

Differential mRNA Expression of Antimicrobial Peptides and Proteins in Atopic Dermatitis as Compared to Psoriasis Vulgaris and Healthy Skin

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Key Words

Antimicrobial peptides • Atopic dermatitis • Psoriasis vulgaris • Human β -defensin • Psoriasin • LL-37 • RNase 7

Abstract

Background: Patients with atopic dermatitis (AD) are prone to have skin infections. We aimed to investigate mRNA expression levels of various antimicrobial peptides and proteins (AMPs) in AD patients, and compare it with psoriasis vulgaris (PV) patients and healthy subjects. **Methods:** Skin biopsies were obtained from healthy subjects and patients with AD and PV. Quantitative real-time RT-PCR was used to determine the mRNA levels of human β -defensin (hBD)-1, hBD-2, hBD-3, LL-37, psoriasin, RNase 7, interferon- γ , and interleukin-10 (IL-10). **Results:** Except for LL-37, mRNA of hBDs, psoriasin, and RNase 7 was significantly higher expressed in AD ($n = 42$) and/or PV ($n = 35$) patients when compared to controls ($n = 18$). While PV lesions showed significantly higher mRNA hBD-2 levels than lesions of AD, the latter was associated with significantly higher mRNA levels of RNase 7 when compared to PV. A significant positive correlation of hBD expression was observed both in AD patients and PV patients. hBD mRNA levels of AD skin correlated with psoriasin and RNase 7 levels. hBD-1 mRNA expression correlated with AD activity and IL-10 mRNA expression. **Conclusions:** Most AMPs investigated in this study proved to be overexpressed in AD as well as PV when compared to controls.

However, a statistically significant difference in AMP mRNA expression between AD and PV was only found for hBD-2 and RNase 7. A moderate-to-strong linear relationship between the mRNA expression of particular AMPs appears to exist in AD, and to a lesser extent in PV as well.

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Introduction

Epithelia not only serve as physical barriers against infection but also mount an innate immune response by producing antimicrobial peptides (AMPs) to neutralize invading microbes. AMPs are abundant and widely distributed effector molecules of the innate immune response from insects to human and are active against a broad spectrum of Gram-positive and Gram-negative bacteria, as well as some fungi and enveloped viruses. The key role of AMPs in mammalian host defense is made evident by mice with disrupted AMP genes that are prone to infection in the affected epithelial organs, including the skin [1, 2]. In humans, the role of epithelial AMPs in host defense is suggested by studies of two major human skin diseases with a defective skin barrier, atopic dermatitis (AD) and psoriasis vulgaris (PV). Both skin conditions are chronic inflammatory disorders that may share some clinical and histological features [3, 4]. However, patients with AD are much more prone to have skin

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infections than are patients with PV, and this difference has now been linked to the much higher expression of AMPs in PV than in AD [5, 6]. Indeed, some of the major AMPs of human skin, such as defensins, were originally isolated from the scales of patients with PV. Although inflamed epithelia are a particularly rich source of AMPs, the molecular events that lead to increased expression of the different AMPs in epithelia in response to infection and inflammation are incompletely understood [7].

The human β -defensin (hBD) constitutes the major class of AMPs found in human epithelia, and three β -defensins – hBD-1, hBD-2, and hBD-3 – are expressed in the skin. During inflammation, the expression of hBD-2 in inflamed skin is induced by interleukin-1 from resident monocyte-derived cells, which establishes a clear link between infection/inflammation and hBD-2 expression. Inflamed skin and other epithelia greatly increase the production of hBD-3, which may be influenced by cytokines, growth factors, and bacterial challenge [1, 2]. The expression of hBD-1 has been deemed constitutive, but there is evidence that it also may be somewhat increased by inflammation [7]. Another AMP of potential importance in human skin defense is the cathelicidin LL-37 which is particularly expressed by keratinocytes and neutrophils at sites of inflammation. In contrast to psoriasis, decreased expression of LL-37 (as well as hBD-2, hBD-3) has been detected in skin biopsies of patients suffering from AD [8–10]. Psoriasin, a protein, also known as S100A7, was originally discovered in psoriatic skin lesions. Keratinocytes seem to secrete psoriasin as their main *Escherichia coli*-specific bactericidal compound in vitro and in vivo. This may explain the natural resistance of skin against colonization by *E. coli* [11]. RNase 7 is a protein that is constitutively produced in healthy human skin. The amount isolated per gram of psoriatic-scale material was found to be higher than isolated from normal stratum corneum extracts [12]. It was therefore suggested that the expression of RNase 7 might be inducible, despite the high level of constitutive expression in healthy skin. As has been found in the case of hBD-2 and hBD-3, it was demonstrated that bacteria can also upregulate the expression of RNase 7 [12]. Pro-inflammatory cytokines, including interferon- γ (INF- γ) and tumor necrosis factor- α (TNF- α), have been shown to induce the expression of AMPs. These Th1 lymphocyte-released cytokines are highly expressed in psoriasis skin, whereas negligible levels of INF- γ and TNF- α have been shown in AD skin [8, 9]. By contrast, Th2 lymphocyte-released cytokines such as IL-10 and IL-13 have been shown to be overexpressed in AD and may cause downregulation of AMPs in this

disease [8, 9]. In this study, we aimed to investigate the mRNA expression of AMPs in the skin of AD patients and compare it with AMP mRNA expression in PV skin and healthy subjects.

Material and Methods

Patients

In this prospective study, we recruited patients diagnosed with extrinsic AD including a history of allergic rhinitis and/or asthma, elevation of total serum IgE, specific IgEs (e.g. house dust mite), and a Six Area, Six Sign, AD score over 30 (SASSAD) [13, 14]. Moreover, we recruited patients diagnosed with PV (plaque type) who had a psoriasis area and severity index over 10 [4]. Study exclusion criteria included frank evidence for skin infection, any internal immunosuppressive or antibiotic therapy within the last 4 weeks, and any topical treatment within the last 2 weeks except for the use of emollients. In addition to the patient groups, healthy, nonatopic individuals were used as controls. The study was conducted in the light of the declaration of Helsinki and followed a protocol approved by our institutional review board. All subjects who participated in the investigation signed an informed consent. In all patients and controls, one 4-mm full-thickness skin specimen was taken from chronic lesional and healthy skin of the upper extremities, respectively.

Real-Time RT-PCR

Quantitative analysis of real-time RT-PCR was performed as previously suggested [15]; total cellular RNA was isolated from skin tissue samples using RNeasy® Lipid Tissue Kit (Qiagen, Chatsworth, Calif., USA) following the manufacturer's protocol. Prior to cDNA synthesis RNA was digested with RNase-free DNase I (Roche Diagnostics, North America). cDNA was synthesized by reverse transcription from DNase I-treated RNA using MultiScribe™ reverse transcriptase enzyme and random hexamers primers (TagMan® Reverse transcription reagents, Applied Biosystems, Foster City, Calif., USA). Real-time PCR was performed using a Power SYBR® Green PCR Master Mix and GeneAmp® Sequence Detection System (Applied Biosystems). PCR primers for AMPs, cytokines, and the housekeeping gene (RPL-38) were designed using the computer program Primer Express (PE Biosystems) and produced by the custom oligonucleotide synthesis service TIB Molbiol (Germany). All primers were checked for cDNA specificity using the BLAST program (<http://www.ncbi.nlm.nih.gov/BLAST/>). The primers for AMPs, IL-10, INF- γ , and RPL-38 are given in table 1. PCR amplifications were performed in a total volume of 25 μ l, containing 5 μ l cDNA sample, 5 μ M of each primer and 12.5 μ l Power SYBR® Green PCR Master Mix. PCR was started with 2 min at 50°C and an initial 10-min denaturing temperature of 95°C, followed by a total of 40 cycles of 15 s of denaturing and 1 min of annealing and elongation at 60°C. In order to identify all amplified PCR products, melting curve analysis was performed. The reaction products were separated by 2% agarose gel electrophoresis. Relative mRNA expression levels were calculated using the comparative $\Delta\Delta C_t$ method as previously suggested by Livak and Schmittgen [16]. Quantities of all targets in the test samples were normalized to the homoge-

Table 1. Primers of AMPs, cytokines and the housekeeping gene used in the RT-PCR study

Primer	Sequence
hBD-1	F 5'-AGATGGCCTCAGGTGGTAACTTT-3'; R 5'-GGGCAGGCAGAATAGAGACATT-3'
hBD-2	F 5'-TGATGCCTCTTCCAGGTGTTT-3'; R 5'-GGATGACATATGGCTCCACTCTT-3'
hBD-3	F 5'-TCCATTATCTTCTGTTTGTCTTGC-3'; R 5'-TTCTGTAATGTGTTTATGATTCTCCAT-3'
LL-37	F 5'-AGGATTGTGACTTCAAGAAGGACG-3'; R 5'-GTTTATTTCTCAGAGCCCAGAAGC-3'
Psoriasin	F 5'-CAACTTCCTTAGTGCCTGTGACAA-3'; R 5'-CCAGCAAGGACAGAACTCAGAA-3'
RNase 7	F 5'-GAAGACCAAGCGCAAAGC-3'; R 5'-CAGCAGAAGCAGCAGAAGG-3'
IL-10	F 5'-GGCGCTGTCATCGATTTCTT-3'; R 5'-CTCTTGGAGCTTATTAAAGGCATTCT-3'
IFN- γ	F 5'-CAGGTCATTCAGATGTAGCGGATA-3'; R 5'-TCTGTCACTCTCCTCTTTCCAATTC-3'
RPL-38	F 5'-TCACTGACAAAGAGAAGGCAGAGA-3'; R 5'-TCAGTGTGTCTGGTTCATTTTCAGTT-3'

	Medians and range of mRNA expression		
	Atopic dermatitis	Psoriasis vulgaris	Healthy controls
hBD-1	2.1 (0.38–35.8)	0.88 (0.18–3.63)	0.72 (0.14–3.2)
hBD-2	1.2 (0.01–47.9)	21.1 (0.32–125.5)	0.004 (0.001–0.027)
hBD-3	3.56 (0.06–24.1)	3.2 (0.42–14.3)	0.059 (0.006–0.12)
LL-37	0.003 (0.001–0.028)	0.004 (0.00–0.06)	0.004 (0.001–0.029)
Psoriasin	168.7 (2.1–874.3)	202.5 (19.3–1926.7)	5.0 (0.31–44.1)
RNase 7	13.4 (0.4–56.9)	4.2 (0.58–20.9)	2.5 (1.1–4.4)
IL-10	0.056 (0.007–0.32)	n.d.	n.d.
IFN- γ	0.002 (0.00–0.029)	n.d.	n.d.

Medians as well as range of mRNA expression levels of AMPs in patients with atopic dermatitis, psoriasis vulgaris, and healthy controls. n.d. = Not done.

neously expressed housekeeping gene RPL-38. The quantities of mRNA levels were determined using the median log transform of the gene expression.

Statistical Analysis

Nonparametric analyses of the real-time RT-PCR results were used because a non-normal distribution was observed in subsets of data (D'Agostino-Pearson test). The Kruskal-Wallis test was applied to test for overall significant differences of data between groups. If significant differences were detected by the Kruskal-Wallis test, a Mann-Whitney test (unpaired data) was used for post hoc analysis after the Kruskal-Wallis test. Spearman's coefficient of rank correlation was assessed within groups (AD, PV, controls, respectively). Bonferroni-Holm correction for multiple testing was applied for pairwise comparisons of groups. Corrected values of $p < 0.05$ were considered significant.

Results

In total, we studied 42 patients (28 men and 14 women; mean age 37.5 ± 9.9 years) diagnosed with extrinsic AD and 35 patients (15 men and 17 women; mean age 43.2 ± 11.3 years) diagnosed with PV. Moreover, 18 healthy individuals (12 men and 6 women; mean age 39.7 ± 10.8 years)

were included as control subjects. Data of the SASSAD score and the RT-PCR results were non-normally distributed. The median SASSAD score was 39.5 (32–78). Medians and range of mRNA expression levels of AMPs and cytokines are described and illustrated in detail in table 1 and figures 1–6, respectively. mRNA of hBD-1 was significantly higher expressed in AD when compared to healthy controls ($p < 0.05$). hBD-1 mRNA levels of AD and PV did not significantly differ ($p > 0.05$), although there was a trend for higher hBD-1 levels in AD. For hBD-2, the highest mRNA levels were observed in PV which significantly differed from AD and controls ($p < 0.05$). hBD-2 mRNA expression in AD also significantly differed from controls ($p < 0.05$). hBD-3 mRNA was significantly higher expressed in AD and PV as compared to controls ($p < 0.05$). However, hBD-3 mRNA levels of AD and PV did not significantly differ ($p > 0.05$). All in all, LL-37 expression was relatively weak in AD, PV, and controls. No significant difference of LL-37 mRNA expression was observed between AD, PV, and controls. In AD and PV, psoriasin and RNase 7 mRNA expression was significantly higher than in healthy controls ($p < 0.05$). RNase 7 mRNA levels of AD skin were significantly higher than those observed in PV

Fig. 1. mRNA expression of hBD-1 in the skin of healthy controls (n = 18) and patients with AD (n = 42) and PV (n = 35). Significant difference between AD and healthy controls (* p < 0.05). Median indicated by lines. Error bars denote range.

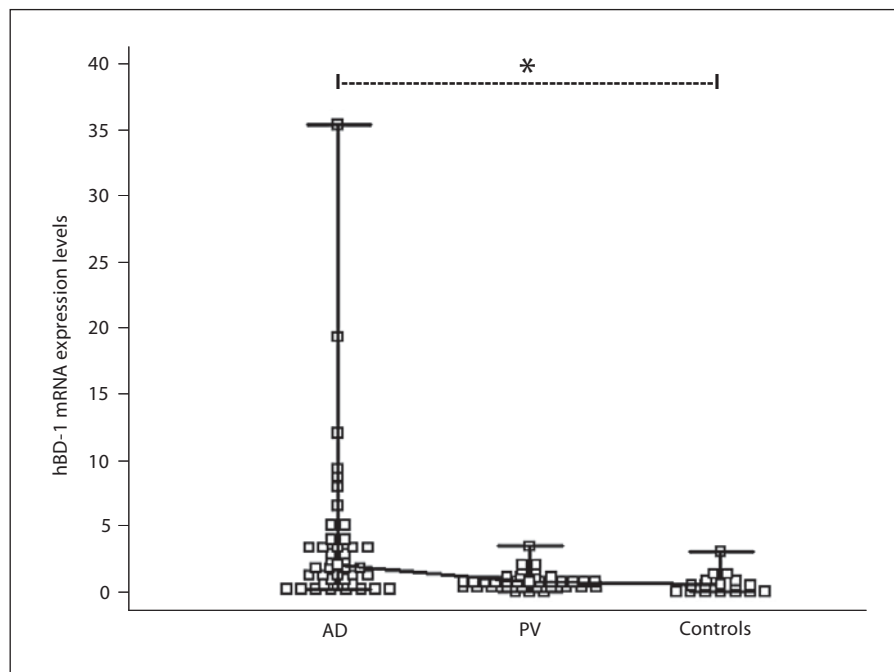
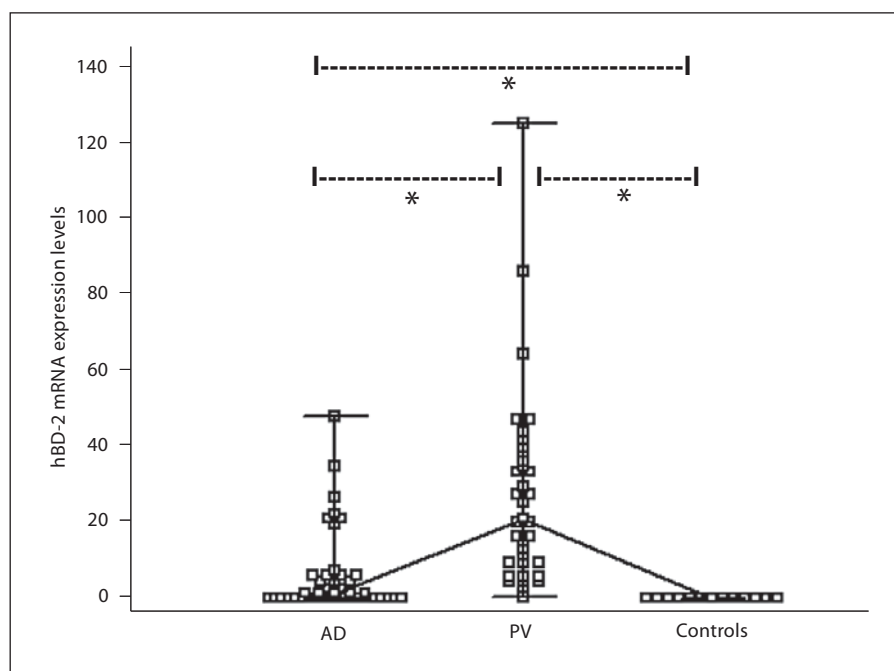


Fig. 2. mRNA expression of hBD-2 in the skin of healthy controls (n = 13) and patients with AD (n = 42) and PV (n = 35). Significant differences between both two groups (* p < 0.05). Median indicated by lines. Error bars denote range.



(p < 0.05). Nevertheless, psoriasin mRNA levels did not significantly differ between AD and PV (p > 0.05).

The SASSAD score weakly correlated with hBD-1 and RNase 7 mRNA expression (r = 0.34, p < 0.05; r = 0.43, p < 0.05, respectively). In AD patients, correlations of mRNA expression between hBD-1, hBD-2, and hBD-3 were significant (r = 0.5 to 0.72; p < 0.05). mRNA levels

of hBDs also significantly correlated with psoriasin (r = 0.5 to 0.8; p < 0.05) and RNase 7 (r = 0.5 to 0.66; p < 0.05) mRNA levels. In AD patients, however, no significant correlation was found between hBD expression and LL-37 (p > 0.05). Expression of INF- γ mRNA levels was very weak in AD skin and did not correlate with AMPs levels. IL-10 weakly to moderately correlated with

Fig. 3. mRNA expression of hBD-3 in the skin of healthy controls (n = 18) and patients with AD (n = 42) and PV (n = 35). Significant differences between AD and controls as well as PV and controls (* p < 0.05). Median indicated by lines. Error bars denote range.

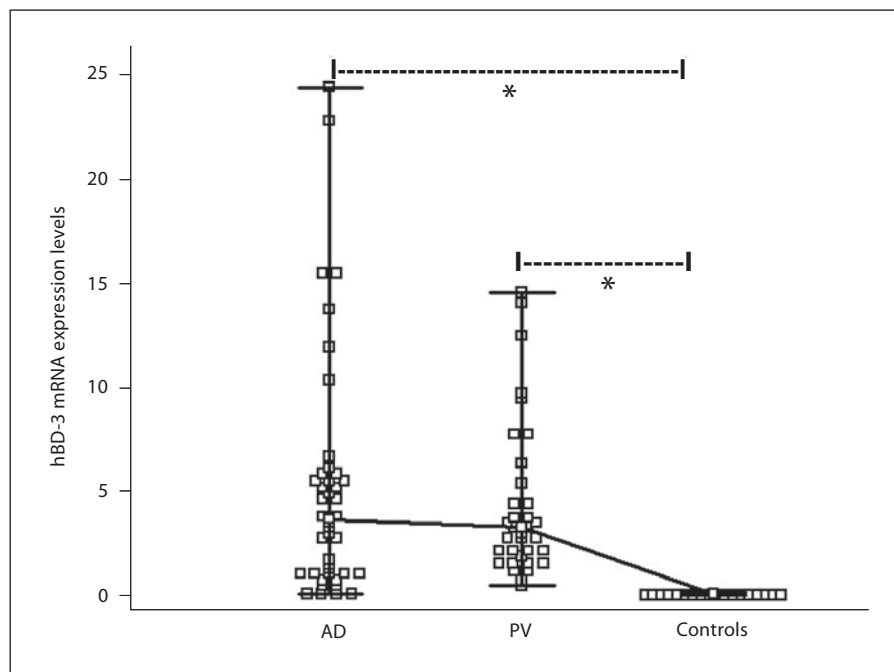
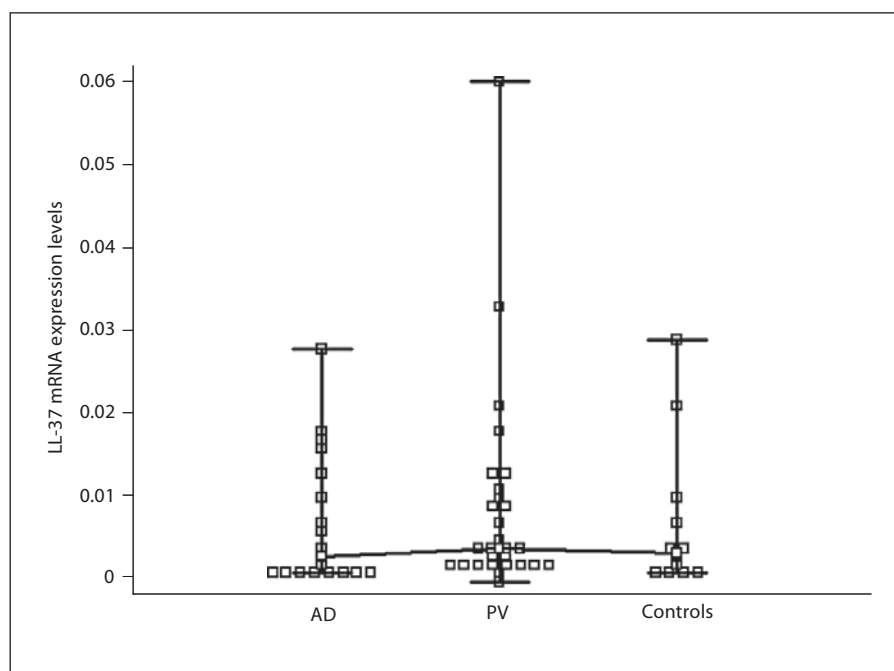


Fig. 4. mRNA expression of LL-37 in the skin of healthy controls (n = 12) and patients with AD (n = 19) and PV (n = 28). There were no significant differences between groups (p > 0.05). Median indicated by lines. Error bars denote range.



psoriasis and hBD-1 ($r = 0.37$, $p < 0.05$; $r = 0.54$, $p < 0.05$). In PV patients, hBD mRNA levels significantly correlated with each other ($r = 0.55$ to 0.72 ; $p < 0.05$). However, there was no significant correlation between hBDs and psoriasis, LL-37, and RNase 7 ($p > 0.05$). In healthy controls, hBDs did neither correlate with each other nor with psoriasis, LL-37, and RNase 7 ($p > 0.05$).

Discussion

AD and PV are two distinct clinical entities characterized by various different clinical and histological features depending on the stage of the lesion. These clinical and histological characteristics are most apparent in fully developed acute lesions. In the chronic phase of the disease,

Fig. 5. mRNA expression of psoriasin in the skin of healthy controls (n = 18) and patients with AD (n = 42) and PV (n = 35). Significant differences between controls and AD and PV (* p < 0.05). Median indicated by lines. Error bars denote range.

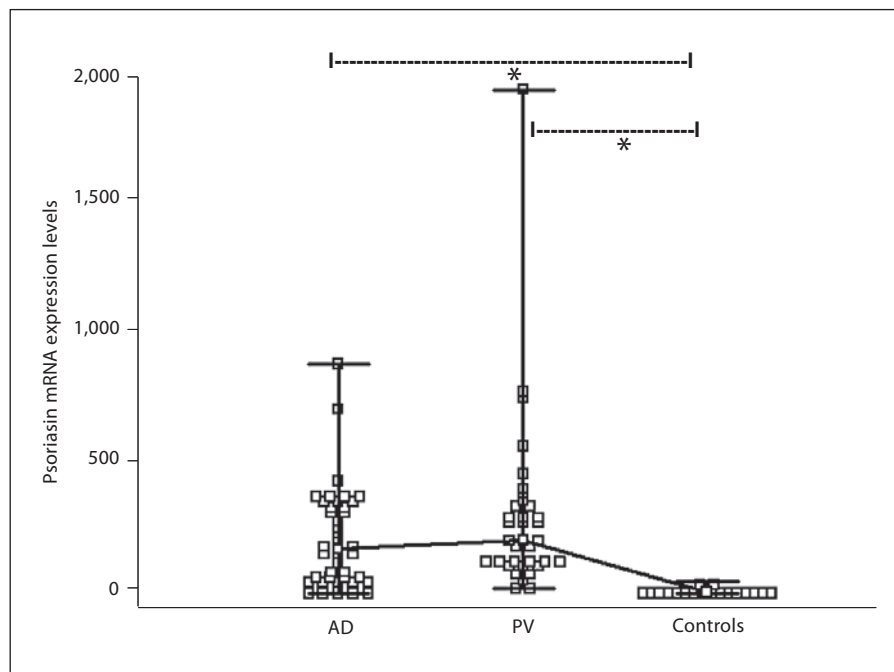
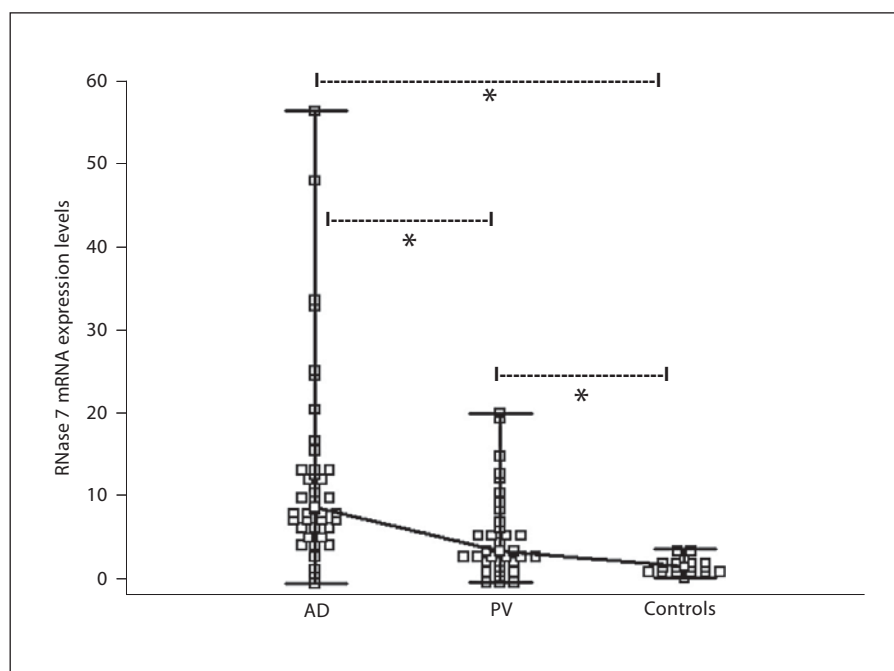


Fig. 6. mRNA expression of RNase 7 in the skin of healthy controls (n = 18) and patients with AD (n = 42) and PV (n = 35). Significant differences between both two groups (* p < 0.05). Median indicated by lines. Error bars denote range.



the differences are less pronounced [3, 4]. However, a striking difference is the high frequency of bacterial colonization of nonlesional AD skin and recurrent skin infections by viral, bacterial, and fungal pathogens in lesional skin of AD patients [5]. In contrast, Henseler and Christophers [6] demonstrated that psoriasis patients have an increased resistance to bacterial and viral infec-

tions compared with AD patients and controls. Recently, the work of the group of Leung has raised the intriguing possibility that the increased susceptibility of AD patients for skin infections may be related to low expression levels of AMPs when compared with psoriasis patients [8, 9, 17]. Particularly, it has been shown that mRNA expression of the two AMPs hBD-2 and LL-37 are significantly

decreased in lesional AD skin compared with lesional psoriatic skin [8].

From the statistical point of view, the validity of previous data on AMP expression in AD and PV is limited because most studies included less than 15 patients per group [8, 9, 17–20]. We therefore aimed to investigate AMPs expression in a larger population. Interestingly, we observed a positive correlation of hBDs both in AD patients and PV patients. hBD mRNA levels of AD skin also positively correlated with psoriasin and RNase 7 levels indicating that the mRNA expression of AMPs may be relatively uniformly orchestrated. Our data confirm results of previous studies showing that hBD-2 appears to be the strongest and most psoriasis-specific AMP known so far [8, 9, 18, 20]. hBD-2 expression is usually very low in normal skin. hBD-2 exhibits antimicrobial activity primarily against Gram-negative bacteria such as *E. coli* and *Pseudomonas aeruginosa*. It is also active against the yeast *Candida albicans* but not against Gram-positive *Staphylococcus aureus*, against which it only demonstrates bacteriostatic activity at very high concentrations [7]. In psoriatic skin hBD-2 is significantly higher induced at the mRNA level when compared to normal skin as well as AD skin [7]. In lesional skin of AD patients, however, hBD-2 is also significantly induced at the mRNA level compared with normal skin. At the protein level, hBD-2 is only occasionally found in the epidermis [18, 20]. Although the expression of hBD-1 has been deemed constitutive [7, 21], our data indicate that it also may be increased by inflammation. In the present study, AD and PV skin showed overexpression of hBD-1 and hBD-3 mRNA compared to healthy skin, respectively. Interestingly, we have also observed that hBD-1 mRNA expression significantly correlates with AD activity and IL-10 mRNA expression. In smaller studies, however, hBD-1 has previously been reported to be decreased in lesional skin of AD patients compared to normal individuals [19], and expressed higher in PV than AD, respectively [9]. Moreover, we found that the expression of hBD-3 mRNA in AD and PV is not different. This is contradictory to previous studies showing that hBD-3 augments in PV and decreases in AD probably due to the abundance of Th2 cytokines that may downregulate hBD-3 induction [9, 21]. Unlike the research group of Leung [8, 9, 17], de Jongh et al. [18] and our working group did not find a significant overexpression of LL-37 in the skin of patients with AD or PV. Thus, the significance of LL-37 in AD and PV remains controversial. The aforementioned discrepancies of data reported in previous studies are likely due to differences in methodology. It is probably of particular

significance whether patients with intrinsic or extrinsic AD are recruited, and whether biopsies are harvested from acute or chronic lesions. The actual microbial colonization of skin investigated is also of importance – most studies including the present trial failed to assess the status of microbial skin colonization by means of appropriate methods. Furthermore, it is of significance whether pure epidermis or full thickness skin samples are investigated by RT-PCR. In the present study, we focused on the evaluation of chronic lesions of extrinsic AD using full thickness skin biopsies. The currently accepted model of AD proposes a predominant Th2 cytokine milieu in the initiating stages of acute AD lesions, and a rather mixed Th1 and Th2 pattern in chronic lesions [3]. The latter may not strictly be dominated by a Th2 cytokine-driven downregulation of AMPs as observed in acute lesions of AD [9, 17, 21].

Little is known about the mRNA expression of psoriasin and RNase 7 in the skin of AD and PV patients [11, 12]. In the present study, we observed a similar overexpression of psoriasin mRNA in AD and psoriatic skin when compared to healthy controls. Notably, mRNA levels of RNase 7 were significantly higher in AD patients as compared to PV patients and controls. Similar to hBD-2 in psoriatic skin, RNase 7 may be a specific AMP in AD skin. RNase 7 exhibits broad-spectrum antimicrobial activity against Gram-negative bacteria (e.g. *P. aeruginosa*, *E. coli*), Gram-positive bacteria (e.g. *S. aureus*, *Propionibacterium acnes*), and the yeast *C. albicans*. RNase 7 possibly represents on a per-molar base, the most potent and efficacious human AMP known so far [12]. RNase 7 is constitutively produced in healthy human skin. However, it has been suggested that the expression of RNase 7 may also be induced by bacteria [12]. Hence, the overexpression of RNase 7 mRNA in lesions of AD may be due to an increased lesional colonization of microbes. Limitations of the present study include the absence of immunohistochemical studies in order to confirm the presence and localization of differently expressed genes on the protein level as well. As also performed in previous studies, we used full-thickness skin biopsies for RT-PCR analysis [8]. Of course, an approach of using pure epidermis would have minimized the contribution of infiltrating cells and cells normally present in full thickness skin biopsies, such as hair follicles, sweat glands, fibroblasts, etc. [18]. For example, cathelicidins and defensins are also produced by mast cells and inflammatory cells, respectively. Nevertheless, the extent of AMP production within subepithelial skin compartments is probably negligible.

In conclusion, we have studied mRNA expression of various AMPs in a relatively large number of patients with AD and PV. Most AMPs investigated in this study proved to be significantly overexpressed in lesions of AD as well as PV. A significant difference in AMP mRNA expression between AD and PV was found for hBD-2 and RNase 7. While PV lesions show significantly higher mRNA hBD-2 levels than lesions of AD, the latter is associated with significantly higher mRNA levels of RNase 7 when compared to PV. Interestingly, hBD-1 mRNA ex-

pression significantly correlates with AD activity and IL-10 mRNA expression. A moderate-to-strong linear relationship between the mRNA expression of particular AMPs appears to exist in AD, and to a lesser extent in PV. From the clinical point of view, our results indicate that hBD-2 mRNA downregulation might be one of the major immunological alterations which is possibly associated with increased susceptibility of AD patients for skin colonization and/or infection by microbes others than *S. aureus*.

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