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Research Article

Evaluation of Hematological, Proinflammatory and Tumor Markers in Bovine Cutaneous Papillomatosis

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Abstract

Cutaneous papillomatosis occurs as benign tumoral skin lesions in cattle. Although this condition is most commonly observed in young animals, it can occur in cattle of any age. Although tumor necrosis factor alpha, high mobility group box-1 protein, myxovirus resistance protein-1 have been investigated in various tumoral conditions in human medicine, the relationship between these markers and other hematological indices have not been investigated in bovine papillomatosis with benign tumoral lesions. The purpose of the study is to determine the levels of these markers and their relationship with each other and with hematological indices. The study comprised two groups: the bovine papillomatosis (BP) group, consisting of animals with papillomatosis, and the control group, consisting of healthy animals. Each group consisted of 10 animals of different breeds and genders with an age of 1-2 years. In the BP group, there were significant differences in total leukocyte count, neutrophil count, monocyte count, and neutrophil-lymphocyte ratio values, which were higher, and lymphocyte-monocyte ratio values, which were lower. The levels of high mobility group box-1 protein, myxovirus resistance protein-1, and tumor necrosis factor alpha were significantly higher in the BP group compared to the control group. In conclusion, this study showed that there were significant differences in hematological, inflammation and tumor markers in cutaneous papillomatosis.

Keywords: Bovine haematological values, Cutaneous papillomatosis, High mobility group box-1, Myxovirus resistance protein-1, Tumor necrosis factor alpha

Introduction

Papillomatosis in cattle typically manifests as benign tumoral structures in the cutaneous and mucosal epithelium. Lesions can occur at any age, although the onset of papillomatosis in cattle is usually around 2 years of age [1,2]. The literature now lists 14 BPV pathotypes, however there might be as many as 20 [3]. The disease spreads through direct contact, contaminated equipment and food, and the use of contaminated syringes. Additionally, inheritance, sunlight, nutritional and hormonal disorders, and immunosuppression are factors that play a role in the pathogenesis of the disease [4]. Arthropods have also been reported to play an important role in transmission. Skin papillomas can spread throughout the body and to the alimentary tract, mammary glands, genital mucosa, and lungs, causing respiratory difficulties, decreased milk yield, and weight loss in animals [5,6]. Inflammation in the tumor area is generally initiated locally by inflammatory proteins, cytokines and immune mediators. These tumor-associated mediators and cytokines are subsequently released into the bloodstream, activating other inflammatory mediators in circulation [7]. In human medicine, the neutrophil-to-lymphocyte ratio (NLR) and lymphocyte-to-monocyte ratio (LMR) are commonly used as prognostic and survival markers in various types of malignant cancers [8,9].

High mobility group box-1 protein (HMGB1) is released from necrotic and inflammatory cells [10]. Elevated levels of HMGB1 have been identified as a marker of cancer. HMGB1 diffuses from tumor cells along with other intracellular molecules [11]. Overaccumulation of HMGB1 has been reported in cervical carcinomas [12]. In addition, HMGB1 increases the stimulation of tumor necrosis factor alpha (TNF-α), interleukin 1 beta and other inflammatory cells, and has many functions such as activation of the innate immune system and dentritic cell activation in cancer [10].

TNF-α is a cytokine that is released as an indicator

of the activation of macrophages and other pro-inflammatory cytokines and is detected in the early stages of inflammation. Additionally, TNF-α plays a crucial role in regulating the immune system [13]. In viral infections, TNF-α exhibits antiviral properties by inhibiting virus replication [14]. TNF-α has been shown to effectively initiate inflammation and plays an active role in repair and regeneration processes following epidermal injury and infection [15].

Myxovirus resistance 1 (Mx1) gene appears to increase in vertebrate viral infections when stimulated by type I and III interferons (IFNs). Myxovirus resistance protein-1 (Mx1) serves as a valuable biomarker for IFN activity and the effectiveness of IFN treatment in specific cancer types [16]. It has been reported that Mx1 is associated with various human cancers. Although Mx1 has been implicated in antitumor activity, it is unclear how it affects immune cells in tumoral conditions [17]. Mx1 proteins are released upon stimulation of IFNs. Additionally, Mx1 proteins exhibit antiviral properties against many viruses, including influenza viruses [18].

The aim of this study is to determine whether markers such as NLR, LMR, TNF-α, HMGB1 and Mx1, which are used as tumor markers in human medicine, can also be used as tumor markers in bovine papillomatosis, a benign tumoral disease, and to investigate the levels of these tumor markers and their correlations with each other and with hematological index values such as NLR and LMR.

Material and Methods

Ethical Approval

The study was approved by Atatürk University Animal Experiments Local Ethics Committee (Decision no: 2023/13, Approval date: 20.11.2023). Before taking blood samples from the animals, an "Informed Consent Form" was obtained from the animal owners.

Animals

The animals used in the BP group consisted of animals brought to the Veterinary Hospital of Atatürk University, Faculty of Veterinary Medicine with complaints of skin lesions. The healthy group was comprised of animals of different breeds and genders, and animals with no pathological conditions in both clinical and hematological examinations were selected. Each group consisted of 10 animals of different breeds and genders.

Clinical and Hematological Examination

Routine clinical examination (examination of lymph nodes, heart and respiratory rate, body temperature, dehydration status, etc.), skin examination and hemogram analysis (Abacus junior Vet 5, Hungary) were performed on both healthy animals and animals with papillomatosis. The diagnosis of papillomatosis was made based on clinical observations. Animals with any other disease in their clinical and hematological examinations were excluded from the study. In order to create uniformity in the clinical examination of animals and to avoid different interpretations, an examination protocol was carried out by a researcher.

Blood Collection and Handling

Blood samples were taken from the jugular veins of the animals using a 20 mL needle (18 G, 1.20 x 38 mm, Berika, Türkiye). Hematological examinations of these blood samples were immediately performed by placing 2 mL into tubes containing ethylenediamine tetraacetic acid (EDTA) and their analyses were performed. For biochemical analyses, 8 mL blood samples were taken into serum tubes (BD Vacutainer® SST™ II Advance, UK) and kept at room temperature for 30 min. The serum tubes were then centrifuged in a refrigerated centrifuge (Beckman Coulter Allegra X-30R, USA) at 3000 rpm for 10 min. The sera obtained were transferred to Eppendorf tubes and stored

Table 1. *Comparison of mean ± SD and median values of hematological index in bovine papillomatosis and control group*

Parameters	**Control Mean±SD**	**BP Mean±SD**	**P**
WBC (×10³/μL)	7.72±1.19	18.76±3.78	<0.001
LYM (×10³/μL)	4.13±0.84	4.60±0.96	>0.05
NEU (×10³/μL)	3.34±0.95	13.22±3.06	<0.001
NLR	0.84±0.29	2.91±0.59	<0.001
LMR	23.51±4.07	6.12±2.01	<0.001
Parameter	**Median (Q1-Q3)**	**Median (Q1-Q3)**	**P/Mann Whitney U**
MON (×10³/μL)	0.15 (0.14-0.24)	0.74 (0.57-0.99)	<0.001/<0.0001

WBC: *Total leukocyte count;* ***LYM:*** *Lymphocyte;* ***MON:*** *Monocyte;* ***NEU:*** *Neutrophil;* ***NLR:*** *Neutrophil to lymphocyte ratio;* ***LMR:*** *Lymphocyte to monocyte ratio. P<0.05 is statistically significant*

Table 2. *Comparison of mean ± SD and median values of inflammation and tumor marker in bovine papillomatosis and control group*

Parameter	**Control Mean±SD**	**BP Mean±SD**	**P**
Mx1 (ng/mL)	8.66±2.59	12.83±3.00	<0.01
Parameters	**Median (Q1-Q3)**	**Median (Q1-Q3)**	**P/Mann Whitney U**
HMGB1(ng/mL)	26.82 (22.55-29.26)	46.42 (35.31-60.41)	<0.01/8.00
TNF-α (ng/L)	118.53 (114.19-132.43)	216.75 (195.36-243.69)	<0.001/<0.0001

BP: *Bovine papillamotosis;* ***Mx1:*** *Myxovirus resistance protein-1;* ***HMGB1:*** *High-mobility group box-1 protein;* ***TNF-α:*** *Tumor necrosis factor alpha. P<0.05 is statistically significant*

Table 3. Spearman correlation relations between values

Parameters	WBC	LYM	MON	NEU	NLR	LMR	HMGB1	Mx1	TNF-α
WBC	1.000	0.519*	0.916**	0.944**	0.864**	-0.842**	0.484*	0.634**	0.622**
LYM		1.000	0.542*	0.328	0.073	-0.292	-0.115	0.149	-0.011
MON			1.000	0.836**	0.728**	-0.928**	0.505*	0.576**	0.671**
NEU				1.000	0.946**	-0.816**	0.582**	0.652**	0.658**
NLR					1.000	-0.764**	0.652**	0.649**	0.711**
LMR						1.000	-0.610**	-0.597**	-0.762**
HMGB1							1.000	0.295	0.713**
Mx1								1.000	0.701**
TNF-α									1.000

WBC: *Total leukocyte count;* ***LYM:*** *Lymphocyte;* ***MON:*** *Monocyte;* ***NEU:*** *Neutrophil;* ***NLR:*** *Neutrophil to lymphocyte ratio;* ***LMR:*** *Lymphocyte to monocyte ratio;* ***HMGB1:*** *High-mobility group box 1 protein;* ***Mx1:*** *Myxovirus resistance protein-1;* ***TNF-α:*** *Tumor necrosis factor alpha. * Correlation is significant at the 0.05 level; ** Correlation is significant at the 0.01 level*

in a deep-freeze refrigerator (Esco Lexicon® ULT Freezer, USA) at -80°C until analysis.

Assays for Serum High Mobility Group Box-1 Protein, Tumor Necrosis Factor Alpha, and Myxovirus Resistance Protein-1

Serum levels of HMGB1, TNF-α, and Mx1 were measured using a commercial ELISA test kit (Bovine BT Lab., Zhejiang, China) following the manufacturer's instructions. The ELISA procedures were also processed and shaped in the ELISA reader device (BioTek μQuant MQX200 ELISA Reader, USA).

Statistical Analysis

A study was conducted on cattle with papillomatosis to evaluate their peripheral blood hematologically and immunophenotypically. Each group consisted of 5 cattle. The study also included a power analysis of hematologic index values (≥95% power ratio (effect size=2.84; α=0.05; distribution ratio=1:1), which revealed that the study could be conducted on at least 4 animals [19]. Power analysis was performed using G*Power® version (3.1.9.4, Franz Faul, Universität Kiel, Germany). Data analysis was performed using IBM SPSS Statistics 27.0.1 software.

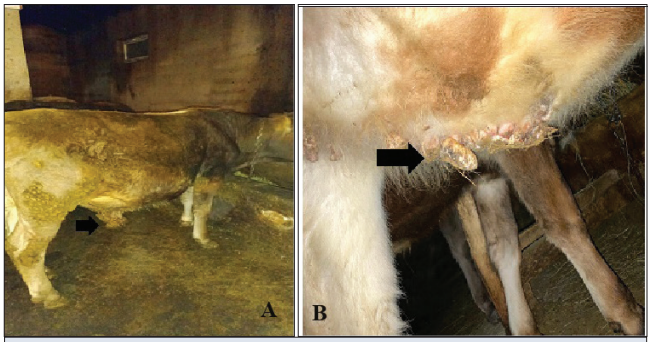

Fig 1. A-B: Umbilical and abdominal papillomatosis lesions. The arrow indicates the papillomatosis lesion

The Shapiro-Wilk normality test was used to assess the normal distribution of the data, and Levene's test was conducted to determine the homogeneity of the data. Descriptive statistics were presented as number of units (n) and mean ± standard deviation ($\bar{x}$±sd) for normally distributed data and as quartiles (Q1-Q3) for non-normally distributed data. To determine the difference between groups, the independent samples *t*-test was used for normally distributed data, and the Mann-Whitney U test was used for non-normally distributed data. Spearman correlation analysis was used to determine the correlation between the data. As stated by Chan [20], a correlation coefficient (*rho*) <0.3 is considered as a very weak correlation, 0.3-0.5 is considered as a moderate correlation, 0.6-0.8 is considered as a strong correlation, and a correlation coefficient value of ≥0.8 is considered as a very strong correlation in Spearman's correlation coefficient analysis.

Results

Clinical and Hematological Findings

In clinical examinations of cattle with papillomatosis, it was determined that cauliflower-like skin lesions were formed in the abdominal region and linea alba region *(Fig. 1)*. Although other clinical examinations (such as respiration rate, pulsation, rectal temperature) of these animals were within the reference range, hematological examinations revealed that the values of total leukocyte count (WBC), neutrophil count (NEU), monocyte count (MON) and NLR were significantly higher in the BP group than in the control group (P<0.001). Additionally, LMR value in the BP group was statistically lower than that in the control group (P<0.001). However, there was no significant difference between the groups in terms of lymphocyte count (LYM) (P>0.05) *(Table 1)* *(Fig. 2)*. Animals in both groups consist of 1-2 years old animals.

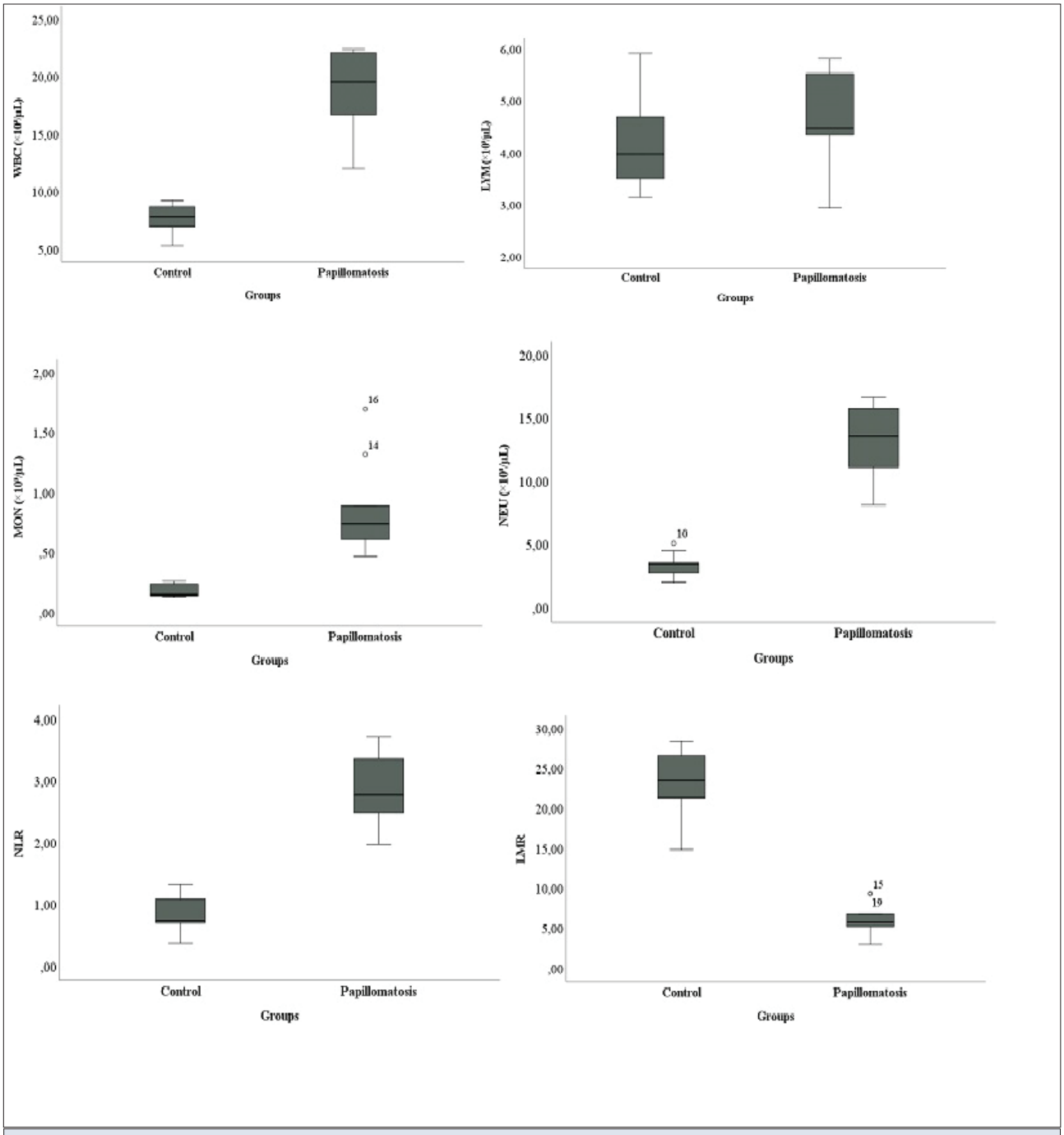

Fig 2. Boxplot diagram of hematological values between groups. Circles indicate outliers

Inflammation and Tumor Marker Findings

Table 2 shows the serum values of HMGB1, TNF-α, and Mx1. Cattle with BP had significantly higher serum Mx1, HMGB1 and TNF-α levels compared to the control group (P<0.01; P<0.01; P<0.001, respectively) *(Fig. 3)*.

Correlation Findings of Hematological, Inflammatory and Tumor Markers

The correlation table for these markers is shown in *Table 3*. WBC was moderately correlated with LYM and HMGB1 (*rho*=0.519; P<0.05, *rho*=0.484; P<0.05, respectively). WBC was found to be very strongly correlated with MON, NEU, NLR and LMR (*rho*=0.916, 0.944, 0.864, and -0.842; all P values<0.01, respectively). WBC showed a strong correlation with both Mx1 and TNF-α (*rho*=0.634; P<0.01; *rho*=0.622; P<0.01, respectively). There was a moderate correlation between the values of LYM and MON (*rho*=0.542; P<0.05). The MON value showed a very strong correlation with both NEU and LMR (*rho*=0.836, -0.928, respectively; both P values <0.01). The MON value showed a strong correlation with both NLR and TNF-α (*rho*=0.728; 0.671, respectively; both P values

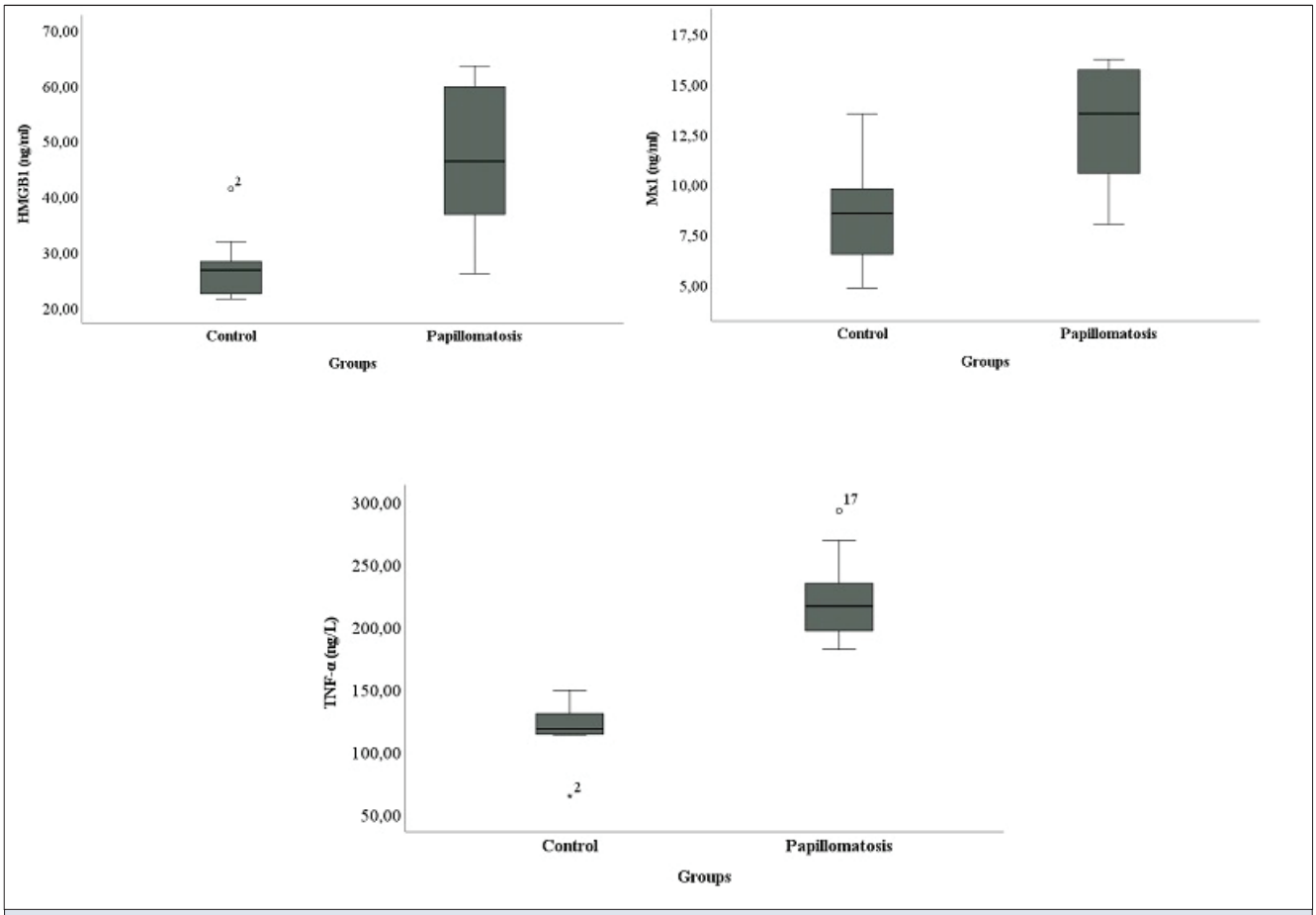

Fig 3. Boxplot diagram of inflammatory and tumor markers values between groups. Star and circles indicate outliers

P<0.01). The MON value showed moderate correlation with HMGB1 (*rho*=0.505; P<0.05) and Mx1 *(rho*=0.576; P<0.01). NEU was very strongly correlated with both NLR *(rho*=0.946, P<0.01) and LMR *(rho*=-0.816, P<0.01). A moderate correlation was found between NEU and HMGB1 *(rho*=0.582; P<0.01), while a strong correlation was found between NEU and Mx1 and TNF-α *(rho*=0.652, 0.658, respectively; both P values <0.01). NLR was found to be strongly correlated with LMR, HMGB1, Mx1, and TNF-α *(rho*=-0.764, 0.652, 0.649, 0.711, respectively; all P values <0.01). LMR was found to have a strong correlation with HMGB1 and TNF-α *(rho*=-0.610, -0.762; P<0.01, respectively). Additionally, LMR was moderately correlated with Mx1 *(rho*=-0.597; P<0.01). Strong correlations were obtained between HMGB1 and TNF-α *(rho*=0.713; P<0.01) and between Mx1 and TNF-α *(rho*=0.701; P<0.01). The correlation of the values is also presented in *Fig. 4.*

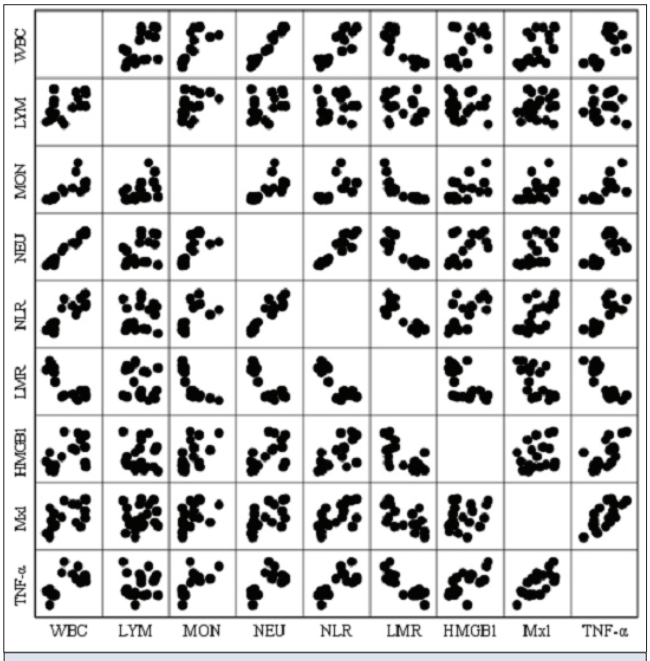

Fig 4. Correlation coefficients among hematolological index, inflamation and tumor markers in cattle with cutaneous papillomatosis. **WBC:** Total leukocyte count; **LYM:** Lymphocyte; **MON:** Monocyte; **NEU:** Neutrophil; **NLR:** Neutrophil to lymphocyte ratio; **LMR:** Lymphocyte to monocyte ratio; **HMGB1:** High mobility group box 1 protein; **Mx1:** Myxovirus resistance protein-1; **TNF-α:** Tumor necrosis factor alpha

Discussion

Bovine papillomatosis is a viral disease caused by bovine papillomaviruses that result in benign tumor-like skin lesions [21]. Skin papillomas in cattle mostly occur on the head and neck, and in some animals, on other parts of the

body [22]. In this study, cutaneous papillomatosis lesions were found on the linea alba and around the abdomen in all animals in the BP group. It was also observed that the age of the animals affected by cutaneous papillomatosis in the current study was between 1-2 years of age.

Imbalances in WBC have been shown to indicate a poor prognosis in hepatocellular carcinoma [23]. However, since there is a balance between neutrophil and lymphocyte counts in maintaining the immune system, polymorphonuclear leukocytes and WBC alone are not reliable prognostic markers [24]. On the other hand, NLR has been shown to be a prognostic marker in certain types of cancer [25]. Lymphocytes actively regulate the immune system in tumoral conditions. Therefore, low lymphocyte levels can trigger both tumor development and increased susceptibility to metastasis [26]. Circulating monocytes have been reported to trigger tumor cell growth and aid in their evasion of the immune system's control [27]. It has been reported that papillomavirus-like particle structures interact with immune system cells such as monocytes, dendritic cells, B lymphocytes, and this is shaped by the effect of the immune response to papillomavirus [28]. Additionally, there is a strong correlation between systemic inflammation and the development of cancer. While the antitumor effects of neutrophils are well-established, it has been reported that an increase in the relative number of neutrophils can also lead to an increase in inflammatory markers, such as anti-apoptotic substances and pro-angiogenic growth factors [29]. IL-6 and TNF-α are the major cytokines that increase the spreading of polymorphonuclear neutrophils [30]. A study on cattle with mastitis reported an increase in NLR levels parallel to the increase in TNF-α, caused by an increase in the inflammatory response [31]. Although the hematological examinations of healthy animals were within normal physiological limits, the study found a higher NLR rate in cattle with cutaneous papillomatosis and a lower LMR rate. It is seen that the reason for the change in NLR ratio here is due to the increase in inflammatory mediators [29,31], and the change in LMR status is due to monocytosis. The significant increase in monocyte levels in the BP group may be due to an immune system response to papillomavirus [28].

During the early phase of inflammation, TNF-α induces other chemokines that direct neutrophils and macrophages towards the skin epidermis. Furthermore, TNF-α contributes to the repair of damaged skin, in addition to initiating the innate immune system and inflammatory phase [32]. A study conducted in cattle with lumpy skin disease (LSD) reported that the increase in proinflammatory cytokine levels (IL-1β, IL-6, and TNF-α) may be caused by the systemic inflammatory response in LSD [33]. Another study conducted in cattle with trichophytosis found a significant increase in TNF-α levels in the patient group compared to the control group, possibly due to the increase in the inflammatory state [34]. In this study, like the previous studies, cattle in the BP group showed a significant increase in TNF-α levels compared to the control group. This may be due to the systemic inflammatory response, as noted by Kamr et al.[33]. The study found strong correlations between HMGB1 and TNF-α *(rho*=0.713; P<0.01), Mx1 and TNF-α *(rho*=0.701; P<0.01), and NLR and TNF-α *(rho*=0.711; P<0.01), suggesting the formation of an inflammatory response in cattle with cutaneous papillomatosis.

Mx1 is an indicator of the antiviral status induced by IFNs in many species. Studies have shown that Mx proteins inhibit the activity of Orthomyxoviridae, Rhabdoviridae, and Bunyaviridae viruses [35,36]. Additionally, it regulates the activity of IFNs in controlling normal and tumor cell metastasis. Mx1 has therefore been shown to be a tumor suppressor and a prognostic marker in tumoral conditions [37]. Evidence of a strong link between Mx1 protein and cutaneous squamous cell carcinomas has been reported due to marked hemostasis impairment caused by mutations (L95P, P96S, and P218S) [38]. A study conducted on patients with prostate cancer found high levels of Mx1. It was concluded that Mx1 plays an important role in suppressing tumor progression in this type of cancer [39]. Mx1 may be an indicator of disease resistance in livestock [40]. In this study, Mx1 levels were significantly higher in cattle in the BP group compared to the control group. It is thought that this may be related to both the ability of Mx1 to inhibit virus replication [36] and its suppressive property in tumoral tissues [39]. In cattle administered lipopolysaccharide (LPS) during early pregnancy, an increase in Mx1 gene expression was observed alongside an increase in TNF-α levels in response to an inflammatory stimulus. It has been suggested by the authors that this may be a regulatory response to the uterine proinflammatory response [41]. In the present study, a strong correlation was found between TNF-α and Mx1 *(rho*=0.701; P<0.01), suggesting that an increase in Mx1 status may serve as a regulatory response against the inflammatory state in cutaneous papillomatosis.

HMGB1 is a proinflammatory cytokine that is released by activated monocytes and dendritic cells or damaged and dead cells [42]. Extracellular HMGB1 has many important functions, such as inducing TNF-α, IL-1β, and other inflammatory cytokines, as well as promoting dendritic cell maturation. These processes play an active role in the chronic inflammatory process in cancer diseases [10]. In viral infections, such as respiratory syncytial virus [43], herpes simplex virus type-1 [44], and herpes simplex virus type-2 [45], HMGB1 levels have been reported to increase in direct proportion to the severity of the inflammatory

condition. Similarly, in a study conducted in cattle with bovine herpes virus-1, HMGB1 levels were shown to increase [46]. In this study, HMGB1 levels were found to be higher in cattle in the BP group compared to the control group, which may be related to the severity of the inflammatory condition, as stated by Workenhe et al.[45]. HMGB1 can activate nuclear factor kappa B (NF-κB), which in turn increases the stimulation of inflammatory cytokines such as TNF-α and IL-1β [47]. The strong correlation between HMGB1 and TNF-α in this study (*rho*=0.713; P<0.01) indicates the severity of the inflammatory state in cutaneous papillomatosis.

There are several limitations in this study. Although clinical findings can be used to diagnose cutaneous papillomatosis, other methods such as histopathology, electron microscopic examination, polymerase chain reaction, and viral genetic analysis can be used to determine the type of virus [48-50]. In the animals included in the study, diagnosis was made only on the basis of clinical findings of cutaneous lesions. Additionally, while the number of animals used in the study was supported by power analysis, it is possible that different results may be obtained in a study with a larger patient group. Finally, only a single blood sample was collected from both patient and control animals in this study. In addition to the markers investigated in the current study for this disease, studies conducted with different inflammatory markers, such as acute phase proteins, and simultaneous investigation of repeatedly taken blood samples may provide additional information about the disease.

In conclusion, this study found significant changes in hematological parameters, HMGB1, and Mx1, which are used as inflammatory and tumor markers, as well as TNF-α, a proinflammatory marker, in bovine cutaneous papillomatosis. These markers may provide useful information in determining the inflammatory status of the disease.

Declarations

Availability of Data and Materials: The datasets used and/or analysed during the current study are available from the corresponding author (Ö. Aydın) on reasonable request.

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