

Missing C-terminal filaggrin expression, NFkappaB activation and hyperproliferation identify the dog as a putative model to study epidermal dysfunction in atopic dermatitis

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Abstract: Filaggrin loss-of-function mutations resulting in C-terminal protein truncations are strong predisposing factors in human atopic dermatitis (AD). To assess the possibility of similar truncations in canine AD, an exclusion strategy was designed on 16 control and 18 AD dogs of various breeds. Comparative immunofluorescence microscopy was performed with an antibody raised against the canine filaggrin C-terminus and a commercial N-terminal antibody. Concurrent with human AD-like features such as generalized NFkB activation and hyperproliferation, four distinctive filaggrin expression patterns were indentified in non-lesional skin. It was found that 10/18 AD dogs exhibited an

identical pattern for both antibodies with comparable (category I, 3/18) or reduced (category II, 7/18) expression to that of controls. In contrast, 4/18 dogs displayed aberrant large vesicles revealed by the C-terminal but not the N-terminal antibody (category III), while 4/18 showed a control-like N-terminal expression but lacked the C-terminal protein (category IV). The missing C-terminal filaggrin in category IV strongly points towards loss-of function mutations in 4/18 (22%) of all AD dogs analysed.

Key words: atopic dermatitis – dog – filaggrin – hyperproliferation – nuclear factor-kappaB

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Background

Filaggrin is a key molecule in epidermal differentiation, the formation of the cornified envelope (CE) and establishment of epidermal barrier function (1). In line with increased transepidermal water loss (TEWL), disturbed differentiation and epidermal hyperproliferation (2,3), loss-of-function mutations in the filaggrin gene (*FLG*) have recently been associated with human AD (4–8). Furthermore, activation of nuclear factor-kappaB (NFkB), which is one of the first responses of keratinocytes to alterations in epidermal barrier function and is often associated with increased epider-

mal proliferation, is considered a critical event in the progression and maintenance of AD (9,10).

Clinical features, histological lesions and immunologic aspects of canine AD show remarkable similarity to human AD (11,12). Reduced expression of terminal differentiation markers and increased TEWL consistent with impaired epidermal barrier function are also features of canine AD. This let us to hypothesize that, similar to human AD, canine AD might be associated with mutations in the *flg* gene, which is highly homologous to its human orthologue (<http://www.ensembl.com>). To the best of our knowledge, *flg* mutations have not been reported so far in canine AD (Appendix S1, suppl. information), and the expression pattern of other CE proteins such as involucrin and loricrin as well as NFkB activation and hyperproliferation have also not been addressed collectively in this canine disorder.

Abbreviations: AD, atopic dermatitis; FLG, filaggrin; NFkB, nuclear factor-kappaB.

Question addressed

The more than 20 different *FLG* loss-of-function mutations described in human patients with AD invariably introduce a premature termination codon, as a result of nonsense or frameshift mutations, resulting in a C-terminal truncation of the filaggrin protein (1,8,13). Establishing a diagnostic sequencing strategy for human *FLG* has been a difficult task because of ten almost identical filaggrin repeats and two half-repeats encoding the >400 kDa profilaggrin protein (13). Hence, the possibility of *flg* mutations resulting in C-terminal protein truncations in canine patients with AD was addressed by other means. We raised a monospecific polyclonal C-terminal antibody to canine filaggrin and screened various breeds by immunofluorescence microscopy for lack of C-terminal versus presence of N-terminal filaggrin protein. We further linked these investigations to comprehensive analyses of CE, proliferation (Ki67 expression) and differentiation markers.

Results

Immunofluorescence microscopy consistently revealed two distinct lines for both, the N-terminal and the C-terminal filaggrin antibody on all biopsies of healthy dogs ($n = 16$) (Figs 1 and S1, suppl. methods). In comparison with haematoxylin and eosin-stained sections (Fig. S2), the inner (i.e. lower) line localized to the stratum granulosum and the outer (upper) to the bottom of the stratum corneum. In analogy to humans, this suggests that the inner line with a granular aspect (Figs 1 (C) and S1; insets) corresponds to immature pro-filaggrin in keratohyalin granules, while the outer line represents mature filaggrin. In addition to this pattern, the N-terminal antibody exhibited strong nuclear labelling consistent with nuclear translocation of the N-terminal filaggrin peptide described in humans (14). In non-lesional skin of 18 AD dogs of various breeds and mixed genders (see Appendix S1), the C-terminal antibody revealed four different highly distinctive filaggrin expression patterns (Fig. 1, I–IV C-terminal filaggrin). Category I was characterized by control-like filaggrin expression levels (3/18), which were visibly lower in categories II (7/18) and III (4/18). The distinctive peculiarity of category III, also observable by haematoxylin and eosin staining (Fig. S2), was the presence of large aberrant cytoplasmic vesicles in non-lesional skin (Fig. 1, III_L, insert). In lesional skin with a stronger hyperplasia, the number of epidermal layers containing these granules and their size further increased (Fig. 1, III_L). Like the C-terminal antibody, the N-terminal antibody revealed reduced filaggrin expression levels for categories II and III (Fig. 1). However, the large vesicles seen with the C-terminal antibody in category III were not

visible with the N-terminal antibody (Fig. 1, III and III_L, inserts). Most strikingly, dogs of category IV (4/18) lacked specific immunolabelling for C-terminal filaggrin in both, non-lesional and lesional skin, but exhibited a strong control-like filaggrin expression with the N-terminal antibody (Fig. 1, IV shows non-lesional skin). Together, these data demonstrate that dogs of category I and II display the same expression pattern for the N- and C-terminal antibody, with overall weaker expression levels in category II. In contrast, dogs of categories III and IV exhibited differences in the staining pattern between the two antibodies. The accumulation of C-terminal but not N-terminal filaggrin in large vesicles may indicate abnormalities in the maturation and/or transport of the C-terminal filaggrin peptide. However, the detection of N-terminal but not C-terminal protein in category IV (4/18) highly supports the possibility of a loss-of-function mutation resulting in a C-terminal filaggrin truncation, analogous to those seen in humans.

Besides filaggrin, the expression of other proteins of the CE such as loricrin and desmoglein-1 (an essential adhesion molecule for epidermal integrity (15)) but not involucrin, was found to be focally reduced in non-lesional skin of all categories (Figs 1, 2a and S3). Moreover, in the case of moderate to strong hyperplasia, in particular in lesional skin, loricrin and involucrin were prematurely expressed. However, while involucrin levels were aberrantly increased, loricrin was reduced (Fig. S3, I_L and III_L).

Compatible with findings in human AD, NFKB and the proliferation marker Ki67 were significantly increased in non-lesional skin of all categories described here (Fig. 2). While these alterations can also be caused by an inflammatory imbalance, they may be a direct consequence of an affected barrier function that can be expected in case of missing or aberrant C-terminal filaggrin expression in categories III and IV.

Conclusion

Our results indicate that abnormalities in filaggrin protein expression are common in AD dogs (15/18), of which 4/18 (22%) present with a lack of C-terminal filaggrin protein, suggesting loss-of-function mutations. The exclusion approach we have designed proves to be a valuable strategy to identify candidate subjects to be sequenced for *flg* loss-of-function mutations and subsequently screen for these mutations within large cohorts of AD dogs. Our study further extends the similarity between human and canine AD to altered expression of epidermal differentiation markers, hyperproliferation and activation of NFKB. These results strengthen that the dog could represent a valuable model to gain further insights into the molecular mechanism of human AD (11).

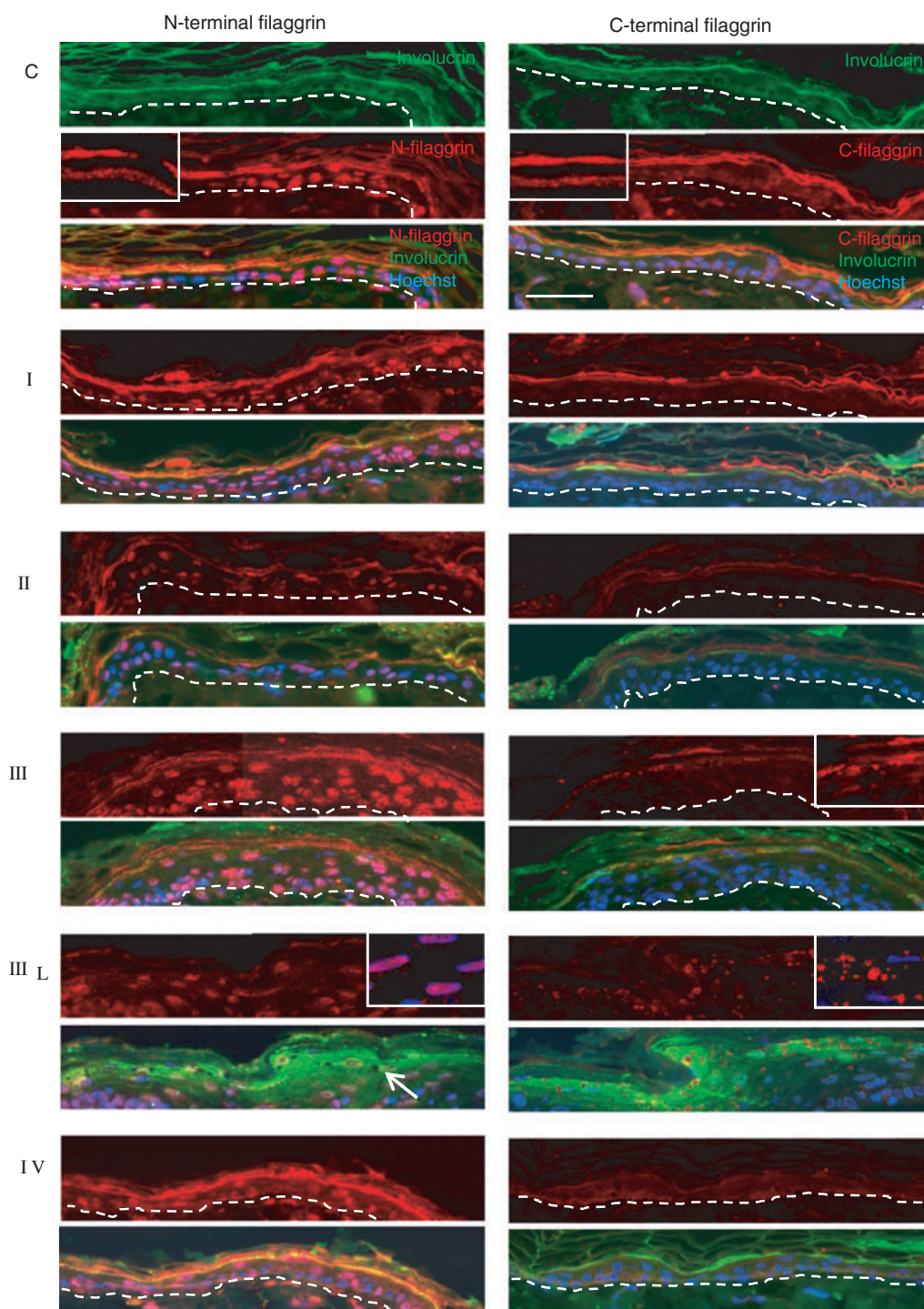


Figure 1. Filaggrin expression pattern in control and AD dogs. Shown are composite micrographs obtained by immunofluorescence microscopy of paraffin sections from control skin (C), non-lesional skin of AD dogs (categories I-IV, shows representative dogs) and lesional skin of one AD dog (category III_L) using the N-terminal (left panel) or C-terminal (right panel) filaggrin antibody. In all sections, immunolabelling for involucrin was merged with that for filaggrin (lower panel of each category). Inserts represent a three times higher magnification with adapted exposure time of a representative area to show the continuous and granular filaggrin lines, respectively, in C, cytoplasmic vesicles in III and their localization outside of nuclei (right) in contrast to the nuclear localization revealed by the N-terminal antibody (left) in III_L. Arrow points to gap in involucrin labelling at the location of vesicles stained with the C-terminal but not N-terminal antibody. Nuclei were counterstained with Hoechst 33258. Note that biopsies were processed simultaneously and photographic procedures held constant to allow comparison of intensities. Scale bar = 100 μ m.

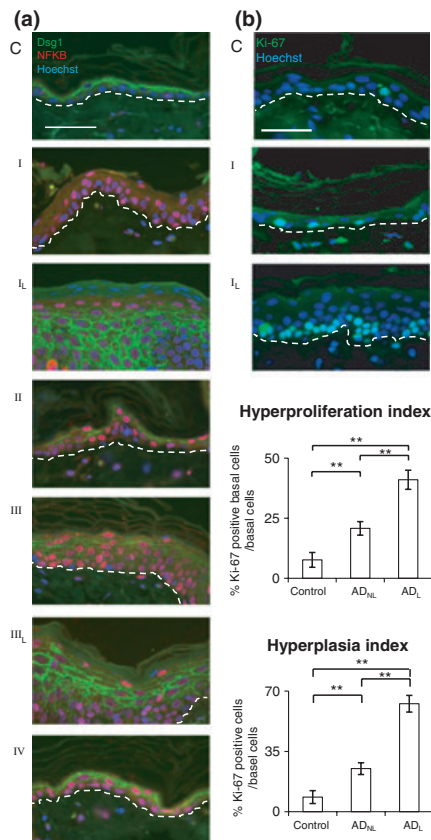


Figure 2. AD dogs exhibit increased NFKB activation, patchy desmoglein-1 expression and hyperproliferation as assessed by immunofluorescence microscopy on paraffin sections. (a) Immunolabelling for desmoglein-1 (green) and phospho-NFKB p65 (red) was merged. Note the general increase in NFKB and focally reduced desmoglein-1 expression in non-lesional skin of AD dogs. (b) Immunolabelling for Ki67 (green) on control, non-lesional and lesional skin. Shown are representative pictures. Biopsies of all dogs were stained and showed increased Ki67 expression (data not shown). For quantification, shown graphically, a subgroup of dogs was randomly selected, control; $n = 5$; AD non-lesional, $n = 6$ (representing all categories described in Fig. 2); AD lesional, $n = 3$. Categories and dogs are as in Fig. 1. Nuclei were counterstained with Hoechst 33258 (blue). The hyperproliferation (Ki67-positive basal cells over total number of basal cells) as well as the hyperplasia index (Ki67 positive over total number of basal cells) is depicted. Bars indicate mean $\pm$ SE. Data with significant P -values $**P \leq 0.01$. Scale bars = 100 μ m.

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Conflict of interest

Irwin McLean has filed patents relating to human filaggrin diagnosis and genetic testing.

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Supporting Information

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

Figure S1. Specificity of filaggrin antibodies. Filaggrin immunofluorescence staining on paraffin sections of a control dog using the pre-immune serum (from the rabbit injected with the C-terminal peptide), the C-terminal serum, and a commercially available N-terminal filaggrin antibody as indicated. Nuclei were counter-stained with Hoechst 33258. Inserts are three-fold magnifications of representative areas and illustrate a double filaggrin line. Note that the N-terminal antibody stains the nuclei.

Figure S2. Hematoxylin and Eosin (H&E) staining of representative sections from skin of humans and dogs from category I to IV. Controls (C) show overviews of normal human and canine skin. Of note is that healthy canine epidermis is thinner than human epidermis but has the same overall architecture. In both, normal human and canine skin, the keratohyalin granules are located in the stratum granulosum underneath the stratum corneum in a discontinuous line. Inserts are three times enlargements of a selected area showing keratohyalin granules. (I–IV) H&E sections corresponding to the four filaggrin categories described by means of immunofluorescence microscopy (Fig. 1). Results obtained in immunofluorescence microscopy reflected the situation observed on H&E sections; dogs of categories I and IV showed a similar distribution pattern and amount of keratohyalin granules to controls, whereas canine patients of category II had less detectable granules. In contrast, dogs of category III exhibited larger granules which were even bigger and expressed over several cell layers in lesional skin. Right panel is a 2.5 fold magnification of left panel. Scale bars = 100 μ m.

Figure S3. Co-staining for loricrin and involucrin assessed by immunofluorescence microscopy on paraffin sections. Staining for involucrin and loricrin are shown separately or were merged as indicated for control (C), non-lesional (I–IV) and lesional skin (II_L, III_L). Exposure time was kept constant except for micrographs with insert. Inserts represent the same exposure time as the other micrographs; while micrographs II_L, III_L in the left panel were reduced and II_L in the middle panel increased to visualize the staining pattern. Nuclei were counter-stained with Hoechst 33258. The same dogs as depicted in Fig. 1 are shown. Scale bar = 100 μ m.

Appendix S1. Supplementary information, Material and Methods.

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SUPPLEMENTAL INFORMATION

It has been communicated that Beagles with high IgE levels, which is characteristic for a group of AD dogs, exhibit reduced filaggrin expression (1). However the reported pan-epidermal staining did not correspond with the expected subcorneal localization of filaggrin {Sandilands, 2009 #7633} and thus precludes further conclusions. So far, a genetic linkage study conducted in AD West Highland White Terriers failed to provide evidence for an involvement of *flg* mutations (3).

Of note is that unlike commonly used rodent models, dogs are physiologically closer to humans, share the same environment and with a largely line bred population structure may facilitate the identification of other causative mutations or modifier genes in AD (4).

SUPPLEMENTAL METHODS

Material

Eighteen dogs of mixed gender suffering from AD were selected by the clinical dermatology service, Vetsuisse Faculty University of Berne. The breeds were: Labrador (6), Golden Retriever (2), Boxer, Jack Russell Terrier, German Shepherd, Hovawart, Bull Terrier, German Wachtelhund, Tervueren, American Cocker Spaniel, Newfoundland and English Bulldog. West Highland White Terriers were excluded from the study as AD dogs of this breed often have more pronounced clinical and histological epidermal changes in response to secondary infections with *Malassezia sp* (5).

The diagnosis of canine AD was based on a combination of compatible history, clinical signs (6), and exclusion of differential diagnoses (e.g. flea allergy, ectoparasite infections and adverse food reactions). Concurrent bacterial or yeast infections had been treated and no anti-inflammatory medication was given for at least 3 weeks prior to biopsy collection. Sixteen age-matched and, where possible, breed matched freshly euthanized dogs without a history and clinical signs of skin problems served as control. These dogs had been euthanized for reasons unrelated to this study. Eight-mm punch biopsies were obtained from both non-lesional and lesional skin in AD dogs. Lesional samples were taken from areas with erythema from the ventral body. Non-lesional samples were selected from unaffected skin on the dorsal thorax in AD and control dogs. All dog owners were informed about the

purpose of the study and had signed a written informed consent. The study had been approved by the local ethical committee on the use of animals, canton Bern, Switzerland (BVET 84/05).

Immunofluorescence microscopy

Formalin-fixed and paraffin-embedded biopsies were deparaffinized and antigens retrieved for all proteins except filaggrin by microwaving 3 times 5 minutes at 720 W in 0.01M sodium citrate buffer. Filaggrin retrieval was done in a pressure cooker 7 minutes in Tris/urea buffer. Slides were incubated with primary antibodies overnight at 4°C in phosphate-buffered saline containing 2mM MgCl₂/2mM CaCl₂ (phosphate-buffered saline) and 1% BSA. Incubation of the secondary antibodies was performed for 1 hour at room temperature and nuclei were counterstained with Hoechst 33258 (B-2883, Sigma, St-Louis, MO) diluted 1:3000. Primary antibodies used were Dsg1/2 (DG3.10, Progen, Heidelberg, Germany), involucrin (0.1 F established in our laboratory (7)), loricrin (PRB-145P, Babco, Richmond, CA), phospho-NFKB p65 (Ser276, Cell Signaling Technology, Beverly MA) and Ki-67 (Zymed Laboratories, South San Francisco, CA). The N-terminal filaggrin antibody (ab24584, abcam, Cambridge, United Kingdom) was raised against DSQVHSGVQVEGRRGH of human filaggrin, and likely recognizes the discontinuous epitope DSSRHSGSH of canine filaggrin. Antibodies against the C-terminal domain of canine filaggrin (DSV-42i and DSV-28i) were raised in rabbits against the C-terminal peptide DSVFVQSQNGSRSHD coupled to keyhole limpet hemocyanin (Moravian Biotechnology, Brno, Czech Republic). The resultant high-titre sera were tested in serial dilutions, and DSV-42i was chosen for further use. Immunofluorescence microscopy was performed as previously described (8). Representative pictures for each category are presented and are from the same dog for all antibody conditions. Note that unless indicated, biopsies were processed simultaneously and photographic procedures held constant to obtain semi-quantitative results.

Proliferation and hyperplastic index

All AD dogs exhibited increased Ki-67 expression. Of each category defined by the C-terminal filaggrin pattern, at least one dog was randomly selected for further analysis. Seven pictures were taken per section and the total number of Hoechst positive nuclei (>300) in the basal cell layer were counted in the controls (n=5), AD non-lesional (n=6) and AD lesional groups (n=3). The ratio of Ki-

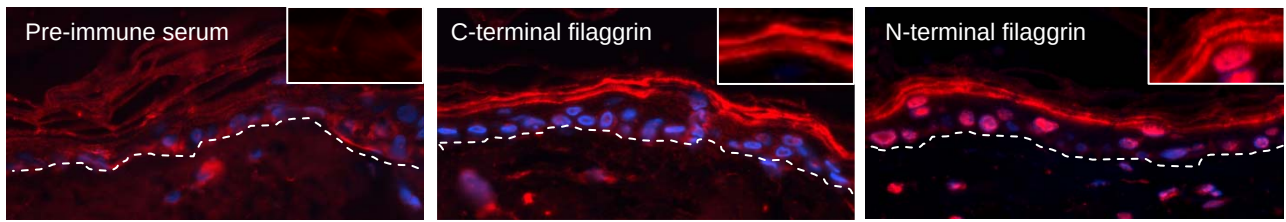
67 stained nuclei in the basal layer over the total number of Hoechst positive nuclei in the basal layer was defined as hyperproliferation index (hyperproliferation if greater than control), and the total number of Ki-67 labeled nuclei over the number of basal cells as hyperplasia index (hyperplasia if greater than control).

Statistics

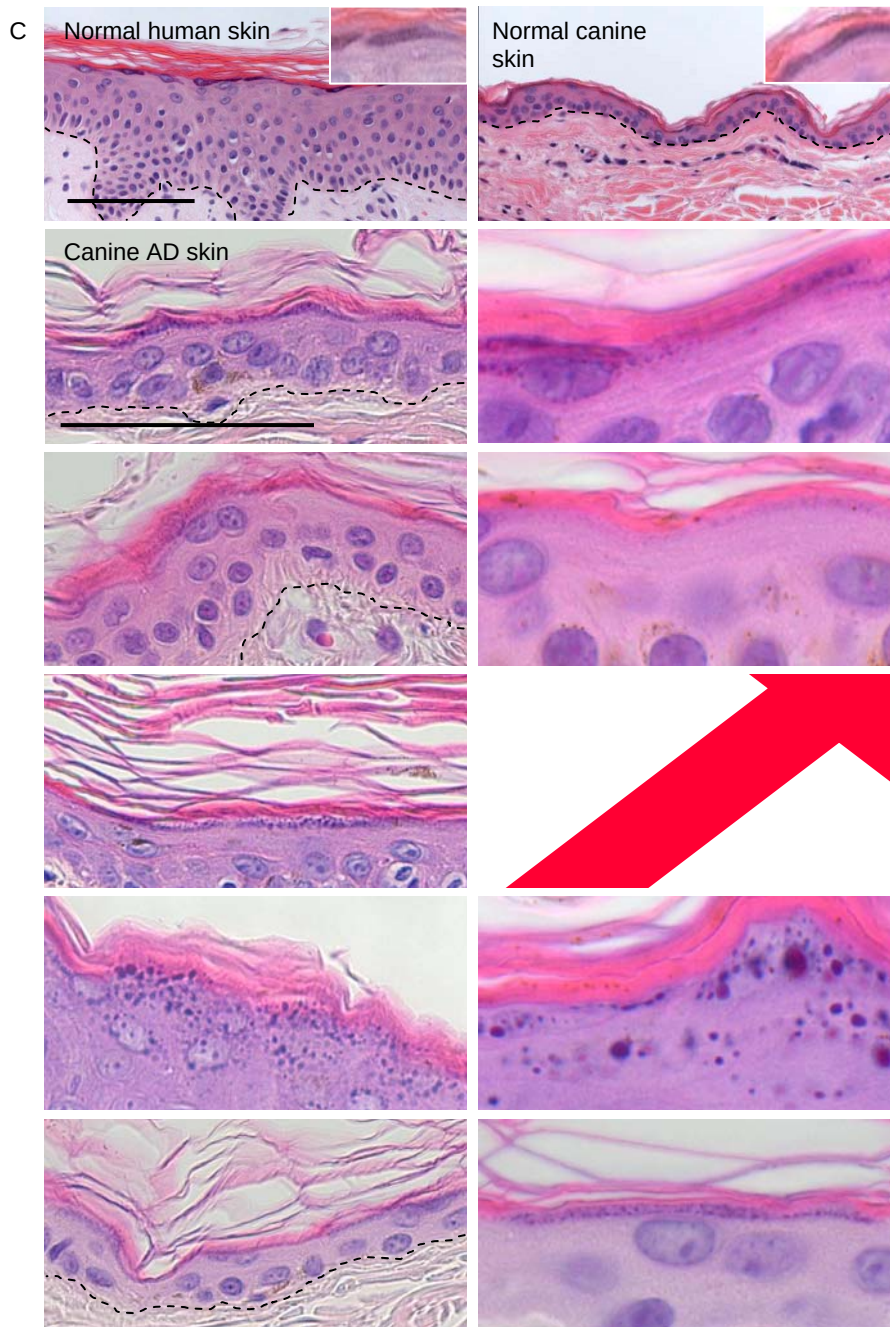
Statistical analysis was performed using the statistics program NCSS (Kaysville, UT). A one-way analysis of variance (ANOVA) was conducted to compare multiple groups and significant differences were assessed by a Kruskal-Wallis test. Comparisons between two groups were analyzed using the Student's t-test with significant differences conferred when $p \leq 0.05$.

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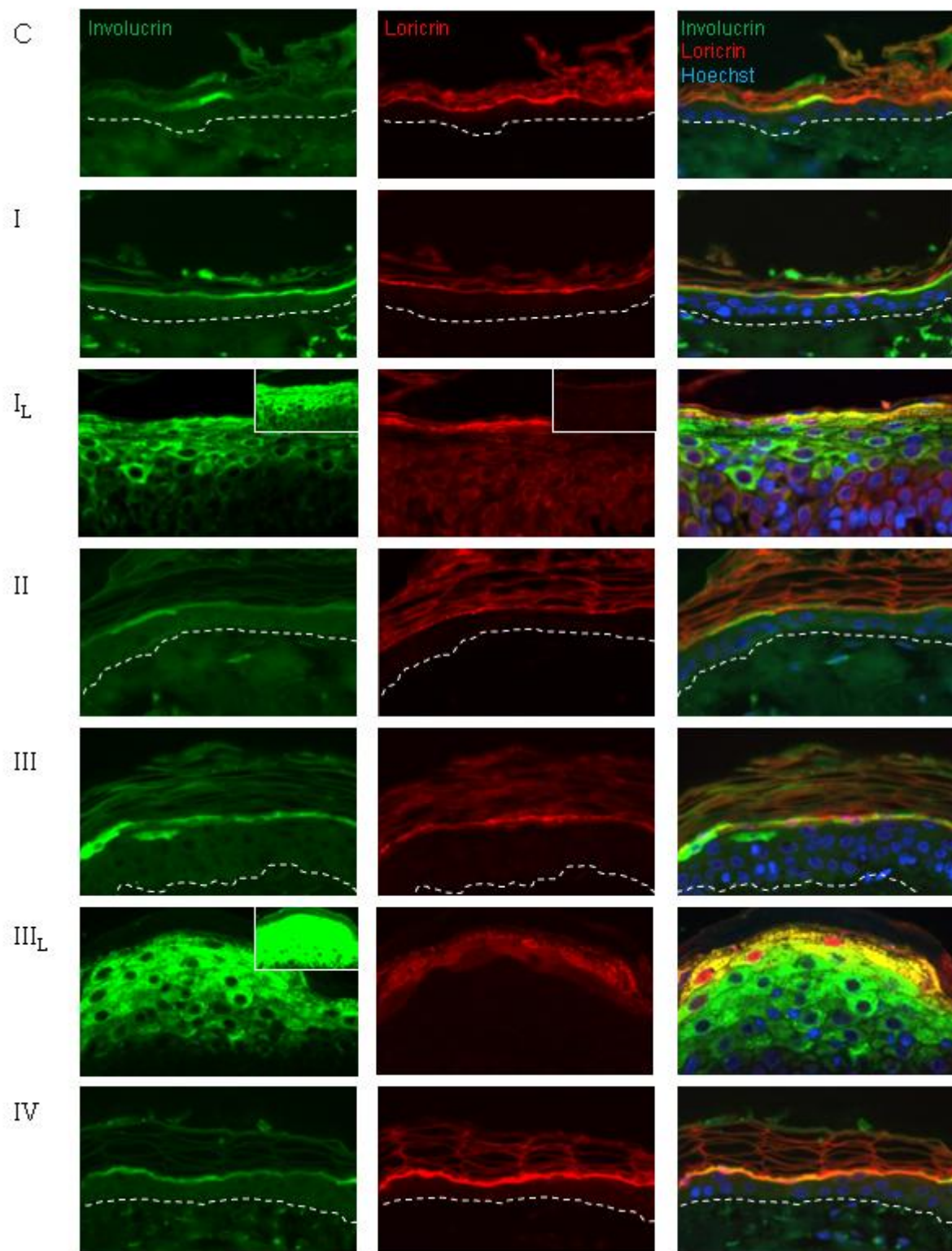


Supplemental Figure 1. Specificity of filaggrin antibodies. Filaggrin immunofluorescence staining on paraffin sections of a control dog using the pre-immune serum (from the rabbit injected with the C-terminal peptide), the C-terminal serum, and a commercially available N-terminal filaggrin antibody as indicated. Nuclei were counter-stained with Hoechst 33258. Inserts are three-fold magnifications of representative areas and illustrate a double filaggrin line. Note that the N-terminal antibody stains the nuclei.



Supplemental Figure 2. Hematoxylin and Eosin (H&E) staining of representative sections from skin of humans and dogs from category I to IV.

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Supplemental Figure 3. Co-staining for loricrin and involucrin assessed by immunofluorescence microscopy on paraffin sections. Staining for involucrin and loricrin are shown separately or were merged as indicated for control (C), non-lesional (I-IV) and lesional skin (IL, IIIL). Exposure time was kept constant except for micrographs with insert. Inserts represent the same exposure time as the other micrographs; while micrographs IL, IIIL in the left panel were

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