RESEARCH ARTICLE

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CD133, CD47, and *PD-L1* Expression in Ovarian High-grade Serous Carcinoma and Its Association with Metastatic Disease: A Cross-sectional Study

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Abstract

Introduction: Ovarian cancer is a primary cause of cancer-related death in women. At the time of diagnosis, the majority of ovarian malignancies had metastasized. It is believed that cancer stem cells (CSCs) and immune evasion play a crucial role in the metastatic process. The objective of this study was to describe the expression profiles of cluster of differentiation *(CD)133, CD47*, and programmed death ligand 1 (*PD-L1*) in high-grade serous ovarian cancer (HGSC) as commonly utilized markers for CSCs and immune evasion. **Material and methods:** Using an immunohistochemical procedure, 51 HGSC tissue samples were stained with anti-*CD133*, anti-*CD47*, and anti-*PDL1* antibodies. The samples contained 31 HGSC with metastases and 20 HGSC absent metastases. The expression of *CD133, CD47*, and *PD-L1* was compared between groups. **Results:** Strong expression of *CD133* and *CD47* was seen in 52% and 66% of tissue samples, respectively. Twenty of the thirty-one patients with metastases had a significant level of *CD133* expression, with a p-value of 0.039. *CD47* expression was increased in 26 of 31 samples with metastatic disease. A 62.7 percent of samples were negative for *PD-L1* expression, significantly inversely correlated with HGSC metastatic disease (p=0.023). Although there was no significant association between CD133, CD47, or *PD-L1* expression and age, Tumor Infiltrating Lymphocytes demonstrated a significantly varied relationship. **Conclusion:** Our findings suggested that expression of *CD133, CD47*, and *PD-L1* may have dynamically increased as the primary lesion progressed to the metastatic lesion, implying that these proteins may be involved in the progression of high-grade serous ovarian cancer from the primary to the metastatic stage.

Keywords: Cancer Stem Cells- Immune evasion- Metastasis- Ovarian Cancer

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Introduction

In 2023, an estimated 13,270 women will die from ovarian cancer in the US and an estimated 19,710 new cases of ovarian cancer will be diagnosed of ovarian cancer [1]. Despite its low prevalence in comparison with breast cancer, ovarian cancer is a highly lethal disease [2]. According to Surveillance, Epidemiology, and End Results (SEER) database, localized stage ovarian cancer has a 5-year survival rate of more than 93%, most patients (75%) are detected in distant stage, with a five-year survival rate of 31% [3].

The complexity of the disease on a clinical, molecular, and prognostic level, as well as evidence of resistance and adverse effects associated with ovarian cancer chemotherapy, has prompted the search for anti-ovarian cancer drug candidates from treatment interventions other than conventional chemotherapy and radiotherapy. The issue of chemotherapy resistance and the high recurrence rate of ovarian cancer presents a significant barrier in managing ovarian cancer therapy and provides an opportunity to develop novel therapeutic approaches. Cluster of differentiation (CD) 47 also known as integrin associated protein (IAP), is a transmembrane immunoglobulin superfamily member. When cancer cells produce *CD47* and interact with a signal regulating protein on the surface of macrophages, they broadcast a "don't eat me" signal that deters phagocytosis [4, 5]. *CD47* expression is high in solid tumors such as ovarian, breast, colon, bladder, glioma, hepatocellular carcinoma, and prostate cancer and has been associated with a poor prognosis [6, 7].

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Programmed death ligand 1 (*PD-L1*), also known as CD274, is activated by its receptor, programmed death 1 (PD-1, CD279), which is found on the cell surface of T cells, B cells, and dendritic cells [8, 9]. *PD-L1* may react directly to integrin receptors, activating intracellular signaling pathways such as the AKT/GSK3 pathway, increasing cells' propensity to migrate and invade [8]. The PD-1 coupling in *PD-L1* inhibits signaling, resulting in diminished effect or differentiation, an asymmetry between immune cell proliferation and T cell death, as well as energy and saturation [10, 11].

CD133 (also known as prominin-1 and AC133) has been identified as a surface marker of cancer stem cells (CSCs) in an increasing number of investigations and has been detected in various solid tumors, including ovarian cancer [12, 13]. Our earlier analysis demonstrated that *CD133* is involved in the carcinogenesis of serous ovarian cancer, with dynamically elevated expression in high-grade serous carcinoma entities [14]. Furthermore, *CD133* induces nuclear factor kappa B and upregulates matrix metallopeptidase-9 to increase cell proliferation, carcinogenesis, tumor invasion, and metastasis [15].

Numerous biomarkers, including *CD133, CD47,* and PDL-1, have been extensively studied to determine their significance in the metastasis and prognosis of ovarian cancer [16-18]. Targeted treatment and immunotherapy are the most current methods being developed. Neuroblastoma, malignant glioma, colorectal cancer, and acute myeloid leukemia are among the solid tumors for which anti-*CD133* targeted therapy has entered Phases I and II of clinical studies [19, 20]. Moreover, *CD-47* and *PD-L1* immunotherapy continue to develop their applications in various solid and hematological cancers [21, 22].

This study aimed to examine the expression of *CD133*, *CD47*, and *PDL-1* in ovarian high-grade serous carcinoma samples to determine the possibility for targeted therapy and immunotherapy. The findings of this study are expected to provide evidence to support the potential of treating ovarian cancer patients with a combination of chemotherapy, multitargeted therapy, and immunotherapy.

Materials and Methods

This work was a retrospective cross-sectional study using an immunohistochemistry assessment of metastatic high-grade serous ovarian cancer (HGSC) and non-metastatic-HGSC. We investigated 51 paraffin-embedded samples consisting of 31 metastatic lesions and 20 non-metastatic of HGSC. All samples were taken from patients in 3 main pathology facilities in Makassar, Indonesia (Wahidin Sudirohusodo hospital, Universitas Hasanuddin hospital, and Sentra Diagnostik Patologia Makassar). Samples were gathered from January 2017 to June 2021. The use of preserved human tissue has been approved by The Research Ethics Committee of the Faculty of Medicine Universitas Hasanuddin, Makassar, Indonesia, number: 310/UN4.6.4.5.31/PP.36/2021. This study has been reported in line with the Strengthening the reporting of cohort studies in surgery (STROCSS) Guidelines [23].

The sampling technique used was consecutive sampling. We selected tissue samples using sequential (non-random) inclusion criteria based on their presence in the case population database. By selecting samples consecutively until the minimum number of metastatic and non-metastatic samples is attained, we ensure that there is no selection bias in our study sample. The sample size formula is modified according to the research design, which may be unpaired analysis with a numerical scale or unpaired analytic numerical analysis [24]. The inclusion criteria are as follows: (i) specimen diagnosed with primary ovarian high-grade serous carcinoma; (ii) patients who have not had chemotherapy; (iii) patients who are not recurring cases; and (iv) both early and late-stage patients were included. The following are the conditions for exclusion: (i) the sample lacks clinicopathological data according to the characteristics of the study; (ii) the sample lacks primary lesions of the fallopian tubes and peritoneum; and (iii) the sample lacks a focused cell tumor of at least 10 high power fields under 400x magnification. No cell block samples were included in the study. Metastatic lesions are characterized by the presence of HGSC on the ovaries in conjunction with the development of the same lesions in the omentum. Non-metastatic lesions are HGSC primary ovarian lesions that are absent from the omentum or other sites. We rated *PD-L1* expressions 1% as negative, >1% to 5% as low, >5% to 50% as moderate, and >50% as high using the *PD-L1* clone 28-8 score method [25].

Immunohistochemistry Protocol

Tissues fixed in paraffin were sectioned at a depth of 3 m to obtain tissue sections. Standard procedures were used to treat the entire slide, and serial sections were used for the immunohistochemistry (IHC) reaction. After deparaffinization in xylene and rehydration in various ethanol concentrations, the slides were submerged for three minutes in 3 percent hydrogen peroxide and then rinsed with tap water. Afterward, samples were microwaved for 10 minutes to promote heat-induced epitope retrieval. After allowing specimens to reach room temperature, they were washed with Tris Buffer Saline and 1% protein blocking solution for ten minutes. After that, all specimens were treated for 90 minutes at room temperature with polyclonal rabbit anti-human *CD133* (1:200), anti-*CD47* (1:200), and anti-*PD-L1* clone 28-8 (1:100) antibodies. The samples were then incubated with the secondary antibody for ten minutes (Ultratek Complete HRP Anti-Polyvalent – LSAB). Diaminobenzidine (DAB) was then used to color the samples. Samples were then rinsed for five minutes in running water before being placed in a solution of Hematoxylin. As a contrast color, Lillie's Mayer was tinted. Lastly, the specimens were overlaid with deck glass and evaluated with the Olympus CX-43 (Tokyo, Japan) Microscope Binocular in greater detail. There was a positive control for each reaction. In addition to incubating the samples, a negative control (the absence of the primary antibody) was used.

Semiquantitative analysis

Two gynecological pathologists independently evaluated *CD133*, *CD47,* and *PD-L1* expression,

calculated by multiplying the percentage of stained cells (extensity) in ten 'hotspot' high power fields (0-100), the intensity (0-3), and the histochemical score (H-Score) (0-300). *CD133* expression is classified as either low or high according to the median H-score used as a cut-off number [26].The expression of *CD47* was quantified by multiplying the proportion of stained cells (0-4) by the intensity of expression (0-3), and the H-Score was calculated (0-12). A score of less than or equal to six is interpreted as a lack of expression and vice versa. The intensity and proportion of membranous or cytoplasmic staining in tumor cells were used to determine the presence of *PD-L1*. 0 indicates no staining; 1 indicates a weak staining intensity of less than 10% or a strong staining intensity < 1%; 2 indicates a moderate staining intensity of more than 10% or a strong staining intensity of 1-10%, and 3 shows a strong staining intensity >10%. These markers will look brown stained on the membrane and cytoplasm of tumor cells when they are overexpressed. All slides were examined under a light microscope at a magnification of x400 (Olympus CX-43).

Statistical Analysis

All data were analyzed using the GraphPad Prism version 6 program. The mean + standard deviation is used to express the values. The Kruskal-Wallis test was performed to determine the mean H-score difference between test groups, followed by the Mann-Whitney test. Statistical significance was defined as a p-value of 0.05.

Table 1. Characteristics of 51 Indonesian HGSC Patients

Characteristics	n (%)
Age (years, mean±SD)	50.37±10
< 50	24 (47.1)
> 50	27 (52.9)
Metastasis	
Yes	31 (60.8)
No	20 (39.2)
TILS (%)	
<5	11 (21.6)
5-10	18 (35.3)
>10	22 (43.1)
PD-L1	
High	3 (5.9)
Moderate	4 (7.8)
Low	12 (23.5)
Negative	32 (62.7)
CD133	
High Expression	27 (52.9)
Low Expression	24 (47.1)
CD47	
High Expression	34 (66.7)
Low Expression	17 (33.3)

Abbreviations, SD, standard deviation; TILS, Tumor Infiltrating Lymphocytes; PD-L1, programmed death ligand 1; CD133, cluster of differentiation 133; CD47, cluster of differentiation 47.

Results

Sample Features

The cumulative number of respondents was 51 cases of HGSC, with 31 (60.8%) metastatic samples and 20 (39.2%) non-metastatic samples. The mean age of the patients was 50.37±10 years, with the over-50 age group having the highest frequency. Additionally, the distribution of Tumor Infiltrating Lymphocytes (TILs) expression was dominated by the >10% group, with as many as 22 instances (43.1 percent). The number of samples expressing high *CD133* and *CD47* was more significant than those expressing low levels (Table 1).

CD133 and CD47 immunoexpression may be shown in (Figure 1A-D), stained brown in the membrane and cytoplasm of tumor cells. These antibodies were generally expressed differently in the samples we studied, with the majority being dominated by robust expression. Additionally, *PD-L1* is expressed dynamically on the membrane and cytoplasm of tumor cells (Figure 2A-D). Compared to metastatic samples, most non-metastatic samples revealed a high level of *PD-L1*.

Table 2. *CD133, CD47*, and *PD-L1* Profile Based on Clinicopathological Parameters in Indonesian HGSC Patients

Characteristics	n (%)	CD133		P*	CD47		P*	PD-L1				P*
		High	Low		High	Low		High	Moderate	Low	Negative	
Age (years, mean±SD)	50.37±10											
< 50	24 (47.1)	12 (23.5)	12 (23.5)	0.692	14 (27.5)	10 (19.6)	0.234	1 (2)	3 (5.9)	6 (11.8)	14 (27.5)	0.645
> 50	27 (52.9)	15 (29.7)	12 (23.5)		20 (39.2)	7 (13.7)		2 (3.9)	1 (2)	6 (11.8)	18 (35.3)	
Metastasis												
Yes	31 (60.8)	20 (39.2)	11 (21.6)	0.039	26 (51)	5 (9.8)	0.001	3 (5.9)	1 (2)	4 (7.8)	23 (45.1)	0.023
No	20 (39.2)	7 (13.7)	13 (25.5)		8 (15.7)	12 (23.5)		0 (0)	3 (5.9)	8 (15.7)	9 (17.6)	
TILS												
<5%	11 (21.6)	9 (17.6)	2 (3.9)	0.046	9 (17.6)	2 (3.9)	0.242	2 (3.9)	4 (7.8)	4 (7.8)	1 (2)	<0.001
5-10%	18 (35.3)	10 (19.6)	8 (15.7)		13 (25.5)	5 (9.8)		1 (2)	0 (0)	7 (13.7)	10 (19.6)	
>10%	22 (43.1)	8 (15.7)	14 (27.5)		12 (23.5)	10 (19.6)		0 (0)	0 (0)	1 (2)	21 (41.2)	

Note, *Chi-Square Test; SD, standard deviation; TILS, Tumor Infiltrating Lymphocytes; CD133, cluster of differentiation 133; CD47, cluster of differentiation 47; PD-L1, programmed death ligand 1.

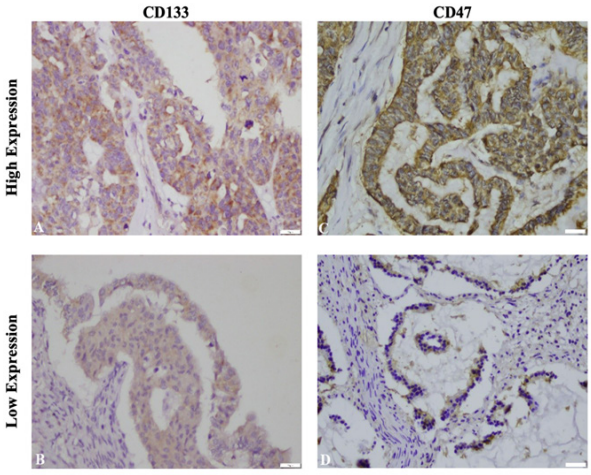

Figure 1. A-D. Representative IHC Immunoexpression of *CD133* and *CD47* in HGSC Tissue. Tumor cells are variably stained, ranging from weak to strong expression, shown by brown staining in some level of intensity. The expression of protein was showed in the membrane and cytoplasm of tumor cells. The images were obtained by Olympus CX41, Objective Lens 40x, Scale bar 10 µm.

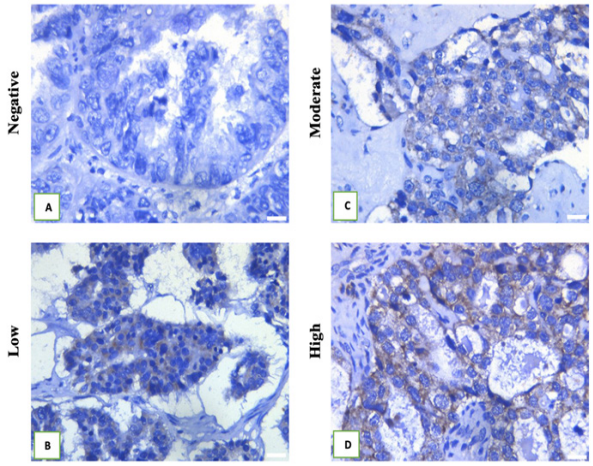

Figure 2. A-D. PD-L1 Immunostaining Was Expressed in the Cytoplasm and Membrane of Tumor Cells. The images above showed representative visualization of HGSC tissue. *PD-L1* was mostly stained in non-metastatic groups rather than metastatic ones. Figures were taken by objective magnification 40x, Scale bar 10 µm (Olympus CX41).

Relationship of CD133, CD47, and PD-L1 expression to metastatic disease of HGSC

As shown in Table 2, there was no significant relationship between age and *CD133* (p=0.692), *CD47* (p=0.234), or *PD-L1* (p=0.645) expression status. TILs parameters demonstrated a variety of relationships with the expression of these markers, with *CD133* and *PD-L1* exhibiting significant expression at p-values of 0.046 and 0.001, respectively. *CD47,* on the other side, did not display a high association with TILs status. All antibodies detected in this sample were significantly expressed when associated with tumor cell metastasis. *CD47* and *CD133* expression were positively associated with metastasis, with p-values of 0.001 and 0.039, respectively, whereas *PD-L1* expression was negatively associated with metastasis.

Correlation among CD133, CD47, and PD-L1 expression in HGSC

Correlation analysis findings for each marker are shown in (Table 3), including the value of r (coefficient correlation) between variables. There was a moderate correlation between *CD133* expression and *PD-L1* expression. Meanwhile, *CD47* showed an abysmal relationship with *CD133* and *PD-L1*.

Table 3. Correlation Matrix among *CD133, CD47*, and *PD-L1* Expression in Indonesian HGSC Patients

	CD133	CD47	PD-L1
CD133			
CD47	0.175*		
PD-L1	0.413*	0.139*	

Note, n, 51; *r, coefficient correlation analyzed by Gamma test; CD133, cluster of differentiation 133; CD47, cluster of differentiation 47; PD-L1, programmed death ligand 1.

Discussion

High-grade serous ovarian carcinoma is the most aggressive form of ovarian cancer, accounting for up to 70% of all cases [27-30]. This study reported that *CD133* is commonly expressed in HGSC-metastatic compared to non-metastatic. High expression of *CD133* indicates poor prognosis in HGSC patients. Numerous investigations have established the complicated role of *CD133* in the ovaries, particularly in ovarian serous carcinoma entities, from carcinogenesis through invasion and metastasis of malignant tumor cells [14, 31, 32]. According to a current CSCs hypothesis, ovarian cancer may be fueled and sustained by a fraction of cells with stem cell features such as infinite proliferative capacity and resistance to therapy [33-35].

Immune evasion is a hallmark feature, as evidenced in various malignancies. *CD47* and *PD-L1* are recognized to play a critical role in cancer cells evading cytotoxic T cell assaults, which results in uncontrolled tumor development during the early stages of invasion and metastasis [36, 16]. Our findings indicated increased *CD47* expression in metastatic HGSC tumor cells compared to non-metastatic HGSC tumor cells. *CD47* initiates epithelial-mesenchymal transition (EMT) and tumor cell motility. *CD47* plays a critical role in blocking macrophages from phagocytosing tumor cells. *CD47* overexpression is related to a worsening disease course and a poor prognosis in high-grade serous ovarian cancer. *CD47* overexpression results in an increase in N-cadherin levels and a decrease in E-cadherin levels, inducing EMT, a process in which epithelial cells differentiate into mesenchymal stem cells, facilitating the separation of tumor cells from the primary tumor and subsequent invasion [8]. Following the loss of E-cadherin, other cadherins (N-cadherin, P-cadherin) will be regulated, resulting in tumor cells becoming more fibroblast-like

and aiding tumor cell spread [37, 38]. *CD47,* an integrin protein, modulates the actin cytoskeleton, encouraging the development of lamellipodia and filopodia at the cellular level. This process is accomplished by activating the protein cdc42, a downstream mediator of *CD47*, and the Src family and MEK/MAPK (mitogen-activated protein kinase/mitogen-activated protein kinase) pathways. Cells mobilize and invade through the development of lamellipodia and filopodia [16].

Our investigation, *PD-L1*, revealed that the HGSC-metastatic group had a higher proportion of weakly and negatively expressed samples. This observation contrasts with previous research in gastric cancer, where *PD-L1* expression is predominantly found in tumors that metastasis to lymph nodes [39-43]. A variety of causes could explain these divergent findings. First, *PD-L1* expression fluctuates in a variety of solid malignancies. Second, the cut-off value for positive *PD-L1* staining varies between investigations. Third, the time and location of the *PD-L1* staining may affect the result [42]. This observation warrants more investigation to acquire a more comprehensive understanding of this issue.

Moreover, we examined the connection between these markers with TILs status, with *CD133* and *PD-L1* indicating a decreased TILs component in tumors with high expression. *CD47*, on the other side, did not demonstrate a significant correlation with TILs status. Our findings imply a trend toward decreased TILs expression in areas of dense tumor cells that express high levels of *CD133* and *PD-L1*. However, we did not see a significant connection between TILs status and the incidence of HGSC metastases in a separate analysis. We hypothesize that the TILs component may contribute to the development of HGSC metastases.

The heterogeneity of the ovarian cancer cell population has been frequently noted in the literature [44, 45]. The complexity and dynamic interactions of molecules involved in carcinogenesis and metastasis warrant further investigation. We attempted to examine the expression correlations of the *CD133*, *CD47*, and *PD-L1* markers. There was a moderate connection between *CD133* and *PD-L1*, with a tendency for *CD133* expression to increase in the group of HGSC tumor cells with high *PD-L1* expression. According to Wei et al. (2019) reports, *PD-L1* can control *CD133* and *CD44* as CSC markers in colorectal cancer by encouraging self-renewal via the HMGA1-dependent signaling pathway [46]. On the other hand, our results indicate a lack of correlation between *CD47* and *CD133*, and *PD-L1*, respectively. These findings require further investigation of more precise intermolecular interaction processes to gain a comprehensive understanding.

Our data highlighted that we employed just IHC and made no comparisons to other protein detection techniques; therefore, caution should be exercised when interpreting our findings. Our data highlighted that we employed just IHC and made no comparisons to other protein detection techniques; therefore, caution should be exercised when interpreting our findings. Furthermore, instead of comparing metastatic and non-metastatic lesions, we suggest comparing primary and metastatic lesions in pairs. Confounding variables associated with disease stage, therapy status, and recurrence must be accounted for to prevent bias in the results.

A limitation of this study due to the limited number of ovarian cancer tissue samples, and the samples might not represent the whole HGSC characteristic; this may lead to selection bias. Larger scale studies using more specific characteristics of HGSC are needed in the future, and treatment based on the traits could be performed. However, our findings can serve as a starting point for further investigation of the possibility of combination therapy in treating HGSC.

In conclusion, our findings suggested that the expression of *CD133*, *CD47*, and *PD-L1* may have dynamically increased as the primary lesion progressed to the metastatic lesion, implying that these proteins may be involved in the progression of high-grade serous ovarian cancer from the primary to the metastatic stage. Despite the limitation of the selection bias of samples, these markers could be explored as potential HGSC treatment targets.

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Author Contribution Statement

RM: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Validation, Visualization, Writing – original draft, Writing – review & editing; DI: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Data curation, Formal analysis, Investigation, Methodology, Project administration, Resources, Software, Validation, Visualization, Writing – original draft; RD: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Resources, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing; YT: Conceptualization, Investigation, Methodology, Resources, Software, Validation, Writing – original draft, Writing – review & editing; BJN: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Resources, Validation, Writing – original draft, Writing – review & editing; MG: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Resources, Supervision, Writing – original draft, Writing – review & editing; SR: Conceptualization, Data curation, Investigation, Resources, Supervision, Writing – original draft, Writing – review & editing; MF: Conceptualization, Investigation, Methodology, Resources, Software, Validation, Writing – original draft, Writing – review & editing.

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None.

Conflicts of interest

The authors declare that they have no conflict of interests.

Ethical approval

The study was approved by the Research Ethics Committee of the Faculty of Medicine Universitas Hasanuddin, Makassar, Indonesia, number: 310/UN4.6.4.5.31/PP.36/2021. We promised that the participants' data were anonymized or maintained with confidentiality, the rights or interests of participants were not invaded, and informed consent was taken from all individual participants.

Consent

The research was conducted ethically in accordance with the World Medical Association Declaration of Helsinki. The patients have given their written informed consent on admission to use their prospective database and files for research work.

Availability of data

The data that support the findings of this study are available from the corresponding author [RM] upon reasonable request.

Study Registration

The study is not registered in any registering dataset.

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