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METHODOLOGY

Serum Onconeural Antibodies in Dogs and Cats for Early Diagnosis of Cancer

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Abstract: Cancer is one of the leading causes of death in dogs and cats worldwide, and effective early detection techniques and reliable therapies are still lacking. Given the high demand for early cancer detection and differentiation in veterinary diagnostics, we developed and validated a new diagnostic approach to assess onconeural antibodies, also known as high-risk antibodies, in dog and cat blood serum samples. We determined the presence of systemic onconeural/high-risk (ONHR) antibodies, their suitability for early cancer diagnostics, and the feasibility of differentiating various malignancies. Our results identified several ONHR antibodies in 0.1 mL of specimens by the immunoblot-based technique, which was confirmed by indirect immunofluorescence assay. The diagnostic performance for detecting identified antibodies has demonstrated >95% sensitivity in dogs, >93% sensitivity in cats, as well as >97% specificity in dogs, and >95% specificity in cats. Thus, our data provide the first proof-of-principle that onconeural antibodies can be detected in dogs and cats, and their identification in serum might serve as a new tool for early cancer diagnosis.

Keywords: onconeural high-risk antibody, early cancer diagnosis, cancer in dogs and cats, cancer detection, cancer differentiation, immunoblot, immunofluorescence

Introduction

Malignant tumors in companion animals are a significant problem affecting the lives and well-being of pets and their owners. According to the Veterinary Cancer Society, cancer is the leading cause of death in 47% of dogs, especially elderly dogs, and 32% of cats.[1] Dogs develop cancer at almost the same rate as humans.[2] Cancer detection at the earliest stage may significantly improve the efficacy of the treatment and survival. Sensitive and specific methods for cancer screening can reduce mortality and improve the quality of life of affected animals.

Recently, two advanced techniques adapted from human cancer diagnostics have been implemented into veterinary medicine. The OncoK9 "liquid biopsy", based on next-generation sequencing, could only differentiate hematological malignancies, required large blood volumes, was costly, and was discontinued from commercial use.[3]

The second, Nu.Q test, measures plasma nucleosome concentration to predict certain cancers but provides only positive/negative results and can be provoked by inflammation, trauma, or other diseases.[4]

To address these diagnostic limitations, we focused on antibody biomarkers, widely used in human medicine[5] but still limited in veterinary diagnostics due to a lack of development and standardization.[6–11] Canine and human cancers share biological similarities,[12] making this approach promising. We investigated onconeural antibodies, produced against neuronal proteins aberrantly expressed in tumor cells. These antibodies are linked to paraneoplastic neurological syndromes (PNS), which may precede cancer diagnosis.[13] Although not always directly pathogenic, they are valuable diagnostic markers.[14]

Onconeural antibodies target intracellular or extracellular (membrane-bound) neuronal or glial proteins. Antibodies against intracellular proteins are more specific for malignancy rather than neurological syndromes and considered "high-risk",[14,15] while extracellular-directed antibodies are more closely tied to neurological diseases.[16] Sometimes, multiple

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antibodies coexist and are associated with the same cancer or PNS.[17] Paraneoplastic disorders are diagnosed in human patients with cancer, but there is a lack of knowledge about these diseases in animals. Even though these syndromes have been reported in dogs and cats, these diseases are usually diagnosed too late after the identification of cancer.

Since ONHR antibody formation usually precedes cancer, its early detection can predict malignancy before clinical symptoms, aiding timely treatment[18–21] and better outcomes for pets.[22–24] Thus, serological detection of ONHR antibodies represents a convenient diagnostic method for early cancer detection and differentiation.

Depending on the type of malignancy, tumor cells may express intracellular proteins/antigens such as amphiphysin, CV2/CRMP5, Ri/NOVA1, Yo/CDR2, Hu/ELAV, recoverin, SOX1, titin, ZIC4, GAD65, and Tr/DNER that are normally restricted to the nervous system.[25–35] These antigens can induce specific antibody formation before tumors are symptomatic. Altered expression, mutations, or posttranslational modifications can create neoantigens, provoking immune responses.[36]

As illustrated in many studies, between 50 and 90% of tumor-associated proteins/antigens in the human body acquire a variety of modifications that alter immunologic processing and presentation.[37] Thus, the modified self-antigens and neoantigens can be recognized as non-self-antigens by the immune system and promote onconeural antibody formation in the blood of patients with different types of tumors and have the potential to be an early sign of cancer. According to the literature, ONHR antibodies were detected in more than 90% of cases with underlying cancer within a few years of the study.[38]

Unfortunately, in veterinary medicine, only a few antibodies to neuronal proteins have been identified.[39–45] In our study, we validated ONHR antibodies in sera of cancer-bearing dogs and cats, confirming their stability and strong association with malignancies. These findings support their use as novel, noninvasive biomarkers for early cancer detection and malignancy-associated neurological diseases in pets.

Materials and Methods

Ethics Statement

Ethical approval was not required for this study, as it used de-identified residual serum samples submitted to Pet Preferred Diagnostics for research purposes and after routine diagnostics, with no additional collection or animal interventions.

Inclusion Criteria

The validation study was conducted on a total of 202 dogs and 50 cats, including 76 presumably healthy dogs and 20 presumably healthy cats with medical records from participating veterinary clinics and the National Institute of Health blood bank. The records included patient information such as age, sex, breed, neuter status, clinical history, and examination details.

Serum samples were collected from dogs and cats with confirmed or suspected malignancies at the time of validation, as well as animals with specific neurological symptoms. Sera obtained from presumably healthy dogs and cats, based on physical examination and clinical history, were also included in the validation study. Animals with conditions such as chronic infections were excluded from this study.

Animal Samples

Serum samples (0.05–0.5 mL) from animals (dogs and cats) with confirmed, suspected cancer, and presumably healthy were prepared from blood using VACUETTE tubes with serum separator. After 30–45 minutes at room temperature (which allows blood to clot), the tubes containing the collected blood were centrifuged at 2000–2500 RPM for 20 minutes to separate the serum. Sera were then transferred to Eppendorf tubes labeled with the patient's name and date of sample collection and shipped to Pet Preferred Diagnostics laboratory to be tested for the presence of onconeural antibodies.

Routine screening for the presence of ONHR antibodies such as anti-Hu, -Yo, -CV2, -Ri, -Tr, -ZIC4, -SOX1, -GAD65, -recoverin, -amphiphysin, and anti-titin (11 ONHR antibodies) was performed by immunoblot and confirmed by indirect immunofluorescence technique (IIFT) using monkey tissue slides.

Protein Sequence Comparison

UniProt Knowledgebase (UniProtKB) has been used for the analysis of ELAV/HuD (ELAV-like protein 4), CDR2/PCD17 (cerebellar degeneration-related protein 2), CRMP5/DPYSL5 (dihydropyrimidinase-related protein 5), NOVA1 (RNA binding protein), DNER/BET (Delta/Notch-like epidermal growth factor-related receptor), ZIC4 (Zinc finger protein), SOX1 (transcription factor protein), GAD65 (glutamate decarboxylase 2), RCVRN/RCV1 (recoverin/cancer-associated retinopathy protein), AMPH/BIN1 (amphiphysin/bridging integrator1/Myc box-dependent-interacting protein 1), and TTN (Titin/Connectin) human, canine, and feline protein sequences. Human, canine, and feline protein sequences were aligned using Clustal Multiple Sequence Alignment. A sequence similarity search query was submitted in the FASTA format.

Immunoblot-Based Assay

Serum samples from dogs and cats were tested using the commercial immunoblot kit (EUROLINE Neuronal Antigens Profile 72 (IgG), DL 1111–1601-72 G; Euroimmun, Lübeck, Germany). Immunoblots were performed on the EUROBlotOne system (Euroimmun). The nitrocellulose test strips with immobilized amphiphysin, CV2, Ri, Yo, Hu, recoverin, SOX1, titin, ZIC4, GAD65 and Tr antigens were incubated with patients' serum according to the manufacturer's instructions, modified and validated by our laboratory to adapt the test kit for veterinary diagnostics.

The strips were washed with a buffer provided in the kit and then incubated with the secondary alkaline-phosphatase conjugated goat anti-Canine IgG (SouthernBiotech, Birmingham, AL, USA) and goat anti-Cat (Bethyl Laboratories, Montgomery, TX, USA) antibodies. The onconeural antibodies in the serum bound to the related antigens were then visualized by NBT/BCIP substrate solution (nitroblue tetrazolium chloride/5-bromo-4chloro-3 indolylphosphate, Euroimmun). The intensity of binding of 11 ONHR antibodies was evaluated using EUROLineScan software (Euroimmun). An ONHR antibody band intensity value between 0 and 7 was considered negative or Class 0, between 8 and 14 was considered borderline or Class (+), between 15–35 and 36–70 as positive or Classes +, ++, and above 70 as strongly positive or Class +++. Strips with no added serum samples were used as negative controls.

Indirect Immunofluorescence Assay

The Neurology Mosaic 8 testing kit (Euroimmun, FA 1111–1005-8) is an indirect immunofluorescence assay used to confirm the presence of autoantibodies against eight targets associated with cancers and neurological diseases. This test detects antibodies against Hu, Yo, CV2, Ri, Tr, ZIC4, GAD65, and amphiphysin.

Serum samples were applied to the slides that were filled with BIOCHIPs – cerebellum and pancreatic sections collected from a monkey, according to the manufacturer's instructions, and were modified and validated by our laboratory to adapt the test kit for veterinary diagnostics. Sera from a dog and a cat previously diagnosed with cancer were used as positive controls. A serum sample from a healthy dog or cat was included in each evaluation as the negative control. After incubation with diluted serum samples (1:10 to 1:100) for 30 min at room temperature, BIOCHIP slides were washed with phosphate-buffered saline/Tween (PBST) for 5 min and developed with secondary fluorescent-labeled antibodies (for dog samples: fluorescein isothiocyanate (FITC)-conjugated goat anti-Dog IgG, Euroimmun; for cat samples: FITC-conjugated goat anti-Cat IgG from Jackson ImmunoResearch Laboratories, West Grove, PA, USA). The BIOCHIP slides were then incubated for 30 min at room temperature and washed with PBST for at least 5 min. Immunofluorescent images were acquired with the EUROStar III Plus fluorescent microscope at ×20 and ×40. Each power field was captured in four quadrants using the same imaging technique. Serum samples from dogs and cats with positive results were retested with positive and negative controls to ensure the repeatability of the results.

Statistical Analysis

Sensitivity and specificity were determined as the fractions of true-positive results in the cancer-diagnosed/detected group and true-negative results in the control group, respectively, and calculated for results where borderline results were interpreted as negative. Confidence intervals (CIs) were determined to be 95% for all sensitivity and specificity

calculations. Microsoft Excel (Microsoft Corporation, Redmond, WA, USA) and GraphPad Prism 10 (GraphPad, San Diego, CA, USA) online tools were used for statistical analyses.

Results

Protein Sequence Alignment

Multiple sequence analysis has demonstrated that Hu, Yo, CV2, Ri, Tr, ZIC4, SOX1, GAD65, amphiphysin, recoverin, and titin proteins shared significant sequence similarity (from 88% for recoverin to more than 99% for most of the analyzed proteins) between the human-canine (Table 1) and between human-feline homologs (Table 2).

Animal Characteristics

The median age of dogs included in the study was 9.2 years (2–20 years range), while the median age of cats was 10.3 years (2–18 years range). The median weight of dogs was 23.6 kg (range: 1.4–56.7 kg), and the median weight of cats was 4.7 kg (range: 2.6–7.7 kg). In dogs, males represented 57% of the population, with 92% spayed females and 74% neutered males. In cats, males represented 59.6%, with 100% spayed females and 100% neutered males. For validation, 70 healthy dogs and 20 healthy cats were included.

Of the 202 tested dogs, 60 were diagnosed with different types of cancers, 66 with suspected cancers, and 76 dogs were presumably cancer-free, healthy controls. Of the 50 tested cats, 19 were diagnosed with various cancers, 11 with suspected cancer, and 20 healthy controls. 64 dog breeds and 9 cat breeds were included in the validation study and are represented in Tables 3 and 4, respectively.

In this validation study, 21 cancer types were diagnosed in dogs and cats (Table 5). Examples of diagnostic findings in cancer-bearing participants and those with suspected cancers are represented in Table 6 for dogs and Table 7 for cats. No statistically significant association was identified in ONHR antibody detection between breed, sex, neuter status, and weight of animals included in the study.

Immunoblot and Indirect Immunofluorescent Test Results

The 11 ONHR antibodies, associated with specific types of cancers in sera from dogs and cats diagnosed with cancers as well as in serum samples from dogs and cats with suspected tumors, were detected independently by two assays. In our preliminary studies, the dilutions of serum and secondary antibodies were optimized for both assays. For immunoblot repeatability, at each dilution, evaluated by analyzing agreement within several replicas used in the run, was >97%, and reproducibility at each dilution, evaluated by analyzing agreement between operators, times, and lots of reagents, was >97%. Indirect immunofluorescence tests also display high inter- and intra-assay and inter-lot reproducibility (>98%) based on multiple analyses of characterized samples over several days or on a single day, correspondingly. Interference with hemolytic, lipemic, and icteric serum did not affect the results for both assays. Some false-positive results confirmed by both methods in dogs and cats (1.6% in dogs and 6.7% in cats) were obtained based on the knowledge that control patients are presumably cancer-free.

Initially, immunoblot-based assays have been used to screen dog and cat serum samples for ONHR antibodies, followed by confirmation with indirect immunofluorescence.

Immunoblot Assay of Dog Sera

Serum samples collected from dogs diagnosed with different cancers were analyzed by immunoblot. The results of the blot strips and antibody profiles are presented in Figure 1A–K for immunoblot and Supplemental Figure 1A–K for software-generated graphical test results. The immunoblot assay revealed the positivity for the investigated 11 ONHR antibodies in 54 dogs diagnosed with specific cancers and 66 dogs with suspected cancers (Table 6). Some serum samples from tumor-bearing dogs were positive for at least one antibody. For example, antibodies against amphiphysin, which can precede cancer for up to five years,[46] have been found in the serum of a dog diagnosed with osteosarcoma (Figure 1A). Figure 1B shows that anti-CV2 ONHR antibodies are related to prostate carcinoma. Anti-Ri antibodies were detected in a dog diagnosed with mammary gland carcinoma (Figure 1C). Figure 1D demonstrates the anti-Yo antibodies, which are also graded as high-risk with a frequency of

Table 1 Comparative Sequence Analysis of Human and Canine Antigen Homologs

Antibody	Protein (Antigen)	Symbol/Gene	UniProtKB ACC/ID Homo Sapience	UniProtKB ACC/ID/RefSeq Canis Lupus Familiaris	Query/Cover (%)	Percent Identity Align Results (%)
Anti-GAD65	Glutamate Decarboxylase 2/65	GAD65/ GAD2	Q05329|DCE2_HUMAN	Q4PRC2|DCE2_CANLF	100%	96.75%
Anti-CV2	Dihydropyrimidinase-Related protein 5	CRMV5/ DPYSL5	Q9BPU6|DPYL5_HUMAN	A0A8C0QDP3|A0A8C0QDP3_CANLF	100%	98.76%
Anti-amphiphysin	Amphiphysin/Bridging integrator 1/ Myc box-Dependent-interacting Protein 1	AMPH/BIN1	O00499|BIN1_HUMAN	A0A8I3S6X5|A0A8I3S6X5_CANLF	100%	97.12%
Anti-Hu	Hu-antigen D (HuD)/ ELAV-like protein 4	ELAV/ HUD	P26378|ELAV4_HUMAN	A0A8COMJG3|A0A8COMJG3_CANLF	98%	99.69%
Anti-Yo	Major Yo paraneoplastic Antigen/CDR2 Cerebellar Degeneration-related Protein 2	CDR2/ PCD17	Q01850|CDR2_HUMAN	A0A8C0RXI0|A0A8C0RXI0_CANLF	99%	93.10%
Anti-Tr	Delta/Notch-like Epidermal Growth Factor-Related Receptor	DNER/ BET	Q8NFT8|DNER_HUMAN	XP_038291591.1 DNER (CANLF)	100%	92.64%
Anti-ZIC4	Zinc finger protein ZIC 4	ZIC4	Q8N9L1|ZIC4_HUMAN	A0A8C0QIH6|A0A8C0QIH6_CANLF	100%	93.41%
Anti-Ri	RNA binding protein NOVA-1/ Paraneoplastic Ri antigen	NOVA1	P51513|NOVA1_HUMAN	A0A8C0S194|A0A8C0S194_CANLF	100%	99.80%
Anti-Titin	Titin/Connectin	TTN	Q8WZ42|TITIN_HUMAN	XP_038440898.1 Titin (CANLF)	100%	93.56%
Anti-recoverin	Recoverin/Cancer-associated Retinopathy protein (Protein CAR)	RCVRN/ RCV1	P35243|RECO_HUMAN	Q8MIH6|RECO_CANLF	100%	88%
Anti-SOX1	Transcription factor SOX-1	SOX1	O00570|SOX1_HUMAN	XP_038287415.1 SOX-1 (CANLF)	100%	99%

Table 2 Comparative Sequence Analysis of Human and Feline Antigen Homologs

Antibody	Protein (antigen)	Symbol/Gene	UniProtKB ACC/ID Homo sapience	UniProtKB ACC/ID/RefSeq Felis catus	Query/Cover (%)	Percent Identity Align results (%)
Anti-GAD65	Glutamate Decarboxylase 2/65	GAD65/ GAD2	Q05329|DCE2_HUMAN	M3VYC1|M3VYC1_FELCA	100%	97.09%
Anti-CV2	Dihydropyrimidinase-Related protein 5	CRMV5/ DPYSL5	Q9BPU6|DPYL5_HUMAN	A0A5F5XRM5|A0A5F5XRM5_FELCA	100%	98.76%
Anti-amphiphysin	Amphiphysin/Bridging Integrator 1/ Myc box-Dependent-interacting Protein 1	AMPH/ BIN1	O00499|BIN1_HUMAN	A0A337SB34|A0A337SB34_FELCA	100%	96.45%
Anti-Hu	Hu-antigen D (HuD)/ ELAV-like protein 4	ELAV/ HUD	P26378|ELAV4_HUMAN	A0A337SS82|A0A337SS82_FELCA	100%	99.08%
Anti-Yo	Major Yo paraneoplastic Antigen/CDR2 Cerebellar Degeneration-related Protein 2	CDR2/ PCD17	Q01850|CDR2_HUMAN	M3XFZ5|M3XFZ5_FELCA	100%	91.63%
Anti-Tr	Delta/Notch-like Epidermal Growth Factor-Related Receptor	DNER/ BET	Q8NFT8|DNER_HUMAN	M3WGD2|M3WGD2_FELCA	98%	93.23%
Anti-ZIC4	Zinc finger protein ZIC 4	ZIC4	Q8N9L1|ZIC4_HUMAN	A0A2I2U6W1|A0A2I2U6W1_FELCA	100%	94.31%
Anti-Ri	RNA binding protein NOVA-1/ Paraneoplastic Ri antigen	NOVA1	P51513|NOVA1_HUMAN	A0A337SFQ8|A0A337SFQ8_FELCA	100%	99.80%
Anti-Titin	Titin/Connectin	TTN	Q8WZ42|TITIN_HUMAN	XP_044889964.1 Titin (FELCA)	100%	94.07%
Anti-recoverin	Recoverin/Cancer-associated Retinopathy protein (Protein CAR)	RCVRN/ RCV1	P35243|RECO_HUMAN	XP_003996258.4 Recoverin (FELCA)	100%	88%
Anti-SOX1	Transcription factor SOX-1	SOX1	O00570|SOX1_HUMAN	XP_023109060.2 SOX-1 (FELCA)	100%	99.74%

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Table 3 Breeds Utilized in the Validation Study: Dogs

A	**F**	**P**
Alaskan Husky	Feist/Labrador mix	Pitbull
American Staffordshire Terrier	French Bulldog	Pomeranian
American Bulldog	**G**	Portuguese Water Dog
Aussie Doodle	German Shepherd	**R**
Australian Shepherd	German Shorthaired Pointer	Rottweiler
B	Glen of Imaal Terrier	**S**
Basenji	Golden Doodle	Samoyed
Beagle	Golden Retriever	Shih Tzu
Belgian Malinois	Great Dane	Shar Pei
Bernese Mountain Dog	Great Pyrenees	Siberian Husky
Black Russel Terrier	**H**	Staffordshire Terrier
Bloodhound	Harrier	Standard Schnauzer
Border Collie	Havanese	Standard Poodle
Boxer	**I**	St. Bernard
C	Italian Greyhound	**T**
Cane Corso	Irish Wolfhound	Toy Poodle
Cavalier King Charles Spaniel	Irish Setter	**V**
Chihuahua	**J**	Vizsla
Cockapoo	Jack Russell Terrier	**W**
Cocker Spaniel	**L**	Weimaraner
Coonhound	Labradoodle	Wheaton Terrier
D	Labrador Retriever	Wire Fox Terrier
Dachshund	**M**	**Y**
E	Maltese	Yorkshire Terrier
English Bulldog	Mini Poodle	
English Greyhound	Mini Schnauzer	
English Mastiff	Mix breed	
Erdel Terrier	Morkie	

Table 4 Breeds Utilized in the Validation Study: Cats

British Sort Hair	Domestic Short Hair	Maine Coon
Domestic Long Hair	European Shorthair	Russian Blue
Domestic Medium Hair	Himalayan	Siamese

>90% of underlying cancer, detected in the serum of a dog diagnosed with urothelial carcinoma, also known as transitional cell carcinoma (TCC), the most common type of bladder cancer in dogs. Anti-Hu antibodies have been associated with lung carcinoma (Figure 1E). Other onconeural antibodies, such as anti-recoverin, have been detected in the serum of a dog diagnosed with an ovarian tumor that is uncommon in dogs (Figure 1F). Antibodies against transcription factor protein SOX1 were found in the serum of a patient diagnosed with pulmonary carcinoma (Figure 1G). Antibodies against the sarcomeric protein titin were related to thymoma (Figure 1H). Anti-ZIC4 antibodies were detected in the serum of a dog diagnosed with thyroid tumor (Figure 1I). Although anti-GAD65 antibodies, considered as low-risk antibodies, were detected in the serum of a dog diagnosed with multiple myeloma (Figure 1J). It was shown that anti-GAD65 antibodies are not highly predictive of paraneoplastic neurological disorders. However, diverse cancer types associated with this antibody detection have been reported.[47] Anti-Tr ONHR antibodies were related to B-cell lymphoma (Figure 1K). Figure 1L and Supplemental Figure 1L show that no ONHR antibody was detected in the serum of a few presumably cancer-free dogs.

Table 5 Cancer Types Diagnosed in Dogs and Cats in the Validation Study

N#	Type of Cancer
1	Basal cell carcinoma
2	Bladder tumor
3	Brain tumor
4	Esophageal cancer
5	Gallbladder tumor
6	Intestinal tumors
7	Kidney tumor
8	Lung cancer
9	Lymphoma
10	Mammary tumors
11	Melanoma
12	Multiple myeloma
13	Neuroblastoma
14	Ovarian tumor
15	Pancreatic tumors
16	Prostate tumor
17	Squamous cell carcinoma
18	Testicular tumor
19	Thymoma
20	Thyroid cancer
21	Uterine tumors

Indirect Immunofluorescence Assay Results of Dog Sera

The detection of ONHR antibodies in the dog sera by the immunoblot-based assays was confirmed by indirect immunofluorescent testing (Figure 2A–K). The IIFT shows the positivity for anti-amphiphysin, -CV2, -Ri, -Yo, -Hu, -SOX1, -ZIC4, -Tr, and anti-GAD65 antibodies in dogs diagnosed with specific cancers, corresponding to results demonstrated in Figure 1. Negative results for cancer-free dogs are presented in Figure 2J and K.

The serum samples from dogs diagnosed with cancers and positive for one antibody, for example, against amphiphysin associated with osteosarcoma, reacted in the presynaptic nerve ends of the granular layer of the monkey cerebellum immobilized on the BIOCHIP slide as shown in Figure 2A. Figure 2B demonstrates the sand-like reaction product of anti-CV2 ONHR antibodies in the granular layer of primate cerebellum developed with serum of a dog diagnosed with prostate carcinoma, which confirmed the presence of these antibodies in the serum sample and the disease status. Anti-Ri antibodies reacting with almost all nuclei of the monkey cerebellum were found in the serum of a dog diagnosed with mammary gland carcinoma (Figures 2C). Figure 2D demonstrates the anti-Yo antibodies reacting with the rough endoplasmic reticulum and Golgi apparatus in the cytoplasm of the Purkinje cells in the sera of a dog diagnosed with urothelial carcinoma. Anti-Hu antibodies reacting with neuronal nuclei have been detected in the sera of dogs diagnosed with lung carcinoma (Figure 2E). The anti-SOX1 antibodies reacted with glial cells within the Purkinje cell layer in the cerebellum of a dog diagnosed with pulmonary carcinoma (Figure 2F). The anti-ZIC4 antibodies, which reacted in almost all neuronal cell nuclei of the granular layer of the monkey cerebellum, were found in the serum of a dog diagnosed with thyroid tumor (Figure 2G). The anti-Tr antibodies reacting with the granular pattern in the Purkinje cell cytoplasm were detected in the serum of a dog diagnosed with B-cell lymphoma (Figure 2H). The anti-GAD65 antibodies were detected in the pancreatic tissue developed with the serum of a dog diagnosed with multiple myeloma (Figure 2I). These antibodies and anti-amphiphysin antibodies reacted with the granular layer of the monkey cerebellum and islet cells on monkey pancreas slides. Thus, only images of anti-GAD65 antibodies reacting with the monkey pancreas were demonstrated due to the possibility of overlapping. Example of negative results for ONHR antibody

Table 6 Examples of Diagnostic Findings in Cancer-Bearing Dogs and Dogs with Suspected Cancers

NN	Breed	Age	M-male; N-Neutered; F-Female; S-Spayed	Cancer Type	Neurological Symptoms	ONHR Antibodies Detected
1	Mix	12 years and 2 months	MN	Prostatic carcinoma	None	Anti-CV2; anti-Yo
2	German Shorthair Pointer	9 years and 7 months	MN	Urotelial carcinoma/Transitional cell carcinoma	None	Anti-CV2; anti-Yo
3	Mix	13 years	FS	Osteosarcoma	None	Anti-amphiphysin, anti-GAD65
4	Bernese Mountain Dog	5 years	MN	Lymphoma	None	Anti-GAD65
5	Wire Fox Terrier	12 years	MN	Lymphoma	None	Anti-Zic 4
6	Mix	10 years and 6 months	MN	Cutaneous T-cell lymphoma	None	Anti-GAD65
7	Poodle	14 years and 8 months	FS	Mammary gland Carcinoma	None	Anti-Yo; anti-GAD65
8	Samoyed	10 years and 1 month	MN	Lung squamous cell carcinoma	None	Anti-CV2; anti-GAD65
9	Golden Retriever	8 years and 3 months	F	Uterin leiomyosarcoma	None	Anti-Tr (DNER); anti-GAD65
10	Golden Retriever	6 years and 5 months	MN	Lymphoma	None	Anti-GAD65
11	Yorkie	15 years and 4 months	FS	Mammary gland Carcinoma	None	Anti-GAD65
12	Mix	11 years and 1 month	MN	T-zone lymphoma	None	Anti-GAD65
13	German Shepherd	6 years	MN	Thymoma	Myasthenia Gravis	Anti-CV2; anti-Yo
14	Boxer	5 years	FS	Mast Cell Tumor	None	Anti-Tr (DNER); anti-GAD65
15	Cockapoo	13 years and 1 month	MN	Thymoma	None	Anti-Yo; anti-Titin
16	Irish Wolfhound	11 years	FS	Lung cancer	None	Anti-recoverin
17	Wheaten Terrier	12 years and 7 months	MN	Lymphoma	None	Anti-CV2; anti-GAD65
18	Mix	15 years	FS	Mammary gland tumor	None	Anti-Tr (DNER); anti-GAD65
19	Basenji	3 years and 4 months	FS	Lung cancer	None	Anti-recoverin; anti-CV2
20	Australian Shepherd	12 years	FS	Thymoma	None	Anti-CV2; anti-Titin
21	German Shepherd	5 years	FS	Squamous cell carcinoma	None	Anti-CV2; anti-Yo
22	Bernese Mountain	7 years and 4 months	FS	Lymphoma	None	Anti-CV2; anti-GAD65
23	Bulldog	5 years and 2 months	FS	B-cell lymphoma	Cerebellar Ataxia	Anti-Tr (DNER); anti-GAD65
24	Jack Russell/Bichon	13 years and 5 months	MN	Transitional cell carcinoma	None	Anti-CV2
25	Mini Schnauzer	13 years and 3 months	FS	Small cell neuroendocrine Carcinoma	None	Anti-GAD65
26	Rottweiler	7 years and 9 months	FS	Thyroid carcinoma	None	Anti-SOX1; anti-Zic 4
27	Staffordshire terrier	15 years and 6 months	FS	Lung adenocarcinoma	None	Anti-CV2; anti-recoverin; anti-Tr/ (DNER); anti-GAD65
28	Miniature Poodle	13 years and 6 months	M	Prostate carcinoma	None	Anti-CV2
29	Golden Doodle	11 years and 8 months	FS	Intestinal adenocarcinoma	None	Anti-amphiphysin; anti-GAD65
30	Pittbull	8 years and 2 months	MN	Lymphoma	None	Anti-CV2; anti-GAD65
31	Pittbull	5 years and 1 month	MN	Lymphoma	None	Anti-CV2; anti-GAD65
32	Labrador Retriever	8 years and 2 months	MN	Transitional cell carcinoma	None	Anti-CV2; anti-ZIC4; anti-GAD65; anti-Tr (DNER)
33	Labrador Retriever	10 years	MN	Lymphoma	Cerebellar Ataxia	Anti-GAD65

(*Continued*)

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Table 6 (Continued).

NN	Breed	Age	M-male; N-Neutered; F-Female; S-Spayed	Cancer Type	Neurological Symptoms	ONHR Antibodies Detected
34	Rottweiler	6 years and 10 months	MN	Osteosarcoma	None	Anti-amphiphysin
35	Labrador Retriever	13 years	MN	Renal cell carcinoma	None	Anti-CV2; anti-Ri; anti-GAD65
36	Bulldog	8 years	MN	Mast cell tumor	None	Anti-recoverin; anti-GAD65; Anti-Tr (DNER)
37	Beagle	15 years	FS	Neuroblastoma	None	Anti-Hu; anti-GAD65
38	Golden Retriever	8 years and 2 months	FS	Brain tumor	None	Anti-Tr (DNER)
39	Golden Retriever	9 years	FS	Thymoma	Myasthenia Gravis	Anti-Titin
40	Wire Hair Fox Terrier	9 years and 1 months	FS	Urotelial carcinoma/Transitional call carcinoma	None	Anti-Yo
41	Dachshund	11 years and 2 months	F	Mammary gland carcinoma	None	Anti-Ri
42	Australian Shepherd	11 years and 1 months	MN	Pulmonary carcinoma	None	Anti-SOX1
43	Cockapoo	12 years and 3 months	MN	Lung carcinoma	None	Anti-Hu
44	Golden Retriever	7 years and 5 months	FS	Thyroid tumor	None	Anti-ZIC4
45	Bernese Mountain Dog	8 years and 7 months	FS	Lymphoma	None	Anti-Tr (DNER)
46	German Shepherd	7 years and 4 months	MN	Multiple myeloma	None	Anti-GAD65
47	Boxer	7 years and 7 months	F	Ovarian tumor	None	Anti-recoverin
48	Mix	7 years	FS	Thyroid cancer	None	Anti-CV2; anti-GAD65
49	Pitbull	12 years 6 months	FS	Lymphoma	None	Anti-ZIC4; anti-GAD65
50	Boxer	9 years	MN	Thymoma	None	Anti-amphiphysin; anti-Titin
51	Mix	13 years	FS	Lymphoma	None	Anti-Hu; anti-GAD65
52	Australian Shepherd	8 years	MN	Pancreatic tumor	None	Anti-recoverin; anti-GAD65
53	German Short Haired Pointer	8 years	FS	Pulmonary carcinoma	None	Anti-CV2; anti-recoverin
54	Golden Doodle	9 years	FS	Thymoma	None	Anti-Titin
55	Alaskan Husky	2 years	MN	Lymphoma	None	Anti-Yo
56	Pitbull	13 years	MN	Multiple myeloma	None	Anti-GAD65
57	Labrador Retriever	10 years	FS	Pulmonary carcinoma	None	Anti-amphiphysin
58	Staffordshire Terrier	6 years	MN	Squamous cell carcinoma	None	Anti-recoverin
59	American Staffordshire Terrier	13 years	MN	Lymphoma	None	Anti-CV2; anti-amphiphysin

Table 7 Examples of Diagnostic Findings in Cancer-Bearing Cats and Cats with Suspected Cancers

NN	Breed	Age	M-male; N-neutered; F-Female; S-Spayed	Cancer type	Neurological Symptoms	ONHR Antibodies Detected
1	DSH	15 years	MN	Small Cell LSA	None	Anti-GAD65
2	DSH	9 years	MN	T-cell LSA	None	Anti-Yo; anti-recoverin; anti-GAD65
3	DSH	9 years	FS	Mammary gland cancer	None	Anti-GAD65
4	DSH	11 years	MN	Lymphoma	None	Anti-CV2; anti-Yo; anti-GAD65
5	DLH	12 years	MN	Lymphoma	None	Anti-Hu; anti-Titin; anti-GAD65
6	DSH	10 years 5 months	MN	Pulmonary carcinoma	Seizures	Anti-CV2; anti-recoverin; Anti-GAD65
7	DSH	8 years	FS	Multiple myeloma	None	Anti-GAD65
8	DSH	10 years and 2 months	F	Mammary gland carcinoma	None	Anti-amphiphysin
9	DSH	12 years	MN	Squamous cell carcinoma	None	Anti-CV2
10	Siamese	7 years and 11 months	FS	T-cell lymphoma	None	Anti-Yo
11	DLH	15 years	MN	Pulmonary carcinoma	None	Anti-Hu
12	British Blue	11 years and 1 months	MN	High-grade lymphoma	None	Anti-ZIC4
13	DSH	16 years and 3 months	MN	Pulmonary carcinoma	None	Anti-Tr (DNER)
14	DSH	5 years	MN	Melanoma	None	Anti-recoverin
15	DSH	10 years	MN	Bladder tumor	None	Anti-Yo; anti-SOX1
16	DSH	7 years and 2 months	MN	Lymphoma	None	Anti-amphiphysin; anti-Tr (DNER); anti-GAD65
17	DLH	9 years	FS	Multiple myeloma	None	Anti-GAD65
18	DLH	15 years	MN	Thyroid adenocarcinoma	None	Anti-SOX1
19	DSH	11 years	MN	Thymoma	Myasthenia Gravis	Anti-Titin

detection in the sera of presumably healthy dogs using IIFT are represented in Figure 2J and K. Other ONHR antibodies, such as anti-recoverin and anti-titin antibodies detected by immunoblot in sera of tumor-bearing dogs, have not been supported by the IIFT study on monkey cerebellum tissue, because they are the antibodies targeting antigens in cells of retina and striated muscles. However, these two types of detected antibodies in the sera of dogs with specific cancers are correlated with clinical evaluation and symptoms associated with the diagnosed cancer types.

Thus, the presence of ONHR antibodies in sera of tumor-bearing dogs or in dogs with cancer suspicions has been confirmed by two assays. The total number of confirmed ONHR antibodies with positive results was 120/126. Test sensitivity, which has been calculated as the percentage of all cancer-diagnosed dogs, was 95.2% (95% CI: 90.7% - 99.3%). No significant difference in sensitivity was found based on the breed, sex, weight, or age status of the cancer-diagnosed dogs. The detection rate was 95.2% (Table 8). In the sera of two presumably cancer-free dogs, onconeural antibodies have been detected. Thus, the overall specificity of this test was 97.4% (95% CI: 94.3% - 99.8%), which has been calculated as the percentage of all presumably healthy dogs where onconeural antibodies have not been detected. The accuracy of ONHR antibody detection in the dog was 96%. Positive predicted values (PPV) and Negative predicted values (NPV) for this test were 98.4% and 92.5% respectively (Table 8).

Immunoblot Assay of Cat Sera

The immunoblot assay shows the positivity for anti-amphiphysin, -CV2, -Yo, -Hu, -recoverin, -SOX1, -titin, -ZIC4, -GAD65, and anti-Tr antibodies in 17 cats with detected specific cancers and 11 cats with suspected cancers (Figure 3A–J for immunoblot, Supplemental Figure 2A–J for software-generated graphical test results and Table 7). For example, anti-amphiphysin antibodies have been detected in a cat with mammary gland carcinoma (Figure 3A), the third most common tumor in cats. Anti-CV2 antibodies were found in a cat with squamous cell carcinoma (Figure 3B). Anti-Yo antibodies have been detected in a cat with renal cell carcinoma (Figure 3C). The anti-Hu antibodies were associated with pulmonary carcinoma (Figure 3D). Anti-recoverin antibodies were found in a cat with melanoma (Figure 3E). Antibodies against SOX1 have been detected in a cat with thyroid adenocarcinoma, which is relatively rare in cats (Figure 3F). Antibodies against titin were associated with thymoma (Figure 3G). The anti-ZIC4 ONHR antibodies were detected in a cat with high-grade lymphoma (Figure 3H). Anti-GAD65 antibodies were related to small cell lymphoma

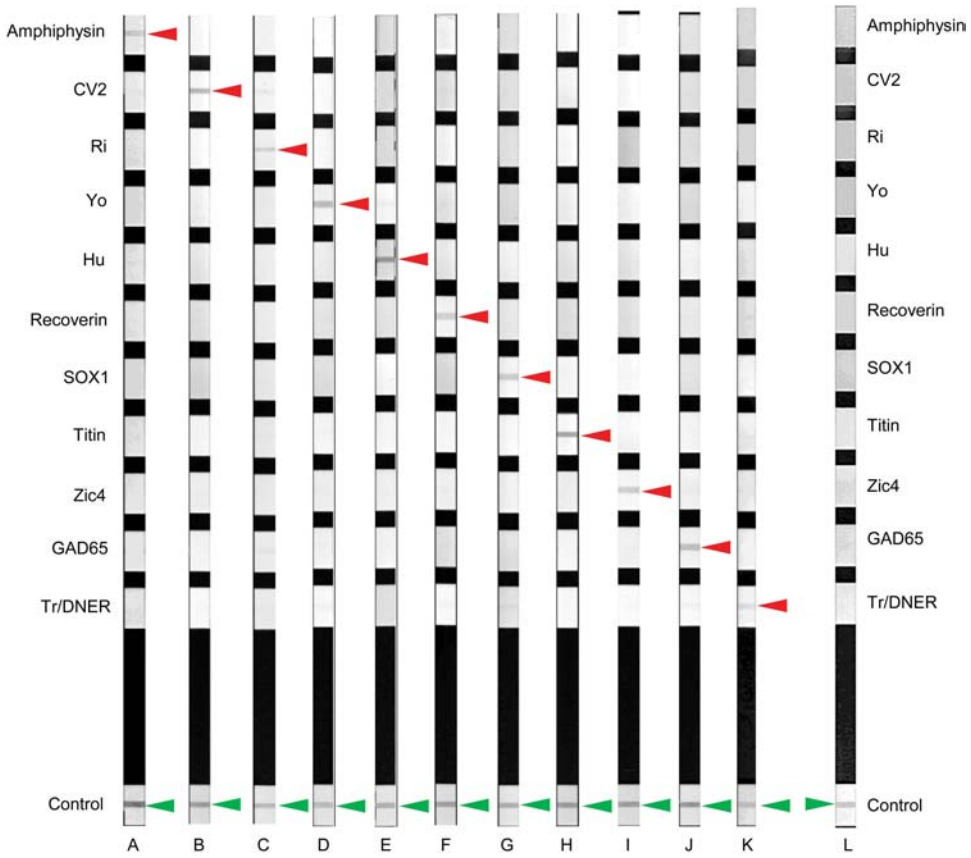

Figure 1 Onconeural antibodies detection in cancer-diagnosed (**A**–**K**) dogs by immunoblot-based assay. Examples of ONHR antibodies (anti- amphiphysin (**A**), - CV2 (**B**), - Ri (**C**), - Yo (**D**), - Hu (**E**), - recoverin (**F**), - SOX1 (**G**), - titin (**H**), - ZIC4 (*I*), - GAD65 (**J**), and – Tr (**K**)) bound to associated antigens immobilized on the strips detected in the sera of cancer-diagnosed dogs. Red arrowheads indicate the bands linked to these antibodies (**A**–**K**). Green arrowheads point to the control bands, indicating an experiment's success. No ONHR antibodies have been detected in the sera of cancer-free (healthy) dogs (**L**).

(Figure 3I). The anti-Tr antibodies were found in a cat with T-cell lymphoma (Figure 3J). Interestingly, anti-Ri antibodies have not yet been found in cat serum samples using an immunoblot-based assay. Figure 3K and Supplemental Figure 2K represent the results of onconeural antibody detection in the serum of presumably cancer-free cats.

Indirect Immunofluorescent Assay Results of Cat Sera

IIFT has confirmed the results obtained by the immunoblot-based assay. ONHR antibodies detected by IIFT in cats with different cancers are represented in Figure 4A–H and Table 7. Negative results for cancer-free cats are presented in Figure 4I and J. For example, anti-amphiphysin antibodies reacted with the presynaptic ends in the granular layer of a monkey cerebellum immobilized on a BIOCHIP slide have been detected in the serum of a cat diagnosed with mammary gland carcinoma (Figure 4A). Anti-CV2 antibodies have demonstrated a sand-like reaction in the granular layer of the cerebellum (Figure 4B) developed in a cat with squamous cell carcinoma. The anti-Yo antibodies reacted with rough endoplasmic reticulum and Golgi apparatus in the cytoplasm of the Purkinje cells (Figure 4C) in a cat with renal cell carcinoma (RCC). The anti-Hu antibodies that reacted with the nuclei on the immobilized cerebellum (Figure 4D) were identified in a cat with pulmonary carcinoma. Antibodies against protein Sox1, which reacted with glial cells within the Purkinje cell layer, were found in the serum of a cat diagnosed with thyroid adenocarcinoma (Figure 4E). The anti-ZIC4 antibodies reacted with the nuclei of the granular layer of cerebellum (Figure 4F) in a cat with high-grade lymphoma. The anti-Tr antibodies were detected in a granular pattern in the Purkinje cell cytoplasm of cerebellum, in a cat (Figure 4G) with T-cell lymphoma. The anti-GAD65 antibodies, which reacted with islet cells on immobilized monkey pancreas (Figure 4H), were detected in cats with small cell lymphoma. The ONHR antibodies, such as anti-recoverin and anti-titin, detected by immunoblot in the sera of cats with cancers, were not confirmed by the IIFT

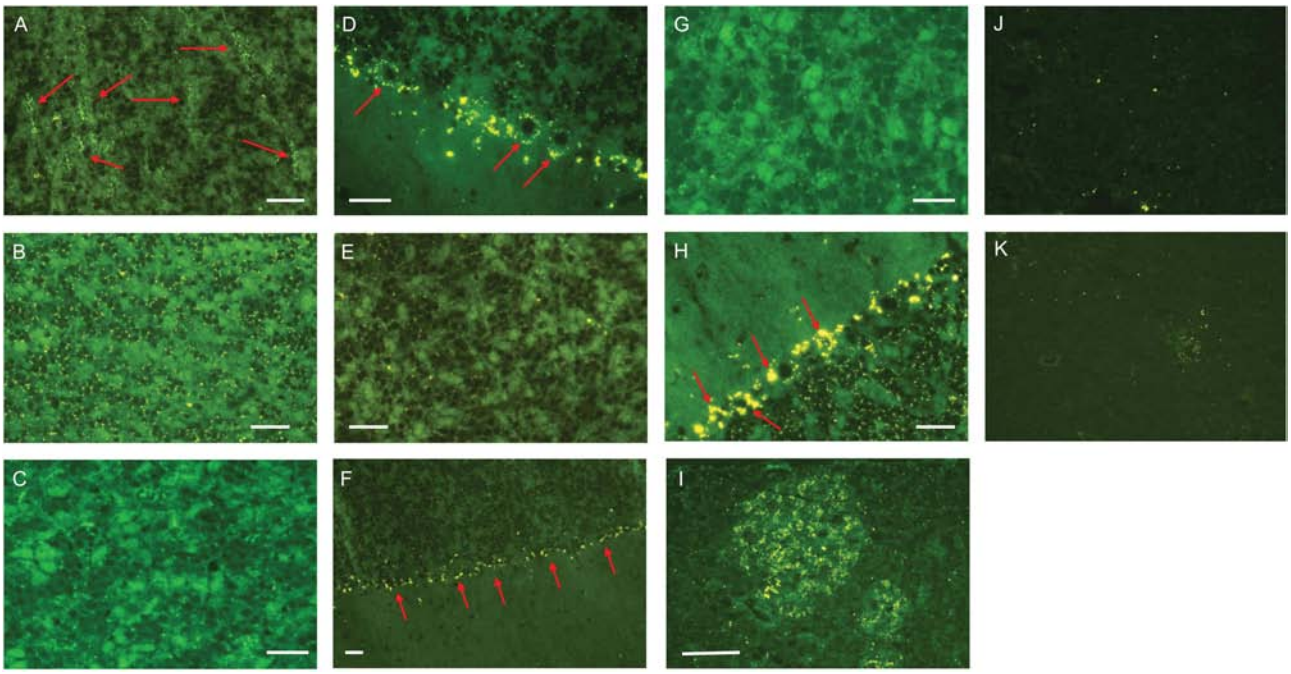

Figure 2 Immunofluorescent detection of ONHR antibodies in the same serum used for the results represented in Figure 1 and collected from cancer-diagnosed (**A**–**I**) and cancer-free (healthy, **J**–**K**) dogs. Sera from cancer-diagnosed dogs demonstrate positive staining for onconeural antibodies on the cerebellum tissue sections (**A**–**H): A** – reaction product observed in the presynaptic nerve ends (arrows) of the granular layer (anti-amphiphysin); **B** – sand-like staining in granular layer (anti-CV2); **C** – reaction product on almost all neuronal nuclei of the granular layer (anti-Ri); **D** – arrows point to the strong positive staining of Purkinje cell cytoplasm (anti-Yo); **E** – reaction product in almost all neuronal nuclei of the granular layer (anti-Hu); **F** – arrows point to the staining in glial cell within the Purkinje cell layer along the border between molecular and granular layers (anti-SOX1); **G** – staining in almost all granular layer nuclei (anti-ZIC4); **H** – strong reaction product in the Purkinje cell cytoplasm (arrows) (anti-Tr). **I** - pancreatic tissue with the reaction product (anti-GAD65). No staining was observed in the sera of cancer-free (healthy) dogs on the cerebellum (**J**) and pancreatic (**K**) sections (corresponds to Figure 1L). Serum dilution is 1:100. Scale bars: 10 μm for **A-H**; 40 μm for **I**.

test due to the localization of their antigens in the retina and striated muscle tissue, but in the primate cerebellum used on BIOCHIP slides for IIFT. However, as in dog cases, all three types of these antibodies detected in the sera of cats diagnosed with specific cancers are correlated with clinical pictures and symptoms associated with the revealed cancer types.

Thus, ONHR antibodies have been detected in cats with cancer and cancer suspicions and confirmed by two different assays, immunoblot and IIFT. The test sensitivity (detection rate), which has been calculated as the percentage of cancer-diagnosed cats (28/30), was 93.3% (95% CI: 88.4% - 98.2%). As with dogs, no significant difference in sensitivity was found based on the breed, sex, weight, or age status of the cats diagnosed with cancer. The detection rate was 93.3% (Table 9). The ONHR antibodies have been detected in the sera of two of 20 presumably cancer-free cats. Thus, the overall specificity of this test was 95% (95% CI: 90.7% − 99.3%), which has been calculated as the percentage of all presumably healthy cats with no onconeural antibodies detected. The accuracy of onconeural antibody detection in the cat's sera was 94%. PPV and NPV for this test were 96.6% and 90.5% respectively.

Detection of Multiple Onconeural Antibodies

Two or more ONHR antibodies were detected simultaneously by immunoblot and IIFT in some samples collected from cancer-diagnosed dogs and cats. Multiple ONHR antibodies can be detected in the same sample because cells of a single cancer can express multiple neuronal antigens, which produce antibodies against several antigens simultaneously, resulting in several ONHR antibodies in a patient's blood. Some ONHR antibody reaction products can overlap on immunofluorescent images, as is the case for anti-Hu, -Ri, and -ZIC4, as well as for anti-Yo and -Tr, and for anti-amphiphysin and -GAD65 due to their identical localization in cell compartments. Therefore, besides cerebellar tissue, the pancreas tissue was used to detect anti-GAD65 antibodies to support their existence in the same serum sample. An example of the evaluation of serum by two assays of a dog diagnosed with lymphoma is represented in Figure 5, and

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Table 8 Calculation of Test Specificity and Sensitivity for Dogs

Dogs	Cancer present	Cancer absent	Total population: 202
Test Positive: **Test negative:**	True positive tests: 120 False negative tests: 6	False positive tests: 2 True negative tests: 74	Total positive tests: 122 Total negative tests: 80
Sensitivity=95.2% Specificity=97.4% Positive predicted values=98.4% Negative predicted values=92.5% Prevalence=62.4%			

a cat diagnosed with transitional cell carcinoma in Figure 6. Multiple ONHR antibodies were detected in a single serum sample of dogs (Figure 7A) and cats (Figure 7B) with cancer. Overall, the frequency of different ONHR antibody detection in the serum of dogs and cats is represented in Figure 8A and B, respectively.

Case Report: Detection of ONHR Antibodies Months Before the Onset of Clinical Signs, During and After Treatment

This case study examines the ability to detect oncogene-specific antibodies before the clinical signs of cancer appear.

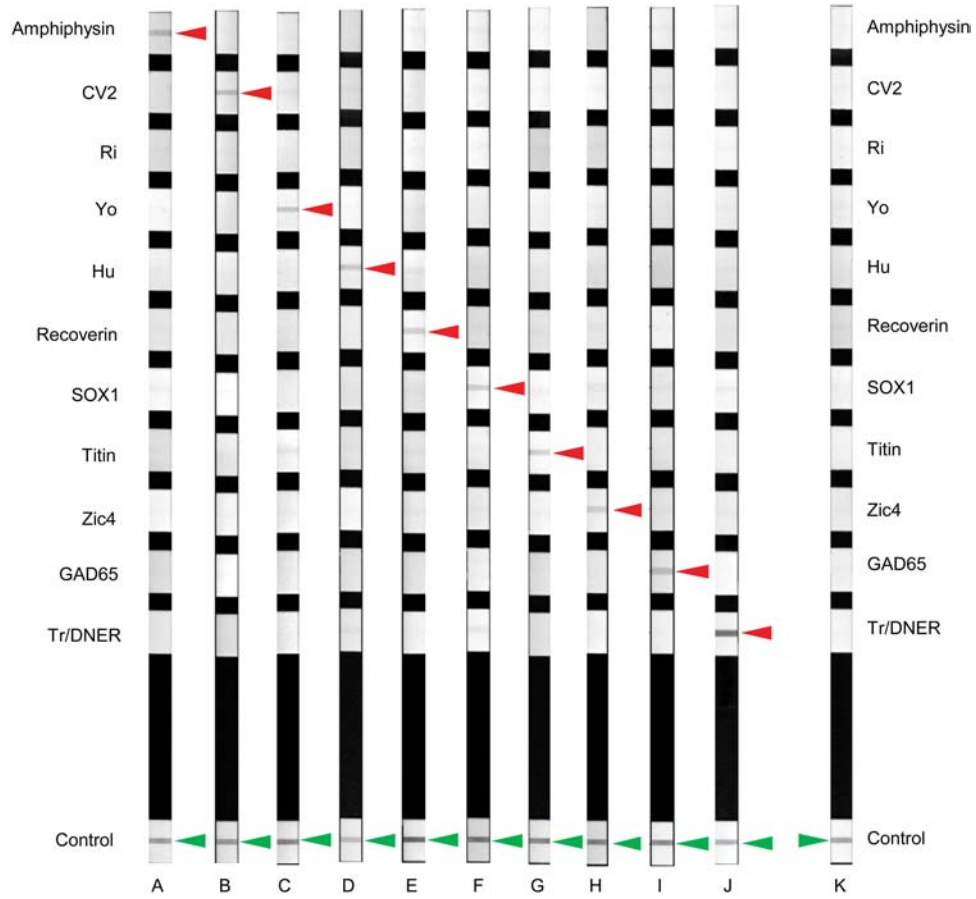

Figure 3 ONHR antibody detection in cancer-diagnosed (**A**–**J**) cats by immunoblot-based assay. Examples of ONHR antibodies (anti-amphiphysin (**A**), -CV2 (**B**), -Yo (**C**), -Hu (**D**), -recoverin (**E**), -SOX1 (**F**), -titin (**G**), -ZIC4 (**H**), -GAD65 (**I**), and -Tr (**J**)) bound to associated antigens immobilized on the strips detected in the sera of cancer-diagnosed cats. Red arrowheads indicate the bands linked to these antibodies (**A-J**). Green arrowheads point to control bands, indicating a successful experiment. No ONHR antibodies have been detected in the sera of cancer-free (healthy) cats (**K**). Serum dilution is 1:10.

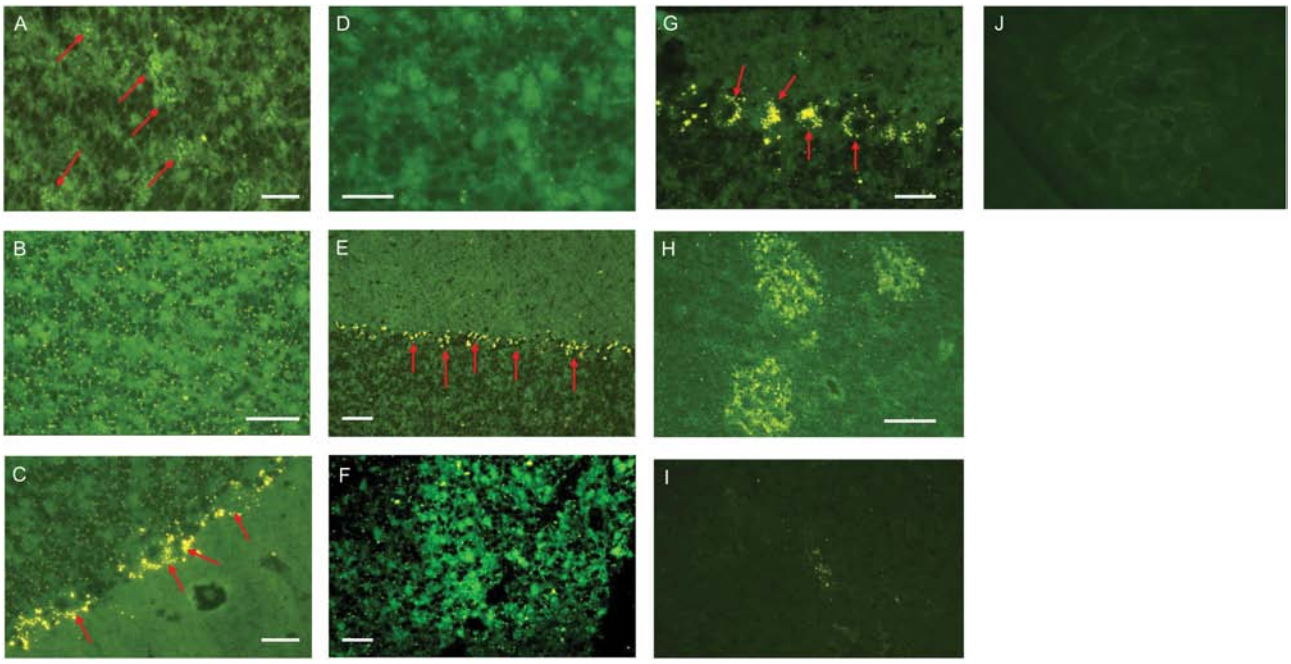

Figure 4 Immunofluorescent detection of ONHR antibodies in the same serum used for the results represented in Figure 3 and collected from cancer-diagnosed (**A**–**H**) cats. Sera from cancer-diagnosed cats demonstrate positive staining for ONHR antibodies on the cerebellum tissue sections (**A**–**G): A** – reaction product observed in the presynaptic nerve ends (arrows) of the granular layer (anti-amphiphysin); **B** – sand-like staining in the granular layer (anti-CV2); **C** – reaction product on almost all neuronal nuclei of the granular layer (anti-Yo); **D** - reaction product in nearly all neuronal nuclei of the granular layer (anti-Hu); **E** – arrows point to staining in glial cell within the Purkinje cell layer along the border between molecular and granular layers (anti-SOX1); **F** – staining in almost all granular layer nuclei (anti-ZIC4); **G** – arrows point to the strong positive staining of Purkinje cell cytoplasm (anti-Tr); **H** - pancreatic tissue with the reaction product (anti-GAD65). No staining was observed in sera specimens of cancer-free (healthy) cats on the cerebellum (**I**) and pancreatic (**J**) sections (corresponding to Figure 3K). Serum dilution is 1:100. Scale bars: 10 μm for **A-G**; 50 μm for **H**.

On October 20, 2023, the serum of a 10-year and 4-month-old spayed female Golden Retriever was subjected to an ONHR antibody screening test due to the dog's age and breed predisposition for cancer. No clinical signs of cancer or cancer suspicions were noticed based on physical examination at that time. The anti-GAD65 ONHR antibodies at the level associated with class "Borderline", which was evaluated as increased but negative, have been detected (Figure 9A and B). However, the level of these antibodies was very high for borderline and close to the class "Positive", which can mean a "Weak positive". Even though the relation of anti-GAD65 antibodies to malignancy is still being discussed, they are associated with a broad spectrum of oncological diseases. Animal patients with high anti-GAD65 antibody levels should first be routinely screened for cancer. Therefore, it was recommended that the early cancer diagnostic test be repeated in six months.

Table 9 Calculation of Test Specificity and Sensitivity for Cats

Cats	Cancer Present	Cancer Absent	Total Population: 50
Test Positive: **Test negative:**	True positive tests: 28 False negative tests: 2	False positive tests: 1 True negative tests: 19	Total positive tests: 29 Total negative tests: 21
Sensitivity=93.3% Specificity=95.0% Positive predicted values=96.6% Negative predicted values=90.2% Prevalence=60.0%			

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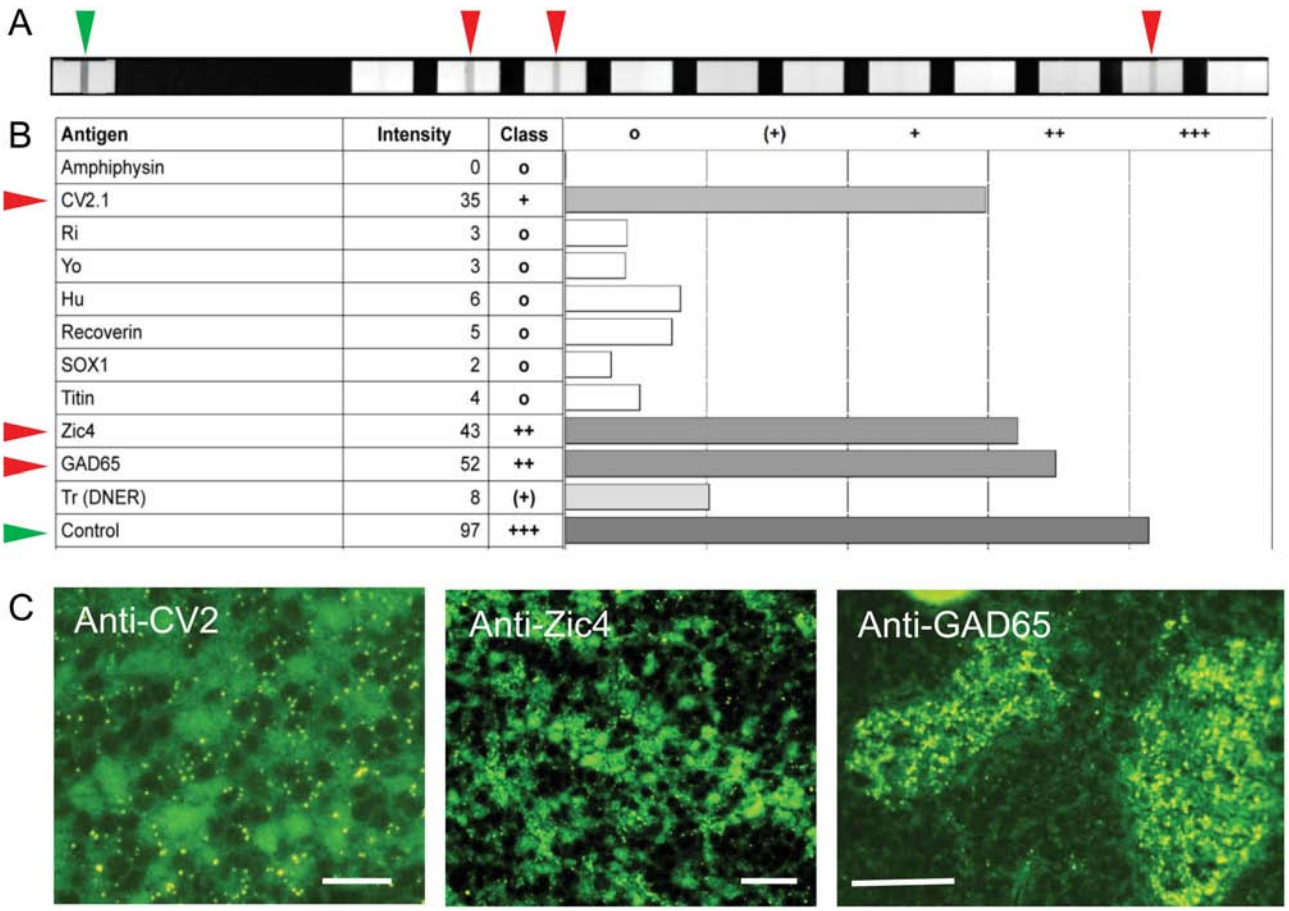

Figure 5 Multiple ONHR antibody detection in a 6-year-old Golden Retriever with lymphoma. **A** and **B** – results of the immunoblot assay. Red arrowheads point to the detected onconeural antibodies (anti-CV2, -ZIC4, - GAD65). The green arrowhead points to the control band. **C** – reaction product of anti-CV2 (sand-like) and anti-ZIC4 (nuclear) staining in substrate cerebellum, and anti-GAD65 in pancreatic tissue, confirming the immunoblot findings. Scale bars: 10 μm for anti-CV2 and anti-ZIC4 images; 50 μm for anti-GAD65.

The next test was performed on April 17, 2024, as recommended. At that time, the dog did not yet exhibit clinical symptoms of the disease except for a slight loss of appetite and slightly reduced activity. The test results revealed a sharply increased level of anti-GAD65 antibodies in the "Strong positive" class (Figure 9C and D). The following chest X-ray demonstrated a mass in the cranial lobe of the left lung, and no distant metastases were found. A fine-needle aspiration from the left lung showed cells immunopositive for cytokeratin, indicating the presence of epithelial cells, which are most likely cancerous, due to cytokeratin, a protein marker commonly used to identify epithelial malignancies such as canine pulmonary adenocarcinoma. There was no positivity to vimentin, which is often associated with invasive behavior in tumors, meaning that cancer cells are not aggressively spreading or metastasizing.

The mass (<3cm) without metastasis was surgically removed in four days. The following veterinary visit registered improved wellness, and a test for onconeural antibody detection was recommended in six months again.

The last test was performed on January 21, 2025. According to the test results, the level of anti-GAD65 antibodies significantly decreased to class "Positive", which demonstrates the success of the treatment and the tumor removal. However, the level of another onconeural antibody, which had not been detected in previous tests, anti-SOX1, started to increase (Figure 9E and F). Following the explanation of the results, the level of anti-SOX1 antibodies belonging to the class "Borderline" is considered as increased but still negative. Considering that the level of these antibodies is relatively high and close to the class "Positive", another screening was suggested to monitor this patient's health.

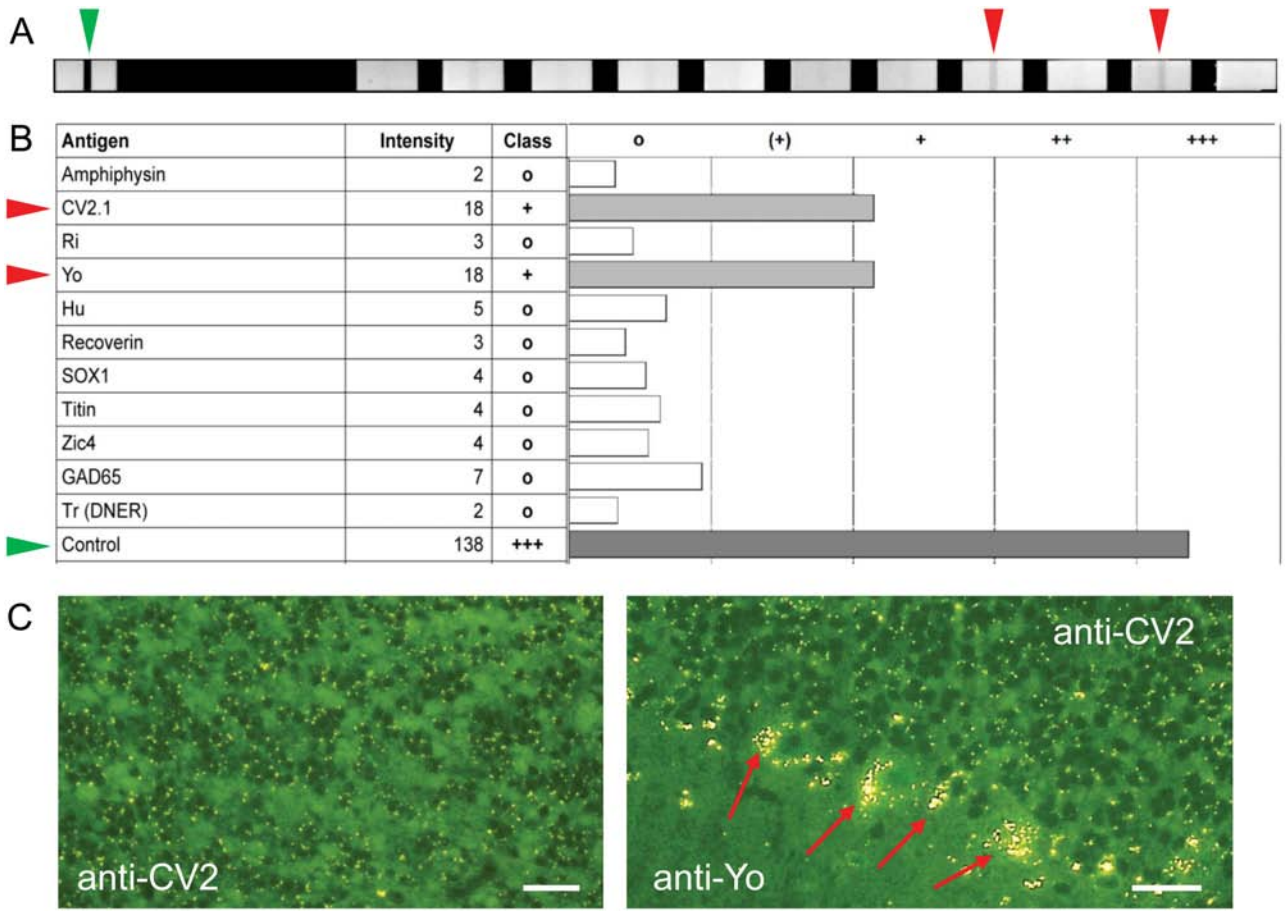

Figure 6 Multiple ONHR antibody detection in an 11-year-old DLH cat with transitional cell carcinoma. **A** and **B** – results of the immunoblot assay. The red arrowheads point to the detected onconeural antibodies (anti-CV2, -Yo). The green arrowhead points to the control band. **C** – reaction product of anti-CV2 (sand-like) and anti-Yo cytoplasmic staining of Purkinje cell cytoplasm (red arrows). Note the sand-like and nuclear staining on the same image, confirming the immunoblot findings. Scale bars: 10 μm for both immunofluorescent images.

Thus, this is the first case report associated with the detection of ONHR antibodies in a dog's serum a few months before the dog developed actual clinical signs. We suggest that the detection of onconeural antibodies can be used to monitor cancer status and treatment in companion animals (Figure 10).

Discussion

Our data provide several essential findings regarding significant sequence similarity between the human, canine, and feline neuronal antigen homologs,[48,49] the discovery of onconeural/high-risk antibodies in dogs and cats blood, their relationship with cancers, and the application of these antibody detections and malignancy differentiation as a test for early cancer diagnosis in veterinary medicine.

In 2021, the International Panel of Neurologists updated and established the new criteria for ranking the onconeural antibodies into a "high-risk" category (>70% associated with cancer, for specific types of cancers it is more than 90%), an "intermediate-risk" category (30–70% associated with cancer), and a "low-risk" category (<30% associated with cancer).[50] According to the new criteria, the diagnosis of cancer requires the presence of high- or intermediate-risk antibodies. All antibodies validated in this study are onconeural antibodies against intracellular neuronal antigens, belong to the "high-risk" category associated with cancers, and can be called onconeural/high-risk antibodies. Anti-GAD65 antibody is the only exclusion that, according to updated criteria, belongs to the low-risk antibody group. Despite this category, the anti-GAD65 alone or with other ONHR antibodies can be widely detected in the sera of patients with various cancers.[51] The significant sequence similarity between the human-canine (Table 1) and human-feline homologs

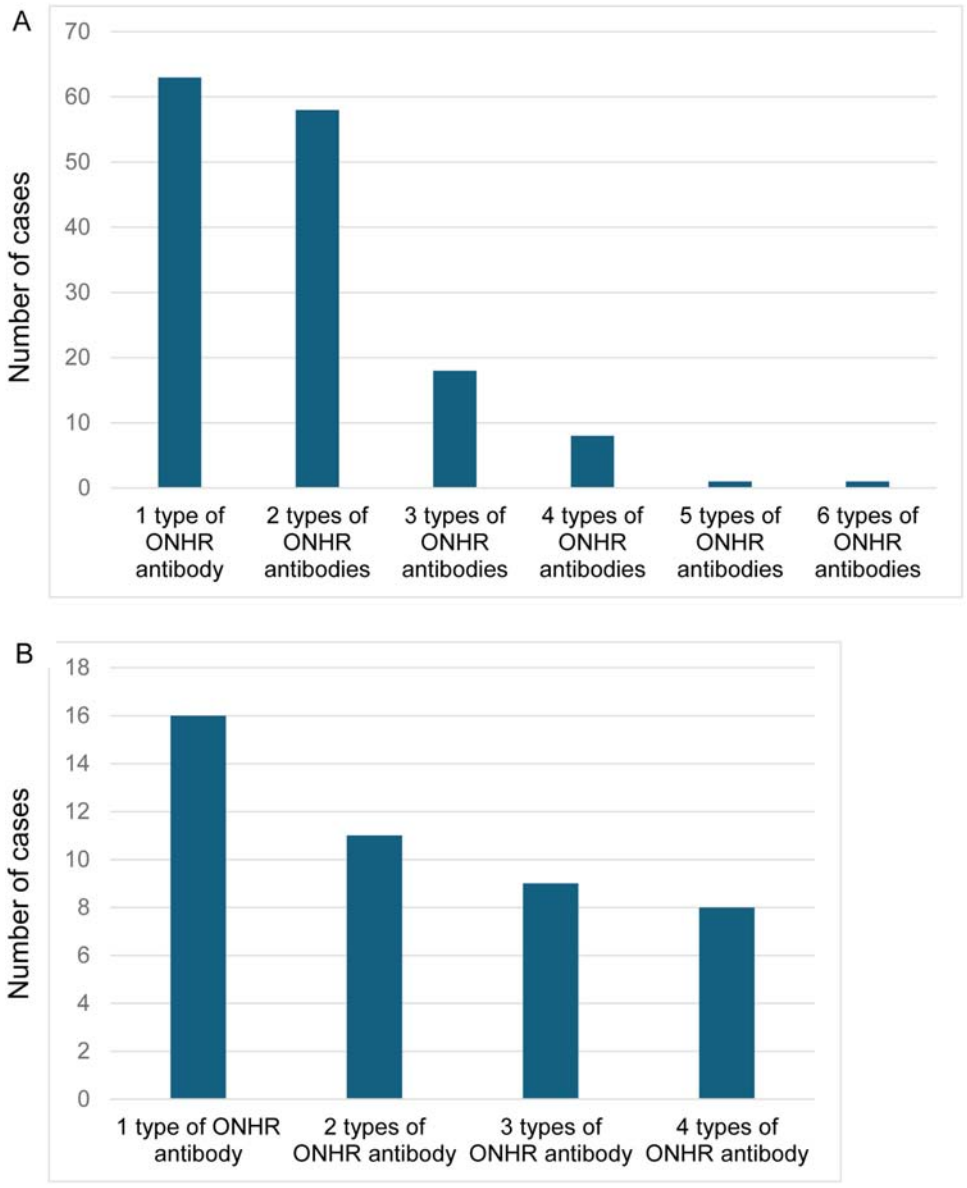

Figure 7 Number of different types of onconeural antibodies detected in the single serum sample from cancer-bearing dogs (**A**) and cats (**B**).

(Table 2) [48,49] and the similarity of the cancer biology between dogs and humans[12] confirmed the importance of the ONHR antibodies as a marker for malignancy and their contribution to PNSs. Before these findings, in veterinary medicine, only a few antibodies against intracellular and extracellular neuronal proteins had been identified.[16,39,52] In this validation study, using immunoblot and IIFT, the presence of 11 ONHR antibodies was identified in serum samples collected from 120 dogs and 28 cats.

Immunoblot is a convenient and rapid method for detecting cancer-related onconeural antibodies, widely used in human diagnostics. Applying this method to veterinary diagnostics can be a game-changer in veterinary medicine because of the lack of non-invasive and precise detection of malignancy. The validation of this convenient method for early cancer diagnostics in dogs and cats has demonstrated a high detection rate of identification of the most common cancers including basal cell carcinoma, bladder tumors, brain tumors, esophageal cancer, gallbladder tumors, intestinal tumors, kidney cancers, lung cancer, lymphoma, mammary tumors, melanoma, multiple myeloma, neuroblastoma, ovarian tumors, pancreatic tumors, prostate tumor, squamous cell carcinoma, testicular tumor, thymoma, thyroid cancer,

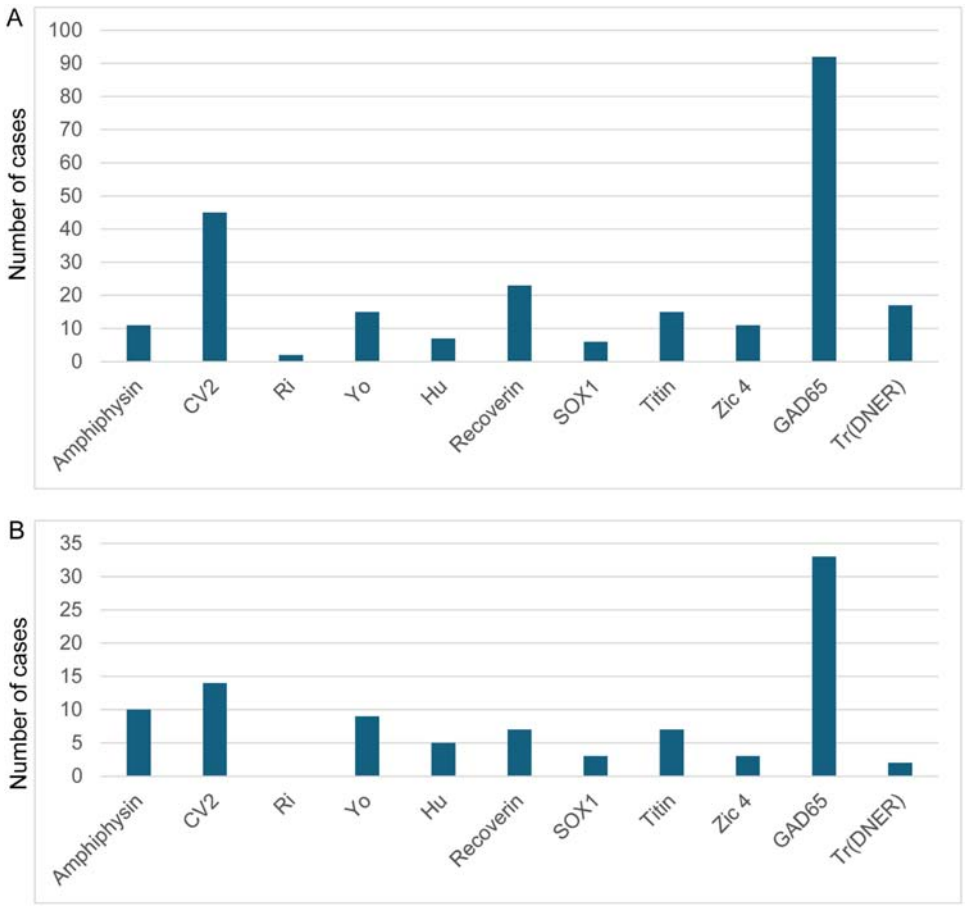

Figure 8 Frequency of different onconeural antibody detection in the serum of cancer-bearing dogs (**A**) and cancer-bearing cats (**B**).

and uterine tumors. Most onconeural antibodies related to specific cancers have been detected by immunoblot and IIFT, with a high correlation between the two assays, and can be used to detect cancers in dogs and cats.

The ONHR antibody test can reveal malignancy months or even years before any signs of the disease show up[53,54] and can also serve as a valuable tool in veterinary diagnostics as a part of an annual wellness exam. Moreover, the test can play a significant role in determining whether dog and cat breeds are predisposed to cancer.[55,56] Asymptomatic healthy control dogs and cats with positive test results were recommended for retesting in 6–24 months to exclude false positivity. During this time of our validation, no cancer signs appeared in these dogs and cats. However, it cannot be excluded that these animals may develop cancer at a later point. All false-negative results in cancer-diagnosed dogs were related to High-Grade Lymphoma in its late stage, which is probably the result of al breached ONHR antibody production.

Some onconeural antibodies have been found to have a higher frequency than others, like, for example, the low-risk anti-GAD65 antibody. However, these antibodies, if found at a high level in animals, may be considered as high-risk. GAD65, the major autoantigen in type 1 diabetes and a variety of neurological disorders, is also highly expressed in different cancers and is involved in cancer cell invasion and migration.[57–60] Although the presence of antibodies against this protein related to malignancy is still discussed, they have been found in association with a broad spectrum of oncological diseases in human patients.[47] Despite anti-GAD65 antibodies being defined as low-risk antibodies, based on our findings, it has been suggested that animal patients with high anti-GAD65 antibody levels should be carefully screened for cancer.[59]

Anti-Ri antibodies target RNA-binding proteins involved in mRNA processing,[61–65] which are often highly over-expressed and associated with the production of onconeural antibodies in many types of human cancers.[66–70]

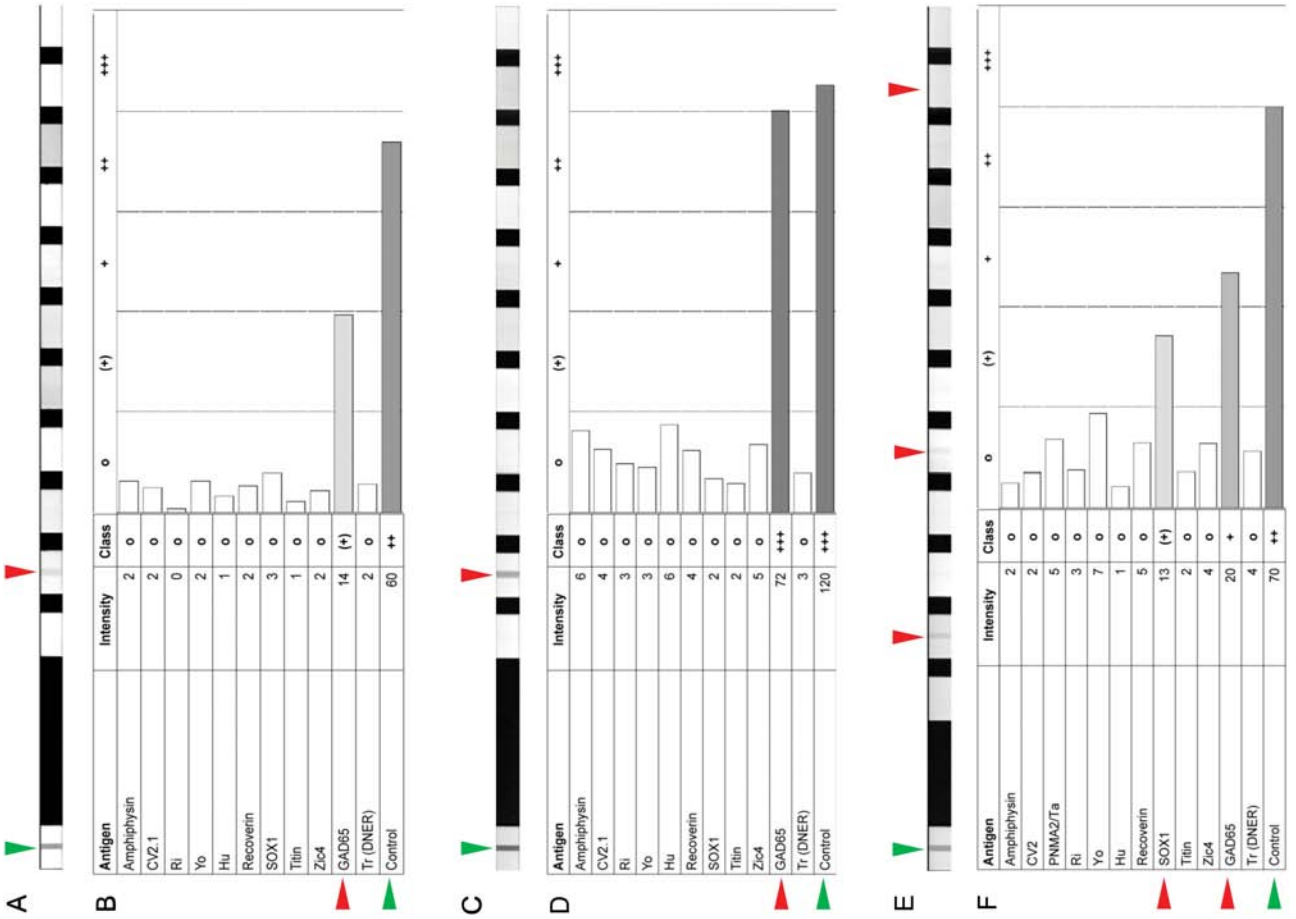

Figure 9 Case report: immunoblot-based assay of serum collected from a 10–year–old Golden Retriever with no cancer suspicions at the time of the first preventative wellness exam. The reason for the exam - the age and breed's predisposition to cancer. **A** and **B** – Results of the immunoblot assay indicate an increased borderline level of anti-GAD65 antibodies (red arrowhead) in the serum of this presumably healthy dog tested on October 20, 2023. **C** and **D** – Results of the immunoblot assay indicate a very high level of anti-GAD65 antibodies in the serum of the same dog during the second screening in 6 months on April 17, 2024. **E** and **F** – results of immunoblot assay indicate a lower anti-GAD65 antibody level, and a slightly increased anti-SOX1 antibody level detected in the serum six months post-surgery (January 21, 2025). Red arrowheads point to the detected ONHR antibodies, the green arrowheads - to the control bands.

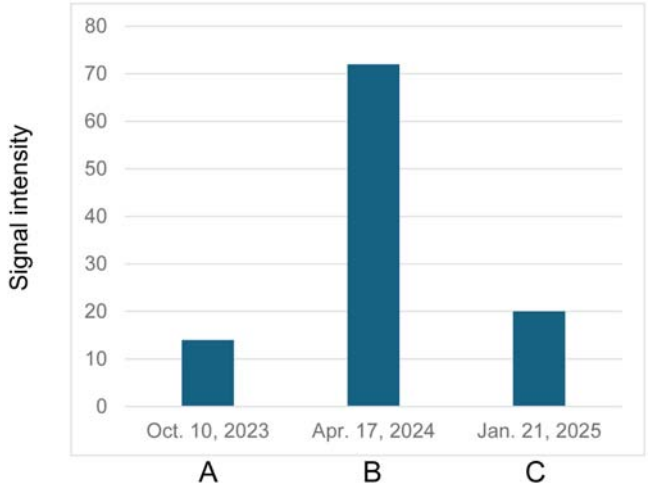

Figure 10 Monitoring of the cancer status and its treatment via anti-GAD65 antibody detection. Level of anti-GAD65 antibodies in the serum of a dog without any cancer symptoms, presumably healthy, that was used as a negative healthy control in this study (**A**). Level of anti-GAD65 antibodies in the dog's serum after six months, when some signs of the disease showed up (**B**). Level of anti-GAD65 antibodies in the dog's serum six months after surgical removal of pulmonary adenocarcinoma (**C**).

Interestingly, in this study, only low levels of anti-Ri antibodies were found in dogs with lung adenocarcinoma, and none were detected in cats. This suggests that these types of cancer may be less common in animals.

The onconeural protein SOX1 is known as a tumor suppressor.[71] However, due to its abnormal hypermethylation of the promoter region in its gene, it causes the inactivation of tumor suppression and induces the development of many tumors.[72,73] The antibodies against SOX1 have also been found in dogs and cats with pulmonary carcinoma, bladder tumors, and thyroid cancer in this validation study.

The detection of antibodies against CV2 in our validation has been chiefly associated with lymphoma, thymoma, and lung cancer in dogs and cats (Table 6 and Table 7). High expression of this onconeural antigen was also revealed in human patients diagnosed with several types of cancer, that presumes the formation of antibodies against this protein in specific conditions,[31,74] and using more cohorts of companion animals may enable the detection of these ONHR antibodies in broader types of dog and cat cancers.

Anti-Yo antibodies have been identified in several types of cancer, such as mammary gland carcinoma, prostatic carcinoma, and urothelial carcinoma in dogs and a few types of lymphoma in cats (Table 6 and Table 7). The CDR2 onconeural protein associated with anti-Yo antibody response is involved in signal transduction and gene transcription. Recent studies have demonstrated that CDR2 is widely expressed in normal and significantly overexpressed in malignant tissues,[75–78] which can trigger an immune response and production of anti-Yo antibodies in pets as well.

The other ONHR antibody we found in dogs and cats is anti-amphiphysin, most often linked to lymphoma and lung cancer in both species, and in two cases to osteosarcoma in dogs (Table 6 and Table 7). Amphiphysin is known to be overexpressed in different cancers,[79–84] and frequently, antibodies against this protein coexist with other onconeural antibodies.[85] According to the International Cancer Genome Consortium, amphiphysin has 26 high-impact mutations in different types of cancer. Thus, overexpression of this protein and its high mutation rate can stimulate ONHR antibody formation and tumor development in dogs and cats. Recent evidence demonstrated high amphiphysin overexpression in osteosarcoma tissues that plays a significant role in the tumor's progression.[86] Since anti-amphiphysin antibodies have been detected in the serum of dogs diagnosed with osteosarcoma, it can be suggested that these antibodies are a potential serological marker of this type of cancer; however, more studies are needed to confirm this assumption.

The onconeural antigen, recoverin, which is expressed in the retina, pineal glands, and neuroectodermal melanocytes, has also been detected in various types of malignant tumors.[87–91] Anti-recoverin antibodies play a notable role in the pathogenesis of immune-mediated retinal degeneration and cancer-associated retinopathy, mainly in human patients with small-cell lung cancer.[90] Onconeural antibodies to recoverin have been detected in the sera of dogs and cats with T-cell lymphoma, pulmonary carcinoma, and others (Table 6 and Table 7). However, additional studies may be needed to detect more cancer types associated with these ONHR antibodies.

Anti-Hu antibodies in this validation were detected in sera of dogs and cats with different types of lymphoma, pulmonary carcinoma, and neuroblastoma (Table 6 and Table 7). It was published that Hu antigen has been expressed in various kinds of cancer, and a few mutations and single nucleotide polymorphisms in the Hu sequence were identified in several tumors in humans,[92–95] which can trigger a process such as immune response and anti-Hu antibody formation in dogs and cats as well.[96–98]

Tr/DNER, a neuron-specific transmembrane protein highly expressed in the Purkinje cells of the cerebellum,[99,100] is a member of the atypical Notch ligand family and binds to the Notch1 receptor.[101,102] DNER is also expressed at abnormally high levels in various cancer tissues and promotes the proliferation, migration, and invasion of cancer cells.[103–105] High levels of DNER expression may cause an anti-Tr antibody production in humans. In this study, anti-Tr antibodies have been detected in uterine leiomyosarcoma, mammary gland carcinoma, lymphoma, mast cell tumors, and other cancers in dog and cat sera.

The antigenic protein, titin, abundant in striated muscle tissues, is a novel oncogenic protein that plays a crucial role in carcinogenesis, being significantly upregulated in 76% of patients with thymoma-associated myasthenia gravis.[106] Although this protein is usually described in association with thymoma, in human patients it has also been related to prostate cancer,[107] breast carcinoma, and small-cell lung neoplasia.[108] A specific somatic mutation in titin detected in lung cancer can trigger onconeural antibody production.[109–111] In our validation study, the anti-titin antibodies were detected in the sera of dogs and cats with thymoma and lymphoma and have also been associated with neurological

diseases such as myasthenia gravis in dogs and seizures other than those caused by myasthenia gravis in dogs and cats (Table 6 and Table 7).

The last antibodies detected in this study are anti-ZIC4 antibodies. The ZIC4 (zinc-finger protein) neuronal antigen is expressed in the central nervous system and plays a critical role in developing the cerebellum. However, this neuronal protein has also been detected in a variety of human cancers.[112,113] It was also shown that ZIC4 is hypermethylated in hepatocellular carcinoma, which can induce an immune response.[114] This study revealed that the highest level of anti-ZIC4 antibodies has been associated with dog thyroid carcinoma, transitional cell carcinoma, and lymphoma. Just four dogs and two cats included in the validation had neurological signs and symptoms (Table 6 and Table 7).

Thus, our findings and previous studies demonstrate that antibodies against intracellular neuronal antigens (onco-neural autoantibodies) have a stronger association with cancers compared with neurological diseases in pets.[114] This validation has revealed that ONHR antibody detection is more cancer-relevant for companion animals than humans. The study confirmed that the ONHR antibodies can be detected many months and possibly years ahead of the related malignancy and that a durable formation of these IgG antibodies to particular onconeural antigens occurs in most cancers.[115] The case reported in this study confirmed the ONHR antibody detection in an early stage of cancer without clinical signs or tumor mass development. As a new observation, the validation has shown that the coexistence of antibodies specific to multiple onconeural antigens in dogs and cats is more frequent than previously assumed.[17,116] Antigen distribution can be triggered by a switch of the antigen expression pattern of the underlying tumor tissue due to a mutagenic process caused by cancer itself, as we discussed above.[117]

In summary, it is strongly recommended that any positive test result be followed up with targeted screening for malignancies due to the detection of high-risk onconeural antibodies that are almost invariably associated with cancers. However, the validation has limitations, including the lack of longitudinal data on autoantibody seroprevalence and dogs' and cats' clinical outcomes and survival. Thus, follow-up examinations for ONHR-antibody-positive patients are needed to provide a more detailed insight into long-term clinical effects.[80,118]

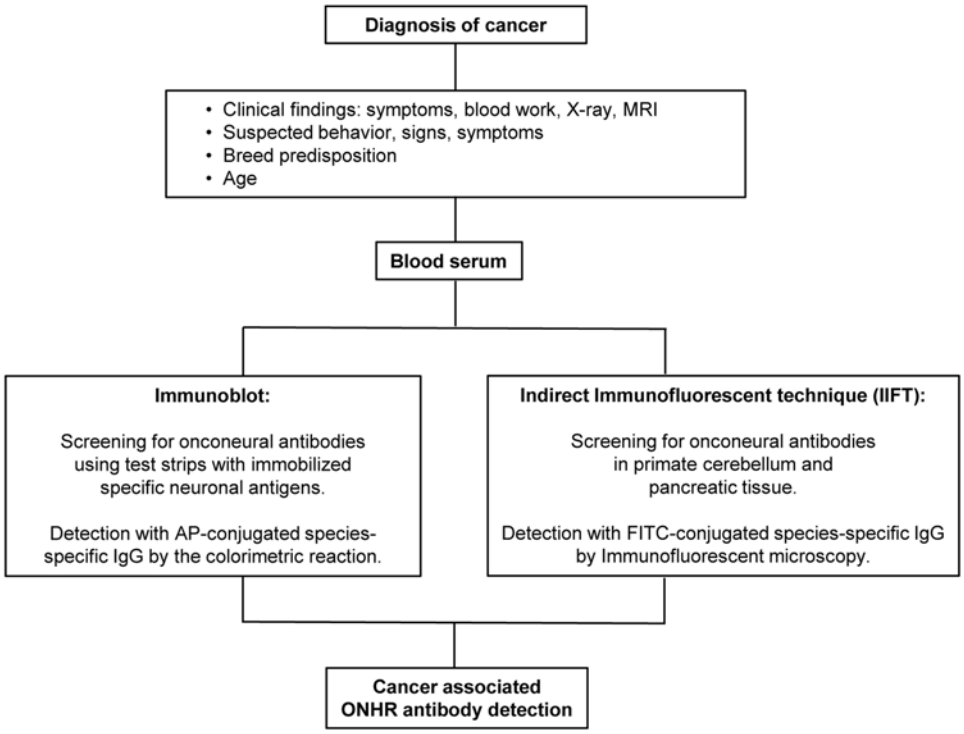

Figure 11 The ONHR antibody detection procedure by immunoblot and IIFT methods leads to a suggestion of cancer.

Conclusion

This study introduces a groundbreaking approach for early cancer detection in dogs and cats using ONHR antibody testing. This ONHR antibody test represents a significant advancement in veterinary oncology, offering veterinarians and pet owners a powerful tool for early cancer detection and differentiation of 21 cancers.

The onconeural antibody profiles observed in canines and felines can predict malignancy and be used as a first-of-its-kind test in veterinary medicine for early cancer diagnostics. This test is the first in the industry for companion animals - dogs and cats. The test can be offered to any dog or cat three years and older as a preventative early screening test during an animal's check-up. The test is highly recommended for all canines and felines two years and older for breeds genetically predisposed to cancer. The amount of serum required for testing, only 0.1 mL, makes this assay suitable for all small dog breeds and cats. A highly specific and sensitive ONHR antibody detection test can serve as an excellent diagnostic procedure (Figure 11) to confirm clinical suspicion, initiate a timely treatment, and monitor post-treatment. The test can also give a good understanding of the etiology of a PNS if it is triggered by a tumor or if it is observed regardless of cancer.

Abbreviations

DLH, domestic long hair; DSH, domestic short hair; FITC, fluorescein isothiocyanate; IgE, immunoglobulin-E; IgG, immunoglobulin-G; IIFT, indirect immunofluorescent test; NPV, negative predicted values; ONHR, onconeural/high-risk antibody; PBST, phosphate-buffered saline/Tween; PNS, paraneoplastic neurological syndrome; SNPs, single nucleotide polymorphisms; PPV, positive predicted values.

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Disclosure

Dr. Marianna Agassandian and Dr. Khristofor Agassandian report the patent 19/197,987 (US application number) and PCT/US2025/29430 (International patent application number) to Agent's registration No. with the Office 41404. The authors report no other conflicts of interest in this work.

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