

# Evaluation of filaggrin 2 expression in dogs with atopic dermatitis before and after oclacitinib maleate administration

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## Funding information

CAPES

## Abstract

**Background:** Canine atopic dermatitis (cAD) is a chronic, inflammatory, multifactorial and pruritic disease. The presence of skin barrier impairment (e.g. filaggrin alterations), along with abnormal immune responses, can negatively impact cutaneous barrier function.

**Objectives:** To evaluate the filaggrin 2 (FLG2) expression in atopic dogs before and after the administration of oclacitinib maleate.

**Animals:** Sixteen privately owned dogs with a diagnosis of cAD and 10 healthy control dogs.

**Materials and Methods:** Oclacitinib maleate monotherapy at 0.5 mg/kg, orally, twice-daily for the first 14 days and once-daily for 16 additional days, was administered to the atopic dogs. Skin biopsies from lesional and nonlesional skin were obtained from atopic dogs on Day(D)0 and D30 and from the same anatomical locations from the control group on D0. Immunohistochemical investigation was performed using a primary custom-made anti-canine-filaggrin 2 polyclonal antibody. Immunolabelled slides were scanned and FLG2 expression was measured. Data were analysed and a  $p$ -value  $\leq 0.05$  was considered statistically significant.

**Results:** There was a higher FLG2 expression in control skin when compared with atopic skin (lesional and nonlesional) on D0 ( $p=0.033$ ). FLG2 expression comparison between control and D30 (nonlesional) did not show a significant difference ( $p=0.509$ ). A significant increase in FLG2 expression in atopic nonlesional skin on D30 compared with nonlesional skin on D0 was also observed ( $p=0.014$ ).

**Conclusions and Clinical Relevance:** Oclacitinib maleate could have a positive impact on cutaneous barrier structure, improving FLG2 expression by decreasing inflammation and cutaneous trauma.

## KEYWORDS

atopic dermatitis, dog, filaggrin, oclacitinib, skin barrier

## INTRODUCTION

Canine atopic dermatitis (cAD) results from the interaction between genetics and environmental factors that shape the immune response and skin barrier function.<sup>1</sup> Epidermal barrier dysfunction occurs in both human AD and canine (c)AD, allowing penetration of irritants, micro-organisms, microbial antigens and environmental allergens. This stimulates the local immune system and induces inflammatory responses.<sup>2</sup> Aberrant lipid composition, defective and decreased structural proteins,

increased skin pH and reduced skin microbiome diversity are some of the known skin barrier alterations present in human patients with AD.<sup>3</sup>

Filaggrin (FLG) belongs to the S100 fused-type protein family along with filaggrin 2 (FLG2), trichohyalin and hornerin, among others, and plays a critical role in epidermal differentiation and cutaneous barrier function.<sup>4</sup> It derives from profilaggrin, a precursor formed by several FLG units and stored in keratohyalin granules of the stratum granulosum.<sup>4</sup> Breakdown of FLG generates natural moisturising factors that maintain hydration and lower pH, and also

contribute to skin antimicrobial protection. FLG deficiency leads to increased epidermal pH, which stimulates serine protease activity to degrade corneodesmosomes and inhibit ceramides production.<sup>3</sup>

Mutations affecting the C-terminal portion of the *FLG* gene are one of the best confirmed risk factors for the development of AD in humans.<sup>5</sup>

In dogs with AD, the disruption of the skin barrier has been hypothesised. Decreased immunohistochemical staining,<sup>5,6</sup> mRNA altered expression, decreased<sup>7</sup> or increased<sup>8</sup> and nonhomogeneous distribution of FLG have been described as potential factors involved in the pathogenesis of cAD.<sup>1</sup> In dogs and humans, FLG and FLG2 have similar locations within the epidermis.<sup>9</sup> In humans, FLG2 is expressed in the stratum granulosum where it is processed into smaller fragments by the protease calpain 1.<sup>10</sup> The amino terminal domain of FLG2 is a component of cornified envelopes and co-localises with corneodesmosin, implying that FLG2 participates in epidermal and stratum corneum adhesion. FLG2 alterations in the skin of atopic humans<sup>10</sup> and dogs<sup>11,12</sup> have been described.

Abnormal immune responses also have been linked to the pathogenesis of AD. In humans, it is documented that T-helper (Th)2, Th22 and Th17 cells are involved in the acute stage of the disease.<sup>11,13</sup> Th2-polarised immune responses have been demonstrated in cAD with increased levels of interleukin (IL)-4, IL-5 and IL-13 in the serum, peripheral blood mononuclear cells and lesional skin.<sup>14</sup> IL-31 also is a key mediator of pruritus in cAD,<sup>15</sup> by activating skin-innervating sensory neurons, as well as by enhancing the release of pro-inflammatory mediators from keratinocytes and immune cells.<sup>15</sup>

It is remarkable that, in addition to *FLG* gene mutations, type 2 inflammatory mediators, as well as inflammation mediated by Th17, Th22 and Th1, can also reduce FLG expression, as reported in humans with AD.<sup>16</sup>

Oclacitinib maleate is a janus kinase (JAK) inhibitor labelled for use in dogs for the control of pruritus associated with allergic dermatitis. It is effective at inhibiting JAK1, which plays a pivotal role in mediating the intracellular signalling of IL-2, IL-4, IL-6, IL-13 and IL-31, cytokines involved in allergy, inflammation and pruritus.<sup>17</sup>

Considering the relevance of Th2 responses in the pathogenesis of cAD and their possible involvement in cutaneous barrier alterations, we hypothesised that FLG2 expression in dogs with AD improves after the use of oclacitinib maleate. Hence, the aim of this study was to evaluate the FLG2 expression in the epidermis of atopic dogs before and after the administration of oclacitinib maleate.

## MATERIALS AND METHODS

### Institutional protocol review and approvals

This study was approved by the Ethics Committee on Animal Use (CEUA) of the Pontifical Catholic University of Paraná PUC-PR (No. 2248). Informed client consent was obtained by signature at the time of enrolment of each dog in both groups.

## Animals

Sixteen privately owned dogs with a diagnosis of AD, comprising seven mixed-breed, five shih-tzus, two Lhasa-apso, one beagle and one golden retriever (age range 1.5–12 years) and 10 healthy control dogs, with no history or clinical signs of skin disease, comprising seven mixed-breed, two Yorkshire terrier and one pug (age range 1–12 years) were included in this study.

Diagnosis of AD was based on compatible history, the presence of five or more signs under Favrot's 2010 criteria<sup>18</sup> and the exclusion of other causes of pruritus, through skin/ear canal cytological investigation and skin scrapings to rule out concurrent infections/infestations. Before enrolment, if present, secondary bacterial and/or yeast infections were addressed by appropriate antimicrobial therapy (topical/systemic medication). An ectoparasiticide (Afoxolaner Nexgard: Boehringer Ingelheim) was administered orally to the atopic dogs, also, according to body weight. No anti-inflammatory medications were given for  $\geq 4$  weeks before inclusion. Oclacitinib maleate (Apoquel: Zoetis) at 0.5 mg/kg, orally, twice-daily for the first 14 days and once-daily for 16 additional days, was administered to the atopic dogs. Concurrent topical therapies (i.e. antiseptics and moisturisers) were not allowed during the study period.

Dogs aged <12 months, weighing <3 kg, with nervous and/or aggressive behaviour, history or evidence of neoplasia, demodicosis and/or severe infections, as well as dogs exhibiting chronic AD (e.g. lichenification) and pregnant/lactating females, were excluded from the study.

## Clinical evaluation

Canine Atopic Dermatitis Extent and Severity Index—4th iteration (CADESI-04)<sup>19</sup> and pruritus Visual Analog Scale (pVAS)<sup>20</sup> evaluations were performed in the atopic dogs on Day (D)0, D15 and D30.

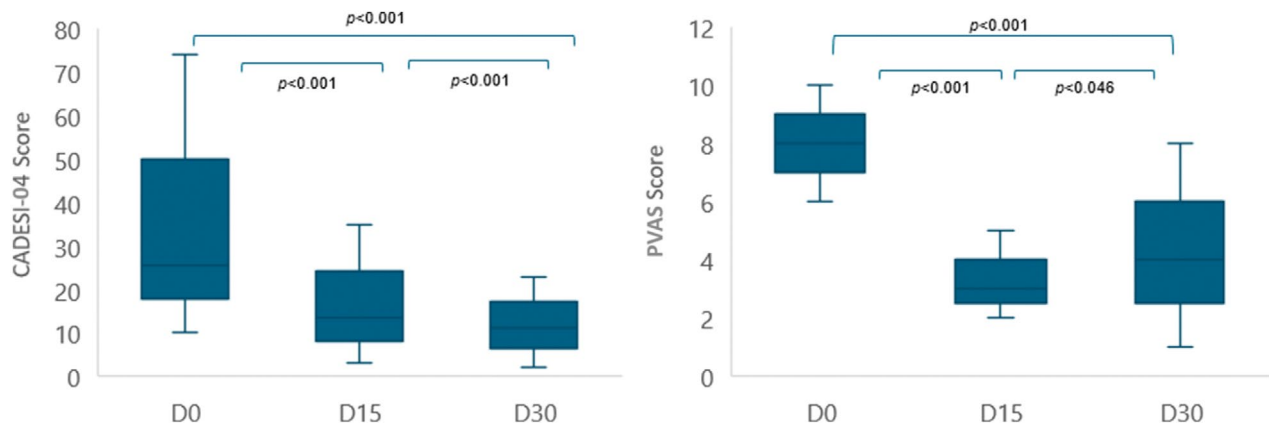
## Skin biopsies

Punch skin biopsies (6 mm diameter) were taken from lesional (erythema—axillae/inguinal area) and nonlesional skin (inguinal area) from the atopic dogs on D0 (before oclacitinib maleate) and D30 (after oclacitinib maleate), and from the same anatomical locations from the control group on D0. Biopsies were obtained after local anaesthesia with 2% lidocaine and were sutured routinely.

## Assessment of cutaneous filaggrin expression by immunohistochemical investigation

### Processing of samples

Skin biopsies from atopic dogs (lesional and nonlesional) obtained on D0 and D30 and from control dogs



**FIGURE 1** Reduction of the clinical scores Canine Atopic Dermatitis Extent and Severity Index—4th iteration (CADESI-04) and pruritus Visual Analog Scale (pVAS) associated with the use of oclacitinib maleate.

obtained on D0 were fixed in 10% neutral buffered formalin for 48 h and then subjected to standard histological processing. Tissue microarrays were prepared to perform immunohistochemical investigation. Slides were deparaffinised and subjected to antigen retrieval at pH9.0. Then, they were quenched in 3% hydrogen peroxide, and a primary custom-made anti-canine-filaggrin 2 polyclonal antibody (University of Florida, USA),<sup>11,21,22</sup> diluted at 1:500, was added for 1.5 h. Histofine 3,3'-diaminobenzoide substrate (Nichirei Biosciences) was used for the visualisation of the immunolabelling, and slides were counterstained with haematoxylin. Between reactions, slides were washed in distilled water and phosphate buffered saline.

### Imaging and image analysis

Immunolabelled slides were scanned (Axio Scan.Z1; Zeiss) obtaining 20 images for each animal using the software ZEN BLUE EDITION (Zeiss). Images were obtained from random areas and analysed blindly, with no interference from the observer. FLG immunolabelling scans were measured through the software IMAGE PRO-PLUS v4.5 (Media Cybernetics), using a colour semiautomated segmentation method, which artificially delimited and quantified the area of tissue immunoexpression. Immunolabelling values were initially obtained in absolute areas ( $\mu\text{m}^2$ ) and then converted into percentages through the formula:  $\% = (\text{immunopositive area} / \text{total tissue area}) \times 100$ . An immunolabelling average for each animal was calculated and analysed statistically.

### Statistics

Normality of data distribution was tested using the Shapiro–Wilk test. Student's *t*-test and ANOVA for independent groups and *t*-test for paired samples were used for parametric variables. Wilcoxon–Mann–Whitney and Kruskal–Wallis tests for independent groups as well as Wilcoxon and Friedman tests for dependent groups were used for nonparametric variables. Spearman coefficient was used to correlate two

continuous nonparametric variables. A *p*-value  $\leq 0.05$  was considered significant. For data analysis, JMP PRO 14.0.0 (SAS Institute) was used.

### RESULTS

A significant reduction in the clinical scores, CADESI-04 ( $p < 0.001$  between D0, D15 and D30) and PVAS ( $p < 0.001$  for D15 and D30 compared with D0 and  $p = 0.046$  between D15 and D30), associated with the use of oclacitinib maleate was observed (Figure 1).

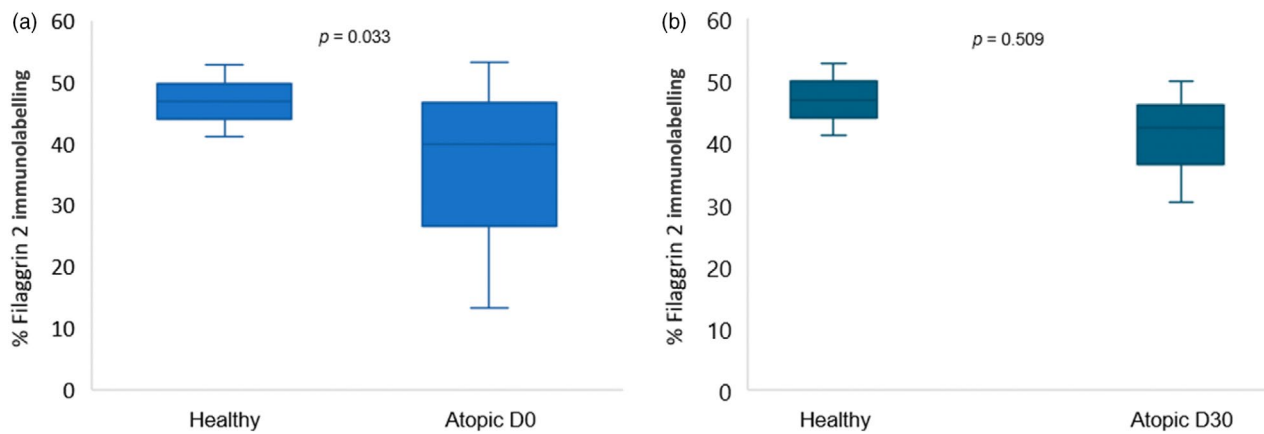
For the percentage of FLG2 immunostaining, there was a higher FLG2 expression in control skin when compared with atopic skin (lesional and nonlesional) on D0 ( $p = 0.033$ ) (Figure 2a). Interestingly, the comparison of FLG2 expression between control and D30 (nonlesional) did not show a significant difference ( $p = 0.509$ ) (Figure 2b). A significant increase in FLG2 expression in atopic skin on D30 compared with nonlesional on D0 ( $p = 0.014$ ) (Figure 3) also observed. Conversely, the comparison between FLG2 immunolabelling of lesional skin on D0 and D30 did not exhibit a significant difference ( $p = 0.688$ ) (see Figure S1).

Spearman's matrix showed no positive correlation between FLG2 expression and clinical scores, CADESI-04 (D0  $\rho = -0.026$ ; D15  $\rho = 0.073$ ; D30  $\rho = 0.289$ ) and PVAS (D0  $\rho = 0.216$ ; D15  $\rho = -0.111$ ; D30  $\rho = -0.058$ ).

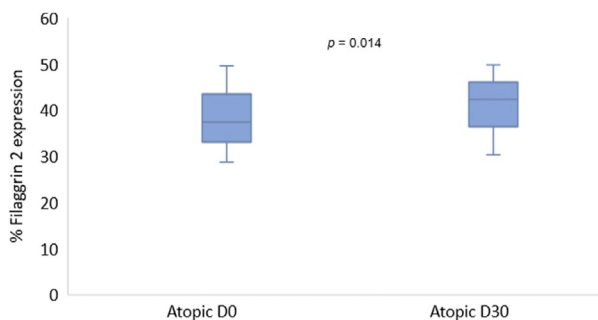
Figure 4 shows representative images of FLG2 immunolabelling in skin sections from normal and atopic dogs (lesional and nonlesional) on D0 and atopic on D30 (nonlesional), after oclacitinib maleate administration.

### DISCUSSION

The results of this study showed significantly higher FLG2 immunostaining in control dogs than in atopic dogs (lesional and nonlesional), as described previously.<sup>5,6,21</sup> Moreover, there was a significant increase in FLG2 expression on D30 nonlesional skin compared with atopic nonlesional skin on D0, suggesting a positive impact of oclacitinib maleate on the physical skin barrier, possibly linked to the blocking effect of the drug on Th2 cytokines. This suggests that cutaneous



**FIGURE 2** Comparisons of filaggrin 2 expression between control and atopic skin (lesional and nonlesional) on Day (D)0 (a), and between control and atopic skin on D30 (nonlesional) after oclacitinib maleate (b).



**FIGURE 3** Comparison of filaggrin 2 expression in nonlesional atopic skin on Day (D)0 (before oclacitinib maleate) and D30 (after oclacitinib maleate).

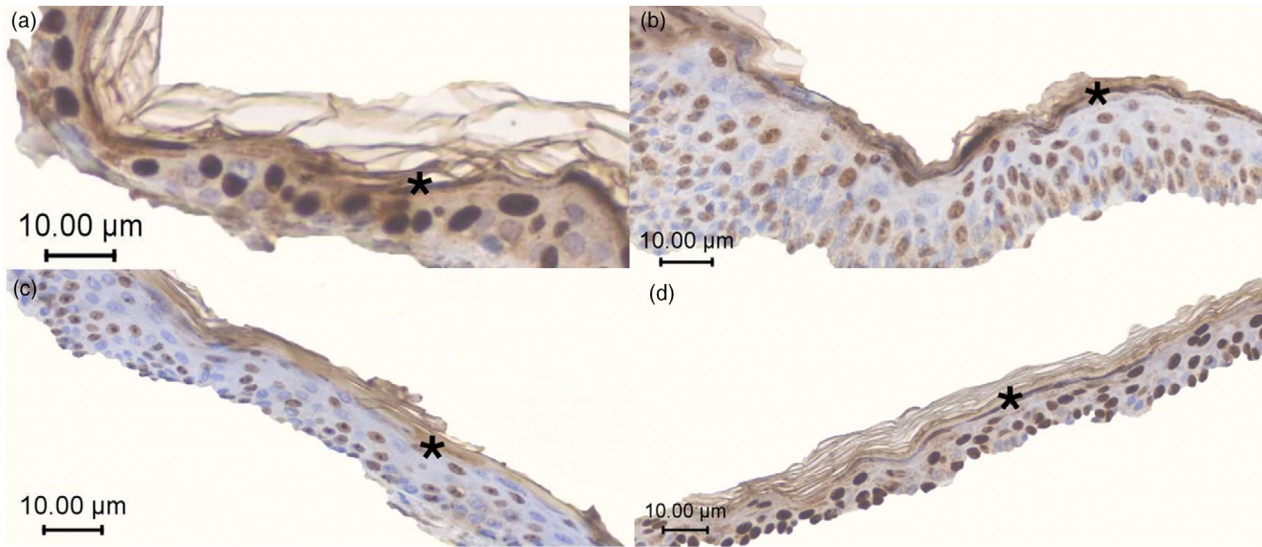
inflammation plays a key role in disrupting the skin barrier in dogs with AD. Another interesting finding was the nonsignificant difference between control and atopic skin on D30; thus, it can be speculated that the use of oclacitinib maleate could improve FLG2 expression in atopic dogs at a similar level to healthy skin. These findings support our hypothesis, specifically in nonlesional atopic skin.

Most humans with AD have altered FLG expression on either a genetic<sup>23</sup> or acquired basis (e.g. Th2 inflammation and repetitive scratching).<sup>16</sup> Alarmins, including IL-33 and TSLP, and type 2 inflammatory mediators, such as IL-4, IL-13 and IL-31, can reduce FLG expression. This has also been described in cutaneous inflammation mediated by Th17 (IL-17), Th22 (IL-22) and Th1 (IL-1 $\alpha$ , IL-1 $\beta$  and tumour necrosis factor- $\alpha$ ). Likewise, other innate immune effector cells, including eosinophils, basophils and mast cells, increase the release of mediators that aggravate inflammation and worsen skin barrier disorders by downregulating stratum corneum structural proteins.<sup>16</sup> Type 2 inflammatory cytokines and alarmins can also contribute to the itch–scratch cycle by activating pruritogenic neurons, intensifying skin barrier disruption and allowing bacterial colonisation, hence perpetuating the inflammatory responses.<sup>16</sup>

Prominent Th2-polarised immune responses also have been demonstrated in cAD with variably increased levels of IL-4, IL-5 and IL-13 in the serum,

peripheral blood mononuclear cells and lesional skin, as well as increased numbers of Th2 cells in the peripheral circulation.<sup>14</sup> However, it is unclear whether decreased FLG expression in atopic dogs is the result of a primary skin barrier defect (genetic) and/or secondary to inflammation.<sup>21</sup> One study showed that treatment of canine reconstructed epidermis with Th2 cytokines decreases FLG immunodetection,<sup>24</sup> suggesting an impact of inflammation on this protein. Other researchers found that treatment with ciclosporin led to an increase in FLG expression compared to that before treatment.<sup>25</sup> Considering these findings, modulation or suppression of Th2 cytokines could be beneficial and secondarily improve skin barrier function<sup>21</sup> as described in the present study with the use of oclacitinib maleate. This could represent additional evidence of the possible involvement of allergic inflammation as a main actor in cutaneous barrier defects in dogs.

Oclacitinib maleate acts as an inhibitor of the function of important pro-inflammatory, pro-allergic and pruritogenic cytokines via inhibition of the JAK signal transducer and activator of transcription signalling pathway.<sup>26</sup> The suppressive effect of this drug on Th2 responses, leading to a reduction in skin inflammation, could improve FLG2 expression in nonlesional atopic skin in this study, after 4 weeks of its use. Conversely, this effect was not statistically significant on lesional skin, implying a promising proactive, not reactive, effect on cutaneous barrier structure, or possibly the need for longer treatments to improve FLG2 expression on the more modified lesional skin. This could be explained by the fact that IL-31 is one of the earliest and most pivotal cytokines involved in acute cAD. One study showed upregulation of messenger RNA encoding IL-31 in the skin of atopic dogs as early as 6 h after allergen challenge, when erythema was not yet evident.<sup>27</sup> However, the same study demonstrated that 48 h after allergen challenge, there was amplification of additional Th2 (IL-4, IL-5, IL-13, IL-31 and IL-33), Th9 (IL-9) and Th22 (IL-22) cytokines, as well as Th2-promoting chemokines,<sup>27</sup> some of them being pathways that escape from the oclacitinib maleate mode of action, which could justify the nonsignificant improvement of FLG2 expression in atopic dogs exhibiting erythema



**FIGURE 4** Representative filaggrin 2 immunolabelling in the stratum granulosum and stratum corneum by immunohistochemical investigation (asterisks). (a) Control skin; (b) atopic nonlesional skin on Day (D)0; (c) atopic lesional skin on D0 (erythema); and (d) D30 nonlesional skin (after oclacitinib maleate). ZEN BLUE EDITION (Zeiss) software.

(lesional skin). Additionally, the description of these transcriptomes<sup>8</sup> could explicate the lack of positive correlation between FLG2 expression and clinical scores (CADESI-04 and PVAS) in this study. Exaggerated inflammatory responses may result in widely diffused secondary changes that influence the AD phenotype, such as suppression of epidermal differentiation genes, including FLG, by Th2, Th22 and Th1 cytokines,<sup>28</sup> as well as excessive degradation of FLG and disorders in the processing of profilaggrin, as described in mice and humans.<sup>29</sup>

The primary custom-made anti-canine-filaggrin polyclonal antibody used in this study was directed to canine FLG2. The *FLG2* gene has similarities in genomic sequence as well as protein distribution with *FLG*, and its participation in skin barrier defects has been proposed in humans and mice.<sup>30,31</sup> A comparison between the updated canine database and its human counterpart suggested that the previously identified canine *FLG* gene<sup>5,7,8,32–35</sup> is more similar to human *FLG2* than to the human *FLG* gene. A recent study evaluated the expression of both filaggrins, FLG and FLG2, in normal and atopic skin biopsies from dogs before and after allergen challenges, by immunohistochemical investigation and Western blot. The authors concluded that staining using an antibody for canine FLG<sup>36</sup> and one for canine FLG2 results in a comparable epidermal location, suggesting a similar distribution, and not a similar behaviour, of these proteins in canine skin.<sup>12</sup> We observed that canine atopic skin has a significantly decreased expression of FLG2, compared to healthy skin. This finding reinforces the likelihood of FLG2 affectations in dogs with AD.

Limitations of this study include the small number of animals, the variability of breeds and the short period of oclacitinib maleate use assessed. However, the differences in FLG2 expression observed between healthy and atopic dogs appear to be similar in all animals, despite the different breeds included, leading to statistically significant results.

In conclusion, we observed a significant increase in FLG2 expression in atopic nonlesional skin after 30 days of oclacitinib maleate administration. These results suggest that this drug could have a positive impact on cutaneous barrier structure, improving FLG2 expression by decreasing inflammation and cutaneous trauma. This could represent an additional benefit of oclacitinib maleate as a proactive therapy, leading to a possible improvement of the skin barrier in dogs with subclinical or mild cutaneous inflammation. Further studies with more animals and more extended study periods are needed to understand the extent to which therapies targeting type 2 inflammatory responses can improve skin barrier function and how they can impact clinical manifestations in dogs with AD.

**AUTHOR CONTRIBUTIONS**

**Wendie Roldan Villalobos:** Conceptualization; investigation; writing – original draft; methodology; validation; writing – review and editing; supervision. **Tássia Ferreira:** Investigation; methodology; validation. **Fernanda Borek:** Investigation; methodology; validation. **Domenico Santoro:** Investigation; methodology; validation. **Lluís Ferrer:** Investigation; validation; writing – review and editing; supervision. **Marconi Farias:** Conceptualization; investigation; methodology; validation; writing – review and editing; supervision.

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**CONFLICT OF INTEREST STATEMENT**

No conflicts of interest have been declared.

## DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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### Résumé

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### Resumen

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### Zusammenfassung

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## SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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