



Bilirubin oxidation derived from oxidative stress is associated with disease severity of atopic dermatitis in adults

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Summary

Background. Bilirubin is an essential antioxidant. Its oxidative metabolites, biopyrrins, are sensitive urinary markers of oxidative stress. Multiple studies suggest that oxidative stress affects the pathogenesis of skin diseases such as atopic dermatitis (AD).

Aim. To examine oxidative stress-induced bilirubin oxidation and its association with AD pathogenesis in adults.

Methods. In total, 11 patients with AD and 7 healthy controls (HCs) were enrolled. Bilirubin oxidation profiles in the combined urine of the patients and that of the HCs were examined using high-performance liquid chromatography (HPLC) and fast atom bombardment mass spectrometry. The concentrations of urinary biopyrrins and serum biomarkers for AD disease severity, such as IgE and thymus and activation-regulated chemokine (TARC)/CCL17, were measured by ELISA to determine correlations between urinary biopyrrins and serum biomarkers. Local bilirubin oxidation in AD skin lesions was assessed by immunohistochemical analyses using two antibodies against bilirubin.

Results. Levels of dipyrrole-monopyrrole-aldehyde, a novel urinary biopyrrin, were higher in patients with AD than in HCs, and increased with disease severity based on the SCORing Atopic Dermatitis (SCORAD) objective scoring system. Additionally, urinary biopyrrin levels correlated significantly with serum IgE and TARC/CCL17 levels. Furthermore, immunohistochemical analyses revealed that biopyrrins were strongly expressed in both infiltrating and resident cells in AD lesions. However, bilirubin was expressed at low levels in the lesions, suggesting that bilirubin oxidation is augmented in AD lesions.

Conclusions. Bilirubin oxidation derived from oxidative stress in the skin lesions can be associated with disease severity of AD.

Introduction

Bilirubin, biosynthesized from heme through the action of heme oxygenase (HO) and biliverdin reductase,^{1,2} was originally identified for its participation in

eliminating unwanted heme. However, recent studies reveal that bilirubin has antioxidant properties both *in vitro* and *in vivo*.^{3,4} The skin, like other organs, is capable of bilirubin generation via heme catabolism, as visually observed with subcutaneous bruising, characterized by colour changes from purple (heme) through blue–green (biliverdin) to yellow (bilirubin).¹ Therefore, bilirubin acts as an essential antioxidant in cutaneous biology and inflammation. Bilirubin reacts with reactive oxygen species (ROS) to form hydrophilic oxidative metabolites that are eventually excreted into

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urine. Yamaguchi *et al.* discovered bilirubin oxidative metabolites in human urine,⁵ which are now collectively called biopyrrins.⁶ Urinary biopyrrins are useful markers for evaluating bilirubin oxidation derived from oxidative stress.^{7,8}

Previous reports demonstrated that bilirubin oxidation is observed under pathological and physiological conditions.^{7–9} For example, increased urinary excretion of biopyrrins was observed under conditions of psychosocial stress, such as public speaking.⁷ In addition, in patients with Alzheimer disease, levels of biopyrrins, but not bilirubin, were locally increased in cerebrospinal fluid.⁹ Furthermore, increased urinary excretion of biopyrrins was observed during heart failure.⁸ These findings suggest that bilirubin can act as an antioxidant in both neurological and cardiovascular systems. However, it remains uncertain whether bilirubin contributes to the pathogenesis of skin disorders.

Atopic dermatitis (AD) is a chronic eczematous condition with multiple aetiological factors, including genetics, fragility of the skin barrier and presence of immune responses to environmental elements.¹⁰ Previous studies suggest that oxidative stress is also implicated in the pathogenesis of AD.^{11–13} Polymorphisms of glutathione S-transferases (GSTs), which are critical enzymes for detoxifying ROS, are associated with susceptibility to AD.¹¹ In addition, urinary levels of 8-hydroxy-2'-deoxyguanosine (8-OHdG), a marker of oxidative DNA damage, were higher in patients with AD than in healthy controls (HCs), showing a strong correlation with disease severity as assessed using the SCORing Atopic Dermatitis (SCORAD) index.¹² Furthermore, Tsukahara *et al.* reported that urinary concentrations of both biopyrrins and 8-OHdG were upregulated in children with acute exacerbations of AD,¹³ and elevated levels of heme oxygenase (HO)-1, which is an enzyme involved in bilirubin generation, were observed in the lesions and sera of patients with AD.^{14,15}

The aim of the present study was to examine oxidative stress-induced bilirubin oxidation and its association with AD pathogenesis in adults. Bilirubin oxidation profiles of patients with AD and HCs were compared using high-performance liquid chromatography (HPLC) and fast atom bombardment mass spectrometry (FAB-MS) analysis. In addition, the correlation between urinary excretion of biopyrrins and serum biomarkers for disease severity, such as IgE and thymus and activation-regulated chemokine (TARC)/CCL17, was evaluated. Finally, the local oxidative metabolism of bilirubin in AD skin lesions was examined using immunohistochemistry.

Methods

This study was approved by the ethics committee of Tokyo Medical and Dental University (approval no. 1763), and all participants provided written informed consent.

Participants

In total, there were 11 patients with AD (9 men, 2 women; median age 40 years) enrolled into the study. The healthy control (HC) group comprised 7 healthy people (4 men, 3 women; median age 39 years) (Table S1).

AD was diagnosed based on the Japanese Dermatological Association Criteria for the diagnosis of AD.¹⁶ Disease severity was classified into three groups using the objective SCORAD index, as follows: mild (score < 15), moderate (≥ 15 to ≤ 40), severe (> 40).¹⁷ None of the patients had mild disease; all patients were categorized as having either moderate ($n = 6$) or severe ($n = 5$) AD. All patients were receiving treatment, including steroid ointment, tacrolimus ointment or an antihistamine. None of the patients was affected with another skin disease or an unstable visceral disease. In addition, during the clinical interview, none of the patients reported having experienced any stressful life events.

Blood and urine samples

Venous whole blood and urine samples were collected from the 11 patients with AD, and urine samples were also obtained from the HCs; as blood sampling is invasive, it was not performed for the HCs. Both blood and urine samples were centrifuged, and supernatants and serum, respectively, were frozen in liquid nitrogen and stored at -80°C until analysis.

Urinary biopyrrin measurements and serum biomarker measurements

To confirm whether urinary excretion of biopyrrins was associated with AD disease severity, urinary biopyrrins and serum biomarkers for disease activity (IgE, TARC/CCL17 and MDC/CCL22)¹⁸ were measured, and the associations between urinary biopyrrins and the serum biomarkers were assessed.

Biopyrrin concentrations in urine specimens were measured using an ELISA kit (Shino-test Corp, Tokyo, Japan) containing the antibilirubin monoclonal antibody (mAb) 24G7⁶ following the manufacturer's protocol. The concentration of urinary biopyrrins was

normalized to the urinary creatinine concentration (U/g Cre).

Serum concentrations of IgE were also measured by ELISA (Abcam, Cambridge, UK), as were serum concentrations of TARC/CCL17 and macrophage-derived chemokine (MDC)/CCL22 (R&D, Minneapolis, MN, USA).

High-performance liquid chromatography and fast atom bombardment mass spectrometry

To determine if bilirubin oxidation is associated with AD pathogenesis, differences in bilirubin oxidation profiles between patients with AD and HCs were examined. Combined urine specimens (25 mL) were loaded onto cartridges (Sep-Pak C18 ENV; Waters, Milford, MA, USA) that were equilibrated with 0.1% trifluoroacetic acid (TFA) solution, and were then eluted with acetonitrile and methanol. Samples were subsequently evaporated under reduced pressure, lyophilized, dissolved in 500 µL methanol, and then subjected to HPLC. Samples were analysed by reverse-phase HPLC (600E-LC system; Waters) as described previously by Yamaguchi *et al.*¹⁹ FAB-MS measurements of biopyrrins were conducted with Nano Frontier LD (HITACHI High-Tech Science, Tokyo, Japan).^{5,20}

Immunohistochemistry

To determine if bilirubin is oxidized into biopyrrins in AD skin lesions, we compared the expression levels of bilirubin and biopyrrins in AD lesions and normal skin by immunohistochemical staining using two mAbs: 24G7 (detects biopyrrins and bilirubin)⁶ and 5M2 (detects only bilirubin).²¹

Skin specimens were obtained from active regions of eczema in patients with moderate or severe AD and from nonlesional skin in an additional control group of patients with noninflammatory skin disease. Tissue sections of formalin-fixed and paraffin wax-embedded skin samples were incubated with a mouse mAb that recognizes both biopyrrins and bilirubin (24G7⁶, 1 : 200) or a mouse mAb against bilirubin only (5M2²¹, 1 : 200), at 4 °C overnight, followed by horseradish peroxidase-conjugated anti-mouse IgG (1 : 500). Sections were then incubated in 3'-diaminobenzidine tetrahydrochloride solution and counterstained with Mayer haematoxylin.

Statistical analyses

Correlations between urinary biopyrrins and each serum biomarker were determined using Spearman

rank correlation coefficient. $P < 0.05$ was considered statistically significant.

Results

Higher levels of a novel biopyrrin, dipyrrole-monopyrrole-aldehyde, were detected in the urine of patients with atopic dermatitis compared with healthy controls

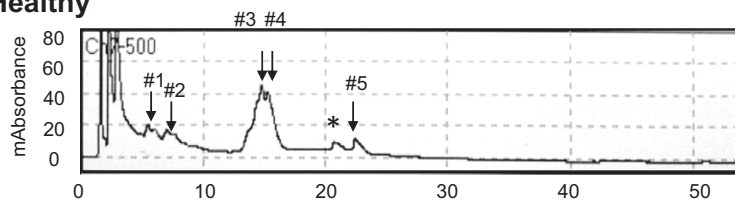
The HPLC elution profiles of all groups contained five peaks (peaks 1–5 in Fig. 1) representing 24G7-reactive bilirubin metabolites. Peaks 3 and 4 had previously been identified as biotripyrrin-a and biotripyrrin-b, respectively⁵; however, the identities of peaks 1, 2 and 5 remained unknown. Therefore, we next accurately measured the molecular weights of peaks 1, 2 and 5 using high-resolution FAB-MS and further determined their structures. Peak 1 represented the molecular formula $C_{17}H_{18}N_2O_4$ [mass divided by charge number (m/z) 314.1267, calculated 314.1296] and peak 5 represented $C_{26}H_{29}N_3O_6$ (m/z 479.2056, calculated 479.2088) by high-resolution FAB-MS. Peak 2 appeared to be a structural isomer of peak 2, based on its spectroscopic properties. As shown in Fig. 2, peaks 1 and 2 were identified as endo-vinyl dipyrrole-aldehyde and exo-vinyl dipyrrole-aldehyde, respectively, while peak 5 was defined as dipyrrole-monopyrrole-aldehyde.

The HPLC profile of the HC group revealed that the levels of biotripyrrins (peaks 3 and 4) were higher than those of the other peaks (Fig. 1a,d), which was consistent with results from HC specimens in our previous report.¹⁹ Intriguingly, dipyrrole-monopyrrole-aldehyde (peak 5) was higher in AD samples compared with non-AD samples (Fig. 1). Further, dipyrrole-monopyrrole-aldehyde (peak 5) urine levels from patients with severe AD were higher than those from patients with moderate AD (Fig. 1b,c). These results demonstrate that bilirubin oxidation into dipyrrole-monopyrrole-aldehyde was elevated in patients with AD.

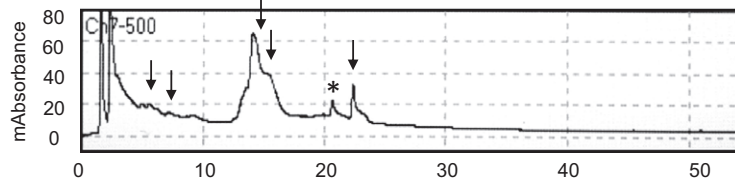
Levels of urinary biopyrrins were correlated with serum IgE and TARC/CCL17 levels

The levels of urinary biopyrrins were slightly correlated with the levels of serum MDC/CCL22, although this was not statistically significant (Fig. 3c). Interestingly, urinary biopyrrin levels showed a positive correlation with serum IgE and TARC/CCL17 expression (Fig. 3a,b). These results demonstrate that the oxidation of bilirubin was associated with AD disease severity.

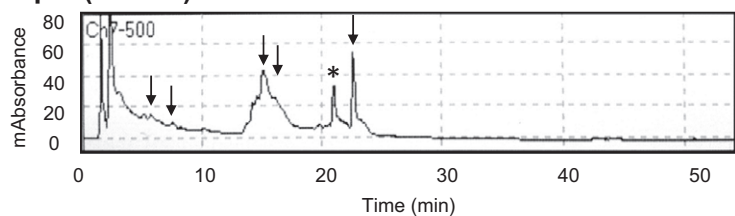
(a) Healthy



(b) Atopic (moderate)



(c) Atopic (severe)



(d)

	#1	#2	#3	#4	#5
(A)Healthy	20.0	17.1	42.9	38.6	11.4
(B)Atopic(moderate)	20.0	14.3	61.4	37.1	31.4
(C)Atopic(severe)	14.3	8.6	25.7	24.3	51.4

(mAbsorbance)

Figure 1 High-performance liquid chromatography elution profiles of urinary biopyrrins in and patients with atopic dermatitis (AD) and healthy controls (HCs). (a–c) Combined urine specimens were collected from three groups of donors: (a) HCs (healthy), (b) patients with moderate AD (moderate), and (c) patients with severe AD (severe). Urine specimens pretreated with Sep-PAK C18 were analysed by reverse-phase HPLC on a Waters 600-LC system. Five peaks of biopyrrins were detected at 500 nm absorbance. *Nonspecific product. (d) Numerical data of the relative intensities of each peak are shown.

Biopyrrins were locally generated in atopic dermatitis lesions

In normal skin, epithelial cells and dermal resident cells stained positive for both 24G7 and 5M2 (Fig. 4), indicating that bilirubin had high expression in normal skin. By contrast, in AD lesions, resident and infiltrating cells stained intensely for 24G7 and only stained weakly for 5M2 (Fig. 4). These observations show that biopyrrins had high expression in AD lesions, but lower levels of bilirubin were expressed in AD lesions compared with normal skin.

Discussion

Previous studies suggest that oxidative stress is implicated in various skin diseases, such as AD.^{11–13} However, it remains unclear whether oxidative stress-induced bilirubin oxidation is involved in the pathological mechanism of AD. In the present study, we provide evidence that bilirubin oxidation is

upregulated in skin lesions of patients with AD and that urinary excretion of biopyrrins is associated with AD disease severity.

Human urine contains various forms of biopyrrins.⁵ In the current study, the profiles of biopyrrins in urine specimens collected from patients with AD and HCs were analysed using HPLC, and the elution profiles for both groups showed five peaks characteristic of biopyrrins, as previously described.¹⁹ Two of the peaks corresponded to biotripyrrin-a and biotripyrrin-b.⁵ Furthermore, we identified three novel bilirubin oxidative metabolites, endo-vinyl dipyrrole-aldehyde, exo-vinyl dipyrrole-aldehyde and dipyrrole-monopyrrole-aldehyde, using FAB-MS. Intriguingly, the levels of dipyrrole-monopyrrole-aldehyde, but not of the other biopyrrins, were significantly higher in the AD than the HC samples, and they increased as disease severity increased, based on the objective SCORAD index. These findings suggest that dipyrrole-monopyrrole-aldehyde is produced under the pathological conditions associated

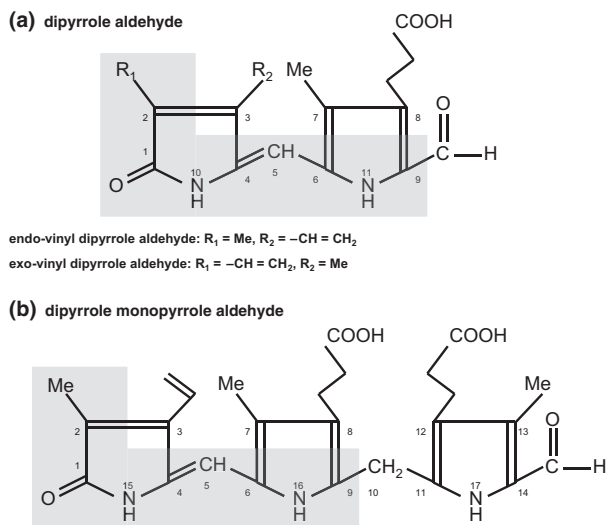


Figure 2 Proposed structures of dipyrrole-aldehyde and dipyrrole-monopyrrole-aldehyde. Proposed structures of (a) dipyrrole-aldehyde and (b) dipyrrole-monopyrrole-aldehyde are shown. Shaded areas indicate the 24G7 antibody epitope.

with AD, although other biopyrrins can be generated under physiological conditions.

In a preliminary study, nitric oxide (NO) treatment induced bilirubin oxidation to form dipyrrole-monopyrrole-aldehyde *in vitro* (data not shown). Although NO is a free radical itself, when its production is increased, it reacts with superoxide to

generate a more reactive oxidant, peroxynitrite.²² NO is generated by three distinct isoforms of the enzyme NO synthase (NOS): neuronal NOS (nNOS), inducible NOS (iNOS) and endothelial NOS (eNOS).²³ The majority of skin-resident cells, including keratinocytes, Langerhans cells and dermal fibroblasts, express iNOS and produce a large amount of NO upon exposure to inflammatory cytokines and bacterial products.²³ Interestingly, high expression of iNOS was detected around the upper dermal microvasculature in AD lesions.²⁴ In addition, plasma levels of nitrate, a metabolite of NO, were increased in patients with AD and were associated with disease severity.²⁵ Taken together, these results indicate that large quantities of NO could be generated in AD lesions, leading to oxidation of bilirubin into dipyrrole-monopyrrole-aldehyde.

Although several previous studies have shown that increased urinary biopyrrin levels are present in both physiological and pathological conditions,^{7–9} little has been reported on the local oxidative metabolism of bilirubin in local lesions. Therefore, this study evaluated whether urinary excretion of biopyrrins directly reflects locally enhanced bilirubin metabolism in patients with AD. Immunohistochemical analysis showed that strong biopyrrin expression was observed in infiltrating and resident cells; however, bilirubin expression was minimally detected in AD lesions, indicating promotion of bilirubin oxidative metabolism into biopyrrins. Listopad *et al.* showed increased gene

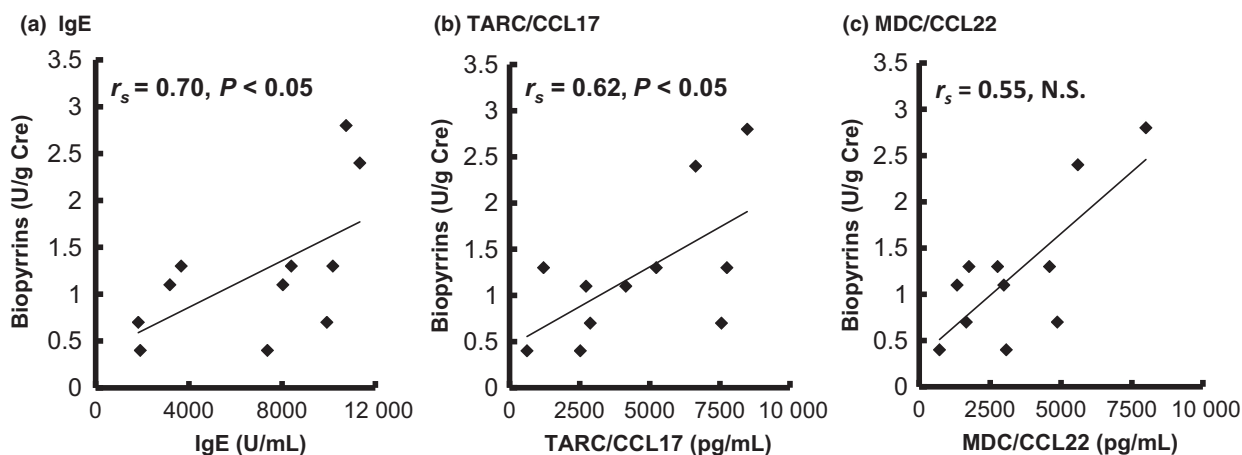


Figure 3 Correlation between urinary biopyrrins and serum biomarkers indicating atopic dermatitis (AD) disease severity. (a–c) Urine and serum samples were collected from 11 patients with AD. Biopyrrin levels in urine were measured by ELISA using the monoclonal antibody 24G7. Serum concentrations of IgE, thymus and activation-regulated chemokine (TARC)/CCL17 and macrophage-derived chemokine (MDC)/CCL22 were also measured by ELISA. The correlations between levels of (a) biopyrrins and IgE, (b) biopyrrins and TARC/CCL17, and (c) biopyrrins and MDC/CCL22 are shown. R values were analysed by Spearman rank correlation tests. $P < 0.05$ was considered statistically significant. NS, not significant.

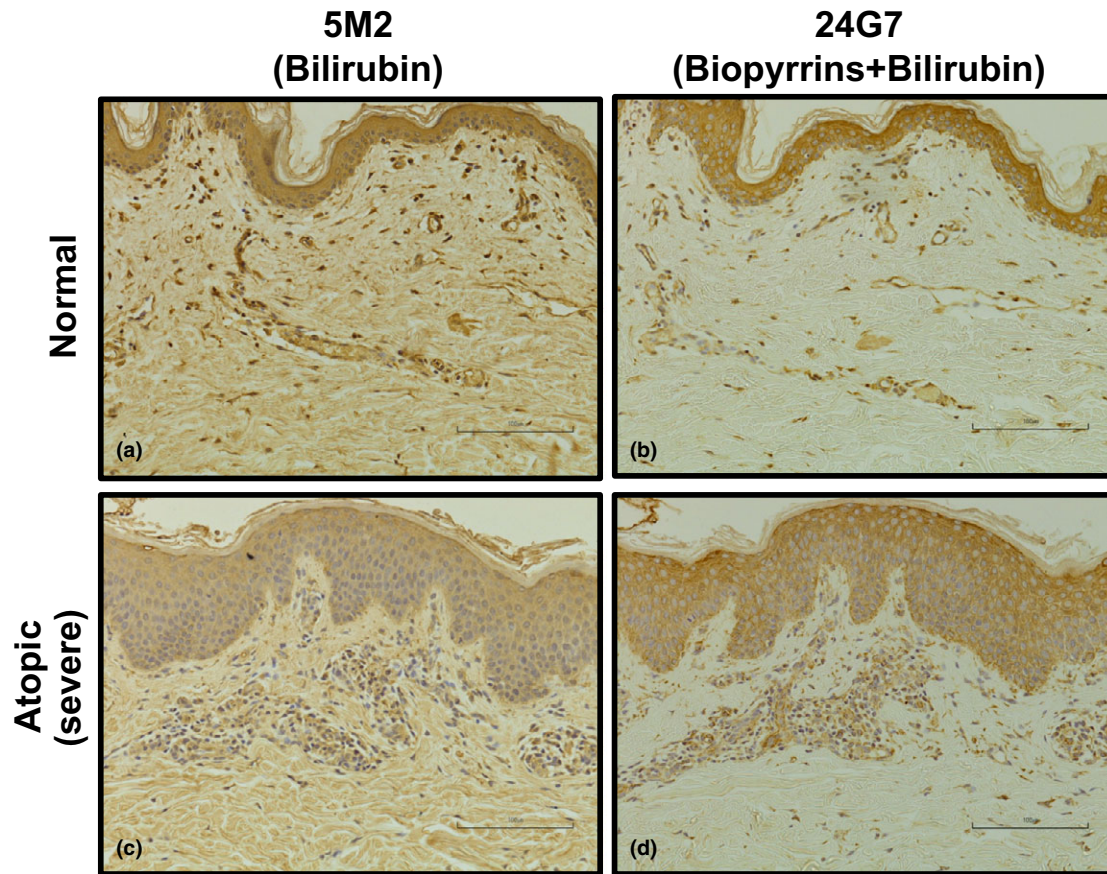


Figure 4 Bilirubin oxidation was upregulated in atopic dermatitis (AD) lesions. Sections of (a,b) normal skin and (c,d) AD lesions were stained with (a,c) 5M2, a monoclonal antibody (mAb) against bilirubin or (b,d) 24G7, a mAb against both bilirubin and biopyrrins, followed by horseradish peroxidase (HRP)-conjugated anti-mouse IgG, with the reaction product visualized as brown staining with diaminobenzidine. Scale bar = 100 μ m.

expression levels of HO-1 in AD lesions compared with nonlesional skin.¹⁴ HO-1 protein expression was also detected in the dermis of patients with AD, whereas it was scarcely detected in that of normal patients.¹⁵ These observations suggest that heme catabolism is accelerated to supply bilirubin for oxidation.

Conclusions

The present study revealed that bilirubin oxidation is upregulated in atopic lesions and that urinary excretion of biopyrrins is associated with AD disease severity. There was also a difference in bilirubin oxidation profiles between patients with AD and HCs. In patients with AD, bilirubin is likely oxidized into the novel biopyrrin dipyrrole-monopyrrole-aldehyde. Thus, it is

important to develop tools to specifically detect dipyrrole-monopyrrole-aldehyde in order to achieve accurate assessment of AD disease severity.

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What is already known about this topic?

- Oxidative stress has been implicated in various skin diseases, such as AD.

What does this study add?

- Bilirubin is oxidized into dipyrrole-monopyrrole-aldehyde in AD.
- Changes in dipyrrole-monopyrrole-aldehyde levels parallel AD skin scores.
- Urinary biopyrrin levels are strongly correlated with serum atopic biomarkers.
- Bilirubin oxidation is augmented in atopic lesions.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Participant characteristics.

	Healthy control (n=7)	Atopic dermatitis (n=11)
Age	40 (36-65)	39 (20-48)
Sex (Male, Female)	(4, 3)	(9, 2)
AD severity		
mild	-	0
moderate	-	6
severe	-	5
Urinary biopyrrin (U/Cre g)	-	1.1 (0.4-2.8)
Total bilirubin (mg/dl)	-	0.7 (0.5-0.9)
IgE (U/ml)	-	8033.2 (1829.2-11335.2)
TARC (pg/ml)	-	4145 (620-8475)
MDC (pg/ml)	-	2982 (712-7992)
Onset of AD		
childhood	-	10
adult	-	0
unknown	-	1
Comorbidities		
asthma	-	7
rhinitis	-	6
food allergy	-	5

Supplementary Table 1