

## A comparative study of epidermal tight junction proteins in a dog model of atopic dermatitis

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**Background** – Tight junctions (TJ) are important for skin barrier function and could be relevant in modulating allergen penetration in atopic dermatitis (AD). Humans with AD have been described to have decreased expressions of some TJ proteins in the skin.

**Hypothesis/Objectives** – This study aimed to investigate TJ protein expression using an experimental AD model in dogs.

**Methods** – Skin biopsies from six atopic (nonlesional skin) and five normal beagle dogs were stained for TJ proteins [zonula occludens 1 (ZO-1), occludin, claudin-1] by immunohistochemistry. Staining intensity was evaluated both objectively using imaging software and subjectively. Six images/sections were randomized and blindly scored by six investigators for intensity, distribution, integrity and staining pattern.

**Results** – The intensity of ZO-1 was significantly decreased in the atopic group objectively ( $P = 0.010$ ) and subjectively ( $P = 0.002$ ) relative to the normal group. Occludin was decreased significantly subjectively ( $P = 0.027$ ) but not objectively. Claudin was not significantly different between groups by either quantification. Additionally, only ZO-1 demonstrated a significantly patchier staining pattern in the atopic group. There was no consistent staining pattern in this study.

**Conclusions and clinical importance** – ZO-1 and occludin, which have not been described to be associated with the development of AD in humans, could play a role in this atopic dog model. Further investigation on the expression and modulation of TJ proteins and their clinical relevance is needed.

### Introduction

Atopic dermatitis (AD) is a chronic relapsing eczematous skin disorder that results from complex interactions between genetic, immunological, epidermal barrier dysfunction and environmental factors. It is a very common allergic skin disease in dogs as well as in humans.<sup>1</sup>

A defective epidermal barrier combined with an abnormal immune response contributes to the pathophysiology of AD.<sup>2</sup> Tight junctions (TJs) are important for cell-to-cell adhesion in epithelia, and are responsible for intercellular sealing and the compartmentalization of extracellular environments.<sup>3</sup> TJs mainly consist of claudins, occludin, junctional adhesion molecules (which are 40 kDa members of the IgG superfamily) and the zonula occludens. According to previous studies,<sup>2,4</sup> TJ protein expression may play a role in the development and progression of human AD. Limited studies exist on expression of TJ proteins in canine AD. In one study, TJ protein expression on patch test sites was reported, but no comparison with normal dogs was done.<sup>5</sup> Additionally, another study investigated TJ proteins in atopic and normal dogs with immunohistochemistry (IHC).<sup>6</sup>

In this pilot study we aimed to investigate epidermal TJs expression with IHC in a canine experimental AD model and compare it to normal dogs.

### Materials and methods

#### Dogs

Atopic dogs ( $n = 6$ ) had previously been epicutaneously sensitized to house dust mite (HDM) *Dermatophagoides farinae* and showed minimal to no clinical evidence of AD when not exposed to HDM, but reacted strongly when challenged with allergen.<sup>7</sup> Healthy, unrelated beagle dogs ( $n = 5$ ) with no history of skin disease and pruritus were included as controls. All of the dogs were housed in the same conditions and feeding regime. The dogs' care and study protocol were conducted in accordance with the guidelines of the Institutional Animal Care and Use Committee (IACUC).

#### Immunohistochemistry and analyses

Skin punch biopsy samples were obtained from nonlesional, nonallergen challenged inguinal skin of the atopic dogs, as well from the inguinal skin of healthy beagle dogs. The tissue samples were fixed in 10% neutral buffered formalin for 48 h and transferred into phosphate-buffered saline (PBS) until dehydrated and embedded in paraffin. Sections were cut at 5  $\mu$ m and placed on Superfrost positively charged slides (Fisher Scientific; Pittsburg, PA, USA). All sections after deparaffination were subjected to antigen retrieval for 10 min using protease from *Streptomyces griseus*, type XIV, 1 mg/ml (Sigma-Aldrich, cat. P5147; St. Louis, MO, USA) for occludin and claudin-1 at 37°C and Tris-EDTA (pH 8) for ZO-1 at 95°C. After another 10 min at room temperature, slides were removed and

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washed in PBS and quenched in 3% hydrogen peroxide in methanol. The procedure was then followed according to the Polymer- HRP kit (cat. QP-400, BioGenex; San Ramon, CA, USA). Rabbit polyclonal anti-ZO-1 Ab (cat. 61-7300, dilution 1:400, Invitrogen; Camarillo, CA, USA), anti-claudin-1 Ab (cat. ab15098, dilution 1:50, Abcam; Cambridge, UK), and anti-occludin Ab (cat. 71-1500, dilution 1:600, Invitrogen) were used as primary antibodies. Visualization was with Nova Red (cat. SK-4800, Vector laboratories; Burlingame, CA, USA) and the counterstain was Mayers haematoxylin (Thermo Fisher; Waltham, MA, USA).

Slides were viewed using a Nikon Lapophot microscope at  $\times 200$  magnification and six pictures per slide were sequentially taken for evaluation.

The intensity of staining in slide photographs was analysed objectively using a computer imaging program (ImageJ® 1.41; <http://rsb.info.nih.gov/ij/>) as previously reported.<sup>8</sup> Intensity was also evaluated subjectively. Subjective evaluation of intensity was scored on a scale of 0–3 ("0 = none, 1 = weak, 2 = moderate, 3 = strong"); the mean was calculated to compare scores between groups. For the distribution, the four layers of the epidermis (stratum corneum, granulosum, spinosum and basale) were considered independently and then evaluated for staining characteristics. Epidermal staining pattern was evaluated as "homogeneous" or "patchy" and cellular staining pattern was scored as "membranous", cytoplasmic" or "nuclear". The staining patterns were calculated by percentage for statistical analysis. All analyses were evaluated blindly by five investigators except for objective intensity.

Data were analysed using the unpaired *t*-test to evaluate the differences between the two groups. All statistical analyses were performed using SPSS v18.0 for Windows (SPSS Inc.; Chicago, IL, USA). A probability of  $P < 0.05$  indicated significance.

## Results

### Intensity

IHC staining showed the strongest expression for ZO-1 among the antibodies tested (Figure 1a). The intensity of ZO-1 was significantly decreased in the atopic group objectively (Figure 1b;  $P = 0.010$ ) and subjectively (Figure 1c;  $P = 0.002$ ) relative to the normal group. Occludin was significantly decreased subjectively (Figure 1c;  $P = 0.027$ ) but not objectively. Claudin-1 was not significantly different between groups by either quantification (Figure 1b,c).

### Distribution, integrity and cellular staining pattern

ZO-1 was expressed mainly in the entire epidermis in normal skin, but there was a significant difference in distribution in the atopic skin, with the expression in the stratum granulosum or stratum corneum only (Figure 2a;  $P = 0.006$ ). Additionally, ZO-1 showed a significantly patchier and less homogeneous distribution when compared to normal skin (Figure 2b;  $P < 0.0001$ ). ZO-1 showed cytoplasmic and variable staining patterns between groups, but there was no significant difference (Figure 2c).

Occludin was expressed mainly in the entire epidermis in normal skin, but was highly variable in the atopic skin. Distribution in the atopic skin was in both stratum corneum and basale, or stratum corneum only. The occludin

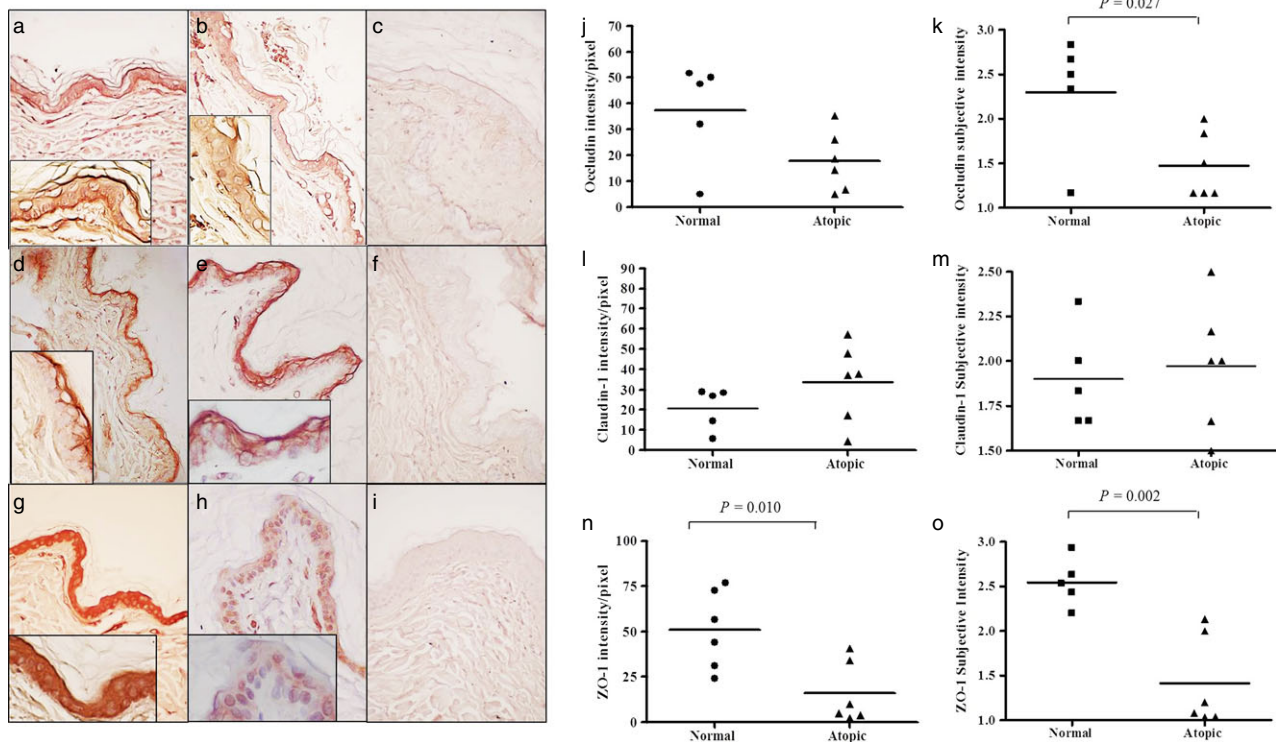


**Figure 1.** Evaluation of immunohistochemistry intensity of tight junction (TJ) proteins in a dog model of atopic dermatitis versus normal dogs. Comparison of immunohistochemistry ( $\times 200$ ;  $\times 400$  high magnification images) for TJ (a–i): occludin (a–c), claudin-1 (d–f) and ZO-1 (g–i). (a), (d) and (g) are for normal dogs; (b), (e) and (h) are for atopic dogs; (c), (f) and (i) are for each negative control. Comparison of objective (with Image J, intensity/pixel) and subjective intensities (j–o): occludin (j,k), claudin-1 (l,m) and ZO-1 (n,o). Significant differences were found between atopic and normal skin for ZO-1 (subjective and objective scoring) and occludin (subjective scoring only). Negative control was PBS treatment instead of antibodies.

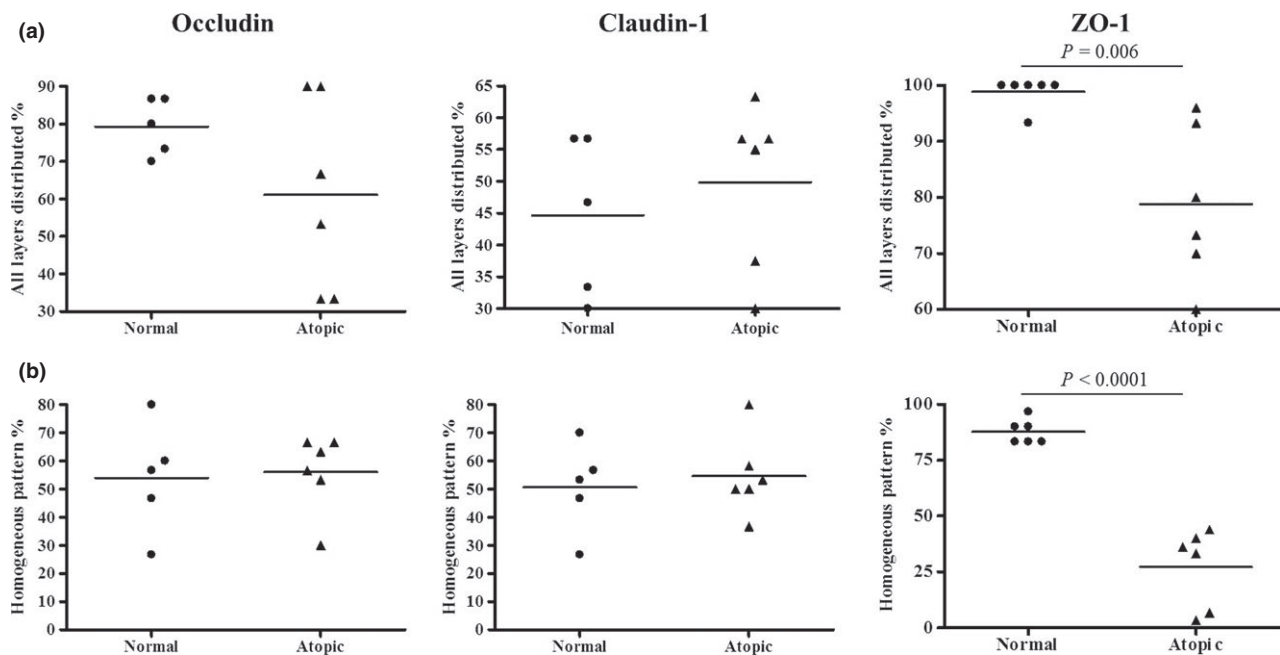


**Figure 2.** Evaluation of distribution, staining homogeneity and subjective cell staining pattern for tight junction (TJ) proteins on immunohistochemistry in the dog model of atopic dermatitis versus normal dogs. (a) Comparison of the percentage of samples demonstrating staining in all epidermal layers. (b) Comparison of the percentage of homogeneous staining pattern for TJ. A significant difference was seen in ZO-1.

showed a cytoplasmic cellular staining pattern in all dogs of both groups and there was no significant difference between groups in cellular staining pattern (Figure 2c). There was a variable pattern of staining throughout the epithelium in both groups.

Claudin-1 showed nonspecific distribution between groups and was expressed in both the stratum corneum and granulosum, or stratum corneum only, by a similar percentage in all layers. Intracellular staining was observed in claudin-1 only, with a similar percentage of cytoplasmic staining pattern in both groups.

## Discussion

In this study, we showed decreased expressions of ZO-1 and occludin in the epidermis of an experimental AD model in dogs. Additionally, we found that ZO-1 was expressed in a significantly more heterogeneous and inconsistent fashion in AD skin than in normal skin. These observations suggest that ZO-1 and occludin, which have not been described to be associated with the development of AD in humans, could play a crucial role in this atopic dog model.

A study in atopic research dogs reported that the integrity of corneodesmosomal and TJ proteins, including claudin-1, was discontinuous in acute challenged atopics compared to vehicle-applied atopic dogs.<sup>5</sup> The authors did not compare these findings with normal dogs. Skin biopsies were taken after acute challenge by patch with house dust mite extract. Therefore, the integrity of the staining pattern could have been a secondary change and partly caused by the allergen-induced inflammation. In our study, the nonhomogeneous staining pattern of TJ proteins was present in nonlesional skin without any exposure to allergen. In our model this could either be a primary barrier defect or secondary to

subclinical inflammation, because nonlesional atopic skin always has some low grade inflammation. Additional studies are needed to pursue the possibility of a primary defect.

A second similar study also compared TJ proteins in atopic and normal dogs.<sup>6</sup> The study showed a defect in claudin-1 expression and no changes in occludin or ZO-1 expression in the nonlesional epidermis of atopic dogs. The authors used only subjective evaluation of TJ protein intensity and distribution, whereas the present study also objectively evaluated the intensity. Additionally staining patterns were not evaluated in the second study. Therefore, the comparison between atopic and normal dogs would be relatively nonspecific and subjective compared to our study.

The expression of TJ protein claudin-1 was significantly decreased or heterogeneous in the skin of atopic dogs in other studies,<sup>5,6</sup> in contrast to the results in our study. This could be related to different protocols in the development of the atopic model or the different skin inflamed status with challenged or nonchallenged subclinical inflammation. Studies in clinical atopic dogs are required to clarify the differences in TJ protein expression compared to normal dogs.

In humans, ZO-1 in the skin was distributed primarily in the stratum granulosum and spinosum, occludin was in the stratum corneum and granulosum, and claudin-1 was distributed in all layers.<sup>9</sup> The present study and other veterinary studies have shown different and variable distributions.<sup>6,11</sup> However, in contrast to our study, occludin is localized exclusively in cell membrane and expressed mainly in the stratum granulosum in humans.<sup>10</sup> In comparative studies,<sup>6,11</sup> occludin in dogs showed cytoplasmic expression and was expressed in all layers in the skin by IHC staining.<sup>11</sup> These differences could be explained by the use of different techniques with different sensitivity

and specificity. In particular, the different antigen retrieval methods for each antibody would affect the differences in distribution and staining patterns. Additionally, the results could be explained by a greater sensitivity of IHC than immunofluorescence in reflecting the dynamic behaviour of TJ proteins.<sup>11</sup> Further staining methods should be tried to confirm the correct distribution in the skin TJ proteins in the AD model.

In summary the changes of TJ proteins in the skin reported in our study could conceivably increase allergen penetration, which could promote allergic inflammation and the development clinical signs. Further investigation into the expression of TJ proteins and their clinical relevance is needed.

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## Résumé

**Contexte** – Les jonctions serrées (Tight Junctions TJ) sont importantes pour la fonction barrière cutanée et pourraient également l'être dans la régulation de la pénétration antigénique de la dermatite atopique (AD). L'homme atopique a été décrit comme ayant une expression augmentée de certaines protéines de TJ dans la peau.

**Hypothèses/Objectifs** – Cette étude porte sur l'expression des protéines TJ à l'aide d'un modèle expérimental d'AD chez le chien.

**Méthodes** – Des biopsies cutanées de six chiens atopiques (peau non lésionnelle) et cinq chiens beagle sains ont été colorées par immunohistochimie pour les protéines de TJ [zonula occludens 1 (ZO-1), occludine, claudine-1]. L'intensité de coloration a été évaluée à la fois subjectivement et objectivement. Six images/sections ont été réparties et notées en aveugle par six investigateurs pour l'intensité, la distribution, l'intégrité et les patrons de coloration.

**Résultats** – L'intensité de ZO-1 était significativement diminuée dans le groupe atopique objectivement ( $P = 0.010$ ) et subjectivement ( $P = 0.002$ ) comparé au groupe normal. L'occludine était diminuée significativement subjectivement ( $P = 0.027$ ) mais pas objectivement. La claudine ne présentait pas de différence significative entre les groupes quelque soit la quantification. En outre, seule ZO-1 montrait un patron de coloration en patch significatif dans le groupe atopique. Il n'y avait pas de patron de coloration significatif dans cette étude.

**Conclusions et importance clinique** – ZO-1 et occludine, qui n'avaient pas été décrit comme étant associé au développement de l'AD humaine, pourraient jouer un rôle dans ce modèle de chien atopique. D'autres études sont nécessaires sur l'expression et la modulation des protéines de TJ et leur importance clinique.

## Resumen

**Introducción** – las uniones oclusivas intercelulares son importantes para la función de barrera de la piel y pueden ser relevantes en la modulación de la protección de alérgenos en casos de dermatitis atópica (AD). Los seres humanos con dermatitis atópica tienen una expresión disminuida de algunas proteínas en estas uniones.

**Hipótesis/Objetivos** – este estudio está enfocado a investigar la expresión algunas proteínas en las uniones oclusivas en un modelo experimental de dermatitis atópica en perros

**Métodos** – muestras de piel de seis perros tópicos (piel sin lesiones) y cinco perros normales de la piel se tiñeron para las proteínas de unión de las uniones oclusivas (zonula occludens -1 (ZO-1), occludina, claudina-1) mediante inmunohistoquímica. La intensidad de la tinción se evaluó objetivamente utilizando un programa de análisis de imagen y subjetivamente. Seis imágenes/secciones fueron distribuidas al azar y valoradas a ciegas por seis investigadores en referencia a distribución, integridad, y patrón de tinción.

**Resultados** – la intensidad de ZO-1 estuvo significativamente disminuida en el grupo atópico tanto de forma objetiva ( $P=0,010$ ) como subjetiva ( $P=0,002$ ) en relación al grupo normal. La ocludina estaba significativamente disminuida subjetivamente ( $P=0,027$ ), pero no objetivamente. La claudina no fue significativamente diferente entre ambos grupos en ningún método cuantitativo. Además, sólo ZO-1 demostró un patrón de tinción más granular en perros atópicos. No hubo un patrón de tinción consistente en este estudio.

**Conclusión e importancia clínica** – ZO-1 y ocludina, que no han sido escritas como asociadas con el desarrollo de dermatitis atópica en humanos, podrían jugar un papel en este modelo de perros atópicos. Hace falta mayor investigación de la expresión y modulación de las proteínas de las zonas de unión oclusivas y de su importancia clínica

## Zusammenfassung

**Hintergrund** – “Tight Junctions” (TJ) sind wichtig für die Schutzfunktion der Haut und könnten bei der Regulierung der Penetration der Allergene bei atopischer Dermatitis (AD) wichtig sein. Bei Menschen mit AD wurde eine verminderte Expression einiger TJ Proteine in der Haut beschrieben.

**Hypothese/Ziele** – Das Ziel dieser Studie war eine Untersuchung der Expression der TJ Proteine mit einem experimentellen AD Modell bei Hunden.

**Methoden** – Es wurden Hautbiopsien von sechs atopischen (nicht veränderte Haut) und fünf normalen Beagles mittels Immunhistochemie auf TJ Proteine gefärbt [Zonula occludens 1 (ZO-1), Occludin, Claudin-1]. Die Intensität der Färbung wurde objektiv mittels bildgebender Software und subjektiv beurteilt. Sechs Bilder/Schnitte wurden zufällig und geblendet von sechs UntersucherInnen auf Intensität, Verteilung, Integrität und Färbeverhalten bewertet.

**Ergebnisse** – Die Intensität von ZO-1 war in der atopischen Gruppe im Vergleich zur normalen Gruppe objektiv ( $p=0,010$ ) und subjektiv ( $p=0,002$ ) vermindert. Occludin war subjektiv ( $p=0,027$ ), aber nicht objektiv vermindert. Claudin war zwischen den Gruppen weder subjektiv noch objektiv signifikant unterschiedlich. Zusätzlich zeigte nur ZO-1 eine signifikant fleckigere Anfärbung als die atopische Gruppe. Es bestand keine konstante Anfärbung in dieser Studie.

**Schlussfolgerungen und klinische Bedeutung** – ZO-1 und Occludin, welche bisher nicht im Zusammenhang mit der Entstehung von AD beim Menschen beschrieben worden waren, könnten in diesem Modell des atopischen Hundes eine Rolle spielen. Es sind weitere Studien über die Expression und die Modulation der TJ Proteine und ihre klinische Relevanz nötig.

## 要約

**背景** – タイトジャンクション(TJ)は皮膚バリア機能にとって重要で、アトピー性皮膚炎(AD)におけるアレルゲンの侵入の調節に関係している可能性がある。ADのヒトでは皮膚におけるいくつかのTJタンパクの発現が減少していると説明されている。

**仮説/目的** – この研究はAD実験犬を用いてTJタンパクの発現を調査することを目的とした。

**方法** – 6頭のアトピー犬(非病変部皮膚)および5頭の正常ビーグル犬から採取した皮膚生検材料を用いて、TJタンパク[閉鎖帯1(ZO-1)、オクルディン、クローディン-1]を免疫組織化学で染色した。染色濃度は画像ソフトウェアを用いて客観的および主観的の両方で評価した。6つの画像/切片を6人の調査者が濃度、分布、整合性および染色パターンに関してランダムおよび盲検的にスコアをつけた。

**結果** – ZO-1の濃度は正常群と比較し、アトピー群において客観的( $P=0,010$ )および主観的( $P=0,002$ )に有意に減少していた。オクルディンは主観的に有意に減少していたが( $P=0,027$ )、客観的には有意でなかった。クローディンはいずれの定量化によっても2つの群の間に有意差は見られなかった。さらに、ZO-1のみでアトピー群において有意な斑状の染色パターンを示した。

**結論および臨床的な重要性** – ZO-1およびオクルディンはヒトのADの発生に関連しているとは記述されていないが、アトピー犬モデルにおいては役割を果たしている可能性がある。TJタンパクの発現および調節とそれらの臨床的な関係に関するさらなる研究が必要である。

## 摘要

**背景** – 紧密链接(TJ)蛋白对于皮肤屏障功能很重要,还与调整异位性皮炎(AD)过敏原渗入密切相关。人AD患者显示某些皮肤TJ蛋白表达减少。

**假设/目的** – 本次研究目的为使用犬AD实验模型评估TJ蛋白质表达。

**方法** – 从六只异位性皮炎患犬(非病变皮肤)和五只正常比格犬上取皮肤活检样本,并使用免疫组化将TJ蛋白[紧密连接1(ZO-1)、闭合蛋白、封闭蛋白-1]染色。着色强度用图像软件客观及主观评估。六个图像/切面随机、盲目派给六位评估者进行评估,评估着色强度、分布、完整性和着色模式。

**结果** – 异位性皮炎组ZO-1的着色强度客观评估( $p=0,010$ )和主观评估( $p=0,002$ )较对照组均显著降低。闭合蛋白主观评估显著降低,客观评估则没有显著降低。此外,异位性皮炎组只有ZO-1表现出显著的点缀着色模式。本次研究中没有一致的染色模式。

**总结与临床意义** – ZO-1和闭合蛋白在人AD中没有研究,但在犬AD模型中起作用。TJ蛋白的表达和调整以及其临床关联仍需进一步调查评估。