

# The Role of Caspase-8, MLKL and RIPK1 in Iraqi Patients' Women with Breast Cancer

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**ABSTRACT: Background:** Breast cancer is the most common type of cancer that affects women's breast tissue, leading to uncontrollably aberrant cell proliferation. **Objective:** This study aimed to investigate the role of Caspase-8, mixed lineage kinase domain-like protein (MLKL), receptor-interacting serine/threonine-protein kinase 1 (RIPK1) in pathogenicity of breast cancer (newly diagnosed and under treatment). **Methods:** A pilot study was conducted in the main medical hospital in Baghdad (Baghdad medical city-oncology teaching hospital). A total of 60 blood samples were collected from women with breast cancer aged 25-65 years old in addition to 28 blood samples were withdrawn from apparently healthy women aged match with patients. Samples were divided into three groups: 30 newly diagnosed patients suffered from breast cancer without treatment, 30 patients under treatment with chemotherapy (Adriamycin and Cyclophosphamide) (60 mg/M<sup>2</sup>) and 28 healthy control individuals. Three mL peripheral blood samples were withdrawn from each participant. The serum was isolated, and an ELISA assay was carried out to determine the serum level of the studied parameters. **Results:** The result showed a significant increase in the serum level of Caspase-8, MLKL and RIPK1 in all patients (newly diagnosed) versus control, and a significant decrease in the serum level of Caspase-8, MLKL and RIPK1 in all patients (under treatment) versus control, the results were (0.12±0.01, 1.11±0.18, 0.54±0.09), (2.35±0.28, 5.98±0.61, 0.72±0.14), and (0.11±0.01, 0.797±0.243, 0.129±0.030) ng/mL respectively. Also, the result showed a significant difference in the serum level of patients according to age, stage, grade and hormonal status of some understudied parameters. **Conclusions:** High serum level of Caspase-8, MLKL and RIPK1 were found in Iraqi females with breast cancer with significant relation depending on grade, stage and hormonal status of breast cancer. While levels for Casp-8, MLKL, and RIPK1 were recorded in patients under treatment, it can be concluded that the studied marker may be considered a good predictor marker for therapeutic response in breast cancer patients.

**KEYWORDS:** Cancer; Breast cancer; Caspase-8; MLKL; RIPK1

## INTRODUCTION

According to the Iraqi Cancer Registry [1], breast cancer represents the first cancer type among the top ten cancers with a number and percentage of 6255(19.74%), and is considered the first type of cancer in Iraqi females with 6,132(34.35%). The distribution of breast cancer in Baghdad was 1839(38.06%) [2], [3]. Apoptosis is an essential physiological precise of cell death that is meant to occur without the discharge of intercellular content and subsequent no activator of cell inflammatory response [4], [5]. However, dysregulation of apoptosis results in significant consequences in carcinogenesis. The imbalance between cell proliferation and cell death is considered the malignant tumor hallmark [6], [7]. Cysteiny aspartate-specific proteinase (Caspase-8) has long been considered a promoter of apoptosis and part of the mechanism by which cytotoxic drugs kill cancer cells. Many studies have confirmed that caspase-8 plays an important role in cancer and it has been proposed the term "PANoptosis" which include three programmed death modes namely pyroptosis, apoptosis and necroptosis [8].

Caspase-8 (a cysteine protease) initiates apoptotic signaling via an extrinsic pathway and it is differently expressed in the peripheral immune system. Since signaling via the death receptor (extrinsic) pathway critically depends on caspase-8, the disturbance of caspase-8 expression or function may contribute to human disease, for example in cancer. Caspase-8 also plays a critical pro-survival function by inhibiting an alternative form of programmed cell death called necroptosis. The low expression level of pro-caspase-8 is therefore associated with the malignant transformation [9], [10].

Caspase-8 was identified as a cysteine protease recruited to the CD95 (Fas/APO-1) death including signaling complex (DISC) [11]. Several studies confined the crucial role of caspase-8 in apoptosis triggered by Fas and by another receptor, including the TRAIL receptor (DR4 & DR5). Upon death receptor stimulation by their relative ligand, caspase-8 is recruited and participates in the assembly of DISC [11], [12]. The conversion from proenzyme to fully active enzyme is promoted by the recruitment of DISC, which allows caspase-8 dimerization, priming a series of auto-processing events at specific acid residues that culminate with the release of large and small subunits [13] which assemble to form the full action tetrameric caspase-8 complex to release from the DISC and its ability to cleave its substrates, which are the key events to initiate the execute the canonical extrinsic apoptotic cascade [12]. Evasion of apoptosis is a well-established hallmark of cancer and contributes both to cancer initiation and development [14], [15]. Tumors can retain or even upregulate caspase-8 expression thanks to inactivating mutation or phosphorylation event that impacts its enzymatic activity and apoptotic function, in addition to caspase-8 inactivating mutation that can inhibit its proteolytic activity [16]–[20].

Recently, necroptosis, as a programmed cell death pathway, has drawn much attention as it has been implicated in multiple pathologies. Pseudo kinase mixed lineage kinase domain-like protein (MLKL) serves as a terminal-known obligate effector in the process of necroptosis [21]. It has been found that cell fate can be determined by a switch from apoptosis to necroptosis via genetic ablation of caspase-8 or by using caspase-8 inhibitors. MLKL, as a pseudo kinase, consists of a c-terminal pseudo kinase domain a two-helix brace or linker, and an N-terminal four-helix bundle (4HB) [22], [23]. The N-terminal region of MLKL consists of a 4HB domain interacting with a two-helix brace. Generally, MLKL disulfide bond-dependent oligomerization and membrane translocation are essential for the formation of membrane pores. The late formation of small pores around 4 nm in diameter is a core event of necroptosis [24].

MLKL activation also leads to the protease (a disintegrin and metalloproteases) ADAM S-mediated ectodomain shedding of cell surface proteins of necroptotic cells. Tumor necroptosis happens in advanced solid tumors, and blocking necroptosis by MLKL deletion in breast cancer dramatically reduces tumor metastasis, it has been suggested that tumor necroptosis can modulate the tumor microenvironment by inhibiting the anti-tumor activity of T cells [25]. The shedding of cell surface proteins by ADAMs promotes necroptosis, cell migration, and inflammation [26], [27].

Necroptosis mostly happens under pathological conditions. For death-receptor-induced necroptosis, the protein kinase receptor-interacting protein kinase 1, 3 (RIPK1, R1PK3) and MLKL. When the activity of cellular inhibitors of apoptosis proteins (cIAPs) and casp-8 are inhibited in cells, the engagement of the death receptor triggers RIPK1 to recruit R1PK3, which in turn recruits MLKL to form a death complex known as necrosome to inhibit necroptosis [28], [29]. In necrosome RIPK3 is autophosphorylated and subsequently, the activated R1PK3 recruits and phosphorylates MLKL. The MLKL oligomerizes and translocates to the plasma membrane to execute necroptosis disrupting plasma and intracellular membrane integrity [30]–[32].

There is growing evidence suggesting that RIPK3 silencing in tumor cells is selected during the process of tumor progression, and RIPK3 down-regulation confers cancer cells to chemotherapeutic resistance in cancer [33], [34]. The current study aimed to investigate the apoptotic marker (casp-8) and necroptotic marker (MLKL, RIPK1) in an attempt to predict the therapeutic response. Especially since necroptosis markers are considered a new therapeutic target to reduce resistance to chemotherapy in cancer patients.

## MATERIALS AND METHODS

### Study Design

The pilot study was carried out at the primary medical facility in Baghdad, namely the Medical City-Oncology Teaching Hospital, from August 2023 to December 2023. The study involved the collection of 88 blood samples divided as 60 samples obtained from women diagnosed with breast cancer and 28 samples obtained from apparently healthy persons whose ages were matched to the patients. The subjects were categorized into three groups: (30) newly diagnosed patients without treatment,

(30) patients who were under treatment with chemotherapy (Adriamycin and cyclophosphamide) at a dosage of 60 mg/M<sup>2</sup>, and (28) persons designated as health control. Specialty physicians evaluated and diagnosed the patients, and the diagnosis of breast cancer was confirmed by mammography and histological findings. The information for each patient which included (grade, stage and hormonal status) was obtained from their reports. Patients with other types of cancer, autoimmune disorders, infectious diseases, severe acute or chronic medical conditions, pregnancy, and breastfeeding were excluded from the study. The study exclusively included female participants.

### Samples Collection

Venus blood specimens (3 ml) were withdrawn from each patient and the control. The blood is put in a gel tube. Then centrifuged for 10 min at 3000 rpm. The serum samples were added Eppendorf tubes and immediately frozen at -20 °C until used.

### Principle of Double Antibody Sandwich ELISA Kits

In this assay, the “Double Antibody Sandwich” technique (ELISA Kits, USNF, USA) is utilized. It involves quantifying the concentrations of Caspase-8, MLKL and RIPK1 in the samples. This is achieved by comparing the optical density (O.D.) of the samples to a standard curve that has been calculated. To ensure accuracy and consistency, all standards, samples, and reagents were meticulously prepared in strict accordance with the test preparation guidelines provided in the kit leaflet.

### Statistical Analysis

The statistical analysis of all data was conducted using the SPSS program. The Independent T-test and Way ANOVA test were employed to determine the P-value using Least Significant Differences (LSD), along with Pearson chi-square and ROC test. All data were reported as the mean standard error ( $\pm$ S.E.), and a P-value less than 0.05 was regarded as a significant difference.

## RESULTS AND DISCUSSION

Table 1 presents the serum level of studied parameters (Caspase-8, MLKL, and RIPK1) in control and patients (newly diagnosed and under treatment). Casp-8 recorded a significant difference among the three groups for the three studied parameters with  $P \leq 0.001$  for each one.

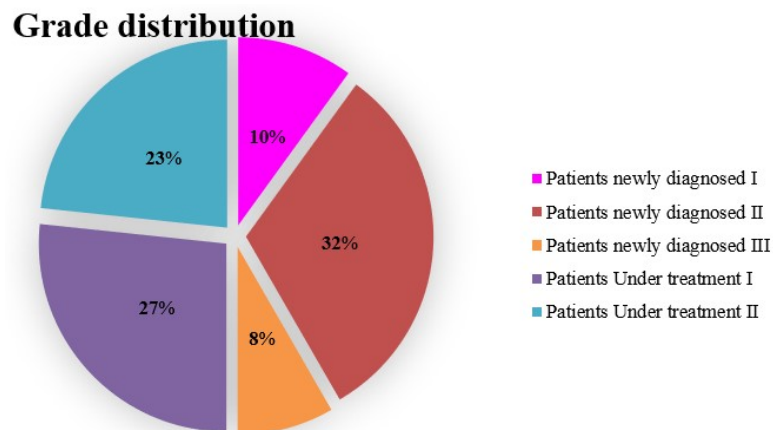
The results were present as mean $\pm$ S. E. for casp-8 were (0.12 $\pm$ 0.01, 1.11 $\pm$ 0.18, 0.54 $\pm$ 0.09) ng/mL for three groups respectively (control, newly diagnosed, under treatment). For MLKL were (2.35 $\pm$ 0.28, 5.98 $\pm$ 0.61, 0.72 $\pm$ 0.14) ng/mL and finally the result for RIPK were (0.11 $\pm$ 0.01, 0.797 $\pm$ 0.243, 0.129 $\pm$ 0.030) ng/mL. There was also a significant difference for all studied parameters between the two patients' groups with P-values 0.001, 0.001, 0.001 respectively.

**Table 1.** Studied parameters of serum level in patients and control

Groups	Parameter Concentration (Mean $\pm$ S.E.)		
	Caspase-8 (ng/mL)	MLKL (ng/mL)	RIPK1 (ng/mL)
Control	0.12 $\pm$ 0.01	2.35 $\pm$ 0.28	0.11 $\pm$ 0.01
Newly Diagnosed patients	1.11 $\pm$ 0.18	5.98 $\pm$ 0.61	0.797 $\pm$ 0.243 <sup>a</sup>
Under Treatment patients	0.54 $\pm$ 0.09	0.72 $\pm$ 0.14	0.129 $\pm$ 0.030
P-value	0.001**	0.001**	0.001**
Between newly and under treatment	-	-	-
P-value	0.001**	0.001**	0.001**

<sup>a</sup> vs. control, \*\*= high significant, NS= no significant

As shown in Figure 1 which presents the percentage of disease grade in two patients' groups (newly diagnosed and under treatment) we noted the high percentage was in newly diagnosed patients with grade II (32%) > under treatment grade II (27%) > under treatment grade III (23%) > newly diagnosed grade I (10%) > newly diagnosed grade III (8%).



**Figure 1.** Percentage distribution of patient groups (newly diagnosed and under treatment) based on disease grade

Table 2 presents the serum levels of studied parameters in studied groups, patients (newly diagnosed and under treatment based on disease grades (I, II, III)). The result presented as mean± S.E. Serum levels for casp-8 in newly diagnosed patients with grade (I, II, III) were (0.59±0.13, 1.06±0.12, 1.90±0.95) ng/mL respectively, with no significant difference P-value= 0.08. For MLKL the three grades were (5.11±1.10, 6.18±0.90, 6.23±0.61) ng/mL with no significant difference P-value=0.79, finally for RIPK for three grades were (0.552±0.393, 1.019±0.357, 0.250±0.085) ng/mL with also no significant difference P-value= 0.47.

The results of (casp-8, MLKL, RIPK) for the patients under treatment based on grade II, III were (0.60±0.15, 0.47±0.08) ng/mL, (0.74±0.21, 0.70±0.17) ng/mL and (0.166±0.049, 0.088±0.030) ng/mL respectively with no significant differences P-value= 0.45, 0.89, 0.2 respectively. In comparison between the two patient groups, it has been noted significant differences in P-value= 0.01, 0.001, and 0.03 respectively for (casp-8, MLKL, and RIPK) based on disease grade II, whereas, no significant differences between the two patient groups based on grade III for casp-8 and RIPK with P-value= 0.2, 0.13 respectively except in MLKL, there was a highly significant difference P-value= 0.001.

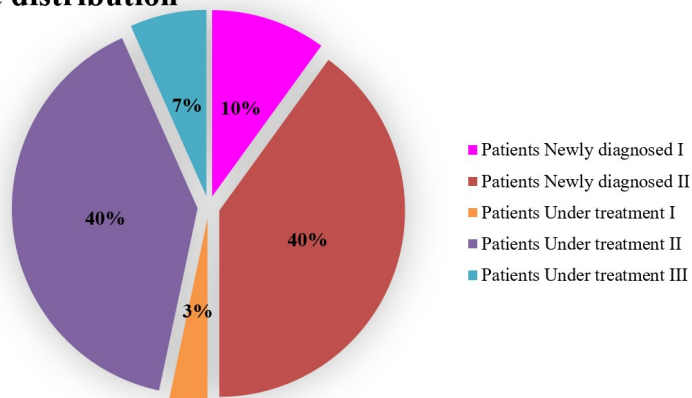
**Table 2.** Serum level of studied parameters in patients (newly diagnosed and under treatment) and control according to disease grade (I, II, II)

Patients groups	Grade groups	Concentration (Mean±S.E.)		
		Caspase-8 (ng/mL)	MLKL (ng/mL)	RIPK1 (ng/mL)
Newly diagnosed patients	I	0.59±0.13	5.11±1.10	0.552±0.393
	II	1.06±0.12	6.18±0.90	1.019±0.357
	III	1.90±0.95 <sup>a</sup>	6.23±0.61	0.250±0.085
<b>P-value</b>		0.08 NS	0.79 NS	0.47 NS
Under Treatment patients	II	0.60±0.15	0.74±0.21	0.166±0.049
	III	0.47±0.08	0.70±0.17	0.088±0.030
<b>P-value</b>		0.45 NS	0.89 NS	0.2 NS
<b>Between newly&amp; under treatment</b>	II	-	-	-
<b>P-value</b>		0.01*	<0.001**	0.03*
<b>Between newly&amp; under treatment</b>	III	-	-	-
<b>P-value</b>		0.2 NS	<0.001**	0.13 NS

NS= no significant, \*\*= high significant

As shown in Figure 2 the percentage of disease stages in patient groups (newly diagnosed and under treatment) we observed that the high percentage was within newly diagnosed and under treatment groups with stage II (40%, 40%) respectively > newly diagnosed with stage I (10%) > patients under treatment with stage III (7%) > patients under treatment with stage I (3%).

**Stage distribution**



**Figure 2.** Percentage distribution of patient groups (newly diagnosed and under treatment) based on disease stages

Table 3 summarizes the serum level distribution of studied parameters (casp-8, MLKL, RIPK) in two patient groups (newly diagnosed and under treatment) based on stage. The result presented as mean± S.E., for newly diagnosed patients with stage I, the results were (0.73±0.14, 6.82±2.29, and 0.562±0.391) ng/mL and for stage II were (1.20±0.22, 5.76±0.55, and 0.856±0.290) ng/mL with no significant differences for three parameters P-value = 0.3, 0.5, 0.63 respectively. For the patients under treatment with stage I, the results were (0.14±0.02, 1.15±0.92, and 0.072±0.010) ng/mL, for stage II (0.55±0.10, 0.61±0.14, 0.053±0.024) ng/mL, for stage III (0.65±0.16, 1.17±0.44, and 0.053±0.024) ng/mL with also no significant differences P-value = 0.44, 0.27, 0.52 respectively. In comparison between two patient groups based on stage, there were no significant differences based on stage I, P-value = 0.6, 0.6, and 0.5 respectively, whereas highly significant differences were recorded between two patient groups based on stage II P-value= 0.004, 0.001, 0.02 respectively.

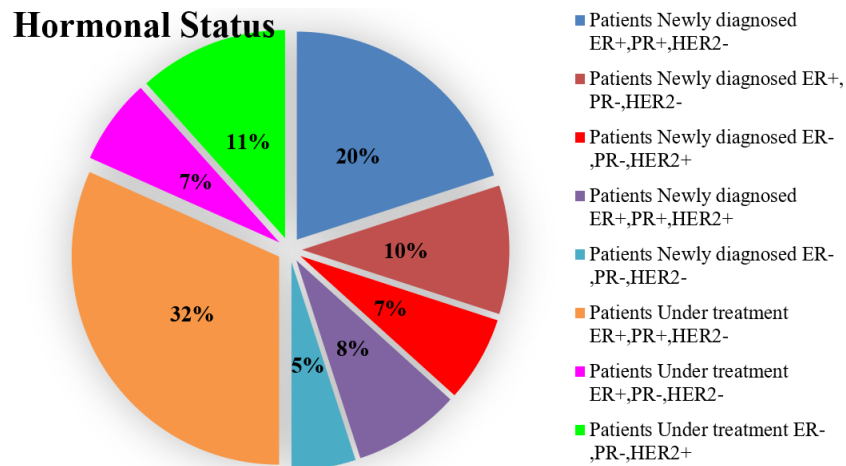
**Table 3.** Serum level of studied parameters in patients (newly diagnosed and under treatment) and control according to stage (I, II, III)

Patients groups	Stage groups	Concentration (Mean±S.E.)		
		Caspase-8 (ng/mL)	MLKL (ng/mL)	RIPK1 (ng/mL)
Newly diagnosed patients	I	0.73±0.14	6.82±2.29	0.562±0.391
	II	1.20±0.22	5.76±0.55	0.856±0.290
<b>P-value</b>		0.3 NS	0.5 NS	0.63 NS
Under Treatment patients	I	0.14±0.02	1.15±0.92	0.072±0.010
	II	0.55±0.10	0.61±0.14	0.147±0.037
	III	0.65±0.16	1.17±0.44	0.053±0.024
<b>P-value</b>		0.44 NS	0.27 NS	0.52 NS
<b>Between newly&amp; under treatment</b>		I	-	-
<b>P-value</b>		0.06 NS	0.06 NS	0.5 NS
<b>Between newly&amp; under treatment</b>		II	-	-
<b>P-value</b>		0.009**	<0.001**	0.02*

<sup>a</sup> vs. stage I in under treatment group, NS= no significant, \*\*= high significant

Figure 3 exposes the percentage distribution of patient groups based on hormonal status. The high percentage was in patients under treatment with hormonal status (ER+, PR+, HER2-) (32%) > newly diagnosed patients (ER+, PR+, HER2-) (20%) > newly diagnosed patients (ER-, PR-, HER+) (11%) > newly diagnosed patients (ER+, PR-, HER2-) (10%) > newly diagnosed patients (ER+, PR+, HER2+) (8%) > newly diagnosed patients and patients under treatment with hormonal status (ER-, PR-, HER2+), (ER+, PR-, HER2-) (7%) > newly diagnosed patients (ER-, PR+, HER2-) (5%).





**Figure 3.** Percentage distribution of patient groups (newly diagnosed and under treatment) based on hormonal status

As exposed in Table 4, hormonal status has no impact on the serum level of all studied parameters in newly diagnosed patients, the results for each hormonal status were (0.93±0.16, 7.20±1.24, 0.615±0.265) ng/mL ER+, PR+, HER2-. For the ER+, PR-, HER2- were (1.61±0.82, 3.35±0.75, 0.529±0.421) ng/mL, for ER-, PR-, HER2+ were (1.10±0.27, 5.20±1.20, 1.061±0.442) ng/mL, for ER+, PR+, HER2+ were (1.01±0.27, 7.29±0.63, 1.866±1.468) ng/mL, for ER-, PR-, HER2- were (0.96±0.10, 5.86±1.02, 0.197±0.094) ng/mL with no significant differences, P-value= 0.74, 0.19, 0.45 respectively. There is also no impact on hormonal status on serum level distribution for studied parameters in patients under treatment. The results for ER+, PR+, HER2- were (0.46±0.06, 0.63±0.18, 0.140±0.043) ng/mL, for ER+, PR-, HER2- were (0.93±0.58, 0.82±0.23, 0.059±0.024) ng/mL and for ER-, PR-, HER2+ were (0.51±0.12, 0.93±0.29, 0.142±0.051) ng/mL, with no significant differences, P-value= 0.2, 0.64, 0.66 respectively.

**Table 4.** Serum level of studied parameters in patients and control according to hormonal status (newly diagnosed and under treatment)

Patients groups	Hormonal status groups	Concentration (Mean± S.E.)		
		Caspase-8 (ng/mL)	MLKL (ng/mL)	RIPK1 (ng/mL)
Newly diagnosed patients	ER+,PR+,HER2-	0.93±0.16	7.20±1.24	0.615±0.265
	ER+,PR-,HER2-	1.61±0.82	3.35±0.75 <sup>b</sup>	0.529±0.421
	ER-,PR-,HER2+	1.10±0.27	5.20±1.20	1.061±0.442
	ER+,PR+,HER2+	1.01±0.27	7.29±0.63	1.866±1.468
	ER-,PR-,HER2-	0.96±0.10	5.86±1.02	0.197±0.094
<b>P-value</b>		0.74 NS	0.19 NS	0.45 NS
Under Treatment patients	ER+,PR+,HER2-	0.46±0.06	0.63±0.18	0.140±0.043
	ER+,PR-,HER2-	0.93±0.58	0.82±0.23	0.059±0.024
	ER-,PR-,HER2+	0.51±0.12	0.93±0.29	0.142±0.051
<b>P-value</b>		0.2 NS	0.64 NS	0.66 NS

<sup>a</sup> vs. ER-PR-HER2+ in newly diagnosed group, <sup>b</sup> vs. ER+PR+HER2- in newly diagnosed group, <sup>c</sup> vs. ER-PR-HER+ in under treatment group

As shown in Table 5, this study demonstrated the correlation between the serum levels of studied parameters measured using ELISA technique. The statistical analysis revealed a significant weak positive correlation was detected between MLKL and Caspase-8 (r=0.267, P=0.011, a significant weak positive correlation was observed between RIPK1 and caspase-8 (r=0.224, p=0.03) and MLKL (r=0.243, 0.02).

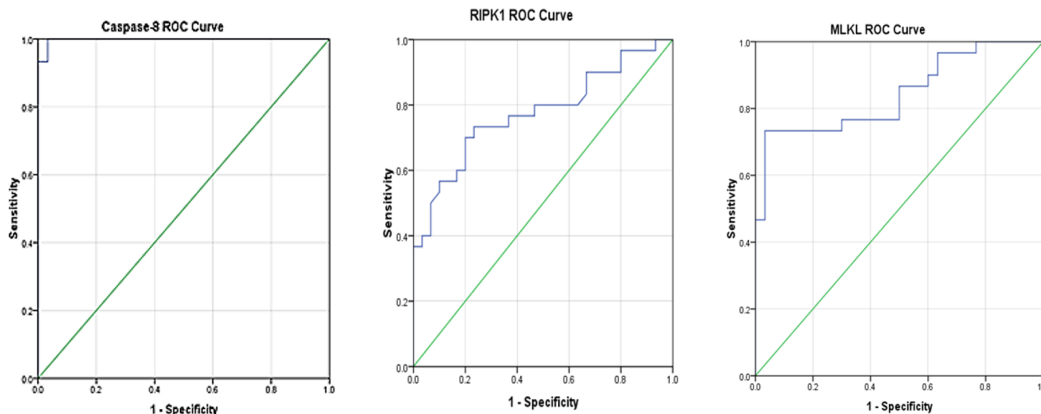
**Table 5.** The correlation between the serum levels of the studies parameters

Parameter		Caspase-8	MLKL	RIPK1
<b>Caspase-8</b>	Pearson Correlation	1		
	Sig. (2-tailed)			
<b>MLKL</b>	Pearson Correlation	0.267*	1	
	Sig. (2-tailed)	0.011		
<b>RIPK1</b>	Pearson Correlation	0.224*	0.243*	1
	Sig. (2-tailed)	0.034	0.021	

Table 6 presents the ROC curve analysis for various parameters, highlighting their diagnostic performance. Caspase-8 demonstrates an impressive Area Under the Curve (AUC) of 0.99, with a cutoff value of 0.22 ng/mL, achieving 100% sensitivity and 97% specificity, indicating excellent predictive accuracy ( $P < 0.001^{**}$ ). MLKL achieves an AUC of 0.84 with a cutoff of 4.45 ng/mL, with a sensitivity of 73% and specificity of 97%, indicating a robust ability to differentiate between conditions ( $P < 0.001^{**}$ ). RIPK1 shows an AUC of 0.77 with a cutoff of 0.138 ng/mL, with a sensitivity of 70% and specificity of 80%, demonstrating good diagnostic capability ( $P < 0.001^{**}$ ).

**Table 6.** The ROC curve summarized the performance and prediction results

Parameters	AUC	Cutoff	Sensitivity	Specificity	P-value
<b>Caspase-8</b>	0.99	0.22	100%	97%	$< 0.001^{**}$
<b>MLKL</b>	0.84	4.45	73%	97%	$< 0.001^{**}$
<b>RIPK1</b>	0.77	0.138	70%	80%	$< 0.001^{**}$



**Figure 4.** The ROC curve

Caspase-8, a key initiator caspase in the extrinsic apoptotic pathway, plays a crucial role in mediating cell death in response to death receptor signals. The significant elevation of Caspase-8 levels in newly diagnosed patients compared to controls is consistent with studies, such as those by [35] and [36], which report increased apoptotic signaling in newly diagnosed various diseases, including cancer and neurodegenerative disorders. These studies support the idea that heightened apoptosis is a hallmark of initial disease stages, contributing to disease pathogenesis. The protein known as MLKL (Mixed Lineage Kinase Domain-Like Protein) is highly involved in the necroptosis process [37]. The increased levels of MLKL in newly diagnosed patients may explained by a genetic ablation of Caspase-8 drugs [21]. The marked decrease in levels of MLKL in the under-treatment patients is in agreement with [38] and [39] that show suppression of necroptosis upon treatment thus making MLKL a possible therapeutic target. RIPK1 (Receptor-Interacting Protein Kinase 1) plays a very important role in modulating both apoptotic and necrotic pathways [40]. It has been found that RIPK1 plays a role in both apoptosis and necroptosis in newly diagnosed patients [38], [41].

The comparison between newly diagnosed and under-treatment patients reveals significant reductions in Caspase-8, MLKL, and RIPK1 levels in under-treatment patients at Grade II. At Grade III,

significant differences are observed for MLKL, but not for Caspase-8, or RIPK1. In newly diagnosed patients, Caspase-8 levels show an increasing trend from Grade I to Grade III, although this increase is not statistically significant ( $P=0.08$ ). This trend aligns with findings by [42], which reported higher Caspase-8 levels in more advanced disease stages, suggesting that Caspase-8 may play a role in exacerbating disease severity through enhanced apoptotic signaling.

In newly diagnosed patients, MLKL levels are raised in grade I as compared with grade III but with no significant ( $P=0.79$ ) as we know that MLKL is the marker of necroptosis which is unregulated in the advanced stages of the disease due to higher levels of necrotic cell death. As for under-treatment patients, there was a little reduction in MLKL level in Grade II compared to Grade III with no statistical impact ( $P=0.89$ ), this is consistent with [38] and [39]. Newly diagnosed patients exhibit variable levels of RIPK1; these levels do not correlate with the patients' grade ( $P=0.47$ ). Compared to newly diagnosed patients, RIPK1 levels in under-treatment patients are reduced, but the difference between Grade II and Grade III patients is not significant. This finding is consistent with other studies, which demonstrated that treatment reduces RIPK1 levels, subsequently decreasing apoptotic as well as necroptotic cell death pathways, potentially limiting inflammation and tumor progression associated with necroptosis [39].

These findings exposed some differences between both groups with more significant differences recorded in the newly diagnosed and under-treatment patients in Grade II but less significant when compared with Grade III ( $P=0.13$ ). The signal indicated that the treatment produced a better outcome in the early stages of the disease than in the advanced stage, and therefore treatment might have a more significant impact on the RIP. In newly diagnosed patients, Caspase-8 levels increase in Stage I as compared to Stage II, though the difference is not statistically significant ( $P=0.3$ ). This trend is consistent with findings by [43], which reported an elevated Caspase-8 level in more advanced stages of various diseases, reflecting increased apoptotic activity as the disease progresses. In under-treatment patients, Caspase-8 levels increase in Stage I as compared to Stage III, though this increase is not significant ( $P=0.44$ ). This observation is consistent with a study by [29], which reported a partial restoration of Caspase-8 levels post-treatment, suggesting that therapeutic interventions may not fully normalize apoptotic signaling in advanced stages [44]. Newly diagnosed patients have a mildly lowered MLKL in Stage I than in Stage II with no statistical difference ( $P=0.5$ ). This observation is in line with the findings by [45] and [41] in a cross-sectional analysis, showing moderate and stable positivity for MLKL across all the phases of the disease. In under treatment patients, the status of the gene product, MLKL, varies, with reduced levels in Stages II and III in comparison with Stage I; and elevated in Stage III. This variability is in line with [39] which showed the oscillating levels of MLKL in post-treatment a hear implying that necroptosis could be differently regulated depending on the stage of the disease and the efficacy of the treatment. For the MLKL, a marked different range has been noted between two patient groups with significant differences  $0=0.001$  recorded between two patient groups with study stage II, and this finding is in line with the study who recorded a decreased level of MLKL in the progressive stages of disease in post-treatment patients RIPK1 has no significant differences between two patients groups.

Hence, in relation to this trend, [46] claims that the level of RIPK1 varies with different stages of the disease, based on its features as the protein that can stimulate both apoptotic and necroptotic processes. In under-treatment patients, the level of RIPK1 protein changes dynamically, the level was higher in patients with stage II compared to stage I, but it has a reduced level in stage III with non-significance. This is in consonance with [47], authors that described variable fluctuations in RIPK1 levels following treatments, in a remark of the dynamism of its regulation during the progression of the disease and treatment. In newly diagnosed patients, Caspase-8 levels exhibit variability across different hormonal statuses, with the highest levels observed in the ER+, PR-, HER2- group and the lowest in the ER+, PR+, HER2- group with no statistically significant ( $P=0.74$ ). This observation aligns with findings by [35], which report variability in Caspase-8 levels depending on hormonal status, reflecting the complex role of hormone receptors in modulating apoptotic pathways. In under-treatment patients, Caspase-8 levels are generally lower across all hormonal statuses, with no significant differences observed ( $P=0.2$ ). This finding goes in line with the works of which reveal the ability of treatment to lower Caspase-8 in practically all hormonal states, which may point to overall suppression of apoptosis in the absence of HR determination.

Unfortunately, the MLKL concentrations in newly diagnosed casualties do not present much distinctness based on the hormonal statuses: the highest content is observed in the ER +, PR +, HER2 -group, and the lowest content in ER +, PR-, HER2-group, but with no statistically significant ( $P=0$ ). The concentration range recorded a highly significant difference between the two patient groups with a grade P-value of 0.001 and thus the hormone appears to play some role in modulating necroptosis. In under-treatment patients, the level of the MLKL protein does not significantly increase depending



on hormonal status, as seen in the data analyzed with a resulting  $P = 0.64$ . The present work is also in line with study by [39] where treatment was seen to have the capacity to bring MLKL levels down irrespective of hormonal status meaning that there is a general downregulation of necroptosis pathways when patients undergo treatment. RIPK1 concentration in the newly diagnosed subject depends on the hormonal status, the highest concentration of RIPK1 is found in the ER+, PR+, HER2+ groups and the lowest concentration is in ER-, PR-, HER2- groups the differences have no significant difference ( $P=0.45$ ).

Interestingly, RIPK1 levels are low in the under-treatment patients and little variability is seen according to hormonal status, and thus no statistically significant differences can be ascertained ( $P=0.66$ ). This is in line with [47] which postulates treatment leads to a decrease in expression of RIPK1 throughout all hormonal ranges hence signaling overall inhibition of cell death pathways after treatment. MLKL has a weak positive correlation with Caspase-8. This association is well supported by the studies conducted by [48] and [49], which demonstrate that though Caspase-8 is associated with apoptosis, it can be involved in necroptotic signaling involving MLKL under stress conditions proposing apoptotic necroptotic crosstalk. RIPK1 had a weak positive relationship with Caspase-8 at Constant  $P < 0.05$  and MLKL. These weak correlations mean that although RIPK1 participates in these processes, its interactions can be controlled by other factors. Caspase-8 demonstrated exceptional diagnostic performance with an Area Under the Curve (AUC) of 0.99, indicating nearly perfect accuracy in distinguishing between affected and unaffected individuals. The cutoff value of 0.22 ng/mL, with 100% sensitivity and 97% specificity, underscores its potential as a highly reliable biomarker. These results align with studies conducted by [36], [50], [51] which highlight Caspase-8's significant role in apoptosis and its diagnostic relevance in various diseases. Because specificity is high, Caspase-8 is specific in terms of correctly identifying non-cases, that is, non-diseased persons, and thus, have low or minimal chances of false positive results.

## CONCLUSION

High serum level of Caspase-8, MLKL, and RIPK1 was found in Iraqi females with breast cancer with a significant relation depending on grade, stage, and hormonal status of breast cancer. The levels recorded a decrement level in patients under treatment, so the studied marker may serve as a predictive marker for therapeutic response.

## SUPPLEMENTARY MATERIAL

*None.*

## AUTHOR CONTRIBUTIONS

*Mays Najem Al-Adilee: Methodology; software; formal analysis; investigation; data curation; writing—review and editing. Hazima Mossa ALabassi: Conceptualization; validation; supervision and project administration.*

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## DATA AVAILABILITY STATEMENT

*None.*

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## CONFLICTS OF INTEREST

*The authors declare no conflicts of interest.*

## ETHICAL APPROVAL

The study secured ethical approval from the Iraqi Ministry of Health—Department of Medical Teaching City Oncology Teaching Hospital (No.26512, 17/7/2023), and College of Education for Pure Science (Ibn Al-Haitham), University of Baghdad (EC-49 on 16/12/2024).

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