

DR. YURIE SHIMODA-KOMATSU (Orcid ID : 0000-0002-7875-8124)

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Title: A novel method to assess the potential role of sweating abnormalities in the pathogenesis of AD

Authors

Yurie Shimoda-Komatsu ^{1*}, Yohei Sato¹, Yoshimi Yamazaki ¹, Ryo Takahashi ², Tetsuo Shiohara ¹

Institutions

¹ Department of Dermatology, Kyorin University School of Medicine,

Mitaka city, Tokyo, JAPAN

² Flow Cytometry Core Facility, Kyorin University Graduate School of Medicine,

Mitaka city, Tokyo, JAPAN

Corresponding author: Yurie Shimoda-Komatsu

Department of Dermatology, Kyorin University School of Medicine

6-20-2 Shinkawa, Mitaka city, Tokyo 1818611, JAPAN

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Phone : 81-422-47-5511 (ext 2881), Fax : 81-422-47-9632

E-mail: kyurie@ks.kyorin-u.ac.jp

Abstract

Background: Although atopic dry skin is believed to be caused by defects in skin genes important for maintaining skin barrier function, the role of sweat in atopic dermatitis (AD) has been apparently underestimated. Given the great capacity of sweat to maintain and increase skin hydration, defective sweating responses may be a logical place to look for changes that predispose individuals to the disease.

Methods: We investigated how disease process and sweating defects progress from early asymptomatic stages to the onset of clinically apparent disease by employing the impression mold technique, which allows an accurate quantification of individual sweat gland/duct activity in relation to skin surface topography. Insensible and sensible sweating responses under baseline conditions and after thermal stimulus, respectively, were measured in various stages of AD patients and healthy controls.

Results: In controls, under baseline conditions sweat ducts/glands at the dermal folds secreted basal levels of sweat (insensible sweating), thereby maintaining skin hydration. Not only such insensible sweating but also sensible sweating markedly decreased even in the earliest asymptomatic stage and the decrease was followed by compensatory hyperhidrosis at the ridge: leakage of sweat into the dermis could represent the initial event resulting in the decreased sweating and inflammation. The defects eventually progressed involving all of the ducts/glands to develop systemic dry skin.

Conclusion: AD skin is characterized by varying degrees of functional impairment of sweat ducts/glands depending on the stage and this defect would be among the reasons for the inability of AD patients to maintain skin hydration.

Key words: atopy, dermcidin, hypohidrosis, sweating responses, thermal stimulus,

Introduction

Disturbance of the epidermal barrier is the most likely candidate that can favor the increased penetration of allergens, thereby facilitating the occurrence of allergic inflammatory reaction in atopic dermatitis (AD) (1-3). AD is now believed to be caused by defects in skin genes that are important maintaining skin barrier function and turgidity (4, 5). Because dry skin caused by defects in these genes is also thought to be responsible for the generation of intense pruritus and the resulting scratching (6), recent research on the pathogenesis of AD has focused on the defects in these skin genes. Thus, a dogma has been established that emphasizes the role of loss-of-function mutations in the gene encoding *filaggrin* (*FLG*), while ignoring the role of sweating on the barrier function and the subsequent development of allergic inflammation. However, given the great capacity of sweat to increase skin surface hydration and to secrete moisturizing factors to maintain skin hydration (7, 8), it is reasonable to expect that the skin surface hydration status of AD patients would be affected by the magnitude of sweating defects as well as by the reduction in ceramides or *FLG*. In support of this possibility, a recent report from Japan has concluded that *FLG* loss-of-function mutations are not a predisposing factor in an Ishigaki Island, a hot and humid environment (9): this result could be interpreted as suggesting the importance of not only environmental factors, such as high humidity, but also sweating. Nevertheless, there have been few studies investigating the role of sweating on the development of AD (10) and such research has been hampered both by the paucity of reproducible quantitative methods to accurately evaluate the activity of each sweat gland/duct in a well-defined location and by the lack of sweat glands/ducts in animal skin, in which a variety of analyses have been performed. In addition, conflicting data regarding whether sweating responses are impaired, normal or enhanced in patients with AD (11-16) would divert the research interest away from investigating the role for sweating responses in the pathogenesis. A consensus has emerged from these studies that sweating responses are impaired and retarded in AD patients (13, 16). However, hypohidrosis in one area may lead to compensatory hyperhidrosis in another (17, 18), indicating that contradictory results

in previous reports might have been explained by such regional differences or differences according to the disease phenotype and stage.

We have recently established a useful method, named the impression mold technique (IMT), which allows an accurate quantification of individual sweat glands/ducts actively delivering sweat in a well-defined location and the volume of sweat they produce (19-21). In this study, we investigated sweating responses to thermal stimulus before and during the ensuing 30 min period in AD patients in different stages. We found that sweating defects in AD initially occurs in some sweat glands/ducts at the dermal folds which could be associated with compensatory hyperhidrosis in those at the dermal ridges and eventually progresses to develop systemic hypohidrosis at the advanced stage characterized by dry skin. Our data substantiate a crucial role for sweating defects that can initiate and drive the disease process forward.

Materials and Methods

Patients and methods

Thirty-three patients with adult-type AD aged 17-54 years, who were diagnosed by experienced dermatologists in accordance with the criteria of Hanifin and Rajka (22), were enrolled in this study (Table S1). All patients had mild-to-severe disease with typical eczematous skin lesions, elevated serum IgE levels and specific serum IgE Abs to aeroallergen. The severity of disease was assessed by the SCORing Atopic Dermatitis (SCORAD) index (23) at the time of enrollment. Twelve unrelated, sex- and age-matched, healthy subjects with no personal or family history of AD, allergic rhinitis or asthma were enrolled in this study and served as healthy controls. Participants provided written informed consent before inclusion. The study was approved by the Institutional Review Board of the Kyorin University Hospital (No. H21-008). This study is based on the Declaration of Helsinki.

Sweating was induced by immersing both lower legs for 30 min in a water bath maintained at 43°C. In some subjects, sweating was also induced by the injection with 50 µl of acetylcholine(Ach) 10%. Sweating responses thus induced was evaluated by measuring the skin surface hydration and IMT (20, 21) (see Appendix S1). Skin surface hydration was measured by the high-frequency conductance of the stratum corneum using an impedance meter (Skicon-200EX, IBS, Hamamatsu, Japan). IMT measurements took place simultaneously on two or three different sites, i.e. the ventral forearm or/and abdomen. These sites were differently chosen depending on subject, because the success of this test was less predictable at more irregular surface contour locations: usually 2 on involved and 2 on uninvolved skin sites, respectively.

Immunohistochemistry

Biopsy specimens from AD patients were obtained, including the lesion itself and uninvolved perilesional skin. Control specimens were recruited from patients with psoriasis vulgaris, including the lesion and uninvolved skin. The specimens were double-stained by anti-dermcidin mAb (sc-33656, Santa Cruz Biotechnology, Santa Cruz, CA) and anti-muscarinic acetylcholine receptor M3 (Ach-RM3) mAb (sc-9108, Santa Cruz Biotechnology) as previously described (21).

Results

Relationship between SSH status and sweating responses evaluated by IMT

Representative silastic impressions from the ventral forearm and abdomen of a healthy control subject before and after thermal stimulus (Figs.1A-C) showed that sweat droplets were distributed evenly and usually occupied each dermal fold in the body, and were rarely detected in the ridges, particularly before thermal stimulus, unlike those in the palms and soles. In IMT measurement, regional differences were minimal when examined in the forearm and abdomen (Figs. 1D and E). These

suggested that even under quiescent conditions before thermal stimulus basal levels of sweat could be excreted at the skin surface: we defined this sweating as basal or insensible sweating,

which may be reflected in the skin surface hydration. We therefore asked whether there could be a true association between the skin surface hydration status and numbers or sizes of sweat droplets detected at either the folds or ridges under quiescent basal conditions. As shown in Fig. S1A, a most significant positive correlation was found between the skin surface hydration status and sweat droplet numbers detected at the folds, indicating that the skin surface hydration status under quiescent basal conditions may reflect basal levels of sweating from the glands/ducts located at the folds. At 15-30 min, sweat droplets were also detected at the ridges: we defined this sweating as inducible or sensible sweating (Figs. 1B-D). These results indicated that sweat ducts/glands located at the folds would serve to maintain skin hydration under basal conditions by ‘insensible’ sweating while those at the ridges would function as a thermoregulation (sensible sweating), suggesting that dry skin observed in AD might be largely dependent on the impaired insensible sweating responses in the ducts/glands located at the folds. Nevertheless, because the skin surface hydration levels have an inverse relationship to SCORAD, particularly at 30 min after thermal stimulus (Fig. S1 D), impaired sensible sweating would also contribute to the severity of the disease.

Impaired sweating responses in the different stages of AD patients

We investigated how disease process and sweating defects progress from early asymptomatic stages to the onset of clinically apparent disease. To this end, AD patients were divided into two groups, based on the different clinical phenotypes and stages, acute and chronic. Patients with active dermatitis characterized by scaling erythematous papules, plaques, lichenoid papules and crusting were evaluated as acute AD: most of them had exudate lesions in the flexural areas, usually for ≤ 5 years (Table S1). “The earliest acute stage” of AD was defined as AD patients, who showed clinical

symptoms only in the limited areas, such as arms, but no apparent clinical symptoms in other areas and who usually developed these lesions up to 2 years before the sweat tests. In contrast, patients characterized by systemic dry skin and relative sparing of eczematous lesions in the flexural area were evaluated as chronic AD.

As shown in Figs. 2B-D, the sweat droplets taken from representative acute AD patients 30min after thermal stimulus (sensible sweating) were distributed much less evenly than those taken from healthy controls (Fig. 2A): in AD they were less numerous but larger in diameter. In the normal-appearing uninvolved skin of the earliest asymptomatic stage and acute stage, a high degree of variability in the number and size was evident, ranging from a simple reduction (Fig. 2B) to larger sizes (Fig. 2C). Importantly, the number of sweat droplets observed at the fold was decreased even at the earliest asymptomatic stage, where skin surface topography was not disrupted yet by disease process (Fig. 2B), indicating that the decrease in sweat droplet numbers at the fold may represent the actual early event that can trigger the onset of clinically apparent disease, but not the result of disruption of skin surface topography. In the involved skin of acute AD patients, the size of the sweat droplets was larger than that in healthy controls, particularly at the ridges: larger sizes of the droplets were detected either in the involved or uninvolved skin of acute AD (Figs. 2C and D). The number of sweat droplets taken from the involved skin sites of acute AD (Fig. 2D) was apparently lower than that in healthy controls. In addition, many of them were located independently of the folds. In contrast, sweat droplets observed in chronic AD (Fig. 2E) were much less numerous in number and smaller in size than those in acute AD.

The histograms of sweat droplet number and diameter in AD

Defects in sensible sweating responses to thermal stimulus in AD patients were also illustrated in the histograms of sweat droplet counts and their diameter from representative AD patients and a healthy control (Fig. S2). In a healthy control, the diameter of sweat droplets was relatively constant, ranging from 80 to 140 μm (Fig. S2A). Representative silastic molds shown in Figs. 3A-E

correspond to the histograms shown in Figs. S2A-E, respectively. In the uninvolved site at the earliest asymptomatic stage of acute AD (Fig. S2B), sweat droplets were more frequently detected at the ridges, where no significant alterations of skin surface topography were detected (Fig. 2B). In the uninvolved site at the relatively advanced stage of acute AD (Fig. S2C), sweat droplets over 160 μm diameter were frequently detected at the ridges, where significant changes in the skin surface topography were already observed despite their uninvolved appearance (Fig. 2C). The involved site of acute AD (Fig. S2D) was characterized by a decrease in the number and an increase in the diameter. In contrast, a more profound decrease in the number and size was observed exclusively in chronic AD (Fig. S2E).

The alterations in the number and size of sweating droplets in relation to skin surface topography in AD patients

We next decided to confirm that sweating defects could initially occur in sweat glands/ducts located at the folds that would serve to maintain skin hydration in many AD patients at different stages. A remarkable decrease in the mean number of sweat droplets was preferentially observed at the folds, regardless of the clinical stage, under baseline conditions (basal or insensible sweating) (Fig. 3A) and 30 min after thermal stimulus (sensible sweating) (Fig. 3C), while a significant increase in the size of the sweat droplets were exclusively detected in acute AD, particularly at the ridges in the uninvolved site (Figs. 3B, D). These results suggest that not only insensible sweating under baseline conditions but also sensible sweating after thermal stimulus significantly decreased and that compensatory hyperhidrosis may occur at the ridges in this phase of AD. Indeed, because the increase in the sweat droplet numbers and diameter detected at the ridges in acute/involved and uninvolved AD skin was higher than that in healthy controls when compared basal sweating with sensible sweating (Fig. 3A vs. C, B vs. D), the inability of sweat ducts/glands at the folds to deliver sweat in AD could be partly compensated by a marked increase in the ability of individual sweat ducts/glands at the ridges in acute AD. We next compared the mean number of sweat droplets

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 $\geq 160 \mu\text{m}$ in diameter in acute AD with that in healthy controls and chronic AD (Fig.3E), indicating that compensatory hyperhidrosis becomes most apparent at the ridges in the uninvolved skin sites of acute AD. The inverse relationship between the number and size of sweat droplets was only observed in the relationship between the number at the folds and the diameter at the ridges in healthy controls, but not in AD (Fig. S3A), suggesting that the decrease in the number cannot be fully compensated by the increase in the diameter in AD.

One may argue that sweating defects thus observed in AD patients are more likely to reflect downstream events which have occurred as the result of chronic inflammation, rather than an early event which specifically contribute to disease progression. We therefore investigated whether sweating defects could be related to the severity of disease. The severity of disease as assessed by SCORAD (23) was positively related to the number of sweat droplets located at the ridges in acute AD, while that was inversely related to those at the folds in chronic AD (Figs. S3 D, E). These results suggest that hyperhidrosis could be preferentially observed in the sweat ducts/glands at the ridges in acute AD, which would serve to aggravate clinical symptoms of acute AD, while systemic hypohidrosis observed either at the folds or ridges in chronic AD could contribute to the progression to the full development of dry skin.

Leakage of sweat into the dermis around the sweat glands/ducts

We next asked whether the decreased sweating observed in AD patients could be due to their inability to produce sweat or to deliver sweat to the surface. Because dermcidin peptides are sweat-specific components but not the components common to other body fluid (24), we investigated whether dermcidin peptides could be immunohistochemically detected in the sweat glands/ducts of AD patients as observed in healthy controls, and, if so, we then asked whether the dermcidin could be also detected in the dermal tissue around the glands/ducts, reflecting leakage of sweat. In the biopsy specimens taken from the involved skin after thermal stimulus, dermcidin antigen was expressed in the cytoplasm of dark cells in the sweat glands of the involved skin of acute AD at much higher

intensity (Fig. 4A) than that in those of other inflammatory disease lesions, such as psoriasis (Fig. 4B). Surprisingly, dermcidin antigen was detected not only within the gland/duct but also in the dermal tissue adjacent to the gland (Fig. 4A) and duct (Fig. 4C), respectively, indicating leakage of sweat either from the glands or ducts into the dermis. In contrast, such leakage of sweat into the dermis near the duct was never observed in the psoriasis lesions even after thermal stimulus (Fig. 4D). These results indicate that the sweat, instead of pouring out and being removed from the skin surface, may diffuse into the dermal tissue around the glands/ducts and that, as a result, the ability to deliver sweat into the surface, but not the ability to produce sweat, could be impaired in AD patients. The diffusion of sweat was most evident in the involved site of acute AD patients, indicating that diffusion of sweat into the dermis would represent the early, or common event, that may favor the continued activation of immune cells. In contrast, expression of Ach-RM3 (Figs. 4A and C) in the eccrine glands was not altered in skin lesions of AD and psoriasis, even after thermal stimulus.

Sweating responses to Ach in AD patients

We next asked whether sweating defects observed in AD could be related to autonomic dysfunction, because previous studies reported impairment of the sudomotor activity of the sympathetic nervous system in AD patients (25). As shown in Fig. S4A, sweating responses to Ach significantly decreased in sweat droplets located at the folds in acute AD when compared with those in healthy controls, a finding similar to thermal stimulus. These results suggested that autonomic dysfunction may play a role in the sweating defects, but not necessarily be the major contributor. Thus, the sweating defects observed in AD would be due to dysfunction of sweat glands/ducts themselves.

Discussion

While sweat is well known to be one of the factors provoking exacerbations of clinical symptoms of AD (26-28), there is no convincing evidence for the protective role of sweat on the development of AD skin lesions except for a recent report describing a protective role of sweating for nonhistaminergic pruritus (29). Indeed, much less attention has been focused on the fact that reduced sweating in AD patients is one of the most important factors, although not enough in itself, that causes the skin dryness: it may contribute more profoundly to skin dryness in AD than the reduction in ceramide and *FLG*, given the great capacity of sweat to increase skin surface hydration. Reflecting such neglect in previous studies on the role of sweat in AD, previous attempts to evaluate sweating disturbance in AD have yielded conflicting results (11-16). One explanation for these conflicting results is that there was no useful quantitative method that allows an accurate assessment of individual sweat glands/ducts actively delivering sweat over a time frame. In this regard, the major advantage of using IMT to evaluate sweating disturbance over the previous methods such as starch-iodine method is its accuracy, sensitivity, and reproducibility.

Although there is controversy regarding whether sweating defects is causative, a cofactor, or just an epiphenomenon of allergic inflammation, our study shows that the sweating defects would initially occur in some sweat glands/ducts at the folds, in which skin surface topography has not been disrupted yet, and be associated with compensatory hyperhidrosis at the ridges in the acute stage, and progress involving all of the glands/ducts to develop systemic hypohidrosis in the chronic stage. Thus, one possible explanation of previous contradictory finding on sweating defects in AD is that this may have been a function of the time point in the stage progression when sweating tests were performed. Because compensatory hyperhidrosis detected in the sweat ducts located at the ridges of acute AD can only occur within 5 years after onset of disease and diminish gradually, previous studies may have missed this phenomenon because of a possible mixture of acute AD and chronic AD. In view of our finding that leakage of sweat into the dermal tissue around sweat glands and ducts, presumably due to

sweat duct fragility, was most evident immediately after thermal stimulus in the involved skin of acute AD, this leakage of sweat may represent the actual early events that may presumably occur well before patients have typical dry skin. While keratotic plugging frequently observed in psoriatic lesions was never associated with the leakage of sweat, no apparent keratotic plugging was histologically identified at any sites in acute AD lesions, in which leakage of sweat was detected. It is therefore unlikely that the leakage of sweat and the decrease in sweating responses are caused by obstruction of the sweat ducts. Thus, we can hypothesize that the sweat duct fragility in AD might be related to putative defective function of tight junction, which can prevent the free diffusion of macromolecules. Indeed, because sweat has been shown to contain various inflammatory cytokines (30-33), it is possible that leakage of sweat into the dermis could initiate a complex sequence of events that promotes eczematous inflammation. In view of a recent study indicating that the areas of anhidrosis on thermoregulatory sweat testing correspond to their symptomatic areas, such as itching and strange sensation (34), itching and strange sensations frequently detected in the AD skin could now be explained by leakage of sweat into the dermis, rather than by small-fiber neuropathy. Given the efficacy of phototherapy for enhancing barrier function in AD (35), it is also likely that phototherapy may act to improve AD symptoms by preventing leakage of sweat. Thus, thinking of sweating defects in AD as a process and time to phenotypic manifestation rather than as a mere result of inflammation would be more important for understanding the pathogenesis of AD. In view of recent studies (36, 37) indicating that histamine can inhibit sweat secretion by suppressing phosphorylation of GSK3 β , a target for phosphorylation by Ach, decreased sweating responses observed in AD patients could alternatively be mediated by histamine.

We demonstrate that sweat droplets located at the folds, but not the ridges, were significantly decreased in number even in the normal-appearing uninvolved skin at the earliest asymptomatic phase of acute AD, suggesting the possibility that the normal functioning of the sweat glands/ducts located at the folds could have been disturbed even in the uninvolved skin while those at the ridges remain

fully functional or more productive. If this is the case, physiological roles for sweat glands/ducts would be different dependent on the location in the skin: for example, the major function of sweat glands/ducts located at the folds is to maintain skin surface hydration (insensible sweating) while that of sweat glands/ducts at the ridges might serve as a backup to impaired function of sweat glands/ducts at the folds. Consistent with this view, compensatory hyperhidrosis observed as sweat droplets $\geq 160 \mu\text{m}$ in diameter was exclusively detected in sweat droplets located at the ridges. Alternatively, sweat glands/ducts located at the ridges might function as a key thermoregulator, unlike those at the folds. Collectively, AD skin is characterized by varying degrees of functional impairment of sweat glands/ducts, at either the folds or ridges, and this defect would be a principal reason for the inability of the host to maintain SSH and lose heat. Because defects in barrier function and hydration could already have been present even in the normal-appearing skin of AD, in which sweating defects can be detected, it is difficult to exclude the possibility that sweating disturbance observed in AD would reflect downstream events which have occurred as the result of defects in barrier function and hydration. To test this possibility, further prospective, long-term studies on sweating responses in infants with single and double allele loss-of-function mutation of *FLG* without inflammation are needed; this study would determine the actual early events that trigger the inflammatory conditions.

Sweating defects in AD, although occurring on a continuum, can be viewed as occurring in 3 temporally overlapping stages. First is the initial stage, in which leakage of sweat into the dermis in some areas due to sweat duct fragility not only provides an inflammatory milieu but also results in the decrease in skin surface hydration. Second is the compensatory stage, in which compensatory hyperhidrosis occurs preferentially at the ridges to maintain temperature, which further exacerbates the inflammatory response. Third is a 'burn-out' process in later stage, in which patients eventually manifest the phenotype with a systemic disturbance in the sweat delivery system no longer associated with compensatory hyperhidrosis; patients we defined as "chronic AD" represent the late or end stage of the disease. Thus, sweating defects appear to follow an ordered developmental progression.

This model for the pathogenesis of AD has significant implications, both in terms of future research and therapies. Therapies directed at correcting the defects at early times while ameliorating inflammatory responses may prove efficacious for preventing further progression of the disease.

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Y. Shimoda-Komatsu, and T. Shiohara conceived the study. Y. Shimoda-Komatsu, Y.Sato, Y. Yamazaki, R.Takahashi, and T. Shiohara all contributed to the design and performance of the experiments. All authors contributed to the preparation and review of the manuscript.

Conflict of interest The authors have no conflicts of interest to declare.

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Figure legends

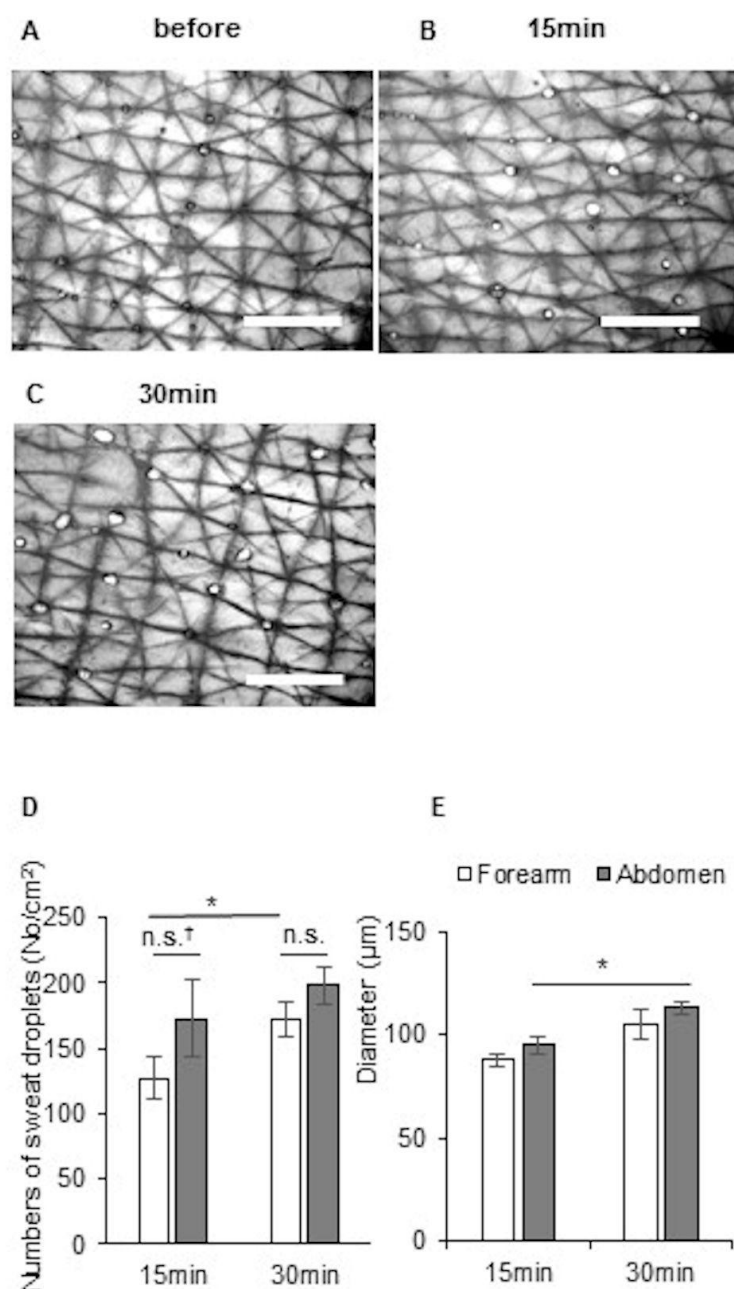
Figure 1. Representative silastic molds from the forearm of a healthy control before (baseline condition) (A) and after (B,C) thermal stimulus. The mean number (D) and diameter (E) of sweat droplets from 12 controls. * $p < 0.05$ †n.s.: not significant Bar=500 μ m

Figure 2. Representative silastic molds from a healthy control (A), 'normal-appearing' uninvolved (B, C) and the involved areas (D, E) of the ventral forearm of AD patients in the acute (B-D) and chronic (E) stages 30 min after thermal stimulus. In some uninvolved areas, samples were also obtained at the earliest asymptomatic acute stage (B). Bar=500 μ m

Figure 3. The mean number and size of sweat droplets at the folds and ridges before (A, B) (insensible sweating) and 30min after (C, D) thermal stimulus (sensible sweating) in controls and AD. E. The mean number of sweat droplets $\geq 160 \mu\text{m}$ at the folds and ridges (sensible), respectively. HC, healthy control; I, involved areas; and U, uninvolved areas. * $p < 0.05$, ** $p < 0.01$,

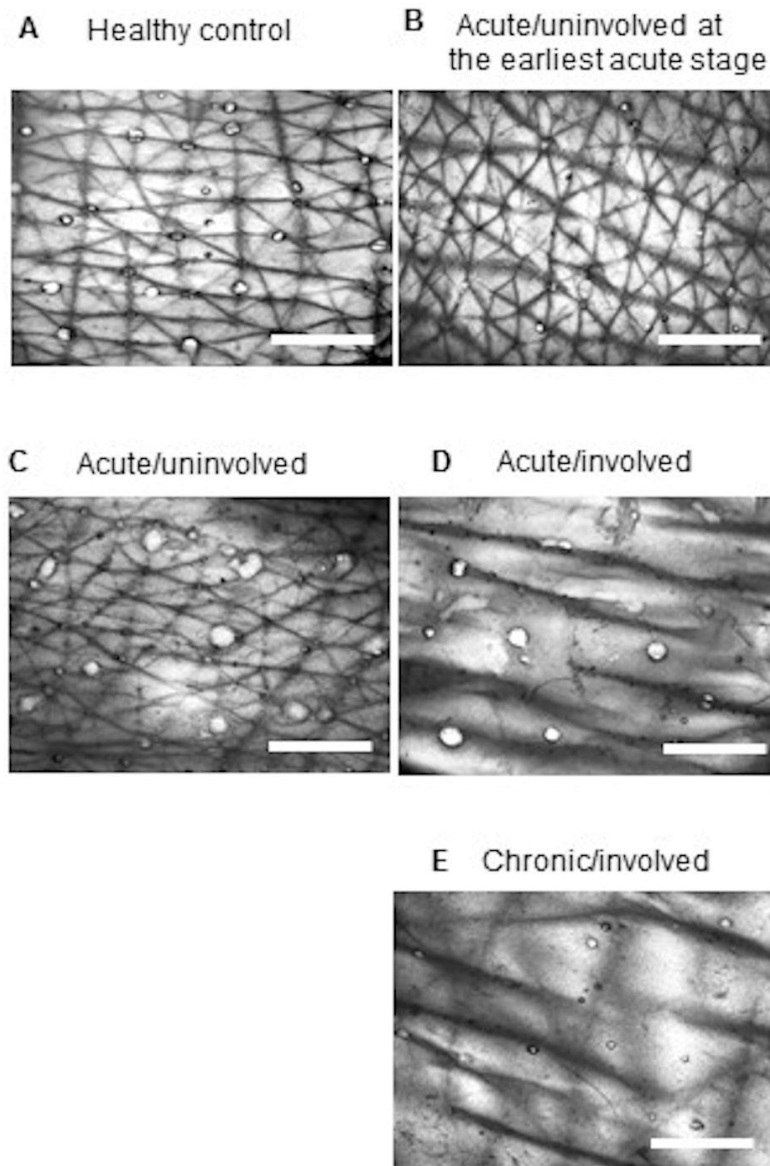
Figure 4. Detection of dermcidin and Ach-RM3 in the involved skin of acute AD lesions and psoriasis lesions. Sections obtained from AD (A and C) and psoriasis (B and D) lesions obtained 30 min after thermal stimulus were double-stained for dermcidin (blue) and Ach-RM3 (red-brown). Bar= $100\mu\text{m}$

Figure 1



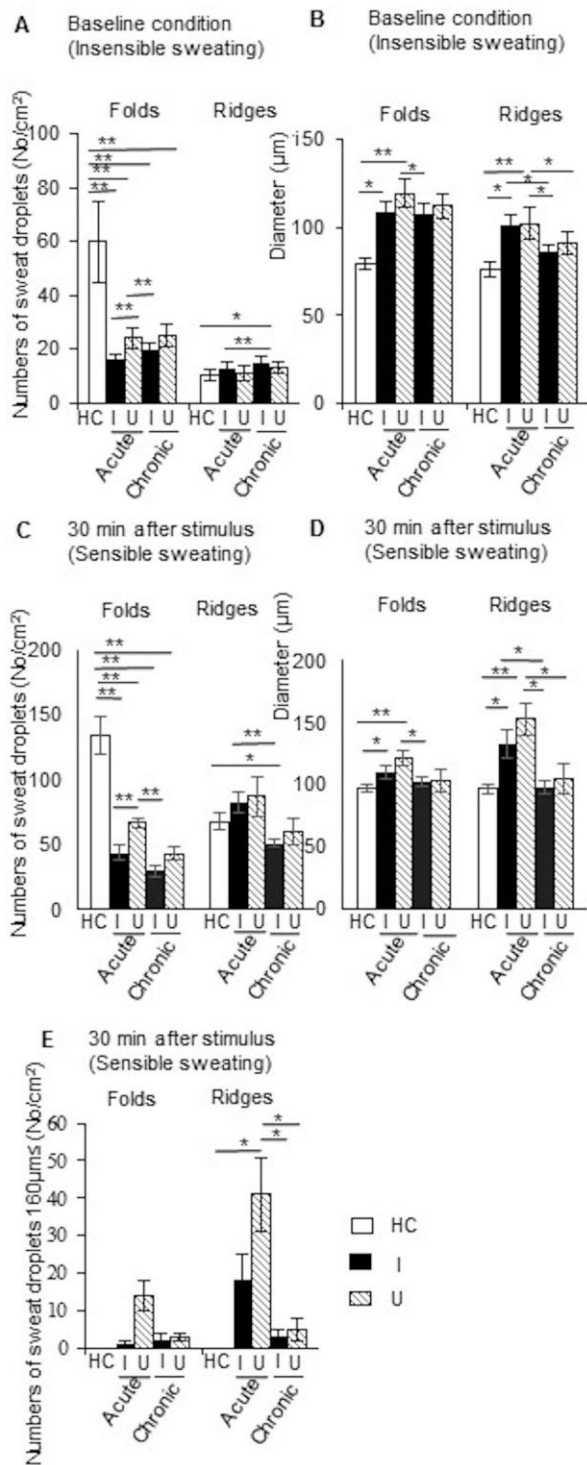
Representative silastic molds from the forearm of a healthy control before (baseline condition) (A) and after (B,C) thermal stimulus. The mean number (D) and diameter (E) of sweat droplets from 12 controls. * $p < 0.05$ †n.s.: not significant Bar=500μm

Figure 2



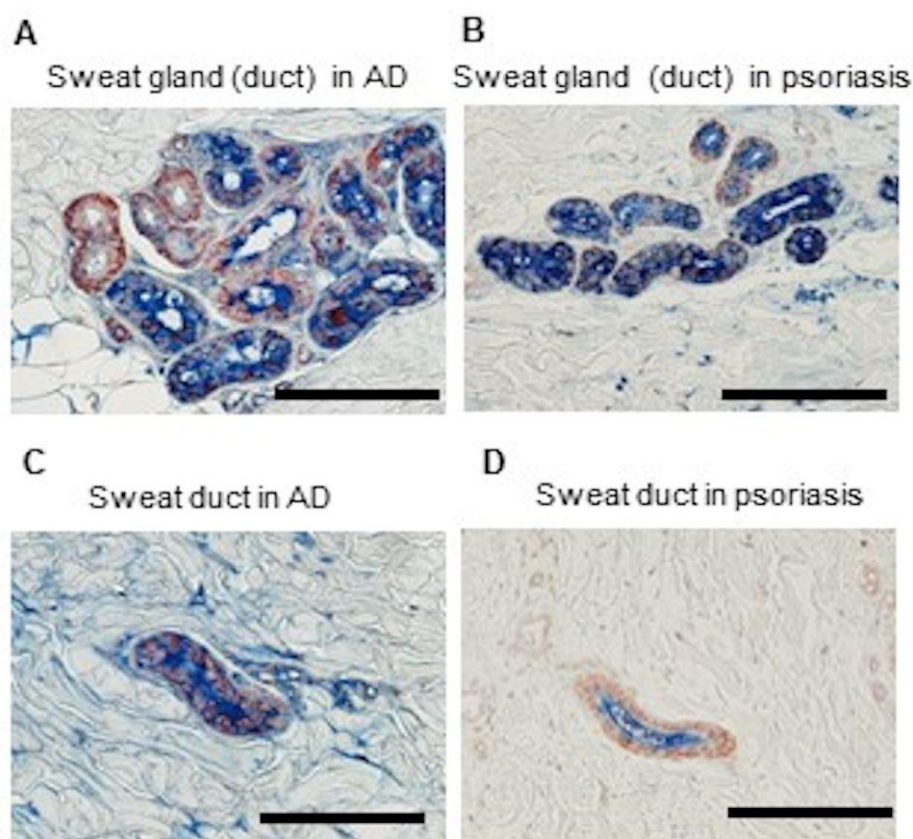
Representative silastic molds from a healthy control (A), 'normal-appearing' uninvolved (B, C) and the involved areas (D, E) of the ventral forearm of AD patients in the acute (B-D) and chronic (E) stages 30 min after thermal stimulus. In some uninvolved areas, samples were also obtained at the earliest asymptomatic acute stage (B). Bar=500 μ m

Figure 3



The mean number and size of sweat droplets at the folds and ridges before (A, B) (insensible sweating) and 30min after (C, D) thermal stimulus (sensible sweating) in controls and AD. E. The mean number of sweat droplets $\geq 160 \mu\text{m}$ at the folds and ridges (sensible), respectively. HC, healthy control; I, involved areas; and U, uninvolved areas. * $p < 0.05$, ** $p < 0.01$.

Figure 4



Detection of **dermcidin** and Ach-RM3 in the involved skin of acute AD lesions and **psoriasis** lesions. Sections obtained from AD (A and C) and **psoriasis** (B and D) lesions obtained 30 min after thermal stimulus were double-stained for **dermcidin** (blue) and Ach-RM3 (red-brown). Bar=100µm