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Albumin Binding Function And Endocan For Early Detection And Progression Of Early Stage Nonalcoholic Fatty Liver Disease (Nafld)

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Abstract:

Background & Aims: Non-alcoholic fatty liver is one of the non-communicable diseases that has spread widely around the world during the recent period.Our aims wereDetermination of the levels of albumin binding function and endocan for early detection and progression of nonalcoholic fatty liver disease.

Methods: Study included 88 patients (29 NAFLD), (29 NASH) and (30 healthy as controls).The ELISA assay was used to determine the endocan serum level, ion metal binding capacity by spectrophotometer, and albumin binding capacity by spectro fluorescence.

Results: The serum level of endocan in control ,NAFLD and NASH were (0.41±0.06 ng/ml) (0.63±0.23 ng/ml) and (1.42±0.54 ng/ml) respectively, with a p value< 0.001 showing a significant difference and high level of endocan concentration in NAFLD and NASH compared with control. The serum level of IMAT in control, NAFLD and NASH were(0.63±0.11IU/L),(0.48±0.05 IU/L) and(0.37±0.07 IU/L) respectively, with a p value <0.001.The plasma levels of ABiC were (192.29±7.79), (170.54±12.29) and (149.81±13.83) respectively, and p value <0.001. The IMAT/Albumin ratios were (1.340.23), (0.920.19), and (0.810.11), with a p value of 0.001. The resultsshows there are significant differences between groups and shows low levels of IMAT ,ABiC andIMAT/Albumin in NAFLD, NASH in comparison with control groups .

Conclusions: Patients with NAFLD and NASH had significantly higher levels of serum endocan than the controls. The level of albumin binding function (IMAT, ABiC, and IMAT/Albumin ratio) decreased significantly in NAFLD and NASH compared with controlbecause of impaired albumin in these pathologies.

Keywords: –Non Alcoholic Fatty Liver Disease- Endocan- Non-alcoholic steatohepatitis -Albumin binding functions.

Introduction:

The common denominator is excess fat in the liver in the two most common and rising forms of chronic liver disease, alcoholicfatty Liver Disease and non-alcoholic fatty liver diseases ,both of which are worldwide public health concerns. Both ALD and NAFLD are becoming more common across the world**(Asrani SK et al.,2019)**.

AFLD is a Hepatic steatosis is caused by chronic alcohol consumption**(Idilman IS et al.,2016)**.Non-alcoholic fatty liver (NAFL) can be classified into NAFLD and non-alcoholic steatohepatitis (NASH).NAFLD is defined by detection of hepatic steatosis, or the presence of macrovesicular fat in >5% of hepatocytes, either by imaging or histology, after exclusion of secondary causes and alcoholic fatty liver disease**(Han MAT et al.,2021).** The most prevalent chronic liver condition and refers to a group of liver disorders that range from basic steatosis to steatohepatitis to severe fibrosis, cirrhosis, and eventually hepatocellular Carcinoma **(Yuet al.,2019)**. NASH is defined as hepatic steatosis with hepatic fibrosis or not, as well as lobular inflammation and ballooned hepatocytes with or without hepatic fibrosis. Specifically, NASH ballooning affects 10% to 25% of people with NAFLD and up to 25% of patients with NASH develop cirrhosis, liver failure as well as, in rare circumstances, hepatocellular cancer**(Francque S et al 2021)**. NASH patients in the early stages of the illness are frequently in good health. Patients begin to suffer symptoms such as tiredness, weight loss, and weakness as the illness progresses or cirrhosis develops**(Goldberg D et al.,2017)**.

Many factors, like obesity, CVD, T2DM, and metabolic disorder, are both risk factors for the development of NAFLD and all-cause mortality. A poor diet and a high intake of saturated fat and processed meat demonstrate the intricate relationship between diet and metabolic liver diseases**(Tana C et al.,2019).**Obesity is a major risk factor for the development of NAFLD.. The World Health Organization (WHO) defines obesity as a BMI more than or equal to 30, while overweight is defined as a BMI larger than or equal to 25(**Younossi ., 2018**). T2DM andinsulin resistance are the major risk factor for the development of NAFLD and accelerates progression to advanced liver disease and increase risk for mortality**(Mantovani A et al.,2021).**The complex interactions between NAFLD, visceral adiposity and insulin resistance make it difficult to distinguish the precise mechanisms underlying the increased risk of diabetes in patients with NAFLD**(Xia M et al.,2019).**NAFLD is closely linked to CVD, which in this demographic is connected with a high risk of morbidity and death**(Henson JB et al.,2020).** NAFLD is linked to a number of subclinical atherosclerosis indicators, including coronary artery calcification, decreased flow mediated vasodilation, arterial stiffness, carotid artery inflammation and thickening of the carotid intima-media, left ventricular hypertrophy, and diastolic dysfunction.In addition to these factors, other factors considered risk factors for NAFLD include (Hypertension, Dyslipidemia and Genetic factors)**(Niederseer D et al.,2020)**.

For clinical purposes, albumin is an important biomarker for liver health because it is the most frequent circulating protein. There are numerous biological activities for albumin, including: antioxidant, oncotic pressure maintenance, anti-inflammatory, molecule transport, and antithrombotic.**(Sun L et al.,**

2019).When liver disease is severe, the liver's ability to synthesize albumin is compromised, resulting in a low level of serum albumin**(Sun et al.,2020).**Endocan:Endothelial cells are a crucial part of the normal vascular wall, which serves as a barrier between the bloodstream and the surrounding tissue. Through the release of vasoactive substances such as nitric oxide, prostacyclin, and endothelin, vascular tone, leukocyte adhesion, and platelet activation are regulated. The endothelium is essential for the proper functioning of the vascular system**(Dallio M et al .,2017)**.

Methods

Case-control research was used in this study that included 88 patients and healthy controls of adults, both males and females.The range of age(30–65) years .The groups divided into as follows: (29 NAFLD), (29 NASH), and (30 healthy as control). The study was executed during the term from the first of December 2020 to the last of May 2021. All samples were collected from the gastrointestinal and hepatology teaching hospital in Baghdad medical city. The information was obtained from the questionnaire paper after receiving the informed permission of all the participants. The study methodology was authorized by the AL-Nahrain University's Ethical Committee. The diagnosis of nonalcoholic fatty liver disease depends on the guidelines for diagnosis and treatment of NAFLD proposed by the fatty liver and alcoholic liver disease study group of the Chinese Liver Disease Association. Fatty liver disease can occur without symptoms. It is generally detected when you do routine liver blood testing. Health care providers may suspect an abnormal test result of fatty liver disease, particularly if the individual is obese. Fat deposits may be shown in imaging examinations of the liver. Some imaging procedures, such as specific ultrasound, MRI, and fibro scans, can assist in identifying liver illness. The physician's diagnosis of NAFLD confirmed the patients' NAFLD diagnoses. Those with liver problems caused by viruses, autoimmune diseases, or drugs were ruled out, as were those with malignant tumors, inflammatory diseases, pregnancy, or heavy alcohol usage**(Sun et al.,2020).**

Serum sample collection

Peripheral venous blood was drawn and diluted to around seven milliliters. Prior to the collection, participants were required to fast for at least 12 hours. Vacuum tubes without additives were used to collect blood samples, which were subsequently centrifuged at 3000 rpm/min for 10 minutes. Prior to analysis, serum was kept at -20°C. About three milliliters of blood are prepared in the EDTA-contained tubes immediately and separated the plasma in the tube for measuring albumin binding function (ABiC) measured by ELISA, and about four milliliters of blood are taken and kept at room temperature in the gel tube for 20 minutes for:

1.Immediate measurements of (ALT, AST, TSB, dTSB, ALB, ALP, LDH, TC, TG, HDL-C, LDL-C, CRP, and GGT) will be done using an appropriate enzymatic colorimetric method.

2.The rest was divided into two parts, the first assayed for serum human endocan and the second for (IMAT). The assay will be measured using enzyme-linked immunosorbent assay (ELISA) kits**.**

Albumin cobalt binding capacity: Cobalt could be used to assess the metal ion-binding capabilities of the N-terminal metal ion binding site, and this is a method for figuring out the amount of serum IMA that is present. **(Lee DH et al.,2017).**

1-Cobalt chloride (1 mg/mL) (Sigma-Aldrich, USA) was added to a 100-uL serum solution in a 96-well plate and incubated for 10 minutes at room temperature (25 °C).
2-After adding the Dithiothreitol (DTT) and incubating the mixture for two minutes with the free cobalt salt (Sigma-Aldrich, USA), we were able to see the reaction begin.
3- To stop the process, added 150 uL saline.
4-Absorbance at 470 nm, the Synergy H1 spectrophotometer (Germany) recorded .
This value was derived by subtracting the absorbance values from both the experimental well and the control well, which had no DTT in them. Less cobalt salt was bound to albumin when absorbance values were high because more free cobalt salt reacted with DTT. The IMA transformed (IMAT) is equal to 1IMA, thus we converted it to be clear. Consequently, greater IMAT values correspond to increased metal ion binding capacities in albumin. Using albumin concentration as a standard, we converted IMAT to an expression called IMAT/albumin**(Sun et al.,2020).**

Site II-specific albumin binding capacity:ABiC is a method for assessing site II specific albumin binding. In a summary, this technique determines the amount of albumin-binding fluorescent marker in the plasma sample that is unbound.

1-Albumin concentrations of 150 mol/L were used in the dilution of plasma samples.

2-The identical quantity of binding site II-specific fluorescent marker was added to the cells twice(DS; Sigma Chemical).

3-Samples separated (ultrafiltration) (Centrisart I, Sartorius Göttingen; cutoff = 20,000 Da)
4- The fluorescence of ultrafiltrate was measured (excitation = 355 nm, emission=460 nm; Fluoroscan, Labsystems, Italy) after the addition of human serum albumin solution as a fluorescence amplifier.
5-A standard reference albumin sample underwent the same treatment. ABiC had as a reference a standardized virus-inactivated human serum preparation derived from pooled human plasma (Biseko, Biotest® Pharma GmbH, Dreieich, Germany). The following equation was used to calculate ABiC:
fluorescence in the ultrafiltrate (reference)

ABiC= $\frac{\text{fluorescence in the ultrafiltrate (sample)}}{\text{fluorescence in the ultrafiltrate (sample)}}$ ×100%.**(Sun et al.,2020).**

Endocan serum level assessment

The manufacturer's instructions (Human Endocan / ESM-1 DIY ELISA Kit, Endomark H1, Lunginnov sas, Lille, France) were followed while testing the sera by enzyme-linked immunosorbent assay (ELISA). To summarize, anti-human Endocan monoclonal antibody was used to bind to Endocan contained in standards, positive controls, and samples before they were introduced to microassay wells. After the incubation process is complete, wash any unbound items that were thrown away. A biotin-conjugated anti-Endocan monoclonal antibody was injected into the wells. This antibody binds to Endocan upon capture in the first step. The lectin-horseradish peroxidase (HRP) conjugate was applied to all wells after incubation and washing. The biotin-conjugate monoclonal antibody is immobilized by HRP, which binds to it. Using a chromogenic reaction of the tetramethylbenzidine substrate solution (TMB) in the presence of HRP, the bound Endocan was measured after incubation and washing. A microplate reader set to 450 nm and a wavelength correction of 630 nm were used to figure out the optical density. Endocan content in diluted samples, standards, and positive controls is proportional to color intensity. Results were derived from a standard curve that was created through the use of linear regression. Endocan levels were measured in triplicate in each serum sample, with the mean value being used as the end result**(Dallio et al.,2017)**

Statistical Studies:

1.As a result, numerical values were presented as mean standard deviation (SD). ANOVA with Student's F test (Student's t test) was used to calculate the individual p-value of the different groups between the control and NAFLD and NASH, and the level of plasma albumin binding function and serum endocan were correlated between them and with the exogenous factor (age, gender, BMI), obesity, height) and with clinical biomarkers (ALT, AST, TSB, dSB, ALP and HDL-C and Glyce A p value of 0.05 or lower was regarded as statistically significant.2. Receiver operator characteristic curve (ROC) used to calculate the cutoff value of plasma albumin binding function and serum endocan.

Results:

The mean and standard deviation for the ages of patients with controls, NAFLD, and NASH showed no significant differences between the three groups. Furthermore, women were significantly more common in the NAFLD group than in the NASH or control groups. The mean BMI in controls was significantly lower than in patients with NAFLD and NASH. In contrast, the mean waist circumference was higher in NAFLD patients than

in controls or the NASH group with significant differences. None of the controls had T2DM, while 41.38% and 34.48% of patients with ANFLD and NASH, respectively, had such a comorbidity, a highly significant difference shown in table 1. LFTs were far higher in patients with NASH than in those with NAFLD, who in turn had far higher LFTs than controls. The only exception was that the AST/ALT ratio was higher in controls (median= 1.21, range= 0.94-1.64) than in NAFLD and NASH patients (median= 0.84 [range= 0.43-1.25] and median= 0.72 [range= 0.51-0.85]).Furthermore, the median albumin concentration in the NAFLD group was 51.14 g/L (range=39.19-60.12 g/L), which was higher than that of the NASH group (median=44.48 g/L, range=35.27-57.48 g/L) with a significant difference. Table (1) shows the lipid profile in different groups. Median serum level of TC, TG and LDL in patients with NASH compared with patients with NAFLD with control with highly significant differences from both NAFLD and NASH groups. In contrast, controls had higher median of HDL(49.55 mg/dl) than either NAFLD group(38.5 mg/dl)or NASH group(31.4 mg/dl)with significant differences.

Table1. Clinical laboratory characteristics and Inflammatory markers(serum levels of endocan, IMAT and ABiC, and IMAT/Albumin ratio in different groups(control,NAFLD and NASH)

Variables	Controls/n=30	NAFLD /n=29	NASH/n=29	p. value
Age(y)	48.67±4.13	49.44±3.58	51.44±3.6	0.212
Range	40-50	41-55	48-61	
Gender(M/ F)	21(70%)/9(30%)	11(37.93%)/18(62.07%)	17(58.62%)/12(41.38%)	0.043
Weight,(kg)	71.8±11.78	77.14±18.46	77.34±14.79	0.288
Range	49-95	55-147	60-117	
Height(cm)	165.7±12.45[a]	157.72±10.29[b]	162.1±9.96[a]	0.024
Range	142-187	143-179	140-177	
BMI(kg/m^2)	25.92±1.77[a]	31.04±6.72[b]	29.35±3.73[b]	<0.001
Range	23.2-29.2	20.55-52.71	23.5-40.5	
Waist. Circ(cm)	83.57±4.72[a]	106.41±15.32[b]	93.55±11.11[c]	<0.001

Range	73.0-92.0	84.0-137.0	77.0-121.0	
Fatty liver index	25.88±8.11[a]	74.55±23.83[b]	71.42±16.31[b]	
Median , Range	26.03(8.66-48.22)	86.1(22.42-98.14)	75.37(40.1-96.0)	<0.001
ALT, U/L	18.92±5.77[a]	44.6±35.1[b]	126.62±16.0[c]	
Median, Range	17.85(11.5-35)	38.9(17.9-215.6)	123.7(98.4-165.7)	<0.001
AST, U/L	23.17±5.7[a]	36.8±32.12[b]	90.9±20.12[c]	<0.001
Median, Range	22.15(15.3-37.8)	31.7(13.9-199.8)	88.9(56.7-128.0)	
ALP, U/L	73.61±21.0[a]	107.43±64.39[b]	185.0±16.75[c]	<0.001
Median, Range	69.6(39.6-132.6)	78.8(41.9-328.3)	187.9(149.9-216.1)	
GGT, U/L	26.82±11.13[a]	48.88±18.68[b]	63.22±8.39[c]	<0.001
Median, Range	23.9(14.0-63.9)	46.1(19.8-112.7)	61.7(46.5-78.0)	
AST/ALT	1.24±0.15[a]	0.84±0.18[b]	0.71±0.11[c]	<0.001
Median, Range	1.21(0.94-1.64)	0.84(0.43-1.25)	0.72(0.51-0.85)	
TSB(mg/dl)	0.4±0.11[a]	0.65±0.34[b]	1.21±0.2[c]	<0.001
Median, Range	0.4(0.24-0.65)	0.52(0.28-1.9)	1.2(1.13-1.29)	
DSB(mg/ml)	0.21±0.05[a]	0.25±0.11[b]	0.65±0.1[c]	<0.001
Median, Range	0.21(0.13-0.31)	0.23(0.11-0.62)	0.61(0.4-0.86)	
TC(mg/dl)	140.78±22.95	167.0±33.54	188.4±12.96	<0.000
Median ,Range	139.9[a](95.4-184.4)	168.4[b](111.6-228.3)	190.3[c](167.8-214.7)	
TG(mg/dl)	97.54±16.4	157.48±29.4	182.05±15.76	<0.000

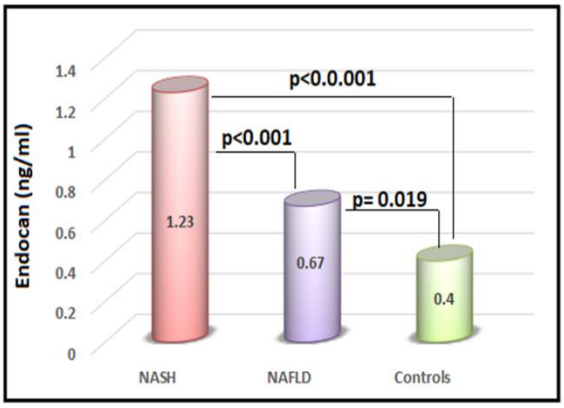
Median ,Range	95.2[a] (75.6-136.3)	149.7[b](112.7-209.0)	180.4[c](148.9-211.4)	
HDL(mg/dl)	50.07±5.82	39.05±9.53	32.61±5.75	<0.000
Median, Range	49.55[a](37.8-60.0)	38.5[b](24.32-60.4)	31.4[c](23.9-44.6)	
CRP(mg/L)	2.65±1.1	5.75±3.44	12.79±3.73	<0.001
Median ,Range	2.05(1.3-5.0)	4.8(2.9-21.0)	11.4 (8.6-22.1)	
LDH, U/L	145.66±10.26	156.79±31.0	187.14±32.9	<0.001
Median	147.0[a]	147.0[a]	191.3[b]	
Range	127.0-170.0	124.0-245.0	125.2-247.38	
Endocan,ng/ml	0.41±0.06	0.63±0.23	1.42±0.54	<0.001
Median	0.4[a]	0.67[b]	1.23[c]	
Range	0.29-0.52	0.1-1.06	0.78-2.42	
IMAT, ABU	0.63±0.11	0.48±0.05	0.37±0.07	<0.001
Median	0.64[a]	0.5[b]	0.38[c]	
Range	0.45-0.89	0.83-0.54	0.25-0.49	
ABiC, %	192.29±7.79	170.54±12.29	149.81±13.83	<0.001
Median	193.8[a]	173.3[b]	155.3[c]	
Range	195.19-177.19	150.0-188.9	125.55-166.71	
IMAT/Albu×10^{-2}	1.34±0.23	0.92±0.19	0.81±0.11	<0.001
Median	1.33[a]	0.97[b]	0.82[c]	
Range	0.89-0.18	0.1-0.18	0.56-0.99	

.Age, gender ,weight ,height, ,BMI body mass index,Waist circumference ,fatty liver index, liver function test(ALT,AST,GGT,DSB,ALB,TSB and ALP), lipid profile(TC, TG, HDL-C and LDL-C), (CRP)c-reactive protein,LDH

lactate dehydrogenase, Endocan, (ABiC) albumin binding capacity, (IMAT) ischemia modified albumintransformed, IMAT/ALB ischemia modified albumin transformed/albumin, (AU)absorbance unit.F-Tests (ANOVA) ,Chi square.Different small letters indicate significant differences.

Inflammatory Markers Serum Levels of Endocan, IMAT, and ABiC

The median concentration of CRP in the NASH group was far much higher than that of the NAFLD group, with highly significant differences. On the other hand, controls and the NAFLD group had similar levels of LDH (median=147 U/L), which was significantly lower than that of the NASH group (191.3 U/L). The median concentration of endocan in patients with NASH was higher than that of controls with significant differences (Table 1, Figure 1). In contrast, the median serum level of IMAT and ABiC in controls (0.64 IU/L and 193.8%, respectively) was higher than that of the NAFLD group (0.5 IU/L, 173.3%, respectively) or the NASH group (0.38 IU/L and 155.3%, respectively) with highly significant differences (Table 1, Figure 2, and 3). Moreover, controls had a higher IMAT/Albumin ratio (1.33) than both the NAFLD and NASH groups (0.97 and 0.82, respectively) as shown in (Table 1 and Figure 4).



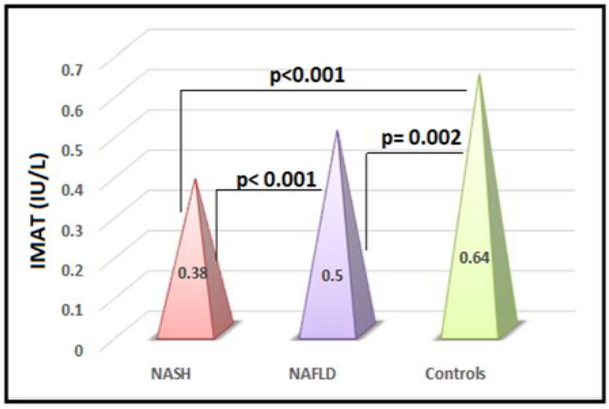
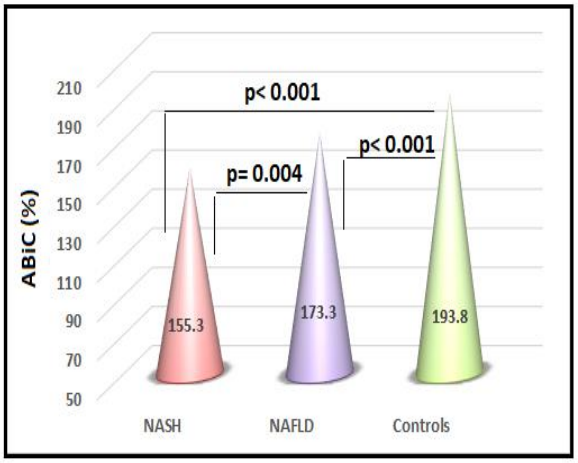


Figure 1: Median level of endocan in patients and controls.Figure 2: Median level of"(IMAT)in patients and controls.



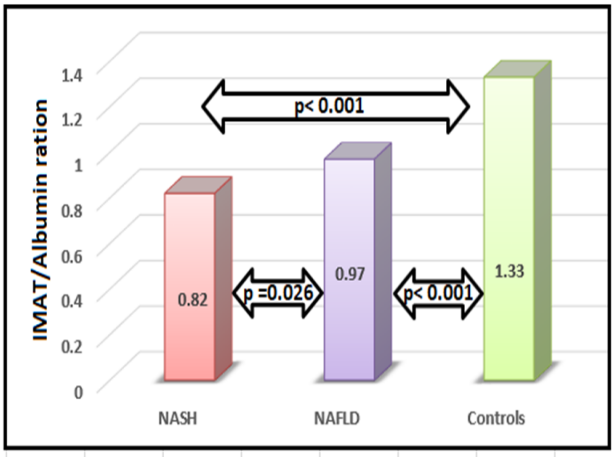


Figure 3: Median level of (ABiC) in patients and controls.Figure 4: Median(IMAT/Albumin)ratio an in patients and controls

Correlations Between Different Variables

Spearaman's correlation was used to explore the possible correlations between markers and other continuous variables in patients and controls. In NAFLD group, LDH demonstrated a positive significant correlation with each of TSB (r= 0.328, p =0.014) and LDL (r= 0.296, p= 0.025), and a negative significant correlation with IMAT (r= -0.325, p= 0.014). Positive significant correlation of CRP with direct SB (r= 0.283, p= 0.036). Endocan displayed a negative significant correlation with each of HDL (r= -0.286, p= 0.030) and ABiC (r=-0.434, p<0.001). Finally, IMAT had negative significant correlation with weight (r= -0.263, p= 0.047) (Table 2).

Table 2: Spearman's correlation between different inflammation markers and other variables in the NAFLD group.

Variables	LDH		CRP		Endocan		IMAT		ABiC	
	r	P	r	P	r	P	r	P	r	P
Age	0.120	0.375	0.090	0.508	-0.084	0.533	0.015	0.910	-0.071	0.597
Weight	-0.007	0.955	-0.012	0.925	0.077	0.560	-0.263	0.047	0.129	0.329
Height	-0.184	0.170	0.020	0.880	-0.098	0.463	0.001	0.999	0.184	0.169

BMI	0.040	0.764	-0.069	0.599	0.148	0.260	-0.170	0.195	-0.096	0.464
WC	0.085	0.523	-0.100	0.452	0.184	0.165	-0.017	0.895	-0.102	0.441
FLI	0.104	0.430	-0.154	0.244	0.207	0.115	-0.165	0.209	-0.200	0.128
ALT	-0.089	0.499	0.030	0.822	-0.237	0.072	-0.145	0.268	0.190	0.148
AST	-0.017	0.895	0.082	0.535	-0.230	0.081	0.149	0.260	0.134	0.311
ALP	0.040	0.764	0.129	0.329	-0.030	0.822	0.185	0.159	-0.082	0.536
GGT	0.142	0.284	-0.127	0.338	-0.156	0.327	0.044	0.736	-0.089	0.499
TSB	0.328	0.014	-0.038	0.778	0.087	0.511	-0.124	0.348	-0.150	0.259
DSB	0.173	0.194	0.283	0.034	0.037	0.778	-0.030	0.822	0.015	0.910
TC	0.164	0.215	0.199	0.133	-0.059	0.652	-0.081	0.536	-0.146	0.268
TG	0.194	0.143	0.243	0.066	-0.074	0.573	-0.101	0.442	-0.042	0.750
HDL	-0.015	0.910	-0.228	0.084	-0.286	0.030	0.131	0.320	-0.027	0.836
LDL	0.296	0.025	-0.017	0.895	-0.111	0.398	0.089	0.499	0.153	0.244
Albumin	0.050	0.707	-0.089	0.499	0.001	0.999	0.032	0.807	0.072	0.586
LDH			0.160	0.229	0.194	0.143	-0.325	0.014	-0.057	0.666
CRP					0.080	0.548	0.067	0.612	0.037	0.778
Endocan							-0.052	0.693	-0.434	0.001
IMAT									-0.015	0.910

In NASH group, CRP displayed a negative correlation with each of weight , BMI , waist circumference and fatty liver index . LDH had also had a negative significant correlation with each of ALT and LDL . On the other hand , endocan showed a positive significant correlation with DSB and a negative

significant correlation with ABiC . Finally, IMAT had a positive significant correlation with albumin as shown in table 3.

Table 3: Spearman's correlation between different inflammation markers with other variables in NASH group.

Variables	LDH		CRP		Endocan		IMAT		ABiC	
	r	P-	r	P-	r	P-	r	P-	R	P
Age	-0.031	0.871	0.091	0.637	0.056	0.771	-0.241	0.209	0.004	0.983
Weight	-0.011	0.954	-0.475	0.009	0.041	0.834	-0.034	0.861	-0.074	0.703
Height	-0.144	0.457	-0.215	0.262	0.077	0.691	0.029	0.833	0.046	0.814
BMI	0.145	0.453	-0.539	0.003	0.071	0.714	-0.137	0.479	-0.124	0.522
WC	0.022	0.908	-0.408	0.028	0.140	0.469	-0.112	0.563	-0.241	0.207
FLI	0.087	0.655	-0.418	0.024	0.067	0.728	-0.079	0.683	-0.133	0.491
ALT	-0.370	0.049	0.202	0.294	0.179	0.353	-0.065	0.739	-0.026	0.892
AST	-0.191	0.321	0.192	0.318	0.281	0.140	-0.048	0.806	0.064	0.741
ALP	0.065	0.738	-0.159	0.411	0.017	0.929	-0.152	0.431	-0.087	0.654
GGT	0.208	0.278	-0.244	0.202	-0.170	0.378	0.025	0.896	0.082	0.672
TSB	0.131	0.497	0.042	0.829	-0.268	0.160	-0.271	0.155	0.189	0.325
DSB	0.133	0.492	-0.014	0.941	-0.397	0.033	-0.133	0.491	0.363	0.053
TC	0.003	0.986	0.038	0.846	-0.203	0.290	-0.001	0.995	-0.029	0.833
TG	-0.043	0.825	-0.049	0.802	-0.043	0.826	0.125	0.518	-0.106	0.585
HDL	-0.288	0.130	0.138	0.474	0.247	0.196	0.005	0.980	0.014	0.943

LDL	-0.447	0.015	0.311	0.101	-0.031	0.872	0.035	0.855	-0.003	0.986
Albumin	0.157	0.417	-0.184	0.339	0.223	0.246	0.772	<0.001	0.136	0.481
LDH			-0.156	0.420	-0.142	0.461	-0.119	0.539	0.151	0.433
CRP					-0.130	0.500	0.029	0.882	0.137	0.479
Endocan							0.318	0.093	-0.657	<0.001
IMAT									0.013	0.946

Diagnostic Value of Inflammatory Markers, Endocan, IMAT and ABiC:

The diagnostic values of inflammatory markers, endocan, IMAT, and ABiC were discovered using a Receiver Operating Characteristic Curve to distinguish between patients and controls, as well as between NAFLD and NASH patient groups. In the context of discrimination between NAFLD patients and controls, the area under the curve (AUC) for CRP was 0.907, 95%CI=0.835-0.980, p<0.001. The sensitivity and specificity of the test at cut off value of CRP = 3.55 mg/L were 93% and 77% respectively. For endocan, the AUC was 0.786, 95%CI= 0.644-0.908, p< 0.001.The sensitivity and specificity of the test at cut off value of endocan=0.47 ng/ml were 69% and 77% , respectively. For IMAT, the AUC was 0.857, 95%CI= 0.755-0.960, p< 0.001.The sensitivity and specificity of the test at cut off value of IMAT= 0.52 were 80% and 73% , respectively. For ABiC, the AUC was 0.945, 95%CI= 0.894-0.996, p< 0.001.The sensitivity and specificity of the test at cut off value of ABiC= 182.17% were 90% and 86% , respectively.For IMAT/Albumin ratio, the AUC was 0.942, 95%CI= 0.883-1.00, p< 0.001.The sensitivity and specificity of the test at cut off value of IMAT/Albumin ratio= 1.05 were 93% and 83% , respectively (Figure 5).

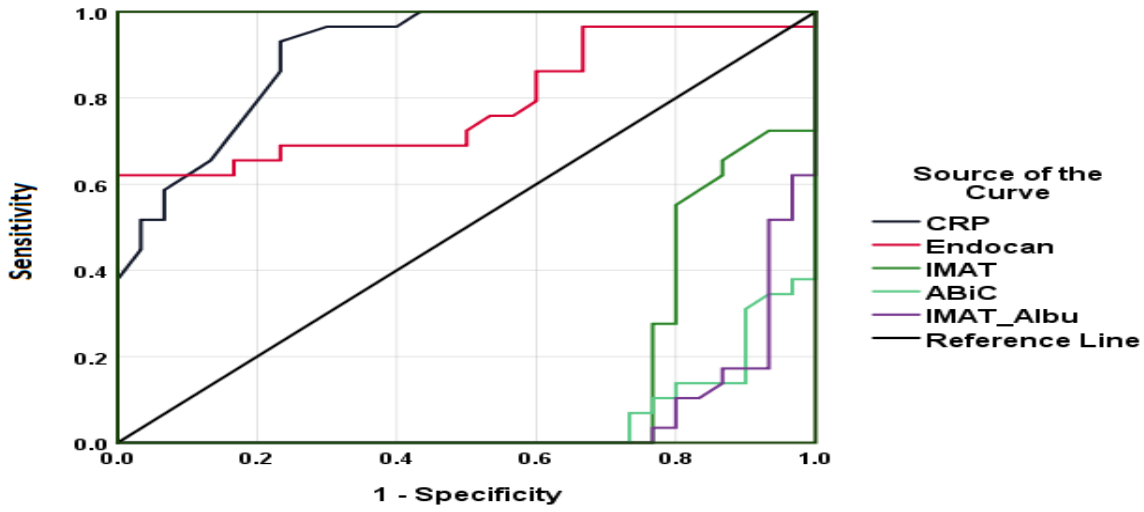


Figure 5: Receiver operating characteristic curve for CRP, endocan, IMAT, ABiC and IMAT/Albumin ratio in the context of discrimination between patients with NAFLD and controls.

In the context of discrimination between NASH patients and controls, the area under the curve (AUC) for CRP was 1.00, 95%CI=1.0-1.0, p<0.001.The sensitivity and specificity of the test at cut off value of CRP = 6.8 mg/L were 100% for both. For LDH, the AUC was 0.868, 95%CI= 0.761-0.975, p< 0.001.The sensitivity and specificity of the test at cut off value of LDH=154.2 U/L were 83% for both. For endocan, the AUC was 1.00 95%CI= 1.0-1.0, p< 0.001. The sensitivity and specificity of the test at cut off value of endocan=0.65 ng/ml were 69% and 77% , respectively. For IMAT, the AUC was 0.982, 95%CI= 0.957-1.0, p< 0.001.The sensitivity and specificity of the test at cut off value of IMAT= 0.48 were 93% for both. For ABiC, the AUC was 1.0, 95%CI= 1.0-1.0, p< 0.001.The sensitivity and specificity of the test at cut off value of ABiC= 171.95% were 100% for both. For IMAT/Albumin ratio, the AUC was 0.987, 95%CI= 0.966-1.00, p< 0.001. The sensitivity and specificity of the test at cut off value of IMAT/Albumin ratio= 0.95 were 100% for both (Figure 6).

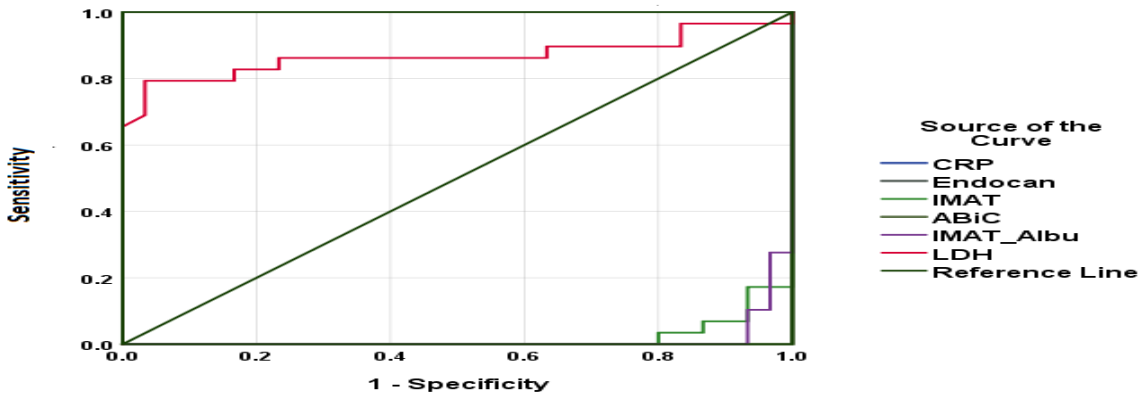


Figure 6: Receiver operating characteristic curve for CRP, endocan, IMAT, ABiC IMAT/Albumin ratio and LDH in the context of discrimination between patients with NASH and controls.

In the context of discrimination between NASH and NAFLD patients, the area under the curve (AUC) for CRP was 0.954, 95%CI=887-1.0, p<0.001. The sensitivity and specificity of the test at cut off value of CRP = 8.3 mg/L were 100% and 93%, respectively. For LDH, the AUC was 0.754, 95%CI= 0.624-0.884, p=0.001. The sensitivity and specificity of the test at cut off value of LDH=157.19 U/L were 79% and 65%, respectively. For endocan, the AUC was 0.956, 95%CI= 0.911-1.0, p< 0.001. The sensitivity and specificity of the test at cut off value of endocan=0.88 ng/ml were 90% and 86% , respectively. For IMAT, the AUC was 0.913, 95%CI= 0.844-0.982, p< 0.001. The sensitivity and specificity of the test at cut off value of IMAT= 0.42 were 83% for both. For ABiC, the AUC was 0.842, 95%CI= 0.742-0.941, p< 0.001. The sensitivity and specificity of the test at cut off value of ABiC= 160.44% were 72% for both. For IMAT/Albumin ratio, the AUC was 0.781, 95%CI= 0.662-0.899, p< 0.001. The sensitivity and specificity of the test at cut off value of IMAT/Albumin ratio= 0.88 were 66% and 69%, respectively (Figure 7).

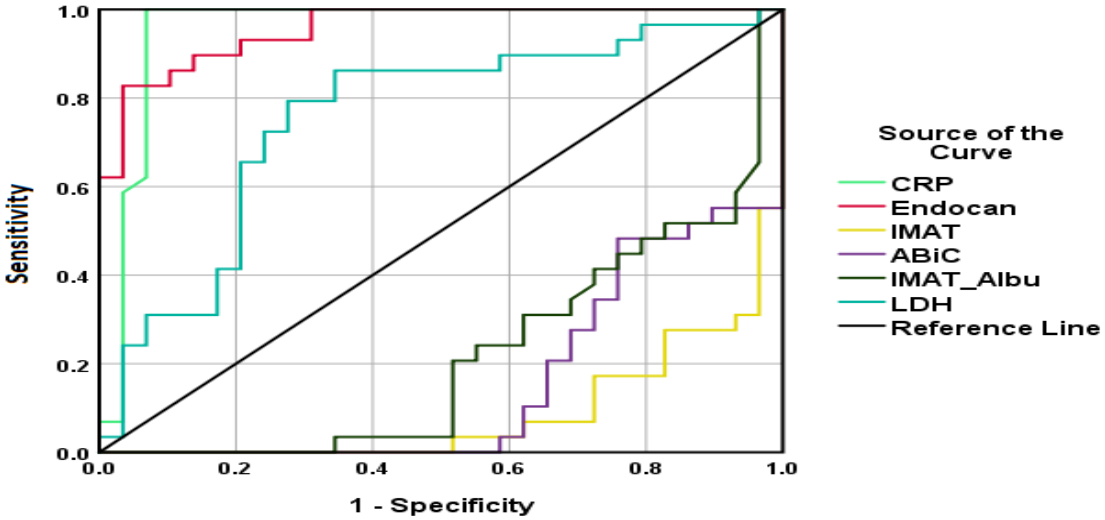


Figure 7: Receiver operating characteristic curve for CRP, endocan, IMAT, ABiC IMAT/Albumin ratio and LDH in the context of discrimination between patients with NAFLD and those with NASH.

Discussion

The results of age showed that there were no significant differences between the study groups. This means that the groups in the study were selected for their ages in a close range to get rid of the age difference problems that may affect the levels of the markers used and hence the nature of the study, as shown in table (1). These results were agreed upon in a study done by**(Sun et al., 2020**). The results of gender show there are no significant differences between females and males in the three groups as shown in table (1), which means that the diseases (NAFLD and NASH) can affect both genders (males and females) with the same effect and are not influenced by physiological changes for both sexes. These results were agreed upon by the study done by **(Sun et al.,2020).** The results of the BMI showed significant differences between the study groups. These results showed BMI is higher in NAFLD and NASH than in control. These results agree with studies done by**(Jance et al., 2019).** Obesity leads to metabolic syndrome, and as we know, is one of the risk factors of non-alcoholic fatty liver disease **(polyzos et al.,2020).** The (WC) results show there are significant differences between the three study groups' control, NAFLD, and NASH. These results show that NAFLD and NASH have a wider waist circumference than the control group. The reason for this may be that an increase in waist circumference leads to obesity and an increase in fat accumulation, which is one of the risk factors that leads to NAFLD. These results agree with studies done by**(Khang et al., 2019).**The Type 2 diabetes results show there are significant differences between the control, NAFLD, and NASH groups , as shown in table (1). These findings show that NAFLD and NASH have higher rates of T2DM than the control group . The reason for this may be that it indicates that T2DM and insulin resistance lead to NAFLD and NASH and are considered the main risk factors**(Younossi ZM. 2018).** FLI is one of these indices developed as a convenient tool based on (BMI, WC, TG, and GGT) levels **(Motamed et al., 2016).** In table 1, the results show there are significant differences between the three study groups. The results also showed the level of FLI in NASH was less than in NAFLD. The reason may be a return to NASH patients' taking drugs or weight loss for treatment of NASH. These results were agreed upon by**(Huang et al., 2015) .**The data regarding LFTs, as well as other clinical parameters, were found to be non-normally distributed. As a result, these data were expressed as median and range and analyzed using the Kruskal-Wallis or Mann Whitney tests.The results of the liver function tests show a high level of (ALT, AST, ALP, GGT, levels of AST/ALT, TSB, and ALB) in NASH compared to NAFLD and the control group. NAFLD levels stay within the normal range and, in some patients, rise two-fold or three-fold in ALT and AST. That means they may have liver damage, which is the main cause of the rise in liver enzymes' function. The results agreed with the study done by**(Sun et al.,**

2020) .The results of the lipid profile show there are significant differences between the three groups. The (TC, TG and LDL) showed an evaluation in patients with NASH and NAFLD compared with the control group and a high level in NASH compared with NAFLD. This study revealed that high levels of TC were associated with increased NAFLD and NASH risk, which agrees with the finding of the significant association between high levels of TC and NAFLD risk, which may be attributed to differences in fat metabolism. TC is highly influenced by the increased fat content of the liver**(Deprince et al.,2020).** The high level of serum LDL was marked as liable to oxidation, which may lead to oxidative stress that leads to molecular and cellular damage that leads to many diseases like NAFLD **(Mohammed ZK et al. 2018).** The results show a lower concentration of HDL-C in NASH and NAFLD compared with control and in NASH less than control. This study's findings, which reveal a link between low HDL-C levels and an increased risk of NAFLD, support the theory that high HDL-C levels protect against the disease, as seen in Table 1.These results agree with those done by**(Erman et al., 2020).** The results show a high level of CRP andsignificant differences between the three groupsNAFLD and NASH compared with control, and in NASH more than in NAFLD. Triglyceride accumulation in the liver increases oxidative stress, which induces more inflammation and results in liver injury**(Lee and D. H., 2017)**. These results agreed with studies done by**(Erman et al.,2020).** As shown in table 1, the results of LDH show there are significant differences between the three groups. These results show high levels of LDH in NAFLD and NASH in comparison with the control group, and higher in NASH compared to NAFLD. These results agree with those of a study done by**(Wu et al.,2018).**The results in table (1) show there are significant differences between the three study groups, which these results agree with those done by **(Dallio M et al., 2017).**The endocan level is high in patients with NASH and NAFLD compared with control. NASH patients saw a greater increase than NAFLD patients. These results disagreed with a study done by**(Erman et al., 2020).**Diabetic patients had much higher endocan levels than nondiabetics, which could explain the discrepancy. Diabetes type II in NAFLD patients caused a statistically significant rise in endocan serum levels, while levels were not substantially higher in NAFLD patients without diabetes than in healthy people**(Dallio M et al.,2017**). In the present study, it was found that (41.38%) of NAFLD patients and (34.48%) of NASH patients have diabetes(Tab 1).The results in (Tab.1) show there are significant differences between the three study groups. The results of ABiC, IMA and IMAT/Albumin show low levels of albumin binding functions in NASH and NAFLD compared with control, and in NASH the levels are less than in NAFLD. IMA increases in diabetes patients **(Reddy et al., 2016**) and, according to the IMAT equation, increasing the level of IMA decreases IMAT, which explains why the values of IMAT in NAFLD and NASH are lower than in other studies done by**(Sun et al., 2020).** These results agree with studies done by**(Sun et al., 2020).**

Normal Range (Cutoff Value) of Endocan Concentration in Serum of Patient Groups (NAFLD, NASH, and Control)

The current study concluded that the cut-off value for serum endocan concentration in (NAFLD) patients compared to healthy controls was 0.47 ng/ml, and the AUC was (0.786), (95%)CI=0.644-0.908, (p< 0.001). The sensitivity and specificity were 69% and 77%, respectively, as shown in figure (5).The cut off value of serum endocan concentration in (NASH) patients compared with healthy controls was 0.65 ng/ml and the AUC was 1.00 (95%). CI=1.0-1.0, (p< 0.001). The sensitivity and specificity were 69% and 77%, respectively, as shown in figure (6).The cut-off value for serum endocan concentration in (NASH) patients versus (NAFLD) patients was 0.88 ng/ml, with an AUC of (0.956), (95%) CI = 0.911-1.0,(p <0.001). The sensitivity and specificity were 90% and 86%, respectively, as shown in figure (7). This means the endocan has good sensitivity and specificity, which may be considered as a diagnostic and prognostic marker for NAFLD patients. This agreed with studies done by**(Dallio M et al., 2017) .**

Determination the Normal Range (Cutoff Value) of Albumin Binding Function of Patients Groups.

The current study concluded that the cut-off value for serum IMAT in (NAFLD) patients compared to healthy controls was 0.52. The (AUC)was 0.857, with a 95% CI=0.755-0.960 and (p value < 0.001).The sensitivity and specificity were 80% and 73%, respectively, as shown in figure (5). The cutoff value of serum IMAT concentration in (NASH) patients compared with healthy controls was 0.48. The (AUC) was 0.982, with a 95% confidence interval of 0.957-1.0 and (p value < 0.001).The sensitivity and specificity were 93% for both. As shown in figure (6).The cut off value of serum IMAT concentration in (NASH) patients compared with (NAFLD) patients was 0.42. The (AUC) was 0.913, (95%)CI = 0.844-0.982,(p< 0.001). The sensitivity and specificity were 83% for both.as shown in figure (7). The current study concluded that the cut off value of plasma ABiC concentration in (NAFLD) patients compared with healthy controls was 182.17%. The (AUC) was 0.945, with a 95% confidence interval of 0.894-0.996 and(p value < 0.001).The sensitivity and specificity were 90% and 86%, respectively, as shown in figure (5). The cut off value of plasma ABiC concentration in (NASH) patients compared with healthy controls was 171.95%. The (AUC) was 1.0, (95%)CI=1.0-1.0, (p< 0.001). The sensitivity and specificity were 100% for both, as shown in figure (6).The cut off value of plasma ABiC concentration in (NASH) patients compared with (NAFLD) patients was 160.44%. The (AUC) was 0.842, (95%)CI = 0.742-0.941, (p< 0.001). The sensitivity and specificity were 72% for both as shown in figure (7).The current study concluded that the cut off value of serum IMAT/Albumin ratio in (NAFLD) patients compared with healthy controls was 1.05. The(AUC) was 0.942, (95%)CI= 0.883-1.00,(p< 0.001). The sensitivity and specificity were 93% and 83%, respectively, as shown in figure (5). The cut off value of the serum IMAT/Albumin ratio in (NASH) patients compared with healthy controls was 0.95. The (AUC) was 0.987, 95%CI = 0.966-1.00,(p< 0.001). The sensitivity

and specificity were 100% for both, as shown in figure (6). The cut-off value for serum IMAT/Albumin ratio in (NASH) patients was 0.88, and the AUC was 0.781, (95%) CI= 0.662-0.899, (p <0.001). The sensitivity and specificity were 66% and 69%, respectively, as shown in figure (7).This means the albumin binding function has good sensitivity and specificity, which may be considered as a prognostic and diagnostic marker for NAFLD and NASH patients in which it was demonstrated that serum and plasma levels of albumin binding function decreased in patients with NASH and NAFLD diseases compared with healthy controls. This agreed with a study done by (**Sun et al., 2020)** .

Correlation Between the Studied Parameters in the NAFLD Group

According to the current findings in the NAFLD group, LDH had a positive significant correlation with both TSB (r= 0.328, p=0.014) and LDL (r= 0.296, p=0.025), and CRP had a positive significant correlation with direct SB (r= 0.283, p=0.036), indicating that elevated LDH and CRP are associated with NAFLD because these markers are anti-inflammatory anshowing thatincrease the risk of NAFLD.Endocan displayed a negative significant correlation with each of HDL (r=-0.286, p= 0.030) and ABiC (r=-0.434, p<0.001) and a negative significant correlation with IMAT (r=-0.325, p=0.014) because endocan concentration increased in NAFLD compared with IMAT, which decreased in NAFLD. Finally, IMAT had a negative significant correlation with weight (r=-0.263, p=0.047) because the increase in weight caused obesity, one risk factor of NAFLD, and IMAT decreased NAFLD, as shown in table 2. This result agreed with the subsequent result obtained by **(Sun et al.,2020)**.

Correlation Between the Studied Parameters in the NASH Group

According to present result in NASH group, CRP displayed a negative correlation with each of weight, BMI, waist circumference and fatty liver index . The reason is that some patients take medications or follow a diet in order to lose weight in order to recover from NASH. LDH also had a negative significant correlation with each of ALT and LDL .Endocan, on the other hand, had a positive significant correlation with DSB but a negative significant correlation with ABiC because endocan increased in NASH but ABiC decreased.Finally, IMAT had a positive significant correlation with albumin. This result agreed with the subsequent result obtained by **(Dallio M et al., 2017),** as shown in table 3.

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