



Direct assessment of skin epithelial barrier by electrical impedance spectroscopy

Arturo O. Rinaldi^{1,2} | Hideaki Morita^{1,2} | Paulina Wawrzyniak^{1,2} | Anita Dreher^{1,2} | Simon Grant³ | Per Svedenhag³ | Cezmi A. Akdis^{1,2}

¹Swiss Institute of Allergy and Asthma Research (SIAF), Davos, Switzerland

²Christine Kühne – Center for Allergy Research and Education (CK-CARE), Davos, Switzerland

³SciBase AB, Sundbyberg, Sweden

Correspondence

Cezmi A. Akdis, Swiss Institute of Allergy and Asthma Research (SIAF), Oberstrasse 22, CH-7270 Davos Platz, Switzerland.
Email akdisac@siaf.uzh.ch

Abstract

Background: Many skin and mucosal inflammatory disorders, such as atopic dermatitis, have been associated with an impaired epithelial barrier function, which allows allergens, pollutants, or microbes to enter the tissue and activate the immune response. The aim of this study was to establish a method to directly assess in vivo the epidermal barrier function by electrical impedance (EI) spectroscopy.

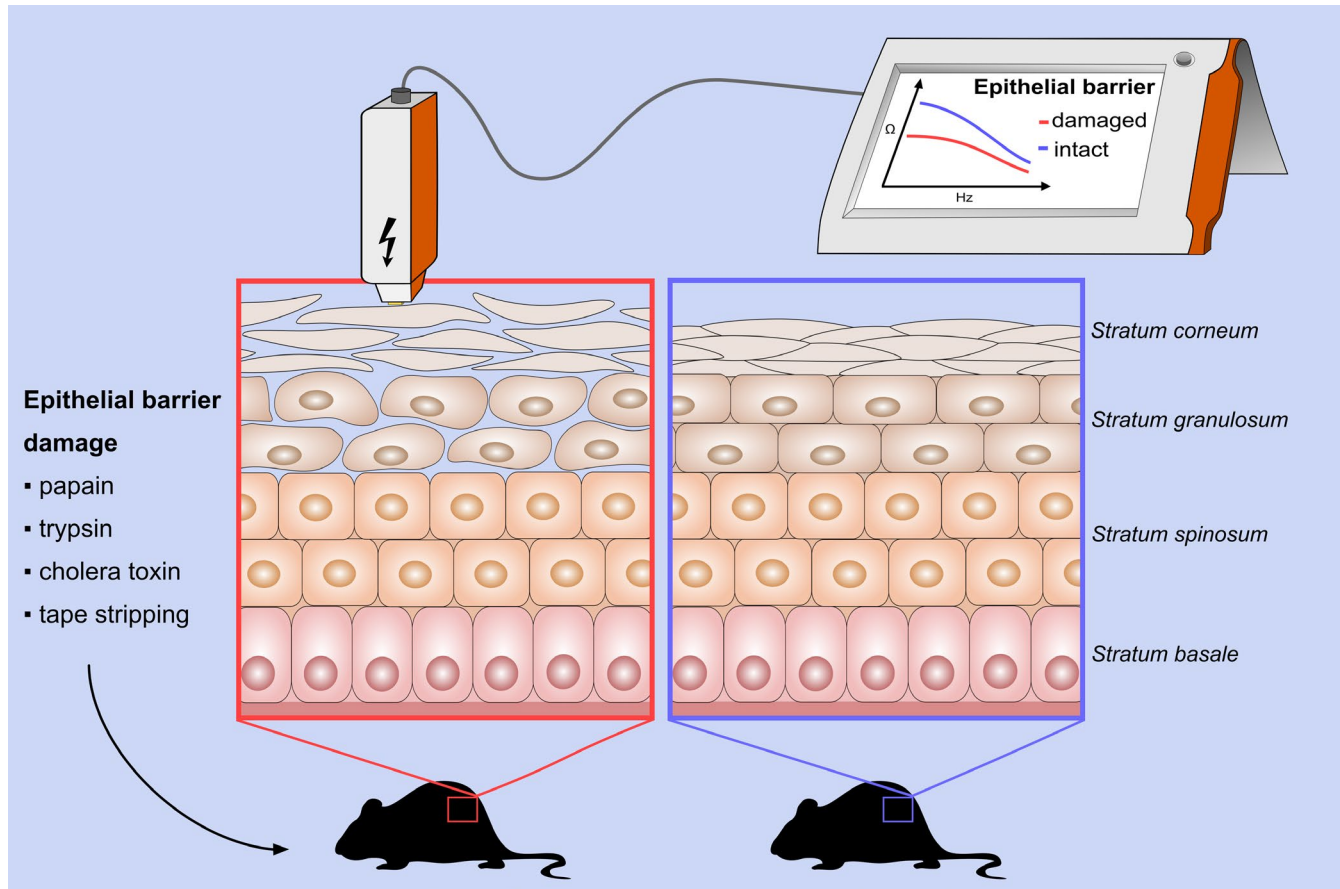
Methods: Mice epidermal barrier was damaged by epicutaneous application of proteases and cholera toxin and by tape stripping. EI and transepidermal water loss (TEWL) were measured before and after the application. Additionally, histological analysis, immunofluorescence staining, and RT-PCR were performed on skin biopsies to evaluate the epithelial barrier.

Results: A few hours after papain application, a dose-dependent reduction of EI was detected, reflecting the decreased barrier function. At the same time, an increase of TEWL was observed, with a significant negative correlation with EI, demonstrating that EI changes were directly linked to barrier defects. Twenty-four and 48 hours after the treatment, EI starts to increase to background levels, indicating tissue healing and restoration of skin barrier. Barrier disruption was confirmed by histological analysis showing an impaired stratum corneum and higher cellular infiltration after papain application. In addition, immunofluorescence staining and RT-PCR showed downregulation of molecules involved in the barrier function, such as filaggrin, occludin, and claudin-1, and mRNA levels of filaggrin, loricrin, and involucrin. Comparable results were observed after tape stripping and cholera toxin treatment.

Conclusion: Electrical impedance spectroscopy is a rapid and reliable diagnostic tool to detect skin barrier defects.

KEYWORDS

atopic dermatitis, electrical impedance, epithelial barrier, papain, skin inflammation



GRAPHICAL ABSTRACT

Mice epidermal barrier was damaged by epicutaneous application of papain, trypsin, cholera toxin, and by tape stripping. Electrical impedance (EI) spectroscopy is a rapid and reliable diagnostic tool to detect skin barrier defects and shows a negative correlation with transepidermal water loss (TEWL). Changes in EI reflect skin barrier damage, as well as tissue healing.

1 | INTRODUCTION

Epithelial tissues consist of layers of specialized cells closely bound together with the primary function to form a physical and chemical barrier between the body and the external environment. The epithelial barrier protects the internal tissues from environmental stresses, by preventing the entry of pathogens, pollutants, toxins, and allergens through skin or mucosae and minimizes water loss.¹ Recent genome-wide association studies have shown that the role of barrier function of the epithelium is essential in several allergic diseases.^{2,3} Barrier defects have been reported in atopic dermatitis (AD), asthma, chronic rhinosinusitis, allergic rhinitis, eosinophilic esophagitis, and colitis.⁴⁻¹¹ The aforementioned defects are a starting point of chronic inflammation and allergen sensitization and allow tissue damaging factors to enter the deeper tissue and thus activate immune and inflammatory responses.^{12,13}

Skin has two barrier structures: the stratum corneum and the tight junctions (TJs).¹² The stratum corneum is the outermost layer of the epidermis, consisting of terminally differentiated keratinocytes, called corneocytes, that form a densely packed and extensively crosslinked lipid-protein matrix. The proteins filaggrin, loricrin,

and involucrin have a pivotal role in skin barrier function by interacting with keratin intermediate filaments.¹⁴ One of the most important components of the epidermis barrier is represented by the TJs, which form intercellular junctions of epithelial cells at the level of stratum granulosum, right beneath the stratum corneum and filaggrin barrier. TJs seal the paracellular space between neighboring epithelial cells in the mucosae and at the level of stratum granulosum in the skin.¹⁵⁻¹⁷ They form large complexes in the cell membrane consisting of transmembrane proteins: the claudin family, the occludin family, the tight junction associated MARVEL protein family and the junction adhesion molecules (JAM). Intracellularly, the transmembrane proteins bind to several scaffold proteins such as the zonula occludens family (ZO) and are consequently connected to the actin cytoskeleton.^{18,19}

So far, it has been possible to assess the epithelial barrier in vitro, by measuring transepithelial electrical resistance (TEER), which represents the opposition of the epithelium to the passage of a steady electrical current. For this purpose, epithelial cells are cultured to an air liquid interface (ALI) in transwell plates. The confluence of the cellular integrity determines a sharp increase in TEER, indicating a low ion flux and a tight epithelial barrier; while the disruption of

junctional complexes results in reduction of TEER. In addition, TEER measurements show good negative correlation with fluoresceinated dextran passage as demonstrated in ALI cultures of different tissues.^{7,20} In vivo, there are few noninvasive methods to assess epithelial barrier function. One of these is the quantification of transepidermal water loss (TEWL) in the skin across the stratum corneum. Although TEWL increases in proportion to the level of damage, it is also affected by environmental factors such as humidity, temperature, season, and moisture content of the skin. Other noninvasive methods in use include the stratum corneum hydration, colorimetry, skin surface pH, and sebumetry. These methods provide information on different characteristics and/or condition of the skin, but they do not directly measure the barrier function.²¹

The aim of this study was to establish a method to assess in vivo the skin epidermal barrier function with a good stability against the influence of environmental factors, which could be used as a diagnostic tool for skin inflammatory disorders with barrier defects, such as AD. AD affects up to 20% of children and up to 4.4% of adults in Europe.^{22,23} Its hallmark features are itch and eczematous skin lesions that manifest often in early infancy with a course of remissions and exacerbations. An impaired epidermal barrier, characterized by defective expression of filaggrins and TJ proteins, has been described.^{24,25} Electrical impedance (EI) spectroscopy is a relatively new technique that has been under investigation as a possible means to characterize skin tumors. It works by transmitting a harmless electrical signal through the skin at several depths and frequencies and measuring the electrical response, influenced by certain properties of tissue integrity. EI is a measure of a material's defense to the flow of alternating currents of various frequencies. Specifically, EI of biological materials can reflect the pathophysiological status of the tissues. Normal and abnormal tissues may differ with regard to cell size, shape, orientation, compactness and structure of cell membranes. In general, EI at low frequencies is related mainly to the resistive properties of the extracellular environment, whereas EI at high frequencies is related both to the resistive properties of intra- and extracellular environments and the capacitive properties of the cell membranes (Figure S1).^{26,27} In some diseases, such as melanoma, the measurements of tissue EI have been used for diagnosis, assessment of disease progression, and evaluation of therapy. We hypothesized that EI spectroscopy might be used in skin and mucosal diseases, such as AD, where epithelial barrier dysfunction is a part of the pathogenesis and where the effects of certain treatments on epithelial barrier need to be monitored.⁴⁻⁷

2 | MATERIALS AND METHODS

2.1 | Mice

C57BL/6 mice were purchased from Charles River. They were housed in a specific pathogen-free environment at AO Research Institute Davos and used for experiments at 8-12 weeks of age. All the animal experiments were carried out in accordance with Swiss

law and approved by the animal experiment commission of the canton Graubünden, Switzerland.

2.2 | Epicutaneous administration of proteases and tape stripping to destroy the epithelial barrier

Mice were depilated on their back skin using an epilation cream (Veet, Reckitt Benckiser). Three days after depilation, 100 μ L of PBS containing different doses of a protease, papain (Sigma; 0.1, 1, and 10 μ g/ μ L), or trypsin (Sigma; 0.5%), or 100 μ L of PBS alone as control, was applied by means of a skin patch. EI and TEWL were measured before treatment and 1, 3, 5, 24, 48, and 72 hours after the treatment. After defined time points, mice were euthanized and skin sections were used for immune histology. C57BL/6 nude mice skin surface was stripped by adhesive tape (3M Scotch Transparent Tape 550-33 m $\times$ 15 mm) (tape stripping). EI and TEWL were measured before and after 5, 10, 15, and 20 tape strips.

2.3 | Electrical impedance measurement

Electrical impedance measurements were made using the Nevisense[®] (SciBase) (Figure S1), an EI spectroscopy device established for skin cancer diagnosis. The overall Nevisense procedure takes a few minutes and consists in (a) moistening the skin with physiological saline solution; (b) performing measurements on the lesion/treated area to be examined. Measurement time is approximately 8 seconds, and the applied pressure was minimal and did not induce any visible tissue damage, such as scaling or bulla formation. EI values at the different depths and frequencies were exported from the device as xml file and analyzed. EI values at the lowest frequency (1 kHz) are plotted according to the different time points. EI is a complex number and, as such, is made of two components, an imaginary unit and a real unit. In cartesian form, EI is defined as $Z = R + iX$, where the real part is the resistance R and the imaginary part is the reactance X (Figure S1D). By plotting the real part on the X-axis and the imaginary part on the Y-axis, Nyquist plots are obtained, where each point of the curve shows the impedance at one single frequency. In this case, the higher frequencies are on the left side of the plot and lower frequencies are on the right side.

2.4 | Transepidermal water loss measurement

Skin TEWL was measured by the "Delfin VapoMeter" (Delfin Technologies), a battery-operated and portable instrument. The core of the VapoMeter is a sensitive humidity sensor that is inside a cylindrical measurement chamber. This chamber is closed by the skin during the measurement time and is unaffected by ambient airflows. The sensor monitors the increase of relative humidity inside the chamber during the measurement. The evaporation rate value (g/ m^2 h) is automatically calculated from the relative humidity increase. Measurements were done always in the same room and on the same body areas where EI measurements were performed.

2.5 | Analyses of skin biopsies

Tissues were embedded in TissueTek (Qiagen) and frozen in liquid nitrogen, and cryosections were cut at 25 μm . TissueTek-embedded sections were stained with hematoxylin and eosin using standard protocols. For immunofluorescence staining, after fixation with paraformaldehyde 4% for 10 minutes, permeabilization with PBS containing 0.1% Triton-X (Acros Organics) and 0.02% SDS (Sigma-Aldrich) was performed for 10 minutes. After blocking with PBS 10% goat serum for 10 minutes at room temperature, sections were incubated with optimally diluted polyclonal rabbit anti-filaggrin (BioLegend), polyclonal rabbit anti-claudin 1 (abcam), and monoclonal antibody anti-occludin (Alexa Fluor™ 488, Invitrogen) at room temperature for 60 minutes and then, in case of a nonlabeled antibody, with a goat anti-rabbit antibody (Alexa Fluor™ 546, Invitrogen) for 45 minutes in the dark at room temperature. For the isotype controls, rabbit immunoglobulin fraction (Agilent, Dako) or mouse IgG1 (Alexa Fluor™ 488, Invitrogen) was used. Finally, slides were mounted with ProLong-Gold antifade reagent (Invitrogen) containing 4',6-diamidino-2-phenylindole for counterstaining of the cell nuclei and examined under a confocal microscope. From the maximum intensity projection of Z-stack, mean fluorescence intensity was quantified by ZEN Blue software (Zeiss).

2.6 | Real-time PCR

Total RNA from mouse skin was isolated using the RNeasy Plus Universal kit (QIAGEN). Complementary DNA synthesis was performed by using a RevertAid RT kit containing random hexamers (Thermo Fisher Scientific), and RT-PCR was performed with target gene-specific primers (Microsynth) and SYBR Green PCR master mix (Thermo Fisher Scientific) on an ABI PRISM 7000 Sequence Detection System (Applied Biosystems). According to the $-2\Delta\Delta\text{CT}$ value, the eukaryotic elongation factor 2 (EEF2) mRNA was used to standardize the quantities of each mRNA.

2.7 | Statistical analyses

Data were expressed as a mean $\pm$ standard error of the mean (SEM). Mann-Whitney U test was used to compare differences between different conditions and time points. Spearman's rank test was performed for correlation analyses. Statistical analysis was performed with GraphPad Prism 7.0 software (GraphPad Software). A P value of <0.05 was considered statistically significant.

3 | RESULTS

3.1 | Reduction of skin electrical impedance after treatment with proteases

Electrical impedance data were obtained by Nevisense® measurements, which are performed at 35 frequencies from 1.0 to 2.5 MHz,

at four different depths. The core of this technology is an extremely sensitive electrode, composed of five bars with a total of 225 microscopic pins, through which the electrical signals pass to measure the EI (Figure S1). To verify the potential use of EI spectroscopy for the in vivo detection of the epithelial barrier function, we treated the skin of C57BL/6 mice with proteases able to compromise the skin barrier function. In addition to EI, we measured in all our experiments TEWL and compared our findings with this already established method to assess the barrier function as a reference in our study.

Since both EI and TEWL measurements require hairless skin, the use of a depilatory cream was necessary. Being aware of the fact that such a cream might damage the epithelial barrier, we first aimed to define the number of days to wait after the hair removal. Therefore, WT C57BL/6 mice were depilated on their back, and EI and TEWL measurements were performed every day for 5 days. No difference was observed from day 1 to day 5 neither in EI nor in TEWL values (Figure S2), suggesting that there was no detectable effect of epilating cream throughout 5 days. To avoid any influence of the depilation on our results, we then decided to perform all our following experiments three days after depilating the mice.

In order to damage the epithelial barrier, we applied the cysteine protease papain topically to the mice back skin in 3 different doses for 1 hour, via skin patches and measured EI 1, 3, 5, 24, 48, and 72 hours after the treatment. As shown in Figure 1A, a dose-dependent reduction of EI was detected as early as 1 hour after the treatment, reflecting the decreased epithelial barrier function. Simultaneously, an increase of TEWL was observed (Figure 1B), showing a significant negative correlation with EI (Figure 1C), thus demonstrating that changes in EI were directly linked to barrier defects. Nevertheless, the correlations seem to be lost after 24 hours, probably because of the early phases of tissue healing and the restoration of the barrier, which might differently influence the two parameters, EI and TEWL. Indeed, EI started to increase twenty-four hours after the treatment, suggesting a restoration of the epithelial barrier. After 1 hour, EI shows a tendency, although not significant, to decrease in the untreated group. We hypothesize this decrease might be induced by the application of PBS. In addition, to show clearly the effect of papain on the EI, we represented it as a vector on a Nyquist plot, by plotting the two components of the EI, the real part and the imaginary part, on the X and Y axes (Figure S3). A significant reduction both in real and imaginary part of EI measurement is observed after increased doses of papain, in line with the results shown in Figure 1A.

Barrier disruption induced by papain was confirmed by histological analysis, which showed an impaired stratum corneum and higher cellular infiltration after papain application (Figure 2A). In addition, we checked the expression of molecules important for the skin barrier function, such as filaggrin or TJ molecules, occludin, and claudin-1 by immunofluorescence staining. Downregulation of the expression of all three barrier molecules was observed in a dose-dependent manner (Figure 2A). The tissue expression level of these proteins seems more similar after the application of the two highest doses of papain. In contrast, EI and TEWL measurements

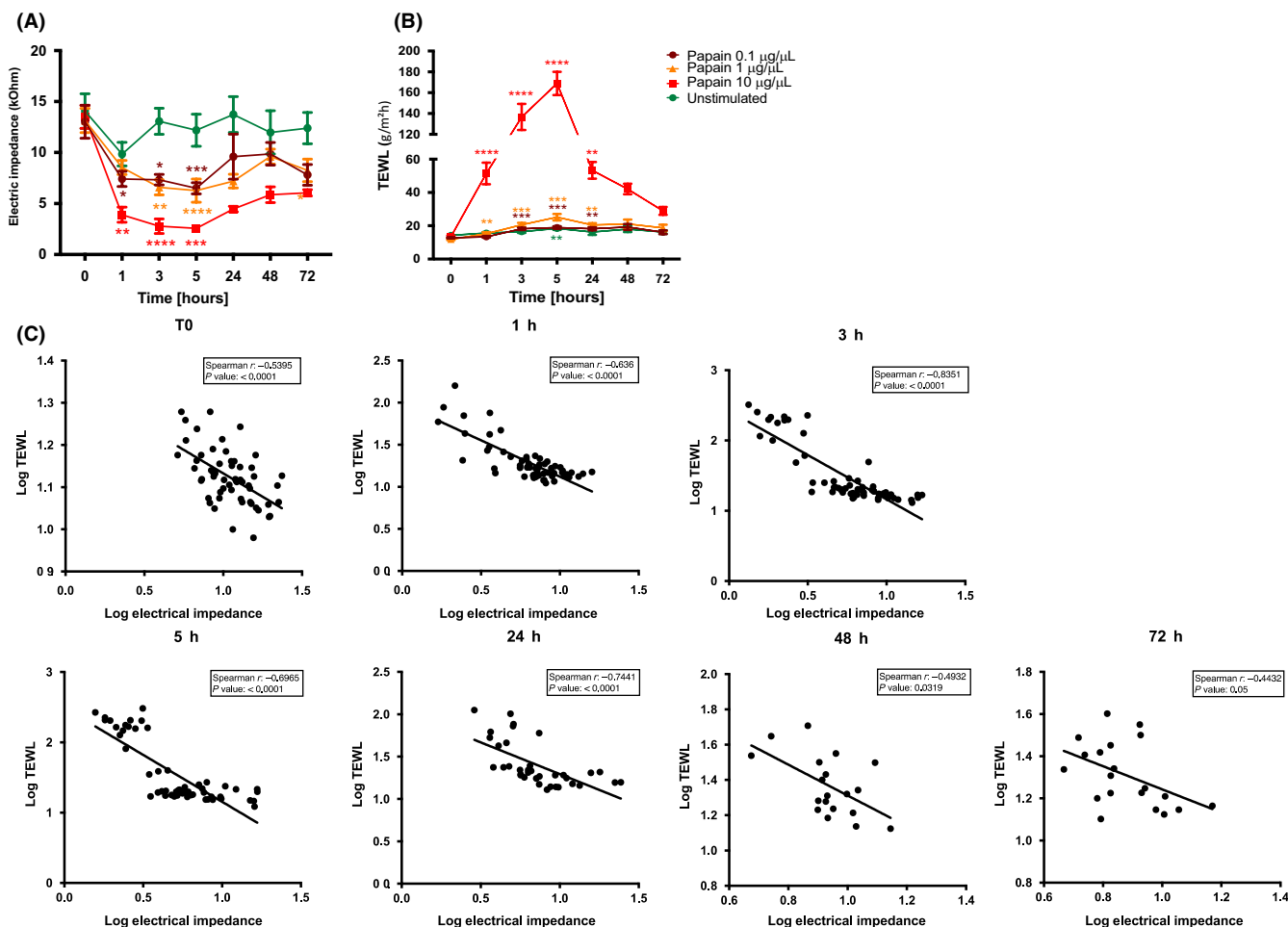


FIGURE 1 Epicutaneous administration of increasing concentrations of papain damages the epithelial barrier, causing a dose-dependent decrease of electrical impedance (EI) and increase of transepidermal water loss (TEWL) of skin. WT C57BL/6 mice were depilated on their back by using a depilatory cream. Three days after depilation, 100 µL of a solution containing different doses of the protease papain (0.1, 1 and 10 µg/µL), or PBS as control (unstimulated), were applied by means of a skin patch. EI (A) and TEWL (B) measurements were performed before treatment and 1, 3, 5, 24, 48, 72 h after the treatment. C, Spearman's correlation between EI and TEWL for all treatments is shown for each time point. Electrical impedance is expressed in kOhm, at the frequency of 1000 Hz. TEWL is expressed in g/m²h. Data are shown as mean ± SD (n = 8). *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$, ****: $P < 0.0001$

were similar after the application of the two lowest doses. This discrepancy suggests the involvement of other proteins, besides the ones we analyzed, in the impairment of skin barrier impairment. Indeed, it was recently reported, in the model of innate lymphoid cell-induced bronchial epithelial barrier damage, that a high number of barrier molecules have been altered.²⁸ Furthermore, mRNA expressions of filaggrin, together with other two essential molecules of the stratum corneum, loricrin, and involucrin, were down-regulated, as an additional evidence for the overall impairment of the stratum corneum barrier function. There was no significant change in the mRNAs of occludin and claudin-1 (Figure 2B). These results confirmed the impairment of the skin barrier function by papain treatment and its detection by EI spectroscopy.

Supporting these data, we tested the effect of another protease, the serine protease trypsin, which was applied epicutaneously to back skin of mice following the same protocol used for the papain application. As shown in Figure 3, a significant decrease of EI and a

simultaneous significant increase of TEWL after trypsin treatment were observed, consistent with the data in papain exposure. Similarly, EI and TEWL values showed a significant inverse correlation.

3.2 | Reduction in electrical impedance after tape stripping

To further investigate the accuracy of epithelial barrier detection, we tested the EI and TEWL methods on nude mice skin after damaging the epithelial barrier by tape stripping, a simple and efficient method through which the layers of the stratum corneum are successively removed by using adhesive films. EI and TEWL measurements were performed before tape stripping and immediately after 5, 10, 15, and 20 tape strips. We detected a significant reduction of EI after tape stripping, reflecting the decreased epithelial barrier function. In parallel measurements, we observed an increase of TEWL, confirming that the skin surface barrier function was reduced (Figure 4).

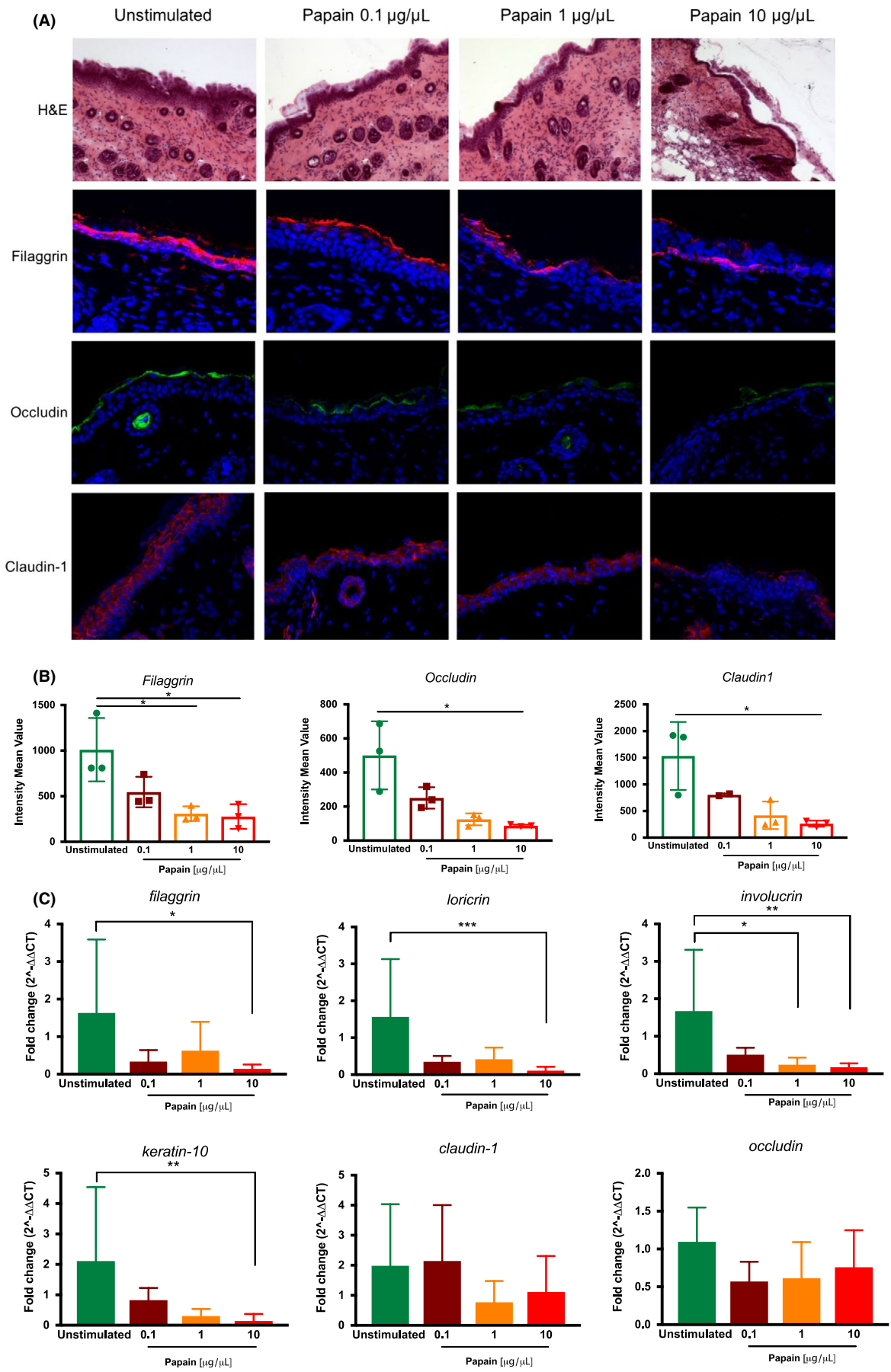


FIGURE 2 Papain downregulates the expression of molecules involved in the epithelial barrier function. A, Hematoxylin and eosin staining, immunofluorescence staining of filaggrin, occludin, and claudin-1 of mice skin after applying epicutaneously 100 μ L of PBS alone as control or 100 μ L of a PBS containing increasing doses of the papain (0.1, 1 and 10 μ g/ μ L). B, Intensity mean values, quantified by ZEN Blue software (Zeiss) and (C) mRNA expression of filaggrin, involucrin, loricrin, keratin-10, claudin-1, and occludin at 5 h after the epicutaneous treatment with different doses of papain

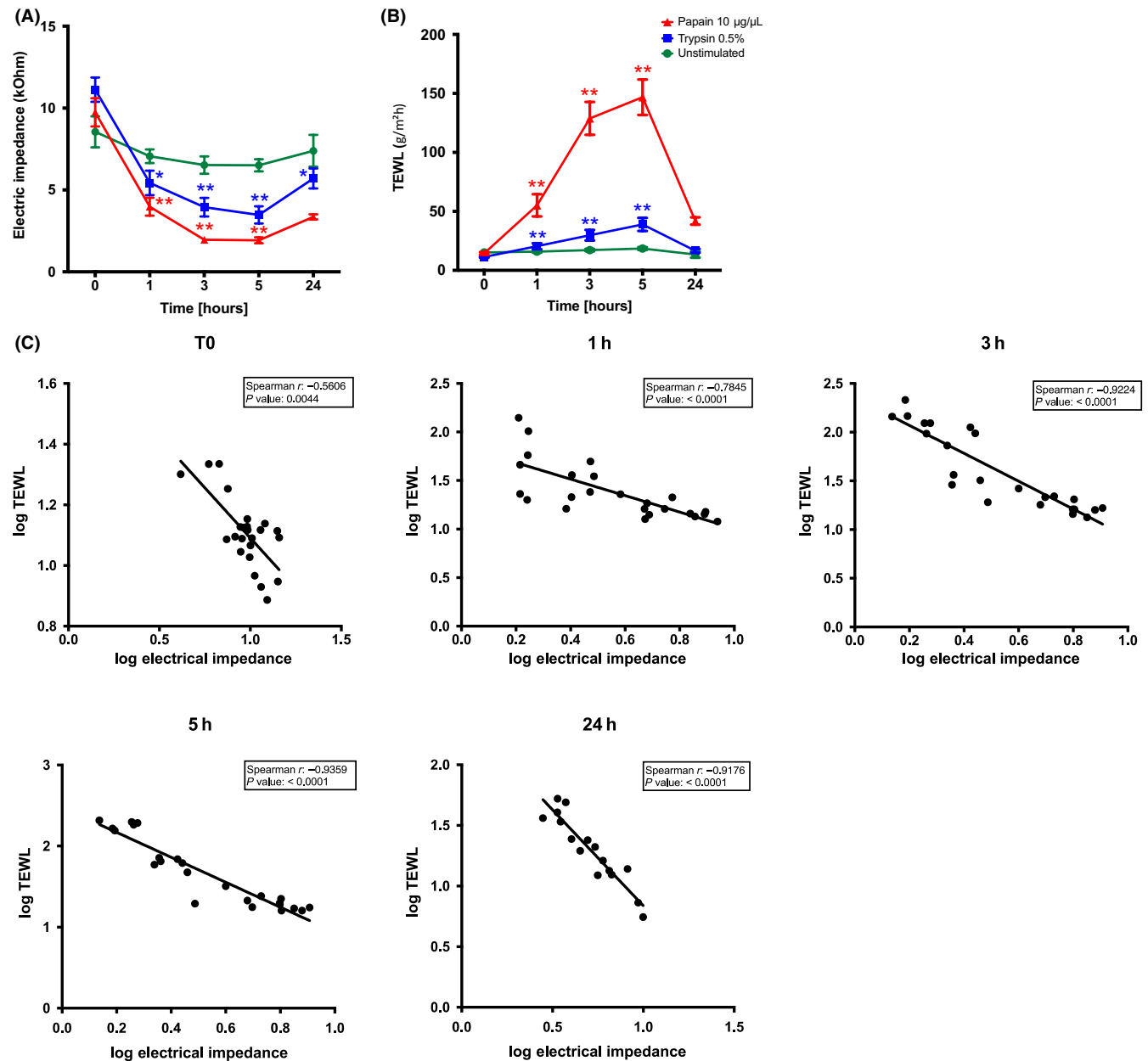


FIGURE 3 Trypsin shows a similar effect on skin epithelial barrier, causing a decrease of electrical impedance (EI) and an increase of transepidermal water loss (TEWL). WT C57BL/6 mice were depilated on their back. Three days after depilation, 100 μ L of a solution containing trypsin (0.5%), or the cysteine protease papain (10 μ g/ μ L), or PBS as control, were applied by means of a skin patch. EI (A) and TEWL (B) measurements were performed before treatment and 1, 3, 5 and 24 h after the treatment. C, Correlation between EI and TEWL is shown for each time point. Electrical impedance is expressed in kOhm, at the frequency of 1000 Hz. TEWL is expressed in g/m²h. Data are shown as mean $\pm$ SD (n = 8). *: P < 0.05, **: P < 0.01

Interestingly, after 10 strips, the TEWL values stop increasing. This is due to the nature of closed chamber devices, in which the chamber gets fully saturated with water vapor in a few minutes. Therefore, accumulation of water vapor in the chamber hinders the diffusion

of water out of skin, invalidating recording of continuous measurements.²⁹ Conversely, NeviSense device for measuring EI allows multiple and fast measurements overcoming the problem of the saturation.

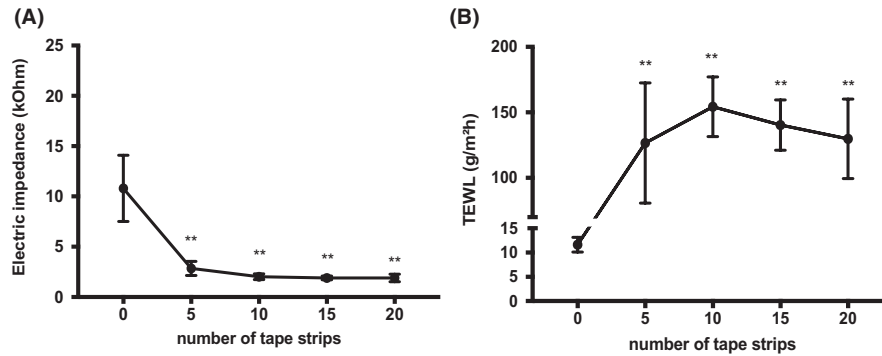


FIGURE 4 Tape stripping leads to a reduction of electrical impedance (EI) and an increase of transepidermal water loss (TEWL) of the skin. C57BL/6 nude mice skin was damaged by tape stripping, an efficient approach through which the cell layers of the stratum corneum are successively removed by using adhesive films. EI (A) and TEWL (B) measurements were performed before tape stripping and after 5, 10, 15 and 20 tape strips. Electrical impedance is expressed in kOhm, at the frequency of 1000 Hz. TEWL is expressed in g/m²h. Data are shown as mean ± SD, n = 12 (A), 5 (B). *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$

3.3 | Reduction of electrical impedance after ex vivo treatment with cholera toxin

In order to induce a different type of damage to the epithelial barrier, back skin from C57BL/6 nude mice was isolated and incubated at 37°C for 1 hour in presence of 2 µg/mL cholera toxin, which is known to be able to disrupt the epithelial barrier function, by affecting the organization of TJs. We observed that, after one-hour cholera toxin treatment, the EI was significantly reduced, in comparison to the control condition with PBS treatment (Figure 5). Once again, our observations indicate the validity of EI spectroscopy as a method to detect the epithelial barrier function.

3.4 | Differential changes in electrical impedance of the superficial skin compared to the deep skin after papain treatment and tape stripping

The different distances between the electrode bars of the EI spectroscopy as well as the lengths of the pins on these electrodes determine the depth of the penetration of the signal (Figure S1). Accordingly, we compared EI values obtained at all of the four different depths. Interestingly, we observed that EI measured at the most superficial layer was significantly lower in comparison to EI measured at the deepest depth. This difference was more pronounced when the epithelial barrier was compromised, both by papain treatment and tape stripping. The same effect was not observed when the skin was treated with PBS (Figure 6). These findings suggest that tape stripping and papain particularly damage the superficial skin layers more than the deeper skin layers.

4 | DISCUSSION

The aim of this study was to investigate the potential role of EI spectroscopy for the detection of the barrier function of skin epithelial tissue. We observed that the impairment of the epithelial

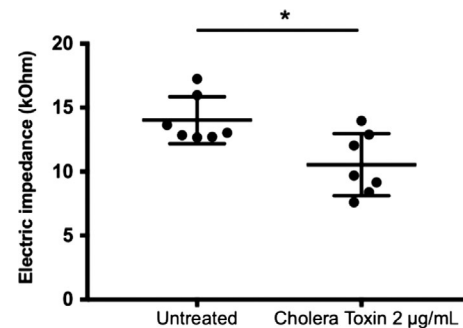


FIGURE 5 Cholera toxin decreases electrical impedance of skin ex vivo. Back skin from C57BL/6 nude mice was isolated and incubated at 37°C for 1 h in presence of 2 µg/mL cholera toxin, a microbial product which can specifically destroy the epithelial TJs. Electrical impedance is expressed in kOhm, at the frequency of 1000 Hz. Data are shown as mean ± SD, n = 5 in A and n = 7 in B. *: $P < 0.05$

barrier function in mice, induced either by administration of papain, trypsin, cholera toxin or by tape stripping, was able to affect the electric conductivity of the skin, causing a decrease of EI, which reflects the weakening of the epithelial barrier. In parallel experiments, the damage of skin barrier function was confirmed by immunofluorescence staining and qPCR, showing that the expression of essential proteins responsible for the skin barrier integrity was downregulated.

One of the proteases used in the present study was papain, a well-characterized cysteine protease mostly concentrated in the skin of papaya fruit, but commonly used in cosmetic, pharmaceutical and food industry. It presents extensive proteolytic activity toward proteins, short-chain peptides, and amino acid esters. It preferentially cleaves peptide bonds involving basic amino acids, particularly arginine, lysine, and residues following phenylalanine.³⁰ Papain has a high potential to drive epicutaneous sensitization: When topically applied, it induces an epicutaneous inflammation and affects the skin barrier, increasing TEWL, degrading TJ proteins, and inducing

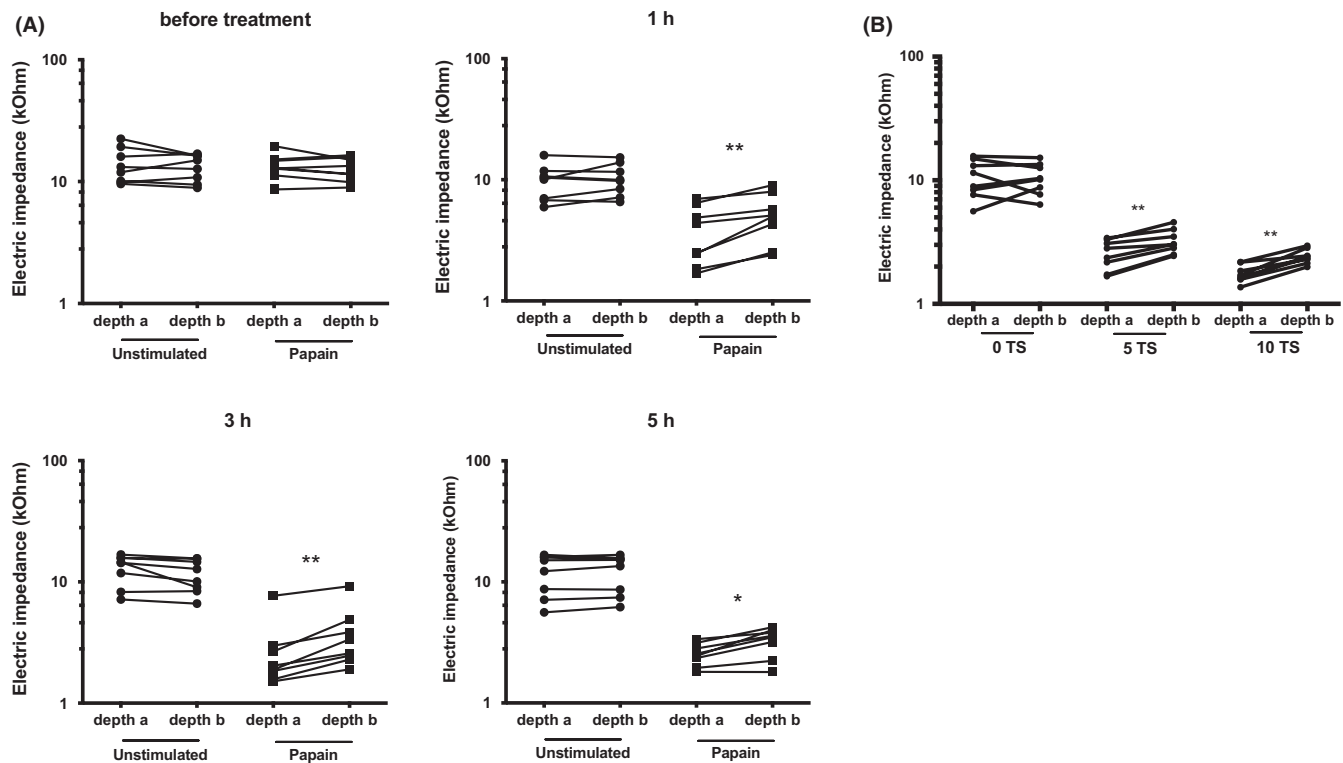


FIGURE 6 Electrical impedance measured at the superficial layer shows a stronger decrease when compared to the deeper layers after papain treatment and tape stripping. Electrical impedance can be measured at several different depths. Electrical impedance, expressed in kOhm at the frequency of 1000 Hz, is shown at depth a, the most superficial depth, and at depth b, the deepest depth, before the treatment with PBS (control) and papain (10 $\mu\text{g}/\mu\text{L}$), after 1, 3 and 5 h from the treatment (A) and before tape stripping and after 5 and 10 tape strips (B). * $P < 0.05$, ** $P < 0.01$

vasodilation and IgE and IgG responses.^{31,32} Therefore, papain seems to mimic the cysteine proteases contained in natural allergens, such as mites and pollen, which have been shown to be able to disrupt the epithelium contributing to the pathogenesis of allergic diseases.³³ In our model, we confirmed the role of papain at damaging the skin barrier function in vivo. After the epicutaneous application of different doses of papain, we observed in mice skin a rapid decrease of the mRNA expression of proteins important for the epithelial barrier, in particular in the stratum corneum, such as filaggrin, loricrin, and involucrin, together with a decreased expression of the protein level of filaggrin, occludin, and claudin-1, as shown by IHC staining. The reason for the decrease of mRNA of some proteins but not others is not clear. It might be due to the induction of cell death caused by papain treatment. In particular, the loss of contact with neighbor cells can induce some death or survival signals that can affect the gene expression of each other. The damage induced by papain was clearly associated to a decrease of EI, which was detectable already one hour after its application in a dose-dependent manner: The strongest decrease was observed with the highest dose of papain. Moreover, EI and TEWL values showed a significant inverse correlation. Comparable experiments were carried out with another protease, the serine protease trypsin. Trypsin hydrolyzes several types of proteins, including food proteins and extracellular matrix proteins, at the carboxyl end of the arginine and lysine residues. It is expressed

in epithelial cells of several tissues: skin, esophagus, stomach, small intestine, large intestine, lung, kidney, liver, and extrahepatic bile duct.³⁴ It has been shown that trypsin stimulation causes an impairment of the epithelial function in ALI cultures, where its application decreases the TEER,³⁵ a well-known described quantitative technique to measure the in vitro integrity of the barrier function.³⁶ In the present study, we observed that the epicutaneous application of trypsin causes an impairment of the epithelial barrier in vivo, associated with a significant decrease of EI and a significant increase of TEWL. Similarly, as after papain application, a strong significant inverse correlation between EI and TEWL has been observed.

Other methods through which we injured the epithelial barrier were tape stripping and cholera toxin application ex vivo. Tape stripping is a technique used in dermatology research for selectively removing the outermost layer of the skin, the stratum corneum, thus affecting the barrier function with a consequent strong increase of TEWL.³⁷⁻³⁹ In the present study, we observed that the application of up to 20 adhesive strips was able to induce a significant decrease of EI together with a consistent increase of TEWL.

Cholera toxin is responsible for diarrhea associated with *Vibrio cholerae* infection, by interfering with endocytic recycling compartment, which regulates the recycling to the plasma membrane. In particular, cholera toxin inhibits Rab11/exocyst-mediated trafficking of host proteins to cell-cell junctions in human epithelial cells, thereby

disrupting barrier function.⁴⁰ Similar to the data obtained after the application of proteases, we observed a significant reduction of EI after one hour of cholera toxin application.

Overall, our data demonstrate that EI spectroscopy is a direct method to assess the epithelial barrier function in vivo. It shows similarity to the TEWL quantification, already extensively described and applied in some allergic disorders, such as AD, whose lesions are characterized by an increase of TEWL.⁴¹ Nevertheless, TEWL has some limitations, not observed in EI. Although TEWL increases in proportion to the level of barrier damage, it is also affected by environmental factors such as humidity, temperature, seasonal variation, and hydration level of the skin.⁴² Therefore, our aim was to find an alternative noninvasive method that could translate in vivo what the TEER represents in vitro. Based on our results, EI spectroscopy represents a good candidate approach for the study and characterization of skin inflammatory disorders, such as AD. Both in lesional and nonlesional skin of some infants, a raise in the TEWL across the stratum corneum has been observed, suggesting the possibility to detect children at risk of AD when TEWL measurements are combined with parental history and detection of filaggrin mutations.^{43,44} EI spectroscopy might integrate with this approach for the prediction of AD in infants, allowing the identification of the risk of the disorder and thus the possibility to apply preventive measures.⁴⁵ Furthermore, AD is a heterogeneous disease with a multifactorial pathogenesis; therefore, it can be classified into various clinical phenotypes, based on severity, onset, atopy, morphology, lesions localization, as well as on the association to concomitant with other atopic or nonatopic disorders.⁴⁶ Some of the abovementioned phenotypes are strongly associated to loss-of-function mutations of genes encoding for proteins important in the skin barrier, for example, filaggrin.⁴³ Therefore, we believe EI would be helpful to identify those phenotypes, thus suggesting a way for the clinical assessment of the nature of the pathology.⁴⁷ Also, the present study gives the first hint that defects in epidermis barrier in different layers such as filaggrin mutations and TJ defects can be discriminated. Although obviously the first prototype has not been developed for this purpose, we detected statistically significant differences between surface barrier and deep barrier changes in response to papain and tape stripping. These findings suggest a possibility for a new concept development in epidermis barrier by using EI spectroscopy. With the improvement and optimization of the length of the pins and the distances between bars in the electrode, EI can be used to differentiate stratum corneum defects, eg. filaggrin defects, from TJ defects in the stratum granulosum.

In conclusion, the present study demonstrates the validity of a novel technique, namely, the EI spectroscopy, in head to head comparison with TEWL, to detect in vivo epidermal barrier changes in mouse skin in response to several enzymes and tape stripping. This opens a new window for human in vivo studies for the early detection of barrier leakiness in babies for prevention as well as monitoring of drug effects. Furthermore, by identifying possible correlations between EI and the innate and adaptive immune responses, immune biomarkers in serum and mutations of genes

associated with the epidermal barrier defects, it may be possible to develop an algorithm through which EI spectroscopy would help for the assessment of the general skin barrier status of patients in an efficient and fast way. Hence, an EI spectroscopy-based tool might help to monitor the effects of drugs on skin lesions and to identify AD patients at risk of relapse, thus indicating the necessity to reinforce the topical therapy to protect or hence skin barrier in order to prevent exacerbations.

CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

ORCID

Arturo O. Rinaldi  <https://orcid.org/0000-0001-8967-3866>

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SUPPORTING INFORMATION

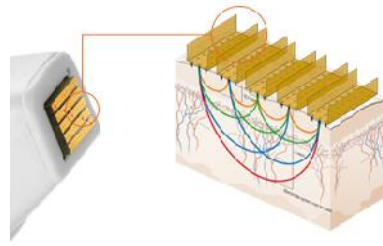
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How to cite this article: Rinaldi AO, Morita H, Wawrzyniak P, et al. Direct assessment of skin epithelial barrier by electrical impedance spectroscopy. *Allergy.* 2019;74:1934–1944. <https://doi.org/10.1111/all.13824>

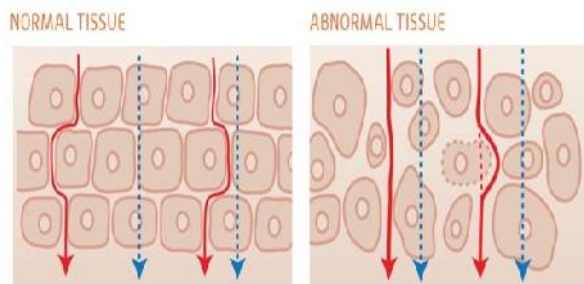
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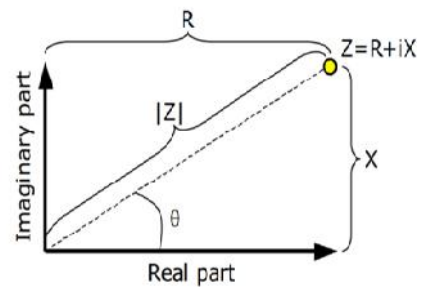
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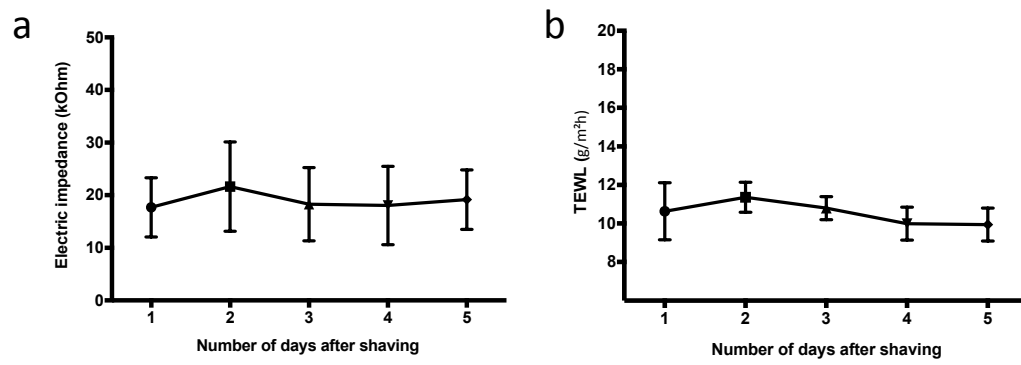
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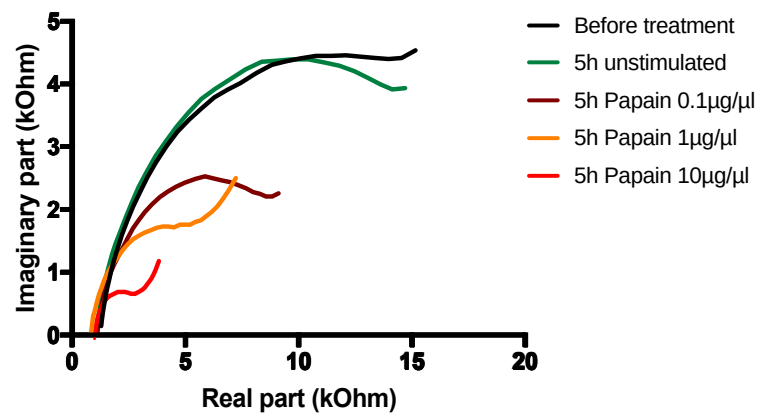
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Rinaldi et al. Supplementary figure 1



Rinaldi et al. Supplementary figure 2



Rinaldi et al. Supplementary figure 3

Gene	Forward (5'-3')	Reverse (5'-3')
m filaggrin	TCA ACC AGT GAT AAG GAG GAA	TCT TGG TCA TCT GGA TTC TTC A
m loricrin	TGG TGC TTC AGG GTA AC	CTT TCC ACA ACC CAC AGG
m involucrin	TCC TGT GAG TTT GTT TGG TCT A	CAC ACA GTC TTG AGA GGT CC
m keratin-10	GGA CCA AGA TAC TAA CAA AAC C	AGA CGA AAG GAC TCT ACC CT
m occludin	CCT ATC TTG GGA GCC TGG ACA T	GAT TGG GTT TGA ATT CAT CAG GTC
m claudin-1	CCA CCA TTG GCA TGA AGT GC	ACT AAT GTC GCC AGA CCT GA
m eEF2 (housekeeping)	CAT GTG GAC CAC GGC AAG TC	GGA GAT GGC GGT GGA TTT GAT

Rinaldi et al. Supplementary table