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Title: Reduced IL-31 receptor alpha splice variant mRNA following allergen challenge in a canine model of atopic dermatitis

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To the Editor,

Interleukin 31 (IL-31) signaling is a target for therapy in atopic dermatitis (AD). Twelve isoforms of the cytokine-specific IL-31 receptor alpha (IL-31RA) produced by alternative mRNA splicing are known to have varied or opposing functions, but the physiological relevance of these has been obscured by the use of qPCR assays which are not specific to one isoform.[1-3] If new treatments are to target IL-31RA, it is important to understand the modulation and likely functions of these different isoforms in allergic inflammation.

When Cevikbas and colleagues examined transcription of two groups of IL-31RA isoforms in skin, they found that while overall IL-31RA transcription was reduced, a subset of transcript variants was increased in the lesional skin of AD patients compared with healthy controls.[4] This subset included both functional IL-31RA isoforms and a non-functional isoform lacking part of the cytokine-binding domain, so exactly which of these are up-regulated in lesional atopic skin, and what effects this may have, remains unclear.[1,4]

Dogs (*Canis lupus familiaris*) spontaneously develop AD which is strikingly similar to its human counterpart, and can be used to answer questions that may be challenging when studying human patients.[5,6]

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Canine IL-31RA mRNA splice variants encode only three isoforms, two full-length and one truncated in the extracellular domain, which correspond to human homologs identified in Cevikbas' up-regulated subset. As in the human full-length IL-31RA, the extracellular region of the canine long IL-31RA isoforms includes a signal peptide and five fibronectin type III (FnIII) domains, the first two of which include the cytokine binding domain in human IL-31RA.[1,2] The short canine IL-31RA is missing the signal peptide and the first FnIII domain, as in its non-functional human homolog.[1] In functional studies of the human homologs of these IL-31RA splice variants by Maier and colleagues, translation of the short isoform was shown to be markedly reduced, and when expressed it was not able to activate STAT signaling in response to IL-31, suggesting a negative influence on IL-31-signalling.[1] The potential role of these transcript variants during allergic inflammation can therefore be clarified by examining their transcription in atopic dogs.

The atopic beagles used in this study have been validated as suitable model for human AD which mimics natural occurring disease, with the advantage that the amount, timing and frequency of allergen stimulation can be controlled and consecutive skin biopsies can be taken.[6] Full-thickness skin biopsies were collected from 10 atopic beagles at baseline (day 0) and at three hours (day 1, early inflammation), three days (peak inflammation) and 10 days (resolution of inflammation) following acute house dust mite (*Dermatophagoides farinae*) allergen stimulation. 50 mg crude *D. farinae* (31 mg/ml in phosphate buffered saline pH 7.2) was gently applied to the inguinal skin on days 1, 2 and 3 to provoke a moderate flare of AD which resolved by day 10. On day 1 and 3, biopsies were taken 3 hours after allergen exposure. The severity of inflammation was assessed using a validated scoring system (canine atopic dermatitis extent and severity index; CADESI-03) prior to collection of each biopsy. IL-31RA splice variant transcription was measured using qPCR assays targeting total IL-31RA transcription, long IL-31RA isoforms mRNA transcript variants X1-4, short IL-31RA isoform mRNA transcript variant X5 or the reference genes CG14980-PB and RPS19. To examine IL-31RA splice variants in chronic inflammation, transcription was similarly examined using archived RNA from skin biopsies taken from two groups of atopic beagles from the same colony: one group of seven dogs following two weeks of chronic allergic

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inflammation (50 mg *D. farinae* applied on days 0, 7 and 14 to induce a continuous allergic flare, biopsies were taken 3 hours after allergen on day 14) were compared with another group of nine dogs prior to allergen exposure. Transcription was also examined using biopsies from nine privately owned healthy dogs.

Both long and short IL-31RA isoforms were transcribed in all the canine skin samples. In non-lesional atopic and normal skin, the short IL-31RA isoform was consistently transcribed at a higher level than the long isoforms (Figure 1). This suggests that the short IL-31RA splice variant has an explicit function in normal canine skin, possibly as a mechanism of post-transcriptional regulation to moderate the response to IL-31 through reduced expression of functional receptors.

During both acute and chronic allergic inflammation, transcription of both long and short IL-31RA isoforms was found to be reduced (Figure 2), and there was no longer a significant difference in transcription between them (Figure 1). A corresponding negative correlation was observed between CADESI-03 scores and transcription of total IL-31RA, both transcript variants, and the ratio of short:long IL-31RA isoform transcripts in the consecutive skin biopsies collected at baseline and over the course of acute allergic inflammation (Supplementary Figure 1). There was no significant difference between the CADESI-03 scores associated with the archived baseline and chronic inflammation samples (Figure 2), probably because these samples were collected from different generations of closely related dogs within the same colony. The younger generation, from which the baseline samples were collected, were able to go outside when the pollen count was low, leading to more variation in baseline CADESI-03, which would explain the non-significant difference compared with the chronic inflammation samples.

The reduced transcription of all canine IL-31RA isoforms in the skin during allergic inflammation is counterintuitive, and suggests that the influence of IL-31 in excess or during inflammation would be undesirable. This is supported by the observed effects of IL-31 on

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organotypic human 3D models generated from primary normal human epidermal keratinocytes and primary dermal fibroblasts, which resulted in a thin epidermal layer and disturbed barrier function.[7] Reduced IL-31RA transcription during allergic inflammation would complement the negative feedback provided by suppressor of cytokine signaling 3 (SOCS3) following stimulation with IL-31. SOCS3 is known to be upregulated in response to IL-31 signaling in both transfected HeLa cells expressing IL-31RA and in human monocyte-derived dendritic cells, and is also upregulated in the skin of both humans and dogs during allergic inflammation.[1,8-10] SOCS3 has been shown to strongly inhibit IL-31RA-induced activation of STAT3 and may also promote degradation of the IL-31RA protein in transfected HeLa cells.[1] Negative feedback via SOCS3 could therefore act in synergy with reduced IL-31RA transcription to prevent excessive IL-31 signaling once inflammation has been initiated. Another function for downregulating IL-31RA which has been proposed is that it could promote T_H2-type inflammation by enhancing the influence of oncostatin M: Bilsborough and colleagues have previously suggested that the exacerbated T_H2-type inflammation in IL-31RA knockout mice may be due to increased availability of OSMRβ to bind with GP130 to make oncostatin M receptor.[11] Oncostatin M has been shown to be produced by T cells in the skin of humans with AD, and to stimulate a gene expression profile in cultured human keratinocytes which is associated with inflammatory infiltration, angiogenesis and hyperplasia as seen in AD.[12]

The stimulus for reducing IL-31RA transcription could be changes in local conditions during inflammation, with reduced transcription reflecting the shift in the local cytokine milieu during T_H2-type inflammation away from interferon gamma (IFNγ), which has been shown to stimulate IL-31RA transcription in keratinocytes.[13]

One area of future study would be to confirm that the short canine IL-31RA splice variant suffers from the same reduced translation and inability to propagate IL-31 signaling as its human homolog. This is highly likely as both arise from a 5' insertion encoding an in-frame premature stop codon. Another potential line of research would be to examine whether IL-

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31RA splice variant transcription can be used as a predictive or diagnostic tool for AD, or is involved in the observed variation in efficacy or rebound pruritus observed following use of anti-IL-31 monoclonal antibody or JAK inhibitor therapeutics in dogs.[5]

This study used a canine atopic model to help clarify the roles of IL-31RA splice variants in the skin during allergic inflammation. Preferential splicing to the presumed non-functional short IL-31RA isoform was identified in healthy skin, whereas during allergic inflammation both functional and non-functional IL-31RA transcription was reduced and this preferential splicing was no longer evident. Regulation of IL-31RA transcription and alternative-splicing to a non-functional IL-31RA isoform are potentially two separate mechanisms by which IL-31 signaling in the skin may be differentially regulated during allergic inflammation or in its absence, respectively.

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Figure 1. Transcription of IL-31RA splice variants in canine skin (N_0, arbitrary fluorescence units calculated using LinRegPCR) normalized to reference genes. Long (●) and short (▲) IL-31RA isoform mRNA in skin biopsies from (a) 10 atopic 2-year-old beagles at baseline and over the course of acute allergen exposure and resolution of inflammation, (b) nine atopic 2-year-old beagles prior to allergen exposure (baseline) and seven atopic 7-year-old

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beagles following two weeks allergen challenge (chronic) and (c) nine normal dogs of varied ages and breeds (p-values < 0.05 annotated).

Figure 2. IL-31RA splice variant transcription in acute and chronic allergic inflammation (N_0, arbitrary fluorescence units calculated using LinRegPCR). Transcription of (a) total, (b) long and (c) short IL-31RA isoforms normalized to reference genes, (d) the ratio of short:long IL-31RA isoform transcripts in skin biopsies, and (e) clinical scores (CADESI-03) at the time of biopsy from (i) 10 atopic 2-year-old beagles at baseline and over the course of acute allergen exposure and resolution of inflammation, and (ii) nine atopic 2-year-old beagles prior to allergen exposure (baseline) and seven atopic 7-year-old beagles following two weeks allergen challenge (chronic) (p-values < 0.05 annotated).

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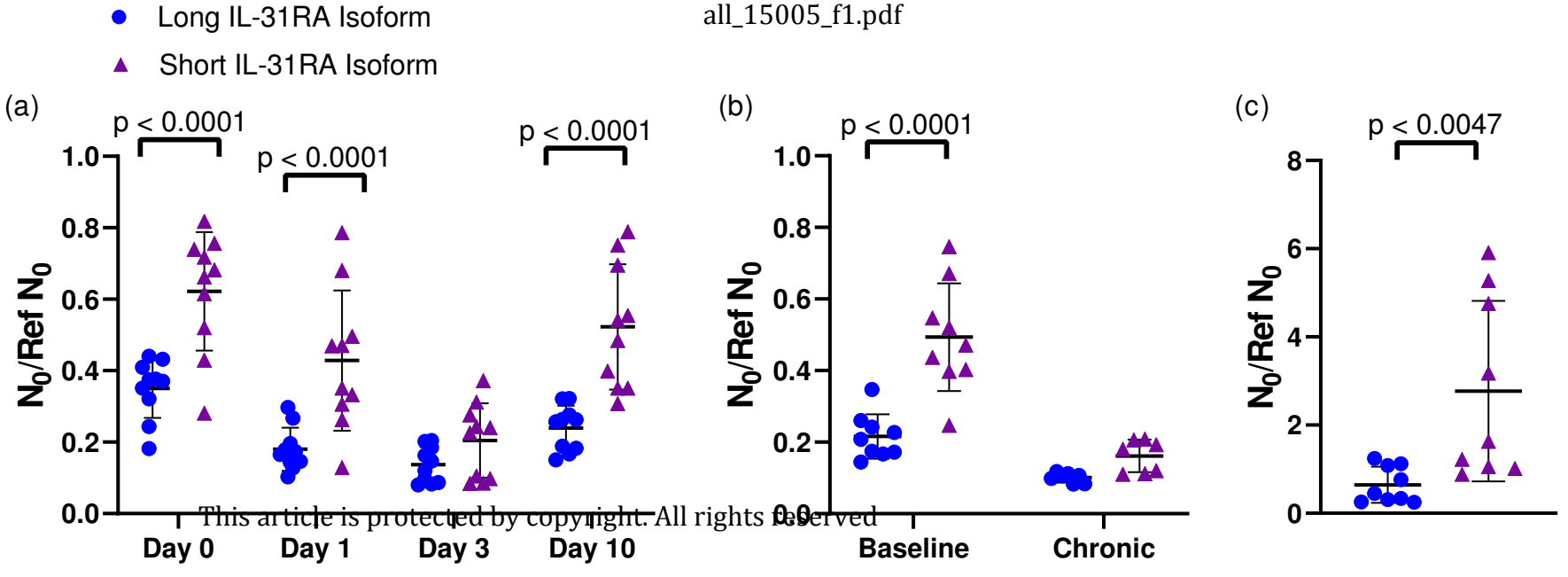

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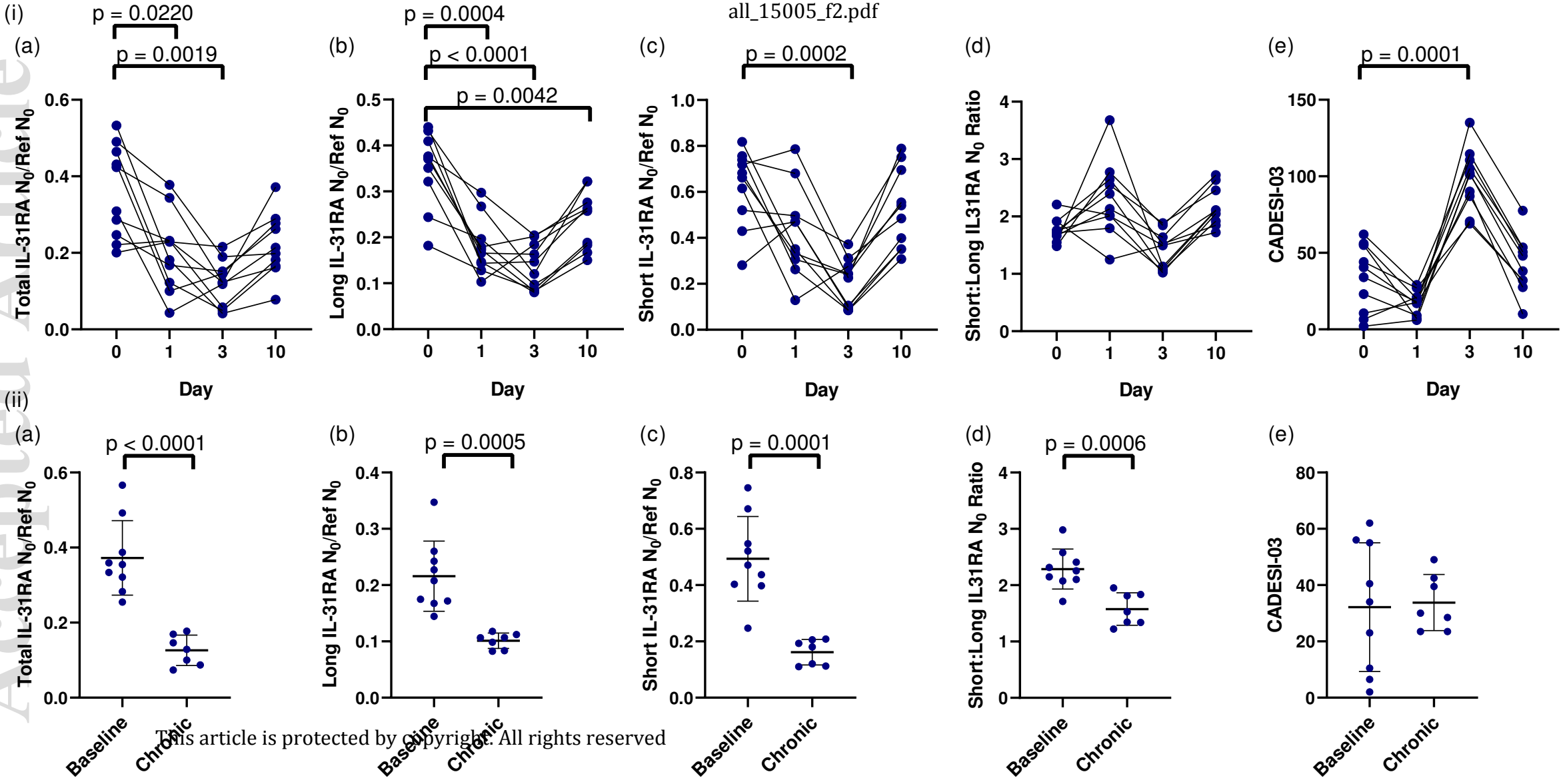

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Reduced IL-31 receptor alpha splice variant mRNA after allergen challenge in a canine model of atopic dermatitis

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Additional background: IL-31 signaling is mediated via a heterodimeric receptor comprising oncostatin M receptor beta (OSMRβ) and the cytokine-specific IL-31 receptor alpha (IL-31RA).[1] Twelve protein isoforms of human IL-31RA produced by alternative mRNA splicing are listed in the UniProtKB database, of which four are known to be capable of activating STAT signaling to varying degrees in combination with OSMRβ, two are likely to be soluble, one possesses a truncated intracellular domain and may be decoy or inhibitory receptor dampening IL-31 signaling, and one is a non-functional receptor truncated in the extracellular N-terminal domain.[1-3] Of the four positively functional membrane-bound isoforms, two of them lack tyrosine residues in their intracellular domain, resulting in reduced STAT3 activation.[1]

Dogs (*Canis lupus familiaris*) spontaneously develop atopic dermatitis (AD) which is strikingly similar to its human counterpart.[4,5] As in humans, IL-31 has been identified as an important cytokine in the pathophysiology of AD in dogs, and is a key mediator in allergic pruritus.[6,7] Levels of IL-31 in serum from laboratory beagles with canine AD correlate with severity of clinical signs, and IL-31 signaling blockade has been demonstrated to alleviate pruritus in canine AD as in human patients.[7-10] Both a caninized monoclonal antibody against IL-31 and a JAK 1 inhibitor which blocks downstream JAK/STAT signaling are accepted and effective treatments for canine AD.[9,11]

The NCBI Nucleotide Database lists only three predicted canine IL-31RA protein isoforms: two full-length long isoforms and one short isoform which is truncated in the extracellular N-terminal region. These isoforms appear to be homologs of the human full-length functional isoform (NCBI Reference Sequence: NM_139017.7) and a non-functional truncated isoform (NCBI Reference Sequence: NM_001242639.2) respectively.[1]

Animals: The atopic laboratory beagles used in this study are sensitized to house dust mite (*Dermatophagoides farinae*) allergen (HDM) and have been validated as a canine model for human AD.[5] The ability standardize the amount, timing and frequency of allergen stimulation, to either induce acute or chronic disease, and to take consecutive skin biopsies, makes this model a valuable tool to investigate modulation of allergic inflammation over time. The procedures used in this study were approved by the University of Florida's Institutional Animal Care and Use Committee and Veterinary Hospitals Research Review Committee.

Ten two-year-old atopic beagles were acutely challenged with 50 mg crude HDM allergen (Greer, Lenoir, NC, USA) daily for three days to induce an acute flare of AD. Baseline skin biopsies (day 0) were collected from the inguinal region prior to allergen exposure, and lesional skin biopsies were collected from the inguinal region three hours (early inflammation), three days (peak inflammation) and ten days (resolution of inflammation) after the first challenge. Archived RNA from skin biopsies collected from seven seven-year-old atopic beagles following chronic allergen challenge was also used.[12] These dogs had been challenged with 50 mg crude HDM allergen at seven-day intervals for two weeks (on days zero, seven and 14). Lesional skin biopsies were collected from the inguinal region six hours after allergen challenge on day 14. These chronic challenge samples were compared with archived baseline samples collected from the inguinal region of nine two-year-old atopic beagles prior to allergen exposure. Full-thickness, 8 mm skin biopsies were collected under local anesthesia, and immediately cut into sections and flash frozen in liquid nitrogen. Severity of AD was assessed using a validated scoring system (canine atopic dermatitis extent and severity index version three; CADESI-03) prior to collection of each biopsy.[13] Skin samples were also collected from four apparently healthy young adult pit bull dogs immediately following euthanasia at the Alachua County animal shelter, and from five healthy privately owned adult dogs (two Labrador retrievers, one greyhound, one beagle and one golden retriever) with no previous history of skin disease. Written owner consent was obtained prior to collecting samples.

RNA extraction and reverse transcription: Frozen skin samples were homogenized in 400 µl PerfectPure Lysis Buffer (5 Prime GmbH, Hamburg, Germany) containing 5 mM tris(2-carboxyethyl)phosphine, then incubated on ice for 10 minutes with 0.5 mg/ml Proteinase K (Meridian Bioscience, Cincinnati, OH, USA). RNA was extracted using 5 Prime PerfectPure RNA Fibrous Tissue kit (5 Prime GmbH, Hamburg, Germany) with DNAse treatment. RNA was quantified using a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, Ma, USA) and stored at -80 °C. Total RNA was reverse transcribed using Superscript Vilo (650 ng RNA; chronic challenge and normal dog samples) or Superscript III (350 ng RNA; acute challenge samples) kits (Thermo Fisher Scientific, Waltham, Ma, USA). RNA-only controls were used to confirm the absence of DNA contamination.

Real-time qPCR assay development: The five canine IL-31RA mRNA sequences listed on the U.S. National Library of Medicine's National Center for Biotechnology Information (NCBI) Nucleotide Database are predicted from the two canine reference sequence genome assemblies updated in January 2021, and are listed in Table 1. IL-31RA mRNA transcript variants X1, X2, X3 and X4 encode long IL-31RA isoforms, X5 encodes the short isoform. These sequences from the two genomes are identical and supported by alignment to RNAseq data.

SYBR Green qPCR assays were developed targeting total, long or short IL-31RA transcripts, CCZ1 vacuolar protein trafficking and biogenesis associated homolog (CG14980-PB), ribosomal protein S19 (RPS19) and ribosomal protein L8 (RPL8) using NCBI's Primer-BLAST tool. Published primer sequences were used targeting ribosomal protein lateral stalk subunit P0 (RPLP0).[14] Specificity was confirmed by melt curve analysis, gel electrophoresis and PCR product sequencing. Primer sequences are listed in Table 1. Stability of the four previously published reference genes for canine skin (CG14980-PB, RPS19, RPL8 and RPLP0) in the chronic challenge, baseline and normal dog samples was examined using RefFinder software.[14-18] RPL8 was found to vary between the groups, so was not used as a reference. Of the remaining three genes, CG14908-PB and RPS19 were found to be the most stable and were used for normalization of the target genes. Assays were run using 300 nM primers, 2.5 µl cDNA and Power SYBR Green PCR master mix (Thermo Fisher Scientific, Waltham, MA, USA) in a total volume of 25 µl, using a StepOnePlus Real-Time PCR System (Thermo Fisher Scientific, Waltham, Ma, USA). Cycling conditions were 10 minutes at 95 °C then 40 cycles of 15 seconds at 95 °C and 1 minute at 60 °C. A calibrator sample was run on each plate and all samples from the same dog were run together on the same plate.

Data analysis: LinRegPCR version 2016.1 was used to calculate mean PCR efficiency for each amplicon/plate, and starting concentration (N_0; arbitrary fluorescence units) for each well, based on the amplification curve.[19,20] Factor-qPCR was used to remove between plate variation.[21] The mean gene of interest N_0 for each sample was normalized to the geometric mean of the reference genes. The normalized N_0 values were then used for statistical analysis. The non-normalized N_0 was also used to calculate the ratio of short:long IL-31RA isoform transcripts. Prism 8 (GraphPad Software Inc., San Diego, CA, USA) was used to identify statistical differences from day 0 or baseline samples, or between transcripts in the same sample, and significance was defined as $p < 0.05$. Statistical analyses used were: normality tests, D'Agostino-Pearson and Shapiro Wilk tests; identification of outliers, ROUT method; significant differences between normally distributed independent groups, t test with Welch's correction; significant differences between non-normally distributed independent groups, Mann-Whitney tests; significant differences between normally distributed non-independent groups, repeated measures one-way ANOVA with Geisser-Greenhouse correction followed by post-hoc Dunnett's test; significant differences between non-normally distributed non-independent groups, Friedman's test and post-hoc Dunn's test; significant differences in transcription between long and short isoforms within the same sample, mixed effects ANOVA with specific post-hoc Bonferroni multiple comparison tests producing adjusted p-values; correlations with CADESI-03 scores, two-tailed Pearson's correlation for normally distributed data and Spearman's correlation for non-normally distributed data.

Supplementary results: During acute challenge, the ratio of short:long IL-31RA isoform transcript variants did not significantly change (Figure 2). In contrast with the acute challenge samples, the ratio of short:long IL-31RA isoform transcripts was lower in the chronic allergen challenge samples (Figure 2), but there was no correlation between CADESI-03 scores and IL-31RA transcription (Supplementary Figure 1).

Supplementary discussion: The findings of this study appear to contrast with observations of Cevikbas *et al*, in which an IL-31RA subset which included both human homologs of these transcript variants was increased in lesional skin from humans with AD compared with healthy controls.[1,22] However, when additional isoforms which are known to have reduced ability to activate STAT signaling were added to the group being studied, they

appeared to account for a much larger proportion of IL-31RA transcription and were significantly reduced in lesional skin, which is similar to our findings.[1,22] Because the former subset included both functional and non-functional isoforms, as well as four other transcript variants, the true pattern of IL-31RA transcription and potential effect on IL-31 signaling in Cevikbas' lesional samples is still unclear.[1,22] Possible explanations for the increased IL-31RA transcription seen in Cevikbas' lesional AD samples include additional transcript variants of unspecified function which are not present in the dog, or differences in lesional age and/or keratinocyte maturation during allergic inflammation.[23]

The marked structural similarity between the canine and the human homolog, and the fact that both splice variants are truncated due to a 5' insertion encoding an in frame stop codon, implies that this transcript may have a conserved role in both humans and dogs.[1]

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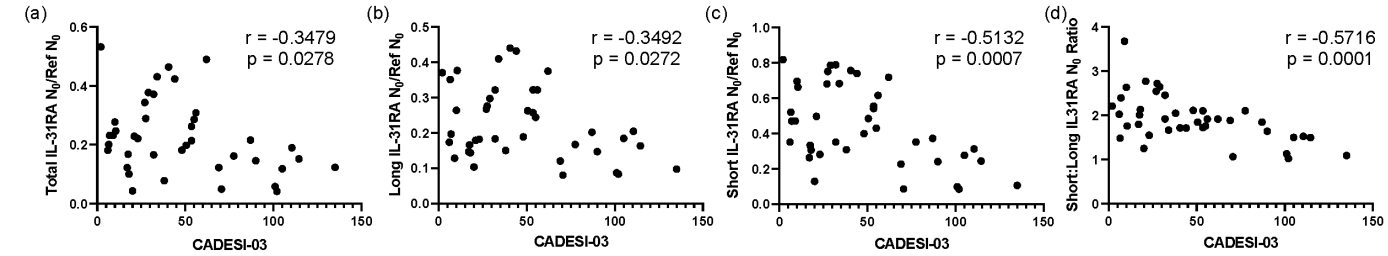
Table 1: qPCR primer sequences. Target mRNA name and NCBI sequence accession numbers for the sequences identified by each primer pair, primer sequences, and whether a primer or the resulting PCR product spans an exon-exon junction.

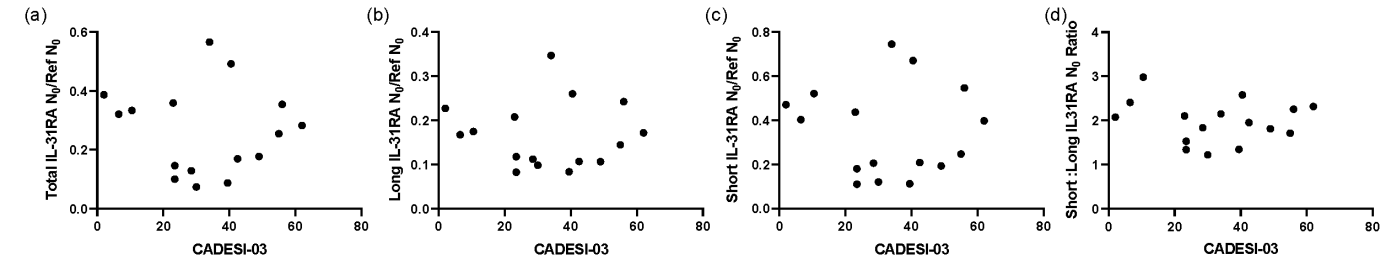
Target mRNA		Accession numbers (NCBI)	Forward and Reverse primers	Product length (base pairs)	Exon-exon junction spanning
Predicted *Canis lupus familiaris* interleukin 31 receptor alpha	Total IL-31RA (all variants)	X5 XM_038459060.1 X4 XM_014108425.3 X3 XM_038459054.1 X2 XM_038459051.1 X1 XM_038459044.1 X5 XM_038658915.1 X4 XM_038658914.1 X3 XM_038658913.1 X2 XM_038658912.1 X1 XM_038658911.1	Forward: TGCCTGCTTTTAGACAGCGA Reverse: GTGTTCAGCCAAGGCAAAGG	177	No
	Long IL-31RA (X1-4)	X4 XM_014108425.3 X3 XM_038459054.1 X2 XM_038459051.1 X1 XM_038459044.1 X4 XM_038658914.1 X3 XM_038658913.1 X2 XM_038658912.1 X1 XM_038658911.1	Forward: TAACTTGGGGATGATGTGGGC Reverse: GTTCTCAGGCTTAGCTGGCA	91	Product
	Short IL-31RA (X5)	X5 XM_038459060.1 X5 XM_038658915.1	Forward: GATGTTATTGGCACCTTCTGGT Reverse: AGCCTGTAGCTCTGTGAGGT	283	Product
Predicted *Canis lupus familiaris* CCZ1 homolog, vacuolar protein trafficking and biogenesis associated	CG14980-PB	XM_038668252.1 XM_038668251.1 XM_536878.7 XM_022419722.2	Forward: CGAGGGCGAGGAGGAAAATA Reverse: CCAACAACTCCTCCTCTTGGT	281	Primer
Predicted *Canis lupus familiaris* ribosomal protein S19	RPS19	XM_005616513.4 XM_038656876.1	Forward: GAAACCCCGTTGCTCCCTTT Reverse: AGCTTCCCAGACTTTTTGAGGA	132	Primer
Predicted *Canis lupus familiaris* ribosomal protein L8	RPL8	XM_853403.5 XM_038685450.1	Forward: GCAAGAAAGCCCAGCTCAAC Reverse: GGGAGATGACAGTCGCGTAG	144	Primer
Canis lupus familiaris ribosomal protein lateral stalk subunit P0	RPLP0	NM_001252137.1 XM_038436104.1 XM_038436105.1	Forward: TTGTGGCTGCTGCTCCTGTG Reverse: ATCCTCGTCCGATTCCTCCG	107	No

Supplementary Figure 1. Correlation of IL-31RA splice variant transcription with clinical scores. Transcription (N_0, arbitrary fluorescence units calculated using LinRegPCR) of (a) total, (b) long and (c) short IL-31RA isoforms normalized to reference genes, or (d) the ratio of short:long IL-31RA isoform transcripts in skin biopsies, correlated with clinical scores (CADESI-03) at the time of biopsy from (i) 10 atopic 2-year-old beagles at baseline and over the course of acute allergen exposure and resolution of inflammation (day 0, 1, 3 and 10), and (ii) nine atopic 2-year-old beagles prior to allergen exposure and seven atopic 7-year-old beagles following two weeks allergen challenge (p-values < 0.05 annotated).

(i)


(a)
r = -0.3479
p = 0.0278
Total IL-31RA N0/Ref N0
CADESI-03
(b)
r = -0.3492
p = 0.0272
Long IL-31RA N0/Ref N0
CADESI-03
(c)
r = -0.5132
p = 0.0007
Short IL-31RA N0/Ref N0
CADESI-03
(d)
r = -0.5716
p = 0.0001
Short:Long IL31RA N0 Ratio
CADESI-03

(ii)


(a)
Total IL-31RA N0/Ref N0
CADESI-03
(b)
Long IL-31RA N0/Ref N0
CADESI-03
(c)
Short IL-31RA N0/Ref N0
CADESI-03
(d)
Short :Long IL31RA N0 Ratio
CADESI-03