International Journal of Advanced Research in Medicine

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Diagnostic accuracy of alpha-fetoprotein combined with neutrophil-to-lymphocyte ratio for hepatocellular carcinoma

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Abstract
Background: One indicator indicative of the existence of a systemic inflammatory response that may be easily and cheaply established by standard blood testing is the neutrophil-to-lymphocyte ratio (NLR). The goal of this investigation was to evaluate the usefulness of NLR in conjunction with alpha fetoprotein (AFP)or to detect hepatocellular carcinoma (HCC).
Methods: This cross-sectional study involved the examination of 100 patients who were categorized into two distinct group. The first group, referred to as group A, consisted of patients who had recently been diagnosed with HCC based on positive dynamic computed tomography (CT) scans. The characteristic property of HCC during CT scans is the existence of arterial enhancement, followed by the tumor's washout in the portal-venous and/or delayed phases. The second group, referred to as group B, comprised 50 control patients who had chronic liver disease, primarily resulting from hepatitis C or hepatitis B virus (HBV) infection, as well as patients with cirrhosis and hepatitis.
Results: A notable and statistically significant reduction in the levels of lymphocytes and neutrophils was seen in participants with HCC as compared to the control group. A noteworthy negative association was seen between NLR and neutrophil count, alpha-fetoprotein (AFP), alanine aminotransferase (ALT) and lymphocyte count. On the other hand, a substantial positive correlation was found aspartate aminotransferase (AST) and the NLR. The combined use of AFP and NLR has shown the ability to identify individuals with HCC with enhanced levels of sensitivity and specificity, achieving 92% and 88% respectively.
Conclusions: Together, AFP and NLR were more effective than each marker alone in distinguishing between liver disease and HCC.

Keywords: Alpha-fetoprotein, hepatocellular carcinoma, neutrophil-to-lymphocyte ratio

Introduction

Cancer of the liver ranks as the sixth most prevalent form of cancer and stands as the third primary contributor to cancer-related mortality on the globe [1]. Hepatocellular carcinoma (HCC) represents the majority (70-85%) of liver cancer cases and is consistently identified at a late-stage disease, which has a poor prognosis. The Five-year overall survival rate for HCC is less than 15% [2].

Currently, medical interventions such as liver transplantation and surgical procedures have shown improved results in the management of early level HCC, leading to a five year overall survival rate over seventy % [3]. Therefore, it is significant to improve clinical outcomes for patients to detect HCC at an early stage. Alpha-fetoprotein (AFP) is extensively used as a blood marker in medical conditions for the purpose of early diagnosis and screening of HCC. Even with a cutoff value of 20 ng/mL, AFP's sensitivity is only around sixty%, and the test's specificity is poor [4, 5]. False-positive and high-negative rates are increased because AFP levels stay within the normal range in fifteen-thirty% of patients with late-stage illness and spike in certain individuals with liver cirrhosis, chronic hepatitis, and other hepatic diseases [2, 6]. As a result, there is a need for innovative biomarkers that may overcome the limitations associated with AFP in order to enhance the improvement of the accuracy of HCC diagnosis and the efficacy of screening. The interaction between cancer cells and the inflammatory microenvironment is of the greatest significance in the onset and advancement of cancer, including key processes such as the facilitation of metastasis, angiogenesis, and proliferation

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[7, 8]. The inflammatory infiltrates presence inside the tumor microenvironment significantly impacts the behavior and biological characteristics of HCC [9, 10].

The neutrophil-to-lymphocyte ratio (NLR) is a statistic that serves as a biomarker of the systemic inflammatory response. It may be easily assessed by normal blood investigations at a relatively cheap cost. The baseline neutrophil-to-lymphocyte ratio (NLR) has been identified as a important diagnostic indicator in many sorts of cancer, such as colorectal cancer [11], renal cancer [12], diffuse large B-cell lymphoma [13, 14], and HCC [15]. The NLR has been identified as a potential diagnostic biomarker for peptic ulcer perforation, as described in earlier investigations [16]. So, the objective of this research was to investigate the potential enhancement of diagnostic accuracy for HCC by combining NLR with AFP. The objective of this research was to evaluate the AFP diagnostic precision in conjunction with NLR for the identification of HCC

Methods and Patients
This cross-sectional research involved the examination of 100 patients who were categorized into two distinct groups. The first group, referred to as group A, consisted of patients who had recently been diagnosed with HCC based on positive CT scans. The characteristic feature of HCC during CT scans is the existence of arterial enhancement, followed by the tumor's washout in the portal-venous and/or delayed phases. The second group, referred to as group B, comprised 50 control patients who had chronic liver disease, primarily resulting from hepatitis C or HBV infection, as well as patients with hepatitis and cirrhosis.

The research occurred from May 2021 to August 2022 after the authorization of the Ethical Committee of the Internal Medicine department, Gastrointestinal and Endoscopy Unit, Tanta University Hospitals. The patients gained informed written consent.

Exclusion criteria were Subjects who did not match the inclusion criteria.

Routine laboratory investigations (complete blood counts with an ERMA INK. (Model PCE-210N) fully automated blood cell counter and liver function tests with an INDIKO PLUS (operating principle: fully automated, sample oriented, random access) were performed on all patients.

Sampling: Under strict aseptic conditions, seven milliliters of blood were drawn from the peripheral vein and then split: The C.B.C. was performed on two ml of blood collected in an EDTA vacutainer tube. A total of five ml of blood was drawn into a plain tube, allowed to clot at room temperature, centrifuged for twenty minutes at four thousand rpm, and the serum was then split into two Eppendorf tubes, one for the liver function test and the other for the alanine AFP investigation.

ALT method: The experiment included the addition of 50 μL of the patient's serum to 500 μL of a working solution, followed by quick mixing. The initial absorbance was measured after a duration of 60 seconds. The timer started concurrently. Perform a reread after 60, 120, and 180 seconds. The average rate of change in absorbance as measured in DA/min was calculated.
Calculations: U/l = 1746 x DA 340 nm /min. Expected values: Femalesup to 31 U/l(up to 0.52 mKat/L), malesup to 41 U/l(up to 0.68 mKat/L)

AST method: The experiment included the addition of 50 μL of the patient's serum to 500 μL of a working solution. The mixture was then quickly mixed, and the initial absorbance was measured after a duration of 60 seconds. The timer started concurrently. Read again after sixty, hundred and twenty and hundred and eighty seconds. the mean absorbance change per minute (DA/min) was determined. Calculation:U/l = 1746 x DA 340 nm /min Expected values:37 oCFemalesup to 31 U/l (up to 0.52 mKat/L), males: up to 37 U/l(up to 0.62 mKat/L)

AFP method: Chemiluminescent microparticle immunoassay was used for the measurement. All reagents were used after being warmed to room temperature (between 18 and 25 °C). One milliliter of distilled water was added to each lyophilized standard. The reconstituted material was let to remain undisturbed for a minimum duration of twenty minutes and was subjected to gentle mixing. The reconstituted standards demonstrated stability for a duration of thirty days when kept in a sealed condition at a temperature range of two-eight °C. The holder was used to secure the required number of coated wells. A volume of twenty μl was dispensed into the appropriate wells for the standard, controls, and specimens. Each well was filled with hundred μl of Zero Buffer and stirred for a duration of thirty seconds. The plate content was incubated at ambient temperature (eighteen-twenty five °C) for a duration of thirty minutes, after which it was afterwards discarded by flicking it into a waste container

The microtiter wells were cleaned and agitated five times using either distilled or deionized water. Subsequently, they were firmly tapped onto absorbent paper or paper towels to eliminate any remaining droplets of water.

A volume of hundred fifty μl of Enzyme Conjugate Reagent was poured into each well. Gently mix for ten seconds. Incubated at room temperature for thirty minutes. The microtiter wells were washed and flicked five times using deionized or distilled water. The wells were carefully struck into absorbent paper to eliminate any remaining water droplets. Hundred μl TMB Reagent were dispensed into each well. Gently mix for ten seconds. Incubated at room temperature for twenty minutes. The reaction ended by the addition of hundred μl of Stop Solution to each well. Gently mix for thirty seconds. till all the blue color converts to yellow color completely. The optical density was measured at a wavelength of four hundred fifty nm using a microtiter reader during a time frame of fifteen minutes.

Statistical analysis
Statistical analysis was done by SPSS v21 (IBM Inc., Chicago, IL, USA). The Kolmogorov-Smirnov test was applied to confirm the normality of distribution. Continuous information were reported as median, IQR and average standard deviation whilst categorical information were expressed as numbers and percentage. The Chi-square test is used to compare distinct groupings of category data. The Fisher's Exact test is used as an replacement to the chi-square test when the predicted count in more than 20% of the cells is less than 5The Student's t-test is a statistical test often used to compare two groups in a sresearch, specifically for normally distributed quantitative data. The Mann–Whitney U test is used to compare two groups using quantitative data that are not regularly distributed.

Significant findings were defined as two-tailed P-values below 0.05.

Results

Table1 illustrated no statistically important variance in terms of age and gender between standard and patient groups.

Table 1: The demographic characteristics of the groups under study.

Variable		Group A (N=50)		Group B(N=50)		Test value	P-value	Sig.
		No.	%	No.	%			
Gender	Male	36	72.0%	32	64.0%	$X^2= 0.735$	0.391	NS
	Female	14	28.0%	18	36.0%			
Age (years)	Average± SD	55.58± 3.60		53.02± 5.92		$Z_{MWU}= 1.782$	0.075	NS

Data are presented as average ± SD or frequency (%).

There was statistically important difference between standard group and patient regarding hepatic disease. Table 2

Table 2: A comparative analysis of liver disease between patients and control groups.

Variable		Group A (N=50)		Group B (N=50)		Test value	P-value	Sig.
		No.	%	No.	%			
Liver disease	HBV	0	0.0%	5	10.0%	$X^2= 8.294$	**0.016**	S
	HCV	8	16.0%	14	28.0%			
	Cirrhosis	42	84.0%	31	62.0%			

Data are presented as frequency (%). HBV, HCC

There was a highly statistically significant decline in neutrophils and lymphocytes levels in HCC patients compared to controls. On the other hand, there was a highly statistically significant increase in NLR in HCC patients compared to controls. There was a highly statistically significant elevate in ALT, AST and AFP levels in HCC patients compared to controls. Table 3

Table 3: Comparison of between control group and HCC patients as regards Neutrophils, Lymphocytes, NLR, ALT, AST and AFP

Variables		Group A HCC Patients (N=50)	Group B Controls (N=50)	Test value	P-value	Sig.
Neutrophils ($\times 10^9$/L)	Mean± SD	0.65± 0.04	5.48± 0.71	$Z_{MWU}= 8.645$	<0.001	HS
	Median (IQR)	0.67 (0.63- 0.68)	5.40 (4.70- 5.80)			
	Range	0.58- 0.70	4.50- 6.50			
Lymphocytes ($\times 10^9$/L)	Mean± SD	0.24± 0.03	4.24± 1.22	$Z_{MWU}= 8.640$	<0.001	HS
	Median (IQR)	0.25 (0.21- 0.26)	3.70 (3.40- 5.40)			
	Range	0.19- 0.29	2.70- 6.50			
Neutrophil-lymphocyte ratio	Mean± SD	2.49± 0.68	1.52± 0.50	$Z_{MWU}= 6.468$	<0.001	HS
	Median (IQR)	2.52 (1.94- 3.10)	1.46 (1.04- 1.67)			
	Range	1.16 - 3.53	0.96 - 2.51			
ALT (IU/mL)	Mean± SD	37.84± 2.47	34.7± 2.41	$Z_{MWU}= 5.559$	<0.001	HS
	Median (IQR)	37.5 (36.0- 39.0)	34.0 (33.0- 34.0)			
	Range	34.0- 43.0	32.0- 40.0			
AST (IU/mL)	Mean± SD	39.34± 4.12	33.12± 4.66	$Z_{MWU}= 5.833$	<0.001	HS
	Median (IQR)	40.0 (35.0- 43.0)	30.0 (29.0- 32.0)			
	Range	34.0- 45.0	28.0- 42.0			
AFP (ng/mL)	Mean± SD	432.33± 157.9	10.30± 9.80	$Z_{MWU}= 6.829$	<0.001	HS
	Median (IQR)	213.4 (45.0- 440.25)	7.80 (7.60- 9.8)			
	Range	5.5- 657.0	5.80- 20.0			

Data are illustrated as mean ± SD or Median (IQR). ALT, AFP, AST

There was important negative relation between lymphocyte and neutrophil- lymphocyte ratio (r=-0.705, p<0.001), Neutrophils (r=--0.495, p<0.001), ALT (r=-0.607, p 0.008) and AFP (r=-0.776, p<0.001) while there was important positive relation between neutrophil- lymphocyte ratio and AST(r=0.478, p<0.001). Picture 1.
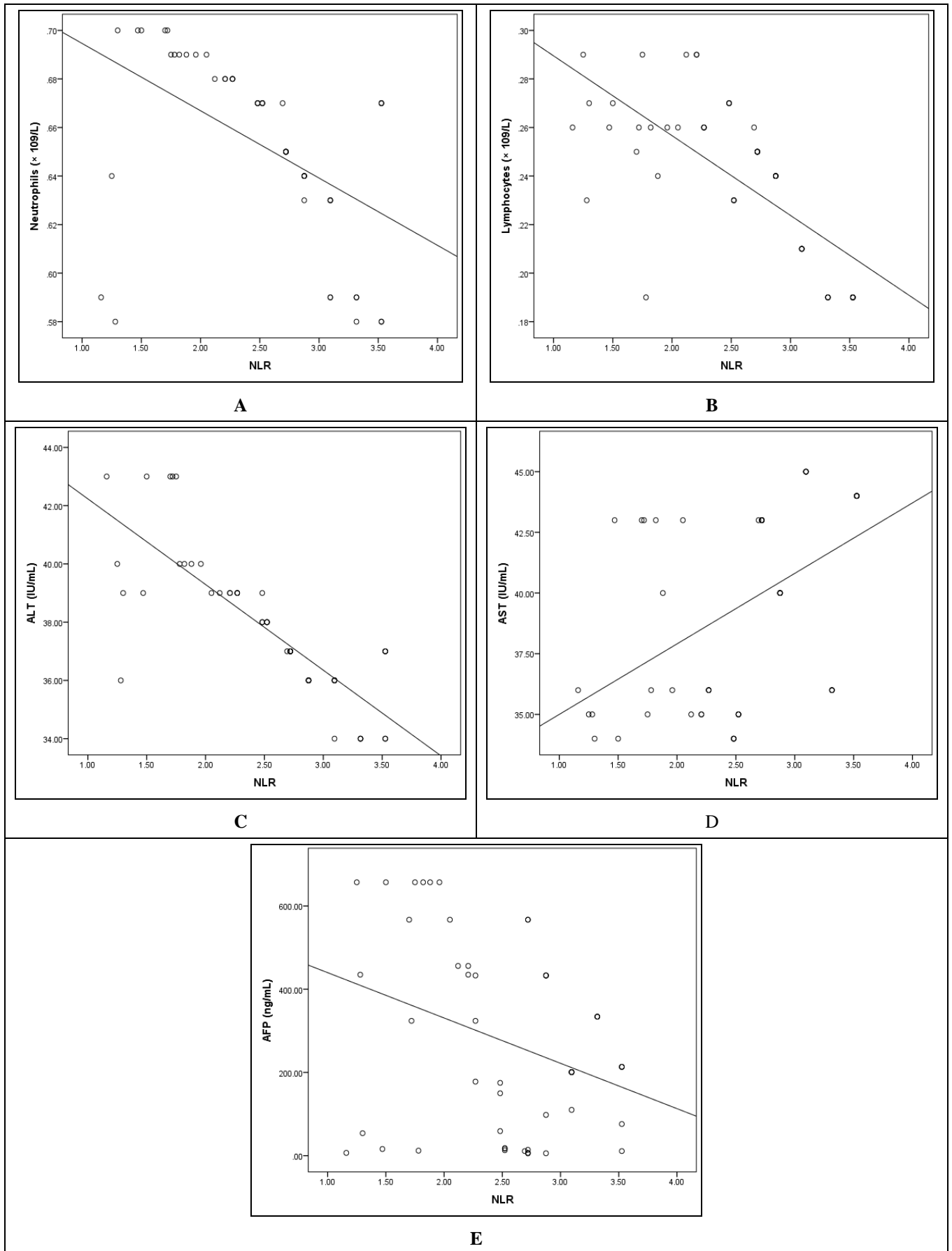
Fig 1: Scatter plot illustrates important negative relation between NLR and (A) Neutrophils, (B) Lymphocyte, (C) ALT, (E) AFP in HCC patients. (D) Important positive correlation between AST and NLR in HCC patints.

There was significant negative link between NLR and AFP in HCC patients. Table 4

**Table 4:** Relationship between variable assessed markers and AFP

	AFP	
	HCC Patients (N=50)	
	R	P- value
Age (years)	-0.117	0.419
Neutrophils (× 109/L)	0.204	0.155
Lymphocytes (× 109/L)	0.254	0.101
ALT (IU/mL)	0.239	0.132
AST (IU/mL)	-0.135	0.349
NLR	-0.324	0.022*

P value< 0.05 is significant r: coefficient of spearman correlation

The use of ROC-curve analysis reveals that AFP in isolation exhibits a sensitivity of eighty six% and a specificity of eighty% in the identification of individuals with HCC, with a cutoff value of thirteen ng/mL. The NLR exhibited a sensitivity of eighty two% and specificity of seventy eight% in identifying individuals with HCC when the cutoff point was set at 1.8.Moreover, the combined use of AFP and NLR has shown enhanced capabilities in identifying individuals with HCC, resulting in an increased sensitivity of ninety two% and specificity of eighty eight%. There was a seventy two% sensitivity and sixty four% specificity for detecting patients with HCC when the AST threshold was thirty five IU/mL, and there was a seventy six% sensitivity and sixty six% specificity for detecting patients with HCC when the ALT cutoff was thirty five IU/mL. However, the sensitivity and specificity for detecting patients with HCC increases to eighty eight% and eighty four%, respectively, when AFP and AST are combined, and to ninety% and eighty six%, when AFP and ALT are combined. Figure 2

A

B

C

D
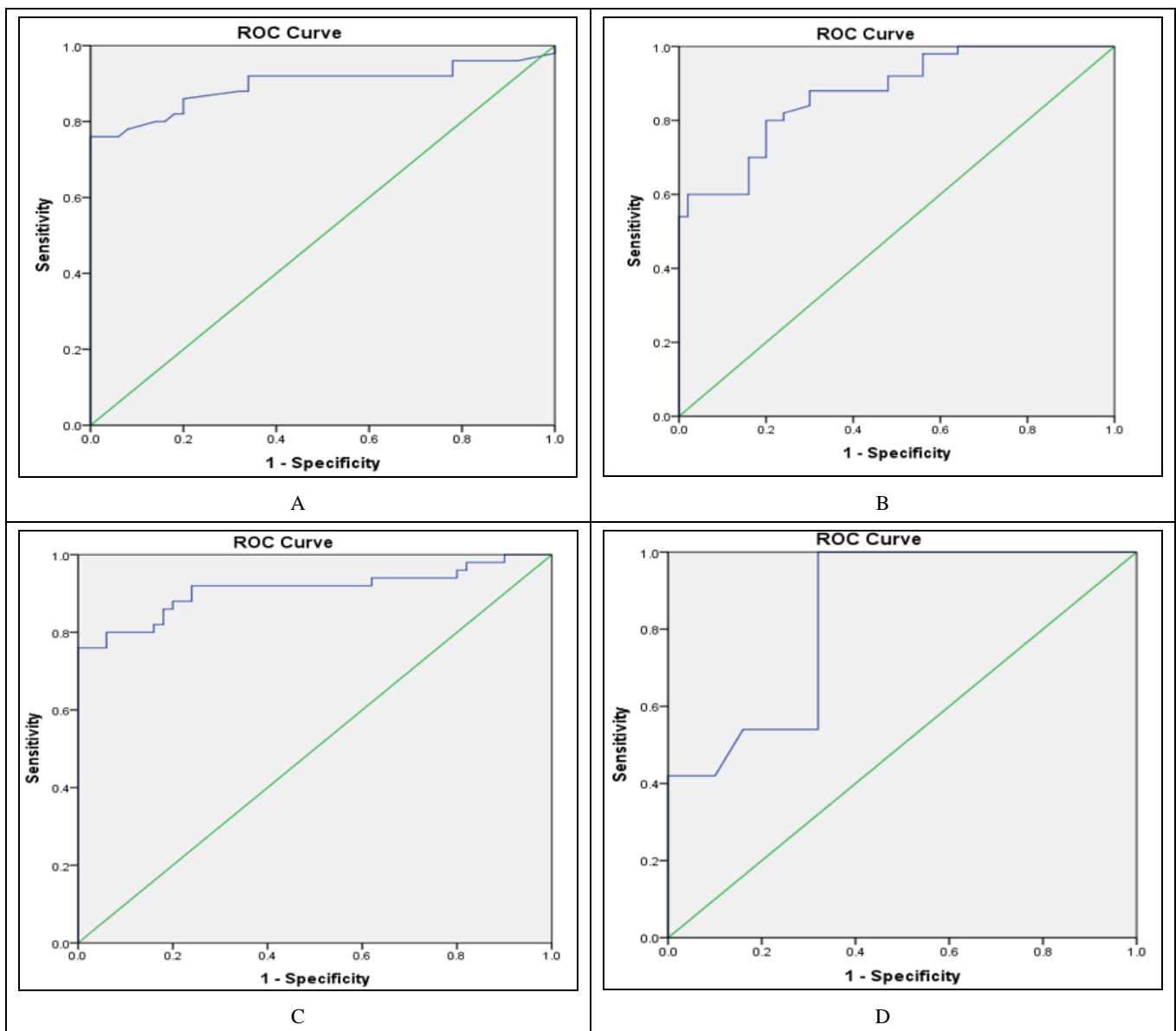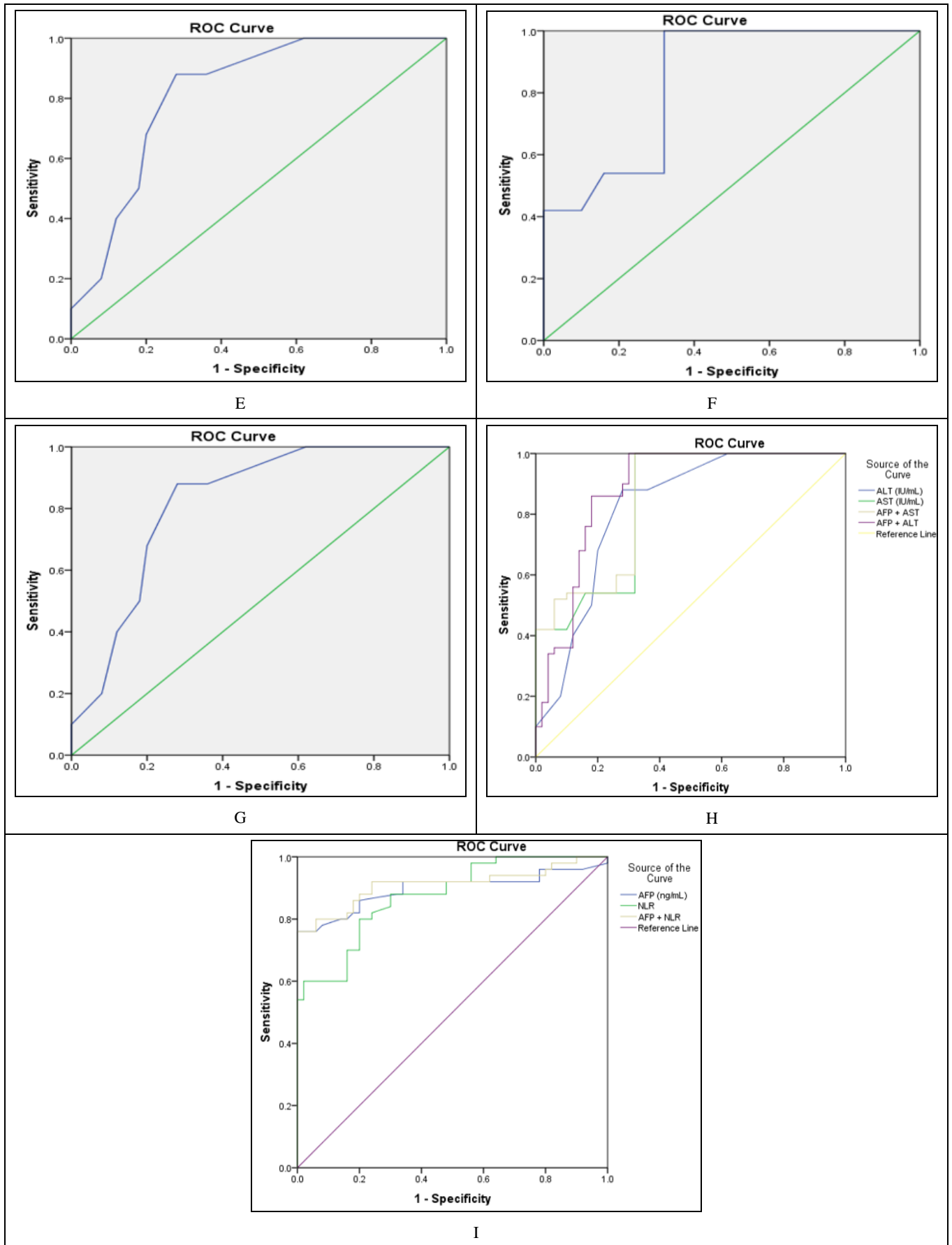
Fig 2: ROC curve for the performance of(A) AFP, (B)NLR,(C) AFP + NLR, (D) AST, (E) ALT, (F) AFP + AST, (G) AFP + ALT, (H,I) different markers in detection of HCC.

Discussion

HCC is a significant contributor to global cancer-related mortality. During the first phase, therapeutic interventions such as, liver transplantation, surgical resection and local ablation have shown the potential to enhance the overall survival of patients [17].

In the present research there was no statistically important variance between control participants and HCC regarding age. The findings of this investigation were consistent with the results reported by J. Hu et al. [18], who observed a mean age of 56.91 ± 10.04 years in the HCC group. However, J. Hu et al. [18] also found a significant difference in age between the control liver disease group and the HCC group. The average age observed in the current research was found to be greater in comparison to the age reported in the study conducted by Shaw et al. (19). But the aforementioned investigation concurred with the present study by indicating that there was no statistically significant gap in mean age between the control group and the group of patients with HCC. In contrast, the mean age observed in the present investigation was found to be lower compared to the findings published in the study conducted by Lai et al. (20).

Within the HCC group, it was shown that 84% of the patients presented with cirrhosis, whereas the remaining 16% of cases were associated with HCV. During the observation period within the control group, it was found that 62% of the patients had cirrhosis, whereas 28% of the cases were diagnosed with HCV and 10% of the cases were identified as having HBV.

In the investigation conducted by Al-Amin Shawon et al., [19], 35 HCC patients (44%) screened negative for both HBV and hepatitis C. In the research conducted by Ho Chu et al. [21], it was found that out of the HCC patients included in the study, 699 individuals (75.1%) were diagnosed with HBV, 101 individuals (10.8%) were diagnosed with HCV, and 131 individuals (14.1%) had HCC caused by other etiological factors. In the research conducted by Lué et al. [22], it was shown that a majority of HCC patients (88.4%) had cirrhosis, with the most prevalent causes of liver disease being alcohol use (39.4%) and infection with the hepatitis C virus (38.6%).

According to research conducted by Lee et al. [23], it was shown that among patients with HCC, the incidence of liver cirrhosis was seen in 96 individuals, accounting for 39.8% of the patient population. Furthermore, a total of 76 individuals, accounting for 31.5% of the whole sample, were diagnosed with chronic viral hepatitis.

The current research demonstrated that there was a highly statistically significant decline in lymphocytes and neutrophils levels in HCC patients compared to controls. In agreement with these findings, Hu et al. (2018) showed in their research that the levels of both variables were significantly lower in HCC patients when compared to the control group with liver disease.

In research conducted by Z. Hu et al. [24], it was shown that there was a notable reduction in lymphocyte levels in group 1 of patients with HCC, with a mean value of 1.73 (range: 1.35-2.18), in comparison to group consisting of patients with cirrhosis, who had a mean value of 2.29 (range: 1.93-2.74). Nevertheless, no statistically significant difference was observed between the two groups regarding of neutrophil levels.

The findings of this research indicate a substantial and statistically significant elevation in the neutrophil-lymphocyte ratio among patients diagnosed with HCC as compared to the control group. According to the findings of Hu et al. (18), the investigation revealed that the NLR values for the control group were 1.851 (1.43–2.53), but for the HCC patients, the NLR values were 3.23 (1.91–6.62). These results correlate with the research conducted by Won

Jeong (25). In the current research, there was a highly statistically significant increase in AST, ALT and AFP levels in HCC patients' comparison to controls. These findings are similar to those obtained by J. Hu et al., [18] Research where HCC patients had higher AFP and AST levels than hepatic disease patients while ALT levels did not differ significantly in contrary to this result. Likewise, Z. Hu et al., [24] The research revealed a substantial and statistically significant elevation in AFP levels within HCC group as compared to the cirrhosis group. According to the current research, When the cutoff threshold was set at 13 ng/mL, the sensitivity and specificity of AFP alone in detecting patients with HCC were found to be 86% and 80% respectively. The NLR has a sensitivity of 82% and specificity of 78% in identifying individuals with HCC when the cutoff point is set at >1.8. Furthermore, the combined use of AFP and NLR has shown enhanced diagnostic capabilities in identifying individuals with HCC, resulting in an increased sensitivity of 92% and specificity of 88%.

The current NLR and AFP cutoff points were lower than those reported in J. Hu et al., [18] research where the ROC curves demonstrated that the AUC values for AFP, and NLR were 0.775, and 0.738 with optimal cut-off values of 24.6 ng/mL, and 2.979, respectively. When applying the common cutoff value of 20 ng/mL for AFP, the AUC was 0.664. The combined use of AFP and NLR had the greatest AUC value of 0.769, exhibiting a considerably greater sensitivity of 0.767 and a lower specificity of 0.773 when compared to the individual use of AFP or NLR.

In the research conducted by Hu et al. (24), it was shown that AFP exhibited a high level of sensitivity and specificity, with values of 78.69% and 74.41% respectively, in distinguishing the HCC group from the control cirrhosis group. On the other hand, NLR demonstrated a sensitivity of 73.91% and a specificity of 69.59%. The use of both NLR and AFP in conjunction resulted in a higher AUC value of 0.856 compared to either NLR or AFP alone. In their work, Xing et al. (26) proposed that the combined use of AFP and NLR might be used as a diagnostic approach for HCC associated with HCV and HBV infections.

In the current research, AST individually can detect patients with HCC with sensitivity and specificity of 72% and 64% respectively when the cutoff point was 35 IU/mL while ALT individually can detect patients with HCC with sensitivity and specificity of 76% and 66% respectively when the cutoff threshold was 35 IU/ML.

In J. Hu et al., [18] research, the ROC curves for serum biomarkers (AST and ALT) for diagnosing HCC revealed that AUC values for ALT, and AST were 0.504, and 0.660, respectively with optimal cut-off values of 111 IU/mL, and 27 IU/mL respectively. ALT demonstrated the highest specificity (0.809) with the lowest sensitivity (0.184).

In the present investigation, combination between AFP and AST can detect patients with HCC with increasing the sensitivity and specificity into 88% and 84% respectively while combination AFP and ALT can detect patients with HCC with increasing the sensitivity and specificity into 90% and 86% respectively. In J. Hu et al., [18] study, AFP + AST combination detected HCC patients with increasing the sensitivity 0.884, while there was no change in the specificity 0.466, compared with AFP or AST alone. When compared to AFP or ALT alone, the AFP + ALT

combination demonstrated greater sensitivity 0.639 but smaller specificity 0.841.

It is recommended that more multicenter studies with a larger sample size be conducted in order to validate the present findings. Furthermore, these studies should aim to evaluate the association between NLR and both aggressiveness and tumor size. NLR is a biomarker that is trustworthy, cost-effective, and non-invasive. It may be quantified during regular examinations. Therefore, we propose that combining aAFP with NLR would enhance the accuracy of HCC diagnosis..

Conclusions
Together, AFP and NLR were more effective than each marker alone in distinguishing between liver disease and HCC

Financial Support and Sponsorship
Nil

Conflict of Interest
Nil

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