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Correlation of Some Apoptotic Markers with Chronic Myelogenous Leukemia in Iraqi Patients

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ABSTRACT

Background: The BCR-ABL gene encodes an oncoprotein with abnormal tyrosine kinase activity affecting cellular proliferation, survival, and apoptosis, is the cause of chronic myelogenous leukemia (CML). A type of programmed cell death termed apoptosis works as a preventative measure against diseases like cancer.

Objective: This study aimed to assess four apoptotic players in CML patients as an attempt to provide new options for targeted therapy, and to examine these proteins as potential predictors for the disease.

Methods: 140 participants were divided into two groups, healthy controls (CT) and people with chronic myeloid leukemia. Healthy samples were 60, while 80 samples were taken from patients who had been diagnosed with CML in the International Hematology Center, Baghdad, Iraq. Using ELISA approach, we measured serum levels of four critical participants of apoptosis (TNFR1, BAX, CASP-9, and CYTO-C) in CML patients and controls.

Results: In comparison to patients, controls had higher median levels of BAX, CYTO-C, and CASP-9. While median TNFR1 levels in patients were greater than in controls. Age-dependently, CT groups had significantly higher levels of BAX, CYTO-C, and CASP-9 than CML groups in the age categories of < 40 years and 40–50 years, in contrast to TNFR1 which was markedly down regulated in CT. According to gender, CT groups had significantly greater levels of BAX, CYTO-C, and CASP-9 than CML, while TNFR1 was significantly higher in CML than CT groups in both genders. BAX, CYTO-C, TNFR1, and CASP-9 are effective predictors in differentiating between CML patients and CT groups according to the results obtained from receiver operating characteristic analysis. Correlation coefficient analysis test revealed that BAX correlated with CYTO-C and CASP-9 in a positive-significant manner. While BAX had a positive, although insignificant correlation with TNFR1. TNFR1 and CASP-9 had a non-significant positive correlation with CYTO-C. Finally, TNFR1 and CASP-9 showed positive but non-significant correlation.

Conclusions: BAX, CYTO-C, TNFR1, and CASP-9, provide potential targets for diagnosis, prognosis, and treatment. As they turned to be excellent predictors in CML.

Keywords: CYTO-C (cytochrome-c), TNFR1 (tumor necrosis factor receptor 1), CML (chronic myeloid leukemia), CASP-9 (Caspase 9).

INTRODUCTION

A translocation between the ABL (Abelson Murine Leukemia) gene on chromosome 9 and the BCR (breakpoint cluster region) gene on chromosome 22 results in the creation of the Philadelphia chromosome, which is the main cause of chronic myeloid leukemia (CML), a myeloproliferative disease. In contrast to ABL1, which encodes a non-receptor tyrosine kinase, BCR is a breakpoint cluster region. The 210-kDa oncoprotein p210 BCR-ABL1 is translated and works as a faulty tyrosine kinase that is constitutively generated, influencing pathways including PI3K/AKT, JAK/STAT, and RAS/MEK and biological processes like cell proliferation and apoptosis [(1)].

The majority of individuals have CML in its chronic phase, which is characterized by symptoms most frequently related with anemia and splenomegaly as fatigue and malaise, can have a cumulative effect that results in pain or early satiety, thrombocytopenia or platelet dysfunction that can also be brought on by CML. As CML enters the rapid or blast phase, symptoms like headache, bone pain, fever, infection, bleeding, and lymphadenopathy become more prevalent [(2)]. Apoptosis is a methodical kind of energy-dependent cell demise that eliminates surplus cells. The pathophysiology of numerous diseases, including cancer, AIDS, and other immunological disorders involves apoptosis. Either the intrinsic or the extrinsic routes of apoptosis are triggered depending on the source of the apoptotic stimuli. A distinct intrinsic apoptotic mechanism dependent on mitochondria was identified by APAF1, the outer membrane of the mitochondria becomes permeable as a result of mitochondrial disruption, and several molecules are released, including cytochrome C. When APAF1 detects cytosolic cytochrome C (CASP 9) the intrinsic apoptotic pathway's initiating caspase, and other proteins come together to form the apoptosome [(3)].

This apoptosome splits CASP 9 into its mature form via an ATP hydrolysis-related mechanism. Mature CASP 9 can activate effector caspases like CASP 3 that are downstream, several BCL-2 family proteins control the permeability of the mitochondria, which permits cytochrome C discharge. [(4)].

The activation of the death-inducing receptors Fas and TNFR starts extrinsic apoptosis, through homotypic connections between their death effector domains (DED), FADD engages the DD of the receptor and recruits CASP 8. When CASP 8 is activated, it cleaves and activates CASP 3 and CASP 7 [(5)]. One form of planned cell death is apoptosis (PCD) that acts as a preventative measure inhibiting the growth of cancer. In

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order to preserve tissue homeostasis, apoptosis eliminates diseased, infected, and senescent cells [6].

In this manner we assessed four apoptotic players in CML patients as an attempt to provide new options for targeted therapy, and to examine these proteins as potential predictors for the disease.

MATERIALS AND METHODS

Collection of Samples

140 participants were divided into two groups, healthy controls and people with chronic myeloid leukemia. Healthy samples were 60 (38 male, 22 female) drawn from volunteers between the ages 30 and 50 in the Blood Bank of Baghdad, Iraq. 80 samples (49 male, 31 female) were taken from patients who had been diagnosed with CML in the International Hematology Center, Baghdad, Iraq. Ages of patients ranged from 30 to 65 years. Four milliliters of blood were taken via venipuncture from each participant. Blood was drawn and placed into gel clotting tubes. Gel clot tubes were centrifuged in a temperature-controlled (4 ^{o}C) centrifuge after being allowed to coagulate for 30 minutes. For ELISA testing, serum was collected, and the test sera were kept frozen at -20 ^{o}C.

ELISA Test:

Using commercially available ELISA kits, the levels of human BAX, CASP-9, CYTO-C, and TNFR1 were determined in both CML patients' and healthy controls' serum. The assay process, and the calculations are all presented collectively because all kits were manufactured by the same company (MyBiosource/USA) and were based on similar principles.

Standards Preparation

Preparation of standard solutions has been executed in accordance with the manufacturer's guidelines: BAX: 0, 5, 10, 20, 40, 80 ng/ml**;** CYTO-C: 0, 315, 625, 1250, 2500, 5000 pg/ml**;** CASP9: 0, 6.25, 12.5, 25, 50, 100 ng/ml; TNFR1: 0, 3, 6, 12, 24, 48 ng/ml**.** The procedure for preparing standards was similar for the four markers examined.

Therefore, we describe a representative method for TNFR1. All of the chemicals in the kit had been brought to room temperature. The highest standard concentration of 48 ng/ml standard stock solution was obtained by combining 120 µl of the lyophilized standard reconstitute (96 ng/ml) with 120 µl of the standard diluent. The standard was then gently swirled and left to stand at room temperature for 15 minutes before being diluted. The duplicate standard points (48 ng/ml) were made by serially diluting the standard stock solution to produce 24 ng/ml, 12 ng/ml, 6 ng/ml, and 3 ng/ml solutions. The zero standard (0 ng/ml) is standard diluent. At -20 ^{o}C, the remaining solutions were kept frozen. Standard solution were diluted as specified in table (1).

Table (1): Standard dilutions for TNFR-1

St. No.	Standard conc.	Suggested dilutions
6	48 ng/ml	120µl original standard + 120µl standard diluent
5	24ng/ml	120µl standard no.6 + 120µl standard diluent
4	12ng/ml	120µl standard no.5 + 120µl standard diluent
3	6ng/ml	120µl standard no.4 + 120µl standard diluent
2	3ng/ml	120µl standard no.3 + 120µl standard diluent
1	0ng/ml	Standard diluent

ELISA Protocol:

Prior to performing the procedure, serum samples, standards and required solutions were brought to room temperature, lightly mixed, and left to equilibrate under room temperature for 30 minutes. The assay procedure was carried out in line with the manufacturer's instructions. The control pits and standard test sample (zero) of the pre-coated plate have been set up and their positions were registered. Standard wells received 50 µl of standard solution. With the exception of the blank control well, 40 µl of sample was added to the sample wells, followed by the addition of 10 µl of anti-TNF-R1 antibody and 50 µl of streptavidin-HRP. The wells were then mixed, the plate was sealed, and the incubation period was set for 60 minutes at 37 °C.

The seal was taken off, and then wash buffer was used to wash the plate five times. Each well received 50 µl of substrate solution A, followed by 50 µl of substrate solution B. The plate was then sealed with a fresh sealer and incubated at 37 °C in the dark for 10 min.

When each well received 50 µl of stop solution, the blue color turned yellow right away. Within 10 minutes of adding the stop solution, the optical density (OD value) for each well was measured using a microplate reader set to 450 nm.

A standard curve was plotted against the standard's concentrations in relation to the measurement of the associated O.D. for calculations (Figure 1):

Related O D at 450 nm = (the O D at 450 nm for each well) -- (the O D at 450 nm of zero well).

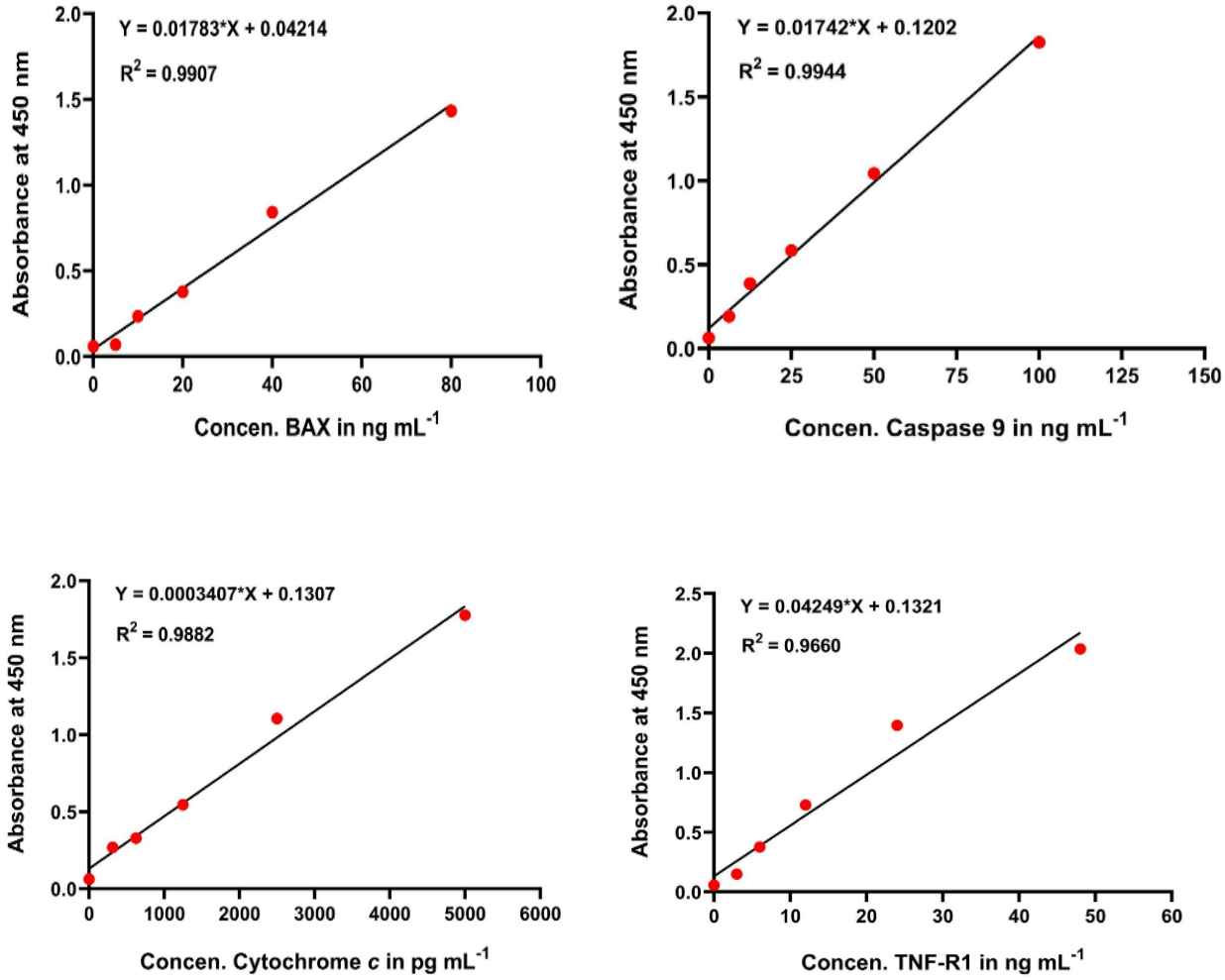

Figure (1): Standard curves for BAX, CYTO-C, TNFR-1, and CASP-9.

Statistical Analysis

Version 22 SPSS for Windows was used to analyze the data (SPSS Inc. Chicago, Illinois, United States).

In order to express categorical variables, numbers and percentages were used, and Pearson & Chi-square tests were used to see whether there were any significant differences.

Parametric variables were given as mean ± standard deviation (SD), the least significant difference (LSD) test was used to determine whether there were any significant differences between the parametric variables. Nonparametric variables were expressed as median and interquartile range (IQR).

The Mann-Whitney U test was performed to determine whether there were any significant differences between the medians of nonparametric variables. To ascertain the area under the curve (AUC), 95% confidence interval (CI), cut-off value, sensitivity, and specificity, receiver operating curve (ROC) analysis was carried out. The Youden index was used to optimize the cut-off value (YI). To assess correlations between variables, the Spearman rank-order correlation analysis was performed.

Ethical Approval:

Both Iraq's Ministries of Higher Education and Scientific Research as well as the Ministry of Environmental and Health have accredited this study as ethical. Prior to enrollment, a written consent of each participant was obtained.

RESULTS

Levels of Apoptosis-Related Indicators and Patient Characteristics

Age and gender-specific classifications of BAX levels were made for groups of CML patients and controls (Table 2). According to age: CT *vs* CML, Significant difference was found in age group < 40 years, in CT 25.5 [IQR: 21.8-28.7] *vs* in CML 11.9 [IQR: 9.7- 14] ng/mL; $p < 0.001$. Significant difference was found in age group 40-50 years, in CT 23.1 [IQR: 21.5-27.7] *vs* in CML 12.1 [IQR: 9.3-13.3] ng/mL; $p < 0.001$. No significant difference was found in age group > 50. Regarding gender, BAX levels were substantially higher in CT than in CML patients in both males 22.8 [IQR: 21.1-27.8] *vs* 11.2 [IQR: 8.8-12.9] ng/mL; $p < 0.001$ and females 25.7 [IQR: 25.5-30.9] *vs* 12.4 [IQR: 9.9-13.6] ng/mL; p 0.001, respectively.

Table (2): Serum levels of BAX in CML patients and controls according to gender and age.

Characteristic		Median (IQR) BAX; ng/mL		*p*-value
Gender	Male	CT	22.8 (21.1-27.8)	<0.001**
		CML	11.2 (8.8-12.9)	
	Female	CT	25.7 (25.5-30.9)	<0.001**
		CML	12.4 (9.9-13.6)	
Age group (years)	<40 years	CT	25.5 (21.8-28.7)	<0.001**
		CML	11.9 (9.7- 14)	
	40-50 years	CT	23.1 (21.5-27.7)	<0.001**
		CML	12.1 (9.3-13.3)	
	>50 years	CT	-	-
		CML	10.4 (7.9-12.5)	

** Significant at the 0.01 level.

Age and gender-specific CYTO-C levels in CML patients and controls were categorized (Table 3). Regarding age: CT *vs.* CML, a significant difference was found in age group < 40 years, in CT 1259 [IQR: 1133-1404] *vs* in CML 278.4 [IQR: 237.4-407.5] pg/mL; $p < 0.001$. Also, a significant difference was found in age group 40-50 years, in CT 1221 [IQR: 1093-1349] *vs* in CML 262.5 [IQR: 226.6-362.6] pg/mL; $p < 0.001$. There was no significant difference in age group > 50. Concerning gender, both genders in CT had substantially higher CYTO-C levels than CML patients, for males 1192 [IQR: 1095-1274] *vs* 277.8 [IQR: 235-514] pg/mL; $p < 0.001$ and for females 1335 [IQR: 1154-1520] *vs* 272.3 [IQR: 261.9-324.6] pg/mL; $p < 0.001$.

Table (3): Serum levels of CYTO-C in CML patients and controls according to gender and age.

Characteristic		Median (IQR) CYTO-C; pg/mL		*p*-value
Gender	Male	CT	1192 (1095-1274)	<0.001**
		CML	277.8 (235-514)	
	Female	CT	1335 (1154-1520)	<0.001**
		CML	272.3 (261.9-324.6)	
Age group (years)	<40 years	CT	1259 (1133-1404)	<0.001**
		CML	278.4 (237.4-407.5)	
	40-50 years	CT	1221 (1093-1349)	<0.001**
		CML	262.5 (226.6-362.6)	
	>50 years	CT	-	-
		CML	273.2 (262.8-572.5)	

** Significant at the 0.01 level

TNFR1 levels in CML patients and controls were categorized by gender and age (Table 4). According to age: CML *vs* CT, significant difference was found in age group < 40 years, in CML 7.2 [IQR: 6.1-8.9] *vs* in CT 3.9 [IQR: 3.5-4.3] ng/mL; $p < 0.001$.

Also, significant difference was found in age group 40-50 years, in CML 6.9 [IQR: 6.6-8.1] *vs* in CT 3.4 [IQR: 2.1-4.4] ng/mL; $p < 0.001$. While, there was no significant difference in age group > 50 years.

Concerning gender, TNFR1 levels were significantly higher in CML patients than in controls in both genders, in males 6.9 [IQR: 6.2-8.3] *vs* 3.8 [IQR: 3-4.1] ng/mL; $p < 0.001$ and in females 7 [IQR: 6.3-8.4] *vs* 4.2 [IQR: 3.8-4.5] ng/mL; $p < 0.001$.

Table (4): Serum levels of TNFR1 in CML patients and controls according to gender and age

Characteristic		Median (IQR) TNFR1; ng/mL		*p*-value
Gender	Male	CT	3.8 (3-4.1)	<0.001**
		CML	6.9 (6.2-8.3)	
	Female	CT	4.2 (3.8-4.5)	<0.001**
		CML	7 (6.3-8.4)	
Age group (years)	<40 years	CT	3.9 (3.5-4.3)	<0.001**
		CML	7.2 (6.1-8.9)	
	40-50 years	CT	3.4 (2.1-4.4)	<0.001**
		CML	6.9 (6.6-8.1)	
	>50 years	CT	-	-
		CML	6.9 (6.2-7.3)	

** Significant at the 0.01 level.

Category-specific CASP-9 levels in CML patients and controls were determined based on age and gender (Table 5). Regarding age: CT *vs.* CML, a significant difference was found in age group < 40 years, in CT 24.2 [IQR: 22.6-31.1] *vs* in CML 8 [IQR: 6.7-9.4] ng/mL; $p < 0.001$. Also, significant difference was found in age group 40-50 years, in CT 24.8 [IQR: 19.2-29.1] *vs* in CML 8.1 [IQR: 5.4-8.5] ng/mL; $p < 0.001$. While, no significant difference was found in age group > 50 years.

Depending on gender, both genders in CT had significantly higher CASPASE-9 levels than CML patients, in males 23.9 [IQR: 21.7-29.9] *vs* 7.7 [IQR: 6.1-9.7] ng/mL; $P < 0.001$, in females 24.9 [IQR: 23.5-32.5] *vs* 8.1 [IQR: 6.7-9.4] ng/mL; $P < 0.001$), respectively.

Table (5): Serum CASP-9 levels in CML patients and controls according to gender and age.

Characteristic		Median (IQR) CASP-9; ng/mL		*p*-value
Gender	Male	CT	23.9 (21.7-29.9)	<0.001**
		CML	7.7 (6.1-9.7)	
	Female	CT	24.9 (23.5-32.5)	<0.001**
		CML	8.1 (6.7-9.4)	
Age group (years)	<40 years	CT	24.2 (22.6-31.1)	<0.001**
		CML	8 (6.7-10.4)	
	40-50 years	CT	24.8 (19.2-29.1)	<0.001**
		CML	8.1 (5.4-8.5)	
	>50 years	CT	-	-
		CML	7.6 (5.7-9.1)	

** Significant at the 0.01 level

Normality testing of apoptosis-related indicators

The Kolmogorov-Smirnov and Shapiro-Wilk tests, two normality checks, were utilized to determine whether the levels of BAX, CYTO-C, TNFR1, and CASP-9 in CML patients and controls were distributed normally.

The four parameter levels were discovered not to fit a normal distribution (non-parametric distribution) (Table 6). Thus, medians and interquartile ranges (IQRs) were used to express these values. By comparing more than two groups using the Kruskall-test Walli's or the Mann-Whitney U test when comparing two groups, significant differences between medians were identified.

Table (6): Normality testing of BAX, CYTO-C, TNFR1, and CASP-9 levels in CML patients and controls groups

parameter	Group	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
		Statistic	DF	*p*-value	Statistic	DF	*p*-value
BAX	Patient	0.136	80	0.02*	0.962	80	0.017*
	Control	0.155	60	0.001**	0.935	60	0.003**
CYTO-C	Patient	0.253	80	< 0.001**	0.788	80	< 0.001**
	Control	0.230	60	< 0.001**	0.814	60	< 0.001**
TNFR1	Patient	0.188	80	0.01**	0.970	80	0.04*
	Control	0.198	60	< 0.001**	0.914	60	< 0.001**
CASP-9	Patient	0.125	80	0.02*	0.133	80	0.03*
	Control	0.120	60	0.03*	0.953	60	0.02*

* Significant at the 0.05 level. ** Significant at the 0.01 level.

Serum Levels of Immunological Parameters

Table (7) and figure (2), displayed the immunological parameters' median values, which were discovered to be as follows: BAX: CML 11.7 [IQR: 9.5-13.2] *vs* CT 25.5 [IQR: 21.7-27.8] ng/mL; $p < 0.001$. CYTO-C: CML 272.3 [IQR: 237.7-395] *vs* CT 1240 [IQR: 1118-1345] pg/mL; $p < 0.001$. TNFR1: CML 7 [IQR: 6.2-8.3 *vs* CT 3.9 [IQR: 3.2-4.3] ng/mL; $p < 0.001$. CASP-9: patient: 7.9 [IQR: 6.5-9.4] *vs* CT 24.3 [IQR: 22.4-31] ng/mL; $p < 0.001$.

Table (7): Serum levels of immunological parameters.

Marker	No. of values	Median LV.	IQR	Std. Deviation	Std. error	*P.* value	Sum.
BAX	Patient 80	11.7 ng/mL	9.5-13.2	3.154	0.3526	$p < 0.001$	**
	Control 60	25.5 ng/mL	21.7-27.8	4.140	0.5345		
CYTO-C	Patient 80	272.3 pg/mL	237.7-395	180.3	20.16	$p < 0.001$	**
	Control 60	1240 pg/mL	1118-1345	308.8	39.86		
TNFR1	Patient 80	7 ng/mL	6.2-8.3	1.623	0.1814	$p < 0.001$	**
	Control 60	3.9 ng/mL	3.2-4.3	0.8439	0.1090		
CASP-9	Patient 80	7.9 ng/mL	6.5-9.4	2.482	0.2775	$p < 0.001$	**
	Control 60	24.3 ng/mL	22.4-31	5.300	0.6842		

* Significant at the 0.05 level. ** Significant at the 0.01 level.

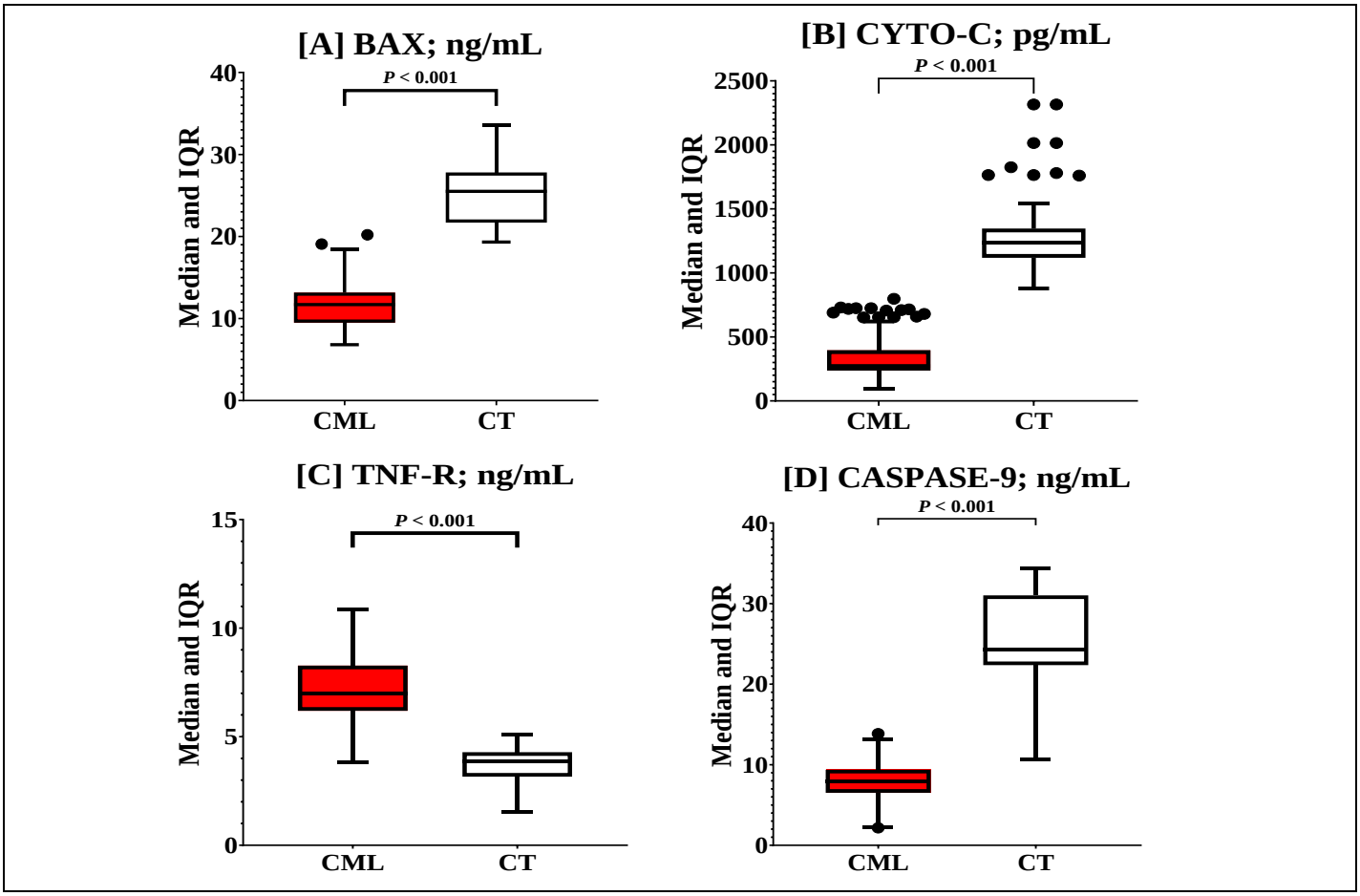

Figure (2): Box and whisker plots of serum BAX (A), CYTO-C (B), TNFR1 (C), and CASP-9 (D) levels in the sera of CML patients and controls groups. Horizontal line inside box indicates median, while whiskers represent interquartile range (IQR). Outliers are shown as black circles.

Immunological parameters Receiver Operating Characteristic (ROC) Curve Analysis

Significance of predictability for BAX, CYTO-C, TNFR1, and CASP-9 in discriminating between CML patients and CT groups was evaluated utilizing ROC curve analysis. The analysis's findings are shown in table (8) and figure (3).

- **BAX**: AUC = 0.999; 95% CI = 0.997-1; $p < 0.001$; cut-off value = 19.6 ng/mL; sensitivity = 98.8%; specificity =96.7% and YI = 0.96.
- **CYTO-C**: AUC = 1; 95% CI = 1-1; $p < 0.001$; cut-off value = 898.2 pg/mL; sensitivity = 100%; specificity = 98.3% and YI = 0.983.
- **TNFR1**: AUC = 0.964; 95% CI = 0.938-991; $p < 0.001$; cut-off value = 3.85 ng/mL; sensitivity = 98.8%; specificity = 45% and YI = 0.44.
- **CASP-9**: AUC = 0.997; 95% CI = 0.992-1; $p < 0.001$; cut-off value = 10.68 ng/L; sensitivity = 85%; specificity = 98.3% and YI = 0.83.

AUC values ranged from 0.50-0.59 (no discrimination), 0.60-0.69 (poor discrimination), 0.70 - 0.79 (acceptable), 0.80 - 0.89 (excellent) or ≥ 0.9 (outstanding). Accordingly, BAX, CYTO-C, TNFR1, and CASP-9 considered excellent predictors of CML.

Table (8): Receiver operating characteristic analysis of immunological parameters.

Parameter	AUC	CI	*P.value*	Cut-off	Sensi.	Spec.	YI
BAX	0.999 (95%)	0.997-1	< 0.001	19.6 ng/mL	98.8%	96.7%	0.83
CYTO-C	1 (95%)	1-1	< 0.001	898.2 pg/mL	100%	98.3%	0.983
TNFR1	0.964 (95%)	0.938-991	< 0.001	3.85 ng/mL	98.8%	45%	0.44
CASP-9	0.997 (95%)	0.992-1	< 0.001	10.68 ng/mL	85%	98.3%	0.83

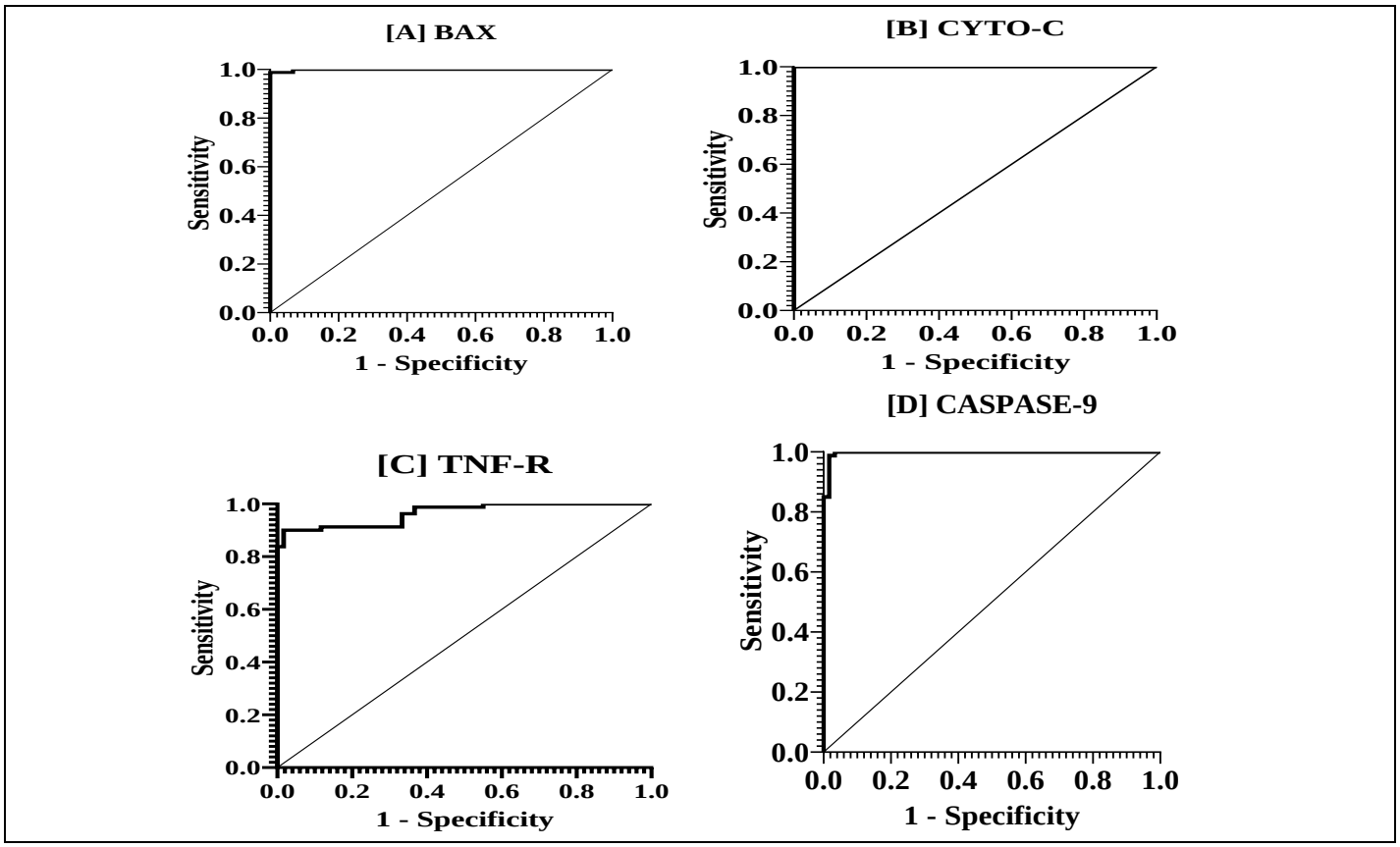

Figure (3): Receiver operating characteristic (ROC) curve analysis of sera BAX (A), CYTO-C (B), TNFR1 (C), and CASP-9 (D) levels in CML patient's vs controls groups.

Correlation Analysis between Immunological Parameters

The correlation coefficient analysis test was used to examine the correlations between the immunological markers BAX, CYTO-C, TNFR1, and CASP-9 in CML patients.

The outcomes are displayed in table (9). BAX was correlated with CYTO-C (rs = 0.234), CASP-9 (rs = 0.55) in a positive manner, and the differences between these three variables were significant. BAX was also positively correlated with TNFR1 (rs = 0.162), but the differences between these variables were not significant. TNFR1 and CASP-9 had positive correlations with CYTO-C (rs = 0.173 and 0.156, respectively), even though the variations weren't statistically significant. TNFR1 and CASP-9 had a positive correlation (rs = 0.106), However, there was no discernible difference (Figure 4).

Table (9): Correlation analysis between serum BAX, CYTO-C, TNFR1, and CASPASE-9 levels in CML patients

Parameters	BAX		CYTO-C		TNFR1		CASPASE-9	
	r	*p* value	r	*p* value	r	*p* value	r	*p* value
BAX	-	-	0.234	0.036*	0.158	0.162 NS	0.55	<0.001**
CYTO-C	0.234	0.036*	-	-	0.173	0.126 NS	0.156	0.168 NS
TNF-R	0.158	0.162 NS	0.173	0.126 NS	-	-	0.106	0.347 NS
CASP-9	0.55	<0.001**	0.156	0.168 NS	0.106	0.347 NS	-	-

NS: Non-Significant. *Correlation is significant at the 0.05 level (2-tailed). **Correlation is significant at the 0.01 level (2-tailed).

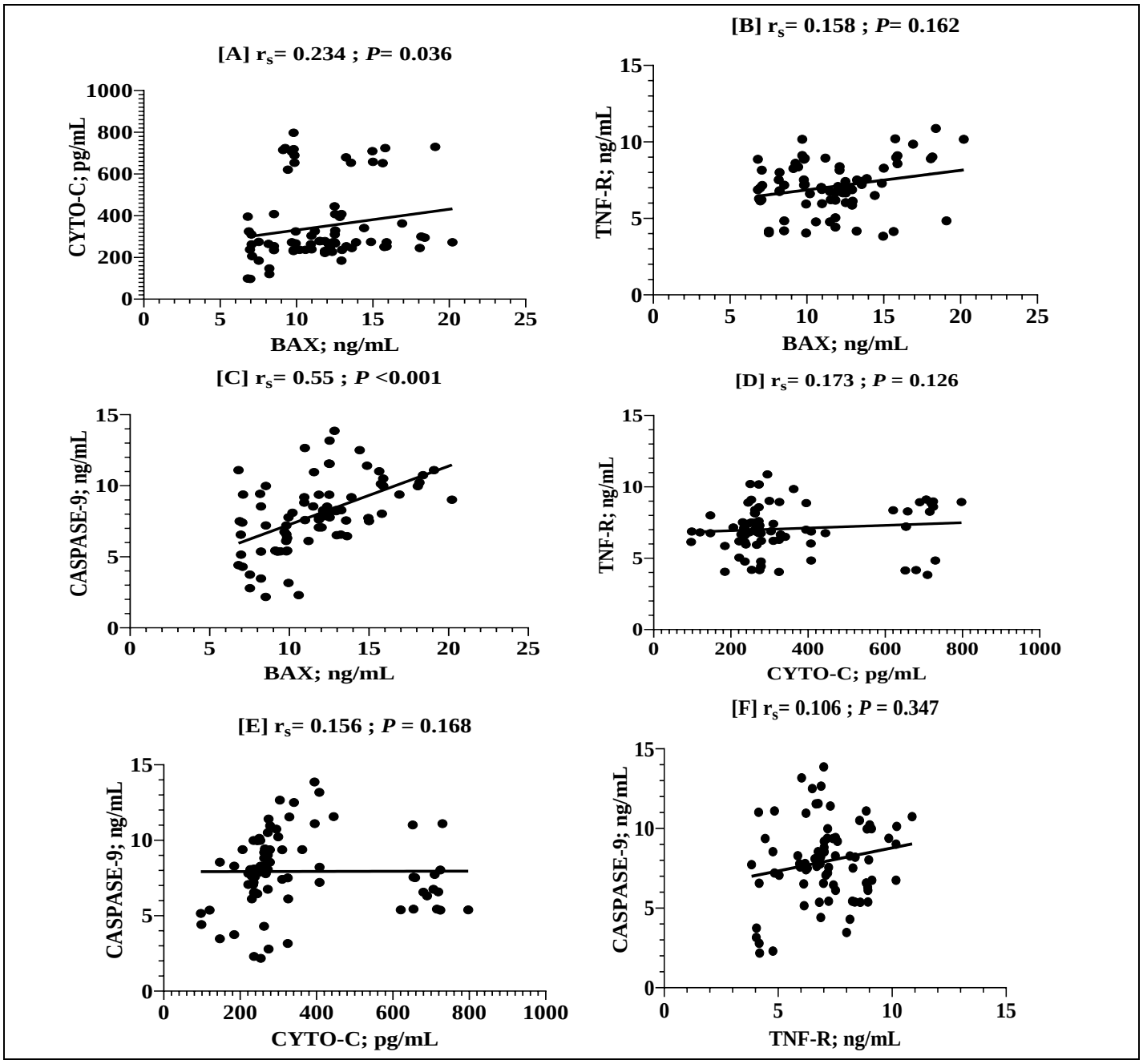

Figure (4): Correlation analysis between serum BAX, CYTO-C, TNF-R, and CASP-9 levels in CML patients

DISCUSSIONS

Age-related physical changes are a result of a loss of homeostasis, which has an impact on the functionality of molecules, cells, and organ systems. Changes in apoptosis can have pathophysiological effects in both aging and related diseases since it is a sentinel homeostatic system [(7)]. Additionally, sexual differentiation has an impact on almost every bodily system and causes noticeable sex differences in growth, longevity, metabolism, and immunity, all of which can affect the progression of cancer, how well it responds to treatment, and how long a person survives. From cellular transformation through all stages of growth, dissemination, and treatment responsiveness, males and females can have fundamentally different defences against cancer. The majority of cancer's hallmarks displayed gender-specific perturbation response patterns particularly in those that include pathways involved in cell proliferation and apoptosis pathways that resist cell death, modulate proliferative signals, and dodge growth inhibitors [(8)]. We found that, in age groups < 40 years & 40-50 years, BAX, CYTO-C and CASP-9 were significantly up regulated in CT than in CML groups. While TNFR1 was significantly down regulated in CT compared to CML group. While, according to gender, BAX, CYTO-C and CASP-9 were substantially higher in both gender in CT than in CML group. While, TNFR1 in both genders was substantially higher in CML than in CT groups.

A type of cell death mechanism, apoptosis eliminates unneeded cells from the body, from cancer to autoimmune illnesses, any aberration in the apoptotic process might result in disease. It is dependent on interrelationships between many molecules, several gene families are involved or work together, including the genes for the B cell lymphoma (Bcl)-2 family, the

caspases, the inhibitor of apoptosis proteins, the tumor necrosis factor (TNF) receptor gene superfamily, and the p53 gene. Cancer is one of the conditions where insufficient apoptosis occurs, resulting in highly proliferating malignant cells. There is evidence to support that BCR-ABL product in CML exert its pathogenicity by promoting cell to survive rather than to proliferate [9]. The inhibition of the mitochondrial release of cytochrome c in reaction to a variety of apoptotic stimuli (including chemotherapy treatments), is mediated by up-regulation of Bcl-2, in cells transformed by BCR-ABL, it was demonstrated that Ras regulates Bcl-2 gene expression, and Bcl-2 has been identified as a downstream target gene of the Ras signaling pathway. It has been shown that ectopic BCR-ABL expression causes Bcl-xL to be upregulated, which contributes to the antiapoptotic impact of BCR-ABL [10]. Subpopulations of both healthy and cancerous cells have been shown to express Fas, a death receptor that is related to the receptor for tumor necrosis factor [11]. Members of the Bcl-2 protein family control the integrity of the mitochondrial membrane, participate in the process of cytochrome c release, and can either act pro- or anti-apoptotic [12]. The overexpression of Bcl-2 proteins, which prevent apoptosis, aids in the development of cancer. Over 3000 cancer specimens from 26 different types of human cancer, including ALL, have somatic copy number variants that are over-represented by the pro-survival genes amplification and pro-apoptotic genes deletion [13]. The crucial stage in the commitment to apoptosis is the activation of Bax and Bak because it is necessary to cause mitochondrial outer membrane permeabilization, which sets off a series of predetermined events, such as the release of cytochrome c, the creation of the apoptosome by APAF-1 and the activation of caspase-9 and effector caspases, which characterize apoptotic cell death [14]. Then, two active members of the pro-apoptotic subfamily, Bak and Bax, assist in the release of cytochrome c. from the mitochondria by permeating the outer mitochondrial membrane [15]. We found that the median levels of BAX were substantially greater in CT than in CML patients (patient: 11.7 [IQR: 9.5-13.2] vs. CT: 25.5 [IQR: 21.7-27.8] ng/mL; *p* 0.001). According to **Frenzel *et al.* [16]** and **Camisasca *et al.* [17]** the equilibrium in respect to expression levels of the Bcl-2 family genes is changed by multiple cancers, and the equilibrium shifts in favor of the pro-survival subfamily member rather than the pro-apoptotic family. In this situation, cancer cells could be able to resist therapeutic drugs and avoid apoptotic signals. Members of the Bcl-2 family are also taken into account in treatment as a result of their therapeutic potential in cancer [18]. Furthermore, **Blombery *et al.* [19]** recently found that an acquired mutation in Bcl-2 has reduced the efficacy of treatment for chronic lymphocytic leukemia. This single mutation dramatically decreased the binding affinity of venetoclax to BCL2, demonstrating the significance of apoptotic genes in human disease. A pro-apoptotic substance termed cytochrome c, which is produced by mitochondria, activates the caspase protein cascade [20]. Pro-caspase-9 is activated by the interaction of cytochrome c with Apaf-1 to produce the apoptosome complex. The final cascade is started when active caspase-9 triggers caspase-3, which causes the nuclear membrane to rupture and the fragmentation of the nucleus [21]. As a result of our research, we discovered that CT median cytochrome c levels were significantly higher than in CML patients (patient: 272.3 [IQR: 237.7-395] vs. CT: 1240 [IQR: 1118-1345] pg/mL; $p \leq 0.001$). This finding can be used in understanding the pathogenesis of CML malignancy by monitoring Cyt c. Serum Cyt-c was examined by **Osaka *et al.* [22]** as potential marker in hemato-malignancies. They clarified that serum Cyt c functions as a marker not only for chemotherapeutic response monitoring but also for spontaneous tumor cell killing. **Eleftheriadis *et al.* [23]** considered cytochrome c as a possible clinically helpful indicator of cellular and mitochondrial damage. They proposed that serum cytochrome c, a straightforward to test for, would be a therapeutically helpful indicator for identifying and judging the severity of abnormalities characterized by cell death).

An anti-neoplastic cytokine, tumor necrosis factor alpha is crucial for the initiation and development of malignant illness. All phases of leukemogenesis, including cellular change, proliferation, angiogenesis, and extramedullary infiltration, are mediated by TNF-a. TNF-a contributes significantly to the tumor microenvironment and aids leukemia cells in immune evasion, survival, and treatment resistance, and it could be a useful target for leukemia treatment [24]. TNF-a receptor 1 and TNF-a receptor 2 are the two different receptors that TNF-a uses to mediate its biological effects. In general, TNF-a or TNFR1 inhibition or deficiency lowers the probability of experimental-malignancies, such as chemically generated liver and skin tumors, indicating that TNF role in the early stages of cancer involves cancer-initiating cells [25]. Due to the fact that DD is only found in TNFR1, TNFR1 frequently participates in TNF-mediated apoptosis (DR1) [26].

When TNF binds to TNFR1, it causes the protein TRADD to be recruited via its DD. Then, procaspase-8, which undergoes proteolytic cleavage to form active caspase-8, is recruited as a result of the interaction between TRADD and FADD. After that, Caspase-8 triggers Caspase-3, which causes apoptotic cell death, only under stressful circumstances, such as those that affect cell metabolism, block cell cycle progression, and impair protein synthesis, TNF causes cell death. Thus, it does not cause apoptosis in unaltered, healthy cells but only in virally infected cells, stressed cells, or altered (cancer) cells [27].

We found that the median levels of TNFR1 were significantly greater in patients when compared to CT, patient: 7 [IQR: 6.2-8.3} *vs* CT 3.9 [IQR: 3.2-4.3] ng/mL; $p < 0.001$. TNF limits normal hematopoietic stem cell (HSC) function based on the expression of

TNF- receptor 1 (TNFR1) and TNFR 2, according to **Pronk *et al.*** [28] an appealing tactic is to target cell surface antigens that have druggable characteristics that are essential for maintaining leukemia. In order to measure the concentrations of certain cytokines and soluble adhesion molecules in AML patients with recently diagnosed disease, **Kupsa *et al.*** [29] used biochip array technology. They came to the conclusion that more study is necessary and that the TNF-type I soluble receptor could be used as a prognostic indicator in AML. The ability of cells with chronic myeloid leukemia to produce TNFR 1 makes it conceivable to use TNFR1 as both a marker and a therapeutic target at the same time.

Caspase-9, which is a protease (cysteine-aspartic) that regulates degenerative tissue aging as well as healthy cell death. It is well known for its function as an intrinsic apoptosis initiator. Cancer has been linked to CASP 9 mutations, a greater risk of disease development if Caspase 9 gene expression is not increased. Less caspase-9 could be a sign that the intrinsic route has been suppressed [30]. Human malignancies are characterized by the evasion of apoptosis, and because caspases play crucial roles in controlling cellular differentiation, proliferation, and apoptosis, their involvement in carcinogenesis has received substantial research [31]. By inducing intrinsic apoptosis that happens when a cell is damaged, such as by oxidative stress, aberrant growth, or genetic instability, caspase-9 can inhibit the growth of tumors. Although some malignancies use caspase-9 inhibition as an apoptosis-evasion tactic [32]. We found that the median levels of CASP-9 were significantly higher in controls compared to the patients (7.9 [IQR: 6.5-9.4] in CML *vs* 24.3 [IQR: 22.4-31] in CT ng/mL; $p < 0.001$). It might be assumed that the death in CML cells is accomplished via the caspase 8 dependent route because we also discovered that TNFR 1 levels were greater in CML patients than in CT. Through the promotion of caspase-8-dependent apoptosis, pharmacological suppression of caspase-9 can aid in sensitizing cancer cells to chemotherapy [33].

By restoring type I interferon production in radiation-damaged cells, **Han *et al.*** [34] noted that the destruction of caspase-9 can make cancer cells susceptible to radiation therapy. They also came to the conclusion in a separate study that cancer cells can sabotage caspase-9 signaling to reduce the immunity inducted via radiation.

By applying ROC curve analysis to evaluate the predicting significance of BAX, CYTO-C, TNFR1, and CASP-9 in discriminating between CML patients and CT group, results showed that BAX, CYTO-C, TNFR1, and CASP-9 are excellent predictors of CML. Furthermore, the correlation between immunological parameters in CML patients revealed that BAX was significantly and positively correlated with CYTO-C and CASP-9, while non-significantly but positively correlated with TNFR 1. Furthermore, TNFR1 and CASP-9 had a positive non-significant correlation with CYTO-C. Finally, TNFR1 and CASP-9 showed a positive non-significant correlation. The malignant transformation of the afflicted cells, tumor spread, and medication resistance can all be caused by flaws in the apoptotic pathways. The evasion of apoptosis in the onset and progression of cancer is mediated by a number of important biological pathways. The main components of the apoptotic pathway that control cell death are the Bcl-2 family of proteins and caspases. Their flaws result in insufficient apoptotic signaling, insufficient apoptosis in cancer cells, and ultimately carcinogenesis. As a result, even though it is the root of the issue, apoptosis can be addressed in cancer treatment [35]. Development of novel drugs that render apoptosis-deficient cells susceptible to apoptosis is greatly facilitated by a profound comprehension of the signaling-processes, specific individual players, and apoptosis-related genes. Our findings, which collectively include BAX, CYTO-C, TNFR1, and CASP-9, provide potential targets for CML diagnosis, prognosis, and treatment as they turned out to be excellent predictors for CML.

CONCLUSIONS

We found that TNFR1, CYTO-C, BAX, and CASP-9 are excellent predictors for CML, and could be addressed in new targeted therapy or monitoring prognosis, and diagnosis of CML. We recommend further investigation into those key players of apoptosis in accordance with CML.

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