Communications

Gingival Crevicular Fluid Zinc- and Aspartyl-binding Protease Profile of Individuals with Moderate/Severe Atopic Dermatitis.

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Abstract: Atopic dermatitis (AD) is a protease-modulated chronic disorder with heterogenous clinical manifestations which may lead to an imprecise diagnosis. So far, there are no diagnostic protease tests for AD. We explored the gingival crevicular fluid (GCF) protease profile of periodontally-healthy individuals with moderate/severe AD compared to healthy controls. An exploratory case-control study was conducted. Matching AD patients (n=6) and controls (n=6) were enrolled at the International Center for Clinical Studies, Santiago, Chile. Complete dermatological and periodontal evaluations (involving the collection of GCF samples) were made. The levels of 35 proteases were analyzed using a human protease antibody array. The GCF levels of zinc-binding ADAM8, ADAM9, MMP8 and Neprilysin/CD10, aspartyl-binding Cathepsin E, and serin-binding Protein convertase9 and uPA/Urokinase proteases were lower in moderate/severe AD patients compared to controls (p<0.05). No inter-group differences in the levels of the other 28 proteases were found. MMP8, Cathepsin E and ADAM9 were the biomarkers with the highest sensitivity and specificity regarding the detection of AD (p < 0.05). The area under receiver operating characteristic (ROC) curve for MMP-8+ADAMP-9 was 0.90. In conclusion, differences in the protease profile between AD and control patients associated with MMP8, Cathepsin E and ADAM9. MMP8, ADAM9 and Cathepsin E may be useful as combined diagnostic and therapeutic biomarkers of moderate/severe AD.

Keywords: Enzymes; Peptide Hydrolases; Metalloproteases; Biomarkers; Gingival Crevicular Fluid; Atopic Dermatitis

1. Introduction

Atopic dermatitis (AD) is the most common chronic inflammatory skin disease in humans worldwide [1]. The prevalence of AD in young adults is high, with an estimated value between 10%

and 34.1%, and its persistence over time is common, greatly affecting the quality of life of those who suffer from it [2].

The etiology of AD is multifactorial and involves the interaction of environmental, genetic, and immune system factors. The pathogenesis of AD is complex and combines cutaneous barrier dysfunction, systemic immune dysregulation, dysbiosis of the bacterial microbiome of the skin and genetic factors. Initially, atopic dermatitis patients have a predisposition to maintain T helper (Th) 2 lymphocyte response, while a change in the response from Th2 to Th1 promotes chronicity of the disease [1,3].

Besides the immune-inflammatory response, the abnormal expression or activity of proteases has been associated with the pathogenesis of AD. Many zinc- and serine-binding proteases are up-regulated or down-regulated in AD lesion/serum samples and seems to play a role in the disruption of the normal skin-barrier function. Also, the increased protease activity in AD leads to permeability barrier dysfunction, inflammation, and defects in the antimicrobial barrier. Because of the proteolytic effects, external antigens of AD can easily invade the epidermis, resulting in dermatitis, coupled with the induction of Th2 cytokines [4-6].

AD clinical manifestations are heterogeneous, and its clinical diagnosis is highly complex. Severely pruritus is the most frequent clinical manifestation and is triggered by heat, perspiration and emotional stress among others [3]. The clinical characterization of AD individuals might not satisfactorily reflect the pathophysiologic profile of patients with AD. The use of biomarkers may help in better defining the clinical heterogeneity of the disease and contribute to its treatment. Currently, there are no biomarkers that can differentiate the disease from other entities or indicate the clinical status of AD in adults[3,7].

Gingival crevicular fluid (GCF) is a transudate harvesting components from local periodontal tissues and serum that can be obtained from the gingival crevice surrounding the teeth. GCF represents a source of a wide range of biological molecular markers allowing the diagnosis, monitoring, prognosis and severity of several diseases in a non-invasive approach[8]. Accordingly, a recent study supports the feasibility of measuring endothelial and placental biomarkers in maternal GCF [9]. Also, biomarkers of inflammation have been considered as biomarker in the progression of coronary heart disease and chronic periodontitis [10]. Therefore, we hypothesize that atopic dermatitis patients show a differential GCF protein profile from healthy individuals, allowing the identification of potential biomarkers for point-of-care monitoring of atopic dermatitis in adults. Hence, the aim of this study was to explore the protease profile of GCF samples in individuals with moderate/severe atopic dermatitis in relation to healthy controls.

2. Materials and Methods

2.1. Study Design

This case control study was approved by the Bioethics Committee of the Faculty of Dentistry of Universidad Andres Bello (No. PROPRGFO_002001980). All individuals were informed about the objective of the investigation and signed an informed consent. All the process was in accordance with the ethical standards of the institutional and/or national research committee and with the 1975 Helsinki declaration, and its later amendments or comparable ethical standards.

2.2. Population and Dermatological Evaluation

Patients with atopic dermatitis and systemically healthy controls evaluated at the International Center for Clinical Studies (CIEC) Santiago, Chile, between March and July of 2019 and selected for convenience. The diagnosis of atopic dermatitis was based on the medical history and physical examination made by a dermatologist. Measured clinical atopic dermatitis variables included: The Scoring Atopic Dermatitis (SCORAD) scale, the Eczema Area and Severity Index (EASI), the Body Surface Area (BSA) index and the Investigator Global Assessment scale for Atopic Dermatitis (IGA). Inclusion criteria were adults with or without clinical diagnosis of AD and otherwise healthy, including the absence of periodontal diseases (gingivitis or periodontitis). Individuals with

periodontal diseases were excluded due to it modify the concentration of biological markers in the GCF [8]. The exclusion criteria were smokers, pregnant women, patients with any concomitant skin pathology, such as Psoriasis, Seborrheic Dermatitis, Contact Dermatitis, or individuals who had been treated with antibiotics, non-steroidal inflammatory drugs, immunomodulators orally or intravenously within the previous 3 months. Included patients were matched by gender and age. Due to the utilization a novel protease screening arrays and nature of this study, no sample size was determined.

2.3. Periodontal Evaluation

Intra-oral clinical examinations were conducted by periodontics specialist at the clinic. The periodontal examination was made with a manual periodontal probe. Clinical in full-mouth recordings at 6 sites in all teeth, including probing depths, the clinical attachment level, and bleeding on probing at the base of the crevice, excluding third molars. Periodontal diseases were defined in accordance with the study by Eke et al [11].

2.4. Sample Collection

GCF samples were collected by a qualified periodontist using sterile periodontal strips (Periopaper®, Interstate Drug Exchange, Amityville, NY, USA). Teeth were isolated with cotton rolls and then carefully dried with an air-syringe to prevent saliva contamination. Samples were collected by inserting the periodontal strips into the gingival sulcus of the first molar of each quadrant for 30 seconds. The four strips obtained per each individual were pooled. Afterwards, GCF samples were stored at -80°C until analysis.

2.5. Protease Antibody Array

Proteins from the GCF were extracted using a protein elution buffer (50 mM Tris-HCl pH 7.5, 0.2 mM NaCl, 5 mM CaCl2, and 0.01% Triton X-100) prepared into sterile tubes. Samples were incubated for half an hour at 4ºC and then centrifuged at 12,000 x g for 5 minutes at 4ºC. The procedure was repeated twice, and then the samples were frozen and kept at -20ºC until analysis, to reach a final elution volume of 160 µL.

50 µL of aliquots were mixed with a cocktail of biotinylated detection antibodies and incubated overnight with the Proteome Profiler Human Protease Array Kit (R&D systems, Minneapolis, USA). The nitrocellulose membranes were washed to remove unbound material. Streptavidin-HRP and chemiluminescent detection reagents were applied, and a signal was produced at each capture spot corresponding to the amount of protein bound. The membranes were exposed to x-ray film for 5 minutes and the signal intensities were quantified using a densitometric software (Bruker MI SE). The signal was measured in pixel density of the pair of duplicate spots representing each protease. A clear area of the array was used as a negative control. The relative expression level (REL) of the proteases was calculated using the following formula: REL = (cytokine signal intensity - mean intensity of negative control/mean intensity of positive control-mean intensity of negative control) x 100 [13].

2.6. Statistical Analysis

Non parametrical analysis was performed with Mann-Whitney U test. The biomarker levels were dichotomized using the median as threshold to determine the diagnostic ability and Odds ratios of each biomarker. The performance discrimination and diagnostic accuracy was evaluated through the construction of a receiver operating characteristic (ROC) curve, by calculating the area under the curve (AUC) of AD patients versus controls after logistic regression modeling. The level of significance was defined as $p<0.05$. The statistical analysis was performed using a statistical software, STATA 13®, Stata-Corp.

3. Results

This study included a total of 12 subjects: 6 healthy controls and 6 individuals with moderate to severe AD. All individuals were adults (≥18 years) with the same age in both groups: the median was 23.5 years and the range were from 20 to 39 years, male, non-smokers and with the same educational level.

Regarding the scoring systems for assessing the signs of AD [14], the results were: for SCORAD 49.5 median (45.8-85.7 range), for EASI 19.95 median (16.8-39.6 range), for BSA 39.8 median (18.5-55.4 range), and for IGA moderate (n=4) and severe (n=2).

In the present study 35 human proteases in GCF samples were evaluated. Results are summarized in Table 1. The intensity signals of the human protease profiles in the GCF of controls and AD individuals are shown in Figure 1. The levels of 35 human proteases analyzed in the GCF were compared between individuals with AD and healthy controls. Results are presented in Figure 2. The levels of 7 proteases, specifically ADAM8 (p=0.0327), ADAM9 (p=0.0094), Cathepsin E (p=0.0282), MMP8 (p=0.0179), Neprilysin/CD10 (p=0.0243), Protein convertase 9 (p=0.0282), and uPA/Urokinase (p=0.0433) were lower in AD patients compared to healthy controls. No inter-group differences were found in the other 28 proteases. The diagnostic ability of ADAM8, ADAM9, Cathepsin E, MMP8, Neprilysin/CD10, Protein convertase 9 (PCSK9) and uPA/Urokinase were further analyzed. Results are presented in Table 2. The biomarkers with the highest diagnostic precision were MMP8 followed by Cathepsin E and ADAM9 (p < 0.05). The other 36 proteases were not evaluated because inter-group differences were not statistically significant (p<0.05). Combining MMP-8 and ADAMP-9, the area under ROC curve was 90.28%, with a sensitivity and specificity of 83.33% respectively, Figure 3. A combined model of MMP8, Cathepsin E and ADAM9 was not considered because it did not converge.

Table 1. Human Protease Array membrane coordinates.

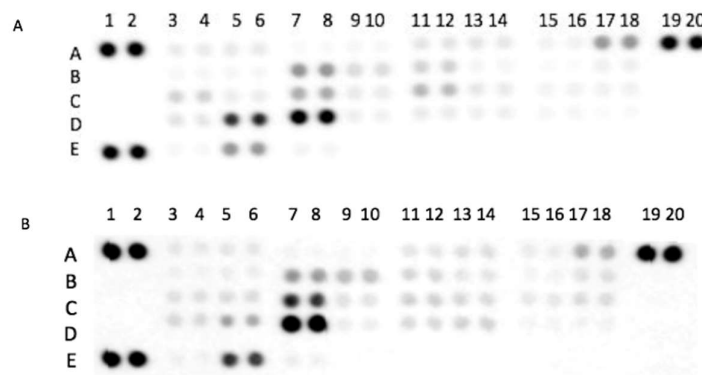|	1 - 2	3 - 4	5-6	7-8	9-10	11-12	13-14	15-16	17-18	19-20
A	RS[1]	ADAM 8	ADAM 9	ADAM TS 1	ADAMTS 13	Cathepsin A	Cathepsin B	Cathepsin C	Cathepsin D	RS[1]
B		Cathepsin E	Cathepsin L	Cathepsin S	Cathepsin V	Cathepsin X/Z/P	DPPIV / CD26	Kallikrein 3/PSA	Kallikrein 5	
C		Kallikrein 6	Kallikrein 7	Kallikrein 10	Kallikrein 11	Kallikrein 13	MMP-1	MMP-2	MMP-3	
D		MMP-7	MMP-8	MMP-9	MMP-10	MMP-12	MMP-13	Neprilysin / CD10	Presenilin	
E	RS[1]	Proprotein convertase 9	Protease 3	uPA / Urokinase	Negative Control					

[1] Reference Spot.

Figure 1. Original membranes of the protease profile in GCF from individuals with (**a**) atopic dermatitis and (**b**) systemically healthy controls (**b**). The following proteases were identified in the membranes: ADAM8 (A3-A4), ADAM9 (A5-A6), Cathepsin E (B3-B4), MMP8 (D5-D6), CD10 (D13-D14), Protein convertase9 (D17-D18) and uPA/Urokinase (E5-E5) with significant differences between both groups. Membranes per group (n=6).
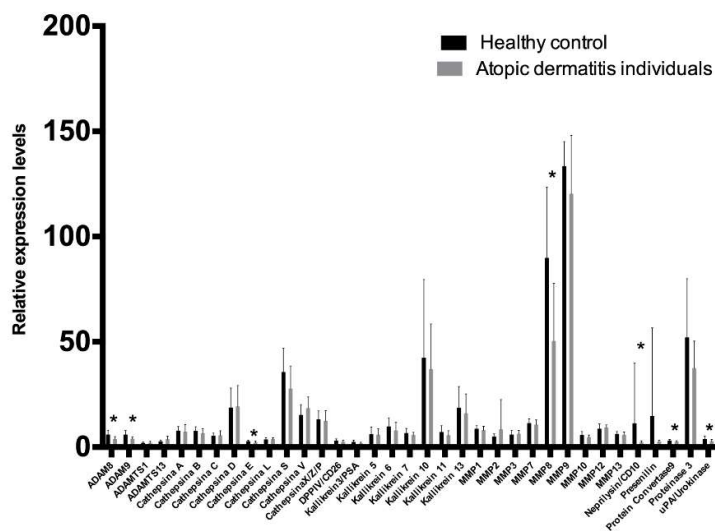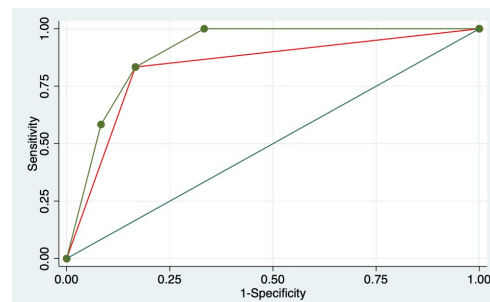

Figure 2. Relative levels of the analyzed proteases in GCF from healthy control and individuals with atopic dermatitis. Error bars represent interquartile range and asterisks $p < 0.05$.

Table 2. Individual Diagnostic Ability an Odds Ratios of Biomarkers.

Marker	Threshold	ROC Area (95% CI)	Sensitivity	Specificity	OR (95% CI)	p value
ADAM8	4,45	0.66 (0.46-0.86)	66,67%	66,67%	4.0 (0.73-21.83)	0,109
ADAM9	4,58	0.75 (0.56-0.93)	75%	75%	9.0 (1.41-57.11)	0.02*
Cathepsin E	2,62	0.75 (0.56-0.93)	75%	75%	9.0 (1.41-57.11)	0.02*
MMP8	63,81	0.83 (0.67-0.98)	83%	83%	25.0 (2.92-213.98)	0.003*
Neprilysin/CD10	2,69	0.66 (0.46-0.86)	66,67%	66,67%	4.0 (0.73-21.83)	0,109
Protein convertase 9	2,84	0.66 (0.46-0.86)	66,67%	66,67%	4.0 (0.73-21.83)	0,109
uPA/Urokinase	3,35	0.66 (0.46-0.86)	66,67%	66,67%	4.0 (0.73-21.83)	0,109

* p value > 0.05.

Figure 3. ROC curves for MMP8 and ADAM9 to evaluate a biomarker's ability for moderate/severe AD status. Lines: *red* MMP8, *green* MMP8-ADAM9, *gray* reference line.

4. Discussion

AD is a chronic inflammatory dermatosis characterized for an abnormal skin-barrier function modulated by proteases, that affects the quality of life of individuals by intractable manifestations and its severity. Although clinical manifestations of AD greatly vary among subjects, currently there are no diagnostic tests which may give a better diagnosis and clinical classification of the moderate/severe AD status.

Emerging evidence has associated several proteases with the defective skin-barrier function in AD [12]. Up to our best knowledge, this is the first study to explore the levels of 35 proteases in the GCF of periodontally-healthy subjects with and without moderate/severe AD, without other diseases, and without smoking habits. In the present exploratory study, we found lower levels of four catalytic-type proteases in patients with moderate/severe AD: zinc-binding metalloproteases ADAM8, ADAM9, MMP8 and Neprilysin/CD10, calcium dependent serine-endoprotease Protein convertase 9 (PCSK9), serine-binding protease uPA/Urokinase, and aspartyl-binding protease Cathepsin E, suggesting these proteases might play an important role in the pathogenesis of AD. Additionally, MMP8, ADAM9 and Cathepsin E showed high sensitivity and specificity as single biomarkers for the detection of moderate/severe AD. In addition, logistic model evaluations showed that the particular combination of MMP8 with ADAM9 raised even more the ability of the proteases

to discriminate between both conditions; suggesting that GCF could serve as a novel source for non-invasive diagnostic biomarkers of moderate/severe AD.

Considering that circulating cytokines and biological molecules from systemic inflammation naturally extravasate into the GCF changing its composition [13], we believe it is plausible that down-regulation of combined proteases, and particularly of MMP8+ADAM9 in the GCF might reflect the clinical status of AD in moderate and severe cases of the disease. Measurement of these biomarker levels in the GCF by dentists and/or dermatologists could be beneficial as a novel chair-side diagnostic tool for AD, since the method is non-invasive and fast taking approximately 2 minutes in total for sample collection, opening the door for future personalized treatment design.

MMP8 and ADAM9 are zinc dependent proteases with a poorly understood role in inflammatory skin diseases. In normal conditions, zinc is involved in the differentiation of keratinocytes and decreases their pro-inflammatory activity. In addition, zinc participates in the expression of filaggrin, one of the key proteins responsible to maintain the skin barrier function. Previous studies have shown a link between zinc concentrations in overall human fluids and AD. In line with our results, lower levels of zinc in serum, erythrocytes and hair have been reported in patients with AD compared to healthy controls. In Spite of the evident and crucial role of zinc in the maintenance of the skin barrier, little is known about the participation of zinc-binding proteases in the pathogenesis of AD [14]. Overall, it is plausible that the decreased expression of zinc dependent proteases results in the loss of epidermal barrier function favoring the development of AD.

Specifically, MMP8 is a collagenase that breakdowns type I collagen and has anti-inflammatory properties [15,16]. Detection of MMP8 in the GCF has been previously studied in patients with marginal and apical periodontitis, both are immune-inflammatory oral diseases characterized by the destruction of the alveolar bone. Levels of MMP8 in the GCF were the highest and could reflect oral-local destructive inflammatory responses [17]. Knockout MMP8 mice with induced periodontitis showed increased alveolar bone loss in relation to uninfected mice. Hereby, MMP8 may act in a protective manner in periodontitis, possibly up-regulating anti-inflammatory mediators. Therefore, the final conclusions of the exact effect or role of MMP-8 in periodontal disease cannot be made[16]. In relation to MMP8 detection in skin, it is highly expressed in the course of skin inflammatory processes, conventionally attributed to production/secretion from Polymorphonuclear neutrophils (PMN), suggesting MMP8 has a role in dermic destruction [18]. Also, it has been reported that MMP8 is required to develop skin pruritus in *in vitro* models [19]. A previous study reported increased levels and activity of MMP8 and MMP9 in skin-wash samples of AD patients versus healthy controls. These authors proposed that MMP8 could play an important role in the pathology of AD and thus, could be useful as a disease biomarker [6]. In our study, we found lower levels of MMP8 in the GCF of AD individuals versus healthy controls. Similarly, a previous study with saliva showed lower concentrations of MMP-8 in drool samples from juvenile periodontitis patients versus individuals with gingivitis and healthy controls, suggesting that MMP8 could have a systemic protective and anti-inflammatory role [15]. In consequence, our novel findings imply that MMP8 could have a key role, at least in part, as a defensive enzyme of the systemic immune response being able to perform its protective role remotely in sites distant from AD skin-lesions. However, more researchers are necessary to further elucidate and confirm the function of MMP8 in AD.

Disintegrin and metalloproteinase (ADAM) 8 and 9 are part of the ADAM family of zinc-binding proteinases that act inducing inflammation or anti-inflammation responses, under specific conditions. ADAM8 and 9 have not been previously studied in AD[20,21]. In this study, we reported lower GCF concentrations of both proteases in patients with moderate/severe AD. In line with our results, a previous study reported higher ADAM9 expression in normal mucosa compared to inflamed gastric mucosa, suggesting that decreased ADAM9 may predispose to chronic mucosal inflammation [21]. In a more recent study, the protease profile of saliva samples from oral squamous cell carcinoma (OSCC) patients was studied. Results from this study showed a higher expression of ADAM9 in OSCC patients compared to healthy controls. Specifically, ADAM9 showed 0.45 and 0.767 of sensitivity and specificity for OSCC, respectively. Since higher concentration of proteases in saliva variated according to the kind of oral disease, the authors considered that the combination of

cathepsin V/kallikrein5/ADAM9 biomarkers was accurate as a diagnostic-biomarker of OSCC [22]. We did could not find literature regarding the detection of ADAM9 in GCF samples, however in a previous study, the GCF levels of ADAM8 in patients with periodontitis were decreased post-treatment with conventional non-surgical periodontal therapy at the moderate and severe sites, suggesting that ADAM8 concentrations in the GCF reflect inflammatory and bone-resorbing activities in the periodontal pocket [23]. Other relevant study reported that transgenic mice that have a hypersensitivity reaction and soluble ADAM8 in their circulation, expressed higher E-selectin mRNA levels in inflammatory skin sites compared to non-transgenic mice. This was also true for the expression of L-selectin in PMN from peripheral blood samples. These results suggest ADAM8 might activate endothelial cells and lead to the up-regulation of E-selectin, thus regulating leukocyte infiltration directly or indirectly. Therefore, it could be expected that ADAM9 and 8 would be detectable in the GCF samples of healthy controls, regulating leukocyte infiltration as a defensive mechanism of periodontal tissues [20]. PMN create a barrier that prevents oral bacteria from reaching the connective tissues of the periodontium, thus helping to preserve and maintain oral health. When neutrophils are deficient, bacteria prosper causing inflammation, and this has been associated with several diseases such as atherosclerosis, diabetes and cancer [24,25]. Altogether, our results suggest that ADAM8 and ADAM9 down-regulation in the GCF could act as a predispose factor of AD.

Cathepsin E is aspartyl-binding protease proposed to further investigate as a single biomarker to determine the moderate/severe AD status. Deficiency of cathepsin E leads to the spontaneous development of AD-like inflammatory skin lesions in mice, along with the systemic accumulation of IL-18 and IL-1, a rise in the ratio of CD4+/CD8+ T cells and the strong polarization of naïve T cells to T helper 2 cells [26]. The mechanism by which cathepsin E deficiency is associated with the development of AD could be explained by the fact that IL-18 and IL-1 are potent inflammatory cytokines. IL-18 induces an increase in the serum concentrations of IgE, IL-4, and IL-13, which are characteristic cytokines of hypersensitivity type 1. In addition, IL-1 accelerates the AD-like inflammation initiated by up-regulation of IL-18 [27]. In line with our results, a clinical study reported higher cathepsin E activity and expression in erythrocyte ghosts of healthy controls versus AD individuals [26], supporting the prospective role of cathepsin E in the systemic response of AD.

Neprilysin / CD10 is the only protease from the rest of the GCF proteases that showed significant differences in their expression between AD patients and controls, that has previous studies in AD. Neprilysin / CD10 is a type 2 cell surface metalloproteinase which protects against excessive skin inflammation by degrading substance P or reducing its levels in the dermal microenvironment [33,34]. A clinical study has also demonstrated higher levels of Neprilysin / CD10 in serum samples of allergy-free versus AD children. This antecedent is in line with our results. Therefore, it is possible that Neprilysin / CD10 plays a key role in downregulating the local inflammation in AD and thus helps to maintain the health of oral tissues. In addition, we also recognized for the first time a possible role of PCSK9 and uPA in pathogenesis of AD [28,29]. Our results are supported in part by previous reports on inflammation: PCSK9 levels were decreased in the saliva and serum of chronic periodontitis and rheumatoid arthritis (RA) patients, respectively, compared to healthy controls [30,31]. These results could suggest that PCSK9 may act locally in AD skin. However, the role of PCSK9 in AD requires further investigations. Moreover, the urokinase plasminogen activator (uPA) modulates immune-inflammatory and fibrinolytic responses [32,33]. A previous study reported increased uPA activity in skin samples from acute eczematous AD patients, especially in the deeper layers of the stratum corneum, compared to healthy skin [34]. Similarly to our results, the peripheral blood of asthmatics has been shown to have lower levels of uPA compared with healthy controls has been demonstrated [35]. However, uPA and suPAR plasma levels did not differ between patients with the atopic eczema/dermatitis syndrome and healthy controls [36]. uPA has been reported to have a central role in regulating the Th1 immune response. An experimental animal study demonstrated that uPA-deficient transgenic mice have an inability to generate IFN-a and IL-12 but have increased levels of IL-5, a type 2 cytokine. In these mice, the macrophages have impaired antimicrobial activity and regional lymph nodes contained fewer cells in infected mice [37].

Considered overall, we believe the decreased level of uPA in GFC in AD patients versus healthy controls may reflect a systemic Th2 response in AD.

In spite the limitations of the present study, including: the small sample size, the recruitment of symptomatic moderate/severe AD patients without other diseases and without treatment for AD, and the use of a very costly laboratory method (owing to the exploratory nature of the study); we were able to discriminate proteases in the GCF between moderate/severe AD patients and healthy individuals for the first time using a protease array system. The results demonstrated that protease spectrum in the GCF of moderate/severe AD patients and healthy individuals significantly differed. These results could be explained due to moderate and severe AD, which causes changes in the circulating/systemic concentrations of these molecules which in turn influence the protease content of the GCF. Overall, results from this exploratory study endorse a differential function of MMP8+ADAM9 among AD patients and healthy controls. Further validation and testing of their combination as a prelaminar and innovative diagnostic biomarker should be carried in future studies.

To conclude, the following proteases: ADAM8, ADAM9, Cathepsin E, MMP8, CD10, Protein convertase 9 and uPA/Urokinase were downregulated in moderate/severe atopic dermatitis patients compared with healthy controls. In addition, the combination of MMP8+ADAM9 may be useful as combined diagnostic biomarker for moderate/severe AD.

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