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Received: 30 October 2020 | Revised: 8 January 2021 | Accepted: 3 February 2021

DOI: 10.1111/vcp.13002

Veterinary Clinical Pathology
An International Journal of Laboratory Medicine
WILEY

ORIGINAL ARTICLE

Comparison of effusion cell block and biopsy immunohistochemistry in mesothelial hyperplasia, mesothelioma, and carcinoma in dogs

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Funding information
Petplan Charitable Trust, Grant/Award Number: 204-417; Petplan Charitable Trust

Abstract

Background: Determining the cause of effusions is challenging and might require a biopsy. Whether cell blocks from effusions are representative of biopsies requires investigation. A previously developed immunohistochemical panel aids in the differentiation of hyperplastic and neoplastic mesothelium in canine biopsies but has not been investigated in effusions.

Objectives: The study aimed to assess cell blocks as an alternative to biopsies and determine whether immunohistochemistry helps distinguish hyperplastic mesothelium, mesothelioma, and carcinoma.

Methods: Effusions and biopsies were collected from five dogs with mesothelial hyperplasia (group MH), six with mesothelioma (group M), and five with carcinoma (group C). Immunohistochemistry (IHC) for cytokeratin, vimentin, Wilm's tumor protein 1 (WT1), desmin, glucose transporter 1 (GLUT1), and insulin-like growth factor II mRNA-binding protein 3 (IMP3) was performed. Sections were scored for staining intensity and the percentage of positively stained cells.

Results: In paired cell blocks and biopsies, vimentin and WT1 staining were positively correlated for intensity and the percentage of positive cells, although not all paired results were identical. The intensity of IMP3 staining in cell blocks was higher in group M than in group C ($P = 0.012$), and WT1 staining was higher in group MH than in group C ($P = 0.020$). For biopsies, the intensity of WT1 staining was higher in group MH than in group C ($P = 0.031$). In group C, WT1 was negative in all cell blocks and biopsies, and desmin was negative in four of five cases.

Conclusions: IHC results for the cell blocks and biopsies were comparable for potentially useful markers, such as WT1, which helped discriminate between groups. IHC provided additional information, although results were not always definitive. Further studies on a larger population are required.

KEYWORDS
canine, carcinoma, cell block, IMP3, mesothelioma, WT1

1 | INTRODUCTION

Body cavity effusions are common in dogs and can occur in mesothelial-lined cavities such as the pericardial, pleural, and peritoneal spaces. Routine investigation includes fluid analysis for macroscopic appearance, a total nucleated cell count (TNCC), total protein (TP) concentration, and cytologic examination allowing classification as a transudate, modified (or high-protein) transudate, or exudate.[1] In veterinary medicine, cytologic abnormalities such as inflammatory cell infiltrates, neoplastic cells, reactive mesothelial cells, and etiologic agents can help determine the cause, but the diagnosis often remains unclear; in one study, the diagnostic sensitivity of effusion cytology for malignancy in dogs and cats was 60%.[2] Determining the nature of atypical cells is a particular challenge. Reactive (hyperplastic) mesothelial cells can exhibit several atypical features and can be difficult to differentiate from mesothelioma and carcinoma on cytologic examination and even histologic examination of biopsies.[3,4] Pericardial fluid also has the disadvantage that mesothelial cells can become highly reactive in this site, sometimes mimicking neoplasia.[5] In human pathology, cytomorphologic features that distinguish reactive mesothelial cells, mesothelioma, and carcinoma have been described for conventional cytology,[6] but the use of cytology alone remains controversial.[7,8] For instance, the sensitivity of cytologic examination for the diagnosis of mesothelioma in human samples varies substantially from 4% to 77%.[9]

Immunohistochemistry (IHC) panels have been developed to differentiate these conditions in human patients.[8,10] Using IHC for glucose transporter 1 (GLUT1) and insulin-like growth factor II mRNA-binding protein 3 (IMP3) in biopsies, a sensitivity of 100% and specificity of 95% were described for distinguishing mesothelioma from reactive mesothelial cells.[11] However, this was based on statistical probability, and IHC might not always be useful in an individual patient.[12] Wilm's tumor protein 1 (WT1), a tumor suppressor gene, is recognized as a reliable marker for mesothelial cells in human pathology, with 70%-95% of mesotheliomas showing positive nuclear staining and lung adenocarcinomas being negative,[8] although it does not distinguish between hyperplastic mesothelium and mesothelioma. Desmin IHC, however, is considered of value in distinguishing hyperplastic and neoplastic mesothelial cells in human patients, with positive and negative staining, respectively, and a sensitivity of 84% and specificity of 94%.[13]

We previously described an IHC panel comprising cytokeratin (CK), vimentin (vim), IMP3, and GLUT1 to aid in distinguishing reactive mesothelial cells, atypical mesothelial proliferation, and mesothelioma in canine pericardial biopsies.[14] Although not definitive in all cases, the panel was considered a valuable addition to routine investigation. Applying this and other IHC panels to cells in cavitary effusions could provide a minimally invasive means of determining the histogenesis of atypical cells. This could be accomplished by IHC on effusions prepared as cell blocks, which has been well established in human pathology for this purpose, including distinguishing adenocarcinoma, reactive mesothelial cells, mesothelioma, and carcinomas.[13,15-18] In veterinary pathology, cell block IHC is a relatively new technique but has been used for distinguishing carcinoma, mesothelial cells, and lymphoma in cavitary effusions in dogs and cats.[19,20] However, our recently described IHC panel to aid in distinguishing benign from malignant mesothelial proliferations in biopsies from dogs[14] has not been applied to cell blocks. Other markers of potential value are WT1 and desmin. WT1 IHC has been validated as a means of distinguishing mesothelial cells from carcinoma in cell blocks from canine patients.[21] It did not distinguish reactive from neoplastic mesothelial cells but was consistently positive in both conditions. Desmin was found to be of limited value in previous studies on canine mesothelioma in immunocytochemistry (ICC)[22] and pericardial biopsies[14] but has not been investigated in cell blocks for distinguishing reactive and neoplastic mesothelium and carcinoma in dogs.

The primary aim of this initial prospective study was to determine whether cell block IHC provides similar results to the corresponding biopsy IHC in canine mesothelioma, mesothelial hyperplasia, and carcinoma using a panel of six antibodies and, therefore, to provide as a proof of principle, the use of cell block IHC of cavitary effusions as an alternative to biopsy. The secondary aim was to assess whether this panel is of value in distinguishing these diseases.

2 | MATERIALS AND METHODS

2.1 | Animals

The dogs were clinical cases referred to the Universities of Edinburgh, Liverpool, and Porto. The study was approved by the Veterinary Ethical Review Committee of each university (approval nos. 11/16, 410, and 237/2017 respectively), and informed consent for the use of excess samples for research was given by all owners. Inclusion criteria were an effusion in one or more of the pericardial, pleural, or peritoneal cavities, in which cells with some features consistent with mesothelial cells (either reactive or neoplastic) or carcinoma were detected on fluid cytology, and where a definitive diagnosis was made on biopsy examination. Exclusion criteria were cases in which the final diagnosis was not completely certain or where insufficient fluid was available. Cases diagnosed on biopsy as atypical mesothelial proliferations, considered to be intermediate between hyperplasia and mesothelioma,[23] were also excluded from the study, as categorization of these proliferations is controversial.

2.2 | Routine fluid analysis

The collection of effusions was carried out by standard methods,[24] and routine fluid analysis was undertaken. The TNCCs were compared between groups. Cytologic examination was carried out on the day of collection by board-certified clinical pathologists at each university after staining direct smears and/or cytocentrifuge preparations and staining with May-Grünwald Giemsa (MGG) or modified Wright Giemsa stains. The designation of the cells in fluids as reactive mesothelial cells, mesothelioma, or carcinoma was based on the experience of the clinical pathologists.

2.3 | Cell blocks and biopsies

The cell blocks were made at the university where each dog was examined, using methods already established at each site. At the University of Edinburgh, effusion fluid (2 mL, although on some occasions less if only a small sample was received) was placed in a 2-mL conical plastic tube (Eppendorf) and centrifuged at 1260*g* for 7 minutes (Biofuge 13, Heraeus GmbH). The supernatant was removed, leaving the cell pellet, and replaced with 2 mL of 10% phosphate-buffered formalin (pH 7.4), mixed using a RotaMix vortexer (Hook & Tucker Instruments Ltd.). The sample was left at room temperature for 1 hour. Centrifugation was then repeated. The supernatant was removed, replaced with 2 mL of a 2% solution of agarose ([Noble agar, cat. No. A543, Sigma-Aldrich Ltd.] dissolved in distilled H_2O and mixed with an equal volume of borate buffer [0.5 M, pH 8.6]) warmed to 50°C. The pellet was vortexed, and then the tube was centrifuged at 1260*g* for 7 minutes. The tube was kept at 4°C for 30 minutes, and then the agarose cell pellet was removed by cutting the tip off the tube and pushing it out at the top. The cell block was cut in two pieces longitudinally, and both halves were processed to a paraffin wax block using standard methods. At the University of Liverpool, a 1.3 mL aliquot of the fluid was placed in a plastic 1.5-mL Eppendorf tube and centrifuged for 5 minutes at 1200*g* (IEC Micromax, Thermo Fisher Scientific). The supernatant was removed, and 0.5 mL of 10% neutral buffered formalin was added, and the pellet was fixed for 24 hours. The cell blocks from the University of Porto were made using microhematocrit tubes, as previously described.[20] No binding agents (agarose or other) are used in the methods established at the Universities of Liverpool and Porto. The cell pellet is typically compact enough to be removed from the Eppendorf tip using forceps (Liverpool) and from the microhematocrit tube using a paper clip (Porto) before being placed into the molten paraffin wax within an embedding mold.

All tissue biopsies used for comparison had been fixed in 10% phosphate-buffered formalin (pH 7.4) and embedded in paraffin wax by routine methods.

2.4 | H&E staining and assessment

Sections of cell blocks and biopsies were cut 4 μm thick and stained with H&E for a description of the cell morphology in the case of the cell blocks, and the tissue architecture of the biopsy sections. Where more than one tissue was sampled, these were processed in the same block. The biopsies were originally examined by the duty pathologists and reviewed by the study pathologists (EM, LR, RM, and MS) at each university.

2.5 | Immunohistochemistry

Sections (4 or 5 μm depending on the laboratory) of cell blocks and biopsies were placed on SuperFrost Plus coated slides (Thermo Electron Ltd.), dewaxed, hydrated, and rinsed in distilled water. The methods for CK, vim, IMP3, and GLUT1 were as previously described.[14] Briefly, nonspecific endogenous peroxidase was blocked with the REAL blocking agent (S202386, Dako Ltd.). All antibodies were diluted in the antibody diluent (S0809, Dako). For CK, a mouse monoclonal anti-cytokeratin antibody, clone MNF116 (M0821, Dako), was diluted 1/50 following antigen retrieval using proteinase K (S3020, Dako). For vim, a mouse monoclonal anti-vimentin antibody clone V9 (NCL-L-VIM-V9, Novocastra Laboratories) was diluted 1/400 following antigen retrieval using high pH antigen unmasking buffer (H-3300, Vector Laboratories Ltd.). Toward the end of the study, sections were stained with a mouse monoclonal anti-vimentin antibody clone V9 (M0725, Dako) diluted 1/7000 due to reagent availability.

For GLUT1, a rabbit polyclonal anti-GLUT1 antibody (15309, Abcam Ltd.) was diluted 1/500, and following antigen retrieval using 0.01 M citrate buffer at pH 6.0. For IMP3, a mouse monoclonal anti-IMP3 antibody clone 69.1 (M3626, Dako) was diluted 1/50 followed by antigen retrieval using high pH antigen unmasking buffer (H-3300, Vector). The sections were then incubated with a secondary antibody (Envision anti-mouse HRP [K4007] or Envision anti-rabbit HRP [K4011], Dako, as appropriate), visualized with the DAB+chromogen (K3468, Dako). The method for WT1 was as previously described[21]; mouse monoclonal anti-human WT1 antibody clone 6F-H (Dako) diluted 1/150 was used, and antigen retrieval was performed in a 100°C water bath, with a polymer detection system (Novocastra). For desmin, a mouse monoclonal anti-desmin antibody clone DE-R-11 (NCL-L-DES-DER11, Novocastra Laboratories) was diluted 1/800 following antigen retrieval in a 100°C water bath in EDTA; the polymer detection system (Novocastra) was also used. All stained sections were counterstained with Harris hematoxylin. Positive controls were canine esophagus (GLUT1), stomach (IMP3), skin, liver, intestine, and kidney (CK), intestine (vim and desmin), human mesothelioma, and kidney (WT1). Negative controls were sections processed with antibody diluent in place of the primary antibody.

The validation of mouse monoclonal anti-cytokeratin antibody clone MNF116 and mouse monoclonal anti-vimentin antibody clone V9 has been previously described for canine tissues.[25,26] The validation of the rabbit polyclonal anti-GLUT1 antibody and mouse monoclonal anti-IMP3 antibody clone 69.1 for canine tissues was previously cited.[14] The validation of mouse monoclonal anti-WT1[21]

TABLE 1 Summary of signalment, diagnoses, and total nucleated cell count of effusions in MH (mesothelial hyperplasia), M (mesothelioma), and C (carcinoma) groups

Group	Age in years (mean and SD)	Sex	Breeds	Effusion nucleated cell count (mean and SD, $\times 10^9$/L)
MH (n = 5)	8.6 ± 1.7	F (1), FN (1), M (2), MN (1)	English cocker spaniel, Labrador, Golden retriever, Great Dane, Rhodesian ridgeback (1 each)	11.2 ± 5.6
M (n = 6)	7.0 ± 3.9	F (1), FN (1), M (4)	Golden retriever (2), Labrador, Border collie, GSHP, Mastiff (1 each)	6.9 ± 5.8
C (n = 5)	12.6 ± 3.3	F (3), M (1), MN (1),	x bred (4), Labrador (1)	10.1 ± 10.9

Note: Group C was significantly older than group M ($P = 0.030$; one-way ANOVA followed by multiple range tests); other age comparisons between groups were not statistically significant. There was no significant difference in total cell counts between groups ($P = 0.612$; one-way ANOVA [cell count not done for one case in group C]) F, entire female; FN, neutered female; M, entire male; MN, neutered male; x bred, crossbred; GSHP, German shorthaired pointer.

and mouse monoclonal anti-desmin clone DE-R-11[27] has also been described for canine tissues.

2.6 | Cell block and biopsy scoring

The sections of blocks and biopsies were scored semiquantitatively for % positive cells as 0 = completely negative, 1 = 1%-25% of cells positive, 2 = 26%-50% positive, 3 = 51%-75% positive, and 4 = ≥ 75% positive. Intensity of staining was scored as 0 = negative, 1 = weakly positive (barely visible specific stain), 2 = moderately positive (between mild and strong), or 3 = strongly positive (comparable with positive control staining). Where more than two tissues were sampled from the same case, these were scored together. The slides were initially scored independently by two pathologists (EM and LR), and a consensus was reached when scores were initially discordant. Intra-observer variation in IHC scoring was assessed separately for the intensity of staining and percentage of positively staining cells for the cell blocks and biopsies using the original scores before modification reached by a consensus. The pathologists were blinded to the initial cytologic diagnosis. In carcinoma cases, scoring was restricted to the main population of interest, that is, the neoplastic cells as identified in H&E sections, and not any mesothelial cells present. When discrepancies in the stain intensity scoring of more than 2 units occurred between cell blocks and corresponding biopsies, restaining was undertaken to ensure that the difference was not due to laboratory error. However, in each case where this was undertaken, the discrepancy was found to be consistent.

2.7 | Statistics

In each disease group, the ages of dogs at the time of clinical investigation were normally distributed (Shapiro-Wilk tests). The average age of dogs and TNCC of the fluids in each group were compared using a one-way ANOVA followed by multiple comparison tests.

To compare IHC scores for paired cell blocks and biopsies, the ordinal scores for the intensity of staining and percentage of cells positive for each antibody marker were compared across carcinoma, mesothelial hyperplasia, and mesothelioma groups using nonparametric Spearman correlation tests.

The linearly weighted kappa values were calculated using the statistical language R, version 3.6.2 (R Foundation for Statistical Computing) to assess intra-observer variability in IHC scoring.

To identify IHC markers that discriminate between disease entities, we compared IHC scores for the intensity of staining and percentage of cells positive with nonparametric Kruskal-Wallis tests followed by Dunn's multiple comparisons tests. Graphs represent the median score as a histogram bar and error bars as the interquartile range. Statistical analysis was carried out using GraphPad Prism 8 software, and statistical significance was set at $P \leq 0.05$ for two-tailed tests.

3 | RESULTS

3.1 | Animals

The signalment of the cases is summarized in Table 1. A range of breeds and sexes were represented, and group C dogs were significantly older than group M dogs ($P = 0.030$). Sixteen cases met the inclusion criteria, including 13 with pleural, two with pericardial, and one with peritoneal effusion. The 16 cases were divided into the following groups on the basis of standard histologic examination of biopsies: mesothelial hyperplasia (MH) (n = 5), mesothelioma (M) (n = 6), and carcinoma (C) (n = 5). For one case of mesothelial hyperplasia and one of mesothelioma, the paraffin wax blocks were lost before WT1 or desmin staining were performed.

3.2 | Cytology

The TNCCs were not significantly different between groups (Table 1). Examples of MGG-stained slides are shown in Figure 1A,C. In group MH, the main cytologic features were individualized, paired, and cohesively clustered, and pseudoacinar arrangements of mesothelial

cells with moderate to marked anisocytosis and anisokaryosis, often with a prominent pink fringe of microvilli at the cell border, considered to correspond with the glycocalyx. Occasionally, pink eosinophilic extracellular material was observed within the cell clusters, corresponding to the so-called collagenous cores. The cytoplasm was medium to deep blue with round to oval, central to eccentric nuclei, stippled chromatin, and variably sized nucleoli. Vacuoles were sometimes present, occasionally displacing the nucleus. Moderate numbers of bi- or multinucleated cells were present, with occasional mitoses.

In group M, mesothelial cells were individualized or in nests, sheets, and pseudoacinar structures with small to large amounts of medium to deep blue staining, sometimes vacuolated cytoplasm. Occasional cells had pink, granular material in the vacuoles. The nuclei were round to oval, medium-sized to very large, and bi- and multinucleation were frequently seen, sometimes with nuclear molding. The nucleoli ranged from one small nucleolus to multiple, large, and prominent nucleoli, and anisonucleoliosis was common. Anisocytosis and anisokaryosis were moderate to marked, and occasional mitoses were seen.

Cytologic findings in group C consisted of individuals and cohesive three-dimensional sheets, nests, and pseudoacinar structures of small to large epithelial cells, with moderate to marked anisocytosis and anisokaryosis. The cells had a moderate to large amount of medium to deep blue cytoplasm, sometimes with vacuoles and/or pink granules, and the nuclei were medium to large and round to oval with stippled chromatin and one to multiple variably sized, round nucleoli. Nuclear molding, multinucleated cells, and mitoses were sometimes observed.

3.3 | H&E staining in cell blocks and biopsies

Samples in group MH were comprised of five incisional biopsies (1 at necropsy) of thickened pleura (3) or pericardium (2). Those in group M were comprised of incisional (3), excisional (2), or Trucut (Merit Medical Systems Inc) biopsies (1) of a thoracic wall mass (2), thickened pleura (2), thickened mediastinum (1), or lung mass (1). Group C samples consisted of five excisional biopsies (4 at necropsy) of a lung

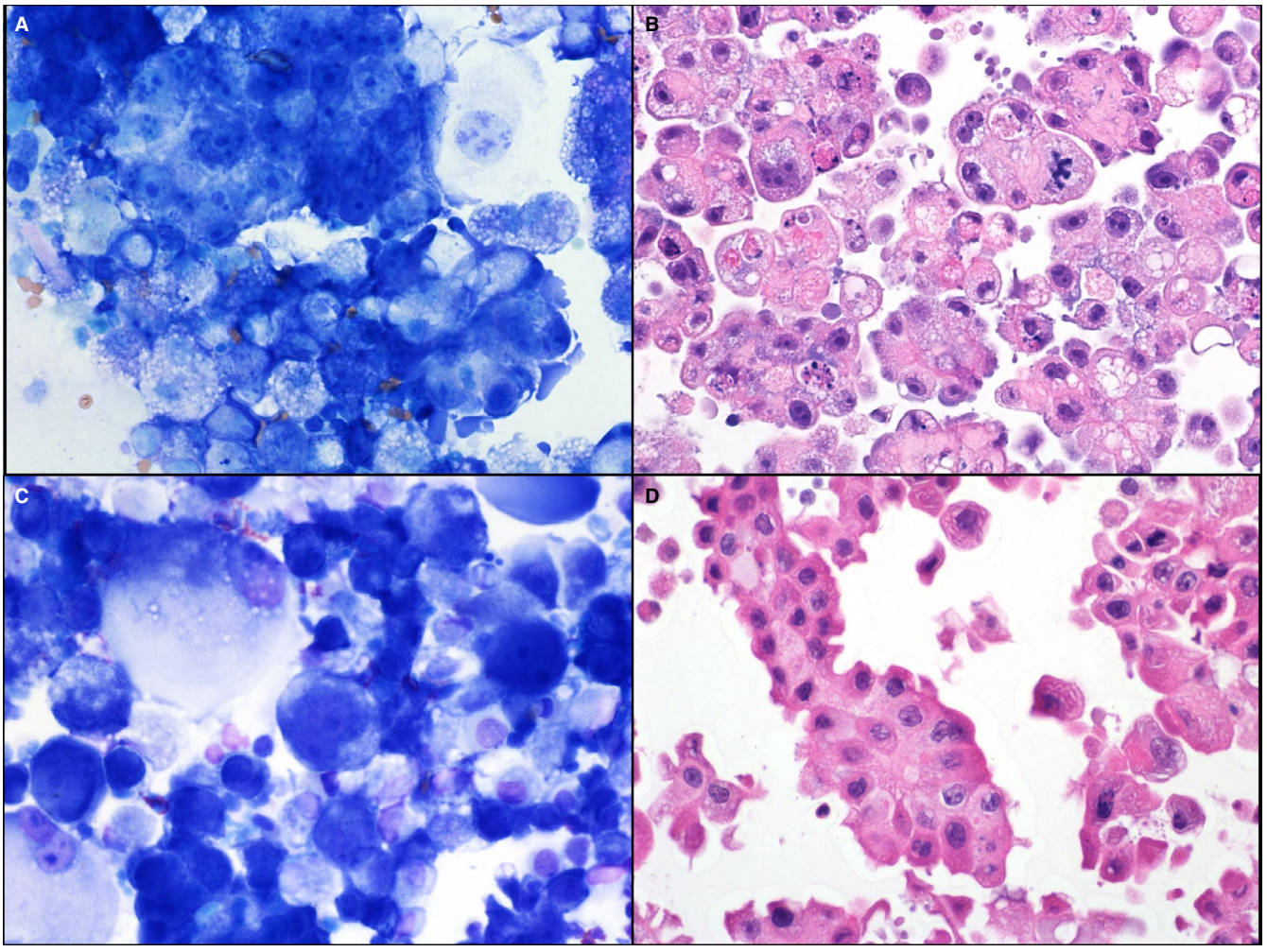

FIGURE 1 Cytology and corresponding cell block preparations of cavitary effusions from mesothelioma and carcinoma cases. A, mesothelioma cytology; B, mesothelioma cell block from the same case as A; C, carcinoma cytology; D, carcinoma cell block. The carcinoma cell block shows tubular "mini biopsies," consistent with adenocarcinoma. Cytologic samples stained with May-Grünwald Giemsa and cell block sections with H&E, ×40 objective

mass (2), mammary carcinoma and pleural mass (2), and prostatic mass with peritoneal nodule (1).

Examples of H&E-stained cell blocks are shown in Figure 1B,D. Sections from group MH contained individuals, clusters, pseudoacinar structures, and rarely more elongated arrangements of reactive mesothelial cells with mild to marked anisocytosis and anisokaryosis. Variable amounts of the cytoplasm and round to oval nuclei were also seen. One or more prominent nucleoli and multinucleation were also occasionally present, whereas binucleation was commonly found. In group M, the arrangement of the clusters were often more irregular than in group MH, although round clusters and pseudoacini were sometimes seen. The cytoplasm was often vacuolated. Multiple nucleoli and bi- or multinucleation were more common in group M than MH. In group C, the findings were variable. Cohesive clusters of medium-sized to large epithelial cells with marked anisocytosis and anisokaryosis were evident. Irregular, elongated, and sometimes pseudoacinar structures, as well as individual cells were also present, with a lack of features (eg, a pink fringe of microvilli at the cell border) that would suggest mesothelial origin. However, the cell morphology based solely on H&E staining was poorly discriminatory between the groups.

The histologic features of the biopsies in groups MH and M were as previously described.[14,23] Cases of atypical mesothelial proliferation, considered to be intermediate between hyperplasia and mesothelioma,[23] were not included in the study. The biopsies in group C presented features typical of carcinoma and consisted of mammary carcinoma (n = 2), pulmonary adenocarcinoma (n = 2), and prostatic carcinoma (n = 1).

After determining the definitive diagnoses by histologic examination, of the five cases in group MH, the original cytologic diagnosis was mesothelial hyperplasia (4 cases) and mesothelial hyperplasia or mesothelioma (1 case). Of the six cases in group M, the cytologic diagnosis was neoplastic effusion (2 cases), atypical mesothelial proliferation (2 cases), carcinoma or mesothelioma (1 case), and mesothelioma (1 case). Of the five cases in group C, the original cytologic diagnosis was carcinoma (4 cases) and mesothelial hyperplasia (1 case). Overall, the sensitivity of fluid cytology for obtaining a correct definitive diagnosis in the study population was 56% (9/16). Due to the nature of the cytologic diagnoses, which sometimes included more than one differential diagnosis, it was not possible to accurately determine false positives and true negatives and hence to accurately calculate specificity.

3.4 | Immunohistochemistry in cell blocks and biopsies

The expected staining pattern for the six IHC methods in each group is shown in Table 2, and the individual dog scores are shown in Table S1. Examples of IHC of cell block sections are shown in Figures 2 and 3, and the summary of the scores for the intensity of staining and percentage of positive cells on IHC for CK, vim, IMP3, GLUT1, WT1, and desmin in the cell blocks and the corresponding biopsies, with statistical comparisons, is shown in Figure 4. Where positive staining was present, CK, vim, and IMP3 showed intracytoplasmic staining; GLUT1 and desmin staining showed both cytoplasmic and membranous staining; and WT1 showed nuclear staining.

In paired cell blocks and biopsies, vim and WT1 staining were positively correlated between the two sample types for intensity and the percentage of positive cells; furthermore, CK and IMP3 staining were correlated for the percentage of positive cells (Table 3). Although these correlations suggest broad comparability of cell blocks and biopsies for this subset of antibody markers, individual pairs of results were not all identical (Figure 4).

Comparisons of markers between disease groups showed that for cell blocks, the intensity of IMP3 staining was significantly higher in group M than in group C ($P = 0.012$), and intensity of WT1 staining and percentage of cells stained for WT1 was higher in group MH than in group C ($P = 0.020$ and $P = 0.006$, respectively). For biopsies, the intensity and percentage of cells positive for WT1 staining were also higher in group MH than in group C ($P = 0.031$ and 0.025, respectively). For desmin, the staining intensity in biopsies was higher in group MH than in group C ($P = 0.025$). WT1 was negative in cell blocks and biopsies from all cases in group C. However, WT1 was also negative in one cell block and one biopsy in group M. Desmin was negative in four of five cell blocks and biopsies from group C compared with one of four cell blocks and none of four biopsies in group MH, and two of five cell blocks and one of five biopsies in group M. In addition, cases in groups MH and M were also occasionally negative for desmin in cell blocks or biopsies (Table S1).

The weighted kappa values with 95% confidence intervals for intra-observer variability were: staining intensity in cell blocks 0.797 (0.638-0.955), percentage of cells stained in cell blocks 0.889 (0.794-0.984), staining intensity in biopsies 0.862 (0.682-1.042), and percentage of cells stained in biopsies 0.896 (0.744-1.048). These values confirmed almost perfect agreement between observers.

4 | DISCUSSION

In this study, the IHC results for cell blocks from cases of mesothelial hyperplasia, mesothelioma, and carcinoma were not significantly different from the corresponding biopsies. The results also confirmed the difficulty in distinguishing these diseases using conventional cytology, especially mesothelioma vs mesothelial hyperplasia, and the potential value of cell block IHC, a minimally invasive technique, using a combination of two recently described IHC panels in dogs.[14,21]

Cytology alone had a diagnostic sensitivity of 56%, slightly lower than a previous report of 60%.[2] The sensitivity in the present study was biased by the relative difficulty in diagnosing mesotheliomas by cytology alone and the overrepresentation of these tumors compared with the prevalence expected in clinical practice. In a series of 304 effusions, mesothelioma comprised only 0.7% of cases,[28] and in a large series of dogs submitted for necropsy, it comprised 0.2% of cases.[29] However, it was clear from the morphologic descriptions

TABLE 2 Expected immunohistochemical staining results in cases of mesothelial hyperplasia, mesothelioma, and carcinoma for each of the six markers used in the study

Marker	Group MH	Group M	Group C	Comments
Cytokeratin	++	++	++	Cytoplasmic
Vimentin	+/++	−/+/++	−	Cytoplasmic; staining in M usually positive but can be variable; in some C, vimentin-positive cells may occur
IMP3	−	++	−	Cytoplasmic
GLUT1	+	+/++	Variable	Cytoplasmic and membranous
WT1	++	++	−	Nuclear
Desmin	++	++	−	Cytoplasmic and membranous

Abbreviations: C, carcinoma; M, mesothelioma; MH, mesothelial hyperplasia.

that there was considerable overlap between categories, especially mesothelial hyperplasia and mesothelioma, confirming the requirement for the use of immunologic techniques.

Previously, ICC, the application of immunological stains to fluid smears, has been used for cavitary fluid analysis in veterinary medicine.[30,31] The main advantages of ICC over conventional IHC on biopsies are the use of a less invasive technique, rapidity of results, and the potential to use antibodies that cannot be used on formalin-fixed tissues.[31] However, ICC has a number of significant disadvantages. Storage times before antigen loss is limited,[32] maintaining a consistent bank of positive control slides is difficult, cell numbers can be low, and the amount of background stain can be high.[33] In addition, as the number of slides could be limited, further material is unlikely to be available from the sample for future diagnostic investigations, for example, further immunostaining.[31] The limitations of ICC have led to the introduction of cell block techniques that allow the advantages of IHC to be combined with a relatively non-invasive method of collection. An additional feature of cell blocks is the potential presence of "mini biopsies," where some architecture is evident in exfoliated tissue fragments,[34] a finding confirmed by our study. We found these structures to be of some value in making differential diagnoses and were helpful in identifying relevant areas for IHC assessment (Figure 1D).

The results in paired cell blocks and biopsies show that vim and WT1 staining were positively correlated between the two sample types for scores of staining intensities and percentage of positive cells. WT1 staining appears to be useful in discriminating between mesothelial hyperplasia and carcinoma. Taken together, these results suggest that IHC of effusions can provide results representative of the underlying abnormalities for some markers. However, it was clear from detailed comparisons between groups that the results for paired cell blocks and biopsies were not identical, and while statistics help identify promising markers for differential diagnosis, they are not absolutely definitive when assessing results in individual patients.[12] In some cases, cell blocks or biopsies gave unexpected results for the disease category. These variations could be due to processing, for example, more prolonged contact with formalin for biopsies compared with cell blocks, differences in the degree to which the cells exfoliate into the fluid, or different antigen expression by exfoliated cells in fluids compared with those in solid tissues. In addition, reactive mesothelial cells were often present in cell blocks from mesothelioma and carcinoma effusions, and some of these cells might have been included in the scoring of the main cell population of interest. However, the fact that the cells tend to be layered in the cell blocks into erythrocytes, neoplastic cells, and smaller mesothelial cells/inflammatory cells aided in differentiation.

Strong CK staining was obtained in all groups, as expected. There were no significant differences between groups for vim staining, although there were trends toward higher staining intensity in groups MH and M than in group C (Figure 4). This is broadly consistent with the expected finding of positive CK and vim staining in mesothelial cells. In most group C cases, the expected positive CK and negative or weak vim staining were obtained in cell blocks and biopsies. Previous studies in cats and dogs have shown CK and vim to be of value in differentiating mesothelial from epithelial cells,[19,35] but these studies did not distinguish hyperplastic from neoplastic mesothelial cells. Similarly, the detection of carcinoma metastasis on bone marrow cell blocks has been described in dogs and cats, but only CK IHC was used.[36] Unexpected results sometimes occurred in the present study, for example, negative staining for vim in one mesothelioma case. However, this is likely due to the altered expression of these antigens by the neoplastic cells rather than technical failure, especially as this individual also had negative vim staining on a biopsy processed on a different day.

Higher WT1 and desmin staining intensity were generally seen in cells of mesothelial origin. It was notable that WT1 was negative in all group C cases. Negative WT1 staining in carcinomas is not surprising given that it is expressed in mesoderm-derived cells,[37] and the value of WT1 for identifying mesothelial cells, either hyperplastic or neoplastic, has been described in people[8] and dogs.[21] However, it is clear that negative WT1 staining was not completely specific to carcinomas as it occasionally occurred in group M. Only nuclear staining for WT1 was considered positive,[21] because cytoplasmic staining is less specific[37] and has been described in canine lymphoma.[38]

Results for desmin were similar to those for WT1 in that carcinomas were usually negative (4/5 cases), whereas desmin was negative in only one of four cell blocks and none of four biopsies in group MH, and two of five cell blocks and one of five biopsies in group M.

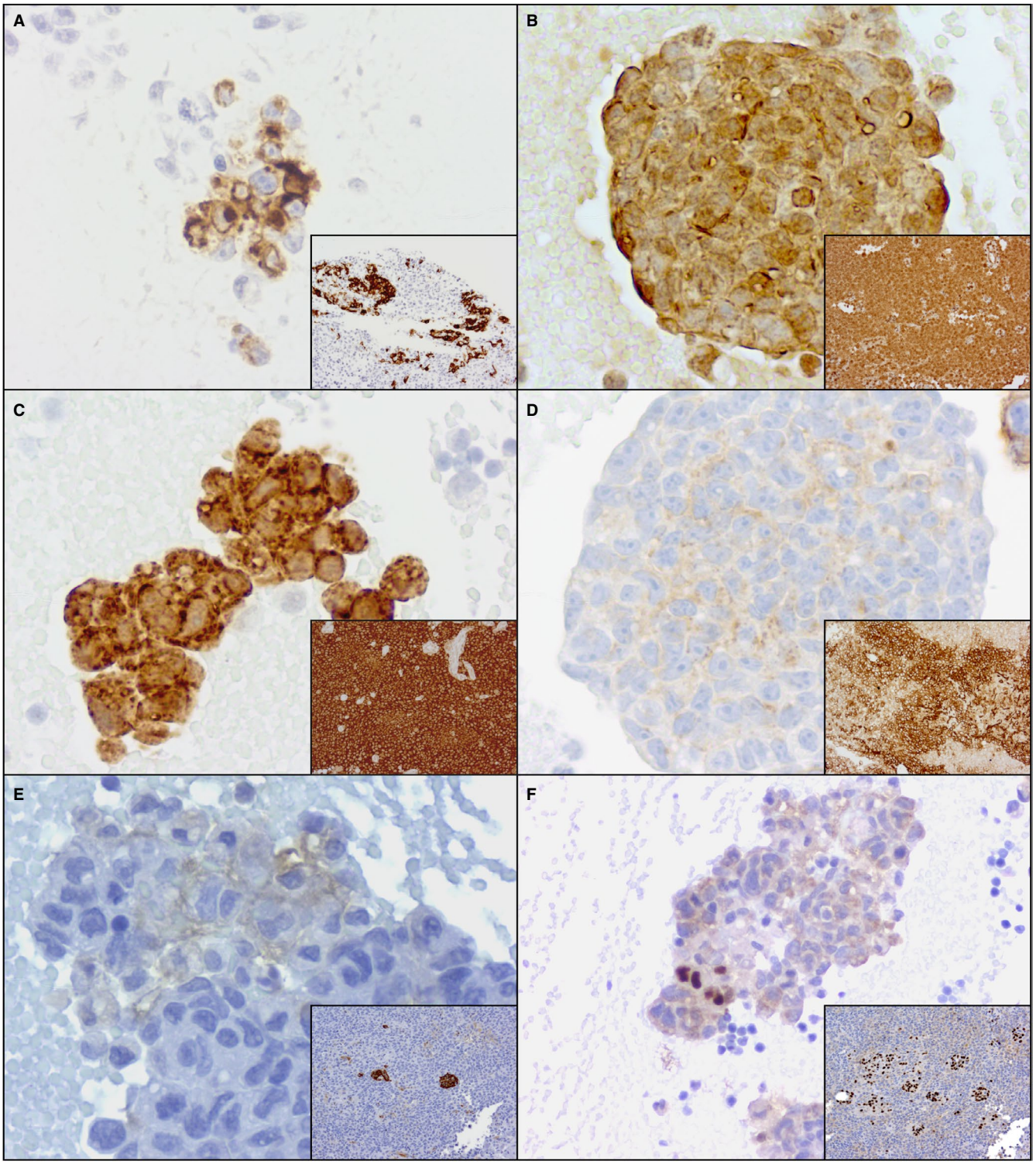

FIGURE 2 A-F, Immunohistochemistry on a pleural fluid cell block and biopsy (insets) from a case of mesothelioma; for each image, the intensity of staining of the cell block is followed by the intensity of the biopsy. (A) Cytokeratin (2,3); (B) Vimentin (2,3); (C) IMP3 (3,3); (D) GLUT1 (1,3); (E) Desmin (1,3); and (F) WT1 (3,3). The results are similar in the cell blocks and biopsies, except for GLUT1 and desmin where staining was more intense in the biopsies. The strong WT1 staining is typical of mesothelial cells and strong IMP3 of mesothelioma (see text for further details). Cell blocks ×40 objective and biopsies ×10 objective

Lack of desmin staining on ICC in canine serosal cavity carcinomas and variably positive staining in cells of mesothelial origin has been previously reported.[35] Desmin is reported to aid in differentiating hyperplastic and neoplastic mesothelial cells in people, with stronger staining in benign mesothelial proliferations,[13,39] but this was not evident in the present study. Furthermore, desmin was ineffective

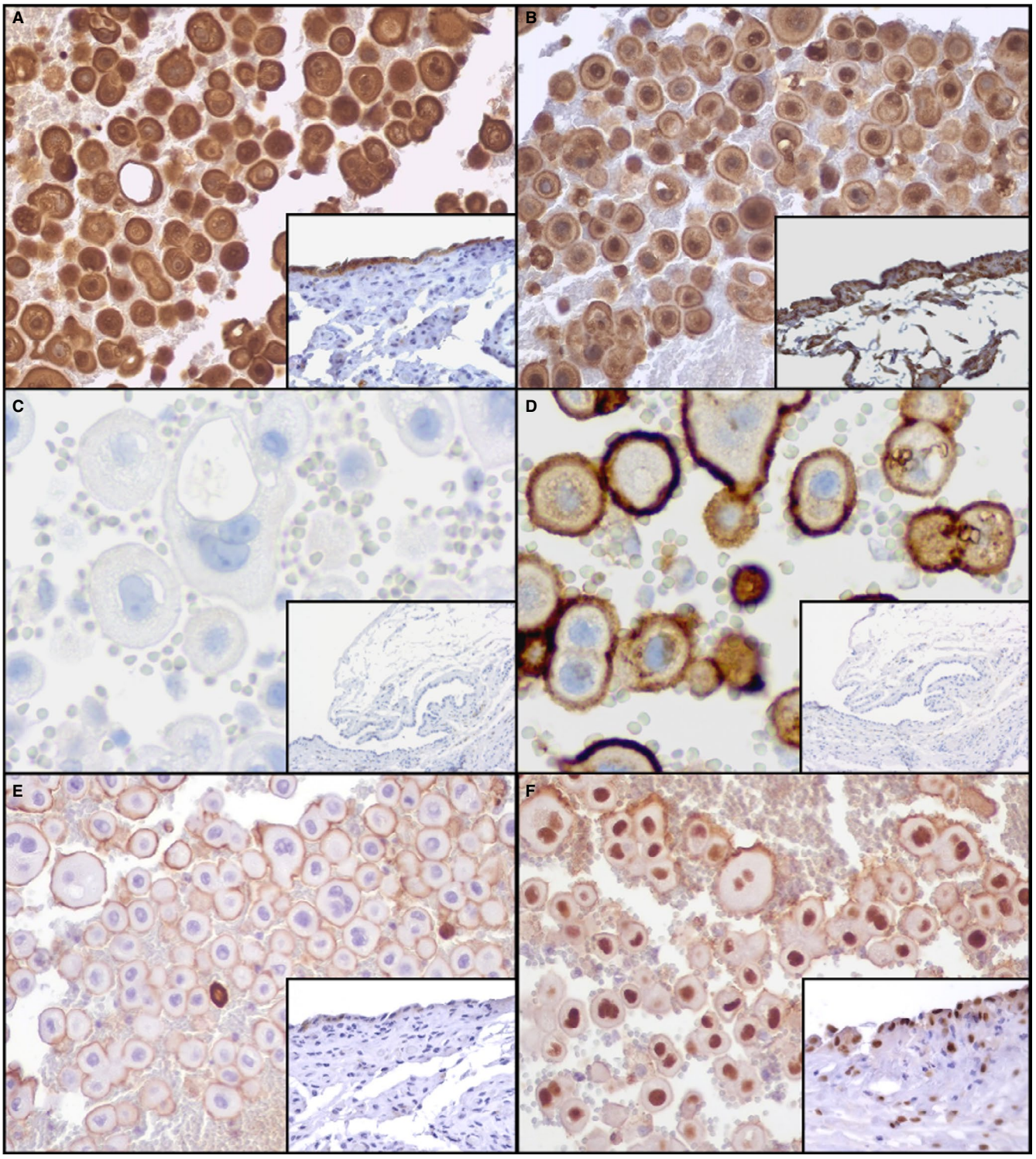

FIGURE 3 A-F, Immunohistochemistry on a pleural fluid cell block and biopsy (insets) from a case of mesothelial hyperplasia; for each image, the intensity of staining of the cell block is followed by the intensity of the biopsy. (A) Cytokeratin (3,3); (B) Vimentin (3,3); (C) IMP3 (0,0); (D) GLUT1 (3,0); (E) Desmin (2,2); and (F) WT1 (3,3). The results are similar in the cell blocks and biopsies except for GLUT1, where staining was more intense in the cell block. The strong WT1 staining is typical of mesothelial cells, and negative IMP3 is typical of mesothelial hyperplasia (see text for further details). Cell blocks ×40 objective and biopsies ×10 objective

in distinguishing hyperplastic mesothelium from mesothelioma in canine pericardial biopsies.[14] Thus, while desmin and WT1 did not distinguish hyperplastic from neoplastic mesothelium, the fact that carcinomas were always (WT1) and usually (desmin) negative indicates that these markers could be of value in IHC panels and might be most value in cases where cytology or standard H&E staining

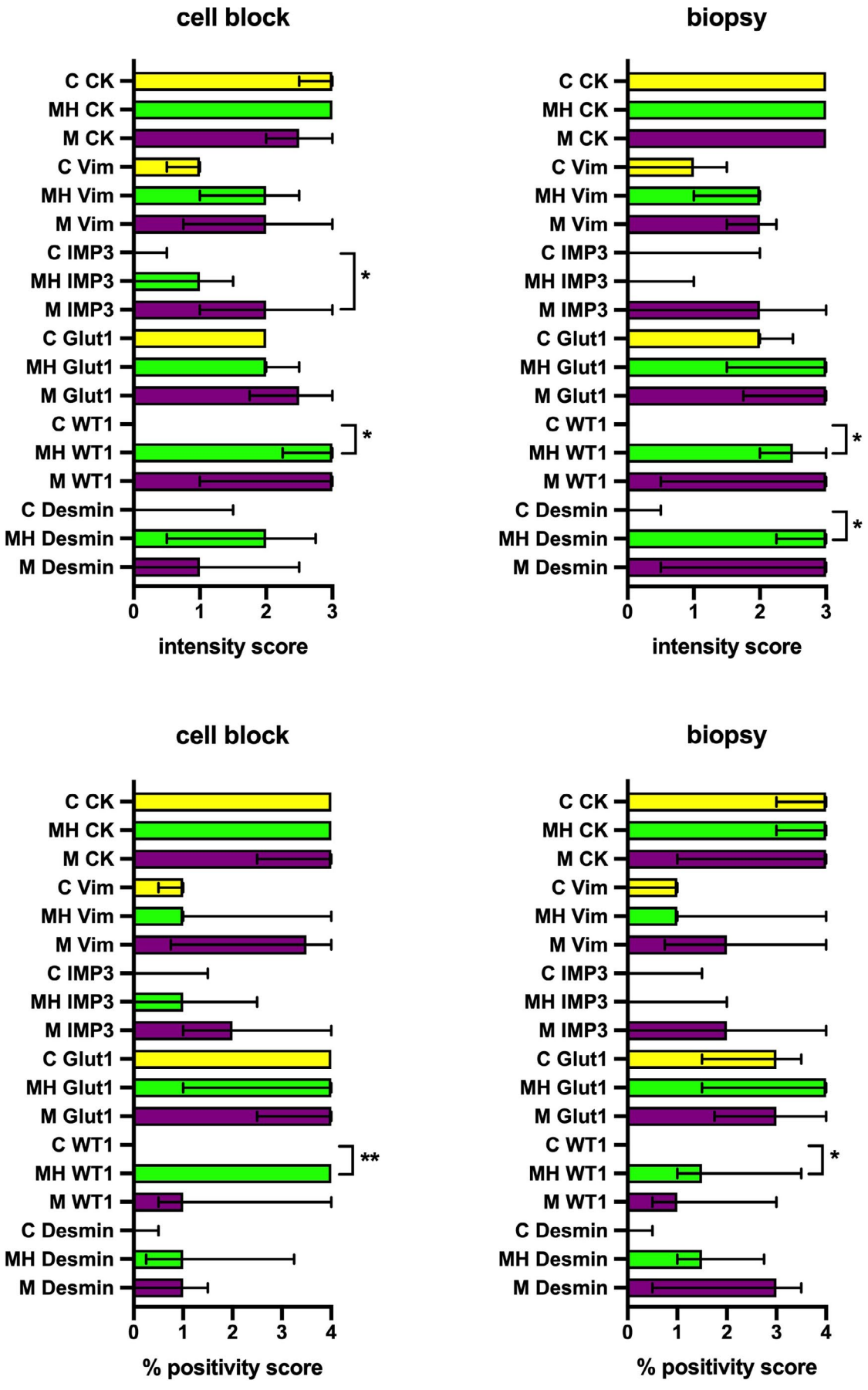
cell block
C CK
MH CK
M CK
C Vim
MH Vim
M Vim
C IMP3
MH IMP3
M IMP3
C Glut1
MH Glut1
M Glut1
C WT1
MH WT1
M WT1
C Desmin
MH Desmin
M Desmin
*
*
0 1 2 3
intensity score
biopsy
C CK
MH CK
M CK
C Vim
MH Vim
M Vim
C IMP3
MH IMP3
M IMP3
C Glut1
MH Glut1
M Glut1
C WT1
MH WT1
M WT1
C Desmin
MH Desmin
M Desmin
*
*
0 1 2 3
intensity score
cell block
C CK
MH CK
M CK
C Vim
MH Vim
M Vim
C IMP3
MH IMP3
M IMP3
C Glut1
MH Glut1
M Glut1
C WT1
MH WT1
M WT1
C Desmin
MH Desmin
M Desmin
**
0 1 2 3 4
% positivity score
biopsy
C CK
MH CK
M CK
C Vim
MH Vim
M Vim
C IMP3
MH IMP3
M IMP3
C Glut1
MH Glut1
M Glut1
C WT1
MH WT1
M WT1
C Desmin
MH Desmin
M Desmin
*
0 1 2 3 4
% positivity score

FIGURE 4 IHC staining scores for antibody markers in cell blocks and biopsies. The histogram bar represents the median and the error bars represent the interquartile range. Purple = Mesothelioma group (n = 6 [n = 5 for WT1 and desmin]); green = Mesothelial hyperplasia group (n = 5 [n = 4 for WT1 and desmin]); yellow = Carcinoma group (n = 5). The sections of blocks and biopsies were scored semiquantitatively for % positive cells as 0 = completely negative, 1 = 1%-25% of cells positive, 2 = 26%-50% positive, 3 = 51%-75% positive, and 4 = ≥ 75% positive. The intensity of staining was scored as 0 = negative, 1 = weakly positive (barely visible specific stain), 2 = moderately positive (between mild and strong), or 3 = strongly positive. Data were analyzed using Kruskal-Wallis tests followed by Dunn's multiple comparison tests. Two-tailed tests: $^{*}P < 0.05$, $^{**}P < 0.01$, all other tests were nonsignificant $P > 0.05$. IHC markers: CK, cytokeratin; Vim, vimentin; IMP3; GLUT1; WT1; Desmin

TABLE 3 Correlations between paired cell block and biopsy IHC scores for combined carcinoma, mesothelial hyperplasia, and mesothelioma cases

Marker	Score	Spearman *r*	95% confidence interval	*P*	No. XY pairs
Cytokeratin	Intensity	NA[a]	NA[a]	NA[a]	16
	% Positivity	0.6978	0.2943 to 0.8901	0.0083	16
Vimentin	Intensity	0.7735	0.4376 to 0.9199	0.001	16
	% Positivity	0.9222	0.7795 to 0.9739	<0.0001	16
IMP3	Intensity	0.4371	−0.09081 to 0.7732	0.0917	16
	% Positivity	0.5985	0.1304 to 0.8484	0.016	16
GLUT1	Intensity	-0.2069	−0.6467 to 0.3361	0.4484	16
	% Positivity	-0.3093	−0.7062 to 0.2354	0.2725	16
WT1	Intensity	0.6234	0.1215 to 0.8714	0.0284	14
	% Positivity	0.8287	0.5193 to 0.9460	0.0022	14
Desmin	Intensity	0.3469	−0.2417 to 0.7488	0.2266	14
	% Positivity	0.2328	−0.3551 to 0.6888	0.4186	14

Note: Correlations between ordinal IHC scores were analyzed with Spearman rank correlation tests.

[a]NA not applicable as the intensity of CK staining was uniformly high.

show clear criteria of malignancy, with the main dilemma of differentiating mesothelioma from carcinoma. Furthermore, as vim staining was not invariably positive in mesothelial tissue and could even be positive in some carcinomas, a panel of CK, WT1, and desmin might be more reliable in this respect.

The results for IMP3 and GLUT1 were not identical to those reported for canine pericardial biopsies in which IMP3 was of some value in aiding in the distinction of mesothelial hyperplasia, mesothelioma, and the intermediate category of atypical mesothelial proliferation.[14] However, the intensity of IMP3 staining was significantly higher in group M than in group C, at least in the cell blocks. There was also a trend toward greater intensity in group M than in group MH, as previously reported.[14] Results for GLUT1 were similar in all three groups and did not appear to be a useful addition to the IHC panel, although GLUT1 is sometimes positive in canine mesothelioma where IMP3 is negative.[14] Thus, there might be justification for including GLUT1 in more extended IHC panels to allow for the identification of such cases. In human pathology, a combination of IMP3 and GLUT1 staining has been recommended for distinguishing hyperplastic and neoplastic mesothelium[10,11] although it could be difficult to interpret the results in individual cases[40]; such difficulties in interpretation are also likely to occur in dogs, as in our study. We, therefore, suggest that a more restricted panel of CK, WT1, desmin, and IMP3 might be of most value in distinguishing mesothelial hyperplasia, mesothelioma, and carcinoma in dogs until more specific markers are identified.

The major limitation of the study was the small number of cases, which reduced the statistical power. This was due to difficulty in obtaining well-documented paired cell block and biopsy samples. The exclusion criteria were rigidly applied to ensure that only confirmed, unequivocal cases were included, which involved discarding many samples where the diagnosis was likely but not confirmed beyond doubt. This approach was considered preferable to including cases that could have been incorrectly classified. In addition, clinicians usually did not proceed with biopsy when useful information had been obtained by effusion cytology combined with IHC on cell blocks. Staining was also undertaken at three laboratories with slightly different methods, although in each individual case, the cell blocks and corresponding biopsies were treated in the same way. Further studies using a larger population are warranted to allow firmer recommendations to be made.

Overall, the differentiation between reactive and neoplastic mesothelium remains challenging, even with the proposed IHC panel. For the purpose of evaluating an IHC panel, we included a similar number of mesothelial hyperplasia, carcinoma, and mesothelioma cases. However, this does not reflect the clinical setting since mesothelioma is a rare neoplasm.

5 | CONCLUSIONS

Immunohistochemistry on cell blocks derived from cavitary effusions is a simple technique that often provided additional information on identifying mesothelial hyperplasia, mesothelioma, and carcinoma. An IHC panel of CK, WT1, desmin, and IMP3 appeared to be of most value, with vim and GLUT1 being less useful. The panel is not of value in every case, but its use on cell blocks can be a first approach before considering surgical biopsy and would have safety benefits, since fluid collection is minimally invasive and performed without general anesthesia. Once the cell blocks are made, additional tests, including further IHC and PCR, can be applied to individual cases, and they can be stored for many years, unlike fluid samples. Establishing a bank of cell blocks and biopsies to use as case controls for IHC staining and scoring and increasing population sizes for future studies will be important to standardize diagnoses and continue the search for more specific markers.

ACKNOWLEDGMENTS

The authors thank Petplan Charitable Trust for their generous funding and to all the clients, clinicians, pathologists, technicians, and nurses involved at the Universities of Edinburgh, Liverpool, and Porto. As a PhD student, Carla Marrinhas benefits from institutional support from ICBAS - U. Porto.

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How to cite this article: Milne EM, Piviani M, Hodgkiss-Geere HM, et al. Comparison of effusion cell block and biopsy immunohistochemistry in mesothelial hyperplasia, mesothelioma, and carcinoma in dogs. *Vet Clin Pathol*. 2021;00:1–13. https://doi.org/10.1111/vcp.13002