UNIVERSITÀ DEGLI STUDI DI MILANO

*Facoltà di Medicina Veterinaria*

*Dipartimento di Patologia animale, Igiene e Sanità Pubblica Veterinaria*
*Sezione di Anatomia Patologica e Patologia Aviare*

*Scuola di Dottorato in Sanità e Produzioni Animali: Scienza, Tecnologia e Biotecnologie*

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# Cell blocks from canine and feline effusions

Dott.ssa Valentina Crippa
Matr. N°: R09473

Docente guida: Prof. Mario CANIATTI
Coordinatore: Prof. Giuseppe Sironi

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# SUMMARY

**Objective**: To evaluate the sensitivity and specificity of a panel of markers in distinguishing mesothelial cells from metastatic adenocarcinoma cells in Cell blocks from canine and feline effusion.

**Methods**: This study included 28 effusion specimens from dogs and cats with a cytological diagnosis of reactive effusion or malignancy of non-hematopoietic origin. Cell Blocks were stained with a standard panel of Vimentin, panCK (MNF116), CK 5/6 and HBME-1 as mesothelial cell markers; Desmin as marker of benign mesothelial cells; Claudin 4 as epithelial marker and CK7/CK20 as a marker of metastasis. Malignancy was confirmed by histologic evaluation; non-malignant conditions were confirmed by histopathology or follow up. Sensitivities, specificities, predictive values and accuracy were calculated.

Results: CK5/6 demonstrated a high specificity (100%) for mesothelium. For the detection of canine and feline mesothelial cells the coexpression of panCK and VIM displayed the best sensibility (94,1%) while HBME-1 was the antibody that presented highest accuracy. Claudin 4 demonstrated a very low sensibility versus canine and feline epithelial cells.

**Conclusion:** The most specific marker, with for the identification of mesothelial cells in canine and effusion, is the Vim/CK coexpression, being CK5/6 the most specific and HBME-1 the marker with the highest overall accuracy. Desmin is a useful marker in discriminating between benign and malignant mesothelial cells. The coordinate expression of CK7/CK20+ has not proved to be useful on the identification of metastatic cells on effusion. The study of Claudin 4 necessitate to be deepened in veterinary medicine. In conclusion, the combination of both cytology and immunohistochemistry studies can greatly enhance the diagnostic accuracy, sensitivity and specificity in malignant effusions.

# INTRODUCTION

## 1. Normal Body Cavity Fluid Formation

A small amount of fluid is normally present within body cavities to provide lubrication between organ surfaces during movement. In the thorax, this fluid also provides mechanical coupling between the chest wall and the lungs allowing for a direct transmission of forces for normal respiration. Normal body cavity fluid is a low protein, serous ultrafiltrate of blood that flows out of arteriolar capillaries through the body cavity and is largely resorbed into the venous capillaries. A smaller portion, approximately 10%, of this fluid is resorbed by the lymphatics. The rate of fluid formation is dependent on Starling's forces (i.e., gradients of hydrostatic and oncotic pressures between the vessels and the body cavities), the degree of mesothelial and endothelial permeability, and the integrity of lymphatic drainage (Dempsey et al., 2011). The serosa is composed of two major elements: mesothelial cells and connective tissue. The mesothelial cell layer is only approximately 2 μm thick and provides most of the diffusion barrier of the serosa. The mesothelium is composed of a thin monolayer of simple, flattened epithelial cells that express both epithelial and mesenchymal markers. These cells line the body wall and organs of the thorax, pericardium, and abdomen. Mesothelial cells also synthesize connective tissue macromolecules and enzymes, participate in transcellular transport, and respond to cytokine and hormonal stimulation. Mesothelial cells have apical microvilli to increase their surface area and produce glycoproteins rich in hyaluronic acid and phospholipids to reduce friction between organ surfaces. There are tight junctions between adjacent mesothelial cells and thus the permeability of the mesothelium is similar to that of the vascular endothelium. The lymphatics communicate directly with body cavities through openings between mesothelial cells called stomas. These stomas are the only way for cells and larger particles to exit cavitary spaces whereas proteins may also exit by solvent drag and transcytosis (Zocchi et al., 2002).

## 2. Pathophysiology of Effusions

Accumulation of fluid within a body cavity results when the rate of filtration of fluid into a space is greater than the rate of fluid resorption from that space. Effusion accumulation is correlated to increased capillary hydrostatic pressure, widening of the oncotic pressure gradient, increased endothelial permeability, increased interstitial hydrostatic pressure, and loss of effective lymphatic drainage. An increase in interstitial hydrostatic pressure narrows the hydrostatic gradient between the cavity and the interstitium resulting in decreased fluid resorption. At times, fluid accumulation can be so severe as to cause tamponade and compromise visceral function. To eliminate effusion accumulation, normal pressure balance,

lymphatic drainage, and permeability characteristics must be restored (Dempsey et al., 2011).

### 3. Effusions that Result from Exfoliation of Cells into a Body Cavity

Exfoliation of neoplastic and/or reactive mesothelial cells into a body cavity can lead to the accumulation of highly cellular effusions. Carcinomas, mesotheliomas, and discrete (round) cell neoplasms (e.g., lymphoma, mast cell tumors, malignant histiocytosis) exfoliate cells into effusions more readily than sarcomas (Dempsey et al., 2011). The number of mesothelial cells lining the peritoneum depends on the fine balance between cell proliferation and cell death. According to studies in human medicine, under normal peritoneal homeostasis, mesothelial cells exhibit limited cell proliferation, with only 0.1%–0.5% of cells in the mesothelium undergoing mitosis at any one time. However, injury to mesothelial cells significantly induces cell proliferation and cell death, thereby altering the structural integrity of the peritoneal membrane. When appropriately stimulated, the mitotic activity of mesothelial cells can be greatly increased. Within 48h of injury to the serosal surface, 30–80% of mesothelial cells at the wound edge and on the apposing surface begin synthesizing DNA. Contact inhibition may be one trigger for this rapid increase in proliferation but soluble mediators released from inflammatory and injured cells are also potent stimulants (Mutsaers et al., 2002). The effects on mesothelial cells of the accumulation of fluid in a serosal cavity include: cell enlargement, transformation from squamous to cuboidal or columnar morphology, increased nucleus/cytoplasmic (N/C) ratio, vacuolation, activation, and loss of microvilli (Di Paolo et al., 1986; Williams et al., 2003). Prolonged exposure of mesothelial cells to the effusion fluid also results in the collapse of cell–matrix interactions and abrogation of cell–cell contact inhibition with subsequent shedding of mesothelial cells into the peritoneal cavity (Whitaker and Papadimitrou, 1985). Injury to the mesothelium triggers events leading to the migration of mesothelial cells from the edge of the lesion towards the wound center and desquamation of cells into the serosal fluid. The fate of these cells after shedding into the peritoneal cavity is still under research. The most accepted hypothesis is that mesothelial cells are still viable in suspension, capable of maintaining function within the peritoneum, instead of being degenerative cells that are destined to apoptosis and removal from the peritoneal cavity by phagocytosis. Currently, most researchers agree that floating cells in effusions will attach and incorporate into the regenerating mesothelium. (Mutsaers et al., 2000; Comer et al., 2002; Mutsaers, 2004).

### 4. Effusion Cytology

Cytologic evaluation of cavitary effusions is considered a standard practice in the diagnostic process, including cell count and biochemical analysis (eg, protein measurement), and is

often useful in differentiating neoplastic and infectious causes of effusion from others; however, the correct determination of the origin of the neoplastic cells is, by far, a very difficult issue for the cytopathologist (Davidson, 2004; Politi et al., 2005). Cytology is known to be a highly specific (99% for canine and 100% for feline effusions) but not very sensitive (64% for dogs and 61% for cats) technique on the diagnostic of effusions (Hirschberger et al., 1999). The major problem for the cytopathologyst is the correct identification of mesothelial cells because they may vary widely in their morphology, so distinguishing macrophages from mesothelial and carcinoma cells may be often challenging (Whitaker, 2000; Addis and Roche, 2009). Mesothelial cells frequently show florid reactive changes in response to many benign conditions such as pulmonary infarction, systemic disease (ie, collagen-vascular diseases), cirrhosis, radiation, underlying neoplasm, chronic inflammation, foreign substance, and infection. The common cytomorphologic features of mesothelial cells in reactive effusion include increase in the cellularity of a monomorphic cell population associated with papillary clusters. The cells are larger than quiescent mesothelium, with some prominence of nucleoli, regular chromatin pattern, and normal nuclear to cytoplasmic ratio (Geisinger, 2004). Reactive mesothelial cells usually appear dark blue and often have an indistinct brush border appearance to their cell membrane. Marked cytologic atypia can also be seen in hyperplastic or reactive mesothelium (Bolen, 1986). The common cytologic features of Malignant Mesothelioma cells are nuclear pleomorphism, macronucleoli, large cellu- lar aggregates, papillary-like tissue fragments, and cell-in-cell engulfment, but Malignant Mesothelioma cells can also be deceptively bland and indistinguishable from benign mesothelial cells using ancillary techniques such immunohistochemistry or FISHare often necessary to reach a diagnosis (Hawang et al, 2016).

**5. Cell blocks technique**

Cytology has been utilized in human medicine since 1854 when reported by Paget for the study of breast tumors (Magalha, 2001; Cassali 2007). Although conventional cytology provides relevant information at the cellular level and demonstrates a close relationship between cell morphology and function (Magalha, 2001; Masserdotti, 2006) there are some major limitations, because features such as surrounding tissue and the architectural pattern of the sampled lesion are not assessed by cytology with a high degree of confidence (Magalha, 2001). In these cases, examination of cytologic smears does not provide sufficient information, increasing the risk of rendering a false-negative or uncertain diagnosis (Handa 2005, Sanchez 2006). Moreover, limited technical skills may lead to erroneous sampling methods for some lesions. Even when sample collection is appropriate, inadequate material may be obtained (Handa, 2005). Finally, blood clots or overlapping cell clusters may interfere with evaluation of cellular detail, making classification and interpretation difficult or

impossible; re-sampling is not always possible (Magalha, 2001, Handa, 2005). To overcome these problems and increase the efficiency of cytologic diagnosis, the cell block technique (CBT), described in 1896, has been used to maximize the information obtained from a small cytologic sample (Kulkarni, 2000; Handa, 2005; Gangana, 2007), thus, the CBT can be considered an “old and forgotten” method. Although the CBT has been used for decades and it has been gained wide acceptance as a diagnostic tool from 1947 (Cassali et al, 2007), there are few reports that have documented its use (Kulkarni, 2000). In human medicine, its main use is in analysis of pleural and peritoneal fluids, bronchial washings, fine-needle aspiration cytology (Nigro, 2007, Cassali et al, 2007, Sanchez et al , 2006) and gynaecological material (Gangane et al, 2007, Nigro et al, 2007). In veterinary medicine, there are few reports of the use of the CBT in a diagnostic or research setting. CB are use maily for CB sections offer advantages over conventional cytological smears with respect to cellular architecture and archival storage. The presence of extracellular matrix, which represents connective tissue between exfoliated cells, is crucial in maintaining tissue architecture; however, extracellular matrix is usually dispersed on conventional smears, distorting cellular arrangments (Masserdotti, 2006). The main advantage of the CBT is that architectural patterns, ie, pavement, honeycomb, acinar, palisade, papillary, trabecular, storiform, and perivascular arrangements of cells (Masserdotti, 2006) are maintained, and relationships between different cell populations may be evident, contributing to the overall representation of tissue. This is particularly true for glandular and mesenchymal neoplasms for which cellular arrangement is a very important clue in determining histogenesis (Masserdotti, 2006). In addition, ancillary diagnostic techniques, such as cytochemistry, immunocytochemistry, and molecular and proteomic analysis, can be applied to cell block (Mansy et al, 2006). They also provide several sections, which can be utilized to perform special stains, immunophenotypic analysis, ultrastructural studies and molecular tests, including cytogenetic and polymerase chain reaction (PCR)- based techniques (Mansy et al, 2004, Mansy et al, 2006). Over the years, many CB techniques have evolved. The basic protocol remains the same with all methods, including a cellular concentration step, followed by processing as for histopathological biopsy tissue. The difficulties in the recovery and processing of small tissue fragments have resulted in alternative manual CB methods which include the use of cell adjuvants, such as agar (Smedts, 2010; Kerstens, 2000), HistoGelTM (Versagi, 2009), gelatin albumin (Koss, 1979), collodion (Fahey, 1993), pre-gelatinized starch (He, 2012), sodium alginate (Noda, 2010) gelatin foam, polyvinyl alcohol foam (Mayall, 2012) and other less practical methods, which include the acetone-melted paraffin technique (Krogerus, 1988) and gelatin capsules (Wen, 2011). Principally, the concentrated sediments are supported by a substance such as agar or a collodion bag . Agar solidifies below 50 °C, and this property of agar is utilized to form the cell pellet. The manoeuvrable cell button is

then embedded in paraffin after processing as for histopathology specimens. If the cellularity is scanty, it is advisable to perform the collodion bag method (Kerstesn, 2000; Versagi, 2009; Fahey, 41). Collodion is a nitrocellulose material, which is used to make blocks of friable tissue, such as brain, in histology laboratories. Hence, the use of a collodion bag, which effectively coats the tube before the cell pellet is added, is associated with advantages of controlling the size of the CB area and cellular concentration. The experience with other rarely used adjuvants is limited in the literature (Jain et al, 2014).

**6. Immunohistochemistry**

Immunohistochemistry (IHC) is a powerful method for localizing specific antigens in formalin-fixed, paraffin-embedded tissues based on antigen–antibody interaction (Taylor and Burns, 1974). The technique is widely used both in diagnostics and research, and its applications continue to be extended because of its ease of use, reliability, and versatility. In IHC an antigen–antibody construct is visualized through light microscopy by means of a color signal. The advantage of IHC over immunofluorescence techniques is the visible morphology of the tissue around the specific antigen by counter- staining. Results of stained IHC markers are reported semiquantitatively and have important diagnostic and prognostic implications. The term “antibody” was coined by Paul Ehrlich in 1891. Immunofluorescence staining on frozen sections based on antigen–antibody interactions was presented by Coons in 1940 (Odell and Cook, 2013). Taylor and Burns developed IHC on routinely processed formalin-fixed, paraffin-embedded tissues in 1974. In 1975 Köhler and Milstein presented the hybridoma technique to produce monoclonal antibodies (mAbs) by fusing an antibody-producing B cell with a myeloma cell that is selected for its ability to grow in tissue culture (Köhler and Milstein, 1975). Prior to this, polyclonal antibodies (anti-sera that contain molecularly different antibodies that target multiple epitopes with varying specificity) were used. These result in higher levels of nonspecific background staining than mAbs. The hybridoma technique enabled the use of mAbs in IHC, with a broad range of antigens and high staining quality. The most common fixative used is formaldehyde, a semi-reversible, covalent crosslinking reagent that can be used for perfusion or immersion fixation for any length of time, depending on the level of fixation required. Other fixatives are available, and their use depends on the antigens that are being sought. This fixation preserves morphologic features but compromises antigenicity to a certain extent. It induces alterations in the tertiary and quaternary structures of proteins but does not cause irreversible reduction or total loss of antigenic determinants in paraffin sections. Therefore, the epitopes of interest remain intact (Dill and Shortle, 1991). For immunohistochemical analysis formalin-fixed, paraffin-embedded tissue should be cut into 4 μm thin sections and mounted on glass slides. Enzyme digestion by trypsin or protease can be used to “unmask” antigens that have been altered by

formalin fixation. The most common antigen retrieval technique to restore the tertiary structure is heating tissue sections in water or buffered solutions (e.g., citrate or EDTA buffer).

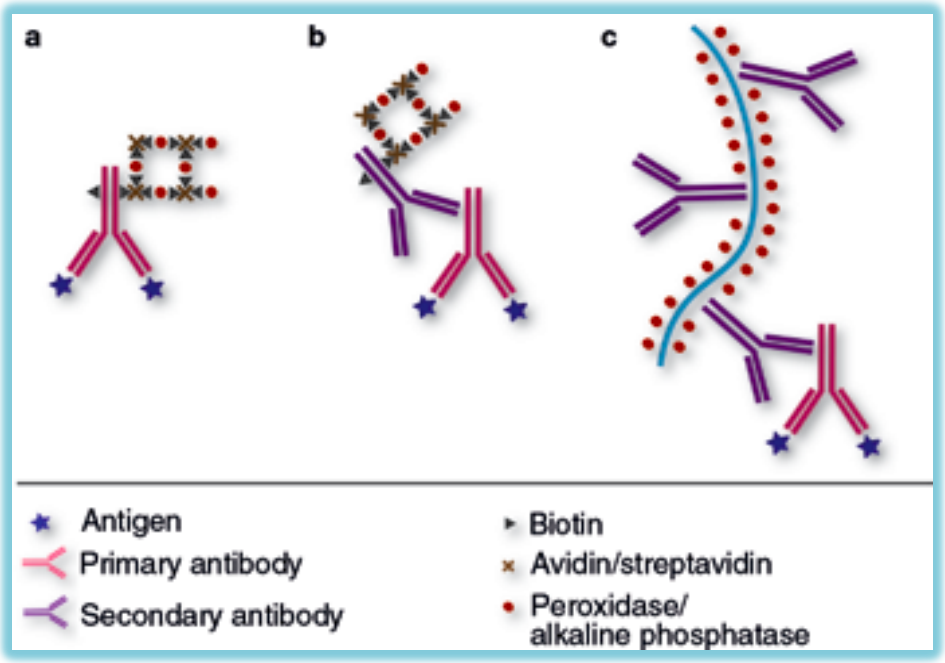


**Figure 1. Schematic diagram of immunohistochemical techniques.(a) Direct method: the antigen-specific primary antibody is biotin labeled. Biotin binds to avidin/streptavidin. Color visualization is achieved through enzymatic reaction of horseradish peroxidase/alkaline phosphatase. (b) Indirect method: the antigen-specific primary antibody is unlabeled. The secondary, biotin-labeled antibody binds to primary antibody. Visualization is achieved accordingly through avidin/streptavidin and peroxidase/alkaline phosphatase complexes. The indirect method increases versatility because unlabeled primary antibodies can be used. (c) Indirect method with polymer chain detection system. Biotin and avidin/streptavidin are replaced by a labeled polymer chain, allowing for increased sensitivity and specificity.**

For the direct method, labeled monospecific antibody is directly applied to the tissue section (Figure 1a) is most frequently conjugated with biotin. Biotin then binds to labeled avidin or streptavidin. Through this second layer of labeling, the staining is amplified. Therefore, the development of these multiple-step detection methods resulted in greatly improved sensitivity of IHC. Thus, these multiple-step detection methods allow for detection of a wide range of antigens in routine diagnostic FFPE tissues. The indirect method uses two layers of antibodies (Figure 1b and 1c). Progression from the one-step direct conjugate method to the multiple-step indirect method greatly increased the versatility of IHC because a wide range of unlabeled primary antibodies could then be used.

Antibody molecules cannot be seen (even under electron microscopy) unless they are labeled or tagged for visualization. Labeling techniques include fluorescent compounds (e.g., for direct immunofluorescence) or active enzymes (for IHC). In IHC, enzymes are added to the tissue sections, and these enzymes bind to the biotin, avidin/streptavidin labeled antibodies; the enzymes used are horseradish peroxidase or calf intestine alkaline

phosphatase (Figure 1a and b). Then chromogens are added to the sections and oxidized by horse-radish peroxidase or alkaline phosphatase, leading to a color reaction. The most widely used chromogens result in red or brown IHC staining. The method shown in Figure 1b is the most widely used; however, newly developed detection systems do not rely on antibody labeling through biotin and avidin/streptavidin. Instead, multiple secondary antibodies and enzymes are linked to a polymer backbone (Figure 1c). These new methods have the advantage of decreased background staining (higher specificity) and increased sensitivity. Double staining (different colors) in one tissue section can be achieved through a combination of two immunoenzymatic systems or one immunoenzymatic system with different substrates. Quality control is essential to ensure that an IHC staining is sensitive and specific, reproducible, and standardized. There can be many pitfalls in IHC (Yaziji and Barry, 2006); therefore, the use of positive and negative controls in each staining run is essential. A positive control is a well-characterized sample that contains the antigen of interest and is stained the same way as the specimen to be checked. The same sample is used for the negative control as for the positive control. It is stained with the same procedure, but the primary antibody is replaced by nonbinding Ig from the same species. Reasons for false-negative results include improper tissue fixation, processing, or pretreatment. False-positive results can occur through nonspecific background staining. The most common cause of this is ionic binding of antibodies to charged connective tissue elements, e.g., collagen fibers. To avoid this, it is recommended that the tissue be incubated with normal serum of the same species as the secondary antibody (blocking). Moreover, endogenous enzyme activity must be blocked (taking into account the fixation and retrieval method) to further avoid false-positive reactions. Undissolved precipitates of chromogen or counterstain can also be mistaken for a positive reaction. Validation of IHC methodologies can be achieved by participation in round robin tests, by staining various tissue and tumor types to determine sensitivity and specificity, or by comparing staining results of different antibodies that recognize similar proteins (Schacht V, 2015).

**7. The choice of the antibodies**

Mesothelial cells are unique since they are derived from the mesoderm and express the mesenchymal intermediate filaments vimentin and desmin, they also express cytokeratins, which are intermediate filaments characteristic of epithelial cells (Ferrandez-Izquierdo et al., 1994; Afify et al., 2002). In veterinary medicine, immunohistochemical procedures are well established and used to characterize several neoplasms (Ramos-Vara et al., 2008), but there are very few studies about the immunoprofile of canine and feline effusion. In human medicine, instead, this field has been largely studied with the aim to differentiate epithelial from mesothelial cells and reactive mesothelial cells from Malignant Mesothelioma. In

histopathology (Tot, 1999; 2001; 2002; Chu et al., 2000; Chu et al., 2002) and cytopathology (Fetsch and Abati, 2001; Davidson et al., 2001; Politi et al., 2005; Sack and Roberts, 1997) markers like cytokeratin (CK) AE1/AE3, CK5/6, vimentin (Vim), desmin (Desm), and HBME-1 have been proven to be useful for the identification of mesothelial cells. Cytokeratins (CK) are intracytoplasmic intermediate filaments expressed in mesothelia, epithelia, and tumours derived from these tissues. Broad-spectrum low-molecular-weight cytokeratins are expressed in both mesothelioma and carcinoma, and therefore have little discriminatory value. However, the CK5/6 pair is almost exclusively expressed in mesothelial derivatives and therefore has the potential to distinguish between carcinoma and mesothelioma. The antibody CK5/6 specifically targets the CK5 moiety of this cytokeratin pair. In 2014 Yan et al demonstrated that the combination with CK5/6 and IMP3 immunostaining is useful to improve the accuracy of cytological diagnoses between reactive mesothelial cells and metastatic adenocarcinoma in pleural effusion with a good sensitivity (78.1%) and specificity (85.5%) for detecting reactive mesothelial cells.

In human medicine, metastatic adenocarcinoma from an unknown primary site is a common clinical problem that leads to extensive and costly clinical and radiological examinations, sometimes with disappointing results. Cytokeratin phenotyping, especially CK20 and CK7, has been proposed to assist and direct the clinical and radiological efforts. Cytokeratin phenotyping also may be beneficial when examining patients with more than one known primary tumours. A proper diagnosis of the primary site is important not only for therapeutic decision-making, but also for correct epidemiological registration, which, in turn, influences the knowledge about the natural history and prognosis of particular tumour types. In 2002 Tot et al published a review of 29 studies showing the practical value of determining CK20 and CK7 in adenocarcinomas. Detection of CK20 in blood, bones (marrow), brain, serous membranes and fluids indicates metastatic tumour spread. The CK20+/7- phenotype indicates metastatic adenocarcinoma, most often from the colon or rectum, not only in bones, brain, or serous membranes, but also in liver, ovaries and lungs. The CK20-/7- phenotype indicates metastatic adenocarcinoma, most often of the prostate, in all the previously mentioned sites.

Another dilemma for both cytopathologists and pathologists is to distinguish reactive mesothelium from Malignant Mesothelioma even in tissue specimens, such as small pleural biopsies. With the aim to solve this problem numerous studies have tested immunohistochemical markers to distinguish reactive mesothelial cells from neoplastic mesothelial cells. The intermediate filament protein desmin is a known marker for smooth and skeletal muscle differentiation. Several studies have reported positive staining of benign mesothelial cells in serous fluid and tissue sections for desmin (Scoones, 1993, Hurlimann, 1994, Wolanski, 1998, Dabbs, 2006). The exact etiology for expression of desmin in

mesothelial cells is not known; however, the multipotential role of mesothelial cells with possible muscle differentiation and coexpression of desmin have been proposed by some studies (Bolen et al., 1986, Afify et al., 2002). In 2010 Hasteh et al confirmed cytoplasmic expression of desmin in reactive mesothelial cells with a sensitivity of 84% and specificity of 94% in cytologic effusion specimens.

HBME-1 is a monoclonal antibody raised from the mesothelioma cell line SPC111 that is suitable for paraffin-embedded tissue. The target epitope is located in microvilli but its exact nature is uncertain. In a review published in 2006, King et al. identified a total of 14 studies evaluating HBME-1, including 769 cases of mesothelioma and 676 cases of carcinoma. Overall sensitivity and specificity were 85% and 43%.

Claudins are a family of 27 proteins that constitute the major components of tight junctions. In the last decade, the role of some claudins in distinguishing mesothelioma from carcinomas has been reported. In 2002, Gordon et al showed a highly differential gene expression of claudin 7 between mesothelioma and lung adenocarcinoma. Some years later, Holloway et al listed claudin 3 and claudin 7 among the highly differentially expressed genes between the 2 pathologies. Simultaneously, Soini et al showed that cldn1, 3, 4, 5, and 7 expression could be used as an adjunct in the differential diagnosis between these tumours. In 2015 Kleinberg et al reported the usefulness of claudin1, 3, and 7 in serous effusion; and Facchetti et al reported the usefulness of cldn4 in pleural and peritoneal biopsies and effusions. Recently, Ordóñez et al (2013), Ohta et al (2013) and Facchetti et al (2007) confirmed the diagnostic utility of cldn4 in distinguishing mesothelioma from carcinoma with 100% specificity and sensitivity (Ohta et al, 2013). Another recent study, published by Chaouche-Mazouni in 2015, demonstrated that Claudin 4 seems to be strongly expressed in 100% of lung carcinoma specimens and completely absent in malignant mesothelioma, confirming once again the results previously obtained and thus highlighting its usefulness in the differential diagnosis between these tumors.

In veterinary medicine published information regarding the immunohistochemical diagnosis of mesothelioma mostly concerns the coexpression of the tumor cells with panCK (AE1/AE3) and vimentin (Reggeti et al., 2005). An immunohistochemical study involving a larger panel of markers, in 10 cases of feline mesothelioma, revealed that all cases coexpressed cytokeratin and vimentin, six showed reactivity to HBME-1, but only two were positive for CK5/6 (Bacci et al., 2006). In 2015 Wallace at al published a study regarding the evaluation of a gel foam cell block technique for converting potential neoplastic cells in cavitary effusions into cell blocks to characterize these further by IHC, but they had a low number of cases and use a small IHC protocol with CD3 and CD79a to immunophenotyping lymphoma's effusions and CK AE1/AE3 and Vimentin to characterize reactive and

carcinoma effusions. Given the lack of data regarding immunohistochemistry of effusion of companion animals, further studies are needed to characterize mesothelial cells.

# AIMS OF THE THESIS

The hypothesis of this PhD work is that immunohistochemistry can reliably help conventional cytology on the achievement of an accurate diagnosis on effusion samples, with the primary goal the identification of the origin of neoplastic cells. In this vein, the main purposes of this work are:

1) To asses the usefulness of cell block preparation with Bio-agar gel for immunohistochemical analysis.

2) To describe and characterize the anti-Claudin 4 in canine and feline effusions as epithelial marker.

3) To evaluate the usefulness of a panel of markers in distinguishing epithelial from mesothelial cell and reactive mesothelial cell from Malignant Mesothelioma:

- Cytokeratin/Vimentin coexpression, CK5/6 and HBME-1 as mesothelial markers;
- Desmin as a marker of reactive mesothelial cell;
- Claudin 4 as epithelial marker;
- Coordinate expression of CK7-/CK20+ as a marker of metastasis of epithelial neoplasm

# MATERIALS AND METHODS

## 1. Effusion cytology

Fluid samples were routinely collected by referring clinicians from the pericardial, peritoneal, and pleural space. Only cases that fulfilled the following criteria were selected: cytological diagnosis of reactive effusion; cytological diagnosis of malignancy of non-hematopoietic origin (carcinoma or mesothelioma); 6 months of follow up or histopathology. Fluids were preserved in EDTA tubes and refrigerated at 4°C. Within 4 hours of collection, fluids were smeared and, for non-turbid fluids with low to moderate cellularity (<5000 nucleated cells/uL), sedimentation slides were also prepared. Samples were air-dried and stained with May-Grünwald Giemsa. Cytomorphological features were assessed. Signalment, clinical information and other diagnostic procedures were recorded. The cytological diagnoses were confirmed by histopathology or follow up information. Necropsy was performed when possible.

## 2. Cell Block preparation

After the smears were prepared, 1 ml of fluid was transferred into an Eppendorf tube, and then centrifuged at 1,500 rpm for 5 minutes. Then, the supernatant was discarded and formalin (4% formaldehyde in phosphate buffered saline) was added to fix cells for at least 12 hours. The tube of Bio-Agar gel was heated at 50°C in a water-bath to liquefy the agar. Formalized samples were put into a Falcon tube and centrifuged at 3,000 rpm for 5 minutes. The supernatant was discarded and 6-10 drops of liquefied Bio-Agar gel were added. The material was gently mixed using a Vortex. Then the tube was placed in a freezer and allowed to solidify for about 3-5 minutes in an upright position. The agar cast containing the sediment that had accumulated on top was removed from the Falcon tube. The top of the cast containing sediment and agar was cut off, placed in a cassette and routinely processed through paraffin.


**Fig. 2- Cell block with bio-agar gel**

### 3. Immunohistochemistry

Formalin-fixed, paraffin-embedded samples were cut using a microtome (4 µm–thick sections) and placed on silane-covered slides. Sections were dried in a stove at 50°C for 30 minutes. Morphological assessment of the samples was obtained by examining sections stained with Hematoxylin-Eosin (H&E).. All slides were stained with anti-pancytokeratin 116, anti-vimentin, anti-cldn4, anti-keratin 5/6, anti-keratin 7, anti-keratin 20, anti-HBME-1 and anti-desmin using an automated immunostainer (BenchMark XT processor, Ventana, Tucson, AZ) that uses a modified technique for the incubation of primary antibodies by reaction streptavidin-biotin-peroxidase and 3'-3- diaminobenzidine as a chromogen.

An external positive control section was included in each immunohistochemical analysis. For the antibody dilution and sources, see Table 1.

**Table 1 – List of specificity, source, clone and dilution of antibodies applied in this project.**

| Antigen | Human specificity | Source | Clone | Dilution |
|---|---|---|---|---|
| PanCK | Epithelial cells | Dako | MNF116 | 1:1500 |
| Vimentin | Mesenchymal cells | Ventana | V9 | prediluited |
| Claudin 4 | Epithelial cells | ThermoFisher | 3E2C1 | 1:80 and 1:40 |
| Desmin | Striated and smooth muscle cells; mesothelial cells | Ventana | DER II | prediluited |
| HBME-1 | Mesothelial cells | Dako | | 1:100 |
| CK 7 | Ductal epithelium | Dako | OV TL12/30 | 1:100 |
| CK 20 | Merkel cell | Dako | Ks 20.8 | 1:100 |
| CK 5/6 | Stratified squamous epithelium Mesothelial cells | Ventana | D5/16 B4 | prediluited |

ThermoFisher Scienntific, USA; Ventana, USA; Dako, Glostrup, Denmark

Sections were deparaffinized with xylene and rehydrated with ethanol. The Ventana Benchmark instrument performed automatically inhibition of endogenous peroxidase using peroxide hydrogen in 3% solution and the antigen retrieval necessary for formalin-fixed sections by using heating or enzymes, see Table 2.

**Table 2 – List of time and methods of antigen retrieval and time and temperature of incubation of primary antibodies applied in this project.**

| | Antigen retrieval | | Incubation of Primary antibody | |
|---|---|---|---|---|
| | **Method** | **Cell conditioning** | **Time (min)** | **°C** |
| PanCK | Protease 1 | - | 60 | 37 |
| Vimentin | Heat | 8'-30' | 30 | 37 |
| Claudin 4 | Heat | 8'-30'-60' | 120 | 37 |
| Desmin | Heat | 8'-30' | 30 | 37 |
| HBME-1 | Heat | 8'-30' | 30 | 37 |
| CK 7 | Protease 1 | - | 30 | 37 |
| CK 20 | Protease 1 | - | 30 | 37 |
| CK 5/6 | Heat | 8'-30'-60' | 30 | 37 |

The Ventana ultraview universal DAB Detection kit (an indirect, biotin-free system for detecting mouse IgG, mouse IgM and rabbit primary antibodies) was used for all samples. The enzyme was directly conjugated to the secondary antibody by long-arm linkers eliminating the polymer backbone that can limit functionality and sensitivity. The smaller multimer molecule minimized steric hindrance and improved sensitivity.

As negative staining controls, the primary antibodies were replaced with the primary antibody diluents. All slides were counterstained with Haematoxylin to visualize the nuclei.

When the staining procedure was completed, in order to preserve for long-term usage and storage and to prevent enzymatic product solubilisation, all samples were mounted with a coverslip with an appropriate mounting that stabilizes the tissue sample and stain.

Immunohystochemical staining was characterized based on pattern (membranar or cytoplasmic). Stain intensity was classified as: – (negative); + (faint); ++ (moderate); and +++ (strong), subjectively assessed in at least 10 mid power fields (x40 objective) in random areas of the specimen. A staining pattern of positive occasional dispersed cells was considered negative. Coexpression of Vim/panCK, and the individual markers HBME-1 and CK5/6 were evaluated as mesothelial markers. Desmin was evaluated for its usefulness on the distinction between reactive mesothelium and mesothelioma. Claudin 4 was evaluated as epithelial marker. Finally, the coordinate expression of CK7-/CK20+ was investigated as a marker of carcinoma cells in effusion.

Sensitivity, specificity, and overall accuracy measurements with positive and negative predictive values from immunohistochemistry were determined using the following equations:

1. Sensitivity = (TP/ (TP+FN)) × 100
2. Specificity = (TN/ (TN+FP)) × 100
3. Overall accuracy = ((TP+TN)/ (TP+TN+FP+FN)) × 100
4. Positive predictive value = (TP/ (TP+FP)) × 100
5. Negative predictive value = (TN/ (TN+FN)) × 100

TP= true positive, TN= true negative
FN= false negative, FP= false positive

# RESULTS

## 1. Clinical Data

28 effusion samples were included in this study. There were 23 canine and 5 feline effusions. Specific site of the effusion and diagnostic groups are reported in table 3. Main represented breeds of dogs were German shepherd, Golden Retriever and Labrador retriever. Cats were mainly Domestic Shorthair.

**Table 3. List of cases, cytological and histological diagnosis**

| | Case n. | Patient | Location | Cytologic diagnosis | Hystology/follow up |
|---|---|---|---|---|---|
| 1 | C1/15 | Cat, DHS, MC 11 Y | pleural | CA vs MM | Lung Carcinoma |
| 2 | C2/15 | Dog, Pinscher, M 11 Y | pleural | CA vs MM | Carcinoma |
| 3 | C3/15 | Cat, DSH, MC 10 Y | pleural | RM | Histiocytic Sarcoma Lung |
| 4 | C4/15 | Dog, Golden Retriever, MC 12 Y | pericardial | RM | Chronic Pericarditis |
| 5 | C8/15 | Cat, DSH, FN , 12 Y | pleural | CA vs MM | Lung Carcinoma |
| 6 | C10/15 | Dog, Labrador Retriever, M 7Y | pericardial | RM | Chronic pericarditis |
| 7 | C11/15 | Dog, Magyar agar, FN 12 Y | abdominal | RM | Adrenal neplasia |
| 8 | C15/15 | Dog, Mixed breed, MC 12 Y | pleural | RM vs MM | Chronic pleuritis |
| 9 | C30/15 | Dog, German Shepherd, M 9 Y | pleural | CA vs MM | Mesothelioma |
| 10 | C31/15 | Dog, Golden retriever, M 7 Y | pericardial | RM | Chronic Pericarditis |
| 11 | C34/15 | Dog, Beagle; M 8 Y | pericardial | RM | Auricular Emangiosarcoma |
| 12 | C39/15 | Dog, Boxer, M 8 Y | pericardial | RM | Idiopathic Pericarditis |
| 13 | C40/15 | Cat, DSH, FN 12 Y | pleural | CA | Lung Carcinoma |
| 14 | C43/15 | Dog, German Shepard, M 11 Y | Pleural | RM | Undifferentiated sarcoma |
| 15 | C44/15 | Dog, German Shepherd M 10 Y | Abdominal | RM | Emangiosarcoma |
| 16 | C50/15 | Dog, German Shepherd F 10 Y | Abdominal | CA | Ovarian Carcinoma |
| 17 | C51/15 | Dog, Golden retriever, F 7 Y | Pleural | MM vs CA | Mesothelioma |
| 18 | C52/15 | Dog, Golden retriever F 7 Y | Abdominal | MM vs CA | Mesothelioma |
| 19 | C53/15 | Dog, Labrador retriever, M, 8 Y | Pericardial | RM | Chronic pericarditis |
| 20 | C54/15 | Dog, Boxer, M 10 Y | Pericardial | RM | Chronic pericarditis |
| 21 | C55/15 | Dog, Pittbull, M 10 Y | Pleural | CA | Lung Carcinoma |
| 22 | C56/15 | Dog, mixed breed, M 8 Y | Pericardial | RM | Chronic pericarditis |
| 23 | C57/15 | Dog | Pericardial | CA | Mucinous adenocarcinoma |
| 24 | C58/15 | Cat | Pleural | CA | Carcinoma |
| 25 | C62/15 | Dog | Abdominal | CA | Carcinoma |
| 26 | C63/15 | Dog | Pleural | CA | Carcinoma |
| 27 | C65/15 | Dog, mixed breed, F 12 Y | Pleural | CA | Lung Carcinoma |
| 28 | C66/15 | Dog, German Shepherd, F 9 Y | Abdominal | MM | Mesothelioma |

**CA=Carcinoma, MM=Malignant Mesothelioma, RM= Reactive Mesothelium**

**Table 4. Selected cases of effusion from dogs and cats. Legend: RM: Reactive mesothelium, CA: Carcinoma, MM: Malignant Mesothelioma**

| | Pleural | | Abdominal | | Pericardic | | n. |
|---|---|---|---|---|---|---|---|
| | Dog | Cat | Dog | Cat | Dog | Cat | |
| RM | 3 | 1 | 2 | 0 | 7 | 0 | **13** |
| CA | 4 | 4 | 2 | 0 | 1 | 0 | **11** |
| MM | 2 | 0 | 2 | 0 | 0 | 0 | **5** |
| | 9 | 5 | 6 | 0 | 8 | 0 | |

In this study we analyzed 13 reactive effusion, mostly pericardial, and 11 carcinoma effusion, five of which were lung carcinomas and 4 malignant mesotheliomas.

## 2. Cytological Features

Reactive effusions were characterized by the presence of a moderate number of mesothelial cells isolated and/or in monolayer cell aggregates. In rare cases of mesothelial proliferation a core of eosinophilic material was seen. Nuclei were round or oval with distinct nuclear membranes, chromatin vesicular or finely granular, and cytoplasm was abundant and deep blue stained. Peripheral cytoplasm was often more deeply stained than perinuclear cytoplasm. At the cytoplasmic margin, microvilli often resulted in fuzzy rim or border. Multinucleation (usually binucleation) was frequently seen. Degenerative cytoplasmic vacuoles sometimes compressed the nucleus, mimicking signet ring cells of adenocarcinoma. Marked reactivity features such as cytoplasmic hyperbasophilia and vacuolation, mitotic activity, multinucleation and macrokaryosis were frequently present in reactive mesothelial cells (fig. 3).

Along with reactive mesothelial cells, a variable number of macrophages and other inflammatory cells were frequently present.

Fig. 3. Reactive Mesothelium

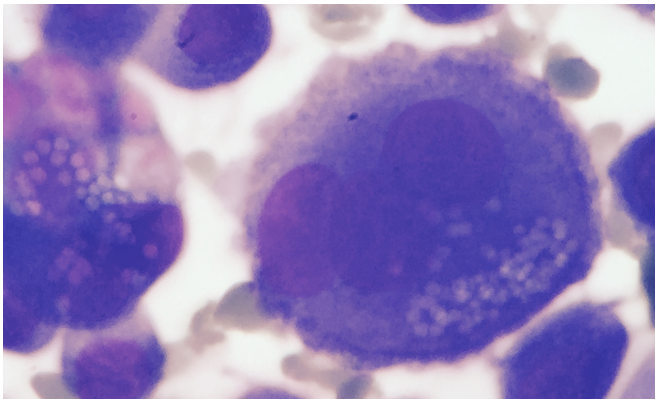

Fig. 3A

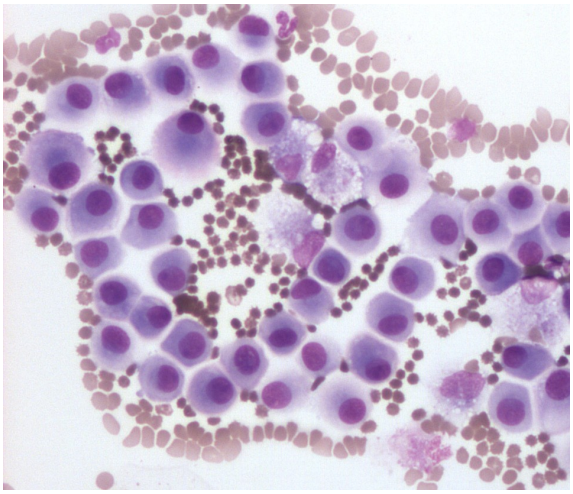

Fig. 3 B

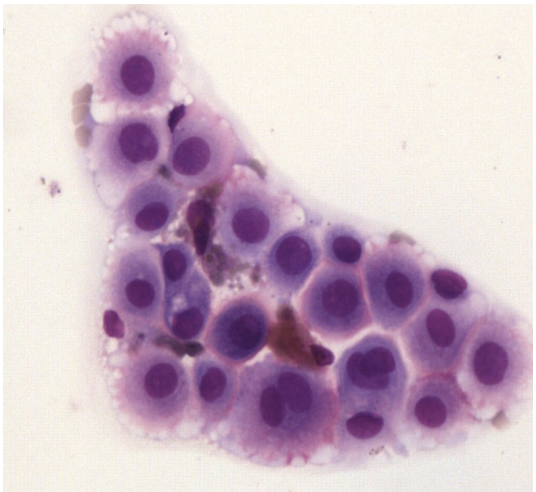

Fig. 3 C

Mesothelioma cases varied in morphology. Some cases had highly atypical groups of cells difficult to distinguish from carcinoma cells, while others had a prevalent population of well-differentiated cells with moderate criteria of malignancy making difficult to make a differential with reactive mesothelium. In Mesothelioma cases large nucleoli, multinucleated cells, cannibalism and mirror-ball-like were frequently observed (Fig.4).

**Fig. 4 – Mesothelioma. A, macroscopic appearance of omentum with numerous confluent neoplastic masses. B-D, cytologic appearance of neoplastic mesothelial cells with moderate to marked malignant criteria.**

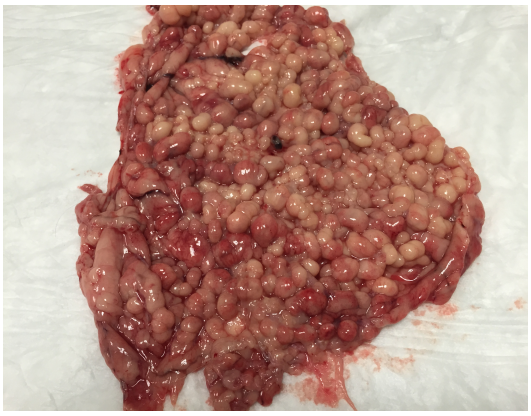

Fig. 4A

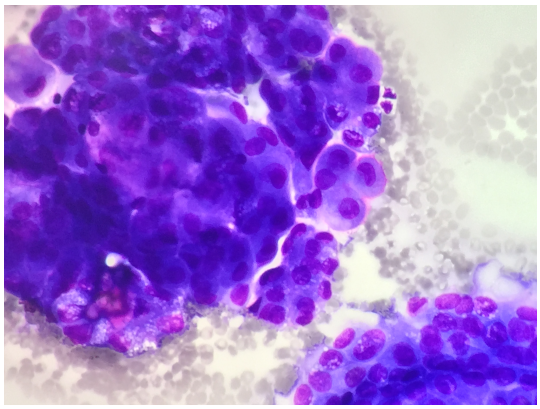

Fig. 4B

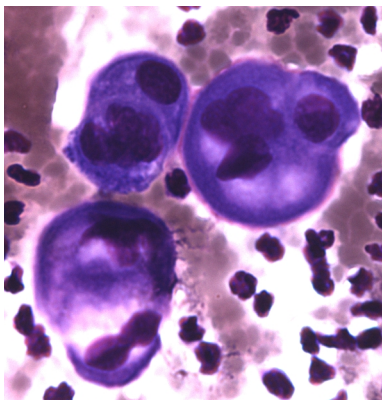

Fig. 4C

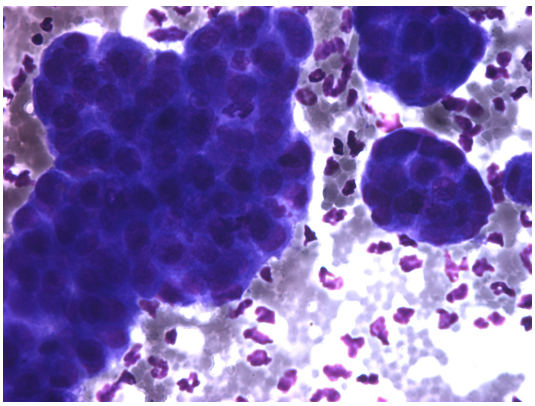

Fig. 4D

Most adenocarcinoma smears showed a population consisting of foreign appearing cells in a background of reactive mesothelial cells. Carcinoma cases were frequently characterized by large groups of cohesive cells in acinar, papillary or tridimensional conformation. Large cells, occasionally with signet ring appearance, were often present. The nuclear/cytoplasmic (N/C) ratio was variable, nuclei varied from central to, most commonly, eccentrically located. Features of malignancy such as anisokaryosis, macrokaryosis and anisonucleolosis were frequently seen (fig. 5).

**Figure 5- Carcinoma. Cytologic appearance of carcinoma cells, in large tridimensional groups (C), small clusters (A) or isolated (B)**

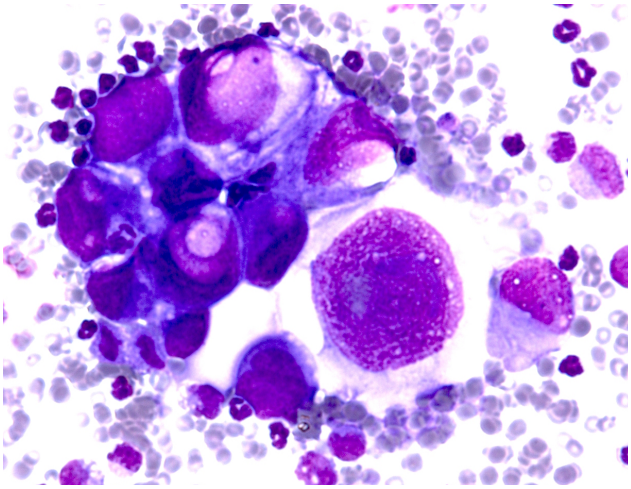

Fig. 5A

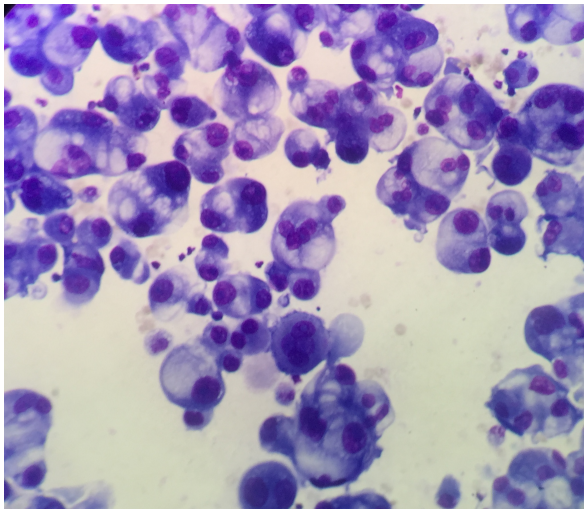

Fig. 5B

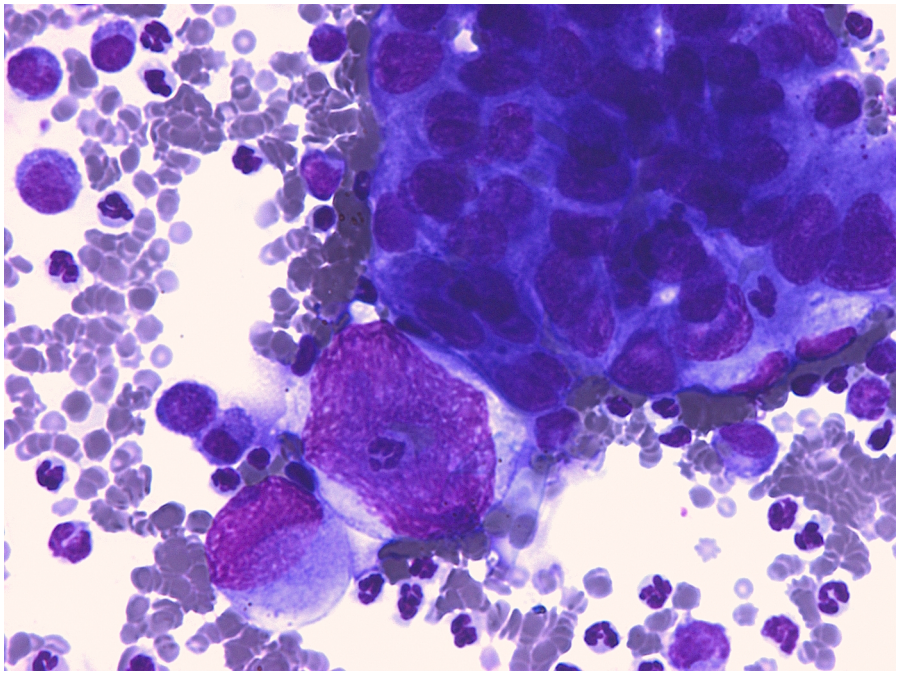

Fig. 5C

### 3. Immunocytochemistry

The immunoreactivity on cytological specimens to the individual antibodies is given in table 4.

**Table 4 Immunohistochemistry results**

| | Case n. | Diagnosis | PanCK | Vimentin | Desmin | CK5/6 | CK7 | CK20 | Cld4 | HBME-1 |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | C1/15 | CA | +++ | - | - | - | + | +++ | - | - |
| 2 | C2/15 | CA | +++ | ++ | - | - | + | - | - | - |
| 3 | C3/15 | RM | - | +++ | - | - | - | - | - | + |
| 4 | C4/15 | RM | +++ | +++ | ++ | - | - | - | - | + |
| 5 | C8/15 | CA | +++ | - | - | - | ++ | + | - | - |
| 6 | C10/15 | RM | +++ | +++ | + | + | - | - | - | + |
| 7 | C11/15 | RM | + | + | - | - | - | - | - | + |
| 8 | C15/15 | RM | +++ | +++ | ++ | ++ | - | - | - | ++ |
| 9 | C30/15 | MM | +++ | +++ | - | ++ | - | - | - | + |
| 10 | C31/15 | RM | ++ | +++ | + | - | - | - | - | + |
| 11 | C34/15 | RM | ++ | ++ | - | - | - | - | - | - |
| 12 | C39/15 | RM | +++ | +++ | ++ | ++ | ++ | ++ | - | - |
| 13 | C40/15 | CA | +++ | - | - | - | - | - | - | - |
| 14 | C43/15 | RM | +++ | +++ | + | + | - | - | - | + |
| 15 | C44/15 | RM | +++ | +++ | + | + | - | - | - | ++ |
| 16 | C50/15 | CA | +++ | - | +++ | - | - | - | - | ++ |
| 17 | C51/15 | MM | +++ | +++ | + | +++ | - | - | - | +++ |
| 18 | C52/5 | MM | +++ | +++ | + | +++ | - | - | - | +++ |
| 19 | C53/15 | RM | + | ++ | + | + | - | - | - | + |
| 20 | C54/15 | RM | +++ | +++ | + | ++ | + | + | - | + |
| 21 | C55/15 | CA | +++ | - | + | - | +++ | +++ | + | - |
| 22 | C56/15 | CA | +++ | +++ | + | ++ | - | - | - | + |
| 23 | C57/15 | CA | +++ | + | - | - | + | - | +/- | - |
| 24 | C58/15 | CA | +++ | - | - | - | - | - | +/- | - |
| 25 | C62/15 | CA | +++ | ++ | - | - | - | - | - | - |
| 26 | C63/15 | CA | +++ | - | - | - | ++ | + | - | - |
| 27 | C65/15 | CA | +++ | - | - | - | - | - | +++ | - |
| 28 | C66/15 | MM | +++ | +++ | - | ++ | - | - | - | ++ |

**Table 6. Sensitivity, Specificity, Positive Predictive Value (PPV) and Negative Predictive Value (NPV) of antibodies for the detection of canine and feline mesothelial cells**

| | PanCK+/Vim+ | HBME-1 | CK5/6 |
|---|---|---|---|
| Sensbility % | **94,1%** | 88,2% | 68,7% |
| Specificity % | 72,7% | 90,9% | **100,0%** |
| PPV % | 84,2% | 93,7% | 100,0% |
| NPV % | 88,8% | 83,3% | 68,7% |
| Accuracy % | 85,7% | **89,2%** | 82,1% |

CK5/6 was expressed in all cases of Mesothelioma. No carcinoma cases were reactive to CK5/6, whereas in reactive mesothelium 8/13 cases (61,5%) showed reactivity. For canine and feline samples, CK5/6 demonstrated a high specificity (100%) for mesothelium. For the detection of canine and feline mesothelial cells the coexpression of panCK and VIM displayed the best sensibility (94,1%) while HBME-1 was the antibody that presented highest sensibility, specificity and accuracy.

**Table 7. Sensitivity, Specificity, Positive Predictive Value (PPV) and Negative Predictive Value (NPV) of Desmin for the detection of canine and feline resctive mesothelial cells**

| | Desmin |
|---|---|
| Sensibility % | 76,9% |
| Specificity % | 73,3% |
| PPV % | 71,4% |
| NPV % | 78,5% |
| Accuracy % | 75,0% |

Desmin was evaluated for differentiation of reactive mesothelial cells from neoplastic cells (both mesothelioma and carcinoma cells). In our study reactivity of desmin was present in all group samples. It was observed in 76,9 % of the reactive mesothelial cells, and in 50% of mesothelioma cases, with a variable staining intensity. Moreover, it was also expressed in 18% of carcinoma cases.

**Table 8. Sensitivity, Specificity, Positive Predictive Value (PPV) and Negative Predictive Value (NPV) of Claudin4 for the detection of canine and feline carcinoma cells**

| | Claudin 4 |
|---|---|
| Sensiblity % | 36,0% |
| Specificity % | 100,0% |
| PPV % | 100,0% |
| NPV % | 61,0% |
| Accuracy % | 53,0% |

Claudin 4 was evaluated as epithelial marker. The reactivity of Claudin is only membranar. In our study Claudin 4 reactivity was seen in low number of carcinoma cases showing a lower intensity compared to the human positive control (Fig.6 A,B). Only in one case we observed a good positivity (Fig. 6 C,D).

**Fig. 6 Claudin 4 reactivity. Membranarian of positive control (human intestine). Positivity in a cell block preparation of a lung carcinoma**

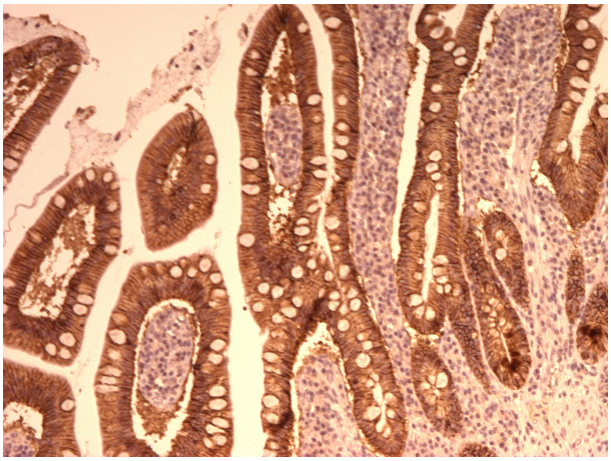

Fig. 6A

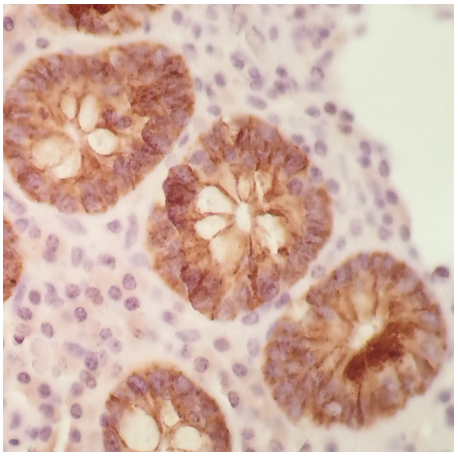

Fig. 6B

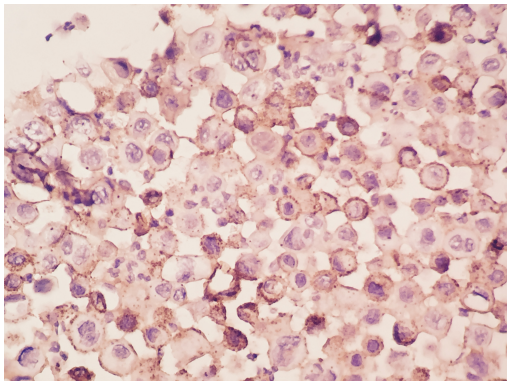

Fig. 6C

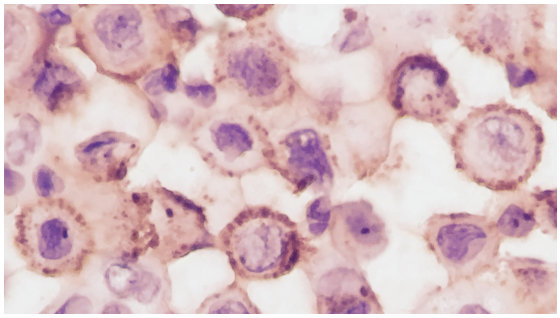

Fig. 6D

Regarding the expression of CK20+/CK7- we did not find any case with this specific pattern in our study. In 7 of 11 cases of Carcinoma we knew the primary origin of the neoplastic process but we did not find any specific correlation between the CK20/CK7 pattern and the primary site of the tumor. We observed two cases of reactive mesothelial cells staining positively with CK20+/CK7+. All Malignant Mesothelioma were negative for both CK20 and CK 7.

**Table 9. Expression of CK 20 and CK7 in Reactive mesothelium (RM), Malignant Mesothelioma (MM) and Carcinoma (CA) effusions**

| | RM | MM | CA |
|---|---|---|---|
| | % | % | % |
| **CK20-/CK7-** | 84,6 | 100 | 45,5 |
| **CK20+/CK7-** | 0 | 0 | 0 |
| **CK20-/CK7+** | 0 | 0 | 18,2 |
| **CK20+/CK+** | 15,4 | 0 | 36,4 |

# DISCUSSION

Evaluation of effusion cytology is one of the most challenging areas of diagnostic cytopathology. Serous effusions are common clinical syndromes that can be divided into benign and malignant. Differentiation between the two kinds of effusions is very important for diagnosis, treatment and prognostic evaluations. Although most cases of effusion cytology can be diagnosed on routine cytological preparations, it is often very difficult to make unequivocal interpretation. The most common difficulty encountered by cytopathologists worldwide is the inability to separate without dispute the exfoliated atypical benign mesothelial cells from metastatic cells of adenocarcinoma in effusion (Henderson et al., 1998; Whitaker, 2000). Very often the presence of a secondary inflammation can modify the mesothelial cells that reacts to the inflammation, so they may vary widely in their morphology, resulting in difficulty for the cytopathology to distinguish between macrophages, mesothelial and carcinoma cells (Mohanty and Dey, 2003). Benign reactive mesothelium undergoes to myriad architectural and cellular alterations in reaction to numerous stimuli. On the other hand, well differentiated or borderline malignant cells can mimick benign cells. Thus, definitive cytological diagnosis of serous effusions is sometimes unattainable on cytomorphologic ground alone. Additionally, other types of tumors may exfoliate into the pleural or peritoneal cavity, being the correct interpretation of the cells dependent upon the cytologist ability to recognize and characterize them (Delahaye et al., 1997; Davidson, 2004; Politi et al., 2005; Addis and Roche, 2009). Moreover, in effusion fluid, the surface tension causes cells to “roundup”, and the native shape of cancer cells in traditional fine needle aspiration cytology (FNAC) cannot be useful to decipher the tumor type. Also, the nutrient-rich effusion fluid causes cells to divide and form proliferation spheres (Whitaker, 2000). A general approach of microscopic evaluation considered useful towards the identification of neoplastic cells in effusion cytology is the “two cell population theory” (Shidham et al, 2010). Although mesothelial cells in effusion fluid present with a wide morphological spectrum, all cells of the same family are similar and demonstrate subtle morphological continuum (Shidham et al, 2010). In contrast, malignant effusions with metastatic tumors to the mesothelial cavities usually show a morphologically alien population. However, in some cases, this distinction can be extremely difficult. In such cases, further evaluation with ancillary tools such as immunocytochemistry may objectively demonstrate the second neoplastic population. (Shidham et al, 2010).

In our study we observed the most pronounced alteration of mesothelial cells in effusions from pericardial origin. The pericardium is a notorious site for mesothelial hyperplasia. These mesothelial cells become very large and basophilic with prominent nucleoli and mitotic figures. In that cases it is often impossible to distinguish reactive mesothelial cells from neoplastic cells. In addition hemangiosarcoma may cause pericardial hemorrage, but neoplasia of mesenchymal origin typically will non shed neoplastic cells (Raskin, 2010).

Cytologic analysis of pericardial effusion does not appear to be highly sensitive for achieving a diagnosis with primary cardiac neoplasms in people. However, it appears to have a moderate to high sensitivity and specificity for metastatic neoplasia in human patients (93.3–100%) (Meyers et al,1997; Wiener et al, 1991).This variable diagnostic yield in human patients is explained by the differences in the underlying etiology of pericardial neoplasia. Human patients more commonly have metastatic carcinoma effusions which can be diagnosed on cytology. Cardiac hemangiosarcoma is the most common malignant cardiac neoplasm in adult human patients. Unlike metastatic carcinomas, diagnosis of hemangiosarcoma is based on open cardiac biopsy or surgical resection of a right atrial mass with no data showing an ability to diagnose this tumor based on effusion cytology alone. The diagnostic capabilities of cytology for cardiac hemangiosarcoma appear to be similar to what is seen in dog (Maisk et al, 2010).

Because of the difficulty in distinguishing reactive mesothelial cells from malignant mesothelioma, several studies have examined different immunohistochemical markers and numerous cell blocks technique in order to improve the diagnostic power by further characterizing the origin of the cohesive cell populations in effusion (Jain et al, 2010). In our study we found bio-Agar cell block method to be a simple, fast, and effective technique of converting fluid-based cytologic samples to a format suitable for IHC staining. Conversion of fluid-based cytologic specimens to histologic preparations for characterization with IHC is becoming increasingly more prevalent in human medicine. The bio-Agar cell block method did not necessitates special equipment and requires few minutes of hands-on preparation time. We found Cell Blocks preparation from effusion to be useful adjuncts to cytologic smear for establishing a more definitive cytopathologic diagnosis. A large number of our Cell Blocks sections showed clearly recognizable normal and abnormal cells with minimal shrinkage and aberration. The cytomorphologic features were well maintained, and staining characteristics of the nucleus, nucleoli, and cytoplasm were sharp and crisp with clear recognition of nuclear and cytoplasmic features closely resembling cells in corresponding May-Grűnwald Giemsa smears (fig 7).

Fig. 7 Epithelial morphology on cytologic smear and on Block preparation

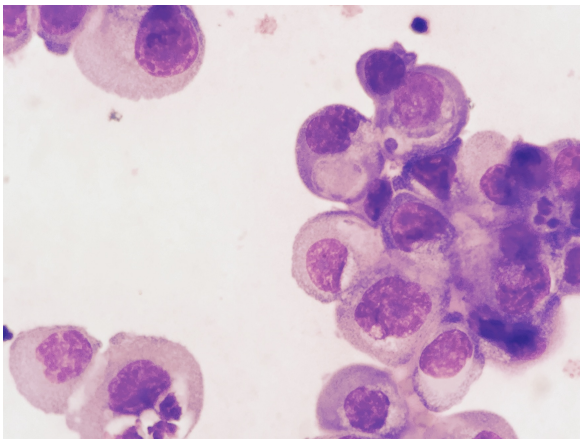

Fig. 7 a

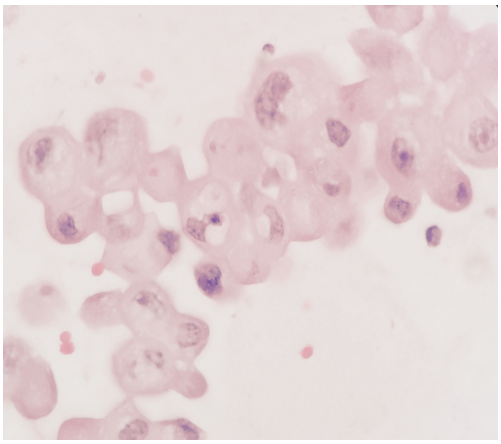

Fig. 7 b

Summarizing, the Advantages of Cell Blocks technique include good morphological interpretation, relative comparability of immunoreactivity results with formalin-fixed paraffin embedded tissue sections, evaluation of many immunomarkers simultaneously, and an archival benefit with availability of material for other types of testing in the future. (Gong et al., 2003; Kim et al., 2009; Shidham et al, 2010).

The disadvantage with the Cell Blocks technique is the delay in diagnosis because of the increased turnaround time and also that the specimen fixed in formalin, alcohol, or paraformaldehyde have excellent cell preservation but need to be processed for antigen retrieval, as it is routinely done for formalin-fixed paraffin-embedded tissues (Valli et al., 2009).

Regarding the immunoistochemical technique, an important point is that attention must be taken on evaluating a case as positive. The positivity must be considered only in cells corresponding to the morphology identified as suspect, and should not be based on the positive staining of other unspecific cells, such as the expression of Vimentin by neutrophils and macrophages. Also, reactive mesothelial cells that are present on carcinomatous effusions can produce a positive staining and interfere with the adequate assessment of the tumor reactivity.

Regarding the antibody panel examined in this PhD study, the coexpression of Vim+/pan CK and the individual markers HBME-1 and CK5/6 were studied as mesothelial markers. Both in human (LaRocca and Rheinwald, 1984) and veterinary medicine (Moroni et al., 2006), cytokeratin and vimentin coexpression within a cell is suggestive of a mesothelial origin, particularly if the filaments are prominent and in a perinuclear distribution (King et al., 2006). This coexpression, however, can be seen in other tumors including anaplastic carcinomas, amelanotic melanomas, renal carcinomas and Sertoli cell tumors (Reggeti et al., 2005).

Vimentin is a group III intermediate filament that primary identifies cells of mesodermal origin. It is expressed by both benign and malignant connective tissue, as well as in benign mesothelial cells and mesothelioma (Afify et al., 2002; Reggeti et al., 2005). Some reactivity in adenocarcinomas has also been reported. Although frequently seen in mesotheliomas, it is less often detected in sections of the epithelial component (Dejmek and Hjerpe, 2000). Vimentin can also be expressed by macrophages and neutrophils (MorVaknin et al, 2003), which are cells frequently present in effusions. In the present study, when analysed together, positivity to Cytokeratin and Vimentin was a very specific marker of mesothelial cell origin. Only 2 carcinomas (one mammary adenocarcinoma and one carcinoma of the lung) demonstrated such coexpression.

HBME-1 (Hector Battifora Mesothelial Cell-1) is a recently available monoclonal antibody that reacts with an unknown antigen on microvilli of mesothelial cells (Politi et al., 2005; Bacci et al., 2006). HBME-1 was originally described as a specific marker of normal and malignant mesothelial cells (González-Lois et al., 2001), but it was later shown to also stain many carcinomas (Longatto Filho et al., 2002; Papotti et al., 2005). In fact, it is reported that HBME-1 is very sensitive for human benign and malignant mesothelium, but also reacts with 15-100% of adenocarcinoma cells (Su et al., 2010). In our study we found a strong positivity only in one case of carcinoma of ovarian origin, whereas HBME-1 showed a good positive reaction in all mesothelial cell (both reactive and neoplastic), with good sensitivity (88,2%) and specificity (90,9%).

Desmin is an intracellular intermediate filament characteristically demonstrated in smooth and skeletal muscle. It has also been described in nonmyogenous tumors, including primitive neuro-ectodermal tumors. It is more commonly expressed in benign rather than malignant mesothelium, producing a cytoplasmic staining pattern (Gill et al., 2000; Davidson et al., 2001; Su et al., 2010). For this reason is used in human medicine to differentiate reactive from malignant mesothelium (Su et al., 2010; Hasteh et al., 2010). Other authors consider that there is not a marker able to precisely differentiate reactive mesothelium from mesothelioma (Hurlimann, 1994). Along with Desmin, other markers as epithelial membrane antigen (EMA), p53 and bcl-2 have been used but so far with confliting results (Cury et al., 1999; Attanoos et al, 2003). In our study, Desmin demonstrated a sensitivity and specificity of 76,9% and 73% respectively to identify reactive mesothelium, so it' a plenty good marker.

Cytokeratins (CKs) are monofilaments intermediate-sized filaments expressed by epithelial and mesothelial cells. The CK5/6 pair is mainly expressed in keratinizing and non-keratinizing squamous epithelium, in the basal-myoepithelial cell layer of the prostate, breast and salivary gland, and in tumours arising in these tissues. Epithelioid or biphasic malignant mesothelioma also usually expresses CK5/6. These keratins are much less frequently identified in sarcomatoid mesothelioma, where only around one third of cases are reported to

stain for CK5/6. In 2006 King et al. founded an overall sensitivity of 83% in studies examining tissue sections of epithelioid mesothelioma.

Studies of surgical specimens have also reported staining of a range of adenocarcinomas for CK5/6. An extensive study by Chu and Weis (2002) demonstrated positive staining in a range of tumours from simple epithelia, as well as a majority of squamous cell carcinoma, salivary gland tumours and mesothelioma cases. Nevertheless, the infrequent expression of CK5/6 by pulmonary adenocarcinomas has made it a useful addition to immunomarker panels used to distinguish these tumours from mesothelioma in tissue specimens. Few studies have been published on the application of CK 5/6 staining in the cytodiagnosis of effusion specimens. In 2000 Whittaker commented on the potential of CK 5/6 in this role, noting 100% staining of 26 mesothelioma cases and no observed positive cases amongst 16 adenocarcinoma specimens. However, in 2004 Han et al. tested 211 cases of metastatic adenocarcinoma in effusion specimens and reported 26% of adenocarcinoma cases to be positive staining. In our study we observed a CK 5/6 positivity in all mesothelioma cases, while carcinomas did not showed reactivity for this marker. So CK5/6 demonstrated to be the most specific marker to detect mesothelial cells.

Claudins are a family of 27 proteins that constitute the major components of tight junctions. In human medicine in the last decade, the role of some claudins in distinguishing mesothelioma from carcinomas has been described in several studies (Facchetti et al, 2007, Jakal et al, 2008, Jakal et al, 2009, Lonardi et al, 2010); in veterinary medicine instead, there are very few studies about utilization of Claudin (Roussel et al, 2014; Washiyama et al, 2014; Jakab et al, 2011) and, to our knowledge, this marker hasn't been investigate in canine and feline effusions. Claudin 4 is reported to be the most interesting marker in distinguishing lung adenocarcinoma from mesothelioma, with 100% specificity and sensitivity (Lonardi et al, 2010). In this study, we found Claudin 4 expression to be absent in both malignant mesothelioma and reactive effusion, but we could not confirm the results obtained in human medicine about the usefulness of this marker to detect epithelial cell in effusion. In our study in fact, Claudin 4 stained positively only 4 of 11 cases of carcinoma, with a very poor sensitivity of 36%. In our study with Claudin 4 we tried different time of incubation (60' and 120') e different dilution of the primary antibody to test the reactivity of canine and feline epithelial cells and we obtained better results with a diluition of 1:40 and two hours of incubation. Given the very good results in human medicine in differentiating epithelial cells from cells of other origin the use of Claudin 4 needs further investigation in order to find the suitable immunohistochemistry procedure for canine and feline samples. In 2014 Roussel et tried to develop an optimal immunohistochemical method for assessment of the expression of TJ proteins in the skin of healthy dogs. Formalin-fixed and paraffin wax-embedded skin biopsy samples from healthy human and canine patients were used. Canine skin samples

were from the inguinal region and the nasal planum. Immunohistochemistry was used to study the expression of zonula occludens-1 (ZO-1), occludin and claudin-1, -4 and -7. They used three different methods of antigen retrieval: the first with protease type XIV from Streptomyces griseus, the second and third with heat but using two different buffer solutions: Ethylenediaminetetraacetic acid (Tris–EDTA, pH 9.0; Diagnostic Biosystems, Pleasanton, California, USA) and citrate (pH 6.0; Diagnostic Biosystems). They obtained the best results with heat-induced antigen retrieval with EDTA (pH 9.0) and the worst results with protease. In our study we used heat for antigen retrieval but a different buffer solution (pH 8, ready to use from Ventana). Afterwards the immunohistochemical procedure for Claudin in canine and feline effusions could be further investigated with the use of different methods of antien retrieval, for example with EDTA.

In human medicine is proved that the detection of CK20 in blood, bones (marrow), brain, serous membranes and fluids indicates metastatic tumour spread (Ramaekers et al., 1990; Tot, 1999; 2001; 2002).. The CK20+/7- phenotype indicates metastatic adenocarcinoma, most often from the colon or rectum, not only in bones, brain, or serous membranes, but also in liver, ovaries and lungs. The CK20-/7- phenotype indicates metastatic adenocarcinoma, most often of the prostate, in all the previously mentioned sites. Absence of strong and diffuse CK20 expression in mesothelium and mesotheliomas is important information because of the well-known differential diagnostic difficulties in discriminating reactive and neoplastic mesothelial cells from cells of metastatic adenocarcinomas (Tot et al, 2002).

In our study, the coordinate expression of CK7-/CK20+ was investigated as a marker of carcinoma cells in effusion. Our results suggest that this panel of markers is not sufficiently specific for the identification of carcinomatous cells having a poor sensitivity. Moreover we determined the exact primary location of only 6 cases of carcinomas and we did not find a specific pattern correlated with a particular primary site. However, in this study the number of cases with known primary site was reduced, and no conclusions should be drawn from these results.

# CONCLUSIONS

From this study, it can be concluded that immunohistochemistry on Cell Blocks preparations is a practical method which substantially improves the diagnostic accuracy of conventional cytology. The coexpression of Vim/PanCK, CK 5/6 and HBME-1 are reliable and effective markers for the identification of mesothelial cells in canine and feline effusions with a high sensitivity and specificity. Claudin 4 was not found to be a useful marker in the differentiation of metastatic adenocarcinoma cells from reactive mesothelial cells in Cell Blocks prepared from canine and feline effusion fluids, despite the good results showed by this marker in human medicine. So the study of Claudin 4 necessitate to be deepened in veterinary medicine. The coordinate expression of CK7-/CK20+ has not proved to be useful on the identification of metastatic cells on effusion. In conclusion, the combination of both cytology and immunohistochemical studies can certainly enhance the diagnostic accuracy, sensitivity and specificity in malignant effusions, but further studies are needed in order to better characterize benign from neoplastic mesothelial cells and malignant mesothelioma vs metastatic carcinoma.

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# PAPERS

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