluence, respectively) samples, as assessed by quantitative real-time PCR. Results are expressed as mean ± *SD*, two additional experiments showed similar results (Figure S2 and S4). (D, F) TRPV3 protein level as determined by Western blot. Optical density was normalized to β-actin. Results are expressed as mean ± *SD* of 3 independent determinations. $^{*}p < 0.05$, $^{***}p < 0.001$ compared to Healthy groups, $\#p < 0.05$, $\#\#\#p < 0.001$ compared with the Non-Lesional group

regions when compared to the AD-L ones (Figure 1D and Figure S4). To our understanding, this is the first report to show that TRPV3 is not only overexpressed in the AD-L but also in the AD-NL epidermis.

These novel data were further assessed in in vitro studies, which were carried out on pre-confluent (70% confluence) and post-confluent (PC2, 2 days in confluence) cultures. Interestingly, in

pre-confluent (proliferating) cultures, the mRNA expression of TRPV3 was found to be significantly higher in cultured keratinocytes isolated from non-lesional areas of the skin (AD-NHEK), but not in cells from AD lesions (AD-HEK), when compared to the level found in keratinocytes from healthy volunteers (NHEK), in only 1 donor (Figure 1E); instead, when compared to NHEK cultures, TRPV3 mRNA levels were significantly less in both AD-NHEK and AD-HEK cells in the other 2 donors (Figure S5). As a marked contrast, in the post-confluent, differentiated cultures (which much better model the *in vivo*-like conditions of the epidermis than the pre-confluent ones), the mRNA expression of TRPV3 was significantly higher in AD-NHEKs in all 3 donors and in AD-HEKs in 2/3 donors than in NHEKs (Figure 1E and Figure S5). Further, in donors 1 and 2, the mRNA expression of TRPV3 was significantly higher in AD-HEKs than in AD-NHEKs whereas, in donor 3, the level of TRPV3 was significantly less in AD-HEKs than in NHEKs and AD-NHEKs (Figure 1E and Figure S5).

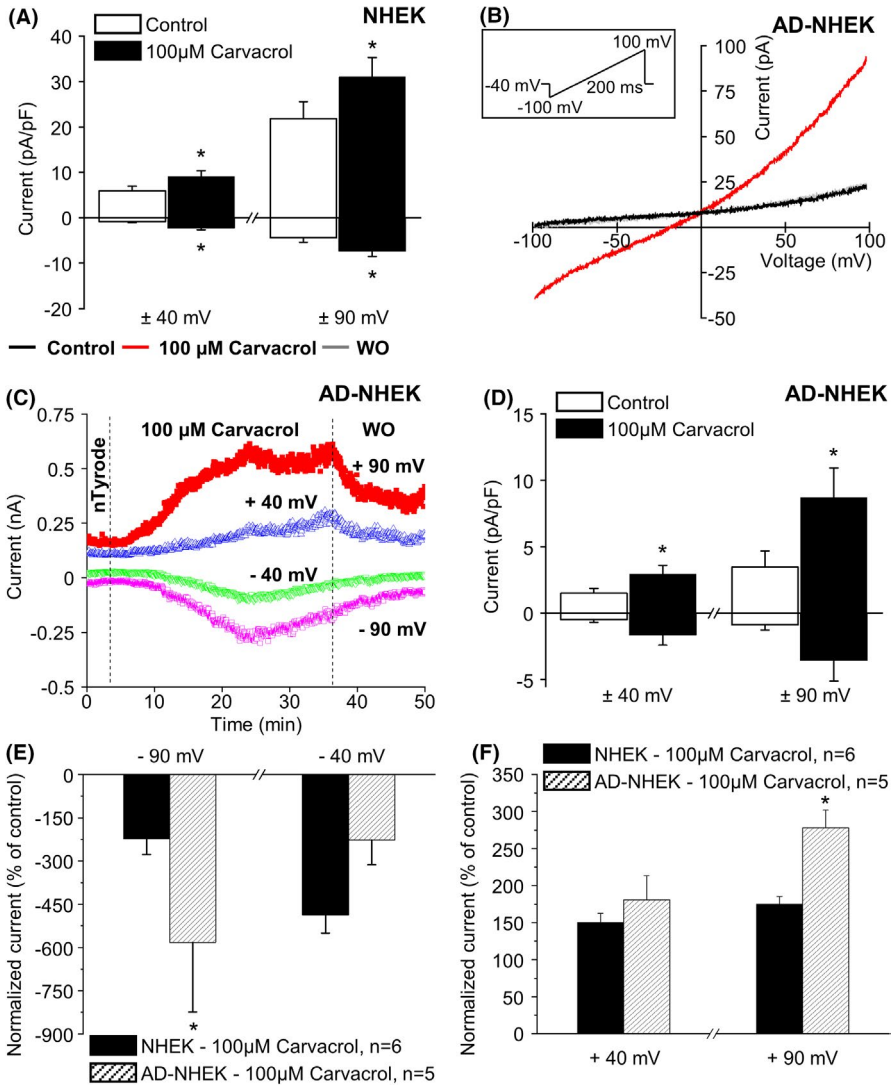

FIGURE 2 Function of TRPV3 is significantly increased in AD patients. (A) Representative I-V curves recorded in AD-NHEK cells in whole-cell patch-clamp configuration, in control (black) and after 100 μM carvacrol (red) and washout (grey). Inset shows the patch-clamp ramp protocol. (B) Statistical analysis of current densities normalized to cell membrane capacitance measured at −40 mV (left side downward), at +40 mV (left side upward), at −90 mV (right side downward) and at +90 mV (right side upward) in control (empty columns) and after 100 μM carvacrol (filled columns) in NHEK cells. Values are mean ± *SEM*, $n = 14$. (C) Representative experiment shows the typical time course of 100 μM carvacrol-induced current, measured at −90 mV (magenta), at −40 mV (green), at +40 mV (blue) and at +90 mV (red). Dashed lines show changes of the experimental solutions. (D) Statistical analysis of current densities normalized to cell membrane capacitance measured at −40 mV (left side downward), at +40 mV (left side upward), at −90 mV (right side downward) and at +90 mV (right side upward) in control (empty columns) and after 100 μM carvacrol (filled columns) in AD-NHEK cells. Values are mean ± *SEM*, $n = 4$. (E, F) Mean values show changes in currents after 100 μM carvacrol treatment. Filled columns show NHEK, while dashed columns show AD-NHEK cells. Currents are displayed as the percentage of the control (no drug), where (E) shows currents at −90 mV and at −40 mV, while (F) shows currents at +40 mV and at +90 mV. Values are mean ± *SEM*, $n = 14$ and $n = 4$ for NHEK and AD-NHEK, respectively. $^*p < 0.05$ compared to control or NHEK groups

Interestingly, in contrast to Western blot findings on shave biopsies (Figure 1D and Figure S4), cultured NHEKs expressed substantial levels of TRPV3 protein; of further importance, this expression was statistically not different from those of the AD-NHEK and AD-HEK cultures, irrespective of their proliferation or differentiation status (pre- or post-confluent cultures) (Figure 1F presenting pooled Western blot densitometry analysis data of the 3 donors and Figure S6 presenting the original Western blots of all donors).

To determine whether TRPV3 is also functionally overexpressed in AD skin, we sought to perform patch-clamp experiments on NHEKs, AD-NHEKs and AD-HEKs. Utilizing the whole-cell patch-clamp configuration, we have previously shown that TRPV3 ion channels can be specifically engaged by the agonist carvacrol resulting in both inward and outward membrane on NHEKs[14]; these findings were confirmed in the current setting (Figure 2A) and extended to AD cultures. Unfortunately, we were unable to form stable seals on AD-HEK cells (in spite of patching more than 150 cells from 3 separate donors), perhaps due to the increased fragility of these keratinocytes.[28] On AD-NHEKs, however, we found that, similar to NHEKs, both inward and outward currents were markedly and significantly ($p < 0.05$) increased by the TRPV3 agonist 100 μM carvacrol which effects were reversible by thorough washout (Figure 2B,C). Currents were measured and normalized to cell membrane capacitance at four different membrane potentials (ie at −90, −40, +40 and +90 mV) in NHEKs (Figure 2A) and AD-NHEKs (Figure 2D), respectively. Statistical analysis of current densities showed a significant current in both cell types at +40 and +90 mVs, while this change was significant at −40 and −90 mVs only in NHEK cells (most likely due to larger sample size in the NHEK group) (Figure 2A,D). Most importantly, the carvacrol-induced normalized currents were significantly greater (at −90 and +90 mV) in AD-NHEK cells compared to NHEKs (Figure 2E,F) suggesting that TRPV3 is functionally overexpressed on AD-NHEKs when compared to healthy NHEKs.

5 | CONCLUSIONS AND PERSPECTIVES

The novel findings of our current study lead to multiple conclusions. Indeed, we provide the first evidence that TRPV3, a prototypic pro-inflammatory ion channel of the human epidermis,[14] is upregulated not only in lesional, but also in healthy-appearing, non-lesional epidermis of AD patients (when compared to healthy skin) which is markedly distinct from normal skin with respect to terminal differentiation and certain immune function abnormalities (Figure 1).[25] Of further importance, we found that the elevated TRPV3 levels in non-lesional (and lesional) AD skin were detected both at the protein and mRNA levels. Previously, TRPV3 expression in non-lesional AD skin samples was assessed only in a very recent study in which the authors reported only insignificant changes between non-lesional AD and healthy skin by RNASeq.[27]

As in practically all studies with human samples and primary cultures from these human specimen, we also observed marked inter-donor variability among the different tissue samples investigated (full-thickness skin and epidermal shave biopsies as well as primary keratinocyte cultures) and the employed technologies (immunohistochemistry, Western blotting, RT-qPCR). Therefore, we extended the expressional analyses with a functional method (ie patch-clamp) which is the most instrumental technology to provide the "real proof" for identifying the augmented function of an ion channel. Possibly most importantly, we found that the agonist-induced TRPV3-mediated ion current is significantly augmented in cultured non-lesional AD keratinocytes (when compared to currents measured on healthy ones) (Figure 2) which data remarkably support the key message of our study.

Taken together, our presentation that TRPV3 is not only upregulated in lesional but also in non-lesional skin parts of AD patients provide valuable preclinical data for those translational and clinical studies that aim at targeting (most probably, inhibiting) the channel in the management of AD and possibly other inflammatory skin conditions.[29]

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CONFLICT OF INTEREST

TB provides consultancy services to Phytecs, Inc. (Los Angeles, CA, USA) and Monasterium Laboratory (Münster, Germany) who had no role in conceiving the study, designing the experiments, writing the manuscript or in the decision to publish it. The other authors state no conflict of interest.

AUTHOR CONTRIBUTIONS

TB and AGS conceptualized the project. NV, ZsP, and KK took part in the investigation. NV and KK performed visualization of data. NV and AGS optimized applied methodology. PPN and AS contributed valuable resources in the form of access to patch-clamp setups and patient samples, respectively. SM performed formal analysis of data. NV and AGS prepared the original draft whereas TB provided invaluable feedback by reviewing and editing the final manuscript. TB supervised the project, who was also responsible for funding acquisition.

DATA AVAILABILITY STATEMENT

Research data are not shared.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

Table S1 Summary of key relevant information of the 14 donors who provided punch biopsies

Table S2 Summary of key relevant information of the 3 donors who provided shave biopsies

Figure S1 Negative control staining of healthy, non-lesional and lesional atopic dermatitis skin sections. Magnification 200×.

Figure S2 TRPV3-specific immunoreactivity, as shown by immunohistochemistry on representative healthy, non-lesional and lesional atopic dermatitis skin sections using three commercially available primary monoclonal antibodies. Magnification 100× (top rows) and 200× (bottom rows). Scale bars =100 μm or 50 μm. (100× and 200×, respectively). Enlarged portions of 200× images are displayed on the far right for all antibodies to better show subcellular localization.

Figure S3 TRPV3 mRNA expression in healthy human (Healthy) as well as non-lesional and lesional atopic dermatitis (Non-Lesional and Lesional) skin tissue, as assessed by quantitative real-time PCR. Results are expressed as mean ± *SD* of three independent determinations. $^{*}p < 0.05$, $^{***}p < 0.001$ compared to Healthy groups, ### marks significant ($p < 0.001$) differences compared to the Non-Lesional group.

Figure S4 Western blot analysis of TRPV3 protein expression on healthy (H), non-lesional (NL) and lesional (L) shave biopsies. Equal loading was assessed by determining expression of β-actin.

Figure S5 TRPV3 mRNA expression in cell lysates of keratinocytes isolated from healthy human (NHEK) and non-lesional and lesional atopic dermatitis (AD-NHEK and AD-HEK, respectively) samples, as assessed by quantitative real-time PCR. Results are expressed as mean ± *SD*. $^{**}p < 0.01$, $^{***}p < 0.001$ compared to respective NHEK group, ### marks significant ($p < 0.001$) differences compared to the AD-NHEK group.

Figure S6 Western blot analysis of TRPV3 protein expression on cell lysates from keratinocytes derived from healthy, non-lesional and lesional shave biopsies. Equal loading was assessed by determining expression of β-actin.

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