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Expression of claudin-1 in canine peripheral nerve sheath tumours and perivascular wall tumours. Immunohistochemical study

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Summary. Aims: A peripheral nerve sheath tumour consists of neoplastic Schwann cells or perineurial cells, or a mixture of Schwann cells, perineurial cells and fibroblasts. The first aim of the present study was to characterise the expression of the claudin-1 tight junction protein in canine intact peripheral nerves, canine benign peripheral nerve sheath tumours (cBPNSTs), such as schwannomas, neurofibromas, perineuriomas and canine malignant peripheral nerve sheath tumours (cMPNSTs), and in different other benign and malignant canine spindle cell tumours. The second aim of the present study was to examine whether claudin-1 can help to distinguish the subgroups of canine perivascular wall tumours. Methods and results: The biopsy and necropsy samples (n=203) included 10 intact peripheral nerves, 20 cBPNSTs (4 schwannomas, 8 neurofibromas, 8 perineuriomas), 16 cMPNSTs, 6 psammomatous meningiomas, 6 dermatofibromas, 6 leiomyomas, 6 myxomas, 4 spindle cell hemangiomas, 2 spindle cell lipomas, 6 fibrohistiocytic nodules, 8 fibrosarcomas, 8 leiomyosarcomas, 6 myxosarcomas, 8 hemangiosarcomas, 8 anaplastic sarcomas, 8 amelanotic spindle cell melanomas, 8 histiocytic sarcomas, 8 spindle cell carcinomas, 8 myoepitheliomas, 8 complex carcinomas, 5 cardiac rhabdomyosarcomas, 4 synovial sarcomas, 5 osteosarcomas, 4 chondrosarcomas and 4 liposarcomas; 31 canine perivascular wall tumours: 10 hemangiopericytomas, 8 myopericytomas, 6 angioleiomyomas, 4 angioleiomyosarcomas, 3 angiofibromas. The immunohistochemical panel consisted of humanized antibodies: anti-claudin-1, anti-neuron specific enolase, anti-S-100 protein, anti-α-smooth muscle actin, anti-vimentin, anti-cytokeratin AE1-AE3, anti-claudin-5, anti-Melan-A and anti-heavy caldesmon, anti-calponin and anti-desmin. The intact perineurial cells, all perineuriomas, neurofibromas, cMPNSTs, spindle cell carcinomas and epithelial components of the complex carcinomas, all hemangiopericytomas and myopericytomas showed claudin-1 positivity. The schwannomas and other spindle shape cell tumours were negative for claudin-1.

Conclusion: Our findings suggest that an antibody against claudin-1, in combination with other antibodies, can be used as a novel diagnostic tool to differentiate canine peripheral nerve sheath tumours from other fusocellular tumours, and anti-claudin-1, together with other antibodies, can also be used to subclassify cBPNSTs. Furthermore, analysis of claudin-1 expression can help to differentiate between subgroups of canine perivascular wall tumours.

Key words: Canine peripheral nerve sheath tumour, Schwannoma, Neurofibroma, Perineurioma, Canine spindle cell tumours, Canine perivascular wall tumours, Hemangiopericytoma, Differential diagnosis, Immunohistochemistry, Claudin-1, Immunohistochemical panel

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Introduction

Canine peripheral nerve sheath tumours (cPNSTs) can often create diagnostic problems (particulary in small, core- and incisional biopsy specimens) because of their cellular origin and histopathological similarities with other canine spindle cell tumours, such as hemangiopericytoma, fibroma, leiomyoma, leiomyofibroma, myxoma, spindle cell hemangioma, spindle cell lipoma, reactive fibrohistiocytic nodule, fibrosarcoma, leiomyosarcoma, myxosarcoma, solid hemangiosarcoma, anaplastic sarcoma, amelanotic spindle cell melanoma, histiocytic sarcoma, spindle cell squamous cell carcinoma, myoepithelioma, complex carcinoma of the mammary gland and meningioma. For definitive diagnosis of cPNSTs, immunohistochemistry can be performed. Previous veterinary studies have described the immunohistochemial phenotype of cPNSTs (Table 1).

Tight junctions are the most apical cell-cell contacts and are important for barrier function in epithelial, mesothelial and endothelial cells (Tsukita et al., 2001). Claudins are integral membrane proteins of tight junction structures. The claudin family consists of at least 24 members (Morita et al., 1999). The differential expression pattern of various members of the claudin family in different human and animal tumours can be used in confirming the histologic identity of certain neoplasms (Jakab et al., 2009a, 2010a,b; Ouban and Ahmed, 2010; Szabó et al., 2009), and not only of epithelial, mesothelial or endothelial tumours. Human

Table 1. Summary of the veterinary immunohistochemical studies of cPNSTs.

Antibody	Expression in cBPNSTs	Expression in cMPNSTs	Authors of veterinary literature
Vimentin	positive (100%)	positive (100%)	Kuwamura et al., 1998; Sawamoto et al., 1999; Chijiwa et al., 2004; Sato et al., 2005; Higgins et al., 2006; Gaitero et al., 2008; Sugiyama et al., 2008; Bergmann et al., 2009; Volmer et al., 2010
NGRF (nerve growth factor receptor)	positive (66%)	positive (64%)	Chijiwa et al., 2004
NSE (neuron specific enolase)	positive (50%)	positive (36%)	Sawamoto et al., 1999; Chijiwa et al., 2004; Gaitero et al., 2008; Sugiyama et al., 2008
α-SMA (alpha smooth muscle actin)	negative	negative	Sawamoto et al., 1999; Chijiwa et al., 2004; Sato et al., 2005; Sugiyama et al., 2008; Bergmann et al., 2009; Volmer et al., 2010
Cytokeratin AE1/AE3	positive (16%)	positive (9%)	Kuwamura et al., 1998; Sawamoto et al., 1999; Chijiwa et al., 2004; Sugiyama et al., 2008; Volmer et al., 2010
MBP (myelin basic protein)	positive (16%)	negative	Kuwamura et al., 1998; Sawamoto et al., 1999; Chijiwa et al., 2004; Sugiyama et al., 2008
PCNA (proliferating cell nuclear antigen)	positive (44,4%)	positive (62,9%)	Chijiwa et al., 2004
S-100 protein	positive (83%)	positive (73%)	Kuwamura et al., 1998; Sawamoto et al., 1999; Chijiwa et al., 2004; Sato et al., 2005; Higgins et al., 2006; Gaitero et al., 2008; Sugiyama et al., 2008; Bergmann et al., 2009; Volmer et al., 2010
GFAP (glial fibrillary acidic protein)	positive (66%)	positive (9%)	Kuwamura et al., 1998; Sawamoto et al., 1999; Chijiwa et al., 2004; Gaitero et al., 2008; Sugiyama et al., 2008; Bergmann et al., 2009; Volmer et al., 2010
Myoglobin	positive (16%)	positive (64%)	Kuwamura et al., 1998; Chijiwa et al., 2004; Sugiyama et al., 2008;
Chromogranin-A	negative	negative	Chijiwa et al., 2004
Factor VIII-related antigen	negative	positive (9%)	Sawamoto et al., 1999; Chijiwa et al., 2004; Sato et al., 2005; Sugiyama et al., 2008; Bergmann et al., 2009
NF (neurofilament protein)	positive	not examined	Sawamoto et al., 1999; Higgins et al., 2006
α-1-chymotrypsin	negative	not examined	Kuwamura et al., 1998
Desmin	negative	negative	Kuwamura et al., 1998; Sato et al., 2005; Bergmann et al., 2009; Volmer et al., 2010
Lysozyme	not examined	negative	Sugiyama et al., 2008
Myeloid/histiocyte antigen	not examined	negative	Sugiyama et al., 2008
Ki-67	positive (3 %)	positive (78%)	Higgins et al., 2006; Sugiyama et al., 2008
Laminin	positive	positive	Higgins et al., 2006; Gaitero et al., 2008
Collagen IV	positive	positive	Higgins et al., 2006; Gaitero et al., 2008
PGP 9.5 (protein gene product 9.5)	positive	not examined	Gaitero et al., 2008
*Claudin-1	positive	not examined	Cornelis et al., 2009

*A single case (perineurioma) was described in a 4 -year-old male Leonberger.

oncopathology studies described claudin-1 positivity in mesenchymal tumours, including epitheloid sarcomas (Smith et al., 2005), Ewing-sarcomas (Schuetz et al., 2005), perineurioma-like low-grade fibromyxoid sarcomas (Thway et al., 2009), dedifferentiated liposarcomas with meningothelial-like whorls which comprised concentric distributions of spindle or epitheloid neoplastic cells (Thway et al., 2011). GyŒrffy (2009) analysed different benign and malignant human spindle cell mesenchymal tumours. Gastrointestinal stromal tumour (GIST), hemangiosarcoma, hemangioma, leiomyosarcoma and leiomyoma showed differential expression of claudins. Leiomyoma was claudin-1 negative, and expressed only claudin-2. Claudin-1 was found positive in leiomyosarcoma only. Leiomyosarcoma and GIST were claudin-2, -3, -4, -5 and -7 positive. Endothelial tumours were claudin-1 negative, and claudin-2, -5 positive (Györffy, 2009). Increased claudin-1 expression, with claudin-1 localized to the nucleus and cytoplasm, but not to the cell membrane, was detected in airway smooth muscle of asthmatic patients (Fujita et al., 2011).

Canine spindle cell tumours are histopathological and differential diagnostic challenges for veterinary pathologists, mainly in the case of small biopsy samples (Bergmann et al., 2009; Gaitero et al., 2008; Ramos-Vara et al., 2010; Regan et al., 2010). In small animal practice a variety of biopsy techniques can be applied, such as incisional-, core needle-, image-guided-, endoscopic biopsy (Carter and Valli, 1990; Fossum et al., 2007). In the last years, with the introduction of several novel markers in veterinary pathology, the role of immunostaining has increased. There are several immunhistochemical markers which enable the correct differential and definitive diagnosis of the biopsy and necropsy samples of canine fusocellulare tumours (Bergmann et al., 2009; Carter and Valli, 1990; Gaitero et al., 2008; Ramos-Vara et al., 2010; Regan et al., 2010). Anti-claudin-1 antibody is one of the most important novel markers which expressed is in human intact perineurial cells and in peripheral nerve sheath tumours (Folpe et al., 2002).

The first aim of the present study was to characterise the expression pattern of the new immunohistochemical marker claudin-1 in canine intact peripheral nerves, BPNSTs and MPNSTs, and different benign and malignant spindle cell tumours. The second aim of the present study was to analyse whether claudin-1 (with other markers) can help to distinguish the subgroups of the cPWTs.

Materials and methods

Samples of canine tumours

Canine tissue samples (total number = 213) were collected between 2004 and 2011 at Szent István University, Faculty of Veterinary Medicine, Department of Pathology and Forensic Veterinary Medicine (Budapest, Hungary). The examined samples comprised 10 intact peripheral nerve samples, 20 cBPNSTs including 4 schwannomas, 8 neurofibromas, 8 perineuriomas (8 samples were primary skin tumours, 10 samples were peripheral nerve neoplasms, and 2 samples were cranial nerve lesions), 16 cMPNSTs (8 samples were from nerves of the brachial plexus, 5 samples were lumbosacral plexus lesions, and 3 samples were cranial nerve tumours), 6 psammomatous meningiomas (all samples were leptomeningeal tumours), 6 dermatofibromas, 6 leiomyomas (all samples were taken from the vagina), 6 myxomas (all samples were taken from the skin), 4 spindle cell hemangiomas (cSCHs) (all samples were dermal tumours), 2 spindle cells lipomas (all samples were subcutaneous tumours), 6 fibrohistiocytic nodules (cFHNs) (all samples were taken from the skin), 8 fibrosarcomas (5 samples were primary skin tumours, 3 were primary oral tumours), 8 leiomyosarcomas (6 samples were vaginal tumours, and 6 were uterine tumours), 6 myxosarcomas (all samples were skin lesions), 8 hemangiosarcomas (sHSAs) (6 samples were spleen tumours, 2 samples were skin tumours), 4 liposarcomas (skin samples), 8 anaplastic sarcomas (all samples were skin lesions), 8 amelanotic spindle cell melanomas (cASCMs) (all samples were oral lesions), 8 histiocytic sarcomas (6 samples were spleen tumours, 2 samples were skin tumours), 8 spindle cell carcinomas (cSCCs) (all samples were skin tumours), 8 myoepitheliomas (all samples were taken from the mammary gland), 8 complex carcinomas (all samples were taken from the mammary gland), 5 cardiac rhabdomyosarcomas, 4 monophasic synovial sarcomas, 5 osteosarcomas (non-productive type from bone), 4 chondrosarcomas, 31 cPWTs (all cPWT samples were skin tumours) were examined. The histopathological diagnosis of the cPWTs was based on an earlier oncopathological study (Avallone et al., 2007). One hundred and fifty-five samples were removed surgically, and 58 samples (10 peripheral nerves, 8 cBPNSTs, 16 cMPNSTs, 6 meningiomas, 5 cardiac rhabdomyosarcomas, 4 synovial sarcomas, 5 osteosarcomas, 4 chondrosarcomas) were collected during necropsy. The samples were fixed in 8% neutral buffered formalin for 24 hours at room temperature, dehydrated in a series of ethanol and xylene, and embedded in paraffin. The 3-4 μm thick sections were routinely stained with hematoxylin and eosin (H&E).

Immunohistochemical analysis

The humanized antibodies used for immunohistochemical analysis *claudin-1* (diluted 1:100, rabbit anti-human polyclonal, Zymed Inc.), *S-100 protein* (diluted 1:50, rabbit anti-ovine polyclonal, DAKO), *neuron specific enolase* (NSE) (diluted 1:100, mouse anti-human monoclonal, DAKO), *α-smooth muscle actin* (α-SMA) (diluted 1:8000, mouse anti-human monoclonal, Sigma), *vimentin* (diluted 1:200, mouse anti-bovine monoclonal, DAKO), *cytokeratin AE1-AE3*

or pancytokeratin (diluted 1:100, mouse anti-human monoclonal, DAKO), *claudin-5* (diluted 1:100, mouse anti-human monoclonal, Zymed Inc.), *Melan-A* (diluted 1:50, mouse anti-human monoclonal, DAKO), *heavy caldesmon* (diluted 1:50, mouse anti-human monoclonal, DAKO), *calponin* (diluted 1:30,000 mouse anti-human monoclonal, Sigma) and *desmin* (diluted 1:400, mouse anti-human monoclonal, Novocastra). For immunohistochemistry 3-4 μm thick sections were cut. The slides for the immunohistochemical reactions were deparaffinized in xylene and graded ethanol. The deparaffinized sections were treated with primary antibody for 60 minutes at room temperature after treatment with appropriate antigen retrieval (Target Retrieval Solution, DAKO, Glostrup, Denmark; pH 6; microwave - 800W - oven for 30 minutes). Immunohistochemical staining was performed using the streptavidin-peroxidase technique. Antigen-bound primary antibody was detected using standard avidin-biotin immunoperoxidase complex (DAKO, LSAB2 Kit). The chromogen substrate was 3,3 - diaminobenzidine tetrahydrochloride (DAB substrate-chromogen, DAKO, Denmark). Mayer's hemalaun was used for counter-staining. The reactions were carried out in a Ventana ES automatic immunostainer (Ventana Medical System Inc., Tucson, AZ, USA) using the reagents provided by the manufacturer. The external positive controls were canine low-grade colorectal adenocarcinoma for claudin-1 (Jakab et al., 2010a), canine chondroma for S-100 (Jakab et al., 2008b), equine peripheral nerve for NSE (Jakab et al., 2008a), canine pericytes for α-SMA and calponin (Jakab et al., 2009b), canine mast cell tumour for vimentin (Jakab et al., 2009c), canine mammary gland cancer for pancytokeratin (Jakab et al., 2010b), canine hemangiosarcoma for claudin-5 (Jakab et al., 2009a), canine melanoma for Melan-A, canine m. arrector pili for heavy caldesmon and desmin (Jakab et al., 2009b).

Immunohistochemical assessment of claudin-1 and S-100 protein

A semiquantitative evaluation of the percentage of positive tumour cells was performed. Ten randomly selected areas of each slide were analysed at high magnification (x400) with one hundred cells counted per field. Reactions were scored claudin-1 positive where linear membrane staining was seen. Reactions were scored S-100 protein positive where cytoplasmic staining was seen. Immunoreactivity was assessed as follows: negative (-) for no immunostaining present; (+) if <25% of tumour cells were positive; (++) if 25-50% of tumour cells were positive; (+++) if 50-75% of tumour cells were positive; and (++++) if 75-100% of tumour cells were positive.

Immunohistochemical assessment of other antibodies

A semiquantitative evaluation of the percentage of positive tumour cells was used. Ten randomly selected areas of each slide were analysed at high magnification (x400) with one hundred cells counted per field. Immunoreactivity was assessed as follows: negative (-) for no immunostaining present; (+) if <25% of tumour cells were positive; (++) if 25-50% of tumour cells were positive; (+++) if 50-75% of tumour cells were positive; and (++++) if 75-100% of tumour cells were positive.

Results

In all canine non-neoplastic peripheral nerve tissue samples the perineurial cells showed intense (++++) *claudin-1* reactivity, while the Schwann cells were negative (Fig. 1A). In regard to the examined cBPNSTs, *schwannomas* showed negativity for claudin-1 (4/4; 100%) (Fig. 1B); *neurofibromas* showed non-diffuse intense (+ to +++) membrane claudin-1 positivity (8/8; 100%) (Fig. 1C); *perinueriomas* showed diffuse intense (++++) membrane claudin-1 positivity (8/8; 100%) (Fig. 1D).

All cMPNST samples (16/16; 100%) were claudin-1 positive: seven cMPNSTs samples (7/16; 43,75%) showed diffuse, intense (++++) membrane claudin-1 positivity (Fig. 2A,B); six samples (6/16; 37,5%) showed diffuse, intense (+++) positivity for claudin-1 (Fig. 2C), and three tumours showed diffuse, intense (++) claudin-1 positivity (3/16; 18,75%) (Fig. 2D). All canine psammomatous meningiomas and also the non-neoplastic peritumoural meningothelial cells were claudin-1 immunonegative. The cSCC samples showed an intense, (+++ to ++++) claudin-1 reactivity in all samples (8/8; 100%) (Fig. 3A). The canine complex mammary gland carcinomas consisted of neoplastic proliferation of the epithelial and myoepithelial cells of the lobules of the mammary gland tissues. In the complex mammary gland carcinoma samples, the neoplastic epithelial cells showed intense claudin-1 positivity (Fig. 3B). The fibromas, leiomyomas, myxomas, spindle cell hemangiomas, spindle cell lipomas, cFHNs, fibrosarcomas, leiomyosarcomas, myxosarcomas, solid hemangiosarcomas, liposarcomas, cASCMs, histiocytic sarcomas, myoepitheliomas and the neoplastic myoepithelial component of the complex carcinomas of the mammary gland, cardiac rhabdomyosarcomas, monophasic synovial sarcomas, osteosarcomas and chondrosarcomas were negative for claudin-1.

The cBPNSTs samples, including *schwannomas*, showed diffuse, intense (+++ to ++++) positivity for *S-100 protein, neurofibromas* showed non-diffuse, intense (++ to +++) S-100 protein positivity, and all *perineuriomas* were negative for this protein.

The claudin-1 (++++) positive seven cMPNST samples showed (+) S-100 positivity. In claudin-1 (+++) six cMPNST samples: four tumours showed (++) S-100 positivity, and two samples showed (+) S-100 positivity. In claudin-1 (++) three cMPNST tumours: two samples showed (+++) S-100 positivity, and one sample showed (+) S-100 positivity.

Complex carcinomas of the mammary gland showed non-diffuse, intense positivtiy for S-100 protein. Psammomatous meningioma, cASCM, myoepithelioma and chondrosarcoma samples showed intense, diffuse (+++ to ++++) positivtiy for S-100 protein. The others samples were negative for this marker. Three (3/4) *schwannomas* were negative for *NSE*, and one (1/4) showed scattered (+) NSE positivity. Six (6/8) *neurofibromas* showed negativity for NSE, and two (2/8) neurofibromas showed scattered (+) NSE positivity. Eleven (11/16) cMPNSTs were negative for NSE, and five (5/16) showed non-diffuse, scattered (+) reactivity for NSE. The histomorphological phenotype of the NSE-positive cells mimicked ganglion cells. These cells were non-neoplastic ganglion cells entrapped in cMPNSTs. The claudin-1 positive perineuriomas, cSCCs, and the other claudin-1 negative samples did not show reaction for this neural marker. Leiomyomas, leiomyosarcomas, myoepitheliomas and cardiac rhabdomyosarcomas showed intense, diffuse (+++ to ++++) *α-SMA* positivity, while complex carcinomas of the mammary gland were intense, non-diffuse (++ to +++) positive for α-SMA. However sPNSTs, meningiomas, fibromas, myxomas, spindle cell hemangiomas, spindle cell lipomas, cFHNs, fibrosarcomas, myxosarcomas, sHSA, cASCM, histiocytic sarcomas, cSCCs, synovial sarcomas, osteosarcomas and chondrosarcomas were α-SMA negative. All biopsy and necropsy samples showed diffuse, intense (++ to ++++) *vimentin* positivity, except the cSCCs, which were negative for this panmesenchymal marker. Non-diffuse weak (+) pancytokeratin positivity was observed in anaplastic

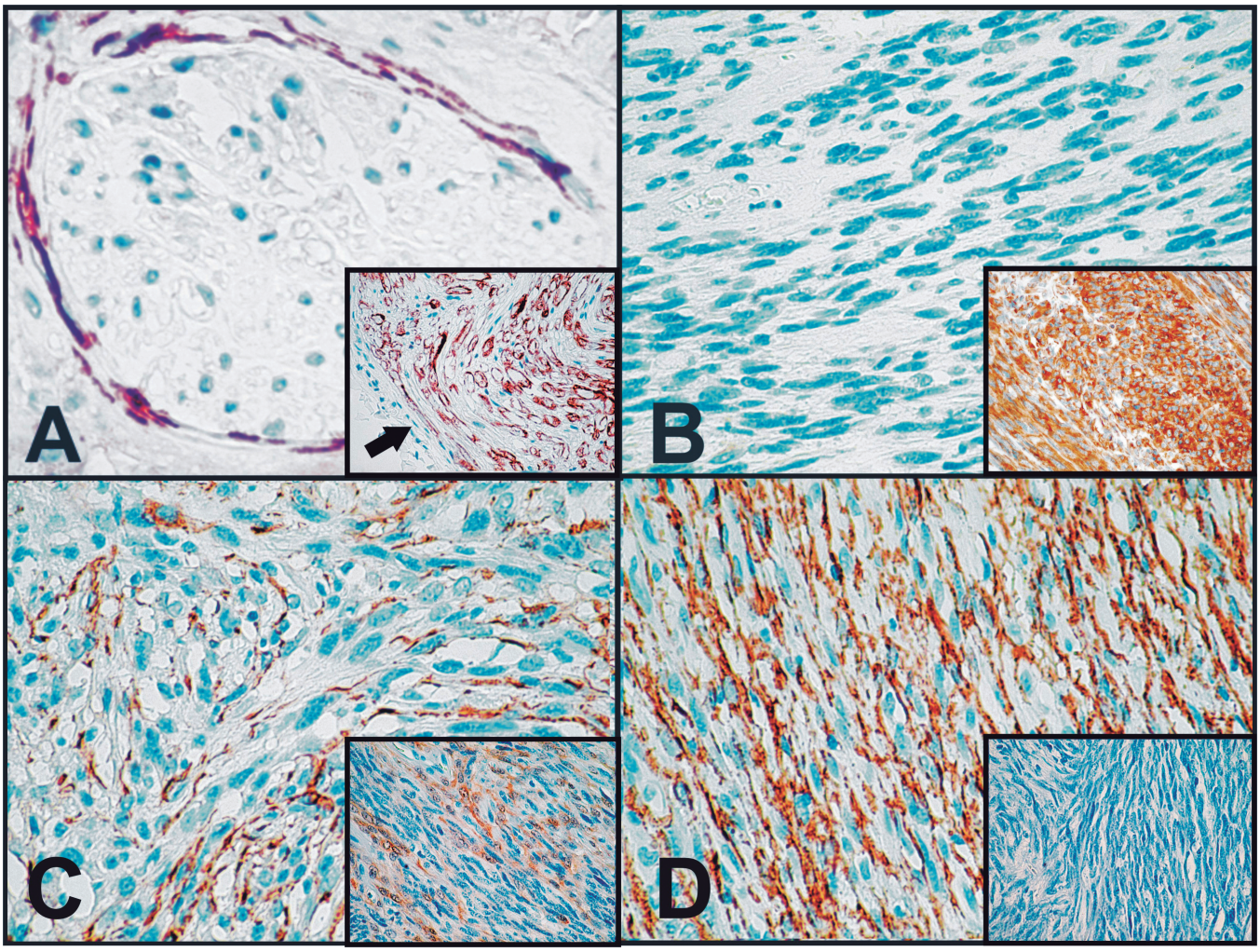

Fig. 1. A. Claudin-1 positive perineurial cells (brown colour) around the claudin-1 negative Schwann cells and axons from canine non-neoplastic peripheral nerve (IHC). Insert: S-100 protein positive Schwann cells and S-100 protein negative perineurial cells (arrow) in canine intact peripheral nerve (IHC). **B.** Claudin-1 negative canine schwannoma (IHC). Insert: S-100 protein (++++) positivity in canine schwannoma (IHC). **C.** Claudin-1 (++) positive canine neurofibroma (IHC). Insert: S-100 protein (++) positivity in canine neurofibroma (IHC). **D.** Claudin-1 (++++) positive canine plexiform perineurioma (IHC). Insert: S-100 protein negativity in canine plexiform perineurioma (IHC). x 400

Table 2. Expression of claudin-1 and other humanized antibodies in cPNSTs and other spindle shape cell tumours (P=positive; N=negative).

Tumour tissue / Antibody	Claudin-1	S-100 protein	NSE	α-SMA	Vimentin	panCK	Claudin-5	Melan-A
1.a. cBPNST: Schwannoma (n=4)	N	P (+++-++++)	N (3/4) P (1/4) (+)	N	P (++++)	N	N	N
1.b. cBPNST: Neurofibroma (n=8)	P (+-+++)	P (++-+++)	N (6/8) P (2/8)(+)	N	P (++++)	N	N	N
1. c. cBPNST: Perineurioma (n=8)	P (++++)	N	N	N	P (++++)	N	N	N
2. cMPNST (n=16)	P (++-+++)	P (+-+++)	N (11/16) P (6/16)(+)	N	P (+++-++++)	N	N	N
3. Psammomatous meningioma (n=6)	N	P (+++-++++)	N	N	P (++++)	N	N	N
4. Fibroma (n=6)	N	N	N	Ne	P (++++)	N	N	N
5. Leiomyoma (n=6)	N	N	N	P (++++)	P (++++)	N	N	N
6. Myxoma (n=6)	N	N	N	N	P (++++)	N	N	N
7. Spindle cell hemangioma (n=4)	N	N	N	N	P (++++)	N	P (++++)	N
8. Spindle cell lipoma (n=2)	N	N	N	N	P (++++)	N	N	N
9. Fibrohistiocytic nodule (n=6)	N	N	N	N	P (++++)	N	N	N
10. Fibrosarcoma (n=8)	N	N	N	N	P (++++)	N	N	N
11. Leiomyosarcoma (n=8)	N	N	N	P (+++-++++)	P (++++)	N	N	N
12. Myxosarcoma (n=6)	N	N	N	N	P (++++)	N	N	N
13. Solid hemangiosarcoma (n=8)	N	N	N	N	P (++++)	N	P (++++)	N
14. Anaplastic sarcoma (n=8)	N	N	N	N	P (++++)	P (+)	N	N
15. Amelanotic spindle cell melanoma (n=8)	N	P (+++-++++)	N	N	P (+++-++++)	N	N	P (++++)
16. Histiocytic sarcoma (n=8)	N	N	N	N	P (++++)	N	N	N
17. Spindle cell carcinoma (n=8)	P (++++)	N	N	N	N	P (++++)	N	N
18. Myoepithelioma (n=8)	N	P (++++)	N	P (++++)	P (++++)	N	N	N
19. Complex mammary gland carcinoma (n=8)	P (+-++)	P (++-+++)	N	P (++-+++)	P (++-+++)	P (++-+++)	N	N
20. Cardiac rhabdomyosarcoma (n=5)	N	N	N	P (+++-++++)	P (++++)	N	N	N
21. Monophasic synovial sarcoma (n=4)	N	N	N	N	P (++-+++)	N	N	N
22. Osteosarcoma (n=5)	N	N	N	N	P (+++-++++)	N	N	N
23. Chondrosarcoma (n=4)	N	P (+++-++++)	N	N	P (+++-++++)	N	N	N
Total number: 168								

Table 3. Summary of claudin-1 and useful antibodies in the differential diagnosis of cPWTs.

cPWT tissue / Antibody	Claudin-1	α-SMA	Heavy caldesmon	Calponin	Desmin	Vimentin
1. Hemangiopericytoma (n=10)	Positive (intense, +++ to ++++)	Positive (+++ to ++++)	Negative	Positive (+++ to ++++)	Negative	Positive (++++)
2. Myopericytoma (n=8)	Positive (weak, scattered +)	Positive (+++ to ++++)	Negative	Positive (+++ to ++++)	Positive (++ to +++)	Positive (++++)
3. Angioleiomyoma (n=6)	Negative	Positive (++++)	Positive (++++)	Positive (++++)	Positive (+++ to ++++)	Positive (++++)
4. Angioleiomyosarcoma (n=4)	Negative	Positive (++++)	Positive (++++)	Positive (++++)	Positive (+++ to ++++)	Positive (++++)
5. Angiofibroma (n=3)	Negative	Negative	Negative	Negative	Negative	Positive (++++)
Total number: 31						

sarcoma; spindle cell carcinomas showed diffuse, intense (++++) *pancytokeratin* positivity, and complex carcinoma showed intense, non-diffuse (++ to +++) positivity for this panepithelial marker. Only the cSCCs and cHSAs and endothelial cells of intratumoural and peritumoural vessels were positive (++++) for *claudin-5*. *Melan-A* positivity (++++) was detected only in cASCM samples (Table 2).

The cHP samples (10/10; 100%) showed an intense (+++ to ++++) membranous positivity for claudin-1 (Fig. 3C,D). The cMPC samples (8/8; 100%) showed a weak, scattered (+) claudin-1 membranous positivity (Figs 4A and 4B). All other cPWT samples, including cALM (6/6), cALMS (4/4) and cAGF (3/3) were negative for claudin-1 molecule. In subgroups of cPWTs, employing the anti-α-SMA, anti-heavy caldesmon, anti-calponin, anti-desmin and anti-vimentin, we achieved similar results to Avallone et al. (2007) in their earlier immunohistochemical study (Table 3).

As internal positive controls for claudin-1 we used the normal follicles in the skin (Fig. 4C), the sebaceous glands and non-neoplastic surface epithelial cells in the skin (Fig. 4D), vagina, uterus, and the peritumoural intact luminal cells of the lobules of the mammary gland. Negative controls for claudin-1 were the intratumoural, peritumoural small arterial type vessels (endothelial and smooth muscle cells), and the tumour infiltrating lymphocytes and macrophages.

Discussion

In the present study we have tested humanized anti-claudin-1 antibody on different canine spindle cell tumours and analysed the usability of this tight junction

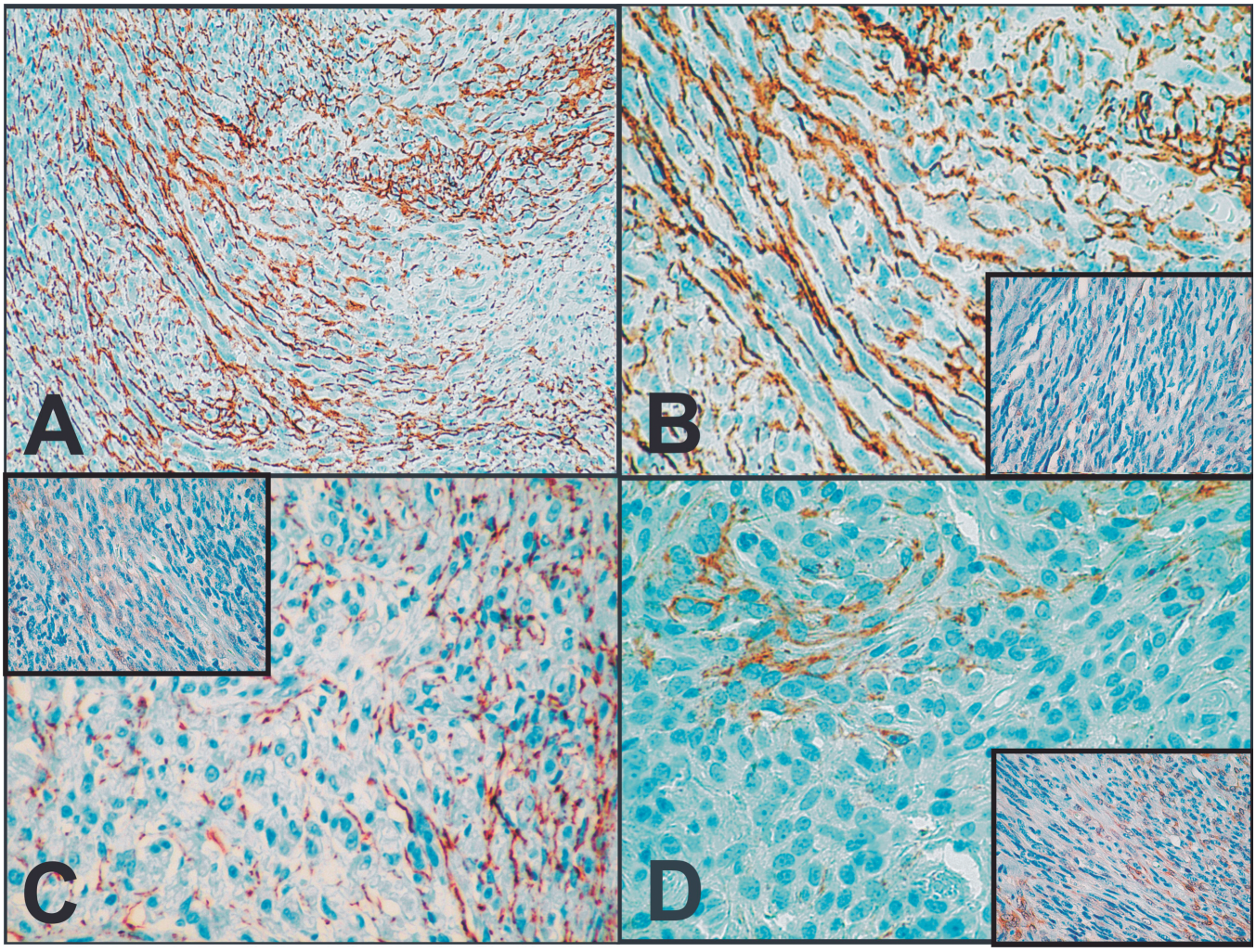

Fig. 2. A, B. Diffuse, ++++ intense claudin-1 positive cMPNST (IHC). Insert: S-100 protein (+) positivity in this cMPNST sample (IHC). **C.** Diffuse, +++ intense claudin-1 positive cMPNST (IHC). Insert: S-100 protein (++) positivity in this sample (IHC). **D.** Diffuse, ++ intense claudin-1 positive cMPNST (IHC). Insert: S-100 protein (+++) positivity in this cMPNST (IHC). A, x 200; B-D, inserts, x 400

protein for differentiation of cPNSTs from other spindle cell neoplastic lesions which show similar histopathological features. This is the first immunhistochemical study of a large series of canine fusocellular tumours, in which the immunohistochemical reactivity of these neoplastic lesions against anti-claudin-1 antibody was evaulated.

In the peripheral nerves myelin is formed by Schwann cells, endo- and epineurium are composed of fibrocytes and collagen and the perineurium contains perineurial cells. Intact and neoplastic perineurial cells are spindle-shaped with pale eosinophilic cytoplasm and fusiform nuclei, a finely distributed chromatin, and with long, non-branching thin cell processes. The perineurial cells contain tight junctions, since the presence of the tight junctions are responsible for the claudin-1 immunopositive staining (Erlandson, 1991). In our present study, canine intact perineurial cells showed intense membranous claudin-1 positivity, while the Schwann cells were negative for claudin-1. Our findings in the canine system are in agreement with those of a previous immunohistochemical study of Folpe et al. (2002) on human perineurial cells, which included twelve perineuriomas, seven firbosarcomas, eight low-grade fibromyxoid sarcomas, three desmoplastic fibroblastomas, seven fibromatoses, nine neurofibromas, and five schwannomas. Claudin-1 expression was detected in 92% of perineuriomas. This was the first human study to decribe expression of claudin-1 in intact perineurial cells and to recommend it as an immunohistochemical marker in the diagnosis of human peripheral nerve sheath tumours (Folpe et al., 2002).

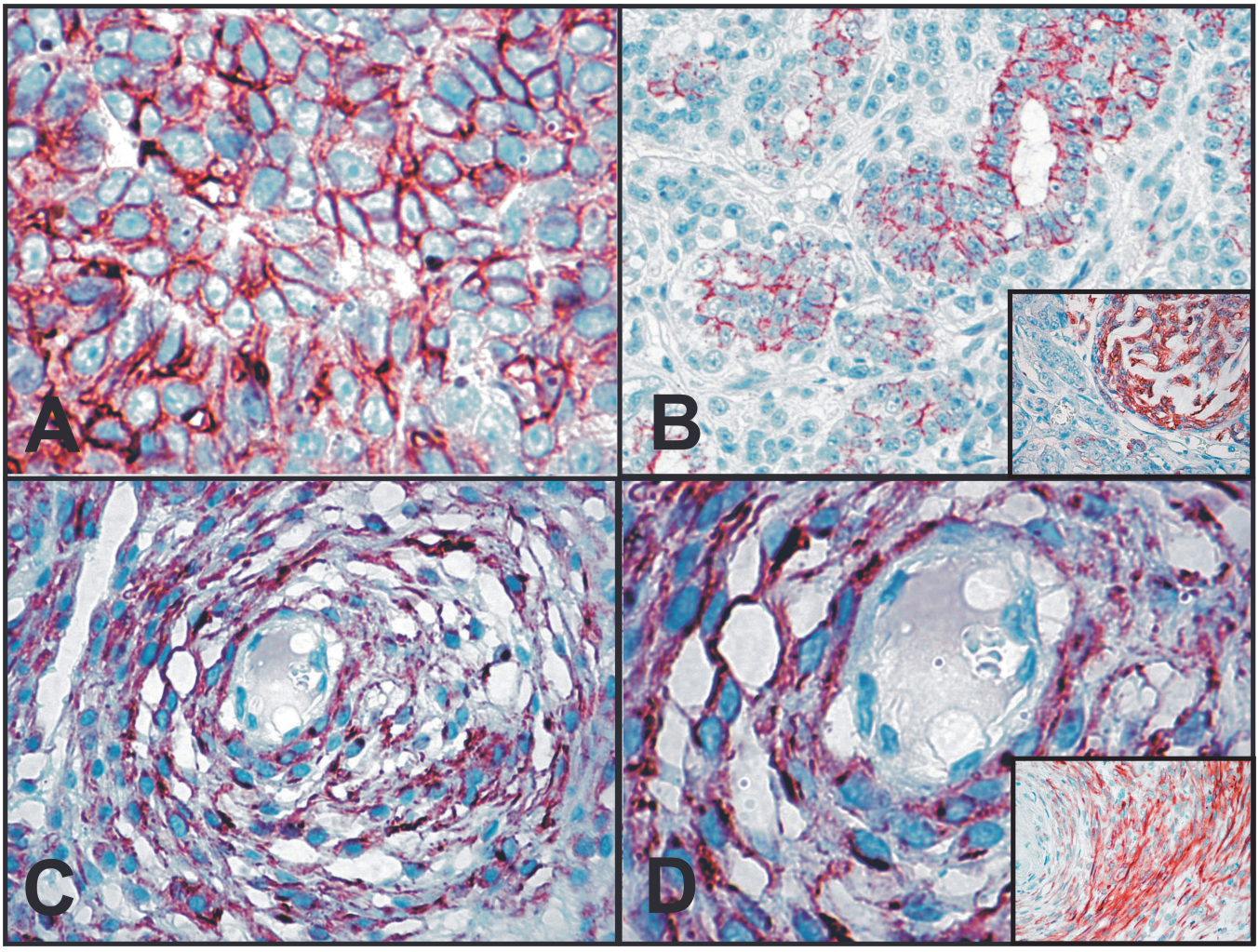

Fig 3. A. Claudin-1 positive cSCC (IHC). **B.** Claudin-1 positive neoplastic luminal epithelial cells admixed with claudin-1 negative neoplastic, spindle-shaped myoepithelial cells in canine complex mammary gland carcinoma (IHC). Insert: S-100 protein positive neoplastic myoepithelial cells in canine complex mammary gland carcinoma (IHC). **C-D.** Claudin-1-positive cHP around the small capillary (IHC). Insert: α-SMA positive cHP (IHC). A, D, inserts, x 400; B, C, x 200

The main subtypes of BPNSTs are schwannoma, neurofibroma and perineurioma. Schwannomas are formed by neoplastic Schwann cells, neurofibromas by neoplastic Schwann cells, perineurial cells and fibroblasts, perineuriomas by neoplastic perineurial cells (Koestner and Higgins, 2002; Piña-Oviedo and Ortiz-Hidalgo, 2008). In veterinary pathology Cornelis et al. (2009) used for the first time claudin-1 for the detection of perineurioma in a 4-year-old male Leonberger (Cornelis et al., 2009). In our present study we analysed 213 canine tissue samples consiting of 10 canine intact peripheral nerves and 203 biopsy and necropsy samples of tumours.

Histologically, cPNSTs are characterized by sweeping fascicles of spindle shaped cells. The classical microscopic appearance of schwannoma shows two distinctive patterns: Antoni A and B areas. The more cellular Antoni A pattern is composed of spindle shape benign neoplastic Schwann cells arranged in interlacing fascicles, with palisading nuclei surrounding eosinophilic areas (Verocay bodies). Antoni B pattern has a looser stroma, fever cells, and myxoid change. Neurofibroma consist of a mixture of three spindle-shaped cell types, namely Schwann cells, perineurial cells and fibroblasts. In perineurioma, the neoplastic perineurial cells form whorls and a vaguely storiform pattern. Individual neoplastic cells are spindled and plump, with a mild amount of pink cytoplasm (Avallone et al., 2007; Koestner and Higgins, 2002; Piña-Oviedo and Ortiz-Hidalgo, 2008). In our study of cBPNST samples, schwannomas were negative for claudin-1, but were diffuse positive for S-100 protein; neurofibromas showed non-diffuse membrane claudin-1 positivity and non-diffuse S-100 protein positivity; perineuriomas

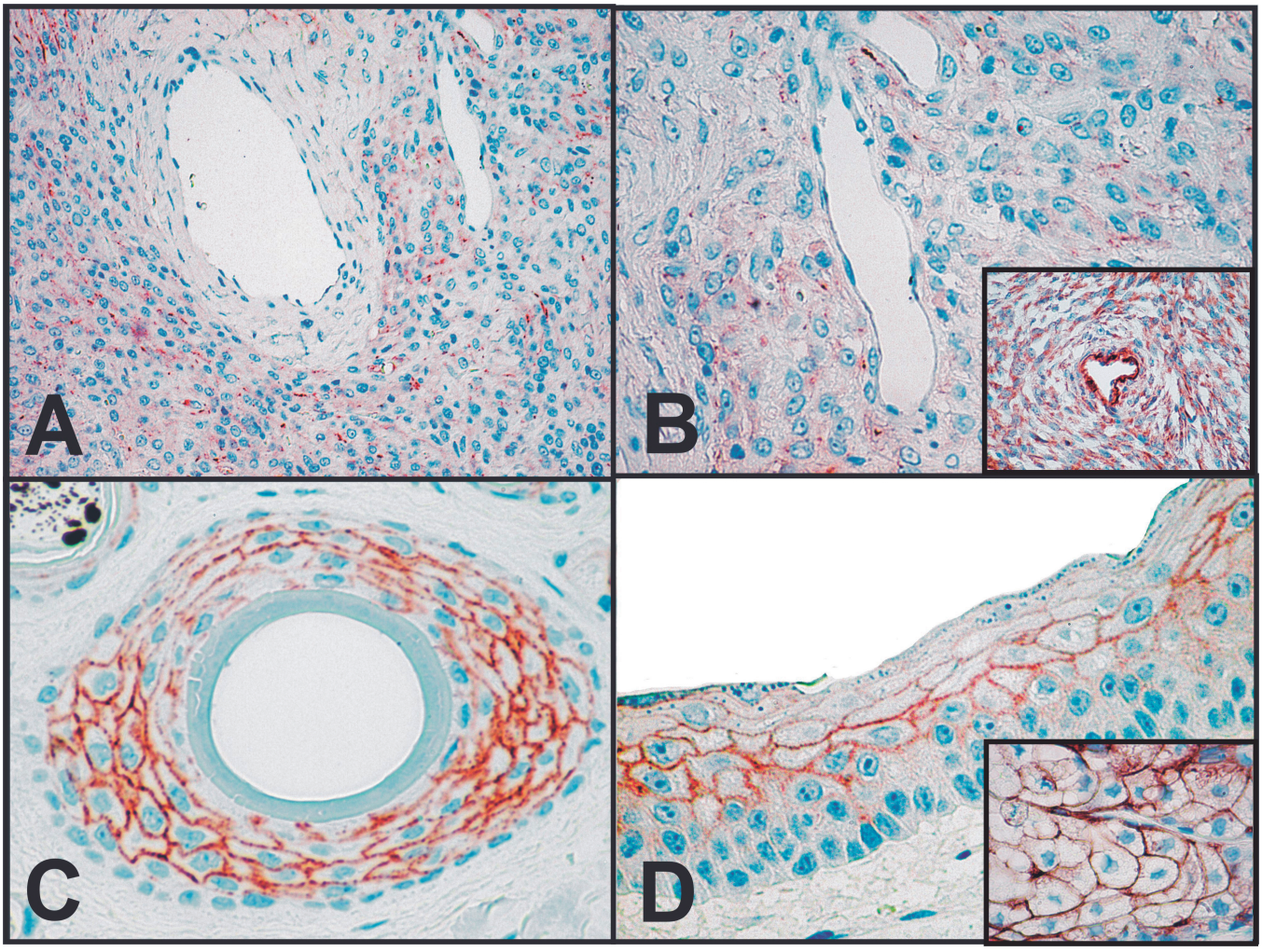

Fig. 4. A, B. Weak (+) claudin-1 positivity in cMPCs (IHC). Insert: α-SMA positive cMPC (IHC). Internal positive controls for claudin-1: **C.** Claudin-1 positive outer root sheath of the intact follicle from the skin (IHC). **D.** A. Claudin-1 positive suprabasal cell layers of the intact skin (IHC). Insert: Claudin-1 membranous positivity in intact sebocytes (IHC). A, x 200; B-D, inserts, x 400

showed diffuse membrane claudin-1 positivity, and S-100 protein negativity. It seems to be that anti-claudin-1 antibody, combined with anti-S-100 protein antibody, can be used for the correct detection of cBNSTs, including *claudin-1 negative/S-100 positive schwannomas, claudin-1 positive/S-100 protein positive neurofibromas, and claudin-1 positive/S-100 protein negative perineuriomas*.

In human oncopathology perineuriomas can be classified into five main groups: intraneural (localized hypertrophic neuropathy), extraneural, plexiform, reticular, sclerosing type. Human perineuriomas subtypes, such as sclerosing pacinian perineurioma (Burgues et al., 2001), lipomatous perineurioma (Zamecnik, 2003), perineurioma with granular cells (Díaz-Flores et al., 1997) and perineurioma with ossifaction have been reported in the last years (Rank and Rostad, 1998). In veterinary oncopathology this nomenclature of perineuriomas is not used. In our study the claudin-1 positive canine perineuriomas showed histologically a plexifrom structure. These tumours consisted of plump spindle-shaped neoplastic cells with an ill-defined, pale eosinophilic cytoplasm and spindled or rounded nuclei. Based on histopathological appearance, we assume that these tumours were canine plexiform perineuriomas. Further investigations are need to define claudin-1 expression pattern in other histotypes of canine perineurioma.

An MPNST (previously called neurofibrosarcoma, malignant schwannoma) consists of a malignant mixed proliferation of Schwann cells, and/or perineurial cells, and/or fibroblasts. The histologic appearance of MPNSTs consists of closely packed or loosely arranged intercaling fascicles of spindle cells that show a wavy pattern (Avallone et al., 2007; Koestner and Higgins, 2002; Piña-Oviedo and Ortiz-Hidalgo, 2008). In our study all cMPNST samples were claudin-1 positive: (1) seven samples (43,75%) showed diffuse, intense (++++) membrane claudin-1 positivity, and these tumours were + S-100 positive; (2) six samples (37,5%) showed diffuse, intense (+++) positivity for claudin-1, and four tumours from these six samples showed (++) S-100 positivity, and two samples showed + S-100 positivity; (3) three samples (18,75%) showed ++ claudin-1 positivity, and two samples from these three tumours showed +++ S-100 positivity, and one sample showed + S-100 positivity. All cMPNSTs showed claudin-1 and S-100 positivity. In our study we did not detect a claudin-1 positive and S-100 negative cMPNST as a true malignant perineurioma. Chijiwa et al. (2004), in their study described three S-100 negative cMPNSTs. Maybe these were claudin-1 positive malignant perineuriomas (Chijiwa et al., 2004).

cPNSTs and other fusocellular tumours can appear histomorphologically very similar, particulary in small biopsy specimens (core-biopsy, Tru-cut biopsy). The distinction between cPNSTs and other spindle-shaped cell tumours is important for the correct pathological diagnosis and appropriate treatment (Gross et al., 2006).

Tight junctions are the most apical cell-cell contacts and are important for barrier function in epithelial, mesothelial and endothelial cells (Tsukita et al., 2001). The question arises as to why claudin-1 expression should be tested in canine mesenchymal tumours. In several human studies it was reported that claudin tight junction molecules can be expressed in non-epithelial, non-endothelial intact or neoplastic tissues (Smith et al., 2005; Győrffy 2009; Thway et al., 2009, 2011). In our present study canine leiomyomas, leiomyosarcomas fibrosarcomas, mysosarcomas, differentiated liposacromas, anaplastic sarcomas were claudin-1 negative. Thus, investigations are needed on claudin-1 expression in poorly differentiated canine liposarcomas. In our study the canine neurofibromas, perineurioma, cMPNSTs, cHPs, cMPCs, cSCCs, complex mammary gland carcinomas showed claudin-1 positivity.

Avallone et al. (2007), in their histopathological and immunohistochemical study, analysed the different subgroups of the cPWT (n=20) including cHP (n=2), cMPC (n=6), cALM (n=5), cALMS (n=2), cAGF (n=1) and adventitial tumour (n=1), using anti-α-SMA, anti-heavy caldesmon, anti-calponin, anti-desmin, anti-vimentin, anti-myosin, anti-smoothelin, anti-pan-actin, anti-laminin, anti-CD34 and anti-CMG-3G5 (Avallone et al., 2007). In the present study we analysed 31 cPWTs with anti-α-SMA, anti-heavy caldesmon, anti-calponin, anti-desmin, anti-vimentin, and anti-claudin-1 antibodies. This group consisted of 10 cHPs, 8 cMPCs, 6 cALMs, 4 cALMSs, and 3 cAGFs. The cHP samples (10/10; 100%) showed an intense (+++ to ++++) membranous positivity for claudin-1, cMPC samples (8/8; 100%) showed weak, scattered (+) claudin-1 positivity, while all other cPWT samples were negative for this antibody.

Pericytes form an incomplete envelopment within the microvascular basement membrane of capillaries and postcapillary venules around the endothelial cells (Díaz-Flores et al., 2009; Lin et al., 2010). cHPs are commonly located on the skin and the subcutaneous tissue of the limbs in canines (Mazzei et al., 2002). There are some case reports on extracutaneous cHPs (Cho and Park, 2006; Vignoli et al., 2008). Myopericytes are intermediate cells between the pericyte and smooth muscle cells. Myopericytoma is a fusocellulare perivascular wall tumour (Mentzel et al., 2006). In human oncopathology it was originally prposed by Requena et al. in 1996, and it has now been endorsed by the World Health Organisation (Folpe and McMenamin, 2002). It was described in canine by Avallonne et al. in 2007. Rajaram et al. (2004) in their study described, 2 of 15 human meningeal hemangiopericytomas (13%) were focally positive for claudin-1 (Rajaram et al., 2004). Hahn et al. (2006) reported that all (5) meningeal hemangiopericytomas were negative in their study (Hahn et al., 2006). In our study we report for the first time that cHPs show intense claudin-1 positivity and cMPCs show weak scattered claudin-1 positivity. It seems that using claudin-1 as the only immunohisto-

chemical marker it is not possible to differentiate between cPNSTs containing perineurial cells, cHPs and cMPCs. Correct differential diagnosis between these three canine fusocellular tumours can be carried out by using two or three antibodies, such as anti-claudin-1, anti-α-SMA and anti-S-100 protein. *Canine neurofibromas* and *cMPNSTs* containing perineurial cells are positive for claudin-1 and S-100 protein, but negative for α-SMA. *Canine perineuriomas* are positive for claudin-1, but negative for S-100 protein and α-SMA. *cHPs* are positive for claudin-1 α-SMA, but negative for S-100 protein. *cMPCs* are weak claudin-1 positive and strong α-SMA positive cPWTs, but negative for S-100 protein. *Canine schwannomas* are negative for claudin-1 and α-SMA, but positive for S-100 protein. It seems that anti-claudin-1 antibody, together with smooth muscle markers, can help distuinguish the claudin-1 positive cHPs from cMPCs and from other claudin-1 negative cPWTs.

cSCC is a rare histotype of the squamous cell carcinoma. Histologically, this tumour is composed of malignant atypical spindle carcinoma cells arranged in a whorled pattern. Spindle cell carcinoma was first reported by Martin and Stewart seventy five years ago (Martin and Stewart, 1935). cSCC may be difficult to differentiate from other spindle- shaped cell tumours, such as cPNST. Here we report that cSCCs are claudin-1 positive malignant tumours. The differentation between cPNSTs and cSCCs is based on at least four antibodies, such as anti-claudin-1, anti-S-100 protein, anti-vimentin and anti-pancytokeratin. *Canine neurofibromas* and *cMPNSTs* containing perineurial cells are positive for claudin-1 and S-100 protein and vimentin, but negative for pancytokeratin. *Canine perineuriomas* are positive for claudin-1, vimentin, but negative for S-100 protein and pancytokeratin. *cSCCs* are positive for claudin-1 and pancytokeratin, but negative for S-100 protein and vimentin. *Canine schwannomas* are negative for claudin-1 and pancytokeratin, but positive for S-100 protein and vimentin.

The complex mammary gland carcinoma is a biphasic, myoepithelial-epithelial tumour in canines. It consists of proliferating neoplastic luminal epithelial cells and spindle- shaped myoepitheliocytes, arranged in a reticulated histological pattern (Hahn et al., 1992). Here we report that the neoplastic luminal epithelial cells of the complex carcinoma were positive for claudin-1, whereas the neoplastic myoepithelial cells and the benign myoepitheliomas were negative for claudin-1. The immunohistochemical differentiation between the cPNSTs and complex carcinomas of the mammary gland is based on at least four antibodies such as anti-claudin-1, anti-S-100 protein, anti-α-SMA and anti-pancytokeratin. *Canine neurofibromas* and *cMPNSTs* containing perineurial cells are positive for claudin-1 and S-100 protein, but negative for α-SMA and pancytokeratin. *Canine perineuriomas* are positive for claudin-1, but negative for S-100 protein, α-SMA and pancytokeratin. The *complex carcinomas* are positive for claudin-1, S-100 protein, α-SMA and pancytokeratin. *Canine schwannomas* are negative for claudin-1, α-SMA and pancytokeratin, but positive for S-100 protein.

Ramos-Vara et al. (2010), consistent with our result, reported that claudin-1 expression was not detected in the outer leptomeninges of canines. In their immunohistochemical study the authors have presented the complete immunomorphological pattern of the different histotypes of the canine meningiomas, using anti-CD34, anti-E-cadherin, anti-claudin-1, anti-glucose transporter I, anti-laminin, and anti-protein gene product 9.5 antibodies (Ramos-Vara et al., 2010). In the present study, all canine psammomatuos meningiomas were negative for claudin-1. A previous study reported that the immunohistochmistry for claudin-1 is only of moderate to low sensitivity in canine meningiomas (Ramos-Vara et al., 2010).

Leotlela et al. (2007) described that in human melanocytic lesions, claudin-1 expression was increased, and its subcellular localization became dysregulated. Benign lesions and less agressive melanomas express claudin-1 in the nucleus, wherears aggressive melanomas have an abundance of claudin-1 in the cytoplasm (Leotlela et al., 2007). Here, in our study, we did not detect mebmranous or cytoplasmic/nuclear claudin-1 positivity in cASCM samples.

Billings et al. (2004) detected that human monophasic synovial sarcomas, compared to biphasic synovial sarcomas, less often expressed claudin-1 (4/14; 28,5%). The glands of human biphasic sacromas expressed claudin-1 (12/13; 92,3%), and the spindle cells of these tumours showed abnormal circumferential membranous expression of claudin-1 (6/13; 46,15%) (Billings et al., 2004). We examined four canine monophasic synovial sarcomas, which were claudin-1 negative. Further studies are needed on claudin-1 expression in canine biphasic synovial sarcomas.

Although claudin-1 was expressed in Ewing-sarcoma samples (19/30; 63%) (Schuetz et al., 2005), the canine osteosarcomas examined in the present study were claudin-1 negative.

In conclusion, the present immunohistochemical study, which is based on humanized antibodies, suggests a new immunohistochemical panel for the correct differential diagnosis between cPNSTs and other spindle-shaped cell canine tumours, particulary in small biopsy specimens. This immunohistochemical panel consists of the following eleven antibodies: *anti-claudin-1, anti-neuron specific enolase, anti-S-100 protein, anti-α-smooth muscle actin, anti-heavy caldesmon, anti-calponin, anti-desmin, ant-vimentin, anti-cytokeratin AE1-AE3, anti-claudin-5* and *anti-Melan-A*. We have detected claudin-1 expression in canine intact perineurial cells, and also in canine neurofibromas and perineuriomas, cMPNSTs containing perineurial cells, cHPs, cMPCs, cSCCs and complex carcinomas. These findings suggest that claudin-1 can serve as a novel marker, and in combination with other antibodies, enables correct pathological diagnosis of canine

fusocellular tumours, and that claudin-1 with other markers can help the differential diagnosis of cBPNSTs, cMPNSTs, and cPWTs.

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