

Skin provocation tests may help to diagnose atopic dermatitis

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To cite this article: Hawro T, Lehmann S, Altrichter S, Fluhr JW, Zuberbier T, Church MK, Maurer M, Metz M. Skin provocation tests may help to diagnose atopic dermatitis. *Allergy* 2016; DOI: 10.1111/all.12995.

Keywords

biomarker; cowhage; flare; histamine; pruritus.

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Accepted for publication 26 July 2016

DOI:10.1111/all.12995

Edited by: Werner Aberer

Abstract

Background: Atopic dermatitis (AD) is a common skin disorder. Its diagnosis relies on clinical judgment. Mild and atypical manifestations may cause diagnostic difficulties. Biomarkers for the differential diagnostic workup of AD are needed.

Objective: To test whether the results of skin provocation with cowhage, an established model of histamine-independent pruritus, and histamine are different in AD patients and healthy subjects and whether these tests may be used as diagnostic markers of AD.

Methods: Twenty-two AD patients and 18 healthy controls were subjected to topical cowhage provocation and skin prick testing with histamine and assessed for differences in the quality, intensity, and persistence of itch, for wheal diameter, volume, and flare size and intensity.

Results: Patients with AD, compared with healthy controls, exhibited significantly smaller histamine-induced flares ($P < 0.01$) and markedly longer itch persistence after provocation with cowhage ($P < 0.01$). Both parameters showed good diagnostic properties for AD (area under the receiver operating characteristic (ROC) curve 0.78 and 0.80, respectively). The persistence of cowhage-induced itch for at least 30 min and a histamine-induced flare of less than 2 cm in diameter were reliable thresholds for the diagnosis of AD. If combinations of the results of both tests were used, their sensitivity and specificity of diagnosing AD were up to 91% and 94%, respectively.

Conclusion: The clinical benefit of cowhage and histamine skin provocation tests should be investigated in further studies. Long persistence of cowhage-induced itch and diminished histamine-induced flare in nonlesional skin may support diagnosis of AD.

Atopic dermatitis (AD) is one of the most common dermatoses, with a lifetime prevalence exceeding 20% in many geographic areas (1, 2). Diagnosing AD is relatively straightforward, but in mild cases and when manifestation or disease course is atypical, differential diagnoses must be considered and diagnostic difficulties may arise (3, 4). The diagnosis of AD is based almost exclusively on a detailed history and clinical assessment (5, 6). Biomarkers for the differential diagnostic workup of AD patients are needed, but are not available

at present. None of the laboratory markers AD patients are commonly tested for are pathognomonic. These include elevated total and specific IgE levels, which are not specific for AD and, by definition, absent in its intrinsic subtype (7, 8).

Histamine skin prick testing induces itch, a flare, and a wheal. Itch intensity and persistence as well as flare and wheal size and persistence are easy to measure. Histamine is included as a positive control in skin prick testing for allergens, one of the most commonly performed tests in patients with suspected AD. Responses to histamine skin prick testing have been reported to be different in AD patients and healthy controls. Histamine-induced flare responses, in several independent studies, have consistently been found to be reduced in AD patients as compared with healthy control subjects (9, 10). In contrast, itch and wheal induced by

Abbreviations

AD, atopic dermatitis; AU, arbitrary units; AUC, area under the curve; HC, healthy controls; PAR, protease-activated receptor; ROC, receiver operating characteristic; SEM, standard error of the mean; VAS, visual analogue scale.

histamine in AD patients and healthy controls have not consistently been found to differ (10–13). As of now, the diagnostic properties of histamine skin prick testing responses including those of itch have not been systematically assessed in AD. As a consequence, it is unclear whether skin prick testing for histamine can help in diagnosing AD.

Skin provocation testing with spicules of a tropical legume cowhage (*Mucuna pruriens*), which contain the protease mucunain, is an established model of histamine-independent pruritus (14–16). A slower decline of cowhage-induced itch intensity during a five-and-a-half-minute observation period has been reported in AD patients compared with healthy controls (14). However, the possibility of application of cowhage as diagnostic tool in AD has not yet been investigated.

Here, we investigated AD patients and healthy controls for differences in itch, wheal, and flare responses to histamine and cowhage skin provocation testing and assessed their diagnostic potential as possible diagnostic biomarkers of AD.

Materials and methods

Study subjects

Twenty-two AD patients (12 females, median age 30 years, range 20–42 years, mean SCORAD $\pm$ SD 34.4 ± 14.6 , mean pruritus intensity extracted from SCORAD (expressed on VAS ranging from 0 to 10) $\pm$ SD 3.9 ± 2.2) and 18 healthy age-matched volunteers (8 females, median age 29 years, range 21–41 years) participated in the study. All patients and healthy volunteers were Caucasian and they were fluent in German. All study participants were examined by a dermatologist to confirm or exclude diagnosis of AD according to the criteria of Hanifin and Rajka. Only patients who had symmetric eczema-free areas on both volar forearms were included. Subjects were not allowed to take any antihistamines for 1 week or receive phototherapy or systemic immunosuppressants for 2 weeks before provocation testing. Topical treatment with corticosteroids or calcineurin inhibitors of the skin of both volar forearms had to be discontinued for at least 2 weeks before provocation testing. Eight AD patients had allergic rhinitis, two had allergic asthma, and two had both. Two patients exhibited intrinsic-type AD. The study was approved by the Ethics Committee of the Charité-Universitätsmedizin Berlin (approval number: EA 4/063/13), and informed consent was obtained from all participants.

Induction of pruritus

As previously described, 40–45 cowhage spicules (kindly provided by Ethan Lerner, Harvard Medical School, Boston, MA, USA) (16) were applied, by gently rubbing for 45 s, to 4-cm² circular areas on the volar skin of forearms (14). Skin prick testing was performed with 10 mg/ml of histamine dihydrochloride and its vehicle as negative control (ALK-Abello, Horsholm, Denmark). Histamine and cowhage were always tested on the opposite forearms, and the side of application (left vs right) was randomized. Patients and healthy controls were randomized to provocation testing at three

different times of the day: 8.00–10.00 a.m., 12.00–14.00 p.m., or 18.00–20.00 p.m.

Skin provocation outcome assessment

Itch was assessed every minute for 30 min after provocation on a 100-mm visual analogue scale (VAS), and the persistence of itch (time from the histamine skin prick or the end of cowhage application to the end of itch sensation) and maximum and overall itch intensity (area under the curve, AUC) were analyzed. In addition, subjects rated the overall intensity of the following Eppendorf Itch Questionnaire descriptors (VAS): itch, urge to scratch, warm, cold, burning, tingling, tickling, pulsating, coming in waves, pricking, piercing, dull, sharp, numbing, pinching, squeezing, and pain (17). Itch qualities were considered as positive, if their reported intensities were significantly stronger after application of histamine or cowhage as compared to negative control.

Wheal and flare parameters were assessed 20, 40, 60, 90, and 120 min after provocation. The largest diameter (DL) and the rectangular diameter (DR) of wheals and flares were recorded with a ruler and used to calculate the surface area ($S = DL/2 \times DR/2 \times \pi$). For planimetric morphometry, digital images of the wheals and flares were analyzed using ImageJ software. Wheal volume was measured using three-dimensional photography and volumetric analyses (PRIMOS; GF Messtechnik GmbH, Teltow/Berlin, Germany). The intensity of the skin erythema was determined by Mexameter (Courage & Khazaka, Cologne, Germany) and was expressed in arbitrary units (AU; range: 0 to 999) as previously described (9). Measurements of flares and wheals showed maximum responses at the 20-minute postprovocation time point, which is why all comparisons between AD patients and healthy controls were performed for this time point.

Statistical analyses

Normality of distribution was tested with the Kolmogorov–Smirnov test. Differences between two groups of parametric and nonparametric variables were tested with the two-sample *t*-test and the Mann–Whitney *U*-test, respectively. Friedman's test with post hoc analysis using Wilcoxon signed-rank test was applied to compare intensities of sensations released by active substances and negative control. Changes in the intensities of the local skin reactions over time were analyzed using ANOVA. If the main effect was significant, post-test with Bonferroni's correction for multiple comparisons was employed to determine at which time points the assessed parameters increased significantly as compared to the initial values. Linear relationships between parametric and nonparametric variables were analyzed using Pearson's and Spearman's correlations, respectively. Logistic regression was used to analyze predictors of AD diagnosis. The area under the receiver operating characteristic (ROC) curve was analyzed to characterize the persistence of pruritus after skin challenge with cowhage and the longest flare diameter after histamine skin prick testing as diagnostic markers of AD. To characterize the overall performance of these two diagnostic markers,

ROC analysis was performed using predicted probabilities. The predicted probabilities were obtained from the logistic regression model, which included persistence of cowhage-induced pruritus and diameter of histamine-induced flare as independent variables. Statistical significance was set at $P < 0.05$.

Results

AD patients have smaller, but not less intensive flare reactions, after provocation with histamine as compared to healthy individuals

Provocation with histamine, but not cowhage, resulted in visible flare responses and significantly increased erythema intensities both in the AD patients and in healthy controls (Fig. 1A–D). Measured at 20 min, flares after skin provocation with histamine were markedly smaller in AD patients than in healthy controls. Comparisons of the longest diameter (means $\pm$ SEM: AD: 18 ± 2 mm vs HC: 32 ± 3 mm,

$P < 0.01$), of the surface area determined by ruler measurements (AD: 280 ± 59 mm² vs HC: 679 ± 100 mm², $P < 0.001$), and of the surface area determined by planimetric morphometry (215 ± 47 mm² vs 518 ± 81 mm², $P < 0.01$; Fig. 1) all showed similar results. The surface areas of flares, determined by ruler and planimetric morphometry, showed a strong correlation with their longest diameter, both in AD patients ($r = 0.862$, $P < 0.001$ and $r = 0.857$, $P < 0.001$) and in healthy controls ($r = 0.933$, $P < 0.001$ and $r = 0.870$, $P < 0.001$). No differences in flare intensity measured with Mexameter (Fig. 1) or in wheal diameter or volume (Fig. 2) were observed between AD patients and healthy controls.

AD patients show longer persistence of itch, but not higher maximum itch, after provocation with cowhage as compared to healthy individuals

Patients with AD exhibited markedly longer persistence of itch after provocation with cowhage (in 14 of 22 AD patients itch persisted at least 30 min) than healthy controls (only in

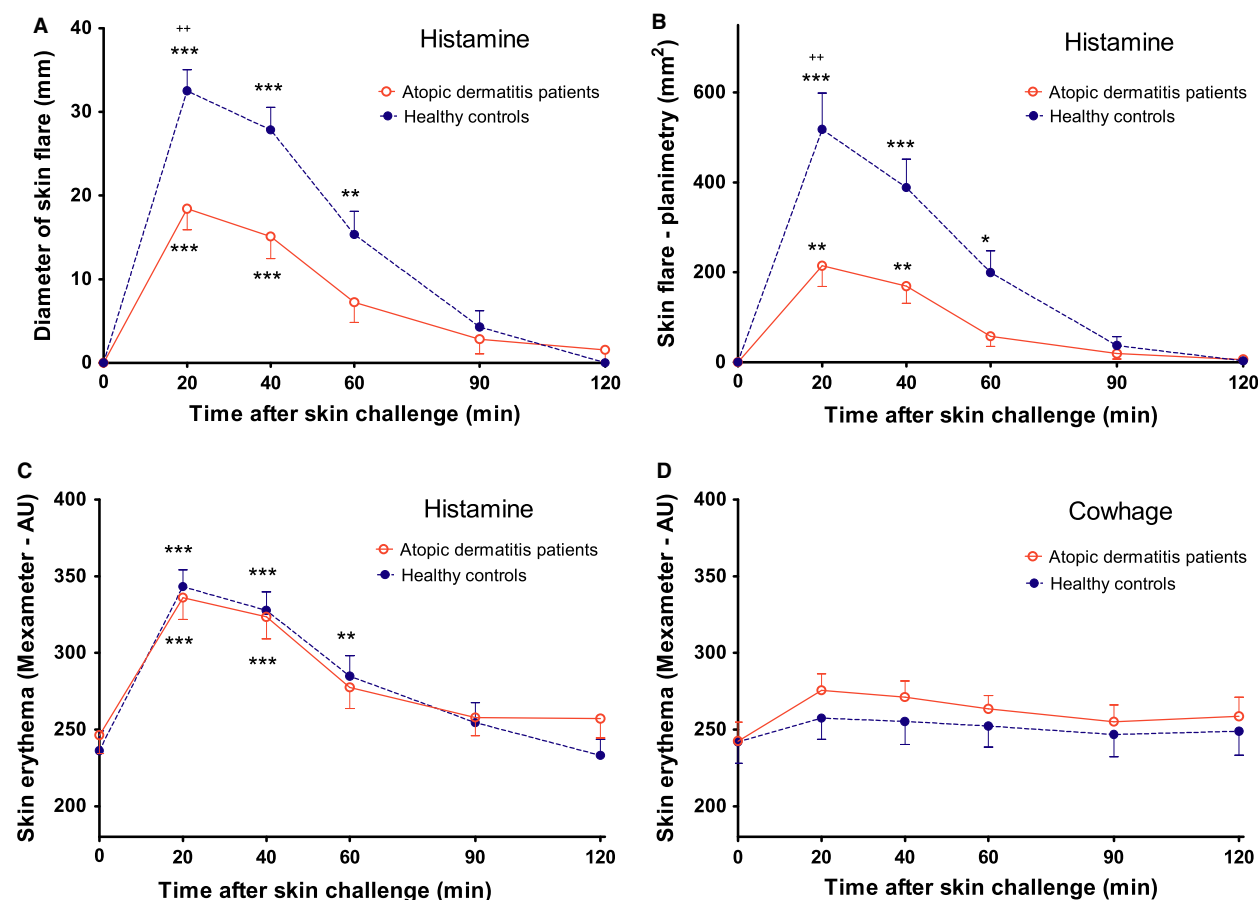


Figure 1 Histamine-induced flare diameter and surface, but not intensity, is bigger in healthy subjects than in atopic dermatitis patients. (A) Longest diameter of flare measured with a ruler after provocation with histamine. (B) Flare area measured planimetric from digital pictures after provocation with histamine. (C) Intensity of the

skin erythema measured with Mexameter after provocation with histamine. (D) Intensity of the skin erythema measured with Mexameter after provocation with cowhage. AU, arbitrary units. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ vs baseline. ++ $P < 0.01$ atopic dermatitis patients vs healthy controls 20 min after skin provocations.

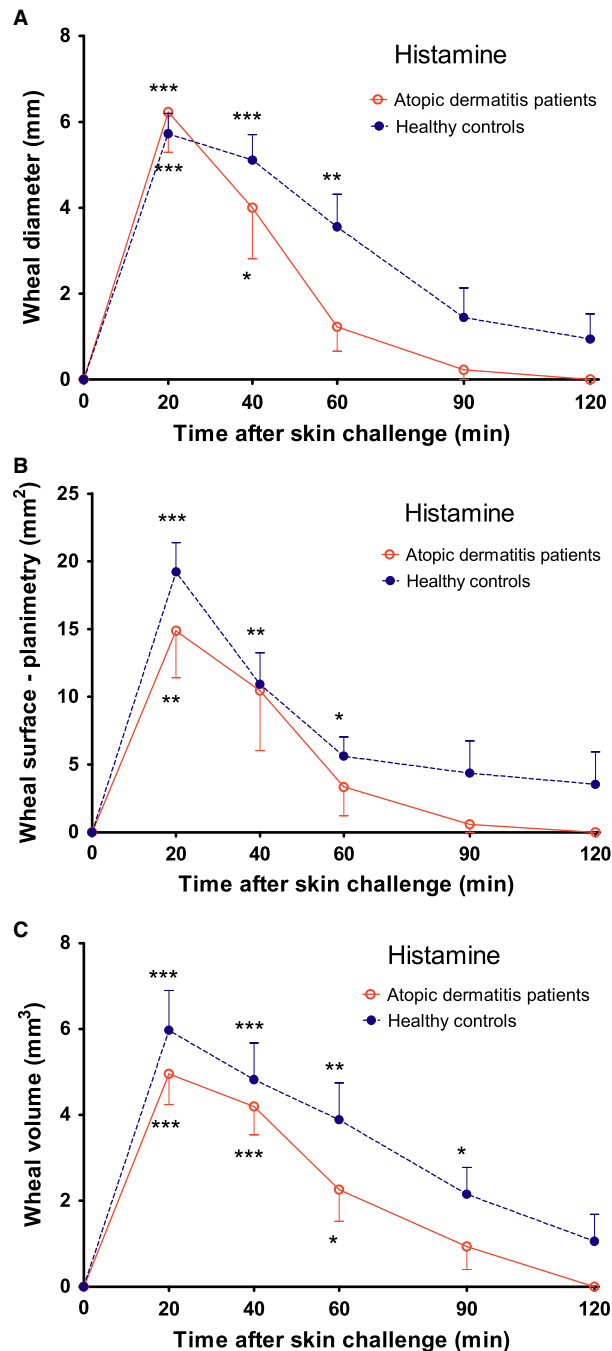


Figure 2 Histamine skin prick test-induced wheals are similar in atopic dermatitis patients and healthy controls. (A) Longest diameter of wheal measured with a ruler. (B) Wheal surface area measured planimetric. (C) Wheal volume measured using three-dimensional photography. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ vs baseline.

three healthy controls itch persisted 30 min, $P < 0.01$; Fig. 3A, B). After provocation with cowhage, AD patients also showed higher overall itch intensity over time expressed

as the area under the curve (AUC) for pruritus as compared to healthy controls (means $\pm$ SEM: 746 ± 138 vs 316 ± 52 , $P < 0.05$; Fig. 3A, C). There was no difference in maximum itch intensity between AD patients and healthy controls (Fig. 3A, D). AD patients and healthy subjects showed no significant differences in any of the analyzed itch parameters after provocation with histamine (Fig. 3E).

Decreased diameter of histamine-induced flare and prolonged persistence of pruritus after application of cowhage discriminate AD patients and healthy individuals

The longest diameter of the histamine-induced flare measured with a ruler 20 min after skin prick testing predicts AD with good discriminative properties (area under the ROC curve 0.80, 95% CI, 0.662–0.936, $P < 0.01$; Fig. 4). Flare diameters shorter than 20 mm allowed the diagnosis of AD with a sensitivity of 0.59 and a specificity of 0.78 (Fig. 4). The surface areas of flares, determined by ruler and planimetric morphometry, showed similar discriminative properties for AD (area under the ROC curve 0.80, 95% CI: 0.660–0.939, $P < 0.01$ and 0.78, 95% CI, 0.628–0.927, $P < 0.01$).

The persistence of itch after provocation with cowhage reliably predicted AD with good discriminative properties (area under the ROC curve 0.78, 95% CI, 0.633–0.930, $P < 0.01$; Fig. 4). Itch for 30 min or longer allowed the diagnosis of AD with a sensitivity of 0.64 and specificity of 0.83 (Fig. 4).

There was no significant correlation between the persistence of itch and the diameter of histamine-induced flare in either AD patients or healthy controls. Both variables, as tested by logistic regression, predicted diagnosis of AD independently. A regression model that included the diameter of histamine-induced flare correctly diagnosed AD in 70% of cases and explained 36% of variance (Nagelkerke $R^2 = 0.358$, $P < 0.001$). A regression model that included the persistence of cowhage-induced itch allowed the correct diagnosis in 75% of cases and explained variance in 31% (Nagelkerke $R^2 = 0.313$, $P < 0.01$). An overall model with both variables correctly diagnosed AD in 80% of cases and explained 52% of variance (Nagelkerke $R^2 = 0.523$, $P < 0.001$).

ROC analyses demonstrated that combinations of both markers, the longest diameter of flare and the persistence of cowhage-induced itch, show even better diagnostic properties than their separate use (area under the ROC curve 0.87, 95% CI, 0.746–0.989, $P < 0.001$; Fig. 4). The sensitivity of correct AD diagnosis when at least one criterion was met, that is, cowhage itch at 30 min or histamine flare diameter less than 20 mm, was 0.91 (specificity: 0.67). The specificity of correct AD diagnosis when the both criteria were met in the same patient was 0.94 (sensitivity: 0.47).

Age, SCORAD, pruritus intensity from SCORAD, diagnosis of asthma or atopic rhinitis, and gender did not differ between subgroups of AD patients divided according to flare diameter (< 20 mm) or persistence of cowhage-induced itch (≥ 30 min).

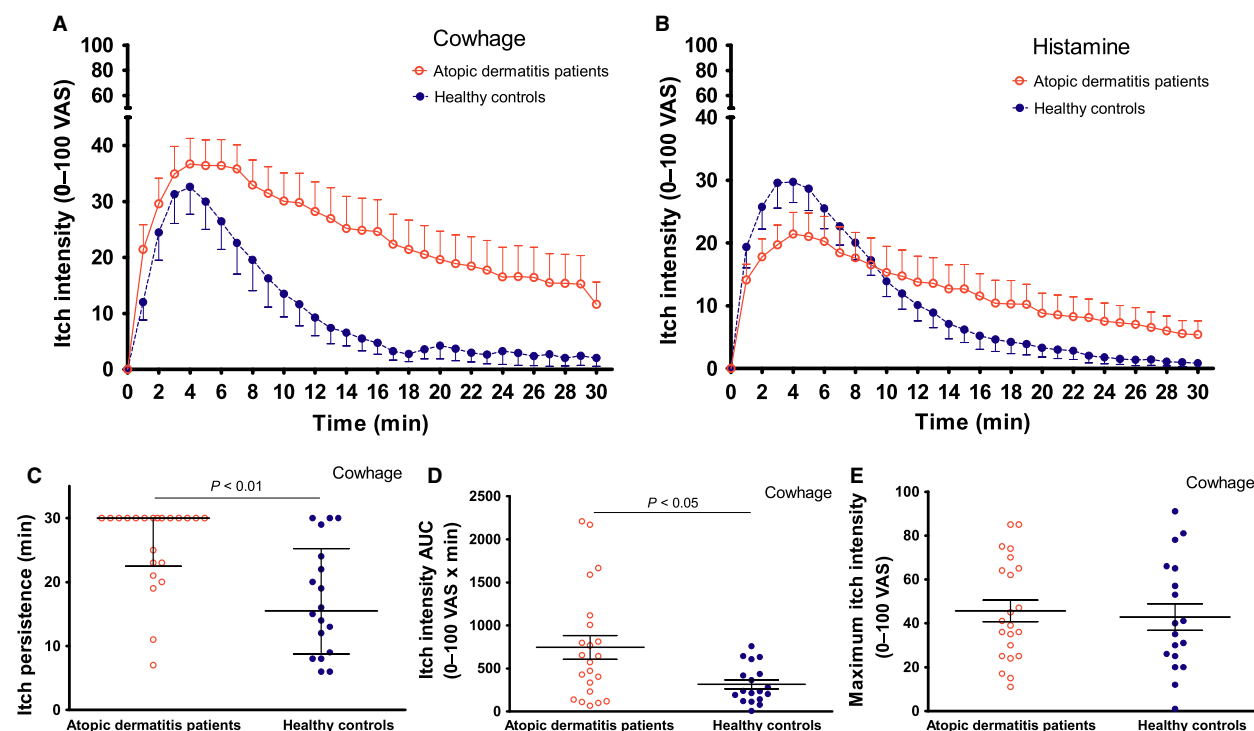


Figure 3 Patients with atopic dermatitis (AD) report prolonged itch after skin challenge with cowhage, but not with histamine, as compared to healthy controls (HC). Parametric variables presented as mean values $\pm$ standard error of the mean (SEM) (A, B, D, E); non-parametric variables presented as median $\pm$ interquartile range (C). (A) Time course of itch intensity ratings on a visual analogue scale (0 to 100 mm) after provocation with cowhage. (B) Time course of itch intensity ratings on a visual analogue scale (0 to 100 mm) after

provocation with histamine. There was no difference in persistence of itch, AUC for itch and maximum itch between patients with AD and HC. (C) Longer persistence of itch after provocation with cowhage in patients with AD than in HC (Mann-Whitney U -test, $P < 0.01$). (D) Bigger area under the curve (AUC) for itch intensity after provocation with cowhage in patients with AD than in HC (Student's t -test, $P < 0.05$). (E) There was no significant difference in the maximum itch between AD patients and HC after provocation with cowhage.

Patients with AD report more different itch qualities than healthy individuals after provocation with cowhage and histamine

After provocation with cowhage, AD patients and healthy controls both reported the following seven sensations: itch, urge to scratch, burning, tingling, pulsating, coming in waves, and pricking. Healthy individuals reported the additional sensation tickling. In contrast, AD patients reported six additional qualities: warm, sharp, pinching, pain, cold, and squeezing (Fig. 5).

After provocation with histamine, both the healthy individuals and the AD patients reported five sensations: itch, urge to scratch, burning, tingling, and pricking. Healthy subjects reported one additional sensation, tickling, whereas AD patients reported six additional qualities: warm, pulsating, coming in waves, pinching, pain, and cold (Fig. 5).

Altogether, more sensations were reported by AD patients compared to healthy controls. The intensities of all itch sensations did not differ between AD patients and healthy controls.

Discussion

The results of our study suggest that the differences in flare and itch responses to histamine and cowhage skin

provocation, respectively, can help to distinguish AD patients from healthy individuals. They also point to distinct differences in AD and healthy skin that could help to better understand the pathogenesis of AD and should be explored for their underlying mechanisms.

Our observation of reduced flare responses in AD patients compared with healthy individuals after skin challenge with histamine is in line with previous reports. Several other studies found that patients with AD develop less erythema in response to histamine (10, 18). In addition, AD patients have also been reported to show smaller flare responses to interleukin-2 and interleukin-31 as well as various neuropeptides including substance P (9, 10, 19). The reasons for this abnormal vascular response in AD patients remain unclear. Tolerance and tachyphylaxis to the tested mediators in atopic skin or abnormal vascular reactivity in AD are possible explanations.

Our results also show that the application of cowhage to the nonlesional skin of AD patients results in a more prolonged itch as compared with healthy individuals. These findings complement and extend the results of a previous study by Papoiu and coworkers (14). In this earlier study, measurements of itch for 5.5 min after induction had pointed to a slower decline of pruritus in AD patients as

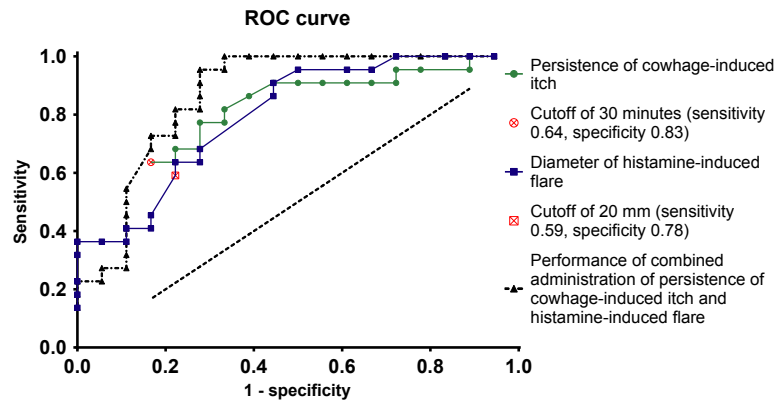


Figure 4 Diameter of histamine-induced flare and persistence of pruritus after application of cowhage discriminate AD patients and healthy individuals. The receiver operating characteristic (ROC) curves for discriminative ability between atopic dermatitis (AD) patients and healthy individuals based on the diameter of histamine-induced wheal and on the persistence of itch after the application of cowhage. Green, continuous line with circles shows area under the ROC curve for persistence of cowhage-induced itch $0.78 \pm 95\%$ CI $0.633\text{--}0.930$, $P < 0.01$. Persistence of itch up to 30 min after application of cowhage or longer enables to confirm the diagnosis of AD

with sensitivity of 0.64 and specificity of 0.83 (empty circle with cross). Blue, continuous line with squares shows area under the ROC curve for diameter of histamine-induced flare 20 min after skin prick testing $0.80 \pm 95\%$ CI $0.662\text{--}0.936$, $P < 0.01$. Diameter of flare shorter than 20 mm enables to confirm the diagnosis of AD with sensitivity of 0.59 and specificity of 0.78 (empty square with cross). Black, dotted line with triangles shows area under the ROC curve for the both parameters combined (the longest diameter of flare and the persistence of cowhage-induced itch, 0.87 , 95% CI, $0.746\text{--}0.989$, $P < 0.001$). CI, confidence interval.

compared to healthy subjects. In our study, the assessment of itch for 30 min revealed profound differences in the time course of cowhage-induced pruritus between AD patients and healthy controls. In accordance with earlier studies, we did not find differences in histamine-induced itch intensity or duration between AD patients and healthy individuals. Previous studies did also not find differences in histamine-induced pruritus intensity, for example, after skin prick testing, iontophoresis or microdialysis in the nonlesional skin of AD patients compared with healthy controls (13, 20, 21), or found even reduced itch ratings in the nonlesional AD skin compared with healthy controls (22) or to patients with urticaria and psoriasis (12). In contrast, in the lesional skin, histamine-induced itch was consistently increased (20, 22, 23). Why cowhage-induced, but not histamine-induced, itch is different in AD patients as compared to healthy individuals remains unknown. Possible explanations include a selective sensitization to protease-activated receptor (PAR)-induced itch in AD, possibly due to the overexpression of PAR-2 in the cutaneous sensory neurons in lesional and nonlesional AD skin (24). Steinhoff and colleagues reported that intracutaneous injection of PAR-2 agonist caused more intense itch in AD patients than in healthy controls (24). Intradermal injection of PAR-2 agonist caused itch lasting only 2–5 min preceded by pain, which is much shorter than the average duration of cowhage-induced itch. Therefore, the cowhage model may be better to differentiate itch responses between nonlesional AD skin and skin of healthy controls. Individual AD patients presented with different persistence of pruritus after provocation with cowhage. It is possible that the seemingly healthy skin of AD patients differs in PAR-2

expression. However, we did not find any clinical correlate of persistence of cowhage-induced itch including clinical severity. Altered responsiveness of the nonlesional AD skin to cowhage may be independent from, or at least not limited to, the overexpression of PARs. Nonlesional, visibly normal AD skin differs from healthy skin at the molecular level due to broad alterations in terminal differentiation and inflammatory genes expression (25).

Regardless of the mechanisms involved in the diminished flare reaction after provocation with histamine, this observation may be practically used to support diagnosis of AD, the more that skin prick testing with histamine as a positive control is routinely performed in patients diagnosed or suspected with other atopic disorders associated with AD such as rhinoconjunctivitis or asthma. Our results show that the widely established skin prick testing appears to bring even more diagnostic information than it was believed to do. Flare size and persistence of itch after provocation of nonlesional skin with histamine and cowhage allowed for the discrimination between AD patients and healthy controls. Histamine prick testing is part of the routine allergological workup of AD patients. Cowhage spicules are easy to administer, safe for topical use, and cheap and, when stored in dry conditions, preserve their properties over long time and, therefore, can be readily included in diagnostic procedures. Importantly, both procedures, histamine skin prick testing and application of cowhage, should be performed on the nonlesional skin, and they do not correlate with severity of AD. This implies that the use of these tests may be helpful also in diagnostically difficult cases, for example, when skin lesions are minimal or absent. Persistence of itch for at least 30 min after provocation with cowhage and histamine-induced flare

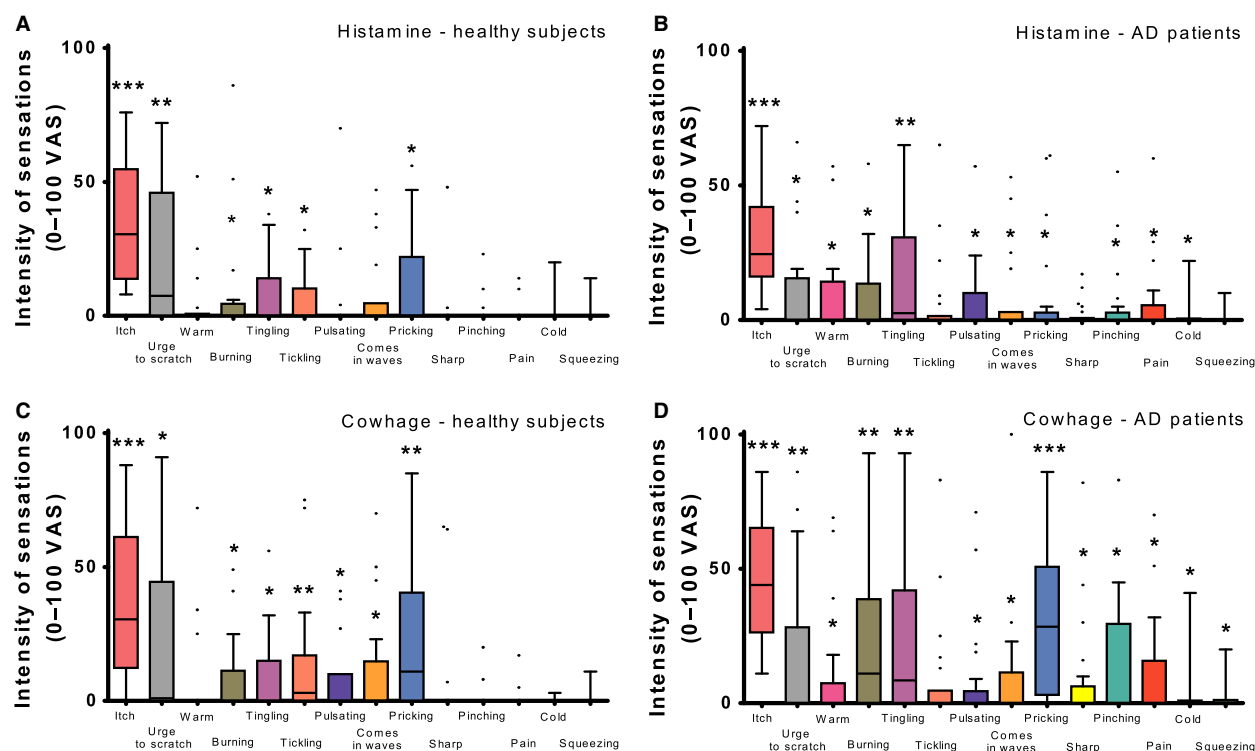


Figure 5 Patients with AD report more different itch qualities than healthy individuals after provocation with cowhage and histamine. Boxplots present interquartile range, the median is indicated by the vertical line and the whiskers present range. The outliers are plotted separately as points. Significance levels (* $P < 0.05$; ** $P < 0.01$;

*** $P < 0.001$) are given for the comparisons with the negative control (skin prick test with saline, Wilcoxon's test). (A) Provocation with histamine in healthy volunteers. (B) Provocation with histamine in atopic dermatitis patients. (C) Provocation with cowhage in healthy volunteers. (D) Provocation with cowhage in atopic dermatitis patients.

diameters shorter than 20 mm show optimal relationship between sensitivity and specificity to diagnose AD and may be used to enhance the diagnosis of AD. The cutoffs may be individually adapted depending on the purpose (i.e., screening vs diagnostic instrument), and the tests may be used separately, but their parallel use improves diagnostic properties.

Differences between AD and healthy controls in the intensity of single sensations in response to skin provocation testing with histamine or cowhage were not observed. In other words, no single pruritus quality allows for the differentiation between AD patients and healthy individuals. In general, patients with AD reported a higher number of perceived sensations after skin challenges, but the assessment of all sensations is too complex to assist with diagnosing AD. The prevalence of different itch qualities in AD was previously investigated, but no data are available to compare AD with other itchy skin diseases (17, 26). Data on the comparison of sensory qualities in experimentally induced itch in AD and in the other skin conditions and healthy controls are missing as well. The qualitative differences in the perception of pruritus may be explained by differences in central processing of itch in AD. Patients with AD reportedly have more brain sites activated on PET scans as compared to healthy controls after histamine iontophoresis in nonlesional skin (13).

It must be noted that patients with skin disorders other than AD were not included in this study. Because of this, no conclusions on the specificity of histamine-induced flare size and cowhage-induced itch duration for AD can be drawn. Additionally, we have only investigated Caucasian skin and have not included children and adolescents. Ongoing and further studies will need to show how the parameters perform as differential diagnostic markers or even as biomarkers.

In conclusion, decreased histamine-induced flare and prolonged persistence of cowhage-induced itch in nonlesional skin in AD may be utilized as diagnostic markers of AD. Whether this also holds true for the differential diagnosis of other pruritic skin diseases remains to be investigated. The tests are easy to perform, fast, cheap, and safe, and may enhance AD diagnosis also when skin lesions are missing. Specifically, our results suggest that the persistence of cowhage-induced itch for at least 30 min and a histamine-induced flare in nonlesional skin of less than 20 mm diameter at 20 min can support diagnosis of AD.

Acknowledgment

We would like to thank Ethan Lerner who kindly provided us with cowhage spicules and Margitta Worm who kindly

supported us in the recruitment of patients. This work was supported in part by the International Institute of Dermatological Allergy (I²DEAL).

Author contributions

Tomasz Hawro designed the study, performed statistical analysis, and drafted the manuscript. Martin Metz and Marcus Maurer designed the study, contributed to interpretation

of the data, and revised the manuscript. Sylwia Lehmann performed the experiments and conducted the literature research. All authors collaborated in retrieval of the data and provided critical review of the manuscript and final approval of the manuscript before submission.

Conflict of interest

None of the authors have any conflict of interest to declare.

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