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J Vet Res 68, 271-278, 2024
DOI:10.2478/jvetres-2024-0035

The diagnostic value of selected immune parameters in peripheral blood of dogs with malignant mammary tumours – a preliminary study

Urszula Lisiecka[1]✉, Piotr Brodzki[2], Anna Śmiech[3], Katarzyna Michalak[1]✉, Stanisław Winiarczyk[1], Beata Żylińska[4], Łukasz Adaszek[1]

[1]Department of Epizootiology and Clinic of Infectious Diseases, University of Life Sciences in Lublin, 20-950 Lublin, Poland
[2]Department and Clinic of Animal Reproduction, University of Life Sciences in Lublin, 20-950 Lublin, Poland
[3]Department of Pathological Anatomy, University of Life Sciences in Lublin, 20-950 Lublin, Poland
[4]Department and Clinic of Animal Surgery, University of Life Sciences in Lublin, 20-950 Lublin, Poland
urszula.lisiecka@up.lublin.pl, katarzyna.michalak@up.lublin.pl

Received: March 28, 2024 *Accepted: June 13, 2024*

Abstract

Introduction: The main adaptive immune cells are T and B lymphocytes and they play key roles in the induction of immune responses against canine mammary tumours. Investigating these cell subpopulations may lead to more precise diagnosis of these malignancies. **Material and Methods:** The percentages of $CD3^+$, $CD4^+$ and $CD8^+$ T cells and of $CD21^+$ B cells in the peripheral blood of bitches with malignant mammary tumours were compared with those in the blood of healthy animals. The phenotypic features of peripheral blood leukocytes were evaluated by flow cytometry. **Results:** There was a significant difference in the mean percentages of $CD3^+$ lymphocytes between healthy (66.7%) and metastatic dogs (46.1%), and between tumour-bearing non-metastatic (66.6%) and metastatic dogs. There was also a significant difference in $CD4^+$ T helper cell percentages between healthy dogs (40.4%) and dogs with metastases (23.2%), and between the latter and dogs without them (35.5%). In the case of $CD21^+$ lymphocyte subsets, a significant difference was noted between healthy animals (10.9%) and those with metastases (20.1%), and between the latter and patients without metastases (8.5%). There were also significant differences in $CD3^+/CD21^+$ ratios between the group with metastases (3.0), the healthy group (7.8), and the group without metastases (8.5). Similarly, a significant difference was noted in $CD4^+/CD8^+$ ratios between animals with metastases (1.4), bitches in the control group (2.2), and dogs without metastases (1.9). **Conclusion:** Peripheral blood leukocyte phenotypic characteristics are putative novel biomarkers. These findings may be useful in future studies improving mammary tumour diagnostic procedures, especially in metastasis detection.

Keywords: canine mammary tumours, flow cytometry, immunophenotyping, lymphocyte subsets, metastasis biomarkers.

Introduction

Canine mammary tumours (CMTs) constitute the most frequent neoplasms in female dogs. The epidemiological, clinical, biological and genetic diversity of these tumours is paralleled by different disease outcomes and choices of appropriate treatment (4, 17). Given the prevalence of CMTs, there is a need to search constantly for more precise diagnostic and prognostic methods that make possible the selection of appropriate therapies. Understanding how the antitumour activity of the immune system in dogs with a particular type of mammary gland tumour differs from this activity in dogs with other types of mammary gland tumour will improve the prediction of the course of a disease and of the response to therapy in specific patients. Cytometric investigation of peripheral blood in dogs with these tumours may be a means of rapid detection of metastases or micrometastases in these animals in the future (25).

There are several studies that have reported changes to the immune status in canine cancers, but most of them are focused on leukocyte immunophenotyping inside the tumour milieu (4, 28). However, cancer is a systemic disease that induces changes to the immune system as a whole. Therefore, an improved understanding of tumour immunology must include not only the tumour

microenvironment but also the systemic immune landscape. Peripheral immune system investigation may facilitate canine mammary tumour diagnosis and prognosis (28).

The main adaptive immune cells are T and B lymphocytes and they play key roles in the induction of immune responses against canine mammary tumours (4). The composition of these cell subpopulations varies depending on the tumour type, and different lymphocyte subsets may cause neoplasms to progress in different ways and fit different prognoses (10). However, the importance of the T-cell-to-B-cell ratio in mammary gland tumours in dogs is not yet clear (25).

The cancer immunosurveillance system involves T cells that identify and eliminate tumour cells, leading to tumour rejection (28). The lineage of canine T cells is defined by the CD3 protein complex as a salient feature; therefore, anti-CD3 antibodies can be used to identify T cell markers in dogs (23). Changes in $CD3^+$ lymphocyte percentages have been detected in dogs with different types of cancers (5), which suggests that the CD3 antigen may constitute a potential marker in canine mammary tumour diagnostic methods. Besides $CD3^+$ lymphocytes, $CD8^+$ cytotoxic T (Tc) cells are crucial to anti-tumour immunity, and the prevalence of such cells in tumour infiltrate means that the reaction of the body against neoplastic cells is mainly cytotoxic (1). Changes in these cell percentages in peripheral blood have been proven in many types of human and canine cancers; therefore, $CD8^+$ Tc lymphocytes may convey valuable information on the immune status of canine cancer patients and may be a potential diagnostic factor in canine mammary tumours (16). Although the majority of T cell-based cancer research focuses on the anti-tumour $CD8^+$ Tc cell immune responses, recent studies have revealed that $CD4^+$ T helper (Th) cells develop and sustain effective anti-tumour immunity, doing so through several different mechanisms (24). For example, $CD4^+$ Th cells aid anti-tumour $CD8^+$ Tc lymphocyte functioning, support $CD21^+$ B cells in antibody production, and indirectly kill autologous tumour cells in a major histocompatibility complex class II–dependent mechanism (24). That is why changes in the cell percentage which comprise this subpopulation may be related to the stage of tumour progression.

The $CD4^+/CD8^+$ ratio gives the proportion of $CD4^+$ Th cells to $CD8^+$ Tc cells and can help predict the likely course of various diseases (12). Many studies have revealed that a decrease in the relative percentage of $CD4^+$ lymphocytes together with an increase in the $CD8^+$ lymphocyte percentage may be related to impaired immune responses (12, 28). This phenomenon is observed in aging and immune senescence, and may lead to increasing incidence and severity of cancer in older dogs (28). Moreover, many cancers are more frequent among human AIDS patients and their $CD4^+$ lymphocyte number and $CD4^+/CD8^+$ ratio decrease significantly during the progression of the disease (22). This suggests that tumour development may be connected with changes in the numbers of specific T lymphocytes. Further, it has been noted that the $CD4^+/CD8^+$ ratio is decreased in many human and animal neoplasms, including canine cancers (6). According to Ostroumov *et al.* (15), $CD4^+$ and $CD8^+$ T lymphocyte interplay exerts a controlling influence on tumour growth. Therefore, this ratio can be considered a potential diagnostic and prognostic factor for canine mammary tumours.

The role of B lymphocytes in cancer progression is still not well established, particularly in canines. There are some articles linking B cells with immunosuppressive or regulatory functions in neoplastic processes (14, 29). In human breast tumours, regulatory B cells may provoke cancer metastasis (14). In other studies, B cells have been described to possess a protective rather than an immunosuppressive function (18). It is possible that this discrepancy is caused by the presence of B cell subsets with distinct phenotypes and different functions. As the processes of tumour eradication and tumour progression are related to the role of canine peripheral B lymphocytes, it is worth knowing whether the number of these cells changes in the course of the neoplastic process (21).

The present investigation aimed to assess the differences between the percentages of selected lymphocyte subsets in peripheral blood from healthy dogs and dogs with two different stages of malignant mammary tumour. In this study, single antibodies were used, which could become simple and relatively cheap diagnostic reagents if they fulfil their promise. The findings of this study concerning the quantity of essential circulating immune cell subtypes in mammary cancer may also improve the prospects of improving treatment strategies.

Material and Methods

Study material. Blood samples were obtained from 20 bitches of different breeds aged over 6 years with spontaneous mammary tumours (Supplementary Table 1). The animals underwent surgery at the Department and Clinic of Animal Surgery and Department and Clinic of Animal Reproduction at the University of Life Sciences in Lublin. All blood specimens were obtained from the laboratory and all of them had previously been used for diagnostic purposes. Information was gathered by a physical examination of each dog with evaluation of the size of the neoplasm and presence or absence of ulceration. A complete blood cell count, serum biochemical profiling and urinalysis were conducted to exclude bitches with concomitant diseases. Radiography was carried out to detect distant metastases. No dogs included in the experimental group had previously been treated with steroids, chemotherapy or radiation therapy. Tissue samples for histological examinations were collected during mastectomy. There was no interference with the standard treatment and no additional procedures

were performed on the animals. The owners of the dogs gave consent for the use of their animals' blood and tissues in this research.

Experimental animals were grouped based on the presence or absence of metastasis. Group WM was composed of 10 bitches with malignant tumours and no evidence of metastasis in lymph nodes (N) or distant organs (M), which were categorised at stage N0M0. Group M comprised 10 bitches with malignant tumours and detectable lymph node metastasis or other distant organ metastasis, which were at stages N1, N0M1 or N1M1 (Table 1). Histological diagnosis was performed to confirm the type of tumour.

The control group (H) contained whole blood samples from 10 healthy female dogs collected for routine diagnostic purposes or deposition in a blood bank. These bitches were at a similar age to the investigated animals and were clinically healthy with normal blood tests, serum biochemical profiles and urinalysis.

The study was conducted in accordance with the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (Directive 86/609/ EEC and its revision in Directive 2010/63/EU).

Flow cytometric immunophenotyping. Peripheral blood samples for cytometric analysis were obtained by venepuncture of the saphenous vein (*vena saphena*) into ethylenediaminetetraacetic acid vacutainer tubes. The blood was collected for a routine blood cell count before surgery. Laboratory procedures were performed within 4 h of blood sampling. The antibodies used in this study were obtained from BIO-RAD (Hercules, CA, USA) or eBioscience (ThermoFisher Scientific, Waltham, MA, USA) (Supplementary Table 2). Before the experiment, titration was conducted to establish the optimal dilutions of antibodies, which ensured optimal direct staining for all of them. Briefly, 100 µL of whole blood sample was incubated with the appropriate amount of antibody for 20 min in the dark. After that, 2 mL of ammonium chloride lysing solution was added to perform erythrocyte lysis. Then, the sample was analysed in a FACSVerse flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA). A total of 10,000 events were gathered in the flow cytometer. Leukocyte subpopulations were gated according to their size and granularity using forward-scatter and side-scatter parameters (Supplementary Fig. 1).

Table 1. Characteristics of canine patients enrolled in the study

Characteristics	Number of patients with different stages of tumour	
	Without metastases (N0M0)	With metastases (N1, N0M1 or N1M1)
G1 complex carcinoma	2	0
G2 complex carcinoma	2	2
G1 tubular carcinoma	3	2
G2 tubular carcinoma	2	3
osteosarcoma	1	3

N0 – no evidence of lymph node metastasis; M0 – no evidence of distant metastasis; N1 – lymph node metastasis; M1 – distant metastasis; G1 – histological grade 1, G2 – histological grade 2

Table 2. Descriptive statistics of variables (full sample set n = 30)

Variable	Health status	Mean	Median	SD	SE	Minimum	Maximum	ANOVA P-value	Tukey pairs	Tukey P-values
% $CD3^+$	without metastasis	66.6	69.3	8.9	2.8	52.7	80.6	0.0003	H *vs* M WM *vs* M	0.001 0.0011
	metastasis	46.1	46.4	14.7	4.7	22.3	68.9			
	healthy	66.7	67.1	9.3	2.9	52.5	85.1			
% $CD21^+$	without metastasis	8.5	8.6	2.5	0.8	5.2	11.9	0.0018	H *vs* M WM *vs* M	0.0139 0.0021
	metastasis	20.1	17.9	10.4	3.3	8.4	38.2			
	healthy	10.9	10.9	4.9	1.6	4.6	21.4			
% $CD4^+$	without metastasis	35.5	34.9	9.5	3.0	22.6	50.4	0.0002	H *vs* WM WM *vs* M	0.0002 0.0061
	metastasis	23.2	25.9	8.3	2.6	10.3	34.6			
	healthy	40.4	42.3	6.2	2.0	31.4	52.0			
% $CD8^+$	without metastasis	20.7	19.4	8.0	2.5	9.5	35.4	No statistically significant difference		
	metastasis	19.3	20.3	9.0	2.8	4.5	36.4			
	healthy	18.4	18.3	2.3	0.7	15.1	21.9			
$CD3^+/CD21^+$ ratio	without metastasis	8.5	8.6	2.8	0.9	4.4	13.3	0.0028	H *vs* M WM *vs* M	0.0125 0.0041
	metastasis	3.0	2.7	2.1	0.7	0.6	8.1			
	healthy	7.8	6.5	4.9	1.5	2.8	18.0			
$CD4^+/CD8^+$ ratio	without metastasis	1.9	1.9	0.9	0.3	0.8	3.3	0.0348	H *vs* M	0.03291
	metastasis	1.4	1.3	0.5	0.2	0.6	2.3			
	healthy	1.2	2.2	0.7	0.2	1.4	3.4			

SD – standard deviation; SE – standard error of the mean; ANOVA – analysis of variance; H – healthy bitches; WM – bitches without metastasis; M – bitches with metastasis

The immunophenotypic features were analysed in fluorescence *vs* side-scatter dot plots. The results were expressed as the percentage of positive cells within gated lymphocytes, as the $CD3^+/CD21^+$ (T/B cell) ratio, and also as the $CD4^+/CD8^+$ (Th/Tc cell) ratio. To assist gating decisions appropriate controls were applied, which were run under the same conditions as the experimental samples. For calibration and validation procedures, BD FACSuite Cytometer Setup & Tracking Research Beads (BD Biosciences, San Jose, CA, USA) and BD Calibrite 3 Beads (BD Biosciences) were used. Analyses for each of the antibodies were carried out on the same, unchangeable protocols, the same instrumental settings, and with the same voltages applied (forward scatter 233.5V, side scatter 354.8V, fluorescein isothiocyanate 501V, phycoerythin 473.1V and allophycocyanin 544.6V). Daily compensation procedures were applied where necessary. All blood samples were run at low flow rate. The results obtained by flow cytometry were confirmed by haematological tests and microscopic evaluation of leukocytes.

Statistical methods and analysis. Statistical analyses were performed using Statistica software version 12.5 (Dell, Round Rock, TX, USA). As the Shapiro–Wilk test proved most of the obtained results to be normally distributed, one-way analysis of variance with a subsequent Tukey post-hoc test were applied to compare the groups. For $CD3^+/CD21^+$ cell ratios where the data were not normally distributed, the Kruskall–Wallis test with multiple comparisons was used to compare the groups. Data were expressed as mean and median percentages of positive cells, and a P-value of < 0.05 were considered statistically significant.

Results

The gating strategy for this experiment is presented in Supplementary Fig.1. The cytograms of CD3 staining are presented in Supplementary Fig. 2. As shown in Fig. 1A, the mean $CD3^+$ lymphocyte percentage in group M was significantly decreased compared to both group H and group WM (P-value < 0.05). However, there was no significant difference between the mean percentages in these latter two groups (40.39% and 35.48%, respectively).

Similarly to the $CD3^+$ lymphocyte percentage results, those for $CD21^+$ also did not differ statistically significantly between group H and group WM. However, a statistically significant increase was discovered in animals from group M over the control dogs' percentage (P-value < 0.05), with mean values of 20.07% and 10.85%, respectively. A significant difference was also noted between the $CD21^+$ lymphocyte percentages in the two groups of tumour-bearing dogs (P-value < 0.05) (Fig. 1B).

Figure 1C shows the $CD3^+/CD21^+$ cell ratio calculated for all groups. The ratio in group WM was significantly increased over that in group H. In contrast, the $CD3^+/CD21^+$ cell ratio was significantly decreased in patients with histological grade G2 tumours from that in group H (P-value < 0.05).

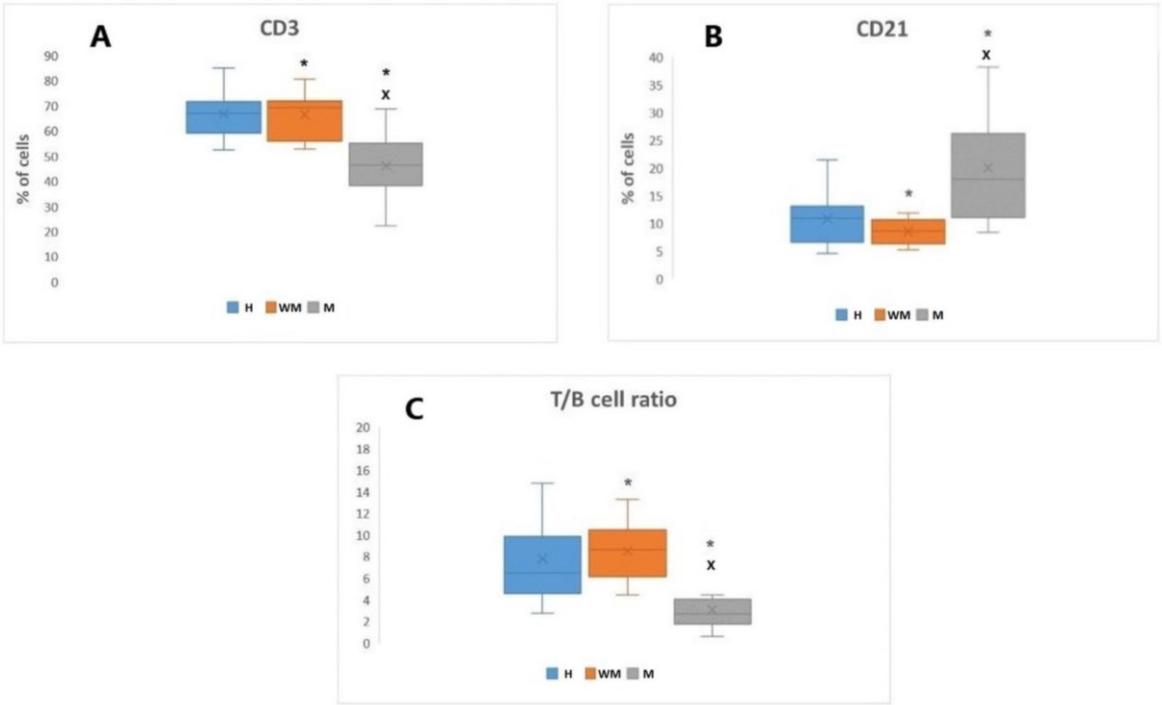

Fig. 1. Frequencies of $CD3^+$ T lymphocytes (A) and $CD21^+$ B lymphocytes (B), and the $CD3^+/CD21^+$ lymphocyte ratio (C) in blood from canine mammary tumour patients and healthy controls. Mean and median percentages of cells in the control group (H), in the group without metastases (WM), and in the group with metastases (M). Boxes cover the 25th–75th percentiles and the median is shown as a line across the box
* – statistically significant difference (P-value < 0.05) between group WM and group M; X – statistically significant difference (P-value < 0.05) between groups WM or M and group H

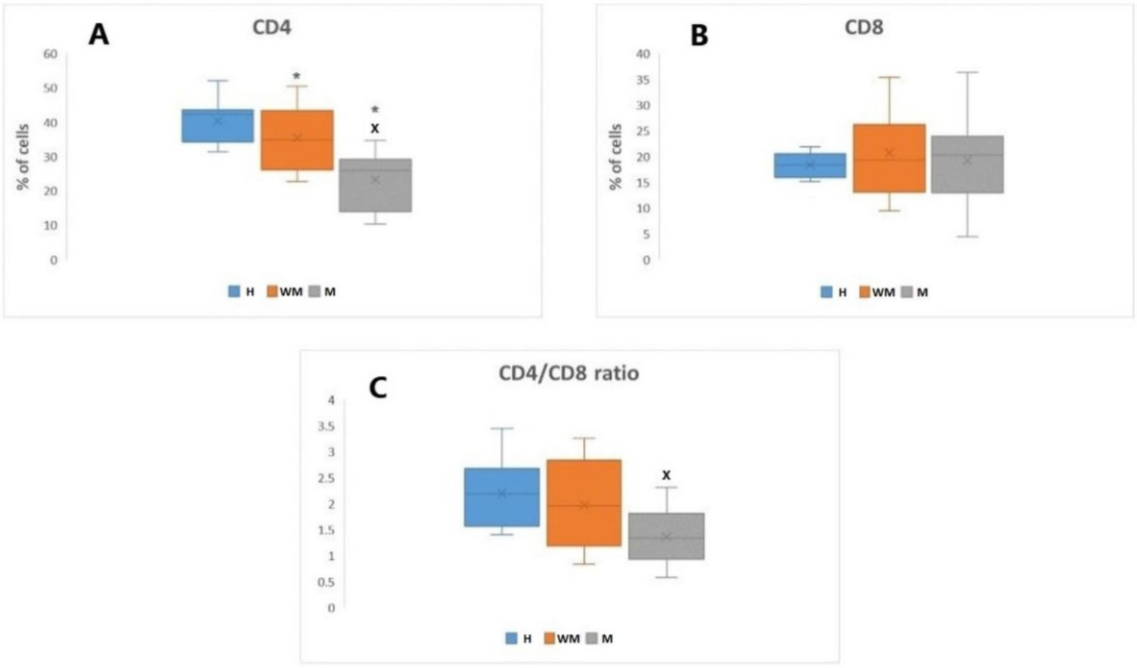

Fig. 2. Frequencies of $CD4^+$ T helper lymphocytes (A) and $CD8^+$ cytotoxic T lymphocytes (B), and the $CD4^+/CD8^+$ lymphocyte ratio (C) in blood from canine mammary tumour patients and healthy controls. Mean and median percentages of cells in the control group (H), in the group without metastases (WM), and in the group with metastases (M). Boxes cover the 25^{th}–75^{th} percentiles and the median is shown as a line across the box
* – statistically significant difference (P-value < 0.05) between group WM and group M; X – statistically significant difference (P-value < 0.05) between groups WM or M and group H

This was caused by the fall in $CD3^+$ lymphocytes and the rise in $CD21^+$ lymphocytes in group M. There was also a significant difference between group WM and group M.

Representative results of CD4 cytometric staining are given in Supplementary Figs 3A–C. The mean $CD4^+$ lymphocyte numbers were compared statistically between all groups. There was no difference between the means of group H and group WM. However, statistically significant differences were observed between the former group and group M and between group WM and group M (P-value < 0.05). The number of $CD4^+$ lymphocytes was lower in group M than in group H and group WM (Fig. 2A).

Representative results of CD8 cytometric staining are given in supplementary Figs 3 D–F. The mean $CD8^+$ cell percentages were comparable between all groups and no statistically significant difference was observed (P-value < 0.05). The means were 18.36% for group H, 20.69% for group WM and 19.25% for group M. However, the variability of group M data was greater than that of group H (Fig. 2B and the standard deviation column in Table 2).

The $CD4^+/CD8^+$ ratio was significantly lower in group M compared to group H (Fig. 2C). This effect was produced mainly by a fall in $CD4^+$ lymphocyte content, because the $CD8^+$ lymphocyte percentage remained at a similar level in all groups.

Discussion

Many studies suggest that tumours are able to exert systemic effects and influence peripheral blood leukocyte composition through tumour-derived cytokines and microvesicles to facilitate progression and metastasis (7, 10). There is growing evidence that tumour development, the recruitment of immune cells into the tumour site, and the presence of tumour-infiltrating leukocytes in tumour milieu are accompanied by changes in the numbers of specific types of leukocytes in peripheral blood (3, 10). The immunophenotyping of peripheral blood lymphocytes described in this article was predicated on the hypothesis that bitches with malignant mammary tumours exhibit an immune profile that is different from that of healthy dogs. Although there are some studies concerning leukocyte immunophenotyping in dogs with mammary tumours, some of the results are contrary; also, there are no standard values for leukocyte percentages in this disease. The innovation of this study lies in its attempt to evaluate single surface proteins to find prognostic and diagnostic markers.

In the present study we documented changes in the parameters of immune status in tumour-bearing dogs with malignant mammary tumours at different clinical stages (Table 2). There were no significant differences in the percentage of $CD3^+$ T lymphocytes between the control group and the group of female dogs with early stage tumours. Contrastingly, in the group of dogs with metastasising tumours, a significant decrease in the percentage of $CD3^+$ lymphocytes was noted. This is in line with the results obtained by Watabe *et al.* (28) and Garcia-Sancho *et al.* (5). The reason for the reduction in $CD3^+$ cells is currently unknown, but may be that cytokines or other immunosuppressive factors secreted by the tumour downregulate the expression of the CD3 protein (28). Another hypothesis is that the decrease in

the percentage of $CD3^+$ lymphocytes in peripheral blood may be due to the infiltration of these lymphocytes into the tumour. A high percentage of $CD3^+$ T lymphocytes has been observed in tumour microenvironment research (20). Given the noted significant difference between group WM and group M, CD3 receptor expression may be useful in differentiating metastasising and non-metastasising tumours and presumably in the prognosis of disease progression in bitches with malignant mammary tumours.

Our findings showed a significant rise in $CD21^+$ peripheral blood lymphocyte percentages in the group of female dogs with metastasising tumours. This is contrary to the findings of Watabe *et al.* (28); however, their research included samples from dogs with various types of cancer, rather than only mammary tumours. In the study of Estrela-Lima *et al.* (4), the percentage of B lymphocytes was also significantly lower in dogs with cancer. This may be explained by the percentage of $CD21^+$ lymphocytes possibly varying depending on the histopathological type of the tumour. The latest studies have proved that B cells can play a dual role in tumorigenesis. Regulatory B cells promote the development of cancer, while other types of B cell act to reduce cancer progression (8). Subsets of B cells may contribute to tumour growth because they promote angiogenesis and are found to be increased in cancer and chronic inflammation (26). The tumour environment is a location where B cells were found to exert immune-regulatory functions, secreting anti-inflammatory cytokines and thus inhibiting anti-tumour immunity (8). Estrela-Lima *et al.* (4) observed high levels of tumour-infiltrating B-cells in animals with worse prognoses (4). Additionally, the rise in the percentage of $CD21^+$ lymphocytes may be evidence of the prevalence of the mechanisms of the Th2 humoral response over the Th1 response in bitches with mammary gland cancer at an advanced stage. The predominance of Th2 mechanisms is related to the downregulation of adaptive immunity, which is involved in an effective anti-tumour response (11). In addition, the prevalence of Th2 responses has been shown to be associated with the chronic inflammatory status of animals and tumour progression (30). From the results of the current research, it can be assumed that B cells contribute to tumour progression, as their increase is associated with the occurrence of metastases in female dogs with malignant mammary gland tumours.

Although there are some studies concerning peripheral lymphocyte phenotyping in tumour-bearing dogs (5, 28) and in bitches with mammary cancers (4, 13), there are few $CD3^+/CD21^+$ cell ratio data. The present study has shown that in the group of bitches with non-metastasising mammary tumours, both $CD3^+$ T lymphocyte and $CD21^+$ B lymphocyte percentages remained unchanged. That is why the $CD3^+/CD21^+$ ratio in the group without metastases was similar to the ratio in the control group. However, in the group with metastases the $CD3^+/CD21^+$ ratio was significantly decreased from the ratios in the control group and the group without metastases. This was due to a significant decrease in the percentage of $CD3^+$ lymphocytes in combination with an increase in the percentage of $CD21^+$ lymphocytes in bitches with metastatic tumours. Therefore, it can be concluded that a downward trend in the percentage of $CD3^+$ T lymphocytes, especially in combination with an upward one in the percentage of $CD21^+$ B lymphocytes, may be helpful in diagnosis and indicate the presence of metastases in female dogs with mammary gland cancers. The dominance of the Th2 humoral response and chronic inflammation in bitches with mammary tumours may be correlated with the advancement of the neoplastic disease and a poor prognosis (1, 5).

In the current research we observed that the percentage of $CD4^+$ lymphocytes in the group of dogs with metastases was significantly lower than that in the group of dogs without metastases and the control group (P-value < 0.05). These findings are consistent with the studies of García-Sancho *et al.* (5) and Watabe *et al.* (28), which identified decreasing $CD3^+$ and $CD4^+$ cell numbers in dogs with different types of cancers. These results suggest that in bitches with malignant tumours at an advanced stage, the immune system is severely affected by the tumorigenic process.

Our findings indicate that there were no statistically significant differences in $CD8^+$ lymphocyte percentages between the investigated groups (P-value < 0.05). These results are similar to those published by Mucha *et al.* (13). The results of other investigations are contrary to ours (5, 28), but those studies were conducted on groups of dogs with different types of tumour. It has been proved that the state of the immune system and the types of cells that are present in the bloodstream are related to the type of neoplasm (27). It is worth emphasising that we did not assess the differences in the activity of $CD8^+$ lymphocytes between the groups of dogs, but only the differences in their percentages. It has been observed in some cancer studies that even if the number of $CD8^+$ T cells remains unchanged, some of them may display features of dysfunction or exhaustion (9).

The $CD4^+/CD8^+$ ratios for the control group, the group with non-metastasising tumours and the group with metastasising tumours were 2.192, 1.967 and 1.361, respectively. The means of this ratio are usually between 1.7 and 2.8 in healthy dogs; the shortening of this ratio in the dogs with tumours may be evidence of immunosuppression (13). The $CD4^+/CD8^+$ ratio was decreased from the control group's ratio in the group without metastases, albeit statistically insignificantly. A significant decrease was observed from the control group's ratio to that of the group of bitches with metastases. Based on these results, it may be concluded that in dogs with malignant mammary tumours, the $CD4^+/CD8^+$ ratio decreases gradually with tumour progression. The noted significant shortening of the $CD4^+/CD8^+$ ratio in the group with metastases was due to a diminution of $CD4^+$ cells, as the percentage of $CD8^+$ cells was similar to that in the control group. A similar ratio change was also observed by

Mucha *et al.* (13), in which metastasising bitches with mammary tumours had the lowest $CD4^+/CD8^+$ ratio. A low $CD4^+/CD8^+$ ratio is associated with immune senescence, activation and inflammation, which may all contribute to carcinogenesis (2). It has been proved that the $CD4^+/CD8^+$ ratio changes during the progression of many human and animal tumours, including those of dogs, and that it may be a potential diagnostic factor (2, 6). With respect to the lymphocyte dysfunction that seems to be present in cancer patients, a tumour-directed immune response involving $CD8^+$ Tc cells, Th1 cells, and natural killer cells appears to protect against tumour development and progression. In contrast, the immune responses that involve B cells, the activation of chronic humoral immunity and/or a Th2 polarised response, and innate inflammatory cells in the tumour can all promote tumour development and progression (1).

Our findings indicate that early-stage malignancies in dogs may not result in any significant changes in leukocyte subpopulations. However, some of the investigated proteins may become potential biomarkers for metastasis and progression in canine mammary tumours. It can be concluded that lower observed percentages of $CD3^+$ T lymphocytes, especially in combination with higher percentages of $CD21^+$ B lymphocytes, may indicate an advanced state of disease and the presence of metastases in bitches with mammary tumours. Additionally, our research revealed that in dogs with malignant mammary tumours, the $CD4^+/CD8^+$ ratio decreases gradually with tumour progression and is not suitable as a diagnostic marker for malignancies, but may be a potential biomarker of mammary tumour metastasis.

Conclusion

Our findings may be useful in tumour metastasis detection, but should be considered preliminary because of the small number of blood samples analysed from bitches with mammary cancer. Lymphocyte immunophenotyping in dogs with mammary tumours can provide a deeper understanding of the staging process and, as a result, it can help in improving staging systems in bitches with mammary tumours and possibly in the development of new diagnostic and prognostic markers.

Conflict of Interests Statement: The authors declare that there is no conflict of interests regarding the publication of this article.

Financial Disclosure Statement: This study was financed as part of the statutory activity of the University of Life Sciences in Lublin.

Animal Rights Statement: Ethical review and approval were not required for this study, because the study had an observational and non-invasive character.

References

1. Carvalho M.I., Pires I., Prada J., Queiroga F.L.: A Role for T-Lymphocytes in Human Breast Cancer and in Canine Mammary Tumors. BioMed Research Intern 2014, 2014, 1–11, doi: 10.1155/2014/130894.
2. Castilho J.L., Bian A., Jenkins C.A., Shepherd B.E., Sigel K., Gill M.J., Kitahata M.M., Silverberg M.J., Mayor A.M., Coburn S.B., Wiley D., Achenbach C.J., Marconi V.C., Bosch R.J., Horberg M.A., Rabkin C.S., Napravnik S., Novak R.M., Mathews W.C., Thorne J.E., Sun J., Althoff K.N., Moore R.D., Sterling T.R., Sudenga S.L.: CD4/CD8 Ratio and Cancer Risk Among Adults With HIV. J Natl Cancer Inst 2022, 114, 854–862, doi: 10.1097/dcr.0000000000001763.
3. Espinoza Vega M.L., Luis Huertas A.L., González Murillo A., Franco-Luzón L., Ramírez Orellana M.: Peripheral blood tumor-infiltrating lymphocytes in a neuroblastoma model. Cir Pediatr 2020, 33, 84–90.
4. Estrela-Lima A., Araújo M.S., Costa-Neto J.M., Teixeira-Carvalho A., Barrouin-Melo S.M., Cardoso S.V., Martins-Filho O.A., Serakides R., Cassali G.D.: Immunophenotypic features of tumor infiltrating lymphocytes from mammary carcinomas in female dogs associated with prognostic factors and survival rates. BMC Cancer 2010, 10, 256, doi: 10.1186/1471-2407-10-256.
5. García-Sancho M., Villaescusa A., Rodríguez-Franco F., Sainz A.: Comparative study of peripheral blood leukocytes in healthy dogs and in dogs with cancer and inflammatory diseases. J Vet Diagn Invest 2014, 26, 282–285, doi: 10.1177/1040638714522464.
6. Hema M.N., Ferry T., Dupon M., Cuzin L., Verdon R., Thiébaut R., Protopopescu C., Leport C., Raffi F., Le Moing V., ANRS CO 8 (APROCO/COPILOTE) study group: Low CD4/CD8 Ratio Is Associated with Non AIDS-Defining Cancers in Patients on Antiretroviral Therapy: ANRS CO8 (Aproco/Copilote) Prospective Cohort Study. PLoS One 2016, 11, e0161594, doi: 10.1371/journal.pone.0161594.
7. Hiam-Galvez K.J., Allen B.M., Spitzer M.H.: Systemic immunity in cancer. Nat Rev Cancer 2021, 21, 345–359, doi: 10.1038/s41568-021-00347-z.
8. Kinker G.S., Vitiello G.A.F., Ferreira W.A.S., Chaves A.S., Cordeiro de Lima V.C., Medina T.S.: B Cell Orchestration of Anti-tumor Immune Responses: A Matter of Cell Localization and Communication. Front Cell Dev Biol 2021, 9, doi: 10.3389/fcell.2021.678127.
9. Li H., van der Leun A.M., Yofe I., Lubling Y., Gelbard-Solodkin D., van Akkooi A.C.J., van den Braber M., Rozeman E.A., Haanen J.B.A.G., Blank C.U., Horlings H.M., David E., Baran Y., Bercovich A., Lifshitz A., Schumacher T.N., Tanay A., Amit I.: Dysfunctional CD8 T Cells Form a Proliferative, Dynamically Regulated Compartment within Human Melanoma. Cell 2019, 176, 775–789, doi: 10.1016/j.cell.2018.11.043.
10. Liang H., Chu X., Zhao J., Xing G., Si Y.: Elevated peripheral blood B lymphocytes and $CD3^+CD4^-CD8^-$ T lymphocytes in patients with non-small cell lung cancer: A preliminary study on peripheral immune profile. Oncol Lett 2018, 15, 8387–8395, doi: 10.3892/ol.2018.8424.
11. Lin C.N., Chien C.Y., Chung H.C.: Are Friends or Foes? New Strategy for Head and Neck Squamous Cell Carcinoma Treatment via Immune Regulation. Int J Head Neck Sci 2017, 1, 105–113, doi: 10.6696/IJHNS.2017.0102.03.
12. McBride J.A., Striker R.: Imbalance in the game of T cells: What can the CD4/CD8 T-cell ratio tell us about HIV and health? PLoS Pathog 2017, 13, e1006624, doi: 10.1371/journal.ppat.1006624.
13. Mucha J., Rybicka A., Dolka I., Szymańska J., Manuali E., Parzeniecka-Jaworska M., Kluciński W., Król M.: Immunosuppression in Dogs During Mammary Cancer Development. Vet Pathol 2016, 53, 1147–1153, doi: 10.1177/0300985816634808.
14. Olkhanud P.B., Damdinsuren B., Bodogai M., Gress R.E., Sen R., Wejksza K., Malchinkhuu E., Wersto R.P., Biragyn A.: Tumor-evoked regulatory B cells promote breast cancer metastasis by

converting resting CD4+ T cells to T-regulatory cells. Cancer Res 2011, 71, 3505–3515, doi: 10.1158/0008-5472.CAN-10-4316.
15. Ostroumov D., Fekete-Drimusz N., Saborowski M., Kühnel F., Woller N.: CD4 and CD8 T lymphocyte interplay in controlling tumor growth. Cell Mol Life Sci 2018, 75, 689–713, doi: 10.1007/s00018-017-2686-7.
16. Qiu J., Zhou F., Li X., Zhang S., Chen Z., Xu Z., Lu G., Zhu Z., Ding N., Lou J., Ye Z., Qian Q.: Changes and Clinical Significance of Detailed Peripheral Lymphocyte Subsets in Evaluating the Immunity for Cancer Patients. Cancer Manag Res 2020, 12, 209–219, doi: 10.2147/CMAR.S221586.
17. Reis L.A., Garcia A.P.V., Gomes E.F.A., Longford F.G.J., Frey J.G., Cassali G.D., de Paula A.M.: Canine mammary cancer diagnosis from quantitative properties of nonlinear optical images. Biomed Opt Express 2020, 11, 6413–6427, doi: 10.1364/BOE.400871.
18. Reuschenbach M., Von Knebel D.M., Wentzensen N.: A systematic review of humoral immune responses against tumor antigens. Cancer Immunol Immunother 2009, 58, 1535–1544, doi: 10.1007/s00262-009-0733-4.
19. Riazi Rad F., Ajdary S., Omranipour R., Alimohammadian M.H., Hassan Z.M.: Comparative analysis of CD4+ and CD8+ T cells in tumor tissues, lymph nodes and the peripheral blood from patients with breast cancer. Iran Biomed J 2015, 19, 35–44, doi: 10.6091/ibj.1289.2014.
20. Saeki K., Endo Y., Uchida K., Nishimura R., Sasaki N., Nakagawa T.: Significance of tumor-infiltrating immune cells in spontaneous canine mammary gland tumor: 140 cases. J Vet Med Sci 2012, 74, 227–230, doi: 10.1292/jvms.11-0118.
21. Sarvaria A., Madrigal J., Saudemont A.: B cell regulation in cancer and anti-tumor immunity. Cell Mol Immunol 2017, 14, 662–674, doi: 10.1038/cmi.2017.35.
22. Serrano-Villar S., Pérez-Elías M.J., Dronda F., Casado J.L., Moreno A., Royuela A., Pérez-Molina J.A., Sainz T., Navas E., Hermida J.M., Quereda C., Moreno S.: Increased risk of serious non-AIDS-related events in HIV-infected subjects on antiretroviral therapy associated with a low CD4/CD8 ratio. PLoS One 2014, 9, e85798, doi: 10.1371/journal.pone.0085798.
23. Szopa I.M., Granica M., Bujak J.K., Łabędź A., Błaszczyk M., Paulos C.M., Majchrzak-Kuligowska K.: Effective Activation and Expansion of Canine Lymphocytes Using a Novel Nano-Sized Magnetic Beads Approach. Front Immunol 2021, 12, 604066, doi: 10.3389/fimmu.2021.604066.
24. Tay R.E., Richardson E.K., Toh H.C.: Revisiting the role of CD4+ T cells in cancer immunotherapy—new insights into old paradigms. Cancer Gene Ther 2021, 28, 5–17, doi: 10.1038/s41417-020-0183-x.
25. Valdivia G., Alonso-Diez Á., Pérez-Alenza D., Peña L.: From Conventional to Precision Therapy in Canine Mammary Cancer: A Comprehensive Review. Front Vet Sci 2021, 8, 623800, doi: 10.3389/fvets.2021.623800.
26. van de Veen W., Głobińska A., Jansen K., Straumann A., Kubo T., Verschoor D., Wirz O.F., Castro-Giner F., Tan G., Rückert B., Ochsner U., Herrmann M., Stanić B., van Splunter M., Huntjens D., Wallimann A., Fonseca Guevara R.J,. Spits H., Ignatova D., Chang Y.T., Fassnacht C., Guenova E., Flatz L., Akdis C.A., Akdis M.: A novel proangiogenic B cell subset is increased in cancer and chronic inflammation. Sci Adv 2020, 6, eaaz3559, doi: 10.1126/sciadv.aaz3559.
27. van der Leun A.M., Thommen D.S., Schumacher T.N.: CD8+ T cell states in human cancer: insights from single-cell analysis. Nat Rev Cancer 2020, 20, 218–232, doi: 10.1038/s41568-019-0235-4.
28. Watabe A., Fukumoto S., Komatsu T., Endo Y., Kadosawa T.: Alterations of lymphocyte subpopulations in healthy dogs with aging and in dogs with cancer. Vet Immunol Immunopathol 2011, 142, 189–200, doi: 10.1016/j.vetimm.2011.05.008.
29. Wei X., Jin Y., Tian Y., Zhang H., Wu J., Lu W., Lu X.: Regulatory B cells contribute to the impaired antitumor immunity in ovarian cancer patients. Tumour Biol 2016, 37, 6581–6588, doi: 10.1007/s13277-015-4538-0.
30. Zhao H., Wu L., Yan G., Chen Y., Zhou M., Wu J., Li Y.: Inflammation and tumour progression: signaling pathways and targeted intervention. Sig Transduct Target Ther 2021, 6, 263, doi: 10.1038/s41392-021-00658-5.
31. Zhao X., Liu J., Ge S., Chen C., Li S., Wu X., Feng X., Wang Y., Cai D.: Saikosaponin A Inhibits Breast Cancer by Regulating Th1/Th2 Balance. Front Pharmacol 2019, 10, 624, doi: 10.3389/fphar.2019.00624.