

Original article

Short title: FXR1 in canine normal tissues and melanomas

Immunohistochemical expression of FXR1 in canine normal tissues and melanomas

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Abstract

FXR1 (Fragile X mental retardation-related protein 1) is a cytoplasmic RNA binding protein highly conserved among vertebrates. It has been studied for its role in muscle development, inflammation and tumorigenesis, being related, for example, to metastasizing behavior in human and canine uveal melanoma. Anti-FXR1 antibodies have never been validated in the canine species. To investigate FXR1 expression in canine melanocytic tumors, the present study tested two commercially available polyclonal anti-human FXR1 antibodies, raised in goat and rabbit, respectively. The cross-reactivity of the anti-FXR1 antibodies was assessed by western blot analysis and the protein was localized by immunohistochemistry in a set of normal canine tissues and in canine melanocytic tumors (10 uveal and 10 oral). Western blot results demonstrated that the antibody raised in rabbit specifically recognized the canine FXR1, while the antibody raised in goat did not cross-react with this canine protein. FXR1 protein was immunodetected, using rabbit anti-FXR1 antibody, in canine normal tissues with different levels of intensity and distribution. It was also detected in 10/10 uveal and 9/10 oral melanocytic tumors. The present study validated for the first time the use of anti-FXR1 antibody in dogs and highlighted different FXR1 protein expression in canine melanocytic tumors, whose significance is undergoing further investigations.

Keywords: dog; eye; FXR1; immunohistochemistry; melanoma; neoplasia; oral cavity; western blot.

Introduction

FXR1 is an autosomal gene encoding for the cytoplasmic RNA binding protein FXR1 (Fragile X mental retardation-related protein 1). FXR1 is a homologue of the Fragile X mental retardation syndrome protein FMR1, and both belong to the family of Fragile X mental retardation-related RNA-binding proteins together with FXR2 (Fragile X mental retardation-related protein 2).^{5,23,25} These proteins are involved in nuclear export, cytoplasmic transport and translation control of target mRNAs.² FMR1, FXR1 and FXR2 are more than 70% homologous in their N-terminal half and have the same functional domains.² The human FXR1 and FXR2 genes are autosomally encoded and are located, respectively, in 3q28 and 17p13.2.²

FMR1 spans about 40 kb encoding a mRNA of 3.9 kb. The N-terminal region has a functional nuclear localization signal (NLS), followed by two KH domains, a nuclear export signal (NES) and an RGG box in the C-terminal region.² Its homologues FXR1 and FXR2 have two KH domains, like FMR1, and a RGG box. FXR1, FMR1 and FXR2 share 63% of amino acid identity in the first half of the protein.¹⁵ Expression of the protein is similar but not overlapping, for example, FXR1 is highly expressed in muscle and heart, where FMR1 is almost absent,² while FXR2 is more expressed than FXR1 in the brain.¹⁵ FXR1 and FXR2 are predominantly cytoplasmic, even if FXR2 and one FXR1 isoform have also nucleolar targeting signal.^{2,15}

FXR1 is highly conserved among vertebrates.² The FXR1 gene is conserved in chimpanzee, Rhesus monkey, dog, cow, mouse, rat, chicken, zebrafish, and frog.⁹ FXR1 is a protein of 621 amino acids with a molecular mass of 69721 Da. Post translational modifications includes dimethylation of Arg-445.¹⁰ BLAST alignment analyses between human and dog FXR1 proteins reveal 99% identity (619/621 amino acids).³ In human, mouse and laboratory models (e.g. frog *Xenopus laevis*), FXR1 is normally expressed in a wide variety of tissues, with the highest level in muscle, heart, and testis,^{2,15,23} and it is

considered to play a role in muscular cell development,⁶ and in inflammatory process by controlling the expression of tumor necrosis factor- α (TNF- α)^{11,14} and regulating monocytes migration.¹⁶ Moreover, FXR1 has been investigated in oncology for its potential role as a key regulator of tumor progression, resulting over-expressed at genetic level, for example, in human lung squamous cell carcinoma, non-small cell lung cancer^{4,21} and colorectal cancer.¹³ It has been supposed that FXR1 affects DNA stability,¹⁸ either regulating target mRNA expression by miRNA pathways⁸ or regulating post-transcription by direct interaction with mRNA.⁶ FXR1 was also demonstrated to be over-expressed in human and canine uveal malignant melanoma with a positive correlation with the metastatic potential of the tumor.^{19,20}

Currently, the expression of FXR1 protein in normal canine tissues has not been investigated and, despite a few molecular studies on gene regulation, there are no data concerning the expression of FXR1 protein in canine melanocytic tumors. To the best of authors' knowledge, anti-FXR1 antibodies were not previously validated in the canine species. Therefore, in the present study, the authors tested the immunoreactivity of FXR1 protein in normal canine tissues and melanocytic tumors.

Materials and methods

Western blot

Canine fibroblasts, canine serum and human serum were used as samples during western blot experiments.

Primary cultures of fibroblasts were established from abdominal full-thickness skin excised from the margins of a specimen obtained during therapeutic surgical procedure, as previously described with slight modifications.⁷ Immediately after collection, skin fragments were placed in PBS on ice; all the fat tissue was removed from the skin with scissors, skin

tissues were trimmed to 0.5 cm × 0.5 cm fragments and finely minced into small pieces evenly distributed onto the bottom of a tissue culture 25-cm² flask in D-MEM Medium with GlutaMax™ (Gibco Thermo-Fisher, Cat. no. 10569-010) supplemented with 10% of Fetal Bovine Serum. After 24 h of culture, when the pieces have adhered, the medium volume was gradually increased to 5 ml. After a week, when a substantial outgrowth of cells was observed, the explants were removed from the center of the outgrowth with a scalpel and the medium was replaced. Fibroblasts were cultured until the outgrowth has spread to cover at least 70% of the growth surface.

All the chemicals and reagents used in this part of study were purchased from Sigma-Aldrich - Merck KGaA, Darmstadt (Germany) except for those specifically mentioned.

Cells at passage 0 were harvested by incubation for 5 minutes on ice with 0.25% trypsin in 0.01% EDTA and collected by centrifugation. Protein extraction was carried out on ice or at 4 °C. Fibroblasts were homogenized in 5 µl/mg TES buffer (10 mM Tris–HCl, pH 7.6; 1 mM EDTA, 0.25 M sucrose) and centrifuged at 10,000 ×g, for 30 min, at 4 °C. Protein concentration values were determined by the Pierce BCA Protein Kit (VWR), using BSA as a protein standard, according to the manufacturer's manual.

A goat anti-FXR1 polyclonal antibody (cat. number ab51970 Abcam, Cambridge, UK), produced against the peptide corresponding to amino acids 317/330 of human FXR1, and a rabbit anti-FXR1 polyclonal antibody (ab50841 Abcam, Cambridge, UK), produced against a synthetic human peptide non-conserved between FXR1 and FXR2, were used throughout the experiments. Since the cross-reactivity of the antibodies had never been validated before in dogs, a preliminary step to assess their possible utilization for canine FXR1 detection was carried out. Serum from human and dog and protein extracted from canine fibroblasts were separated on a 12% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and blotted onto nitrocellulose membrane. Before gel

separation, 1 μ l β -mercaptoethanol (Sigma Aldrich, Saint Louis, MO, USA) was added to each sample.

Immunolabeling was performed using primary antibodies at 0.25 μ g/ml for 1 h at room temperature, while an anti-goat IgG labelled with peroxidase (Sigma-Aldrich, Saint Louis, MO, USA) and an anti-rabbit IgG labelled with peroxidase (Vector Laboratories, Burlingame, CA, USA), were used as secondary antibodies (1:5000 dilution for 45 min at RT), respectively. Antibodies were diluted using Roti®-Block 1X (Carl Roth, Karlsruhe, Germany). Immunoreactive bands were visualized by enhanced chemiluminescence (ECL) using Immobilon Western Chemiluminescent HRP Substrate (Millipore, Billerica, MA, USA). In order to further assess the specificity of the secondary antibodies, western blotting experiments were repeated replacing the primary antibodies with PBS and using only the respective secondary antibodies labeled with peroxidase (1:5000 dilution for 45 min at RT), to perform a negative control and detect any possible non-specific bands. Human serum was used as positive control. Fibroblasts were used as negative control, since these cells do not express FXR1 protein.²⁴

Immunohistochemistry

Immunohistochemical expression of FXR1 protein was tested in canine normal tissues and melanocytic tumors. A pool of canine tissues was examined, using microarrays of normal tissue created by assembling multiple formalin-fixed samples (5 x 5 x 3 mm each) in the same paraffin block. Tissues examined included stomach, intestine, pancreas, liver, thyroid, adipose tissue, lung, kidney, testis, brain, heart, adult and fetal skeletal muscle. Immunohistochemistry was also performed on conventional tissue sections of canine oral (n = 10) and uveal (n = 10) melanocytic tumors.

Serial sections were cut 4 µm thick and mounted on poly-lysine coated slides (Menzel-Gläser, Braunschweig, Germany). Immunohistochemical staining with the standard avidin-biotin-peroxidase complex (ABC) method was performed.

Preliminary set up of the immunohistochemical protocol compared different heat-induced antigen retrieval methods (microwave oven, pressure cooker, water bath) and serial concentration of the primary antibody. The chosen protocol applied to the sections was the following: sections were deparaffinized in xylene and rehydrated through a descending series of ethanol concentrations. The endogenous peroxidase activity was blocked with 0.3% H₂O₂ in methanol for 30 min. Antigen retrieval was performed by heating the slides in citrate buffer solution (pH 6.5) in a water bath at 95C for 30 minutes, followed by cool down in buffer at RT for 30 minutes. Sections were therefore incubated for 20 min at RT with normal goat serum (1:70) to block any nonspecific protein binding. Serial sections were incubated at 4C overnight in a humidified chamber with the primary antibody: rabbit polyclonal anti-FXR1 (ab50841 Abcam, Cambridge, UK), 1:100 dilution.

Sections were then rinsed in Tris buffer solution, and incubated with the secondary anti-rabbit biotinylated antibody (1:200, 30 minutes RT) (Vector Laboratories, Burlingame, CA, USA), followed by rinsing in Tris buffer and therefore incubation with the ABC reagent (30 minutes RT) (Vector Laboratories, Burlingame, USA). The chromogen 3-amino-9-ethylcarbazole (AEC) (Vector Laboratories, Burlingame, CA, USA) was applied for 15 minutes and, after rinsing in tap water, slides were counterstained with Mayer's hematoxylin (Diapath srl, Martinengo, Italy) for 2 minutes. Slides were therefore dried and mounted in aqueous mounting agent (Aquatex, Merck, Darmstadt, Germany).

Negative controls for the technique were carried out by replacing the primary antibodies with rabbit IgG (Santa Cruz, Dallas, TX, USA).

Fibroblasts were used as negative controls. Canine fetal muscle was used as positive control.

Immunolabeling was semi-quantitatively scored with postexamination masking method¹² by two pathologists (LN, CG) simultaneously. At the time of samples evaluation, a prior extensive revision of human literature was intentionally not performed to avoid bias in the scoring of tissues. In normal canine tissue and melanomas, the percentage of positive cells was recorded (<10%, 10-30%, 30-50%, 50-80%, >80%), and the intensity of single cell staining was scored from 1+ (mild) to 3+ (marked). A 3+ signal equated to an intensity easily seen on low magnification, whereas a 1+ signal was seen on high magnification, and 2+ bordered between a strong and a weak staining signal.¹⁷ The immunoreactive score of Remmele and Stegner (IRS score) was calculated combining the intensity and ratio of positivity as follows:²² IRS score (0 - 12) = percentage of positive cells (with: no positive cells= 1, <10% positive cells= 1, 10-50 % of positive cells= 2, 50-80% of positive cells= 3, >80% of positive cells= 4) * intensity of staining (with: no positive cells= 0, 1+/mild = 1, 2+/moderate= 2, 3+/marked= 3).

Results

Western blot

Western blotting experiments were carried out to determine whether the antibodies cross-reacted with the respective canine protein. The rabbit anti-FXR1 antibody cross-reacted with a band of 60-70 kDa, corresponding to the predicted molecular weight (predicted molecular weight: 68 kDa, band detected by the producer: 65 kDa) (Fig. 1). Canine fibroblasts did not show any cross-reactivity. In the negative control for the secondary antibody, the incubation with the lone anti-rabbit secondary antibody did not show reactive bands (supplementary material). Conversely, goat anti-FXR1 antibody showed bands lower than expected MW (predicted molecular weight: 68 kDa, band detected by the

producer: 80 kDa) (supplementary material). These results indicated that rabbit anti-FXR1 polyclonal antibody specifically recognized the canine FXR1 protein and did not react with other canine serum proteins, while goat anti-FXR1 appeared non-specific.

Immunohistochemistry

Based on western blot results, rabbit anti-FXR1 antibody was used to perform immunohistochemical tests.

Different normal canine tissues stained positive, as detailed in Table 1. Staining was always intra-cytoplasmic and its intensity varied from mild (1+) to marked (3+) (Fig. 2).

In details, in the gastrointestinal tract, the mucosal epithelium exhibited strong immunoreactivity in the stomach (parietal/chief cells) and intestine (enterocytes), whereas the connective tissue of the lamina propria and the muscular layers were not stained. In the liver, hepatocytes were strongly positive and ductal epithelium stained mildly for FXR1 expression. Pancreatic acini stained moderately. In the lung, pneumocytes stained mildly and bronchial epithelium intensely; in the kidney, tubular epithelial cells and the pelvis urothelium were diffusely and moderately positive, whereas glomeruli did not stain. Thyroid follicular epithelial cells were diffusely and strongly positive. Neurons in the brain stained intensely for FXR1. Adult striated skeletal muscles was diffusely, moderately FXR1 positive, striated cardiac muscles was mildly positive. Canine fetal skeletal muscle was intensely immunoreactive. In blood vessels, the endothelium multifocally stained moderately positive for FXR1, while the smooth muscle of the tunica media was mostly negative, and only occasionally mildly positive. An intense signal was detected in Leydig cell of the testis, whereas Sertoli and seminal cells were always negative. Stromal connective tissue were consistently and diffusely negative for FXR1.

Concerning melanocytic tumors, nine (9/10) oral melanomas were FXR1 positive (Table 2, Fig. 3A-B). The immunostaining was always intracytoplasmic. Four cases were above 80% of positive neoplastic cells, one ranged 50-80%, two 30-50%, one 10-30% and one case was negative. Immunohistochemical staining intensity ranged, in all cases but one, from intense to moderate. Ten (10/10) uveal melanocytic tumors were positive to FXR1 immunostaining. The cases were homogeneously and variably distributed among the semi-quantitative classes of evaluation (Table 2, Fig. 3C-D). Three cases were above 80% of positive neoplastic cells, four varied between 30-50% and three had less than 10% of positive neoplastic cell. Three cases were intensely positive, six moderately, and one case was mildly positive. In tissue circumscribing tumors, scattered melanocytes with dendritic appearance were recognizable and they did not stain. No immunolabeling was observed in negative controls.

Discussion

FXR1 is a cytoplasmic RNA binding protein whose codifying gene's over-expression has been related to tumor progression in different human cancers,^{4,13,21} and more specifically to metastasizing behavior in human and canine uveal melanoma.^{19,20}

In the current literature, few studies investigated FXR1 protein localization in normal human tissue,^{1,21} and in different tumors.^{4,13,20,21} Detailed tissue expression of FXR1 has been described in the Human Protein Atlas.²⁴ To the best of authors' knowledge, no data are available concerning the tissue immunolocalization of FXR1 protein in the canine species. Moreover, commercially available anti-human antibodies were not previously validated in dogs.

The commercially available anti-human polyclonal anti-FXR1 antibodies tested in the present study were predicted to react with the canine species. On western blotting, a rabbit anti-FXR1 antibody showed a band with a molecular weight consistent with what expected as per manufacturer's indications (predicted molecular weight: 68 kDa, band detected by the producer: 65 kDa) and therefore it was considered to react specifically with human and canine serum. Moreover, the polyclonal antibody rabbit anti-FXR1 is raised against an immunogen sequence within amino acids 338-513, a sequence that is not present in the FXR2 sequence, which is characterized by a homology of 70% with FXR1,² further endorsing the specific binding of this antibody to FXR1 protein. The goat anti-FXR1 antibody recognized a band with a MW lower than expected and it was therefore considered not specific and excluded from further analysis.

In the present study, the specific tissue immunolocalization of FXR1 protein was investigated for the first time in the canine species. On immunohistochemical examination, FXR1 protein was detected in normal canine tissues with different degrees of intensity and resulted to be present in at least one tissue or cell population in all organ tested. These results in canine tissues were consistent with the immunohistochemical expression of FXR1 in human tissues as described in the Protein Atlas.²⁴ Connective tissue in the dog was not immunoreactive: this finding was consistent with the predicted results according to western blot results and human data.²⁴ An intense FXR1 expression was particularly observed in canine fetal skeletal muscle, testicular interstitial cells of Leydig and neurons. These results were consistent with the human literature, where FXR1 is reported to be expressed in different tissues,^{1,9} even if studies on FXR1 expression in normal tissue mostly relied on investigations of gene or mRNA expression.^{9,23} Anyway, canine and human tissues exhibited also slight differences in FXR1 expression, for example in the testis, where the spermatic line resulted negative, while it is reported to be strongly

positive in humans. It may be reasonable to hypothesize that tissue-specific isoforms of FXR1 exists and that they probably have a different distribution and expression in different species. Since 1995, RT-PCR analyses on human tissues revealed that different isoforms of FXR1 exist in brain and testis, presumably suggesting a tissue-specific alternative splicing.²³ Isoforms share extensive regions of identity and vary only in specific domains.²³ This fact probably account for tissue-specific function of the protein,^{15,23} thus explaining subtle differences in the expression between humans and dogs.

FXR1 gene has been previously shown to be over-expressed in a subset of human and canine uveal malignant melanomas with higher tendency toward a metastasizing behavior.^{19,20} However, to the best of authors' knowledge, there is no knowledge about the expression of the FXR1 protein in canine melanomas and no data indicating if FXR1 is peculiar of uveal melanomas or it is also expressed in melanomas arising in different body sites. In the present study, melanoma of the oral cavity was selected because it can be an aggressive tumor, but its progression and metastasizing behavior are not always predictable based only on histomorphological findings. Interestingly, in the cohort of cases investigated in the present study, FXR1 immunostaining was observed in all but one tested oral melanomas, with different levels of staining intensity and percentage of positive cells (results ranged from a negative case up to over 80%). These data indicated that FXR1 protein is actually expressed also in canine oral melanomas and the results may suggest to further investigate the possible relation between the immunohistochemical expression and the biological behavior.

In the cohort of uveal melanocytic tumors examined, all cases were positive for FXR1 even though with different levels of protein expression in terms of staining intensity and number of stained neoplastic cells. The immunohistochemical expression of FXR1 in melanoma

should be compared, in the future, with the follow-up data, in order to determine if it may provide a prognostic meaning, as already demonstrated for FXR1 gene.^{19,20}

Conclusions

In the present study, two anti-FXR1 protein antibodies were tested and the rabbit polyclonal anti-FRX1 showed a more specific binding to the canine FXR1 protein. The distribution of positive immunohistochemical signal obtained with rabbit anti-FXR1 antibody may indicate its potential ability to discriminate among different classes of protein expression possibly related to the tumor behavior. Further studies are ongoing to correlate FXR1 immunostaining with a prognostic significance in canine melanomas.

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Competing Interests

The authors declare they have no competing interests with respect to the research, authorship, and/or publication of this article.

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Author contributions

All authors (LN, ATM, CL, AML, DS, CG) contributed to the experimental design, writing, and editing of the manuscript. AML cultured fibroblast cells, LN, ATM and CL performed the western blot, LN and CG performed the histology and immunohistochemistry. All authors have read and approved the final manuscript.

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Figure legends

Figure 1. Human and canine sera and canine fibroblasts, detection of FXR1 protein, western blot analysis. Rabbit polyclonal anti-FXR1 antibody ab50841.

Figure 2. Normal tissues, dog. Immunohistochemical staining anti-FXR1, rabbit polyclonal anti-FXR1 antibody ab50841, ABC method, AEC chromogen. A: skeletal muscle, diffuse moderate to intense immunostaining of skeletal fibers, bar = 25 μ m. B: testis, intense immunostaining of interstitial cells, bar = 25 μ m. C: kidney, moderate reactivity of tubular epithelium and no reactivity of renal glomeruli, bar = 25 μ m. D: kidney, intense immunostaining of the urothelium, negative staining of connective tissue, bar = 50 μ m. E: stomach, intense immunoreactivity of parietal/chief cells, bar = 25 μ m. F: lung, intense staining of bronchial epithelium and mild scattered staining of pneumocytes, bar = 100 μ m. G: brain, intense reactivity of neurons, bar = 50 μ m. H: liver, diffuse moderate immunoreactivity of hepatocytes and mild reactivity of ductal epithelium, bar = 50 μ m.

Figure 3. Melanoma, dog. Immunohistochemical staining anti-FXR1, rabbit polyclonal anti-FXR1 antibody ab50841, ABC method, AEC chromogen. A-B: oral melanoma, diffuse intense to moderate staining of neoplastic cells. C-D: uveal melanoma, intense staining of neoplastic cells, multifocal to scattered. Bars = 12.5 μ m.

Figure 1 Supplementary material. Human and canine sera, western blot analyses. A: Rabbit polyclonal anti-FXR1 antibody ab50841, detected band around 65 kDa (left), and negative

control (right). B: Goat polyclonal anti-FXR1 antibody ab51970 (left), and negative control (right).

Tables legends

Table 1. Immunohistochemical expression of FXR1 in a series of normal canine tissues.^a

^a Intensity of the staining: - = negative, 1+ = mild, 2+ = moderate, 3+ = intense.

Table 2. Immunohistochemical expression of FXR1 in ten canine oral and uveal melanocytic neoplasms.^a

^a Intensity of the staining: 1+ = mild, 2+ = moderate, 3+ = intense.

Tables

Table 1

Tissue		Intensity	% positive cells	IRS score
	Follicular epithelial			
Thyroid	cells	3+	50-80	9
Pancreas	Acinar cells	2+	30-50	4
	Islet cells	3+	50-80	9
Liver	Hepatocytes	3+	>80	12
	Ductal epithelium	1+	50-80	3
Intestine	Enterocyte	3+	>80	12
	Lamina propria	-	0	0
	Muscular layer	-	0	0
	Serosal layer	1+	30-50	2
Stomach	Parietal/chief cell	3+	>80	12
	Mucus cell	-	0	0
	Connective tissue	-	0	0
	Muscular layer	-	0	0
Adipose				
tissue	Adipocytes	-	0	0
Lung	Bronchial epithelium	3+	>80	12
	Pneumocytes	1+	30-50	2

	Cartilage	1+	30-50	2
Kidney	Glomeruli	-	0	0
	Tubular epithelium	2+	50-80	6
	Interstitialium	-	0	0
	Urothelium	2+	>80	8
Brain	Neuron	3+	>80	12
	Striated cardiac			
Heart	myocytes	1+	50-80	3
Testis	Leydig cells	3+	>80	12
	Spermatoc line	-	0	0
Skeletal				
muscle	Myocytes	2+	>80	8

Table 2

#	Oral melanomas			Uveal melanomas		
	% positive cells	intensity	IRS score	% positive cells	intensity	IRS score
1	negative	negative	0	>80	3+	12
2	>80	3+	12	30-50	2+	4
3	30-50	2+	4	<10	1+	1
4	30-50	2+	4	<10	2+	2
5	10-30	1+	2	30-50	2+	4
6	>80	3+	12	30-50	2+	4
7	<10	2+	2	30-50	3+	6
8	>80	3+	12	>80	3+	12
9	50-80	2+	6	<10	2+	2
10	>80	3+	12	>80	2+	8

Figure 1

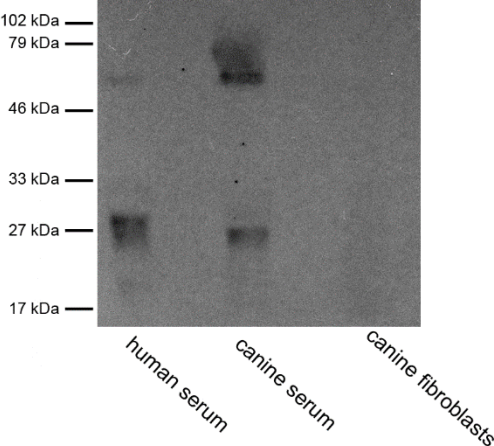


Figure 2

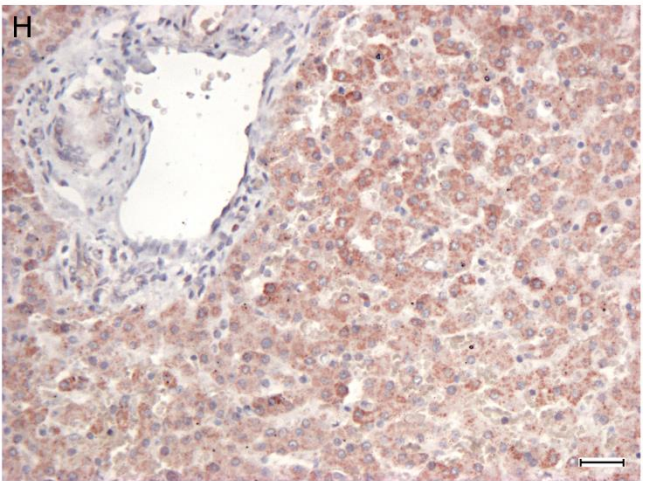
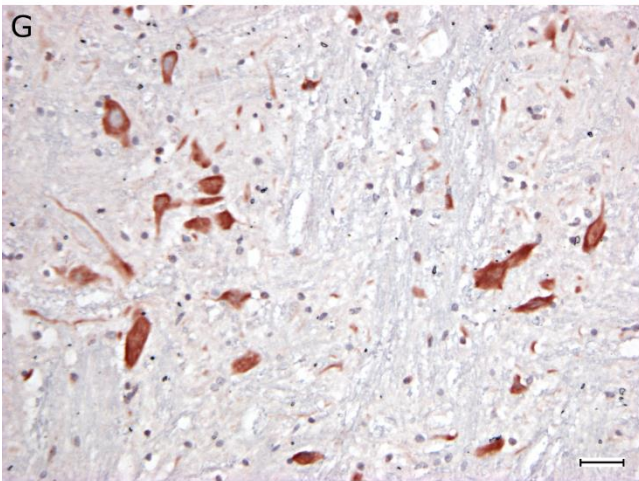
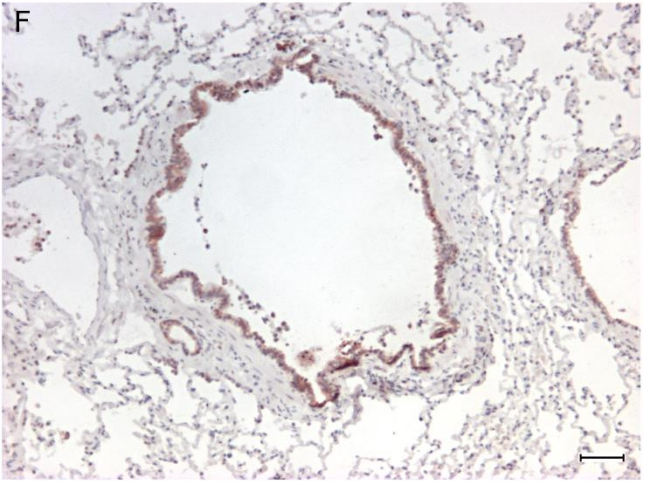
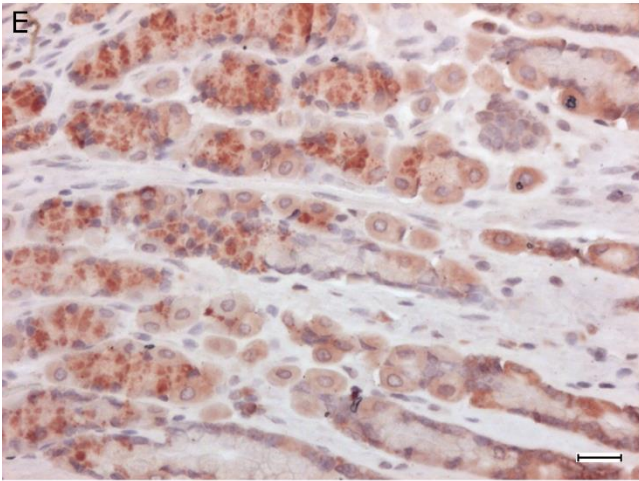
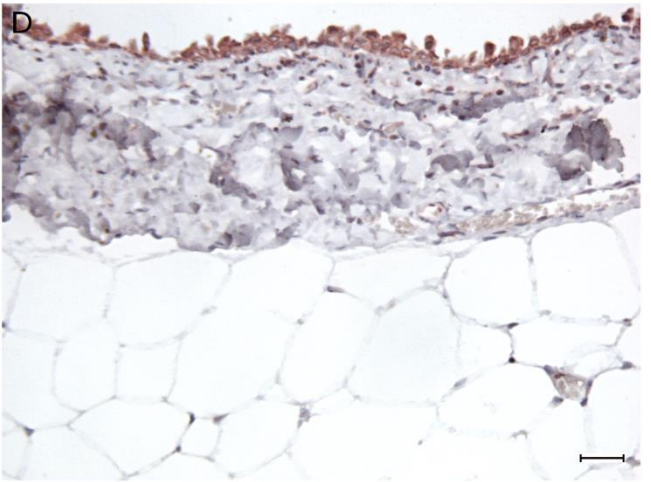
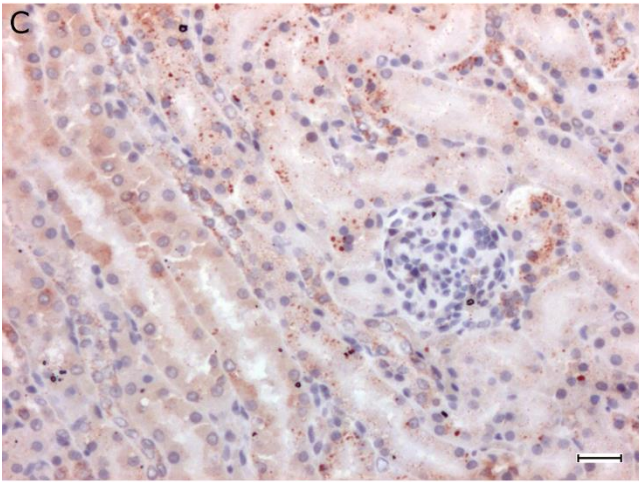
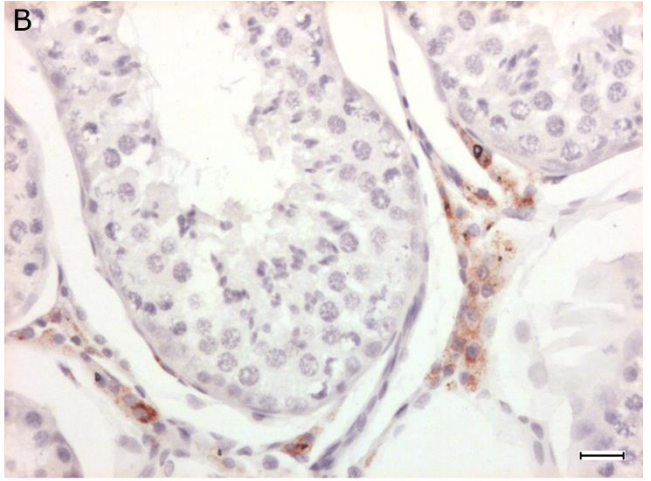
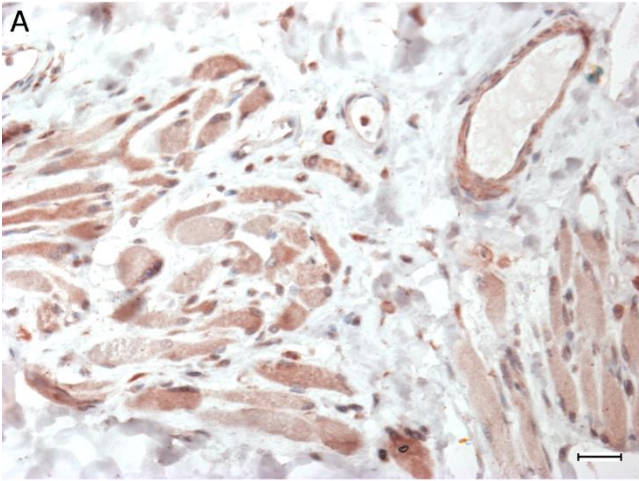
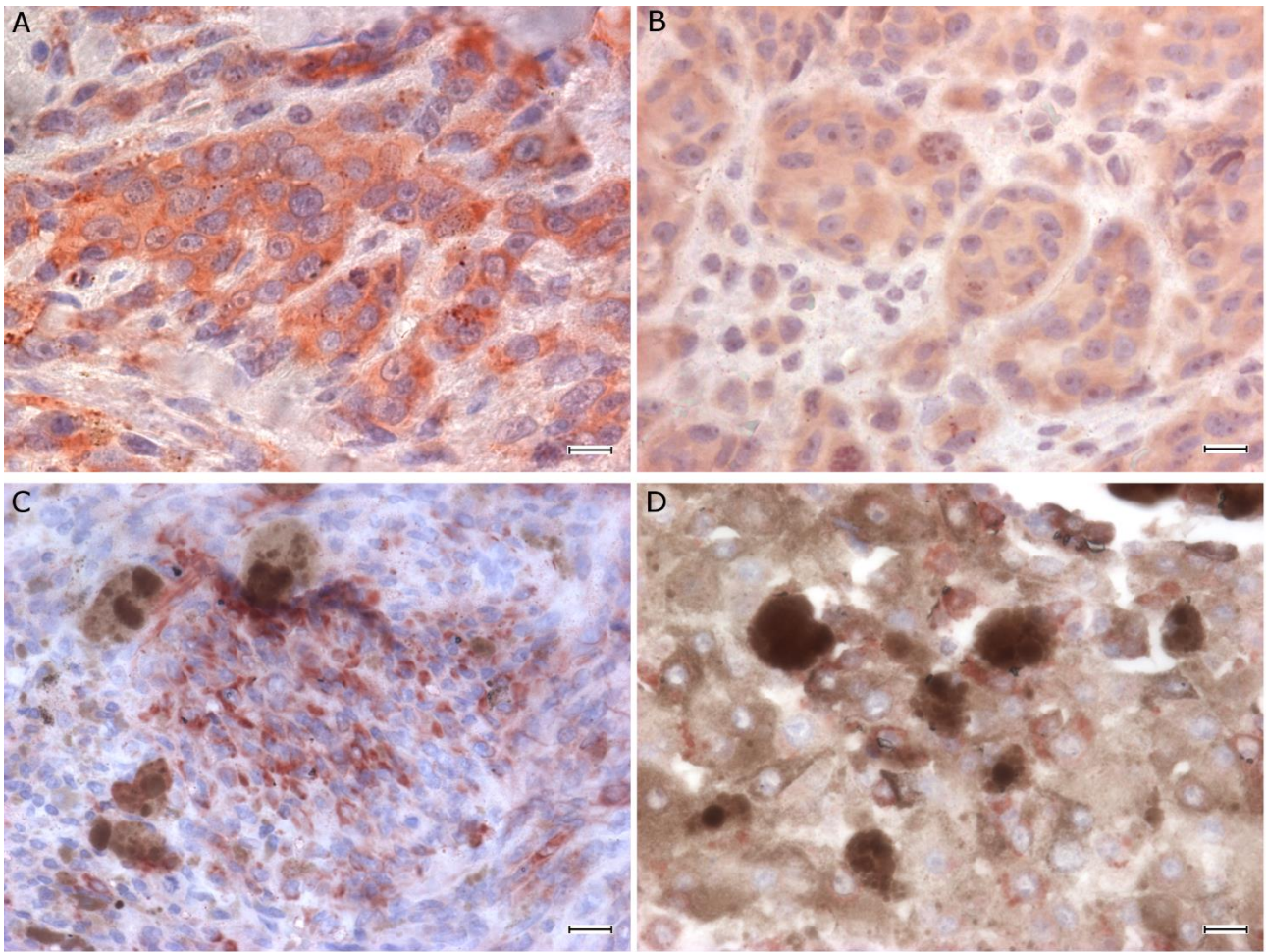


Figure 3



Supplementary material

