RESEARCH ARTICLE

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The Interplay of CD8+ TILs and Microvascular Density: A Novel Prognostic Indicator in Colorectal Adenocarcinoma

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Abstract

Objective: The purpose of this study was to investigate the association of $CD8^+$ tumor-infiltrating lymphocytes ($CD8^+$ TILs), microvascular density (MVD), and vascular endothelial growth factor (VEGF) with the TNM (Tumor-Node-Metastasis) stage, as well as to analyze their interrelationships in colorectal adenocarcinoma. **Methods:** This cross-sectional study involved 50 FFPE samples of colorectal adenocarcinoma from the Laboratory of Anatomical Pathology at Dr. Soedarso Hospital. Microvascular density (MVD) was assessed on hematoxylin eosin (H&E) slides, while $CD8^+$ tumor-infiltrating lymphocytes ($CD8^+$ TILs) and vascular endothelial growth factor (*VEGF*) expression were evaluated using immunohistochemistry (IHC). Correlation analysis was conducted between the biomarkers and the TNM stage, including its components depth of tumor invasion, lymph node status, and distant metastasis using the Chi-square test. Spearman's correlation was used to assess the relationships among $CD8^+$ TILs, MVD, and VEGF. **Results:** High *$CD8^+$ TILs* expression was significantly associated with negative lymph node status ($p = 0.047$; OR = 0.31, 95% CI = 0.098–1.001), absence of distant metastasis ($p = 0.008$; OR = 0.130, 95% CI = 0.025–0.680), and low TNM stage ($p = 0.011$; OR = 0.221, 95% CI = 0.067–0.727). The distribution of high MVD was correlated with deeper tumor invasion ($p = 0.004$; OR = 9.036, 95% CI = 1.741–46.890), positive lymph node status ($p < 0.001$; OR = 10.286, 95% CI = 2.768–38.215), and high TNM stage ($p = 0.002$; OR = 6.612, 95% CI = 1.924–22.728). High expression of VEGF showed a significant correlation with deeper tumor invasion ($p = 0.036$; OR = 0.675, 95% CI = 0.544–0.837). Spearman's test revealed a negative correlation between $CD8^+$ TILs and MVD ($r = –0.280$, $p = 0.049$), and a positive correlation between MVD and VEGF ($r = 0.303$, $p = 0.032$). **Conclusion:** High $CD8^+$ TILs and low MVD are favorable prognostic factors in colorectal adenocarcinoma, whereas increased MVD and VEGF expression indicate tumor aggressiveness and enhanced angiogenesis. The combination of immune and angiogenic biomarkers with TNM staging could improve prognostic evaluation in colorectal adenocarcinoma.

Keywords: Colorectal adenocarcinoma- CD8+ TILs- MVD- VEGF- TNM Stage- cancer

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Introduction

The colorectal adenocarcinoma was the third most common cancer worldwide in 2022, with a mortality rate of 462,252 cases [1]. According to results, mortality and 5-year survival rates for colorectal adenocarcinoma depend on various prognostic factors, one of which is the TNM (Tumor-Nodes-Metastasis) stage [2, 3]. Overall survival (OS) decreases with increasing TNM stage [3, 4].

The cancer cell development is influenced not only by genetic alteration or mutation but also by the tumor microenvironment (TME) within the stromal area of the tumor [5, 6]. TME is a dynamic environment composed of multiple elements, such as the immune and angiogenic systems [5, 7]. CD8+ tumor-infiltrating lymphocytes (TILs), components of the immune system around tumors, are essential for recognizing and eliminating tumor cells [8, 9]. High CD8+ TILs expression is correlated with better prognosis in several types of cancers, including colorectal adenocarcinoma [10, 11].

The angiogenic components in the TME play an essential part in cancer progression by forming new blood vessels that promote tumor cell growth and increase the incidence of metastasis [5, 9]. Microvascular density (MVD) and vascular endothelial growth factor (*VEGF*) are pro-angiogenic factors linked to poor cancer outcomes. High MVD increases the risk of angioinvasion and nodal involvement, while high *VEGF* relates to tumor invasion

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depth [12, 13].

Various components in the TME influence and interact dynamically [5]. However, there have been few studies directly examining the relationship between immune and angiogenic system components in colorectal adenocarcinoma. Therefore, this study aimed to analyze the relationship between *CD8+ TILs, MVD,* and *VEGF* expression with TNM stage, as well as its components (depth of tumor invasion, lymph node status, and distant metastasis) in colorectal adenocarcinoma, and to investigate the interrelationship among these markers to better understand the immune-angiogenic crosstalk in colorectal adenocarcinoma progression.

Materials and Methods

This retrospective cross-sectional study included 50 formalin-fixed paraffin-embedded (FFPE) tissue samples from patients with colorectal adenocarcinoma at Dr. Soedarso Hospital's Laboratory of Anatomical Pathology, Pontianak, between January 2020 and December 2023. The samples were from resection tissue, and contained adequate tumor cells for hematoxylin-eosin (HE) and immunohistochemistry (IHC) examination. Furthermore, it also had TNM stage data based on the American Joint Committee on Cancer (AJCC)-8, which includes the depth of tumor invasion (pT), lymph node metastasis status (pN), and distant organ metastasis status (pM). Observation of HE and IHC slides was conducted by two pathologists in a blinded manner. This research received ethical clearance from the Health Research Ethics Committee of Dr. Soedarso General Hospital (Approval No. 96/RSUD/KEPK/XI/2024).

Immunohistochemical Procedure

Formalin-fixed paraffin-embedded sections were cut into 4 µm thick slices, deparaffinized in xylene, and rehydrated through graded ethanol solutions. Antigen retrieval was performed using heat-induced epitope retrieval in Tris-EDTA buffer pH 9.0 at 95–98 °C for 30 minutes. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide for 5 minutes, followed by protein blocking for 5 minutes.

The samples were then incubated with anti-*CD8* mouse monoclonal antibody (clone IHC542, GenomeMe, Canada; dilution 1:200) and anti-*VEGF* mouse monoclonal antibody (clone IHC682, GenomeMe, Canada; dilution 1:200) at room temperature for 60 minutes. After primary antibody incubation, sections were treated with a polymer-based detection system for 10 minutes and visualized using 3,3′-diaminobenzidine (DAB). Slides were counterstained with Mayer's hematoxylin, dehydrated, cleared, and mounted. Tonsil tissue was used as a positive control for *CD8* expression, and kidney tissue was used as a positive control for *VEGF*.

MVD Assessment

The observations were carried out on HE slides with a 40x objective magnification. The entire tumor stroma area within the slide was observed, and hot-spot areas containing a high MVD were selected. The MVD was assessed by calculating the average blood vessel count in three fields of view in the hot-spot area [7]. The median value obtained was used as a cut-off point (12.5) to divide the data into low and high groups. The criteria for blood vessels observed were having a diameter of less than 8 erythrocytes, thin-walled, and not being located in sclerotic or necrotic areas.

Assessment of CD8+ Tumor-Infiltrating Lymphocytes Expression

The *CD8+ TIL* expression was identified on lymphocyte cell membranes within the tumor stroma using semiquantitative IHC staining. To minimize sampling bias, five random fields of view were assessed at 40× objective magnification [14], selected according to a standardized zigzag (serpentine) pattern. The microscope stage was moved from left to right across the tissue section and then shifted downward before being moved in the opposite direction, allowing systematic coverage of the slide while maintaining random field selection. The average value derived from these selected fields was calculated to evaluate $CD8^+$ TIL expression following the approach of Fathima et al. [15], with minor modifications. The resulting median value (42.8) was used as the cutoff to stratify samples into low- and high-expression groups. $CD8^+$ TIL levels were classified as 'high' when exceeding the median and 'low' when falling below it.

Assessment of VEGF Expression

The *VEGF* was assessed in the cytoplasm of tumor cells using IHC staining. The assessment was performed semi-quantitatively using the H-Score method, which involves multiplying the percentage of stained cells by the staining intensity (0 for no staining, 1 for light staining, 2 for moderate staining, and 3 for strong staining). The H-Score results were classified as negative (0-50); weak (51-100); moderate (101-200); and strong (201-300) [16].

Statistical Analysis

Statistical analyses were conducted using SPSS version 25. The Chi-square test was employed to assess the associations between $CD8^+$ TILs, MVD, and *VEGF* with tumor depth of invasion, lymph node status, distant metastasis, and TNM stage. For variables showing significant associations, Odds Ratios (ORs) and their corresponding 95% confidence intervals (95% CIs) were calculated to quantify the strength of these relationships. The association between $CD8^+$ TILs expression and *VEGF*, as well as MVD, was evaluated using Spearman's correlation test. A p-value of <0.05 was considered statistically significant.

Results

Clinicopathological characteristics

A summary of the clinicopathological characteristics in this study is presented in Table 1. The sample in this study was divided into four groups, including stage I (24%), stage II (26%), stage III (26%), and stage IV (24%). In this study, the mean age of the patients was 59.2 years (range = 25-79 years), with the majority being male (58%). The

Table 1. Clinicopathological Characteristics

Variables	n (%)
Age (Years)	
< 60	23 (46)
≥ 60	27 (54)
Mean±SD 59.2±12.02	
Sex	
Male	29 (58)
Female	21 (42)
Tumor depth invasion	
pT1	3 (6)
pT2	10 (20)
pT3	32 (64)
pT4	5 (10)
Lymph node metastasis status	
Negative	27 (54)
Positive	23 (46)
Distant metastasis	
Negative	38 (76)
Positive	12 (24)
Stages	
I	12 (24)
II	13 (26)
III	13 (26)
IV	12 (24)
CD8+ TILs Expression	
Low	25 (50)
High	25 (50)
MVD	
Low	25 (50)
High	25 (50)
VEGF Expression	
Negative	0 (0)
Weak	4 (8)
Moderate	36 (72)
Strong	10 (20)

CD8+ TILs, CD8+ Tumor-infiltrating lymphocytes; MVD, Microvascular density; *VEGF*, vascular endothelial growth factor

majority of the sample was pT3 (64%), without lymph node metastasis (54%), and without distant metastasis (76%) (Table 1).

Relationship between CD8+ TILs Expression and TNM Stage

The *CD8+ TILs* were expressed on the membranes of lymphocytes in tumor stroma at various stages of colorectal adenocarcinoma (Figure 1). The Chi-square test results showed that high *CD8+ TIL* expression in tumor areas was significantly correlated with negative lymph node metastasis status (p = 0.047; OR=0.31, 95%CI = 0.098-1.001), no distant metastasis (p = 0.008; OR=0.130, 95%CI=0.025-0.680), and low TNM stage groups (p = 0.011; OR=0.221. 95%CI=0.067-0.727). However, CD8+ TIL expression showed no correlation with tumor invasion depth (p = 0.107) (Table 2).

Relationship between MVD and TNM Stage

The amount of MVD was assessed in the stromal area surrounding the tumor (Figure 2). Tumors with high MVD showed higher odds and were associated with deeper tumor invasion (p = 0.004; OR=9.036, 95%CI= 1.741-46.890), positive lymph node metastasis (p = 0.000; OR=10.286, 95%CI = 2.768-38.215), and high TNM stage (p = 0.002, OR=6.612, 95%CI=1.924-22.728). High MVD correlated with deeper tumor invasion, positive lymph node metastasis, and high TNM stage (III-IV). No significant correlation was observed between MVD and distant metastasis (p = 0.508) (Table 2).

Relationship between VEGF Expression and TNM Stage

The expression of *VEGF* was evaluated in the cytoplasm of colorectal adenocarcinoma cells (Figure 3). Most *VEGF* expression was moderate (72%), and none were negative. Strong *VEGF* expression was significantly associated with deeper tumor invasion (p=0.036; OR=0.675, 95%CI= 0.544-0.837). In contrast, *VEGF* expressions were not significant correlation with lymph node metastasis (p=0.321), distant metastasis (p=0.619), and TNM stage (p=0.480) (Table 2).

Relationship between CD8+ TILs and VEGF Expression with MVD

The Spearman's correlation test showed a negative correlation between *CD8+ TILs* expression and MVD (p = 0.049; r = -0.280). These results show that increasing

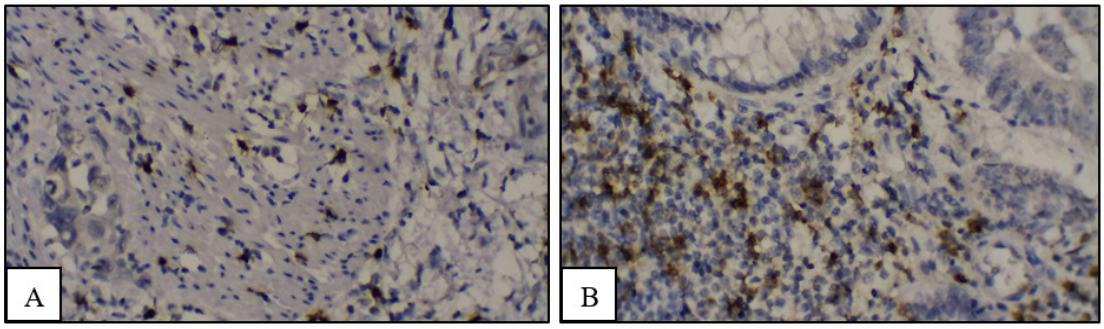

Figure 1. Expression of *CD8+ TILs* in Tumor Stroma Area. (A) Low group of *CD8+ TILs.* (B) High group of *CD8+ TILs* (IHC, Objective 40x).

Table 2. Relationship between *CD8+ TILs, MVD*, and *VEGF* Expression with Clinicopathological Parameters

Variables		CD8+ TILs Expression		p-value	OR (95%CI)	MVD		p-value	OR (95%CI)	VEGF Expression		p-value	OR (95%CI)
		Low(n)	High(n)			Low (n)	High (n)			Weak - Moderate (n)	Strong(n)		
Tumor depth invasion													
	p T1- p T2	4	9	0.107	0.339 (0.088-1.300)	11	2	0.004*	9.036 (1.741-46.890)	13	0	0.036*	0.675 (0.544-0.837)
	p T3- p T4	21	16			14	23			27	10		
Lymph node metastasis													
	Negative	10	17	0.047*	0.314 (0.098-1.001)	20	7	0.000*	10.286 (2.768-38.215)	23	4	0.321	2.02 (0.49-8.32)
	Positive	15	8			5	18			17	6		
Distant metastasis													
	Negative	15	23	0.008*	0.130 (0.025-0.680)	20	18	0.508	1.556 (0.419-5.779)	31	7	0.619	1.47 (0.31-6.90)
	Positive	10	2			5	7			9	3		
Stages													
	I-II	8	17	0.011*	0.221 (0.067-0.727)	18	7	0.002*	6.612 (1.924-22.728)	21	4	0.480	1.65 (0.41-6.78)
	III-IV	17	8			7	18			19	6		

CD8+ TILs, CD8+ Tumor-infiltrating lymphocytes; MVD, Microvascular density; VEGF, vascular endothelial growth factor; *Significant p-value (p<0.05); OR, odds ratio; CI, confidence interval.

Figure 2. Microvascular Density Evaluation in Hot-Spot Areas. (A) Low microvascular density. (B) High microvascular density (HE; Objective 40x).

CD8+ TILs expression is consistent with decreasing MVD counts. Conversely, there was a positive correlation between MVD and *VEGF* (p=0.032; r=0.303), showing that the higher the MVD count, the stronger the *VEGF* expression (Table 3).

Discussion

Most of the patients in this study were male (58%), with a mean age of 59.2 years, ranging from 25 to 79 years. The youngest patient was 25 years old. The incidence of colorectal adenocarcinoma in young patients is correlated with various factors, including Lynch syndrome, a family history of colorectal adenocarcinoma, dietary patterns, and childhood obesity [17, 18]. The prognosis of colorectal adenocarcinoma in young patients is generally poorer, as it is frequently associated with poorly differentiated histological grade and advanced stage at diagnosis [18,

Table 3. Relationship between *CD8+ TILs* and *VEGF* Expression with MVD

Variables	MVD		p-value	r
	Low	High		
CD8+ TILs expression				
Low	9	16	0.049*	-0.280
High	16	9		
VEGF expression				
Weak	4	0	0.032*	0.303
Moderate	18	18		
Strong	3	7		

CD8+ TILs, CD8+ Tumor-infiltrating lymphocytes; MVD, Microvascular density; *VEGF,* vascular endothelial growth factor; *Significant p-value (p<0.05); r = correlation coefficient

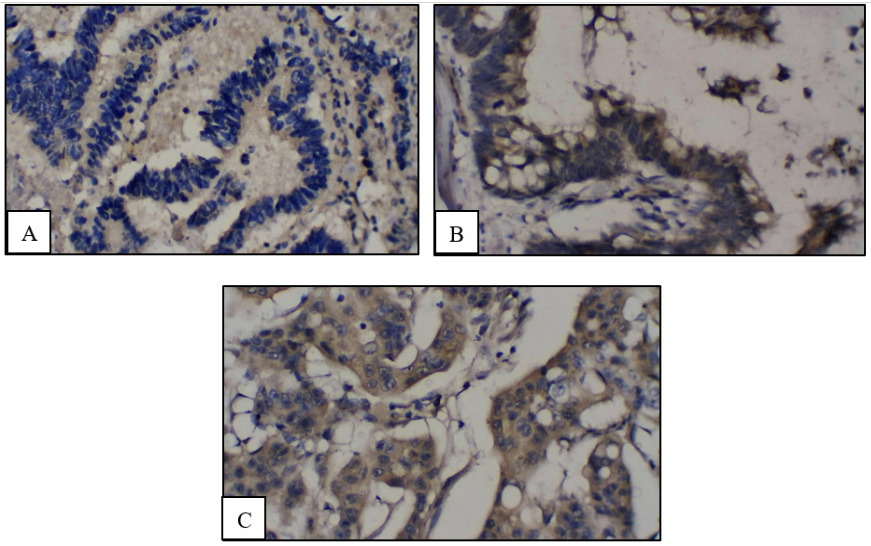

Figure 3. Expression of *VEGF* in Cytoplasm of Tumor Cells. (A) Weakly staining. (B) Moderate staining. (C) Strong staining (IHC, Objective 40x)

19].

The AJCC-8 TNM staging, which assesses the combination of depth of tumor invasion (pT), lymph node metastasis status (pN), and distant organ metastasis (pM), correlates with the prognosis and treatment options for colorectal adenocarcinoma. The results of Hong et al.'s study [20] showed a decrease in OS with increasing stage, namely 94.7% in stage I and only 31.5% in stage IV. This study reported that the majority of the tumor were pT3 (64%), which is consistent with studies by Nugrahani et al. [21] and Hashmi et al. [22]. High pT is associated with poorly differentiated tumor and perinodal spread, and is molecularly associated with the consensus molecular subtype 4 (CMS) or the poor-prognosis mesenchymal subtype [22, 23]. The majority samples showed no evidence of nodal involvement (54%) and distant metastasis (76%). The metastatic ability of tumor cells is influenced by several molecular mechanisms, including the WNT/β-Catenin, transforming growth factor-β (TGF-β) pathway, and mutations in KRAS and BRAF that affect the PI3K/AKT signaling pathway, as well as the hepatocyte growth factor (HGF)/MET pathway through *CD44* expression [24, 25].

Tumors with high *CD8+ TILs* in our study demonstrated a significant protective association, characterized with absence of lymph node metastasis, lack of organ metastasis, and lower TNM stage groups (stage I-II). These results show that *CD8+ TILs* play an essential part in the host-immune response, which can suppress the development and spread of tumor cells, consistent with the studies of Xiao et al. [26] and Kasurinen et al. [11]. Xiao et al. [26] study showed that patients with high *CD8+ TILs* had more prolonged recurrence-free survival (RFS) compared to the low CD8+ TILs group. *CD8+ TILs* are crucial lymphocyte cells in the immune response, as they possess antitumor capabilities by recognizing tumor cell antigens and subsequently destroying them through cytotoxic mechanisms [8,9]. The assessment of CD8+ TILs along with program death-1 (PD-1) and program death ligand-1 (PD-L1) is also the basis for selecting therapy for colorectal adenocarcinoma, particularly immunotherapy with immune checkpoint inhibitors such as pembrolizumab and nivolumab [27]. PDL-1 is a molecule expressed by tumor cells that binds to PD-1 on the T lymphocyte cell membrane, thereby suppressing the ability of T lymphocytes to destroy tumor cells [27, 28].

The results of the MVD calculation in this study showed a significant association with several clinicopathological variables. High MVD was more common in the tumor with deeper invasion, lymph node metastasis, and high TNM stage (stage III-IV). These results are in line with those of Den Ulil et al. [29], which showed a higher prevalence of high MVD in stage III than in stage II tumor, and those of Cho et al. [7], which showed a link between MVD and the occurrence of metastasis in colorectal adenocarcinoma. This study indicates that MVD assessment can also be performed on simple staining, such as HE, making it easily applicable to routine pathology reports of colorectal adenocarcinoma.

Another angiogenic component observed in this study, namely *VEGF* expression, showed significant correlation with deeper invasion, which supports its contribution to tumor progression. These results are in line with the study carried out by Hutajulu et al. [13], who found a significant correlation between *VEGF* expression with deeper tumor invasion and rectal location. Interestingly, *VEGF* expression in this study had a positive correlation with the number of MVDs. A study conducted by El Sabaa et al. [30] on cervical carcinoma also yielded similar results, namely an increase in *VEGF* expression in conjunction with an increase in the number of MVDs. *VEGF* plays a critical role in tumor cell growth by promoting the formation of new blood vessels that supply nutrients and oxygen to tumor cells, while also facilitating the spread of tumor cells to other organs [31–34].

TME components, including immune and angiogenic factors, interact with each other. This study shows a correlation between increased CD8+ TILs and decreased MVD. This result is in line with the study conducted by Yugawa et al. [35] on intrahepatic cholangiocarcinoma

samples, which showed that patients with low MVD had higher CD8+ TILs and lower numbers of Foxp3+. Angiogenesis in the tumor produces blood vessels that contain few pericytes and have imperfect basement membranes, thereby increasing interstitial fluid pressure accompanied by downregulation of intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1), which results in impaired migration of T lymphocyte cells towards the tumor [36]. In addition, tumor-associated endothelial cells (TEC) in the TME can influence the immune response in colorectal adenocarcinoma by producing FasL, which can eliminate CD8+ TILs. However, some TEC subsets can increase *CCL8* expression, which in turn can enhance CD8+ TIL infiltration around the tumor [33]. Endothelial cells will also increase the migration of myeloid-derived suppressor cells (MDSC) to the stroma area of tumor, which can suppress CD8+ TILs through the expression of arginase-1, IL-10, cyclooxygenase-2 (COX-2), PDL-1, and cytotoxic T lymphocyte-associated protein 4 (CTLA-4) [8]. Hypoxic conditions in the tumors also trigger the expression of inhibitory molecules, such as PDL-1, and cytokines, including IL-6, TGF-β, and Tregs, which reduce the T lymphocyte cell response [8, 36]. *VEGF* can also indirectly increase PD-1 expression, resulting in a decrease in the ability of CD8+ TILs to fight the tumor cells [36].

This study has several limitations that should be acknowledged. First, the relatively small sample size may limit the statistical power and the precision of the estimated associations. Second, the single-center, retrospective design introduces potential selection bias and limits the ability to control for unmeasured confounders. Future studies with larger, multicenter cohorts and prospective designs are recommended to validate these findings and to provide more robust prognostic evaluations.

In conclusion, the study showed the significance of immune–angiogenic interplay in colorectal adenocarcinoma. High CD8+ TILs were associated with negative nodal and distant metastasis, as well as a lower TNM stage, supporting their role as a favorable prognostic factor. In contrast, increased MVD and *VEGF* expression correlated with more advanced disease and emphasizing the contribution of angiogenesis to tumor progression. The integration of immune and angiogenic biomarkers with conventional TNM staging may improve prognostication and provide a rationale for combined therapeutic strategies targeting both immune activation and angiogenesis.

Author Contribution Statement

All authors contributed to the study's concept and design. HFT: data collection, analysis, and manuscript writing. GM and DGAS: concept, manuscript review, editing. MM: literature search.

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Ethical Declaration

This study was approved by the Health Research Ethics Committee of Soedarso's General Hospital (No.96/RSUD/KEPK/XI/2024).

Conflict of Interest

All authors declare no conflict of interest.

Data Availability

The data of this study can be obtained from the corresponding author upon reasonable request.

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