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WENDIE ORIANA ROLDÁN VILLALOBOS

Avaliação da expressão da filagrina cutânea em cães com dermatite atópica antes e após a administração de maleato de oclacitinib.

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

Curitiba, 2024

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ATA Nº 051 E PARECER FINAL DA DEFESA DE TESE DE DOUTORADO EM SAÚDE, TECNOLOGIA E PRODUÇÃO ANIMAL INTEGRADA DA ALUNA WENDIE ORIANA ROLDAN VILLALOBOS

Aos trinta dias do mês de abril do ano de dois mil e vinte e quatro, às 9h, na sala virtual do Zoom, realizou-se a sessão pública de defesa de tese da doutoranda Wendie Oriana Roldan Villalobos, intitulada: **"AVALIAÇÃO DA EXPRESSÃO DA FILAGRINA CUTÂNEA EM CÃES COM DERMATITE ATÓPICA ANTES E APÓS A ADMINISTRAÇÃO DE MALEATO DE OCLACITINIB"**. A doutoranda concluiu os créditos exigidos para obtenção do título de Doutora em Ciência Animal, Área de Concentração em Saúde, Tecnologia e Produção Animal, segundo os registros constantes na secretaria do Programa. Os trabalhos foram conduzidos pelo Professor Orientador e Presidente da banca, Dr. Marconi Rodrigues de Farias (PUCPR), auxiliado pelos professores doutores Pedro Vicente Michelotto Júnior (PUCPR), Herberto José Chong Neto (UFPR), Vânia Oliveira de Carvalho (UFPR) e Daniel Guimarães Gerardi (UFRGS). Procedeu-se a exposição da tese, seguida de sua arguição pública e defesa. Encerrada a fase, os examinadores expediram o parecer final sobre a tese, que foi considerada APROVADA.

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Proclamado o resultado, o Presidente da Banca Examinadora encerrou os trabalhos, e para que tudo conste, eu Caroline Nocera Bertton, confiro e assino a presente ata juntamente com os membros da Banca Examinadora.

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SUMÁRIO

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FORMATO DA TESE

A presente tese é composta por capítulos. O capítulo 1 apresenta uma introdução e contextualização do tema através de uma revisão de literatura, sobre a filagrina e o seu papel na Dermatite Atópica, formatada nas normas da revista científica “Journal of Veterinary Internal Medicine”, a qual será submetida. O capítulo 2 trata-se do artigo científico completo, avaliando a expressão da filagrina na pele de cães atópicos, antes e depois da administração de maleato de oclacitinib, formatado nas normas da revista Veterinary Dermatology para o qual será submetido. O capítulo 3 finaliza esta tese com as conclusões gerais, considerações finais e impacto e perspectivas do trabalho.

RESUMO GERAL

A filagrina (FLG) é um componente crítico no envelope corneificado (EC), na camada externa da epiderme. A Dermatite Atópica (DA), a principal doença relacionada com deficiência de FLG, é uma dermatopatia crônica, inflamatória, multifatorial e pruriginosa. A presença de alterações na barreira cutânea, bem como de respostas imunes anormais podem afeitar negativamente a função da barreira, ocasionando anormalidades nos lipídeos extracelulares e nas proteínas de junção e estruturais, incluindo as filagrinas. O objetivo de este estudo foi avaliar a expressão da FLG na epiderme de cães atópicos antes e depois da administração de maleato de oclacitinib. Dezesseis cães com diagnóstico de DA e 10 cães hígidos (controle) foram incluídos no estudo. Maleato de oclacitinib (Apoquel: Zoetis, Brasil) foi administrado nos cães atópicos na dose de 0.5 mg/kg, via oral, a cada 12 horas durante 14 dias e a cada 24 horas durante 16 dias adicionais. CADESI-4 (Canine Atopic Dermatits Extent and Severity Index) e pVAS (Pruritus Visual Analog Scale) foram avaliados nos cães atópicos nos dias 0, 15 e 30. Biopsias de pele lesional (eritema- axila/virilha) e alesional (virilhas) foram obtidas dos cães atópicos nos dias 0 e 30 e dos cães hígidos, das mesmas localizações, no dia 0. As amostras foram processadas através de imuno-histoquímica utilizando um anticorpo policlonal “custom made” anti-filagrina canina (1:500). As lâminas imunomarcadas foram escaneadas (Axio Scan.Z1, Zeiss, Jena, Germany) e 20 imagens de cada cão foram analisadas, utilizando o software ZEN Blue Edition (Zeiss, Jena, Germany). A expressão da filagrina foi mensurada através do software Image Pro-Plus versão 4.5 (Media Cybernetics, Rockville, MD), usando um método de segmentação semiautomatizada por cores, para ser expressa em percentagens. Os dados foram analisados estatisticamente e um valor de $p \leq 0.05$ foi considerado significativo. Houve uma redução significativa dos escores clínicos, CADESI-4 e pVAS, entre os dias 0 e 30 ($p<0.001$). Houve um aumento na expressão da FLG na pele dos cães controle quando comparada com a pele atópica (lesional e alesional) ($p=0.033$). Interessantemente, não se observou diferença significativa na expressão da FLG entre o grupo controle e o dia 30 ($p=0.509$). Foi observado um aumento significativo na expressão da FLG na pele atópica alesional no dia 30 quando comparada com o dia 0 ($p=0.014$). Os resultados de este estudo sugerem que o

maleato de oclacitinib pode ter um impacto positivo na estrutura da barreira cutânea, melhorando a expressão das filagrinas através da diminuição da inflamação e o trauma cutâneo. Isso poderia representar um benefício adicional do maleato de oclacitinib como terapia proativa, resultando possivelmente em uma melhora da barreira cutânea. Estudos adicionais são necessários para compreender as possíveis vantagens das terapias dirigidas às respostas inflamatórias do tipo Th2 na função da barreira cutânea e como isso poderia impactar as manifestações clínicas da DA em cães.

Palavras-chave: Filagrina, canino, cão, dermatite atópica, barreira cutânea, maleato de oclacitinib, epiderme.

ABSTRACT

Filaggrin (FLG) is a critical component of the cornified envelope (CE) in the outer layer of the epidermis. Atopic dermatitis (AD), the main disease associated with FLG deficiency, is a chronic, inflammatory, multifactorial, and pruritic dermatopathy. The presence of skin barrier impairment and abnormal immune responses can negatively impact cutaneous barrier function. This may lead to abnormalities in extracellular lipids, junctional and structural proteins, including filaggrins. The aim of this study was to evaluate the filaggrin expression in the epidermis of atopic dogs before and after the administration of oclacitinib maleate. Sixteen privately-owned dogs with a diagnosis of AD and 10 healthy control dogs were included in this study. Oclacitinib maleate (Apoquel: Zoetis, Brazil) at 0.5 mg/kg, orally, q12h for the first 2 weeks and q24 h for 2 additional weeks, was administered to the atopic dogs. CADESI-4 (Canine Atopic Dermatitis Extent and Severity Index) and pVAS (Pruritus Visual Analog scale) evaluations were performed in the atopic dogs on day 0, 15 and 30. Skin biopsies from lesional (erythema- axillae/inguinal area) and non-lesional skin (inguinal area) were obtained from atopic dogs on days 0 and 30. Skin biopsies from the same anatomic location were collected from the control group on day 0. Immunohistochemistry was performed using primary custom-made anti-canine-filaggrin polyclonal antibody (1:500). Immunolabeled slides were scanned (Axio Scan.Z1, Zeiss, Jena, Germany) and 20 images from each dog, using the software ZEN Blue Edition (Zeiss, Jena, Germany), were analyzed. FLG expression was measured through the software Image Pro-Plus version 4.5 (Media Cybernetics, Rockville, MD), using a color semiautomated segmentation method, and then expressed in percentages. Data were analyzed statistically and a p value ≤ 0.05 was considered statistically significant. There was a significant reduction of the clinical scores, CADESI-4 and pVAS, between day 0 and day 30 ($p< 0.001$). There was a higher FLG expression in control skin when compared with atopic skin (lesional and non-lesional) ($p=0.033$). Interestingly, FLG expression comparison between control and day 30 did not show significant difference ($p=0.509$). A significant increase in FLG expression in non-lesional atopic skin on day 30 compared with day 0 was also observed ($p=0.014$). The results of this study suggest that oclacitinib maleate could have a positive impact on cutaneous barrier structure, improving

filaggrins expression by decreasing g 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. Further studies are needed to understand to what extent therapies targeting type 2 inflammatory responses can improve skin barrier function and how it can impact clinical manifestations in dogs with AD.

Keywords: Filaggrin, canine, dog, atopic dermatitis, cutaneous barrier, oclacitinib maleate, epidermis.

CAPÍTULO 1

Artigo a ser submetido para publicação na revista científica "Journal of Veterinary Internal Medicine"

Filaggrin in atopic dermatitis: What do we know?

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ABSTRACT

Filaggrin (FLG) is a critical component of the cornified envelope (CE) in the outer layer of the epidermis. Atopic dermatitis (AD), the main disease associated with FLG deficiency, is characterized by cutaneous barrier defects, enhanced allergen penetration, susceptibility to cutaneous bacterial colonization and infection, and cutaneous inflammation. Loss-of-function mutations in the FLG gene has been identified as the major genetic predisposing factor for AD in humans. Other factors such as Th2-mediated cutaneous inflammation and increased protease activity can also lead to reduced FLG expression in atopic skin, even in patients not carrying a FLG mutation. There are several studies regarding the role of FLG in canine atopic dermatitis, which showed alterations in FLG mRNA expression, FLG cutaneous expression, and gene mutations in some breeds. The aim of this article is to provide a review including current and relevant information regarding FLG, from the protein function in the skin physiology to its role in the pathogenesis of AD.

Keywords: Filaggrin, canine, dog, atopic dermatitis, cutaneous barrier, epidermis.

RESUMO

A filagrina (FLG) é um componente crítico no envelope corneificado (EC), na camada externa da epiderme. A Dermatite Atópica (DA), a principal doença relacionada com deficiência de FLG, é caracterizada por defeitos na barreira cutânea, facilitando a penetração de alérgenos, a susceptibilidade à colonização e infecção bacteriana e a inflamação cutânea. As mutações de perda de função no gene da FLG, têm sido

identificadas como o principal fator genético predisponente para o desenvolvimento de DA em humanos. Outros fatores como a inflamação cutânea mediada por respostas Th2 e o aumento na atividade das proteases, podem levar a diminuição na expressão da FLG na pele atópica, mesmo em indivíduos sem mutações no gene da FLG. Há vários estudos relacionados com o papel da FLG na DA canina, os quais tem mostrado alterações na expressão do mRNA da FLG, na expressão cutânea da FLG, e mutações genéticas em algumas raças. O objetivo do presente artigo é oferecer uma revisão de literatura, incluindo informação atual e relevante sobre a FLG, desde a sua função na fisiologia da pele até o seu papel na patogênese da DA.

Palavras-chave: Filagrina, canino, cão, dermatite atópica, barreira cutânea, epiderme.

1. INTRODUCTION

The primary function of the skin, the largest organ of the body, is to act as a protective barrier between the host organism and its external environment (1). Epidermis is a complex, dynamic, self-renewing barrier. Cell proliferation in the epidermis is limited to epidermal stem cells in the basal layer. After cell division, daughter cells exit the cell cycle and migrate to form the spinous cell layers, where cell junctions are strengthened, and additional keratin proteins are expressed. Closer to the skin surface, the cells of the granular layer contain dense cytoplasmic granules mainly composed of profilaggrin, with other protein components required for the formation of squames, the flattened, dead cells of the outermost stratum corneum (SC). Two important functions of this stratified, cornified squamous epithelium are to prevent water loss through the huge surface area of the body and to block the entry of foreign substances (pathogens, antigens, allergens, and chemical irritants) from the external environment (2)

Profilaggrin is expressed during the epidermal differentiation, in the granular cell layer. The cytoplasm of the epidermal granular cells is packed with dense-staining keratohyalin granules (KHG), which act as a reservoir of inactive profilaggrin, along with other proteins that are important for squame formation and maturation, such as loricrin. The transitional zone is essentially the uppermost layer of viable granular cells that are in the final process of differentiation into the flattened, tightly packed, and chemically cross-linked anuclear keratinocytes (squames), which make up the SC.(2) After release

in the cytoplasm, in response to increased Ca^{2+} levels, profilaggrin is dephosphorylated and cleaved by proteases which leads to the production of filaggrin (FLG) monomers. The monomers aggregate keratin intermediate filaments forming tight bundles, and induce the collapse and flattening of corneocytes, an essential feature of cornified layer formation (3). Finally, FLG is deaminated and totally degraded by several proteases in the upper SC to release its constitutive amino acids, part of the 'natural moisturizing factors' (NMFs), which contributes to epidermal hydration and barrier function (3,4).

An association between loss-of-functions of the FLG gene in both, atopic dermatitis (AD) and ichthyosis vulgaris, has been documented in Caucasians (5,6), Asians (7), and Afro-Americans (8). FLG loss-of-function mutations are still the major genetic predisposing factor identified for the development of AD, as patients with these mutations are 3 to 5 times more likely to develop the disease (7). However, other factors like Th2-mediated cutaneous inflammation (9), as well as increased protease activity, can result in reduced FLG expression in lesional skin (10).

The aim of this article is to provide a review including current and relevant information related to FLG, from the protein function in the skin physiology to its role in the pathogenesis of AD in humans and dogs.

2. FILAGGRIN FUNCTION IN SKIN PHYSIOLOGY

The epidermal differentiation complex (EDC) is a chromosomal region comprising over fifty genes encoding proteins involved in the terminal differentiation and cornification process of keratinocytes (11). This includes genes for cornified envelop proteins such as loricrin, involucrin and small proline-rich proteins (SPRRs), as well as genes encoding calcium-binding proteins of the S100A family, late cornified envelope (LCE) and multifunctional proteins such as FLG and trichohyalin (12).

FLG was initially isolated from a protein fraction of the SC and identified as a basic histidine-rich protein (13). Proteins from this fraction were shown to aggregate with keratin filaments to form macrofibrils in vitro cell-free experiments (14). These cationic structural proteins, which associate specifically with intermediate filaments but not with other types of cytoskeletal proteins, were nominated as filaggrins (for filament

aggregating proteins) (14). Filaggrins are distinct proteins among species. For instance, rat and mouse filaggrins have different molecular weights (48 and 30 kDa, respectively) and different number of amino acids but show similar functional properties (15).

FLG is a critical component of the cornified envelope (CE) in the outer layer of the epidermis. It is synthesized initially as profilaggrin, an approximately 500 kDa highly phosphorylated, histidine rich polypeptide, which consists of an N-terminal S100 calcium-binding domain and a downstream B-domain, while the central region comprises a tandem organization of repeat units of the FLG polypeptide (16). Profilaggrin processing requires dephosphorylation in KHG, and specific furin cleavage (figure 1), separating the N-terminal from the FLG repeat domain. The N-terminal translocates into the nucleus of transition keratinocytes (1,17) where it localizes to apoptotic nuclei containing fragmented DNA before the formation of enucleated SC in normal epidermis.(12) During this process, the N-terminal is further cleaved by endoproteinases into A and B subdomains. Simultaneously, remnant protein is processed into oligomeric, and then single FLG repeats dissociated from KHG (12). Several proteases participate in this process, including profilaggrin endopeptidase 1, matriptase 1 and channel-activating protease 1 (prostasin, CAP1) (18,19)

During the post-translational processing of profilaggrin, the FLG polypeptides, each approximately 35 kDa, are proteolytically cleaved, followed by dephosphorylation (16) to produce various numbers of FLG monomers (figure 1) depending on the species; 10 to 12 in humans, 16 to 20 in mice (1,20), and 4 in dogs (21).

In dogs, profilaggrin is located in KHG of the stratum granulosum cells, and FLG in the matrix of the lower SC (21,22). No labelling is observed in the upper SC, probably because of the total degradation of FLG and the formation of NMFs (23) This localization pattern was evidenced using immunohistochemistry (IHC) (21,22) and post-embedding immunoelectron microscopy (22). One study, using IHC (24), reported that patterns of FLG expression were similar in different breeds (poodles, golden retrievers, shih-tzus, pugs and labradors) and body locations (16 different regions). However, this result is questionable according to some authors, in view of the pan-epidermal staining pattern

obtained, suggesting that the rabbit polyclonal anti-FLG antibody used was not specific for canine FLG (23).

FLG aggregates the keratin intermediate filaments into higher molecular parallel structures (16,17). At this phase, caspase 14 and calpain 1 degrade FLG peptides into free hydrophilic amino-acids or amino-acids by-products (figure 1). Histidine, the primary product of FLG processing, is further converted into urocanic acid (UCA) by histidase (l-histidine ammonia lyase) (25). UCA and other derivatives, such as pyrrolidone carboxylic, modulate pH levels and protect the skin from UV light (2,26).

FLG transcription is controlled by the transcription factors AP1, Skn1a/I and Oct1 – two POU-domain proteins and glucocorticoids (1,27). Moreover, the 'homeodomain-only protein' HOP positively regulates FLG (28,29).

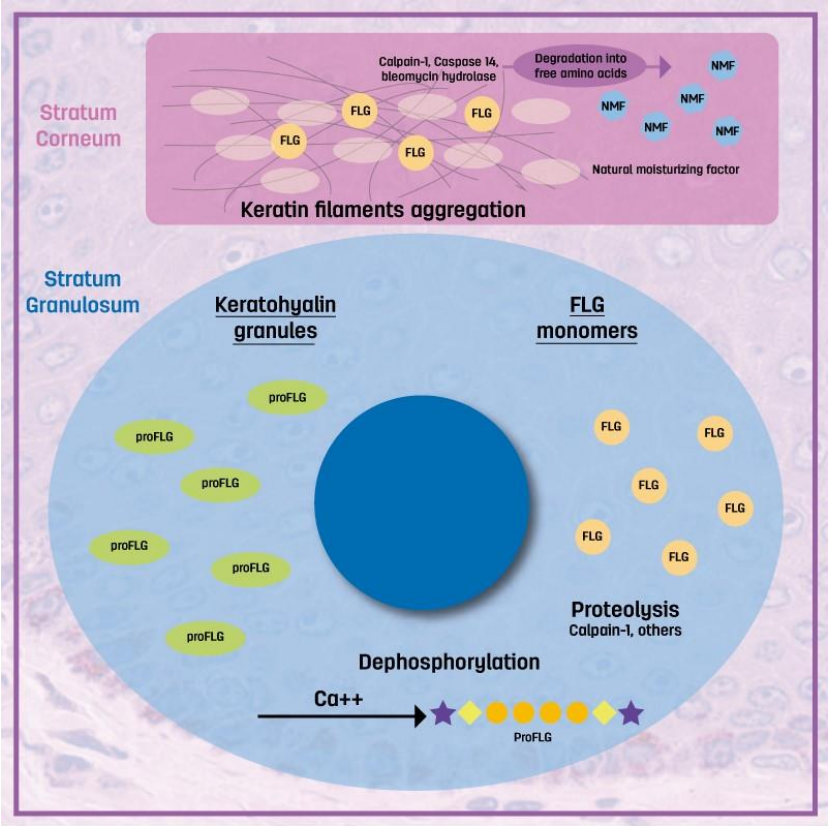

Figure 1. Profilaggrin (ProFLG) processing and filaggrin (FLG) metabolism. ProFLG is located in keratohyalin granules of the stratum granulosum cells, and FLG in the matrix of the lower stratum corneum. After release in the cytoplasm, in response to high Ca^{2+}

levels, ProFLG processing requires dephosphorylation in keratohyalin granules, and specific cleavage by proteases (e.g., calpain 1) to produce FLG monomers. FLG monomers aggregates the keratin intermediate filaments into higher molecular parallel structures. Subsequently, FLG peptides are degraded into free hydrophilic amino-acids or amino-acids by-products, forming the natural moisturizing factors (NMF).

Filaggrin-2 (FLG2) is another member of the S100 fused-type protein family (30). The deduced amino acid sequence of 2391 residues shows typical structural features of the ‘‘fused-type’’ S100 protein family members. This repetitive domain of FLG2 contains two types of tandem repeats, each 75–77 amino acids in length. The A-type repeats (A1–A9) are similar to the repeats of hornerin (50–77% identity) and the B-type repeats (B1–B14) are similar to the repeats of FLG (28–39% identity). FLG2 was detected in the granular and horny layers of normal stratified epithelium, which is the same pattern of distribution as FLG (31). Functionally, FLG2 seems to evolve to form the cornified envelope and NMFs in equal proportions, whereas most FLG is degraded to form NMFs (32,33). The first half of FLG2 is cross-linked to the envelope whereas the second half is degraded in the upper SC (33).

In a reconstructed epidermal model where the expression of FLG2 was downregulated by lentivirus mediated shRNA interference, the levels of FLG processing enzymes were reduced, resulting in decreased levels of FLG derived free amino acids. These results demonstrated that FLG2 has a key role in the processing of FLG to NMFs in the epidermis of human skin (34).

Seemingly, induction of FLG2 and FLG expressions may be coordinated. A study showed that the signals of FLG2 seemed to correspond to KHG and were generally co-localized with those of FLG (5).

Recent research suggests that the N-terminal domain of FLG2 regulates the activation of skin aspartic acid protease (SASPase), which is believed to be a key enzyme involved in FLG processing during epidermal terminal differentiation, regulating a key event upstream of FLG processing to NMFs in the human epidermis (35).

3. FILAGGRIN ALTERATIONS IN ATOPIC DERMATITIS

AD, the main disease associated with FLG deficiency, is characterized by cutaneous barrier defects, enhanced allergen penetration, susceptibility to cutaneous bacterial colonization and infection, and cutaneous inflammation driven by type 2 helper T (Th2) cells (36). Although AD was, for many years, considered to be a primarily immunologically driven disease with a secondary barrier defect (the inside-outside hypothesis), some researchers had hypothesized that the primary defect was in the skin barrier (the outside-inside hypothesis) (2,37). The remarkable association of FLG mutations with AD has validated the outside-inside hypothesis (2)

In 2006, the first loss-of-function mutations in the FLG gene were identified as the cause of ichthyosis vulgaris (6) and, as the major genetic predisposing factor for AD in humans (5).

FLG deficiency negatively influence several pathways relevant to skin barrier function, including disruption of keratinocyte differentiation, impaired corneocyte integrity and cohesion, impaired tight-junction formation (38) decreased water retention (39) altered lipid formation (40) and enhanced susceptibility to cutaneous infection.(38,41,42). In addition, reduced levels of NMFs increase the normally acidic stratum corneum pH (38), activating pH-sensitive serine proteases, leading to premature degradation of corneodesmosomes and activation of interleukin (IL)-1α and IL-1β (43,44). FLG deficiency has also been shown to impair the lipid profile and alter acidification pathways in in vitro skin models (40). Moreover, FLG mutations have been reported to be associated with atopic asthma, allergic rhinitis, nickel allergy and food allergy in humans (45), suggesting that FLG mutation-associated SC barrier defects lead to increased percutaneous allergen exposure (43)

FLG mutations are neither necessary to cause AD, and other factors could play an important role. For instance, Th2-mediated cutaneous inflammation can result in reduced FLG expression in lesional skin, even in patients not carrying a FLG mutation (9). Inflammatory responses may lead to widespread secondary changes that further contribute to the AD phenotype, such as suppression of epidermal differentiation genes, including FLG, by Th2, Th22 and Th1 cytokines (9,43,46). IL4 and IL13 may inhibit FLG

expression through down-regulation of keratinocyte differentiation by modulating the calcium-sensing protein S100A11 (47). In addition, increased protease activity during inflammatory responses can lead to excessive degradation of FLG due to alterations in the processing of profilaggrin (10). Environment could also play a key role, not only in the development of the atopic disease but also directly in FLG expression (7). For instance, increase in the external relative humidity and exposure to the sun or irritants can reduce epidermal FLG levels and lead to an acquired FLG deficiency (7,48).

3.1 FLG mutations in canine atopic dermatitis

FLG loss-of-function mutations have been identified as major risk factors for the development of AD in people, however, this does not appear to occur in most breeds of dogs (49). Research toward establishing a correlation between the severity of clinical signs and gene expression in the skin of atopic dogs has shown that genes relevant to skin barrier formation and immune function were altered (50). One study genotyped 97 single nucleotide polymorphisms (SNPs) in 25 candidate genes in 659 dogs across 8 breeds from three locations (United Kingdom, United States and Japan). Only one SNP within the Thymic Stromal Lymphopoietin (TSLP)-receptor was associated with all 8 breeds. Five SNPs within FLG, DPP4, MS4A2, and INPPL1 were associated with canine AD, but only in certain breeds from different locations. Though these associations are broadly similar to human AD the variability of results across the breeds and locations demonstrates that a candidate gene approach using mixed breeds from different locations is not appropriate (49)

Another research extracted gDNA from blood samples of 49 atopic and 30 non-atopic West Highland White Terriers (WHWT). Results showed that the haplotype frequencies did not differ significantly between affected and unaffected animals and excluded a large causative role for the canine FLG orthologue in atopic WHWT (51) Likewise, investigations using DNA samples from 96 WHWT with AD and 87 non-atopic controls WHWT showed that AD in this breed is associated with a region on CFA3 that contains several candidate genes. The authors also pointed out that a homozygous variant in the F2R gene present in multiple breeds that also suffer from AD warrants further evaluation (52). Another group analyzed SNPs coming from 35 atopic and 25 non-atopic WHWTs.

Their findings suggested that a major locus for canine AD in WHWTs may be located on, or in close proximity to an area on CFA 17 (53). A study made in Thailand assessed whether FLG SNP was associated with a susceptibility to canine AD in small breed dogs, comprising 21 Poodles, 17 Shih tzus and 3 Pugs. Twelve of these subjects were dogs with atopy and the remaining 39 samples were healthy controls. Results identified a novel repeated fragment and several new SNPs in the FLG gene of small breed dogs, nevertheless, the authors remarked the need of repeat the study with a larger population (54)

3.2 FLG cutaneous expression in canine atopic dermatitis

Although the expression of FLG in canine AD has been studied, the information regarding this matter is still unclear. Some researchers have attempted to demonstrate the possible involvement of FLG expression alterations in atopic dogs. For instance, one study showed that IHC staining intensity in the epidermis from 11 atopic and 4 healthy Beagle dogs was lower, both at baseline and after allergen challenge exposure (55) However, some authors have questioned these results, arguing that all the epidermal layers were immunostained, showing that the anti-FLG antibody used was nonspecific and cross-reacted with additional keratinocyte proteins (23)

Another study analyzed skin biopsies from 18 atopic and 16 control dogs from 12 different breeds through comparative immunofluorescence microscopy with an antibody raised against the canine FLG C-terminus region of the profilaggrin molecule. Results indicate that abnormalities in FLG protein expression are common in AD dogs (15／18), of which 4／18 (22%) presents a lack of C-terminal FLG protein, suggesting loss-of-function mutations (56)

3.3 FLG mRNA expression in canine atopic dermatitis

Regarding the expression of FLG mRNA, a study in atopic WHWT dogs found a peak association signal located more than 10 Mb from the canine FLG gene locus on CFA 17. This suggests that, unlike human AD, the orthologous canine FLG gene may not be a major genetic determinant for the development of the disease in this breed of dogs. (57) Conversely, other authors found a significant upregulation of FLG mRNA in lesional

atopic skin and also a trend to its upregulation in nonlesional atopic skin, as compared to healthy control skin (24)

In a study that challenged sensitized non-atopic beagles with a *Dermatophagoides farinae* (Df) extract, no significant changes in FLG mRNA expression were observed in skin biopsies (58)

Other researchers showed a significant upregulation of FLG mRNA in experimentally challenged atopic beagles, suggesting that increased protein degradation may lead to an increase in FLG gene expression due to a feedback mechanism. This study also confirmed that canine FLG is characterized by a ~54 kDa protein identified by western blotting, confirming that canine FLG repeats are larger than its human counterpart (59)

3.4 FLG metabolism alterations in canine atopic dermatitis

It is well known that two main steps have been identified in FLG metabolism: the breakdown of profilaggrin into FLG monomers, which involves several serine proteases including calpain-1, matriptase, furin, profilaggrin endoproteinase and kallikreins (21,32,60), and the final proteolytic cleavage of FLG into free amino acids and small peptides (NMFs) (18) involving enzymes like caspase-14, calpain-1 and bleomycin hydrolase (32,61,62). These enzymatic reactions, fundamental for the cutaneous homeostasis, are regulated by several protease inhibitors (62). Disruption of profilaggrin degradation to FLG may lead to abnormal barrier function and keratinization process with accumulation of profilaggrin (18,62) and a reduced breakdown of FLG may cause impaired skin hydration and UV light protection, due to reduced NMFs formation (61,63). Any change in this complex proteolytic balance can cause skin hydration abnormalities, hyperkeratosis, and contribute to AD, as it has been shown in humans (61,64).

Some studies have investigated the possible alterations of FLG metabolism in atopic dogs. One of them included 8 healthy and 8 atopic colony dogs. Biopsies from non-lesional atopic skin showed a marked decrease in caspase-14 staining (65). The other one demonstrated that calpain-1, caspase 14, furin and matriptase are expressed in all layers of the epidermis of atopic and normal dogs. Furthermore, the semi-quantitative assessment of the epidermal expression of 3 of 4 enzymes tested (calpain-1, caspase-

14 and matriptase) was higher in nonlesional atopic skin compared to healthy control skin, suggesting that changes in the metabolism of FLG may be present in atopic skin in the absence of evident inflammation (66). It is unknown whether this down or up-regulation of enzymes involved in FLG metabolism has any consequences for FLG degradation and NMFs production, since FLG expression has not been studied in parallel (23).

3.5 Immune responses alterations affecting FLG in canine atopic dermatitis

As stated above, it is remarkable the influence of Th2 responses in FLG expression, as the genetic predisposition does not sufficiently explain the development of eczema in all individuals with AD (46). Type 2 inflammatory mediators, including IL-4, IL-13, IL-31, IL-33, and TSLP, can reduce FLG expression. This has also been observed in skin inflammation mediated by Th17 (IL-17), Th22 (IL-22), and Th1 (IL-1α, IL-1β, and TNFα). Moreover, the recruitment of additional innate immune effector cells, including eosinophils, basophils, and mast cells, increase the release of mediators, such as histamine, that not only exacerbate inflammation but also worsen skin barrier disruption by downregulating SC structural proteins. (67) Type 2 inflammatory cytokines and alarmins can also promote the itch-scratch cycle by activating pruritogenic sensory neurons, which have IL-4, IL-13, IL-31, IL-33, and TSLP receptors, thus, exacerbating skin barrier disruption and promoting bacterial colonization, thus, perpetuating the inflammation. (67)

Exposure to IL-22 cytokine has been demonstrated to downregulate profilaggrin mRNA expression in keratinocytes by some authors, who also showed that the expression of genes involved in enzymatic processing of profilaggrin as well as the generation of NMFs were altered (46). IL 22 is secreted by Th22 (68) and Th17 cells (69) and other cellular sources as NK cells (70). IL-22 secretion by peripheral blood mononuclear cells and CD4+ T cells could be enhanced by exotoxins present in *Staphylococcus aureus*, (71) a bacterium frequently observed to colonize skin of atopic people.(46)

Causes of altered FLG expression in atopic dogs are still unclear. Whether these changes are primary or secondary to inflammation remains a matter of debate.(66) A model of reconstructed canine epidermis treated with proinflammatory Th2-type

cytokines, exhibited a high decrease in the anti-FLG immunolabeling (22), highlighting the possible negative impact of inflammation in the epidermal barrier structure. Another study showed that a single epicutaneous allergen challenge led to a transient and reversible decrease in skin surface NMFs and its components, suggesting that some of the skin barrier anomalies seen in atopic dogs likely develop secondarily to the underlying cutaneous allergic inflammation.(72)

3.6 FLG2 in canine atopic dermatitis

It is known that more than one FLG-type protein exists in dogs, although the exact function of FLG2 in dogs is not clear (23). The loss-of-function of FLG reported in people with AD (5), induced not only by the genetic risk factors, but also by proinflammatory cytokines, has been suggested to modulate the expression of both, FLG2 and FLG in atopic skin lesions (31).

Certain studies have addressed the FLG2 expression in canine AD. One study using an experimental model of canine AD did not find any correlation between FLG2 expression through IHQ, and the severity of the lesions (73) Another study showed that the early probiotic (*Lactobacillus rhamnosus*) exposure did not alter FLG2 expression before or after allergen challenge when compared to controls (74). Investigations on canine filaggrin mRNA and protein expression in the skin of experimental atopic beagles resulted in an increase of FLG2 mRNA expression in the atopic skin compared to healthy controls after allergen challenge. Interestingly, this increase of FLG2 mRNA was not accompanied by an increase in FLG2 immunolabeling through IHQ, suggesting an increase in protein degradation or post-transcriptional alteration (59). Additionally, in an experimental model of canine AD, FLG2, but not FLG, was decreased in the allergen exposed skin of sensitized dogs (58). A very 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 immunohistochemistry and Western blot. These authors concluded that staining using an antibody for canine FLG and one for canine FLG2 yields a similar epidermal location, suggesting the similar distribution of these two proteins.(75)

4. CONCLUSIONS

The causes of epidermal barrier alterations are complex, and include a combination of structural, genetic, environmental, and immunological factors.

FLG is a pivotal protein in the differentiation of the epidermis and the formation of the SC, essential to prevent water loss and to avoid the entry of pathogens, antigens, allergens, and chemical irritants. It is well known that FLG loss-of-function mutations represents the most significant predisposing factor for the development of AD in people, although, these mutations are still unclear in dogs.

Other factors affecting the expression of FLG in atopic skin include Th2 responses, which can down-regulate keratinocyte differentiation, and increased degradation of FLG by proteases, which lead to an increased expression of FLG mRNA, possibly in an attempt to compensate this degradation. These factors could better explain the FLG abnormalities found in the skin of atopic dogs, according to several studies, without excluding the possible role of a genetic background.

FLG2, a described member of the S100 protein family, which helps to form the cornified envelope and NMFs in the epidermis of human skin, seems to play a relevant role to maintain the normal function of the epidermal barrier in dogs, and could also be abnormal in atopic dogs.

Since data available concerning the role of FLG in canine AD is limited and difficult to interpret due to variations in populations, breeds, and environment conditions, compared to humans, it is important to highlight the need to continue studying this issue, in order to advance in the understanding of the cutaneous barrier dysfunctions in dogs with atopic dermatitis.

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CAPÍTULO 2

Artigo a ser submetido para publicação na revista científica "Veterinary Dermatology."

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

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Abstract

Background: Canine atopic dermatitis (AD) is a chronic, inflammatory, and pruritic disease. Skin barrier impairment and abnormal immune responses can negatively impact cutaneous barrier function, leading to abnormalities in lipids and proteins, including filaggrins.

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

Animals: Sixteen privately-owned dogs with a diagnosis of AD and 10 healthy control dogs.

Materials and Methods: Oclacitinib maleate at 0.5 mg/kg, orally, q12h for the first 2 weeks and q24 h for 2 additional weeks, was administered to16 atopic dogs. Canine Extent and Severity Index (CADESI-4) and Pruritus Visual Analog Scale (pVAS) evaluations were performed in the atopic dogs on day 0, 15 and 30. Skin biopsies from lesional and non-lesional skin were obtained from atopic dogs on days 0 and 30, and

from the same anatomic location from the control group on day 0. Immunohistochemistry was performed using primary custom-made anti-canine-filaggrin polyclonal antibody. Immunolabeled slides were scanned and analyzed. FLG expression was measured and expressed in percentages. Data were analyzed statistically and a *p* value ≤ 0.05 was considered statistically significant.

Results: There was a higher FLG expression in control skin when compared with atopic skin (lesional and non-lesional) (*p*=0.033). FLG expression comparison between control and day 30 did not show significant difference (*p*=0.509). A significant increase in FLG expression in non-lesional atopic skin on day 30 compared with day 0 was also observed (*p*=0.014).

Conclusions and clinical importance: Oclacitinib maleate could have a positive impact on cutaneous barrier structure, improving filaggrins expression by decreasing inflammation and cutaneous trauma.

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Introduction

Canine atopic dermatitis (AD) results from the complex interaction of genetics and environmental factors that shape the immune response and skin barrier function.[1] The skin barrier plays a key role in AD, and epidermal barrier dysfunction occurs in both human and canine AD, allowing penetration of irritant substances, microorganisms, microbial antigens, and environmental allergens. This, in turn, stimulates the local immune system and induces a type 2 inflammatory response.[2]

The epidermal barrier is composed of the stratum corneum (SC) and tight junctions (TJs). Epidermal barrier impairment in AD can result from altered lipid composition,

dysfunctional and decreased structural proteins, increased skin pH and reduced skin microbiome diversity.[3]

Filaggrin (FLG) is a structural protein that is fundamental in forming the cornified cell envelope and maintaining intercellular cohesion. It derives from a large precursor, named profilaggrin (proFLG), formed by several FLG units and stored in keratohyalin granules of the stratum granulosum.[4] Breakdown of FLG generates natural moisturizing factors that preserve hydration, lower surface pH and contribute to skin antimicrobial defense. FLG deficiency leads to increased epidermal pH, which promotes serine protease activity to degrade SC desmosomes and inhibit ceramides (CER) production.[3] Loss-of-function 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.[5]

In dogs, the disruption of the skin barrier, as a predisposing factor of AD, has been hypothesized. Decreased immunohistochemical staining [5,6] and mRNA overexpression,[7] along with a nonhomogeneous distribution of FLG in canine skin, have been suggested as a potential involvement in the pathogenesis of AD.[1]

In dogs and humans, two FLG-type proteins have been described with similar location within the epidermis and possibly overlapping functions.[8] In humans, filaggrin 2 (FLG2) is one of the most described members of the S100 fused-type protein family. It is expressed in the granulous layer of the epidermis where it is processed to smaller fragments by the protease calpain 1.[9] The amino terminal domain of FLG2 is a component of cornified envelopes and co-localizes with corneodesmosin, indicating that FLG2 plays a role in epidermal and SC adhesion. FLG2 alterations in the skin of atopic humans[9] and dogs[10,11] have been documented.

Aberrant immune responses have been also linked to the pathogenesis of AD. In humans, it is believed that involvement of T helper (Th)2, Th22 and Th17 cells occurs in the acute stage of the disease, and the involvement of Th2, Th22 and Th1 cells occurs in the chronic phase.[10,13] Prominent Th2-polarized immune responses have been demonstrated in canine AD with variably increased levels of interleukin (IL)-4, IL-5, and IL-13 in the serum, peripheral blood mononuclear cells, and lesional skin.[14] IL-31, has also emerged as an important mediator of pruritus in canine AD.[15] IL-31 mediates pruritus directly by activating somatosensory neurons that innervate the skin, as well as

indirectly by upregulating the release of proinflammatory mediators from keratinocytes and immune cells.[15]

In humans with AD, it is remarkable that, besides FLG gene mutations, type 2 inflammatory mediators, including IL-4, IL-13, IL-31, IL-33, and Thymic Stromal Lymphopoietin (TSLP), as well as inflammation mediated by Th17 (IL-17), Th22 (IL-22), and Th1 (IL-1α, IL-1β, and TNFα), can also reduce FLG expression.[16]

Oclacitinib maleate is a janus kinase (JAK) inhibitor approved for use in dogs and labeled for the control of pruritus associated with allergic dermatitis and the control of AD in dogs. Oclacitinib maleate is most effective at inhibiting JAK1, which plays a key role in the mediating the intracellular signaling of IL- 2, IL-4, IL-6, IL-13, and IL-31, cytokines involved in allergy, inflammation, and pruritus.[17]

The aim of this study was to evaluate the FLG 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 enrollment of each dog in both groups.

Animals

Sixteen privately-owned dogs with a diagnosis of AD, comprising 7 Mixed-breed, 5 Shih-tzus, 2 Lhasa-apso, 1 Beagle and 1 Golden retriever (age range 1.5-12 years) and 10 healthy control dogs, with no history or clinical signs of skin disease, comprising 7 Mixed-breed, 2 Yorkshire terrier and 1 Pug (age range 1-12 years) were included in this study.

Diagnosis of AD was based on compatible history, the presence of 5 or more signs under Favrot's 2010 criteria,[18] and the exclusion of other causes of pruritus, through skin and ear canal cytology and skin scrapings to rule out concurrent infections/infestations. Prior to enrollment, if present, secondary bacterial and/or yeast infections were addressed by appropriate antimicrobial therapy (topical/systemic medication). An

ectoparasiticide (Afoxolaner- Nexgard: Boehringer Ingelheim, Brazil) was orally administered as well, according to body weight. No anti-inflammatory medications were given for at least four weeks prior to inclusion. Oclacitinib maleate (Apoquel: Zoetis, Brazil) at 0.5 mg/kg, orally, q12h for the first 14 days and q24 h for 16 additional days, was administered to the atopic dogs. Concurrent topical therapies (i.e., antiseptics, moisturizers) were not allowed during the study period.

Dogs less than 12-months old, with body weight < 3 kg, nervous and/or aggressive behavior, 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 of the study.

Clinical evaluation

Canine Atopic Dermatitis Extent and Severity Index (CADESI-4)[19]
and Pruritus Visual Analog Scale (pVAS)[20] evaluations were performed in the atopic dogs on day 0, 15 and 30.

Skin biopsies

6-mm punch skin biopsies were taken from lesional (erythema- axillae/inguinal area) and non-lesional skin (inguinal area) from the atopic dogs on days 0 (before oclacitinib maleate) and 30 (after oclacitinib maleate). Skin biopsies from the same anatomic location were collected from the control group on day 0. Biopsies were obtained after local anesthesia with 2% lidocaine and sutured routinely.

Assessment of cutaneous filaggrin expression by immunohistochemistry

Processing of samples

Skin biopsies from atopic dogs (lesional and non-lesional) obtained on days 0 and 30 and from control dogs obtained on day 0, were fixed in 10% neutral buffered formalin for 48 h and then subjected to standard histological processing. Tissue microarrays (TMA) were prepared to perform immunohistochemistry. Slides were deparaffinized and subjected to antigen retrieval at pH 9.0. Then, were quenched in 3% hydrogen peroxide, and primary custom-made anti-canine-filaggrin polyclonal antibody (renamed FLG2,

University of Florida, United States),[10,21,22] diluted at 1:500, was added for 1.5 h. The Histofine DAB (3,3´-Diaminobenzoide) substrate (Nichirei Biosciences) was used for the visualization of the immunolabelling, and slides were counterstained with hematoxylin. Between reactions, slides were washed in distilled water and phosphate buffered saline (PBS).

Imaging and image analysis

Immunolabeled slides were scanned (Axio Scan.Z1, Zeiss, Jena, Germany) obtaining 20 images for each animal using the software ZEN Blue Edition (Zeiss, Jena, Germany). Images were obtained from random areas, and analyzed blindly, with no interference of an observer. FLG immunolabeling were measured through the software Image Pro-Plus version 4.5 (Media Cybernetics, Rockville, MD), using a color semiautomated segmentation method, which artificially delimited and quantified the area of tissue immunoexpression. Immunolabeling values were initially obtained in square micrometers (μm²) and then converted in percentages through the formula: % = (immunopositive area/ total tissue area) x 100. An immunolabeling average for each animal was calculated and analyzed statistically.

Statistics

Normality of data distribution was tested using the Shapiro-Wilk test. T test and ANOVA for independent groups and T test for paired samples were used for parametric variables. Mann-Whitney and Kruskal-Wallis tests for independent groups as well as Wilcoxon and Friedman tests for dependent groups were used for non-parametric variables. Spearman coefficient was used to correlate 2 continuous non-parametric variables. A p value ≤ 0.05 was considered significant. For data analysis, JMP™ Pro 14.0.0 (SAS Institute, Raleigh, NC) was used.

RESULTS

A significant reduction of the clinical scores, CADESI-4 (p<0.001 between day 0,15 and 30) and pVAS (p<0.001 for days 15 and 30 compared with day 0 and p=0.046 between day 15 and 30) associated with the use of oclacitinib maleate was observed (figure 1).

For the percentage of FLG immunostaining, there was a higher FLG expression in control skin when compared with atopic skin (lesional and non-lesional) on day 0 (p=0.033) (figure 2). Interestingly, comparison of FLG expression between control and day 30 did not show significant difference (p=0.509) (figure 2). A significant increase in FLG expression in atopic skin on day 30 compared with non-lesional atopic skin on day 0 (p=0.014) (figure 3) was also observed. Conversely, comparison between FLG immunolabeling of lesional skin on day 0 and day 30 did not exhibit significant increase (p=0.688).

Spearman´s matrix showed no positive correlation between FLG expression and clinical scores, CADESI-4 (day 0 ρ= -0.026; day 15 ρ= 0.073; day 30 ρ=0.289) and pVAS (day 0 ρ=0.216; day 15 ρ= -0.111; day 30 ρ= -0.058).

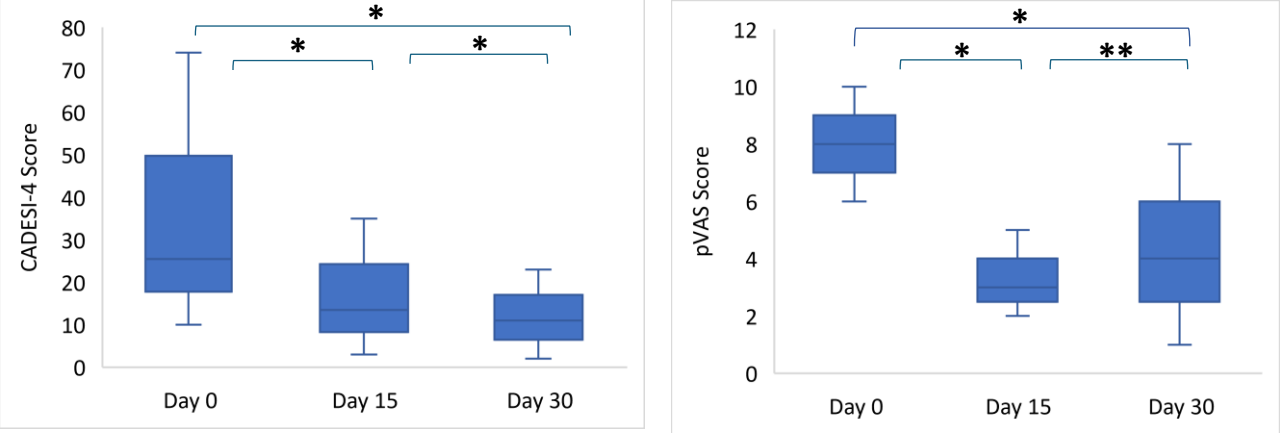

Figure 1. Reduction of the clinical scores CADESI-4 (*p<0.001) and pVAS (*p<0.001; **p=0.046) associated with the use of oclacitinib maleate.

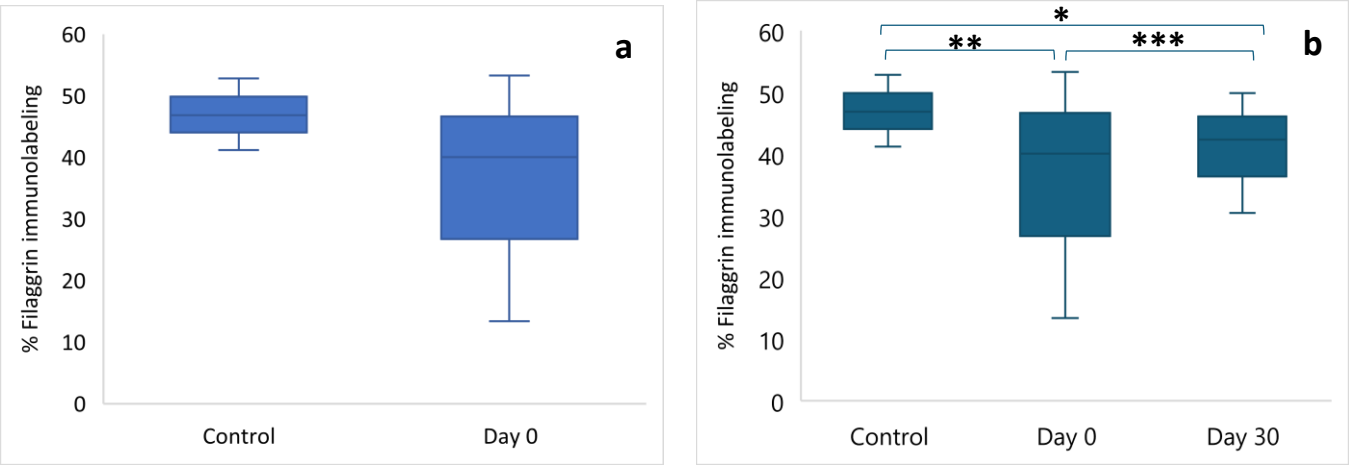

Figure 2. Comparisons of filaggrin expression between control and atopic skin (lesional and non-lesional) on day 0, *p*=0.033 (a), and between control, atopic skin on day 0 (lesional and non-lesional) and atopic skin on day 30 (after oclacitinib maleate), **p*=0.509; ***p*= 0.033; ****p*= 0.445 (b).

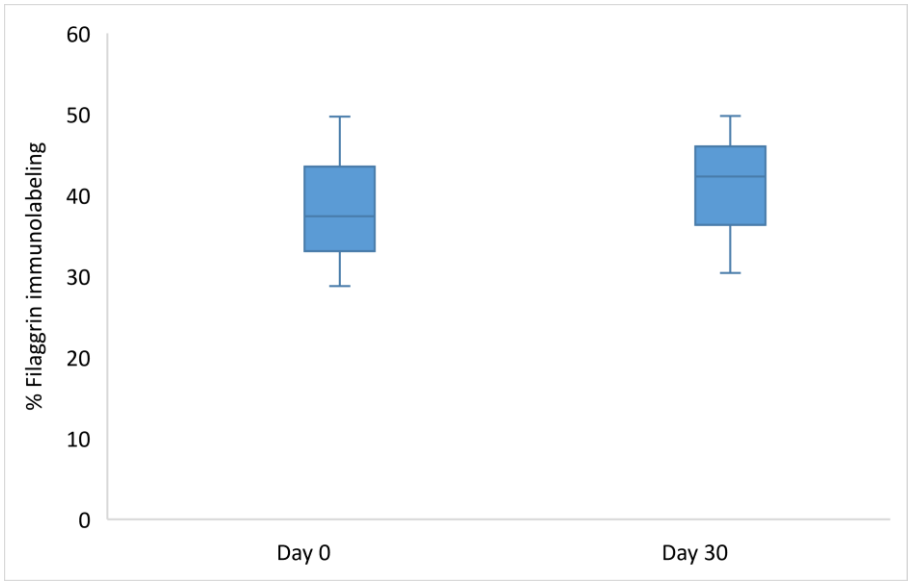

Figure 3. Comparison of filaggrin expression in non-lesional atopic skin on day 0 (before oclacitinib maleate) and day 30 (after oclacitinib maleate), *p*= 0.014.

Figure 4 shows representative images of skin sections from normal, atopic dogs (lesional and non-lesional) and day 30, after oclacitinib maleate.

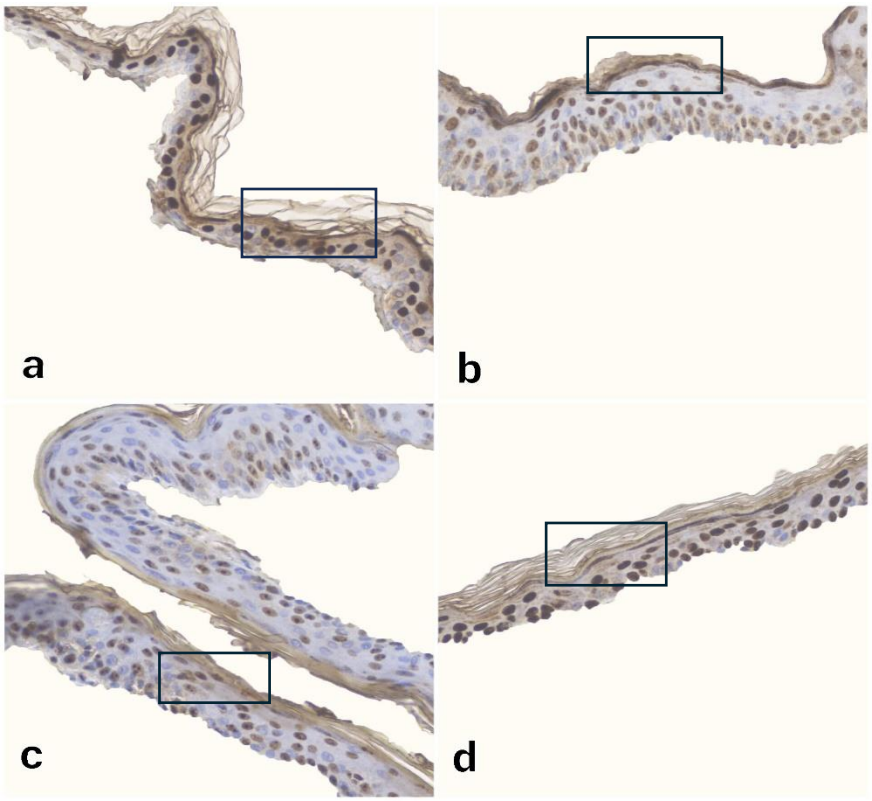

Figure 4. Representative filaggrin immunolabeling in the stratum granulosum and stratum corneum by immunohistochemistry (black rectangles). (a) Control skin (b) atopic non-lesional skin- note the staining reduction compared to control (c) atopic lesional skin (erythema) – note the staining reduction compared with control and non-lesional skin (d) day 30 (after oclacitinib maleate) – note the staining similar to control. Images obtained from the Software ZEN Blue Edition (Zeiss, Jena, Germany).

DISCUSSION

FLG forms part of the S100 fused-type protein family together with FLG2, trichohyalin, hornerin and others, and plays a key role in epidermal differentiation and cutaneous barrier function.[4]

The results of this study showed a significative higher FLG immunostaining in control dogs compared with atopic dogs (lesional and non-lesional), as previously described.[5,6,21] Moreover, there was a significant increase in FLG expression on day 30 compared with atopic non-lesional skin on day 0, suggesting a positive impact of

oclacitinib maleate on physical skin barrier. Another interesting finding was the non-significant difference between control and atopic skin on day 30, so, it can be speculated that the use of oclacitinib maleate could improve FLG expression in atopic dogs at a similar level to healthy skin.

The majority of humans with AD have altered FLG expression on either a genetic[23] or acquired basis (e.g., Th2 inflammation, repetitive scratching, detergent use, low humidity, exogenous or endogenous proteases, air pollution).[16] Alarmins such as IL 33 and TSLP and type 2 inflammatory mediators, including IL-4, IL-13, and IL-31, can reduce FLG expression. This has also been observed in skin inflammation mediated by Th17 (IL-17), Th22 (IL-22), and Th1 (IL-1α, IL-1β, and TNFα). Likewise, the recruitment of additional innate immune effector cells, including eosinophils, basophils, and mast cells, increase the release of mediators, that not only exacerbate inflammation but also worsen skin barrier disruption by downregulating SC structural proteins.[16] Type 2 inflammatory cytokines and alarmins can also promote the itch-scratch cycle by activating pruritogenic sensory neurons, exacerbating skin barrier disruption and promoting bacterial colonization, thus, perpetuating the inflammation.[16]

Prominent Th2-polarized immune responses have also been demonstrated in canine AD with variably increased levels of interleukin 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.[14] Although, whether decreased FLG expression in atopic dogs is the result of a primary skin barrier defect (genetic) and/or secondary to inflammation,[21] remains a matter of debate.

A study showed that treatment of canine reconstructed epidermis with Th2 type cytokines induces a marked decrease in FLG immunodetection[24] 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.[25] Considering these findings, modulation or suppression of Th2 cytokines could be beneficial, and secondarily improve skin barrier function[21] as described in this study with the use of oclacitinib maleate. This could represent additional evidence of the possible role 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 (STAT) signaling pathway.[26] The suppressive effect of this drug on Th2 mediated immunity, leading to reduction in skin inflammation, could improve FLG expression in non-lesional atopic skin in this study, after 4 weeks of its use. Conversely, this effect was not sufficiently significant on lesional skin, implying a promising proactive, but not reactive effect on cutaneous barrier structure.

This could be explained by the fact that IL31 is one of the earliest and major cytokines involved in acute canine AD. One study showed upregulation of mRNA encoding IL-31 in the skin of house dust mite (HDM) sensitized experimental atopic dogs as early as six hours after allergen challenge, when erythema was not still visible, continuing to increase and becoming the most upregulated gene at 24 hours post-challenge. At 48h, there was further amplification of additional Th2 (IL4, IL5, IL13, IL31, and IL33), Th9 (IL9), and Th22 (IL22) cytokines, as well as Th2-promoting chemokines such as CCL5 and CCL17,[27] some of them being pathways that scape from the oclacitinib maleate mechanism of action.

In addition, the description of these transcriptomes,[27] could also explain the lack of positive correlation between FLG expression and clinical scores (CADESI-4 and pVAS) in this study. Increasing inflammatory responses may lead to widespread secondary changes that further contribute to the AD phenotype, such as suppression of epidermal differentiation genes, including FLG, by Th2, Th22 and Th1 cytokines,[28] as well as excessive degradation of FLG and alterations in the processing of proFLG as described in mice and humans.[29]

The primary custom-made anti-canine-filaggrin polyclonal antibody used in this study was directed to canine FLG2. FLG2 gene shares similarities in genomic sequence as well as protein distribution with FLG, and its involvement in skin barrier alterations has been hypothesized in humans and mice.[30,31] In dogs, an updated version of the canine database and its comparison with the human database suggested that the previously identified canine FLG gene, reported in previous studies,[5,7,32–36] 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 immunohistochemistry and Western blot. The authors concluded that staining using an antibody for canine FLG[37] and one for canine FLG2 yields a similar epidermal location, suggesting a similar distribution of these two proteins in canine skin.[11]

In conclusion, the results of this study suggest that oclacitinib maleate could have a positive impact on cutaneous barrier structure, improving filaggrins 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, and perhaps, minimizing the risks of flares. Further studies, with more animals, and more extended study periods, are needed to understand to what extent therapies targeting type 2 inflammatory responses can improve skin barrier function and how it can impact clinical manifestations in dogs with AD.

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CONSIDERAÇÕES FINAIS

A FLG é conhecida por ser uma proteína chave na formação da barreira cutânea. A DA é a principal doença relacionada com alterações na FLG em humanos, fato que tem gerado interesse crescente no estudo da barreira cutânea e especificamente no papel da FLG na DA em cães. Atualmente, sabe-se que há mais do que um tipo de FLG na pele dos cães, incluindo a FLG2. As duas filagrinas são expressas nas camadas mais superficiais da pele, e têm um papel na diferenciação da epiderme e na hidratação cutânea. A patogenia multifatorial da DA canina faz necessária a implementação de uma terapia multimodal, utilizando agentes tópicos e sistêmicos, sendo um dos principais objetivos do tratamento a rápida redução do prurido e da inflamação da pele. É bem sabido que a expressão da FLG na pele pode ser alterada não só pelos fatores genéticos, mas também pela inflamação cutânea mediada pelas respostas Th2. Nesse sentido, os inibidores da janus quinase (JAK), tem se tornado uma opção interessante conhecida por controlar o prurido e a inflamação nos cães atópicos. O maleato de oclacitinib, primeiro inibidor JAK utilizado na medicina veterinária, bloqueia a via JAK/STAT através da inibição direcionada à enzima JAK1, inibindo assim a ação de citocinas pró-inflamatórias, alergênicas e pruritogênicas, o que poderia ter benefícios adicionais na estrutura barreira cutânea, o qual foi mostrado nos resultados de este estudo.

Além da observação de uma redução significativa dos escores clínicos, CADESI-4 e pVAS, entre os dias 0 e 30 ($p<0.001$), foi evidenciado um aumento na expressão da FLG na pele dos cães controle quando comparada com a pele atópica (lesional e alesional) ($p=0.033$). Um outro achado interessante mostrou que não houve diferença significativa na expressão da FLG entre o grupo controle e o dia 30, após o uso do maleato de oclacitinib ($p=0.509$), sugerindo que a medicação conseguiria restaurar a expressão da FLG a níveis perto da normalidade. Adicionalmente, foi observado um aumento significativo na expressão da FLG na pele atópica alesional no dia 30 quando comparada com o dia 0 ($p=0.014$). Levando em conta os dados obtidos na pesquisa, sugere-se que o maleato de oclacitinib pode ter um impacto positivo na barreira cutânea, melhorando a expressão das filagrinas através da diminuição da inflamação e

o trauma cutâneo. O maleato de oclacitinib poderia então apresentar benefícios adicionais como terapia proativa. Estudos adicionais são necessários para compreender as possíveis vantagens das terapias dirigidas as respostas Th2 na estrutura e função da barreira cutânea e como isso poderia controlar ou diminuir as manifestações clínicas da DA em cães.

ANEXO 1.

Pontifícia Universidade Católica do Paraná
Pró-Reitoria de Pesquisa, Pós-Graduação e Inovação
Comissão de Ética em Pesquisa no Uso de Animais

PARECER CONSUBSTANCIADO DA CEUA

TÍTULO DA PESQUISA	**Avaliação do microbioma cutâneo e dos níveis de calprotectina fecal em cães com dermatite atópica antes e após a administração de Oclacitinib e probióticos.**		
Nº DO PARECER / VERSÃO	2248		
PESQUISADOR RESPONSÁVEL	Marconi Rodrigues de Farias		
ESPECIE DO ANIMAL		**Nº DE ANIMAIS**	30 animais
NOME COMUM DO ANIMAL	Cão	**Nº SISBIO** (animais de vida livre)	*Não se aplica*
SEXO / IDADE / PESO	15 machos e 15 fêmeas Idade: entre 1 a 7 anos Peso: entre 3 a 20 kg	**ATIVIDADES** (animais de vida livre)	*Não se aplica*
ORIGEM DO ANIMAL		**GP TAXONÔMICOS** (animais de vida livre)	*Não se aplica*
DATA DE INICIO DA PESQUISA	Janeiro 2022	**LOCAL (IS)** (animais de vida livre)	*Não se aplica*
DATA DE TÉRMINO DA PESQUISA	Agosto 2022	**Nº SISGEN**	*Não se aplica*
APRESENTAÇÃO DO PROJETO	Projeto já aprovado pela CEUA Estudo relativo à dermatite atópica canina (DAC) como uma dermatopatia inflamatória multifatorial, crônica e pruriginosa. Sem predisposição de sexo, cujos sinais podem iniciar antes do primeiro ano de idade. Além dos sinais e manifestações clínicas na pele, também podem estar presentes a otite e algumas anormalidades gastrintestinais como vômitos ou diarreias e a disbiose. Apresenta as respostas imunológicas à exposição do animal aos alérgenos ambientais e alimentares e suas implicações em testes laboratoriais tais como as da calprotectina e ILs. Apresenta também as alterações gastrointestinais dos microbiotas associada à DAC e suas variações Metodologia: Critério de inclusão: cães co prurido crônico, primário, perene, de intenso à grave, em regiões interdigitais e superfícies ventrais, perioral, perianal e otite crônica intermitente ou persistente, excluídos outras dermatopatias pruriginosas. Controle de ectoparasitas e microrganismos. Divisão em 3 grupos iguais: Grupo 1: administração de Oclacitinib na dose de ,5 mg / kg BID por 14 dias e depois SID; Grupo 2: administração de probióticos SID por 30 dias; Grupo 3: grupo controle formado por cães hígidos. Exames do microbioma e da calprotectina fecal de todos os cães nos dias 0 e 30 para grupo 1 e 2 e apenas dia 0 no grupo 3. Coleta: feita com swab na região interdigital, friccionando-se 20 vezes em cada face em uma área de 8cm2 nos locais selecionados. Serão coletados dois swab por área corpórea. , armazenados em temperatura ambiente e enviados para laboratório. O sequenciamento será efetuado por PCR. A amostra de fezes será coletada diretamente do reto dos cães e armazenados em potes de coleta refrigerados de 2 a 8º C e enviados ao laboratório. Emenda do projeto: Avaliação da expressão da filagrina na pele dos cães com DAC, através de estudo imuno-histoquímico de biopsias de pele coletadas antes e depois da administração do Oclacitinib Também serão alterados os números de cães: 15 no grupo experimental 1 e 5 no grupo controle. Não serão incluídos os cães do grupo de probióticos. A biópsia será realizada por punch de 5mm com anestesia local subcutânea com lidocaína 2% sem epinefrina.		
OBJETIVO DA PESQUISA	Avaliar o microbiota cutâneo e os níveis de calprotectina fecal em cães com dermatite atópica antes e depois da administração de Oclacitinib e probióticos orais. Objetivos específicos – comparar a população microbiana cutânea em cães com dermatite atópica, antes e depois da administração de Oclacitinib e probióticos. Avaliar se há mudanças favoráveis na diversidade do microbioma cutâneo nos cães com dermatite atópica após a administração de Oclacitinib e probióticos. Avaliar os níveis de calprotectina fecal em cães com dermatite atópica antes e depois da administração		

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	de Oclacitinib e probióticos. Avaliar se a administração de probióticos orais pode ter efeitos positivos no CADESI 4 e nos níveis de prurido alérgico. Avaliar se existe relação entre os níveis de claprotectina fecal, a severidade das lesões, o nível de prurido e a diversidade no microbioma cutâneo nos animais avaliados.
RISCOS E ATITUDES MITIGATÓRIAS	Coleta de material em espaço interdigital: leve desconforto Efeitos colaterais já conhecidos dos medicamentos: Oclacitinib e probióticos: vômito, diarreia e diminuição do apetite.
CONSIDERAÇÕES SOBRE A PESQUISA	O pesquisador principal realizou as mudanças no projeto devido à impossibilidade na consecução do orçamento (público ou privado) para a avaliação do microbioma cutâneo e da calprotectina fecal. O eixo central da pesquisa continua sendo a Dermatite Atópica Canina e o avanço dacompreensão dos benefícios adicionais que pode ter o oclacitinib sobre outros aspetos bem reconhecidos da patogênese da doença, como as alterações na barreira cutânea.
CONSIDERAÇÕES SOBRE OS TERMOS DE APRESENTAÇÃO OBRIGATÓRIA	Todos os termos, adequadamente preenchidos, foram entregues
RECOMENDAÇÕES	Sem recomendações
CONCLUSÕES OU PENDÊNCIAS E LISTA DE INADEQUAÇÕES	Nada a declarar.
CONSIDERAÇÕES FINAIS	
SITUAÇÃO DO PARECER	**APROVADO**

CURITIBA, 26 de maio de 2021

PROF. DR. SÉRGIO LUIZ ROCHA
Coordenador CEUA-PUCPR

A validade deste documento pode ser confirmada pelo e-mail ceua@pucpr.br informando o número do parecer.

ANEXO 2.

TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO

CEUA No. 2248

TÍTULO DO PROJETO/AULA	Avaliação da expressão da filagrina cutânea em cães com dermatite atópica antes e após a administração de maleato de oclacitinib
NOME DO PESQUISADOR PRINCIPAL	Marconi Rodrigues de Farias
NOME DOS DEMAIS PARTICIPANTES DA EQUIPE	Wendie Roldán Villalobos, Tássia Sell Ferreira, Fernanda Borek
INSTITUIÇÃO DA CEUA QUE APROVOU	CEUA PUCPR – PUCPR

Natureza da pesquisa/aula:

O Sr. (Sra.) está sendo convidado (a) a autorizar a participação de seu (s) animal (is) nesta pesquisa que tem como finalidade avaliar a expressão da filagrina, uma proteína fundamental para a correta função da barreira cutânea, em cães com dermatite atópica, antes e depois da administração de maleato de oclacitinib. O maleato de oclacitinib é um fármaco utilizado para o controle do prurido alérgico nos cães. Os cães participantes do projeto serão distribuídos em 2 grupos: grupo 1 (controle - animais hígidos), e grupo 2 (animais que receberão maleato de oclacitinib). Todos os cães serão avaliados para obter informação referente ao exame clínico geral e dermatológico. No grupo 2 será feita uma avaliação da extensão e severidade das lesões, assim como do nível de prurido através de dois escalas criadas para tal fim (CADESI-4 e pVAS, respetivamente). Nos 2 grupos, serão coletadas biopsias de pele (axilas ou virilhas), previa preparação do local com anestesia local subcutânea (lidocaína 2%), utilizando punch de 6 mm. Nesse momento, os tutores dos cães do grupo 2 serão orientados sobre o protocolo de administração do maleato de oclacitinib (Apoquel®) via oral, que será subministrado na dose de 0.5 mg/kg de peso a cada 12 horas durante os primeiros 14 dias e na mesma dose a cada 24 horas

nos 16 dias seguintes, totalizando 30 dias. Os cães do grupo 2 serão reavaliados nos dias 15 e 30 posteriores ao início do tratamento, incluindo a informação referente às escalas CADESI-4 e pVAS. Igualmente, no dia 30 serão coletadas novas biopsias de pele de cada animal.

Identificação do(s) animal(is):

Espécie do animal	
Sexo	
Raça	
Quantidade	

Envolvimento na pesquisa:

Ao participar deste estudo o Sr. (Sra.) permitirá que o (a) pesquisador(a)/professor(a) realize o exame clínico geral e dermatológico do cão participante, assim como a avaliação da extensão e severidade das lesões cutâneas e o grau de prurido. Do mesmo jeito, o Sr (Sra.) permitirá a coleta de biopsias de pele das axilas ou virilhas do cão (2 amostras), previa preparação do local com anestesia local subcutânea (lidocaína 2%), utilizando punch de 6 mm. O Sr. (Sra.) tem liberdade de se recusar a participar da pesquisa/aula, sem qualquer prejuízo para o seu animal. Sempre que quiser poderá pedir mais informações sobre a pesquisa/aula através do telefone do(a) pesquisador(a)/professor(a). Se necessário, poderá entrar em contato com Comissão de Ética no Uso de Animais (CEUA PUCPR: 41 - 3271-2292).

Riscos e desconforto aos animais:

A participação nesta pesquisa não traz complicações legais. Os riscos inerentes da pesquisa são:

A coleta das biopsias de pele pode gerar um leve desconforto no cão, aclarando que não é um procedimento invasivo, devido a que precisa unicamente do uso de anestesia local. A técnica de coleta é rápida e o tamanho das amostras não supera os 6 mm (2 amostras/animal). O uso de antibióticos e medicações para a dor, posteriores ao procedimento não são necessárias. O maleato de oclacitinib é um fármaco que possui

um alto nível de segurança. No entanto, os efeitos secundários mais frequentes do maleato de oclacitinib reportados pela literatura científica incluem vômito, diarreia e diminuição do apetite, com uma baixa percentagem de aparição.

As atitudes mitigatórias para esses riscos serão: O ambiente da sala de procedimentos será adequado, para oferecer tranquilidade no momento das coletas. Serão colocadas mantas na mesa de procedimentos para gerar mais conforto e será garantida uma manipulação adequada por parte dos pesquisadores durante todo o processo, desde a preparação do paciente para a aplicação da anestesia local, até a obtenção das amostras e cuidados imediatamente posteriores. Os cuidados nos 7-10 dias posteriores à coleta das biopsias por parte dos tutores, são muito importantes para a correta evolução da cicatrização. O tutor deve manter vigilância dos pontos de sutura diariamente, no intuito de identificar inflamação, presença de secreções ou perdida prematura dos pontos, além de garantir um lugar com higiene adequada para o cão, nos dias posteriores ao procedimento. Os tutores poderão entrar em contato com os pesquisadores principais em qualquer momento para informar sobre a evolução do seu cão, mesmo que para manifestar se o animal apresentou algum tipo de efeito secundário à medicação ou alguma complicação no local de obtenção das amostras de pele, visando obter orientações apropriadas para cada caso. Os procedimentos adotados nesta pesquisa obedecem aos princípios éticos no uso de animais, elaborados pelo Conselho Nacional de Controle de Experimentação Animal (CONCEA), sobre a utilização de animais em atividades educacionais e em experimentos que envolvam espécies definidas na Lei 11.794/2008.

Confidencialidade:

Todas as informações coletadas neste estudo são estritamente confidenciais. Somente os pesquisadores/professor(a)(s) terão conhecimento dos dados.

Benefícios:

Esperamos que este estudo traga informações importantes sobre a expressão da filagrina na pele dos cães com dermatite atópica, antes e depois da administração de maleato de oclacitinib, além de esclarecer se há mudanças favoráveis na expressão dessa proteína,

que poderiam favorecer a estrutura e a função da barreira cutânea no paciente alérgico. O conhecimento que será construído a partir desta pesquisa permitirá contribuir no avanço da compreensão dos benefícios adicionais que pode ter o maleato de oclacitinib sobre outros aspetos já bem reconhecidos da patogênese da dermatite atópica canina, como as alterações na expressão e na estrutura da filagrina, visando oferecer uma melhor qualidade de vida nos pacientes afeitados.
O pesquisador/professor(a) se compromete a divulgar os resultados obtidos.

Pagamento:
o Sr. (Sra.) não terá nenhum tipo de despesa para participar desta pesquisa, bem como nada será pago por sua participação. Os custos da avaliação da expressão da filagrina na pele (grupos 1 e 2) e do maleato de oclacitinib utilizado para os 30 dias de tratamento (grupo 2) ficarão por conta da pesquisa. Outros procedimentos, exames laboratoriais e similares não ficarão por conta do projeto.

Apoio técnico da equipe de pesquisa/aula:
O Médico Veterinário responsável pelo (s) seu (s) anima (is) será o (a) Dr (a) Marconi Rodrigues de Farias, inscrito (a) no CRMV sob o n 06804. Além dele, a equipe do Pesquisador Principal, Wendie Roldán Villalobos e Tássia Sell Ferreira também se responsabilizarão pelo bem-estar do (s) seu (s) animal (is) durante todo o estudo e ao final dele. Quando for necessário, durante ou após o período do estudo, você poderá entrar em contato com o Pesquisador Principal ou com a sua equipe pelos contatos:
Tel. de emergência: (41) 988798549
Endereço: Rua Rockefeller 1311
Telefone: (41) 3207-3273

Após estes esclarecimentos, solicitamos o seu consentimento de forma livre para a participação de seu(s) animal(is) nesta pesquisa/aula. Preencher, por favor, os itens que se seguem: A confidencialidade dos seus dados pessoais será preservada.

DECLARAÇÃO DE CONSENTIMENTO

Fui devidamente esclarecido (a) sobre todos os procedimentos deste estudo, seus riscos e benefícios ao (s) animal (is) pelo (s) qual (is) sou responsável. Fui também informado que posso retirar meu (s) animal (is) do estudo a qualquer momento. Ao assinar este Termo de Consentimento, declaro que autorizo a participação do (s) meu (s) animal (is) identificado (s), a seguir, neste projeto. Este documento será assinado em duas vias, sendo que uma via ficará comigo e outra com o pesquisador.

Data:
Assinatura do Responsável:
Assinatura do Pesquisador Responsável:
Identificação do (s) animal (is) (repetir tantas vezes quantos foram os animais):
Nome:
Espécie:
Raça:

ANEXO 3.

Ficha de Avaliação Dermatológica

Paciente:_____ Data: _____/_____/____

Tutor:_____Fone: _____

Sexo: M ( ) F ( ) Peso: _____ Estado reprodutivo: ( ) Inteiro ( ) Castrado Idade: _____

Procedência:_____ Cor/tipo da pelagem:_____

Raça:_____

Tem acesso à rua? ( ) Sim ( ) Não No. de passeios/dia: _____ Início dos sinais clínicos: _____

Presença de ectoparasitas? Sim ( ) Não ( ) - Pulgas ( ) Carrapatos ( ) Piolhos ( ) Outros ( )

Controle com antipulgas: Sim ( ) Não ( )

Produto:_____Frequência:_____ Último uso: _____

Alimentação: ( ) Ração - qual? _____ ( ) Caseira - composição: _____

_____

_____

Nível de prurido (1-10): _____ (no momento da consulta)

Sinais gastrointestinais:

Vômito ( ) Diarreia ( ) Flatulências ( ) Prurido perianal ( ) Motilidade intestinal aumentada ( )

Conjuntivite ( ) Espirros ( )

Ambiente: ( ) Casa ( ) Apartamento ( ) Intradomiciliado ( ) Extradomiciliado

Convivencia com outros animais: Sim ( ) Não ( ) Quais? _____

( ) Contato com tapetes/carpetes ( ) Cobertor/manta ( ) Contato com grama

( ) Sobe em cama/sofás ( ) Usa roupas pet ( ) Dorme com tutor ( ) Usa cama pet

Banhos: ( ) Casa ( ) Pet shop ( ) Frequência: ( )Semanal ( )Quinzenal ( ) Mensal Outro: _____

Data do último banho:_____

Qual shampoo usa: _____

Qual hidratante usa:_____ ( ) não usa

Medicações em uso no momento: Indicar dose e frequência

_____

_____

_____

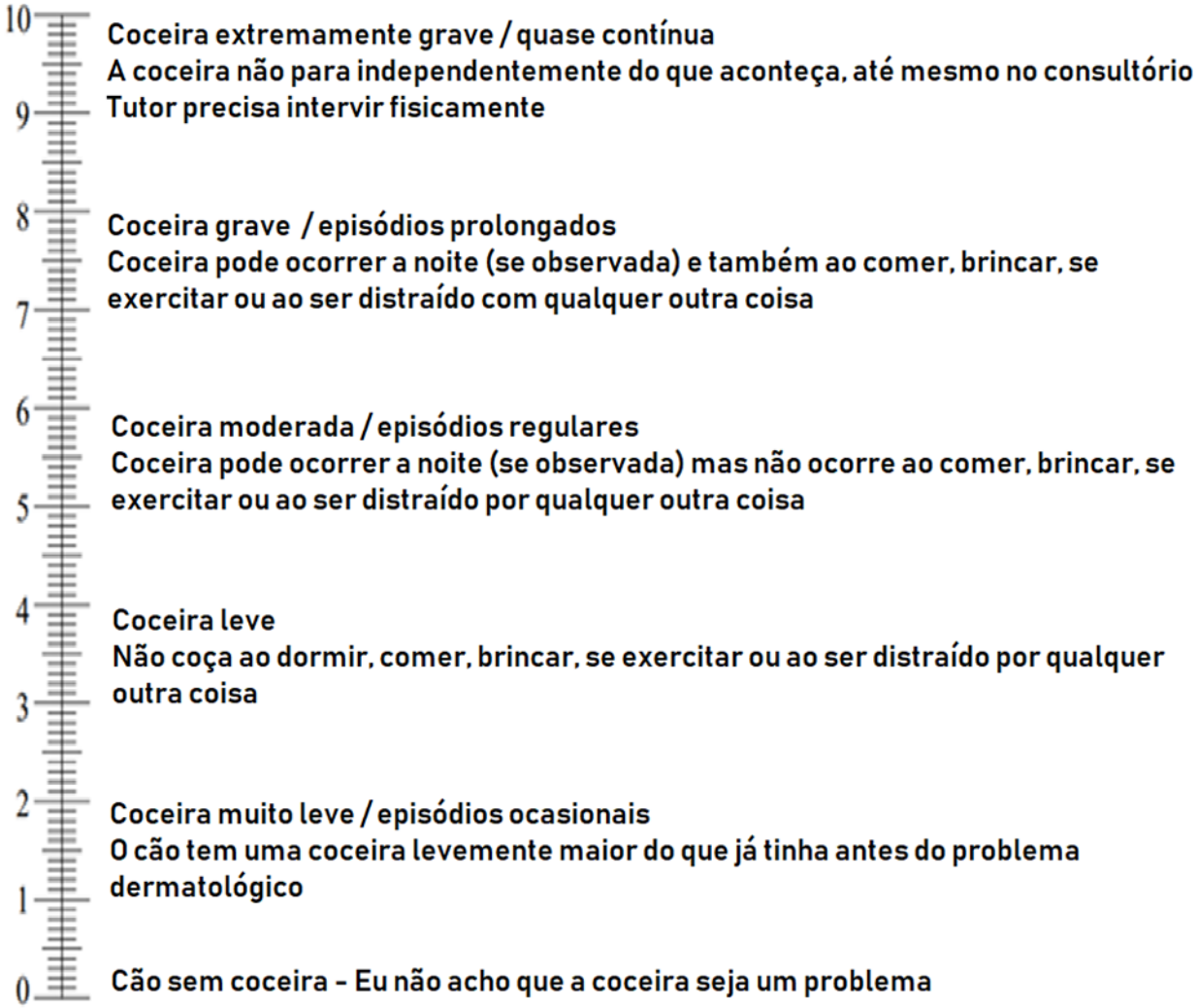
Raspado de pele: ( ) negativo ( ) positivo:_____

Citologia: ( )realizada ( ) não realizada

local: ( ) perioral ( ) axila ( ) virilha ( ) interdigital ( ) flexura anticubital ( ) orelha ( )

Achados citológicos:_____

ANEXO 4. - Escala Visual de Prurido (Rybnícek et al., 2009)



ANEXO 5. Canine Atopic Dermatitis Extent and Severity Index - CADESI-4 (Olivry et al. 2015)

CADESI-04			Eritema	Liquenificação	Escoriação e/ou alopecia	TOTAL
Área Perilabial (dir. + esq.)		1				
Pina côncava	Esquerda	2				
	Direita	3				
Axila	Esquerda	4				
	Direita	5				
Membros torácicos – dorsal e palmar	Esquerda	6				
	Direita	7				
Membros pélvicos – dorsal e plantar	Esquerda	8				
	Direita	9				
Flexura do cotovelo	Esquerda	10				
	Direita	11				
Metacarpo palmar – do carpo aos coxins	Esquerda	12				
	Direita	13				
Flancos	Esquerda	14				
	Direita	15				
Região inguinal	Esquerda	16				
	Direita	17				
Abdome		18				
Períneo (da vulva/escroto ao ânus)		19				
Cauda Ventral		20				
Grau de cada local e cada tipo de lesão: Nenhuma = 0; Leve = 1; Moderado = 2; Severo = 3.			**Total de pontos:** 20x3x3=180