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Non-histaminergic and mechanical itch sensitization in atopic dermatitis

Running head: Intra- and extra-lesional sensitization for itch in atopic dermatitis

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Abstract

Chronic or episodic severe itch is recurrent in atopic dermatitis (AD). Non-histaminergic neuronal itch pathways are suggested to dominate in AD itch, contributing to an "itch-scratch-itch cycle" that prolongs and worsens itch, pain, and skin lesions. We hypothesized that non-histaminergic neuronal sensitization contributes to itch in AD. Hence, we compared sensitivity to thermal, mechanical, and chemical pruritic stimuli in AD patients and controls. The study comprised 25 AD patients with chronic itch and 25 healthy controls. Questionnaires on itch characteristics were administered, and sensory tests were conducted intra-lesionally, extra-lesionally, and in homologous areas of controls. Thermal and mechanical quantitative sensory testing (QST) as well as histamine and cowhage provocations were performed. Subsequently, hyperknesis and vasomotor reactivity were assessed. Average itch and associated pain among AD patients were 60.7±4.3 and 39.7±5.2 (VAS_{0-100}), respectively. Patients experienced significantly higher itch from cowhage both intra- and extra-lesionally compared to controls, whereas histamine-evoked itch intensity was not significantly different between groups. No group differences were found for thermal QSTs or pain evoked by itch provocations. Patients had decreased mechanical detection thresholds intra-lesionally and increased mechanical pain sensitivity intra- and extra-lesionally. Lastly, patients exhibited intra- and extra-lesional hyperknesis prior to chemical itch provocations and augmented hyperknesis following itch provocations. Increased itch to a non-histaminergic pruritogen (but not histamine) suggests pathway-specific itch sensitization in AD while increased susceptibility to mechanically-evoked itch and pain, particularly intra-lesionally, suggests sensitization of normally non-pruritic mechano-sensitive circuitry. Drugs targeting the non-histaminergic ($PAR2/TRPA1^{+}$) itch-pathway and itch sensitization are promising for treating AD itch.

1. Introduction

Atopic dermatitis is a chronic pruritic inflammatory skin condition characterized by lesions with erythema, exudation, excoriations, lichenification, and xerosis as well as chronic or episodic itch and cutaneous pain.[18,25,33,86,90] The disease is common, can be debilitating for affected patients, and is often difficult to treat adequately.[68,90] Pathoetiologically, AD is associated with genetic, immunological, environmental and skin barrier factors.[32,86] Recently, neuronal sensitization has also been proposed as a disease contributor.[41,87,97] Knowledge on the neurophysiological basis of itch has been greatly expanded in the last decade as parallel peripheral pathways of itch transduction have been discovered and explored.[6,45,60,84] In humans, the two most well studied pathways of itch rely on mechano-insensitive C-fibers (CMi) transmitting histaminergic itch and polymodal C-fibers (PmC) transmitting non-histaminergic itch.[36,45,60] To a certain extent, these fibers express different molecular transducers important for itch signaling; e.g., CMi fibers express histamine receptor 1 and PmC fibers express proteinase-activated receptor-2 (PAR2), which tryptase and mucunain activate to induce itch.[31,39]

It is well established that following acute pain as well as in inflammatory and neuropathic pain conditions, peripheral nociceptors may exhibit increased sensitivity to various stimuli. At the same time,

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the spinal processing of these nociceptive signals can be facilitated or disinhibited.[15,53,56,77] These processes are proposed to contribute to the aggravation and chronification of pain in patients and mediate clinical epiphenomenona such as hyperalgesia and allodynia to mechanical and thermal stimuli.[77,94] It is currently unknown whether similar processes contribute to increasing itch severity in patients suffering from chronic inflammatory itch conditions, such as AD. The indirect evidence of neuronal sensitization in AD includes: 1) sustained itch after resolution of active lesions,[17] 2) itch in response to normally innocuous mechanical stimuli (such as certain fabrics),[40,92] 3) poor correlation between objective disease severity (e.g., SCORAD) and itch intensity,[23] and 4) case-based indications of an antipruritic effect of centrally acting anti-hyperalgesic drugs,[71] and 5) altered expression of molecular transducers on peptidergic cutaneous fibers.[83]

Neuronal sensitization to histamine-induced itch and skin reactivity in AD have been studied extensively.[68,83,91,92] Generally, itch in response to histamine provocation is unchanged or even decreased in non-lesional AD skin versus healthy skin, and varying results have been derived from intra-lesional histamine provocations in AD.[5,38,41–43,48,67,91] This is consistent with the fact that antihistamine treatment has little or no effect on clinical itch severity in AD and thus histaminergic signaling is considered to have little implication on the pathophysiology.[68,90,93]

Recently, a non-histaminergic model of itch, relying on the naturally occurring PAR2 agonist, mucunain from the cowhage plant, has been re-introduced. This type of evoked itch is entirely resistant to antihistamines[45,60] and evokes mild nociceptive sensations akin to those associated with itch in AD.[52,79] Accordingly, it has been suggested that this model may much more accurately mimic itch in AD and other conditions than the rigorously studied histaminergic models of itch.[34,59,67]

The aim of this study was to compare sensory sensitivity to pruritic chemical, thermal, and mechanical stimuli between AD patients with chronic itch and healthy controls (HCs), investigating both lesional and non-lesional skin areas.

2. Methods

2.1. Participants

Study subjects comprised 25 patients with AD (25.2±0.9 years, 10M/15F) and 25 healthy age- and gender-matched volunteers (26.3±1.3 years 14M/11F). All AD patients fulfilled the diagnostic criteria of the UK Working Party and were initially diagnosed by a dermatologist. Patients were only included if they presented chronically (>6 weeks[82]) pruritic atopic dermatitis with mean daily itch rated above 3 ($NRS_{0\text{-}10}$), lesions manifesting on the upper extremities, and an eczema-free skin area also on the upper extremities. Use of antihistamines was discontinued 48 hours prior to the experiment and all topical agents and emollients were discontinued 24 hours prior to the experiment. To enable the study of neuronal sensitization in patients with chronically itchy, treatment-resistant AD *in situ*, patients were instructed to continue their usual treatment regimen uninterrupted, with the exceptions stated above. Prior to enrollment, all participants were explicitly informed, verbally and in writing, that they were free to withdraw from the study at any time and that any procedure they deemed intolerable would be ceased immediately.

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2.2. Study procedure

All subjects provided written informed consent prior to experimental procedures, and the regional ethics committee approved the study (N-20150058). Fig. 1 provides an overview of the conducted experimental procedures. Prior to the experimental session, AD patients were evaluated using *Scoring Atopic Dermatitis* (SCORAD)[1,46] and completed a Danish version of the *Eppendorfer Itch Questionnaire*[23] as well as the adapted *Danish Itch Severity Scale questionnaire*[99] (initially developed by Yosipovitch *et al.* (2001)[98], deriving descriptors from the *McGill Pain Questionnaire*). Thereafter, two 4 x 4 cm square areas were marked on the upper extremities: one in a representative atopic lesion and one in an area of eczema-free skin. Scabbed and significantly exudative and lichenificated lesions were avoided; if not possible, an area in the margin of such lesions was used. The anatomical locations of these areas were mirrored in the HC group. All experimental procedures were subsequently conducted within these two areas (with the order of lesional vs. non-lesional being randomized) following the sequence outlined in Fig. 1. For all outcomes, comparisons were made between lesion/non-lesional sites in AD and to anatomically corresponding control areas in HCs. All sessions were conducted in the same temperature-controlled laboratory at ~21-22 °C. AD patients and healthy controls were enrolled and tested in parallel to avoid potential seasonal biases[55].

2.3. Quantitative sensory testing (QST)

The applied QST protocol was partly derived from the guidelines of the German Research Network on Neuropathic Pain (DFNS).[73] The verbal instructions (in Danish) for participants from the DFNS protocol were derived from the supplementary materials of Olsen *et al.* (2014).[65]

2.3.1. Thermal detection and pain thresholds: Tests for cold detection threshold (CDT), warmth detection threshold (WDT), cold pain threshold (CPT), and heat pain threshold (HPT) were performed using a Medoc Pathway (Medoc Ltd, Ramat Yishay, Israel) equipped with a 3 × 3 cm advanced thermal stimulator probe with a baseline temperature of 32°C. Ramping stimuli of 1°C/s were delivered until the subjects identified the associated threshold (first perception of cold or warmth and first perception of cold- or heat-induced pain) by pressing a button. Thereafter the temperature of the probe returned to the baseline temperature at a rate of 5°C/s. The results were calculated as the arithmetic mean of the thresholds from three repeated ramps.

2.3.2. Mechanical detection, pain threshold, and sensitivity: To determine the mechanical detection threshold (MDT), a set of 20 calibrated Von Frey filaments (North Coast Medical, Gilroy, CA, USA) with exerted forces ranging from 0.078 mN to 2.9 N was applied over five ascending/descending series of stimuli. The subjects were asked to report upon any sensation from the area. The final MDT was calculated as the mean of the values obtained in each of the five series of stimuli. The mechanical pain threshold (MPT) was evaluated using a set of seven weight-calibrated pinprick stimulators (MRC Systems, Germany) with weights from 8 to 512 mN (Log2). During five ascending/descending series of stimuli, the subjects reported when a perception of 'sharpness' or 'pricking pain' was first sensed. The final MPT was calculated as the mean of the values obtained in the five series of stimuli. The mechanical pain sensitivity (MPS) was assessed to detect pinprick hyperalgesia to suprathreshold stimulation. The seven pinprick stimuli were applied in ascending order, and the subject was instructed to rate pain intensity of each stimulus on a numerical rating scale ($NRS_{0–10}$, 0 = *no pain*, 10 = *worst imaginable pain*) allowing the use of decimals. The final MPS was calculated as the arithmetic mean of two consecutive series. Wind-up ratio (WUR) was assessed using the pinprick stimulator one intensity above the

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individual average MPT. The subjects were asked to rate the pain intensity following a single stimulus and thereafter the last of a subsequent series of 10 consecutive stimuli (1 stimulus/second). This procedure was repeated twice, and a mean ratio was calculated.

2.4. Chemically provoked itch

2.4.1. Itch induction: Two types of itch provocations were conducted. Histaminergic itch was evoked using intra-epidermal punctures of 1% histamine hydrochloride with standard 1mm skin prick test (SPT) lancets (Allergopharma, Hamburg, Germany). A drop of histamine solution was placed in the predetermined area and an SPT lancet was pricked through the histamine into the epidermis using a 120 g weight-calibrated device (Aalborg University, Denmark) for 1–2 seconds.[9,13] Non-histaminergic itch was induced using cowhage spicules, which contain the PAR2 agonist mucunain. The spicules were prepared immediately before administration under a stereomicroscope (Seben Incognita microscope, Seben GmbH, Berlin, Germany) using a negative grip tweezer (Electron Microscopy Science, Dumont, Switzerland). Approximately 45 spicules were applied to the skin and gently rubbed with the experimenter's fingertip for 15-20 seconds to ensure insertion (the delivered volume of mucunain using this method has been calculated to 15–30 ng).[67] Histamine solution and cowhage spicules were stored at 4 °C between sessions but always taken out well in advance of experiments and applied when at room temperature. Both of these human surrogate itch models have previously been utilized and found to be reliable.[6,13]

2.4.2. Assessment of evoked itch and pain: Intensity of itch and pain was assessed using two visual analogue scales (VAS), one for each sensory quality, following each itch provocation. Two computerized 100-mm VAS ranging from 0 to 100 (eVAS Software, Aalborg University) installed on a Samsung Note 10.1 Tablet (Samsung, Seoul, South Korea) were used. The subjects were instructed to report the occurrence and intensity of the aforementioned sensations continuously throughout the 6-minute, 0.2 Hz sampling. On the VASs, 0 indicated *"no itch"*/*"no pain"* and 100 indicated *"worst imaginable itch"*/*"worst imaginable pain*". The subjects were instructed that itch and pain might, or might not, occur following any of the administered provocations and instructed only to rate itching/painful sensations and not innocuous associated sensations such as tingling or warmth. They were also instructed to disregard the mild initial pricking pain associated with insertion of spicules and the SPT puncture. From the VAS/time data, temporal itch and pain intensity profiles were generated, and mean as well as individual peak itch/pain intensity were calculated.

2.4.3. Assessment of hyperknesis at baseline and after itch provocations: Cutaneous mechanical stimuli delivered by a needle or a thin filament typically induces a pricking sensation occasionally followed by mild itch both in healthy controls and to a greater extend in chronic itch patients.[20,40] Following an experimental itch provocation, the surrounding skin area becomes increasingly itchy in response to this type of mechanical probing [52,78,80]. In the present study, sensitivity to mechanically evoked itch was tested before and ~12 minutes after each itch provocations with 3 von Frey filaments; (9.8, 13.7, and 19.6 mN, from North Coast Medical, Gilroy, CA) by stimulating with each filament 2 × 3 times, for 2-3 seconds, each time instructing the subject to report the resulting itch intensity on a numerical rating scale ($NRS_{0\text{-}10}$, with same outer labels as the previously described VAS). The von Frey stimuli were delivered immediately next to the sites of itch provocation (0.5-2 cm distance) within the predefined 4 × 4 cm areas, but never directly within wheal reactions or the area of spicule insertion.

This technique and the choice of von Frey filaments have previously been described in details.[7] Subjects were instructed before the onset of data collection that: 1) itch is defined by inducing a desire to scratch the probed area and hence this should be the hallmark of their rating, 2) itch may or may not occur in

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response to the stimuli, and 3) itch could occur during the stimulus itself or immediately after. Non-uniform terminology is currently being used to describe itch-associated mechanical dysesthesias; *alloknesis* (itch in response to a normally non-itching stimuli) and *hyperknesis* (increased itch in response to a normally itch or pain-evoking stimuli).[7,10,30,47,49] Since the presently applied methodology is designed to induce mild itch in HC skin and elicits mild itch in majority of HCs prior to itch provocations, we apply the term *mechanically evoked itch* when referring to such data obtained in normal skin of HCs. *Hyperknesis* is used specifically when referring to any observed increase in mechanically evoked itch rating, probed as described above. This definition is a line with the originally proposed nomenclature.[49,50,80].

2.5. Inflammation imaging and wheal measurements

Superficial blood perfusion was assessed at baseline and following the histamine provocation to measure the neurogenic inflammatory response. Cowhage produced no discernable flare. The measurements were conducted using a MoorFLPI-1 (Moor Instruments Ltd, Axminster, UK) with a 35-cm distance between the camera and the skin, exposure time of 8.3 ms, and 160 units of gain. The FLPI data were analyzed using MoorFLPI Review V4.0 proprietary software. The induced increases in average and peak superficial blood perfusion within the 4 x 4 cm pre-marked areas were used as proxies for inflammation intensity. Moreover, the histamine-evoked flare area was calculated as the area of ≥30% perfusion rate compared to the surrounding background in accordance with previously described methodology.[7,8,65] Wheal was measured by the longest diagonal and the orthogonal diagonal approximately 15 minutes after the histamine pricks were conducted in accordance with standardized recommendation for SPT.[35]

2.6. Statistics

Statistical analyses were performed using SPSS (version 23, IBM Corporation, Armonk, NY) and GraphPad Prism (version 6.0, La Jolla, CA). Sample size estimations were based on previous studies and test-retest reliability data. The obtained data are presented as arithmetic means ± standard error of the mean (SEM), unless otherwise stated. Data were tested for normality using visual inspection and, if unclear, the Shapiro-Wilk normality test. Peak and mean of itch/pain were extracted from temporal VAS-recordings and compared between groups. The primary statistical analyses for all outcome measures, with the exception of hyperknesis, MPS, and FLPI (repeated parameters), were performed with independent sample t-tests adjusted according to Levene's variance test and corrected for multiplicity using the Holm-Sidak approach. For MPS, hyperknesis and FLPI, repeated measures ANOVAs were constructed with the within-subject factors *stimulus* (MPS; 7 levels, one per stimulator) and *time* (hyperknesis and FLPI; 2 levels, before and after itch provocation) and the between-subject factor *group* (all three outcomes; 2 levels, AD vs. controls). Mauchly's test of sphericity was utilized, and in cases where sphericity was violated, the Greenhouse-Geisser correction was applied. An unplanned within-subjects analysis (paired t-test) was conducted specifically for HPT as a difference were evident from the main between-subjects comparison. A P-value of ≤ 0.05 was considered significant for all analyses.

3. Results

All subjects in both groups completed all study procedures without the occurrence of immediate or delayed adverse reactions or withdrawals. For most parameters, no or very modest, insignificant differences were observed within the two HC skin areas, showing limited combined differences associated with sensory topography between the investigated sites within the groups.

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3.1. Severity and characteristics of itch and atopic dermatitis

The AD patients reported moderate-to-severe daily mean itch (60.7 ± 4.3 VAS_{0-100}) as well as mild-to-moderate pain (39.7 ± 5.2, VAS_{0-100}) associated with their skin lesions. The itch characteristics, presented as medians and quartiles on a Likert $Scale_{0-4,}$ were most consistently described as: "*warm*" = 3 (3-4), "*burning*" = 3, (3-4), "*searing*" = 3 (2-4), and "*stinging*" = 3 (1.25-4). The most prevalent perceived aggravating factor was "*warmth*" (3, 3-4), and "cold" was most consistently described as alleviatory = 3 (3-4). The most frequent emotional descriptors were: "*annoying*" = 4 (3.25-4), "*bothersome*" = 4 (3-4), and "*my only desire: no itch*" = 4 (3-4). Significant sleep interference from itch was reported = 3 (3-4). The mean Itch Severity Scale score was 12.5 ± 0.4 (0-21 scale), and the average SCORAD was 35.4 ± 3.1 (0-103 scale). The most common lesional anatomical area was in and around the flexural area of the elbow. Qualitatively, a majority of patients reported that they were under the impression that the cutaneous pain occurring on their upper extremity lesions was, mainly or entirely, a self-inflicted consequence of scratching.

3.2. Quantitative sensory testing

3.2.1. Thermal detection and pain thresholds: No significant differences or trends were observed with regards to any thermal detection or pain thresholds between the AD and control group in lesional or non-lesional skin areas (see Table 1). In the AD group a within-subjects analysis for HPT in lesional (41.2 ± 0.8°C vs. non-lesional skin (43.2 ± 0.9°C) demonstrated relative heat hyperalgesia intra-lesionally (P = 0.009, uncorrected).

3.2.2. Mechanical detection, pain thresholds and mechanical pain sensitivity: Intra-lesionally, the AD group exhibited significantly increased MDT compared to the control group, indicative of tactile hypoesthesia (Table 1). This difference was not present when comparing non-lesional skin of the AD group to corresponding area in HCs. No significant differences were observed for MPT, but a trend towards decreased MPT in lesional skin of the AD group was observed (Table 1). No significant differences were observed for the wind-up ratio, known as a perceptual correlate of temporal pain summation. Sensitivity to supra-threshold mechanical pain pinprick stimuli was significantly increased in both lesional ($P < 0.01$, *group* main effect) and non-lesional skin ($P < 0.05$, *group* main effect) of the AD patients (see Fig. 2A and B). This result indicates mechanical hyperalgesia manifesting to supra-threshold stimuli intensities within and beyond the eczematous skin areas.

3.3. Itch provocations

3.3.1. Histamine-induced itch: Histamine-induced mean and peak itch intensities did not differ significantly between AD patients and HCs, regardless of whether the provocations were performed in lesion or non-lesional skin (Fig. 3A and B). However, a notable trend towards increased itch responses in lesional AD skin compared to homologues HC skin was observed for both mean and peak itch intensities (both: P = 0.07), and these differences were only rendered insignificant when correcting for multiple comparisons. The sensitivity to histamine-induced itch in non-lesional skin of AD patients vs. HCs was highly similar (P = 0.74 for mean itch, and P = 0.98 for peak itch). Histamine provocations induced mild pain in a few individuals, but mean peak pain intensity scores were never >10 (VAS_{0-100}), regardless of whether provocations were conducted intra- or extra-lesionally.

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3.3.2. Cowhage-induced (non-histaminergic) itch: Cowhage-evoked mean and peak itch intensities were significantly increased in lesional AD skin vs. corresponding skin in HCs (both: $P < 0.01$), see Fig. 3C and D. For instance, the mean itch in response to cowhage was 63% higher in the AD patients (48.3 ± 3.9, $VAS_{0\text{-}100}$) compared to the HCs (30.4 ± 3.9, $VAS_{0\text{-}100}$). A similar finding was made when comparing non-lesional AD skin to corresponding skin in HCs for the mean cowhage-evoked itch ($P = 0.03$); however, here the peak itch intensity difference was not statistically significant ($P = 0.087$). Temporal itch intensity profiles following cowhage provocations are shown in Fig. 3C and 4D. Cowhage induced mild pain in a minority of subjects in lesional (peak scores: AD group, 15.1 ± 4.3; HC group, 10.2 ± 2.4), and non-lesional skin (peak scores: AD group, 12.3 ± 2.5; HC group, 11.5 ± 3.4), but no significant differences were present between groups.

3.3.3. Mechanically evoked itch and hyperknesis: 59.7% of the 300 von Frey triplicate stimulations delivered the skin of HCs prior to any itch provocations were rated as itching (≥0.5 on $NRS_{0\text{-}10}$), while the equivalent percentage in AD was 73.3% ($P < 0.001$). Patients with AD exhibited significantly more intense itch in response to von Frey stimuli in both lesional and non-lesional skin prior to itch provocations (AD group: 2.4 ± 0.3, HC group: 0.9 ± 0.1). Following histamine and cowhage-induced itch provocations, both the AD and HC group developed significantly hyperknesis; however, this facilitation was more pronounced in the AD group (Fig. 4A and B). AD patients also displayed significant hyperknesis in lesional skin following cowhage (4.0 ± 0.4 vs. 2.4 ± 0.3 at baseline, $NRS_{0\text{-}10}$) and histamine (4.2 ± 0.5, $NRS_{0\text{-}10}$, both: $P < 0.01$), and significant increases of less magnitude were also observed in corresponding control skin areas in HCs following histamine (1.8 ± 0.3 vs. 0.8 ± 0.1 at baseline, $NRS_{0\text{-}10}$, $P < 0.01$) and cowhage (1.5 ± 0.3 $NRS_{0\text{-}10}$, $P < 0.05$). Similar but less pronounced results were present for extra-lesional skin (Fig. 4B); however, here the histamine provocation did not produce significantly more hyperknesis in AD patients compared to HCs. In summary, AD patients displayed hyperknesis at baseline, and following itch provocations (both histamine and cowhage intra-lesionally, and only cowhage extra-lesionally) the AD group developed more pronounced hyperknesis than the HCs.

3.4. Neurogenic inflammation and wheal reactions

No differences were observed for superficial blood perfusion in non-lesional areas at baseline. AD patients expectedly had increased blood perfusion levels in lesional skin compared to corresponding control areas ($P < 0.01$), compatible with the observed erythema. Following histamine provocations, both HCs and patients with AD exhibited visually perceptible flare reactions immediately surrounding the SPT site. Analysis of the FLPI images showed that the flare reactions in non-lesional skin were more developed (higher peak intensities and larger areas) in HCs (both: $P < 0.05$), indicating that this neurogenic response pattern might be blunted in AD patients. FLPI analysis of lesional recordings following histamine provocations did not reveal group differences of peak perfusion responses. Mean perfusion assessment as well as area quantification techniques could not be reliably applied because of significant ceiling effects (presumably both physiological and technological) observed in the lesional skin of the AD patients (Fig. 5, column a-b, row 4-6). No group differences were found in relation to wheal sizes when comparing lesional or non-lesional areas. However, three abnormally large wheals, >40 mm^2 and >5 standard deviations higher than average HC wheals, as well as 4 satellite wheal reactions (separately developed wheal reactions several cm away from histamine prick site) were observed in lesional skin of the AD patients only.

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4. Discussion

This study demonstrates for the first time that AD patients with chronic itch exhibit selective intra- and extra-lesional hypersensitivity to cowhage provocations (non-histaminergic itch). Moreover, AD patients have exaggerated responses (sensitization) to pain-evoking as well as itch-evoking mechanical stimuli both intra- and extra-lesionally, indicative of pinprick hyperalgesia and hyperknesis, respectively.

4.1. Itch and pain in atopic dermatitis

The AD group reported moderate to severe chronic itch with a rated severity equivalent to previous studies[23,25,64]. Notably, 23 of 25 patients reported the presence of pain, and the combined average daily pain was 39.7 ± 5.2 (VAS_{0-100}). While the frequency of cutaneous pain in atopic dermatitis has previously been investigated (Brenaut *et al.* (2013) report that ~87% of AD patients experience associated pain[18] and O'Neill *et al.* (2011) report a 57.3% prevalence of pain in AD[64]) the intensity of the pain has, to our knowledge, not previously been assessed. It is unclear whether scratching chiefly drives the cutaneous pain and/or if it is spontaneously occurring. Given that both histaminergic and non-histaminergic models of itch generally produce mild spontaneous pain and hyperalgesia, it is likely that both of these mechanisms are involved.[10,13,36,52,79] The quality of itch in AD was generally reported as having a warm, pricking, searing quality and as being intensified by warmth as well as alleviated by cooling, all of which is well aligned with findings from previous studies.[23,25,64] While aggravation of itch by warmth is speculatively proposed to rely on TRPV1/4-mediated signaling either summating with pruriceptive activity at the primary afferent level or converging onto pruriceptive pathway SDH neurons,[3,4] alleviation by cooling is likely predominantly mediated by spinal gating of pruriceptive signaling, arising from activity of TRPM8-positive cold-receptive Aδ-fibers.[11,19,96] An alternative explanation, perhaps particularly relevant for warm-induced aggravation of itch, is that simple physical factors such as temperature-induced modulation of neuronal membrane potential, channel-kinetics or receptor-agonist interactions causes thermally induced itch aggravation/inhibition.[21,22,29] Lastly, although sweat has been proposed as a potential link between feeling warm and concurrent itch exacerbation, a recent study failed to show pruritogenic or itch sensitizing properties of sweat.[62]

4.2. Thermal quantitative sensory testing is normal in AD

Standardized thermal QSTs yielded mean thresholds comparable to those found in the normative dataset both when conducted intra- and extra-lesionally [65,72,73], however a paired analysis of HPT in lesional vs. non-lesional AD skin did indicate mild heat hyperalgesia. One previous study found minor but significant impairments in warmth and cold detection thresholds in AD patients, while a similar recent study failed to detect significant differences in thermal sensitivity.[69] Taken together with the present results, this indicates that alterations in thermal detection and pain sensation are likely not a prominent feature in AD as is the case, e.g., for certain neuropathic[16,56] and musculoskeletal pain etiologies.[28,89] Notably, a previous study found that noxious suprathreshold heat stimuli evoke itch in AD, conceptually corresponding to heat-induced alloknesis.[40] This type of sensory assessment is conceivably a more valuable assessment in AD patients than standardized thermal QST.

4.3. Intra-lesional hypoesthesia in AD

The MDT was found to be increased intra- but not extra-lesionally within the AD group. The MDT is a perceptual correlate of Aβ-mechanoreceptor function and as such signifies tactile sensitivity.[54,72,73] Two explanations to the reduced tactile sensitivity are most plausible: 1) prolonged scratching could cause

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cutaneous nerve fiber density decrease, perhaps affecting mechano-sensitive units,[70] 2) the finding reflects an indirect effect of skin barrier alterations, i.e., lichenification, excoriation and scaling, reducing responses to light von Frey stimuli (although attempts were made not to directly stimulate scabbed or extensively excoriated, exudative, and lichenificated areas). In either event, evidence suggests that the loss of tactile sensitivity might have functional implications in AD. For example, a study found that the itch-alleviating effect of scratching is blunted in lesional skin of AD patients compared to HCs,[42] and innocuous mechanical stimulation likely contributes to scratch-mediated itch alleviation.

4.4. Intra- and extra-lesional pinprick hyperalgesia in AD

The MPT was overall on par with previous studies in HCs.[65,72] While insignificant, a trend was observed towards reduced MPT in intra-lesional sites compared to homologous sites in HCs, indicative of mechanical hyperalgesia. More considerably, MPS was increased, particularly in intra-lesional skin, but also to a lesser extent extra-lesionally, when comparing AD patients with the HCs. This sensory parameter has not previously been assessed in AD. The mechanism behind hyperalgesia to suprathrehsold pinprick also affecting non-lesional skin can only be speculatively accounted for. Conceivable mechanisms could involve: 1) peripheral sensitization of mechano-sensitive primary afferents (C- and Aδ-fibers), as indicated by increased responses to cowhage provocations. Evidence suggests that AD patients have increased circulatory concentrations of several pro-inflammatory cytokines, chemokines, and neurotrophic factors, including, e.g. CCL1, interleukin-2 and NGF.[37,63,85,88] In humans, intradermal NGF has been experimentally shown to cause both prolonged mechanical hyperalgesia and increased itch sensitivity to cowhage in the absence of inflammation.[74,75] Hence, increased systemic NGF-levels could explain these two findings co-occurring extra-lesionally in AD patients in the present study. Lastly, permanent serum concentration changes of chemokines and cytokine might lead to increased sensitivity of peripheral nerves.[24,37,58] For instance, CCL1 has been shown to sensitize nociceptors in mice,[2] CCL11 (also increased in serum of AD patients and correlated with severity)[44] is up-regulated in rodent models of cutaneous hyperalgesia,[24] and various chemokines and cytokines are known to be capable of inducing long-lasting increases in sensory nerve excitability and conductivity, e.g. by modulation of Na_V- and/or TRP-channel expression.[24,58] However, such mechanisms would have to be relatively selective since, e.g. heat pain thresholds and histaminergic itch did not differ significantly between groups, 2) prolonged afferent pruriceptive barrage causing a generalized sensitization of mechanoreception, 3) skin barrier alterations either associated with AD itself or as a consequence of prolonged usage of topical corticosteroids. The additional sensitization observed intra-lesionally is proposed to mechanistically resemble the localized hyperalgesia repeatedly shown in HCs immediately following an itch provocation.[6,52,66,78] Ikoma *et al.* (2004) also used noxious pin-prick stimuli in AD patients and found increased itch responses inside and immediately outside of lesions. However, in this study, the difference only manifested in increased ratings of itch (akin to what we observed in response to von Frey filaments during the dedicated hyperknesis assessments), while no differences were observed for pain. This discrepancy is likely related to the much higher maximal stimulus intensities used in the present study.

4.5. Increased sensitivity to chemical itch provocations

In the present study, very similar itch responses were observed following histamine provocation between non-lesional AD and HC skin. This is in line with most previous studies conducting such itch provocations, although outlying studies do exist wherein both increased and decreased sensitivity have been described.[5,38,41–43,48,67,91] In lesional AD skin, previous studies have found either no or moderate increases in histamine itch responses[5,38,41,42,76], in line with the strong trend observed in the present study. Hence, while histamine signaling appears to be slightly sensitized in the lesional skin, it is unaltered or

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even decreased in non-lesional skin. The intra-lesional sensitization to histamine (insignificant when multiplicity corrected) coincided with mild heat hyperalgesia. Mechanistically, this suggests that in AD kin, modestly sensitized TRPV1-signaling associated with the histaminergic CMi-fiber pathway of itch could contribute to augmented itch in response histamine. This could perhaps also explain the intra-lesional heat-pain evoked itch observed in a previous study in AD patients.[40]

In contrast to histamine provocations, cowhage provocations have not previously been conducted in lesional AD skin. Presently, we show that patients display increased sensitivity to cowhage-induced itch not only when applied intra-lesionally, but also in extra-lesional skin. The prominent itch responses to non-histaminergic chemical pruritic stimulation suggest that pathway-specific itch sensitization may be implicated in the sensory symptomatology of AD. Conflicting evidence exists regarding sensitivity to cowhage-induced itch in non-lesional AD skin. Papoiu *et al.* (2011) found no differences between HCs and AD patients, but the sample size was modest and the evoked itch was unusually strong in both AD and HCs, so a ceiling effect could have been present.[67] Oppositely, a recent paper with a larger sample size found increased itch in response to cowhage in AD patients akin to the results of the present study and suggested that cowhage (and histamine) provocations might have diagnostic value for AD.[34] Lastly, a study injecting the chemical PAR2-agonist SLIGKV found increased itch responses in patients with AD compared to HCs.[83] The presence of intra- and extra-lesional hypersensitivity to cowhage-induced, non-histaminergic itch suggests that new pharmaceuticals targeting PAR2 and, importantly, its downstream mediator TRPA1, could be effective antipruritics in AD. Several such drug candidates are currently under development.[14,95] The findings also lend mechanistic support to the notion that antihistamines are ineffective as antipruritics in AD.[27,68,90] The blunted flare reactions in non-lesional skin of patients with AD following histamine provocations has previously been reported[34,41] and could account for the slow decline in histaminergic itch observed in AD patients in several studies by hampering local tissue clearance of introduced histamine.[7,34,41]

The present evidence cannot be applied to interpret the relative peripheral vs. central contribution to the observed sensitization. However, it is most feasible that the sensitization found to both chemical and mechanical stimuli in otherwise asymptomatic skin is driven by central mechanisms, while the 'added' sensitization observed within lesions is likely initiated by peripheral mechanisms associated with ongoing inflammation, itch and pain. The increased responses to intra-lesional tests could also be related to a stronger localized segmental sensitization (perhaps particularly pertinent for hyperknesis) still mediated on a spinal level.

Hyperknesis to von Frey stimuli in AD

Mechanical pinprick hyperalgesia is pronounced in certain pain populations,[56,57] and thus it is pertinent to study the parallel phenomenon, *hyperknesis*, in relation to itch. When stimulating with von Frey filaments in a pre-optimized force range[7] prior to itch provocations, significantly higher itch ratings were found in the AD group intra- and extra-lesionally (Fig. 4). This is aligned with a previous study in AD patients using custom-made weighted needles to conduct a similar assessment[40] as well as case reports in neuropathic itch patients.[12] This mechanically evoked itch hypersensitivity was aggravated following subsequent itch provocations, most prominently in lesional skin, but also in non-lesional skin, where cowhage elicited significantly stronger hyperknesis in AD patients compared with the HC group. It is unclear which neuronal structures convey mechanically evoked itch. Selective tactile C-fibers have been proposed,[30] but Aδ- and PmC-fibers seem to be more probable candidates: the Aδ-nociceptors because of their involvement in pinprick hyperalgesia[100] and the PmC-fibers because of the delayed onset of itch

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following mechanical stimuli.[40] It is likely that increased responsiveness to mechanical itch stimuli involves a C-fiber mediated sensitization of central pruriceptive neurons, which then in turn receive convergent input from relevant mechano-sensitive units, thus mediating an augmented sense of mechanically evoked itch akin to the mechanism involved in pinprick hyperalgesia.[49,81]

Assessment of mechanical pain hypersensitivity has been widely utilized in clinical pain research and therapy as a tool to assess potential sensitization, and recent studies indicate that it could be a valuable guide and predictor of therapeutic responsiveness to analgesic drugs.[16,26,56,57,72] The clinical utility of assessment of hyperknesis and alloknesis as well as itch sensitization in general (e.g., sensitization to chemical provocations) remains to be explored. Clearly, antipruritic therapeutic measures should focus on reducing local inflammation and targeting the underlying cause. However, tentatively, AD patients displaying no signs of sensitization might respond favorably to peripherally acting anti-inflammatory and immune-modulatory drugs, while patients exhibiting significant itch sensitization may benefit more from antipruritic therapy that also inhibits central processing of itch.

Because pruriceptive afferents generally also responds to application of algogens (causing pain) and as such are nociceptors, a theory has recently been proposed that neuronal encoding of itch may rely on a high *spatial contrast* from afferent input. That is, if a few cutaneous nociceptors are activated while neighboring units remain silent, itch is perceived, while more uniform nociceptive activation causes pain perception[51,60,61,78] (the spinal or supra-spinal filtering mechanism for such encoding is unknown). The combined findings of intra-lesional decreased tactile sensitivity increased MPS, increased itch in response to cowhage and von Frey stimuli could perhaps be interpreted in the light of this theory. I.e. increased MDT suggests fiber denervation (aligned with studies showing decreased intra-epidermal density in AD skin[70]) this could facilitate increased spatial contrast from the cowhage provocations and von Frey itch probing. Since increased mechanical pain sensitivity was also observed these findings could collectively be interpreted as a combination of activation of fewer nociceptive endings concurrently with stronger activation of the remaining endings due to sensitization.

Conclusion

In summary, AD patients display aberrant somatosensory sensitivity to distinct chemical and mechanical stimuli. This enhanced sensitivity is not restricted to the lesional skin areas and thus presumably involves both centrally and peripherally mediated sensitization mechanisms. Investigated for the first time, marked intra- as well as extra-lesional hypersensitivity to cowhage-induced non-histaminergic itch was shown. Moreover, increased sensitivity to pain- and itch-evoking mechanical stimuli, but not to histamine-induced itch, was found. This suggests the importance of PAR2-positive PmC-nociceptors in initiating and maintaining chronic itch in AD and highlights this pathway as a potential future therapeutic target.

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Figure and table legends

Figure 1. Flowchart of experimental protocol. A total of four itch provocations were conducted (two in lesional and two in non-lesional skin) in a randomized order, with provocations always alternating between the two areas. The order presented in the picture represents an example (greyed out provocations not conducted). The entire experimental session lasted approximately 3 hours. Abbreviations: AD = Atopic Dermatitis; CDT = Cold Detection Threshold; CPT = Cold Pain Threshold; eVAS = Electronic Visual Analog Scale; FLPI = Full-Field Laser Perfusion Imaging; MPT = Mechanical Pain Threshold; MPS = Mechanical Pain Sensitivity; NRS = Numerical Rating Scale; HPT = Heat Pain Threshold; WDT = Warmth Detection Threshold; WUR = Wind-Up Ratio.

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Figure 2A and B. Mechanical pain sensitivity (MPS) in lesional (**A)** and non-lesional skin (**B**) of AD patients (red) and corresponding sites in healthy controls (blue). Asterisks to the left of the stimulus response curves indicates a significant *group* main effect (scores of entire stimuli series), while asterisks immediately above data points indicates post hoc group differences (*group* x *stimulus* interaction) for ratings produced by each of the pinprick stimulators. Abbreviations: AD = Atopic Dermatitis; HC = Healthy Control. Significance indicators: * = $P \leq 0.05$, ** = $P \leq 0.01$.

Figure 3A-D. Temporal profiles of itch intensity elicited by histamine (**A** and **B**) and cowhage provocations (**C** and **D**) in lesional (**A**) and non-lesional skin (**B**) as well as in respective control sites (**C** and **D**). Rating frequency was down sampled from 1/5 sec (0.2 Hz) to 1/15 sec (0.067 hz) by averaging 3 consecutive ratings for improved overview. Note that while histamine-induced itch was not significantly increased in lesional AD skin (**A**), a considerable tendency was observed ($P = 0.07$). Statistical results were based on mean scores for the 0-6 min period. Abbreviations: AD = Atopic Dermatitis; HC = Healthy Control. * = $P \leq 0.05$, ** = $P \leq 0.01$.

Figure 4A and B. Mechanically evoked itch in lesional (**A)** and non-lesional skin (**B**) of AD patients (red) and corresponding sites in healthy control (blue). The sensitivity to mechanically evoked itch was assessed at baseline as well as following histamine and cowhage provocations. Abbreviations: AD = Atopic Dermatitis, HC = Healthy Control. * = $P \leq 0.05$, ** = $P \leq 0.01$.

Figure 5. A representative series of Full-Field Laser Perfusion (FLPI) images recorded at baseline (column **a** and **b**) and following histamine provocations (column **c** and **d**), signifying "endogenous" skin inflammation in atopic dermatitis and the neurogenic inflammation evoked by histamine in both groups. Images in row no. **1** through **3** are from non-lesional skin areas and corresponding control areas, while images in row no. **4** through **6** are recorded in lesional skin areas and corresponding control areas. Column **a**, row no. **4** (dorsal wrist/hand)**, 5** (lateral aspect of elbow crease) and **6** (medial aspect of elbow crease), show typical FLPI images of atopic dermatitis lesions. Note: 1) the light speckled inflammation pattern (e.g., column **a**, row no. **1** and **2**), recognizable with FLPI, often observed in AD even in skin that appears normal by visual inspection; 2) the blunted flare response to histamine observable in non-lesional skin of AD patients (compare column **b**, row no. **1-3** to column **d**, row no. **1-3**), particularly clear when measuring the intensity of blood flow immediately next to the skin prick test site; 3) that when comparing it is difficult to gauge the inflammatory response evoked by histamine. Abbreviations: Arb = Arbitrary.

Table 1. Results from the quantitative sensory testing in lesional as well as non-lesional skin and statistical outcomes. Abbreviations: AD = Atopic Dermatitis; CDT = Cold Detection Threshold; CPT = Cold Pain Threshold; MDT = Mechanical Detection Threshold; mN = miliNewton, MPT = Mechanical Pain Threshold; HC = Healthy Control; HPT = Heat Pain Threshold; WDT = Warmth Detection Threshold; WUR = Wind-Up Ratio. * = $P \leq 0.05$, [§] = insignificant trend.

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Table 1

QST parameter	Lesional skin			Non-lesional skin		
	AD	**HC**	*P*-value	**AD**	**HC**	*P*-value
CDT (°C)	30.0 ± 0.2	30.4 ± 0.2	*P* = 0.16	29.9 ± 0.3	30.2 ± 0.2	*P* = 0.40
WDT (°C)	34.0 ± 0.4	34.1 ± 0.1	*P* = 0.73	34.9 ± 0.3	34.8 ± 0.2	*P* = 0.76
CPT (°C)	18.5 ± 1.8	17.3 ± 1.4	*P* = 0.61	16.0 ± 1.8	17.4 ± 1.3	*P* = 0.56
HPT (°C)	41.2 ± 0.8	41.9 ± 0.6	*P* = 0.51	43.2 ± 0.9	41.4 ± 0.6	*P* = 0.10
MDT (mN)	**3.4** ± **0.1**	**3.1** ± **0.1**	*P* = 0.049*	3.2 ± 0.8	3.2 ± 0.7	*P* = 0.66
MPT (mN)	133.0 ± 19.6	182.3 ± 21.3	*P* = 0.09§	188.5 ± 24.3	185.2 ± 18.5	*P* = 0.92
WUR (ratio)	1.9 ± 0.14	2.1 ± 0.19	*P* = 0.38	1.8 ± 0.14	2.1 ± 0.17	*P* = 0.30

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Figure 1

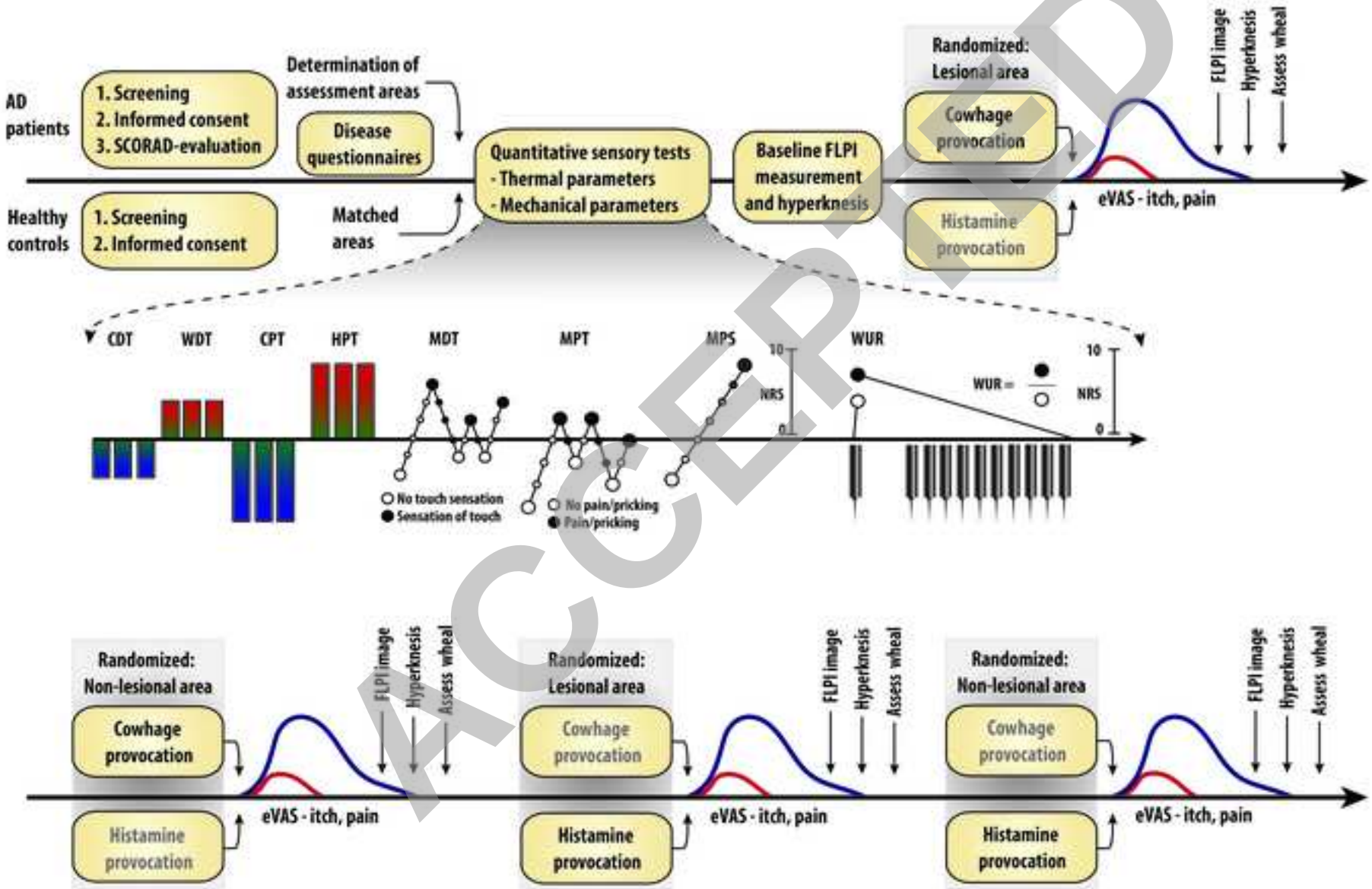

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Figure 2

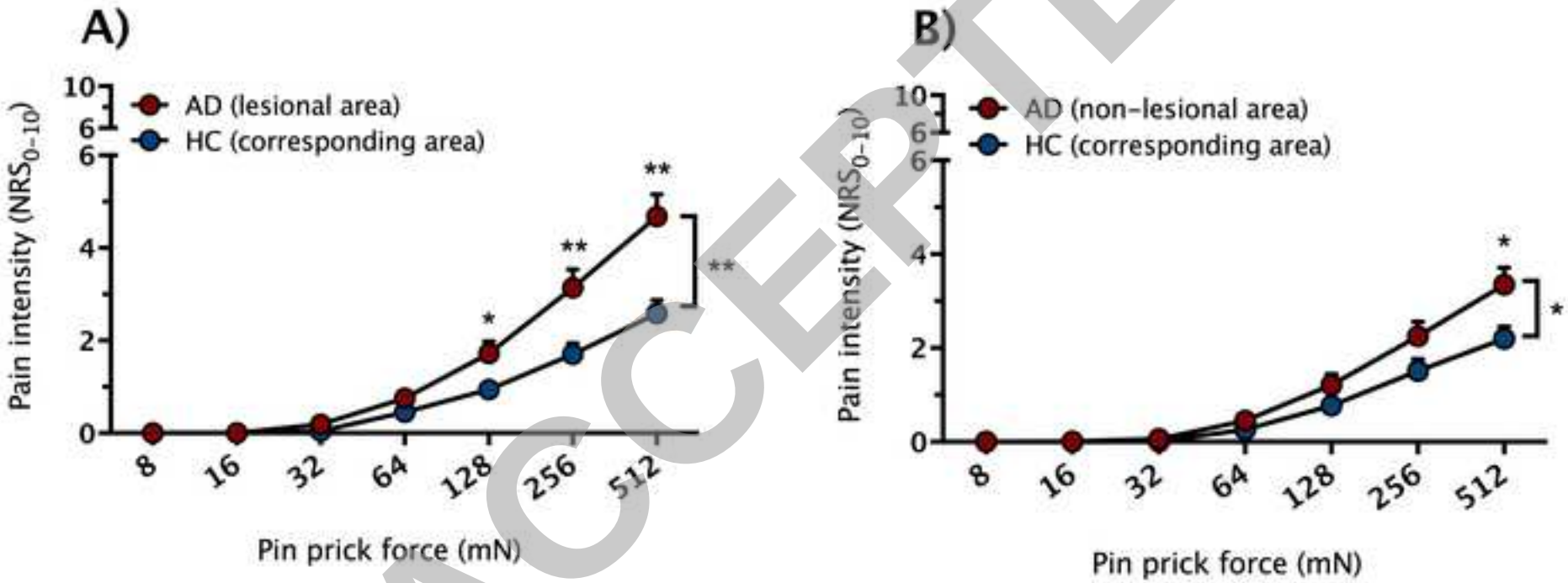

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Figure 3_revised

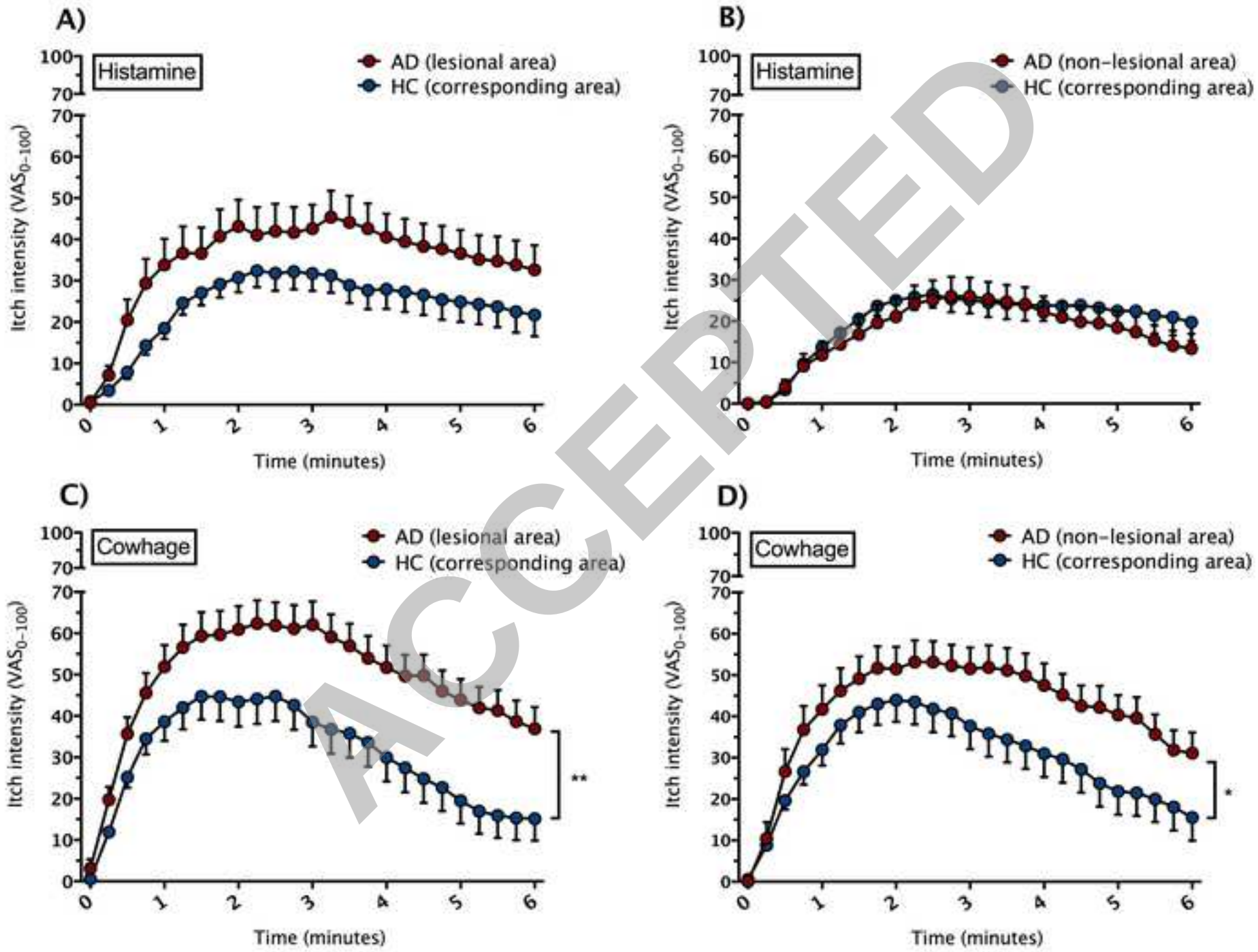

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Figure 4

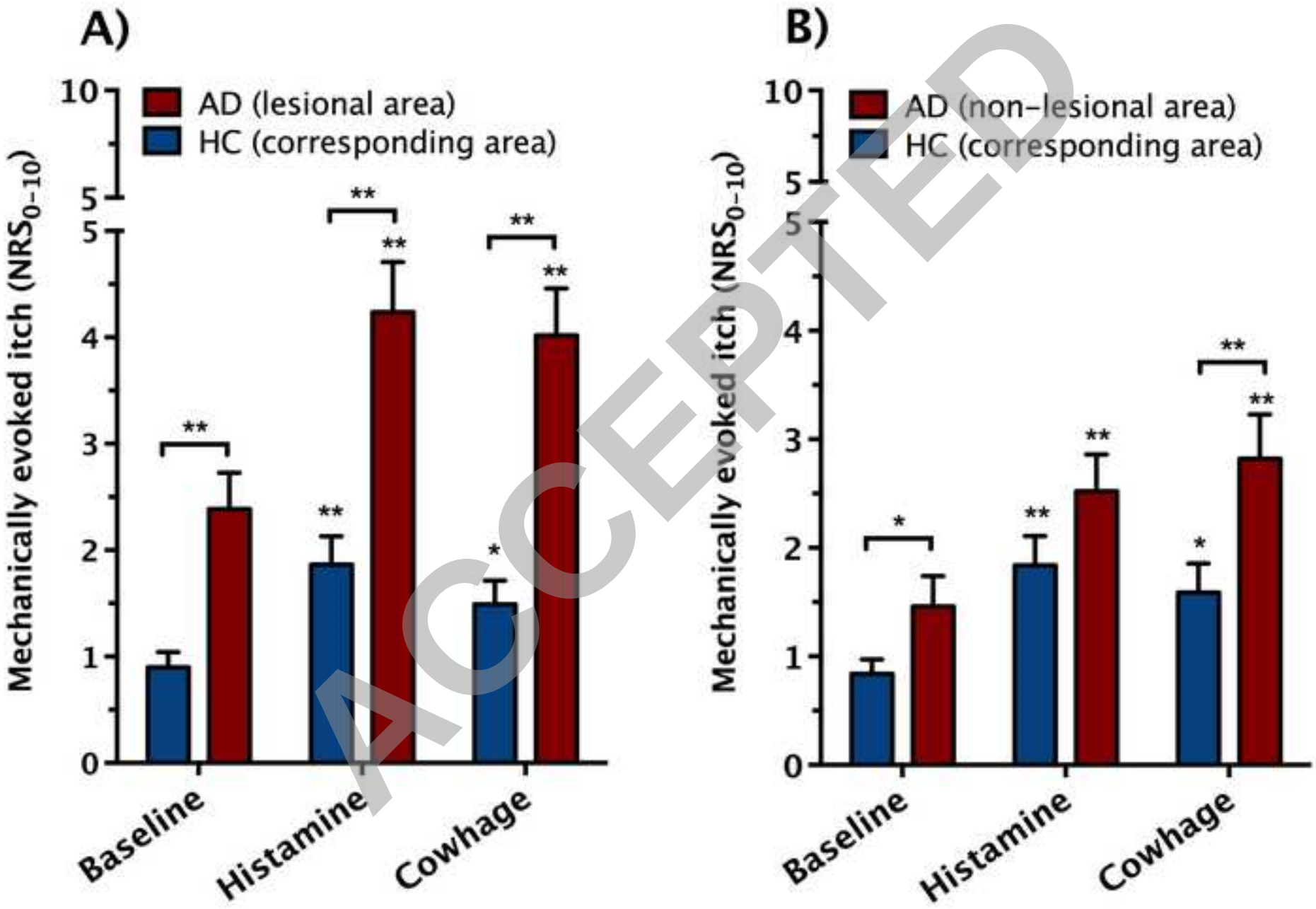

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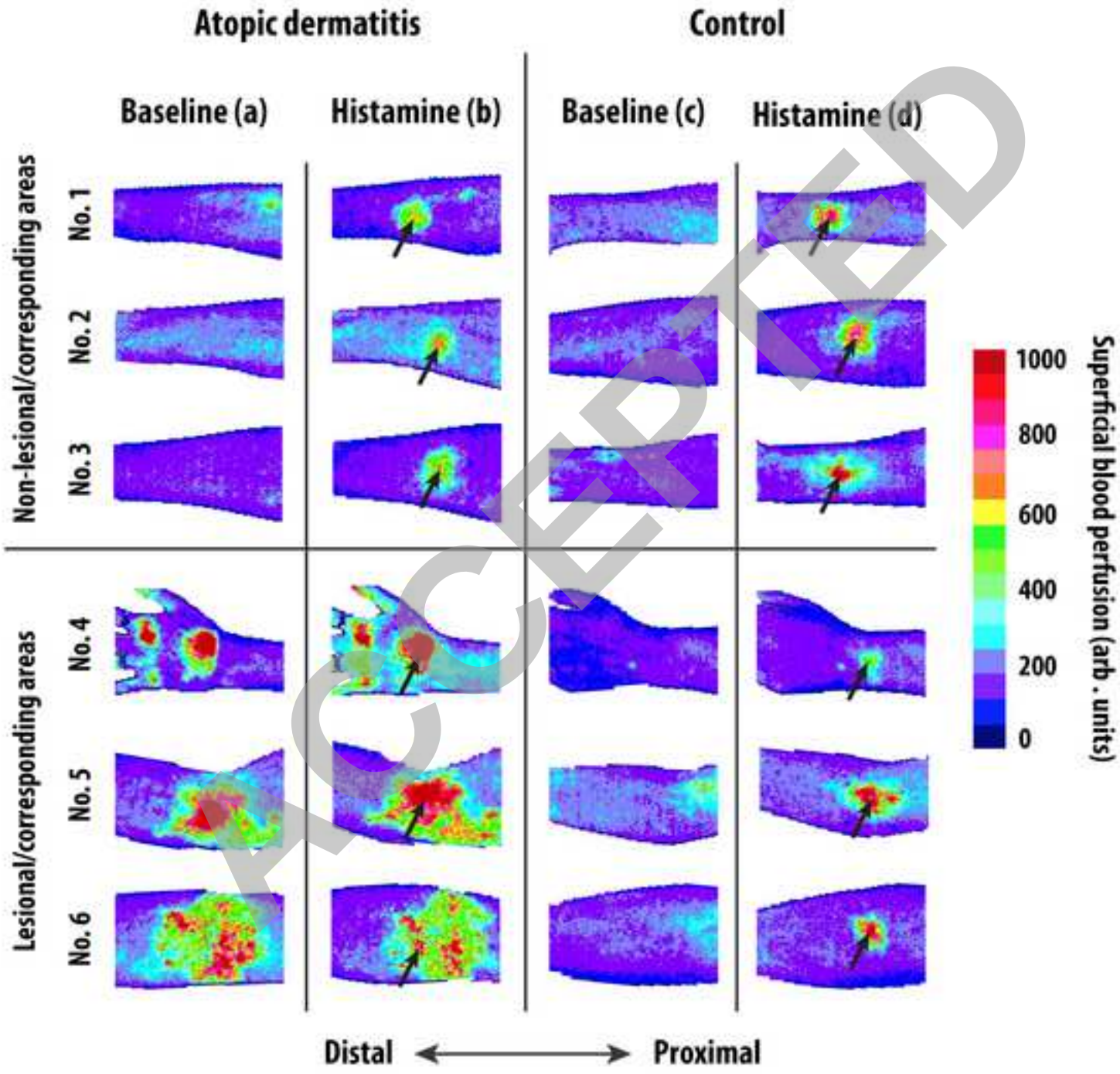
Figure 5_revised



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