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Original Research

Phylogeny and Differential Gene Expression Profile of IL-1R8 in Normal Dog Tissue

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Abstract

Interleukin-1 Receptor 8 (IL-1R8) is a transmembrane protein of the IL-1 receptor family and represents an important regulator of the balance of innate and inflammatory responses. Depending on the immunological insult, IL1-R8 protects from the immunopathology or impairs the protective immune response against the insult. The expression pattern of IL-1R8 in dog tissues is unknown. Given the relevance of inflammatory diseases in dog, the aims of this study were to identify the sequence, analyze the phylogenesis and investigate the differential expression and distribution of IL-1R8 in a wide panel of non-pathologic tissues and organs by means of quantitative Real-Time PCR and uncover species-specific peculiarities. In *Canis lupus familiaris,* the IL1-R8 gene maps on chromosome 18, and includes ten exons. We first compared the coding sequence of dog IL-1R8 with sequences of other carnivors. Phylogenetic analysis revealed that IL-1R8 shares significantly high sequence homology with IL-1R8 of other canids particularly fox, sharing a common progenitor. Our study demonstrated that IL-1R8 is highly expressed in pancreas, considerably expressed in kidney, heart, liver, skeletal muscle, thymus, salivary gland, lymph node and lung. Interestingly, the expression pattern disclosed a unique profile for canine tissues when compared to tissues from other animal's species. Imbalance of pro-inflammatory response leads to a vicious loop whither pro-inflammatory signaling and injury sustain each other and booster the disease. Therefore, it is crucial to investigate key regulator molecules such as IL-1R8, which functions both in homeostasis and disease and has potential to be a valid diagnostic, prognostic and therapeutic biomarker.

KEYWORDS
Canine, Dog, IL-1R8, Innate immunity, Receptor

INTRODUCTION

Interleukin-1 Receptor 8 (IL-1R8), aforetime TIR8 (Toll/Interleukin-1 Receptor 8) or SIGIRR (Single-Immunoglobulin-Interleukin-1 Related Receptor), is a transmembrane protein pertaining to the IL-1 receptor family. IL-1R8 is an essential immune regulator of inflammation, recognized for its role in quite many pathological statuses, either infectious or induced by sterile inflammation (Molgora *et al*., 2018). The signaling pathway of the inflammatory responses needs to be carefully regulated at several levels and by multiple mechanisms in order to maintain inflammation under control. Regulation relies on extracellular and intracellular activities, among which are the ones played by IL-1R8. More than one mechanism of action has been discovered for this receptor, which works as a negative regulator of the pathway of signal transduction of different members of the IL-1/TLR (Toll-like receptor) superfamily: IL-1R1, IL-18R, TLR3, TLR4, TLR7, TLR9 (Turin and Riva, 2008; Molgora *et al*., 2018). One of the most recently revealed mechanism consists in the binding to the strong anti-inflammatory and immunosilencing cytokine IL-37, which is triggered by numerous pro-inflammatory stimuli and performs multiple functions, including the suppression of pro-inflammatory cytokines and the swap of the immune cells from pro- to anti-inflammatory phenotype (Dinarello, 2010; Peng *et al*., 2020). The cytokine IL-37 encompasses a structure similar to IL-18, thereby the extracellular domain of IL-37 is capable to link to the alpha chain of IL-18 receptor (IL-18Rα) generating a dimeric molecule. Subsequently, IL-1R8 gets attached to the dimer IL-37-IL-18Rα on the cell surface forming a tripartite complex IL-37-IL-18Rα-IL-1R8, which transduces anti-inflammatory stimuli by the hampering of NF-kB (Nuclear factor kappa B) and MAPK (Mitogen-activated protein kinase) and the turn on of Mer-PTEN-DOK (Receptor tyrosine kinase expressed in monocytes, epithelial, and reproductive tissues-phosphatase and tensin homologue-Downstream of kinase adapter protein) pathways (Su and Tao, 2021). In addition, IL-1R8 together with IL-1R5/IL-18Rα, was proved to act as co-receptor for the anti-inflammatory cytokine IL-37 (Nold-Petry *et al*., 2015). Therefore, IL-1R8 could be considered a marker for multiple kinds of immunopathologies, such as lupus erythematosus (Lech *et al*., 2008; Lech *et al*., 2010; Wang *et al*., 2015), rheumatoid arthritis (Wang *et al*., 2018), psoriatic arthritis (Batliwalla *et al*., 2005; Giannoudaki *et al*., 2021) and

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multiple sclerosis (Gulen *et al.*, 2010). Beyond these functional mechanisms, IL-1R8 has been demonstrated to lower NF-kB and JNK (Jun N-terminal kinase) triggering and thus inhibiting signaling transduction patterns downstream ILRs and TLRs by means of blockage of MyD88 (Myeloid differentiation primary response 88) homodimerization or retention of the Myddosome complex (Gong *et al.*, 2010; Guven-Maiorov *et al.*, 2015). Furthermore, the extracellular domain of IL-1R8 is capable to block the interplay of IL-1R1 with IL-1R3 (Qin *et al.*, 2005) and TLR3 transduction signaling by hampering of TRAM (Trif-Related Adapter Molecule) homodimerization and TRIF (TIR-domain-containing Adapter-inducing interferon beta)-TRAM binding (Guven-Maiorov *et al.*, 2015). Lastly, IL-1R8 plays a crucial role in the modulation of the mTOR (mammalian Target of Rapamycin) pathway in lymphoid and non-lymphoid cells too (Gulen *et al.*, 2010).

IL-1R8 is strongly conserved in the time scale of evolution from amphibians to humans (Riva *et al.*, 2009; Gong *et al.*, 2010). It is expressed by numerous cell types, most of all in epithelial cells and lymphoid organs, but diverse patterns of expression have been evidenced in animals of various species (Turin *et al.*, 2008; Riva *et al.*, 2010; Turin *et al.*, 2014a). The receptor is also expressed during the ontogenetic stage (Turin *et al.*, 2014b).

The human gene coding for IL-1R8 comprises ten exons, it is located on chromosome 11 and alternative splicing brings to different isoforms, whose function is unknown (Villa *et al.*, 2017).

The mouse gene maps on chromosome 7 and encompasses 9 exons (Molgora *et al.*, 2016).

IL-1R8 is fully characterized in mouse, humans and some other mammalian species, but it is so far scarcely known in dog and largely unknown is the expression pattern of IL-1R8 in canine tissues. Given the relevance of inflammatory diseases in dog, the aims of this study were to identify the sequence, analyze the phylogenesis and investigate the differential expression and distribution of IL-1R8 mRNA in a wide panel of non-pathologic tissues and organs by means of quantitative Real-Time PCR to finally uncover species-specific peculiarities in the expression pattern. A deepen knowledge of IL-1R8 in dog could be helpful in further investigations that could define this receptor as a reliable marker in pathological conditions of dogs such as IBD (Inflammatory Bowel Disease) and lymphomas (Riva *et al.*, 2022).

MATERIALS AND METHODS

Samples

This research was conducted on the biopsies obtained immediately after euthanasia from 2 crossbreed medium-size 3 and 8 years old female dogs under traumatic death (no pathologies). None of the animals had been treated prior to the traumatic death. The study was approved by the Ethical Committee of the University of Milan (Protocol number 14/2022). Samples (kidney, pancreas, heart, liver, thymus, spleen, submandibular salivary gland, mesenteric lymph node, trachea, lung, esophagus, stomach, gallbladder, small intestine, colon, caecum, skin from the back, aorta arteria, cava vein, brain, cerebellum, mammary gland, urinary bladder, skeletal muscle from biceps femoris and bone marrow) collected from each animal for RNA purification, were rapidly put in sterile vials containing 1 ml of RNA Later (Qiagen, Hilden, Germany) and kept at 4 °C for 24 h and then at -20 °C until use.

RNA purification

Total RNA was purified starting from the samples using the guanidine isothiocyanate methodology with slight modifications. In brief, the specimens were homogenized in 3 ml of guanidine isothiocyanate (4 M) utilizing a rotor-stator homogenizer (Ultra Turrax T25 Ika-Werke, Staufen, Germany). The obtained lysate was ultracentrifuged at 113,000 x g at 18 °C for 14 h on a 5.7 M cesium chloride solution (Optima TL ultracentrifuge, Beckman Instruments, Inc. Palo Alto, CA, USA). The pellet (RNA) was resuspended in distilled water and dry ice precipitated for 2 h after addition of absolute ethanol and sodium acetate 3 M pH 5.4. The RNA pellet collected after centrifugation in a microcentrifuge (Eppendorf, Hamburg, Germany) at 13,000 x g for 30 min, was resuspended in distilled water and saved at -20 °C. RNA was quantified utilizing a spectrophotometer (BioPhotometer, Eppendorf, Hamburg, Germany) at 260 nm wavelength.

Reverse Transcription and Real-Time PCR

An equal amount (1 μg) of total RNA for each specimen was reverse transcripted to cDNA using the High Capacity cDNA Archive kit including random hexamers (Applied Biosystem, Foster City, CA, USA). The cDNA obtained was saved at -20 °C until Real-Time PCR tests.

Real-Time PCR assays were performed using the cDNA from each sample as template in optimized 25 μl reaction volume in MicroAmp optical 96-well plates. Duplicates of each cDNA sample were assayed with 2X Power Syber Green PCR Master Mix (Applied Biosystem, Foster City, CA, USA) and primers 300 nM each. The dog specific primer sets were selected by Primer Express using as target the canine sequence homologous to human and mouse *IL-1R8* gene accessible in the NCBI nucleotide sequences database with accession number XM_038423988.1 (Gene Bank). Canine *IL-1R8* primer sequences were forward 5′-CTACACATCCTCCTCAGACAC-3′ and reverse 5′-GTGTCCGAGGGCCTATCTTTG-3′. To adjust for slight differences in purified mRNA amounts and cDNA synthesis performance in quantitative Real-Time PCR tests, primers for determination of the canine housekeeping gene beta-actin were selected as well by Primer Express on the sequence accession number AF021873.2 (Gene Bank), forward 5′-TCCCTGGAGAAGAGCTACGA-3′ and reverse 5′-CTTCTGCATCCTGTCAGCAA-3′. Each primer was custom generated by Invitrogen (Carlsbad, CA, USA). Two no-template controls (NTC) were added in any plate. Real-Time quantitative PCR was performed in the 7000 Sequence Detection System (Applied Biosystem, Foster City, CA, USA) at the thermal cycle protocol of 10 min at 95 °C succeeded by 40 cycles of 15 s at 95 °C and 1 min at 60 °C. The assessment of quantity was achieved by applying an algorithm to the results analyzed by the software of the 7000 Detection System (Applied Biosystem, Foster City, CA, USA). Finally, the *IL-1R8* mRNA expression of any biopsy was normalized using the calculated beta-actin cDNA expression (mean) of the same specimen and run.

Sequence analysis

Dog *IL-1R8* nucleotide sequence (EST) was retrieved from the NCBI (National Center for Biotechnology Information) database (https://www.ncbi.nlm.nih.gov/gene/100686933). The homology of mRNA sequences of *IL-1R8* of different species was analyzed using NCBI Blast software (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The phylogenetic tree of *IL-1R8* coding sequences was built by the software on the http://www.phylogeny.fr/simple_phylogeny.cgi website. The domain structure of the dog *IL-1R8* protein was studied by the SMART program (http://smart.embl-heidelberg.de).

RESULTS

IL-1R8 mRNA sequence analysis

Dog *IL-1R8* gene maps on chromosome 18 and it is organized in 10 exons coding for a 411 amino acid-long protein.

The NCBI database contains 3 predicted sequences of dog *IL-1R8* messenger: variant 1 (GI: 1953308849), variant 2 (GI: 1953308851), and variant 3 (GI: 1953308853). These records are obtained from a genomic sequence (NC_051822.1) annotated utilizing gene prediction system, Gnomon sustained by mRNA and EST evidences. Variant 1 and Variant 3 are 99% homologous except for a 12-nucleotide gap (at nucleotide 481). Variant 2 is longer than the other two variants, presenting a not homologous 1057 base pair sequence at the 5'-end and a homologous (100%) 1538 base pair sequence at the 3'-end. All the three variants code for the same protein (411 amino acids-long).

IL-1R8 protein sequence analysis

The analysis by the SMART program of the protein structure of the predicted IL-1R8 of dog demonstrated the presence of an extracellular Ig-like (Immunoglobulin-like) C-2 type domain (from amino acid 24 to 106), a transmembrane domain (from amino acid 119 to 141), a TIR domain (from amino acid 165 to 311) and a long C-terminal tail without predicted domain structure (Fig. 1).

Phylogenetic study of dog IL-1R8 mRNA

IL-1R8 is known to be highly conserved among vertebrates (Riva *et al*., 2009; Gong *et al*., 2010). This study confirmed the high level of homology of human *IL-1R8* with domestic mammal species: dog, cat, cow, pig, horse, and rabbit that seem to be evolved from a common progenitor. Conversely, mouse, chicken, xenopus and zebrafish are confirmed to be more distant to the domestic mammals and humans (Fig. 2a). Indeed, dog *IL-1R8* mRNA shows 82.4% of homology with humans. Then, the phylogeny of the receptor among the carnivores was also investigated. Dog *IL-1R8* is very close to *IL-1R8* of other canids such as foxes and presents a common progenitor with felids such as cats and big felides (Fig. 2b). A more distant progenitor seems to be shared with ursid, mustelid and phocids (Fig. 2b). *IL-1R8* orthologues have been identified in 420 organisms (https://www.ncbi.nlm.nih.gov/gene).

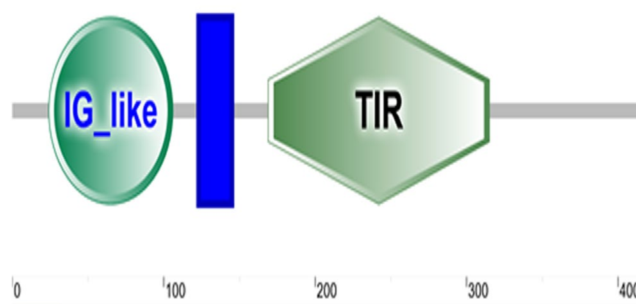

Fig. 1. Structure of predicted dog IL-1R8 protein. Domains in the protein were predicted by the SMART program.

Dog IL-1R8 mRNA is expressed at the highest level in pancreas

The results of Real-Time PCR assays for *IL-1R8* expression obtained in duplicate for each target organ or tissue of the two dogs were averaged after normalization to the beta-actin (housekeeping) gene expression and reported in Fig. 3. The highest expression of *IL-1R8* was detected in pancreas, followed by kidney, heart, liver, skeletal muscle, thymus, salivary gland, lymph

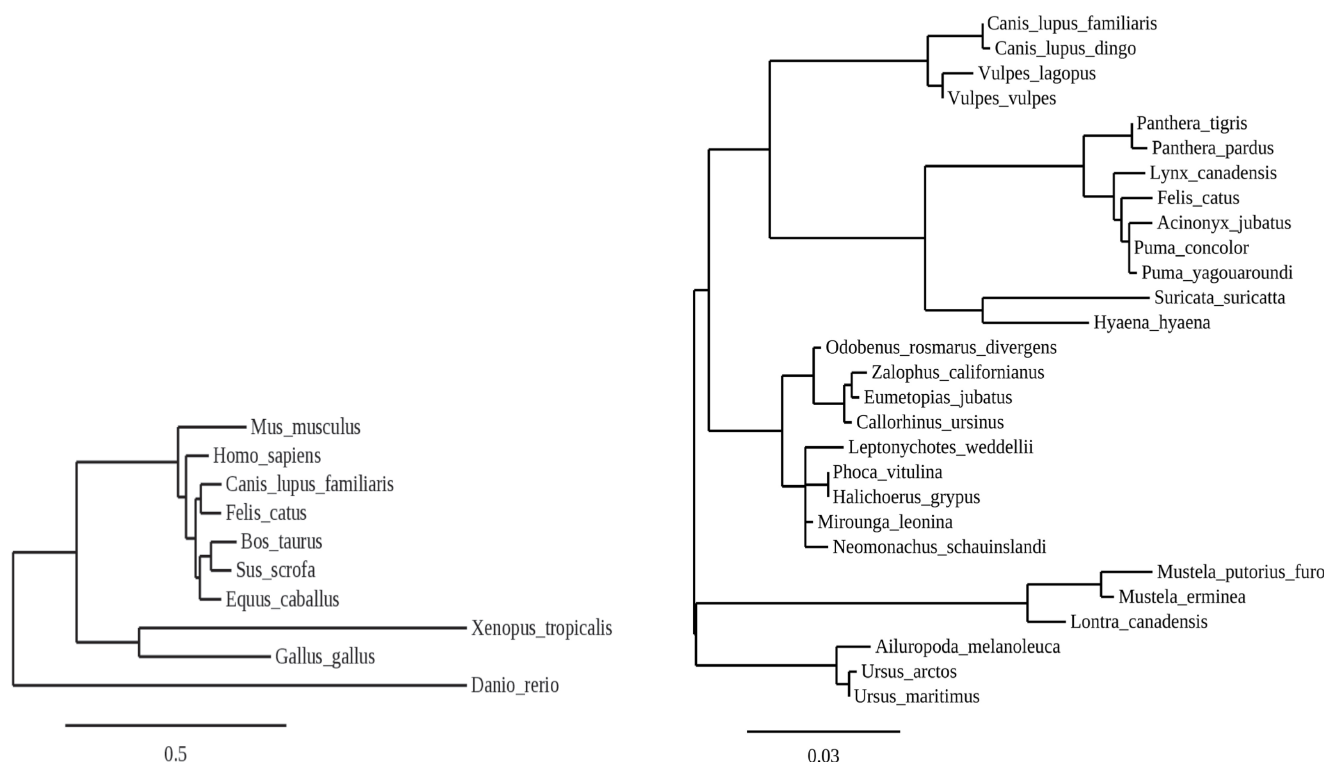

Fig. 2. Phylogenetic trees of *IL-1R8* of different species. (a) Phylogenetic tree generated after *IL-1R8* mRNA sequences alignment of numerous species (humans and domestic animal species); (b) Phylogenetic tree generated after *IL-1R8* mRNA sequences alignment of some carnivores. Both trees were obtained utilizing the free software accessible at http://www.phylogeny.fr/simple_phylogeny.cgi web site. This cladrogram represents a Neighbour joining tree without distances corrections (Dereeper *et al*., 2008).

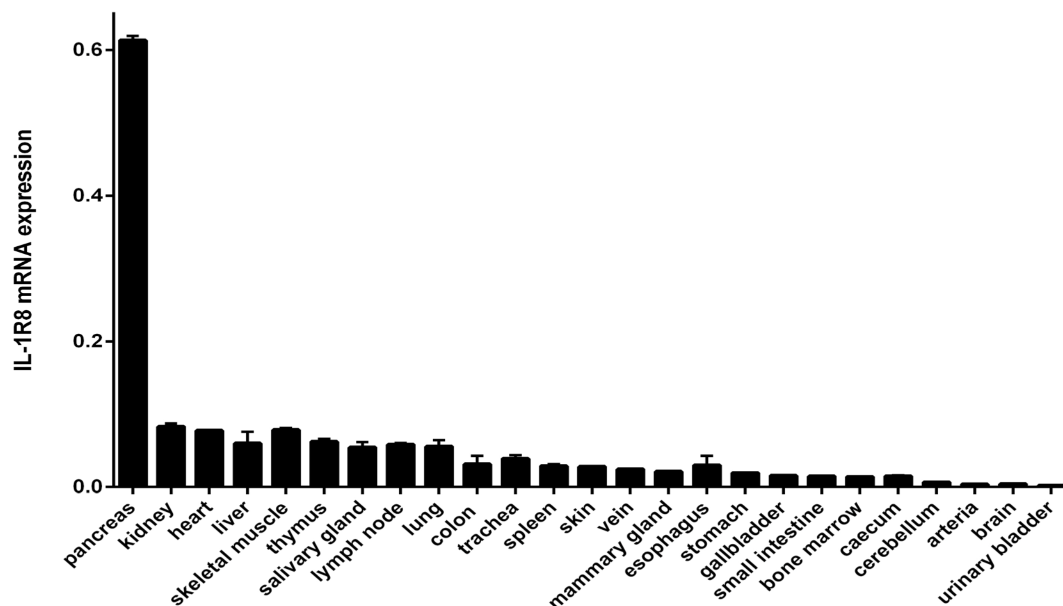

Fig. 3. *IL-1R8* gene expression level in dog tissues. *IL-1R8* mRNA expression was analyzed by means of Real-Time PCR in a wide panel of tissues and organs of dog. The gene expression level of the target gene was normalized to beta-actin gene expression and the results are presented as Arbitrary Unit ($2^{-\Delta Ct}$). Data are expressed as average of samples collected from 2 dogs, and two independent analyses per tissue or organ per dog (total 4 samples per organ).

node and lung. Lower expression was detected in colon, trachea, esophagus, spleen, skin, vein, mammary gland, stomach, gallbladder, small intestine, bone marrow and caecum. A very low or trascurable expression was observed in cerebellum, arteria, brain and urinary bladder.

DISCUSSION

IL-1R8 has a crucial function as modulator of the inflammatory responses, which need to be carefully regulated at several levels and by multiple mechanisms in order to avoid abnormal reactions. The authors identified the sequence and analyzed the mRNA expression pattern of *IL-1R8* in a wide panel of normal canine organs and tissues.

A high degree of homology is known to exist between the cDNA sequences of vertebrates, including fish (Molgora *et al.*, 2018). This study confirmed a very high homology among the *IL-1R8* sequences of domestic mammals, whereas a further progenitor with mouse, poultry, amphibians and fishes. This could be due to a similar microbiological pressure on species that closely shared the same environment, such as humans and domestic mammals. Moreover, a common emergence of IL-1R8 in canids and felides could be due to the eating habits of carnivores compared to omnivores and herbivores.

A high degree of conservation was also demonstrated between the expression patterns among vertebrates, characterized by strong expression in kidney and gastrointestinal tract (Turin *et al.*, 2008; Riva *et al.*, 2009; Riva *et al.*, 2010; Turin *et al.*, 2014a). These evidences suggest high conservation in the time scale of evolution, both with respect to sequence and pattern of expression, an unraveling reflecting the conservation of the regulatory role of this molecule in modulating inflammatory responses along the evolutionary scale (Feng *et al.*, 2016).

Analogously to humans and mice, canine *IL-1R8* mRNA was detected in the gastrointestinal tract and kidney, but interestingly, the highest expression was detected in pancreas. This is a species-specific peculiarity and could be associated to the high predisposition of dog to develop pancreatic inflammation, mainly based on sterile aethiology (Xenoulis, 2015). Species-specific peculiarities were demonstrated also in previously studied species, as for instance high expression of this receptor in liver, pancreas and female reproductive tract for avian, in lymph nodes, thymus and thyroid for bovine and liver, pancreas and lymph nodes for caprine species respectively (Riva *et al.*, 2010; Turin at al., 2014a; Filipe *et al.*, 2019).

Considerable expression of *IL-1R8* mRNA was also detected in canine kidney, heart, liver, thymus, salivary gland, lymph node and lung. Lower expression was revealed in colon, trachea, esophagus, spleen, skin, vein, mammary gland, stomach, gallbladder, small intestine, bone marrow and caecum and a very low or trascurable expression was observed in cerebellum, arteria, brain and urinary bladder.

After demonstrating the link in human and animal models between IL-1R8 and numerous pathologies involving either infections, sterile inflammation or cancer, it has been recently reported a correlation between the receptor expression and canine acute and chronic enteropathies by the same authors (Riva *et al.*, 2022). It is therefore important to characterize the expression of this fine tuner of the inflammatory response in normal canine tissues and organs. *IL-1R8* is indeed a regulator of physiological besides pathological conditions.

In conclusion, to the best of the authors' knowledge, these findings reveal for the first time the expression pattern of *IL-1R8* in tissue and organs of normal dogs. A ubiquitous expression of *IL-1R8* was observed, with species-specific peculiarities.

The epithelium of gastrointestinal tract, which is in close contact with microorganisms is characterized in dog as well as in other animal species by a sustained expression of *IL-1R8* that could control an inflammatory reaction, which may become harmful for the animal. Nevertheless, *IL-1R8* is expressed at the maximal level by pancreas.

Hence, IL-1R8 can be considered an interesting pathway marker, which deserves to be investigated and which may have relevance for the enhancement of diagnostic, therapeutic and prognostic approaches in dogs.

CONCLUSION

The specie-specific pattern of expression of *IL-1R8* in dogs with highest levels in pancreas suggests deepening the role of the receptor in physiology and pathology of that organ. Indeed, pancreatitis is a common pathological condition in dogs with different clinical appearances and difficult diagnostic tools. IL-1R8

may be a new diagnostic marker of dog pancreatitis and could help in the definition of new pathological grading criterion.

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INSTITUTIONAL REVIEW BOARD STATEMENT

The study was conducted in accordance with the Declaration of Helsinki and approved by the Institutional Review Board (or Ethics Committee) of University of Milan (protocol code 14/2022 approved 11/03/2022).

CONFLICT OF INTEREST

The authors declare no actual or potential conflict of interest, including any financial, personal, or other relationships with other people or organizations within three years of beginning the submitted work that could inappropriately influence, or be perceived to influence, their work.

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