Serum Zonulin Levels as New parameter into Non-Alcoholic Fatty Liver Disease and Coronary Artery Disease

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Abstract:

Background: Zonulin is a protein called a prehaptoglobulin that modulates intercellular tight junctions and was first identified as a critical regulator of intestinal permeability by rupturing tight junctions. It has been suggested that zonulin contributes to the pathophysiology of a number of chronic inflammatory disorders, including autoimmune, metabolic, and allergy conditions.

Aim: This study aimed to study the correlation between serum zonulin in each of coronary artery disease (CAD) and compared with that in non-alcoholic fatty liver (NAFLD).

Materials and Methods: This study included 180 samples divided into four groups, 45 patients of coronary artery (Chronic) with non-alcoholic fatty liver, 45 patients with only coronary artery disease, 45 patients with non-alcoholic fatty liver, and another 45 samples as apparently healthy control people with match age ranged between 40 – 70 years and they were obtained from Al-Hussein Medical City, Kerbala Health Directorates / Kerbala – Iraq during the period from (Sep., 2022 to May 2023). The bio-markers investigation was performed in research laboratories at the university of Kerbala's college of medicine's department of chemistry and biochemistry / Iraq which include Zonulin, lipid profile, and liver function test which were determined by using various biochemical techniques including ELISA. The subjects' body weight and height were measured in order to calculate the body mass index.

Results: There was a significant positive correlation between zonulin and body mass index in both patient groups coronary artery disease and non-alcoholic fatty liver patients ($p \leq 0.001$). Area under curve (AUC) for zonulin in CAD+NAFLD was 0.941[95% CI (confidence interval) = 0.907-0.975, Sensitivity = 0.93, Specificity% = 0.83, Cut-off point = 69.79], in CAD was 0.636 [95% Cl (Confidence interval) =0.554-0.719, Sensitivity= 0.75, Specificity = 0.6 Cut-off point = 65.395], and in NAFLD was 0.392 [95% Cl (Confidence interval) =0.314-0.501, Sensitivity = 0.57, Specificity= 0.3, Cut-off point = 57.313].

Conclusion: Zonulin was positively correlated with both coronary arteries and non-alcoholic fatty liver and may be act as a good diagnostic biomarker.

Keywords: Coronary artery disease, non-alcoholic fatty liver, zonulin.

الزونولين هو بروتين الذي ينظم الوصلات الضيقة بين الخلايا وتم تحديده لأول مرة كمنظم لنفاذية الأمعاء عن طريق تمزيق الوصلات الضيقة. وقد اقترح أن الزونولين يساهم في الفيزيولوجيا المرضية لعدد من الاضطرابات الالتهابية المزمنة، بما في ذلك أمراض المناعة الذاتية، والتمثيل الغذائي، والحساسية.

الهدف: هدفت هذه الدراسة إلى دراسة العلاقة بين الزونولين في الدم في كل من أمراض الشريان التاجي ومقارنتها مع تلك الموجودة في الكبد الدهني غير الكحولي.

المواد والطرق: شملت هذه الدراسة 180 عينة مقسمة إلى ثلاث مجموعات، 45 مريضاً يعانون من مرض الشريان التاجي (المزمن) مع الكبد الدهني غير الكحولي، 45 مريضاً يعانون من مرض الشريان التاجي فقط، 45 مريضاً يعانون من الكبد الدهني غير الكحولي، و45 اصحاء تراوحت أعمارهم بين 40 – 70 سنة وتم الحصول عليها من مدينة الامام الحسين الطبية، مديرية صحة كربلاء / كربلاء – العراق خلال الفترة من (سبتمبر 2022 إلى مايو 2023). تم إجراء فحص المؤشرات الحيوية في مختبرات الأبحاث في

كلية الطب بجامعة كربلاء قسم الكيمياء والكيمياء الحيوية / العراق والتي تشمل الزونولين وملف الدهون واختبار وظائف الكبد والتي تم تحديدها باستخدام تقنية الايلايزا .وتم قياس وزن الجسم والطول للمشاركين من أجل حساب مؤشر كتلة الجسم.

النتائج: كان هناك ارتباط إيجابي كبير بين الزونولين ومؤشر كتلة الجسم في كلا المجموعتين من المرضى مرضى تصلب الشريان التاجي ومرضى الكبد الدهني غير الكحولي ($p<0.001$). كانت المساحة تحت المنحنى للزونولين فيCAD + NAFLD 0.941[فاصل الثقة 95٪ (فاصل الثقة) = 0.907-0.975، الحساسية = 0.93، الخصوصية٪ = 0.83، نقطة القطع = 69.79]، في CAD كانت 0.636 [95 %) Clفاصل الثقة) = 0.554-0.719، الحساسية = 0.75، الخصوصية = 0.6 نقطة القطع = 65.395]، وفي NAFLD كانت 0.392 [95%) Clفاصل الثقة) = 0.314-0.501، الحساسية = 0.57، الخصوصية = 0.3، نقطة القطع = 57.313].

Introduction

The most prevalent cardiovascular disease (CVD), coronary artery disease (CAD), is caused by atherosclerosis, which narrows the arterial lumen and reduces blood flow to the distal myocardium, leading to ischemia. It is the biggest cause of death globally, and in recent years, its incidence has risen in low- and middle-income nations **[1].** Coronary artery disease is typically brought on by the development of plaque within the intima of the vessel wall. A plaque is defined as a fatty substance that forms within the intima and is associated with severe inflammation, especially if the inflammation is ongoing. The cardiomyocytes are consequently challenged in receiving enough blood, oxygen, and nourishment **[2].**

NAFLD is defined by the imagistic or histological presence of hepatic steatosis and the lack of other secondary causes of hepatic fat accumulation such as considerable alcohol use and other reasons. Histologically, when at least 5% of the liver cells on a liver biopsy show signs of fat, the condition is regarded to have NAFLD, and at least 30% of the liver cells show signs of fat are considered severe. NAFLD is regarded as the hepatic manifestation of metabolic syndrome and is frequently related with metabolic risk factors such as obesity, dyslipidemia, hypertension, and diabetes, as well as being a risk factor for type 2 diabetes mellitus (T2DM) and cardiovascular disease (CVD) **[3]**

Zonulin is a 47 kDa protein that regulates paracellular permeability in the colon. The protein is released by the liver, gut, and various other tissues, circulates in the blood, and links to receptors on ileum and jejunum enterocytes. Connection to these receptors results in reversible modification of intercellular tight junctions, increasing small intestine paracellular permeability **[4]**

Recent years have seen a number of research studies looking at serum zonulin as a non-invasive marker of intestinal permeability (IP). Numerous clinical illnesses have been observed to increase circulating zonulin, typically displaying a positive connection with body mass index (BMI), suggesting the significance of obesity in modifications to IP. These conditions include autism, type 1 and type 2 diabetes, metabolic syndrome, and inflammatory bowel disease [**5].**

Materials and Methods

The presented case-control study included four groups of (180) samples: (45) patients have chronic coronary artery disease and non-alcoholic fatty liver (grade one), (45) patients have only coronary artery disease, (45) patients with non-alcoholic fatty liver, and (45) apparently healthy people matched in regard with the patient's sex and age. The work was started in Sep., 2022 and finished in May, 2023. Patient samples were collected from Kerbala Health Directorates / Al-Hussein Medical City / Kerbala, Iraq. Chronic coronary artery disease was diagnosed by a specialist doctor based on signs and symptoms, review of an echo, CCTA, and catheterization. By using abdominal ultrasonography, fatty liver disease was identified. The Declaration of Helsinki, adopted by the World Medical Association, guided the conduct of this study. The University of Kerbalaa's medical college's ethical committee has given its approval. Participants' informed consent was gained when the study's outline was explained to them.

Five milliliters of blood were drawn from all fasting subjects by using a sterile disposable syringe. Blood was put into a gel tube, then separated into three Eppendorf tubes and stored at -20 °C in the deep freezer. One tube was used to measure serum zonulin by using the enzyme-linked immuno sorbent assay (ELISA) technique, one tube was used to measure lipid profile, and one tube was to measure liver function test by using the colorimetric method. Zonulin was measured using (Melsin Medical, China), and lipid profile/liver function test was measured by GIESSE Diagnostics kit. All biomarker investigations were carried out in research labs at the University

of Kerbala in Kerbala, Iraq, in the department of chemistry and biochemistry and the faculty of medicine. The subjects' body weight was measured using a body scale. Height was determined by measuring the length from the soles of the feet to the highest point of the head. Weight was measured in kilograms (kg) and height was measured in meters (m). The following equation was used to compute body mass index (BMI):

$$\textbf{BMI (kg/m}^2\textbf{) = weight / (height)}^2$$

Patients were classified into normal (BMI 18.5-24.9), overweight (25-29.9), and obese (30-34.9) **[6].**

Acute coronary syndrome, kidney illness, cirrhosis, stroke, skeletal muscle injury, and cancer were all considered exclusion criteria for this study.

Participants were registered and handed a file containing information such as their name, age, gender, weight, height, smoking status, and existing chronic conditions (diabetes mellitus, hypertension).

The correlation between biochemical indicators and disease was investigated. The diagnostic performance of the markers in patient groups was evaluated using receiver operating characteristics (ROC) curves.

Statistical Analysis

Data from each group was analysed using descriptive statistics. Scale variables were represented by mean standard deviation, whilst values for categorical variables were represented by n (%). The Shapiro-Wilk test was used to assess the data distribution's normality on a numerical level.

The existence of significant variations in categorical variables between the parameters was confirmed by analytical statistical analyses. All hypothesis tests were regarded as statistically significant if their two-sided p-values were less than 0.05.

Receiving operating characteristic (ROC) analysis was used to find the ideal cut-off with the highest specificity and sensitivity for important instances.

Results and Discussion:

The demographic characteristics of patients & control groups were summarized in Table (1). The mean age of the patients groups was (55.64, 55.27, and 55.24) respectively years old and for the control group (54.8) years old. Overall, the results indicated that most of the patient's samples were obese. Sex distribution among the studied groups was: 70.4% male, 29.6% female. The disease history of the patients was collected through a student self-report questionnaire, indicated that about 57.7% of them have DM and 53.3% have hypertension. Also, 45.1% of them were taking lipid-lowering agents.

Table (1): The demographic characteristics of patients and control groups.

`Characteristics		**CAD+NAFLD N = 45 (Mean ± SD)**	**CAD N = 45 (Mean ± SD)**	**NAFLD N = 45 (Mean ± SD)**	**Control N = 45 (Mean ± SD)**
Demographic	Age, year	55.64±8.46	55.27±8.39	55.24±9.17	54.06±9.10
	Sex, (Male/Female)	(32/13)	(33/12)	(30/15)	(32/13)
	BMI(kg/m$^{2)}$	30.01±4.01	27.17±4.30	30.83±2.56	25.06±9.10
	WC(cm)	109.33±17.50	99.87±10.94	105.82±7.93	91.29±7.27
Medical History	Smoking, **(Yes/No)**	(11/34)	(8/37)	(11/34)	-

	T2DM, **(Yes/No)**	(23/22)	(17/28)	(10/35)	-
	Hypertension **(Yes/No)**	(25/20)	(25/20)	(8/37)	-
New Marker	Zonulin, **(ng/ml)**	94.80 ± 18.35	73.26± 10.40	63.42±7.51	54.59± 5.13
Lipid Profile	Total Cholesterol, **(mg/dl)**	119.03 ± 26.27	119.13 ± 30.60	183.29±30.50	130.35 ±19.90
	Triglycerides **(mg/dl)**	131.60 ± 58.53	126.29 ± 55.52	169.92±73.68	124.71 ± 34.94
	HDL-C, **(mg/dl)**	46.89 ± 3.43	47.51 ± 3.62	48.31± 2.17	56.76 ± 9.60
	LDL-C, **(mg/dl)**	85.70 ± 22.37	90.66± 15.73	112.31±32.32	65.50± 19.70
Liver Function Test	Albumin, **(g/dl)**	4.30 ± 0.22	4.35 ± 0.21	4.25± 0.19	4.17± 0.13
	ALT, **(U/L)**	45.39 ± 25.79	22.56 ± 6.34	33.54± 6.97	27.59 ± 11.94
	AST, **(U/L)**	30.14 ± 13.74	23.69 ± 5.21	45.23± 10.0	27.35 ± 7.12
	ALP, **(U/L)**	210.11 ± 51.20	159.40 ± 45.70	202.64±35.36	184.88 ± 38.06
	Total serum Bilirubin **(mg/dl)**	0.235 ± 0.124	0.71± 0.27	0.914 ±0.42	0.68± 0.28

DM: diabetes mellitus; HTN: hypertension; SD: standard deviation; BMI: body mass index, WC: waist circumference.

The male percentage was 70.4% and the female was 29.6% as showed in figure (1-A). According to numerous research, there are sex differences in CAD patients as a result of male patients' greater rates of infection. These sex differences are mostly caused by social behavior and biological causes. Men are thought to smoke more frequently than women for a variety of social reasons and the biology of humans **[7].** Men are more likely than women during the reproductive years to have NAFLD, both in terms of prevalence and severity. NAFLD does however happen more frequently in women after menopause, indicating that estrogen may be protective. The primary NAFLD risk factors also differ according to gender. Male participants had a higher prevalence of hepatic tumors than female subjects did **[8].**

It had been noticed that most patients were in the age group 51-61 (43.3%) and age group ≥62 (35.6%) as showed in figure (1-B). A research by Benjamin in 2019 showed that when compared to other populations, older persons have a significantly greater prevalence of the majority of CVDs **[9]**. Through this study, it had been noticed that a large percentage of patients were overweight (37.03%) and obese (44.4%). As known obesity is one of the main risk factors for both coronary artery and non-alcoholic fatty liver **[10].**

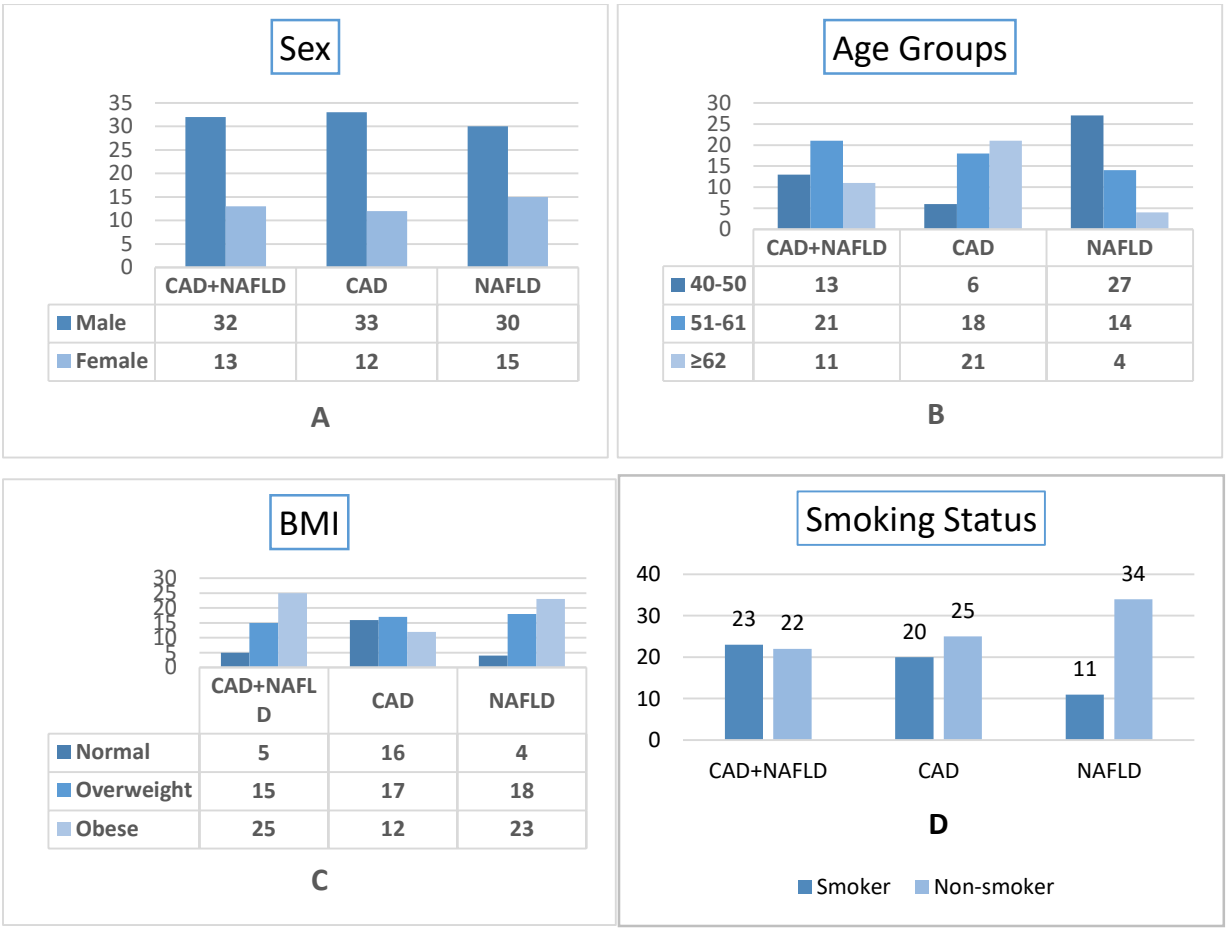

Figure (1): Distribution of patient samples according to (A)sex, (B)age groups, (C)BMI, and (D)smoking status.

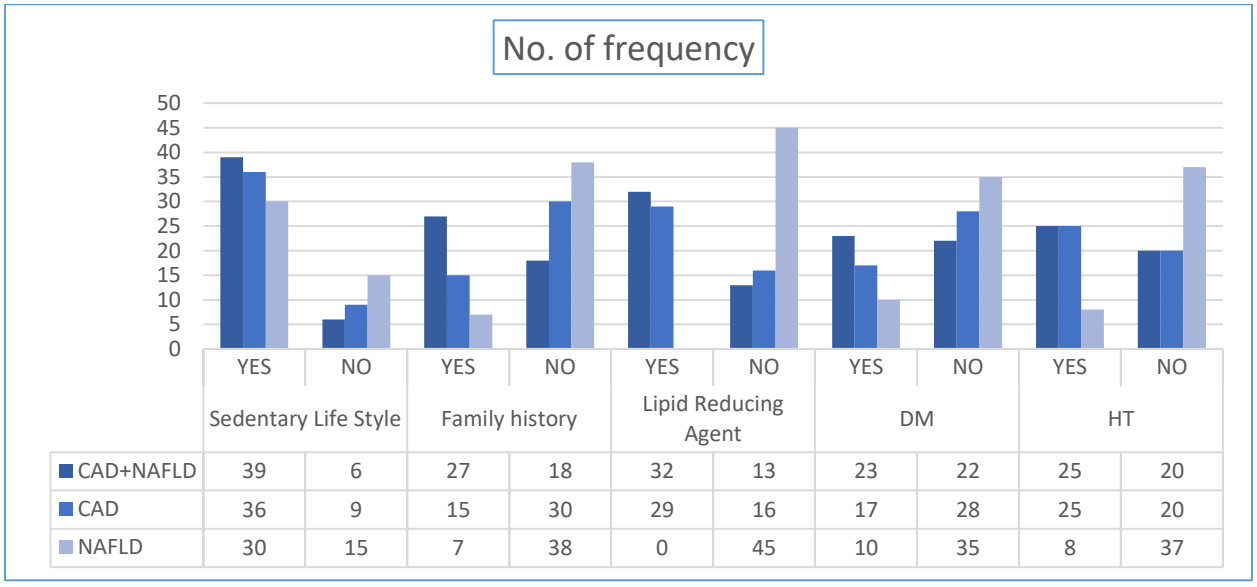

Figure (2): Distribution of patients samples according to the history of participants.

As shown in figure (2), the results indicated that the levels of lipid profiles were normal or nearly normal range in patient groups (CAD+NAFLD and CAD), this is due to drugs used in the hospital.

The gold standard for treating dyslipidemia is statin medication. Statins are prescribed as the first-line pharmacological therapy for the reduction of cardiovascular **[11].** In the fatty liver group, there is little elevation in lipid profile level. Individuals in the non-alcoholic fatty liver group had a higher total cholesterol, triglycerides, LDL, and lower HDL as compared to the healthy group [**12]**.

Figure (4), showed the distribution of Albumin, ALT, AST, ALP, and total bilirubin. There is a slight elevation in AST, ALT, and ALP concentrations. The liver enzymes were a little high in the non-alcoholic fatty liver group. NAFLD patients have LFTs that are normal or very close to normal. Transaminases may be somewhat elevated in patients (ALT > AST) **[13]**.

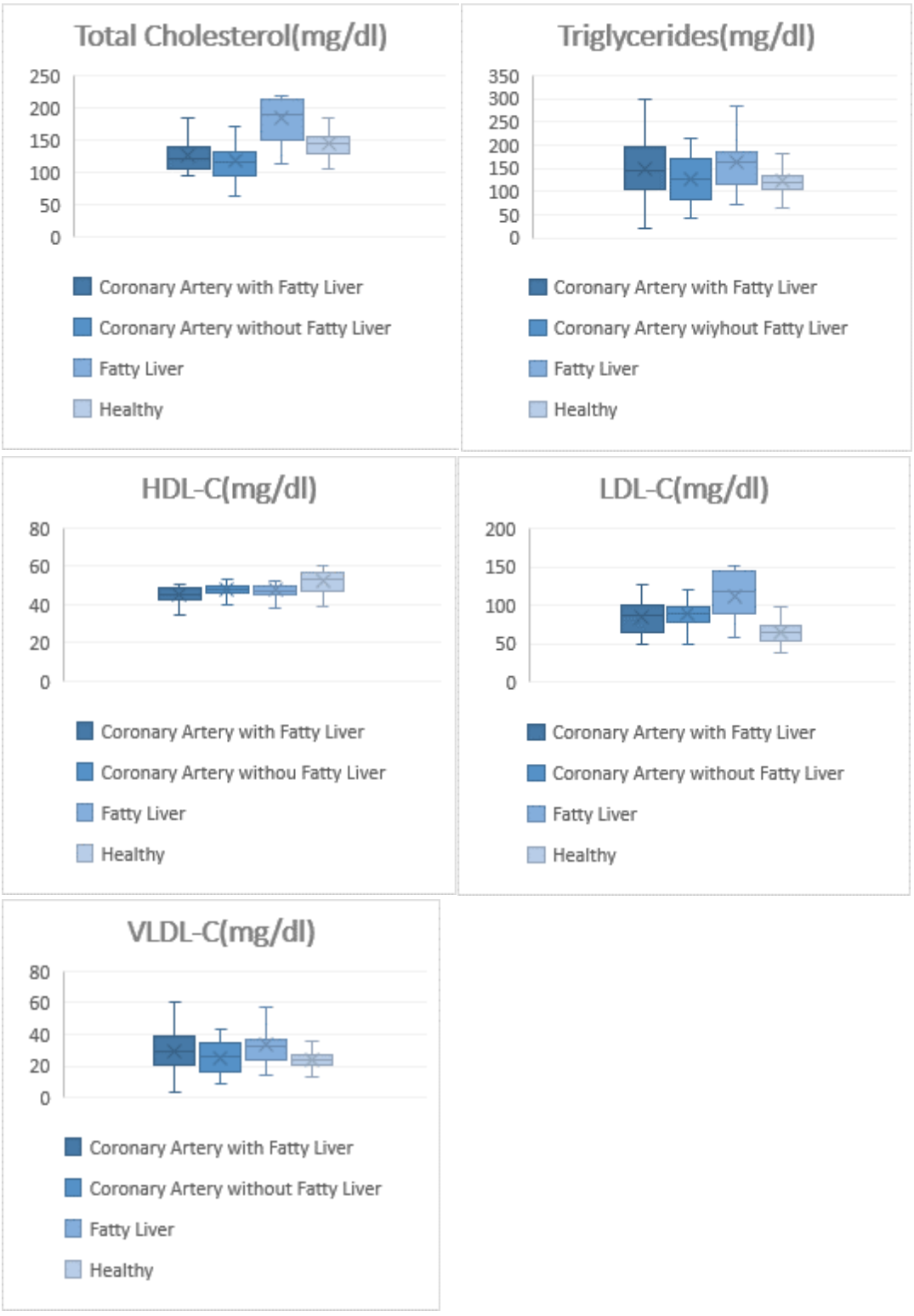

Figure (3): Boxplot of the Distribution of total cholesterol, triglycerides, HDL, LDL, and VLDL in patients and Healthy groups.

Figure (4): Boxplot of the distribution of serum Albumin, AST, ALT, ALP, and total bilirubin in patients and Healthy groups.

Table (2): The Comparison of zonulin levels in patients and healthy groups.

Parameter	CAD+NAFLD Mean±SD	CAD Mean±SD	NAFLD Mean±SD	Healthy Mean±SD	P-value
Zonulin (ng/ml)	94.80± 18.35	73.42±10.39	63.42±7.51	54.59 ± 5.13	**≤0.001[S]**
ANOVA-test was *: significant at p ≤ 0.05					
N: number of cases; SD: standard deviation; S: significant					

In Table (2) and Figure (5), significantly higher levels of zonulin in patient's groups (94.80), (73.42), and (63.42) respectively compared to the control group (54.59). The release of zonulin is accompanied by a rise in intestinal permeability as a result of the loosening of the tight junctional complex's zonula occludens-1 **[16]**. Zonulin pathway activation may facilitate the host's innate immune response to changes in the microbiome ecology (small intestine bacterial proliferation and/or dysbiosis)., which serves as a defense mechanism that "flushes out" microorganisms **[17]**. Rising intestinal permeability causes the passage of bacterial substances such as LPS and metabolic products such as short-chain fatty acids into the systemic circulation, which eventually results in a low-grade inflammatory status **[16].**

Zonulin level was significantly higher in CAD group. According to the present findings, increased zonulin in atherosclerosis may enhance intestinal IP and allow bacteria movement from the intestine to the blood. The mechanisms behind bacteria's role in atherosclerosis are complex. Previous research has primarily focused on indirect pathways. These innate immune receptors are activated by microbes, which raises inflammation, inhibits reverse cholesterol transport, and boosts insulin resistance, hyperlipidemia, and vascular inflammation **[18]**.

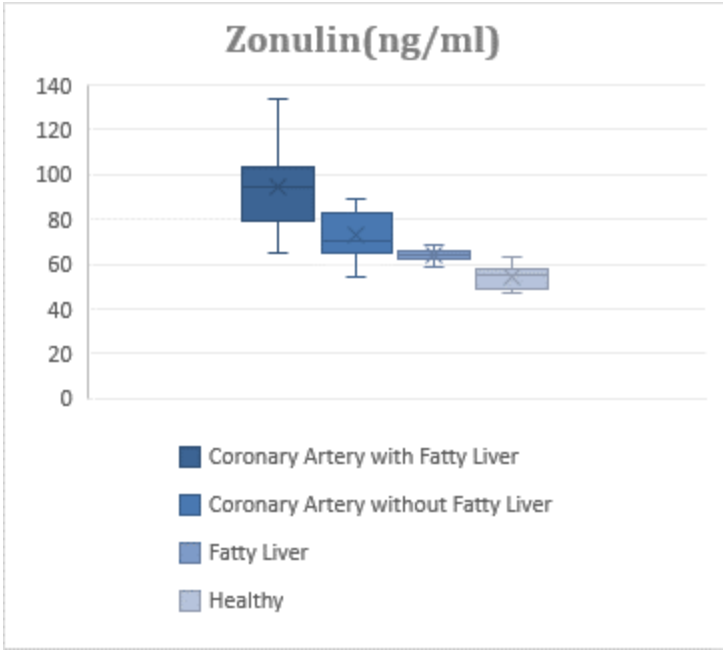

Figure (5): Boxplot of serum zonulin in patients and healthy groups.

Table (3): Correlations between zonulin and parameters in patient's groups.

Parameters	Zonulin(ng/ml)					
	Coronary Artery With Fatty Liver		Coronary Artery		Non-Alcoholic Fatty Liver	
	r	P	r	p	r	p
BMI(kg/m^2)	0.758**	**<0.001[S]**	0.6**	**≤0.001[S]**	**0.540****	**≤0.001[S]**
WC(cm)	0.428**	**0.009[S]**	0.224	0.292[NS]	**0.588***	**0.035[S]**
TC(mg/dl)	0.232	0.126[NS]	0.1	0.699[NS]	0.156	**0.305[NS]**
TG(mg/dl)	0.007	0.962[NS]	-0.028	0.858[NS]	**0.503****	**≤0.001[S]**
HDL(mg/dl)	0.1	0.802[NS]	-0.132	0.782[NS]	-0.067	**0.660[NS]**
LDL(mg/dl)	0.1	0.145[NS]	0.1	0.838[NS]	0.114	**0.454[NS]**
Albumin(g/dl)	0.123	0.474[NS]	-0.252	0.095[NS]	-0.349*	**0.019[S]**
AST(U/L)	-0.1	0.606[NS]	0.1	0.608[NS]	0.107	**0.485[NS]**
ALT(U/L)	-0.127	0.407[NS]	0.258	0.087[NS]	-0.053	**0.730[NS]**
ALP(U/L)	-0.234	0.123[NS]	-0.171	0.261[NS]	-0.341*	**0.022[S]**
TB(mg/dl)	0.1	0.661[NS]	0.090	0.557[NS]	-0.010	**0.947[NS]**

TC; total cholesterol; TG; triglycerides: WC; waist circumference: TB; total bilirubin.

Table (3), showed that in a coronary artery with fatty liver group, there was a significant positive correlation between level of zonulin and BMI (P≤0.001), WC (P=0.009), and GDF-15 (P≤0.001), and a significant negative correlation between zonulin level and osteocalcin (≤0.001).TC, TG, HDL, LDL, Albumin, and TB have a non-significant positive correlation, while AST, ALT, and ALP have a non-significant negative correlation.

In coronary artery without fatty liver group, there was significant positive correlation between level of zonulin and BMI (P≤0.001, and a significant negative correlation between zonulin and osteocalcin (P=0.033). WC, TC, LDL, AST, ALT, and TB have a non-significant positive correlation, while TG, HDL, Albumin, and ALP have a non-significant negative correlation.

In a fatty liver group, there was a significant positive correlation between level of zonulin and BMI (P≤0.001), WC (P=0.035), TG (P≤0.001), and GDF-15 (P=0.003), and a significant negative correlation between zonulin level and Albumin (P=0.019), ALP (P=0.022), and osteocalcin (P=0.014). TC, LDL, and AST have a non-significant positive correlation, while HDL, ALT, and TB have a non-significant negative correlation.

Table (4): Area under curve, cut-off, sensitivity, and specificity of zonulin obtained by ROC curve in CAD+NAFLD group.

Test Variable	Cut-off points	Sensitivity	Specificity	AUC	CI (95%)	p-value
Zonulin (ng/ml)	69.79	0.93	0.83	0.941	0.907-0.975	≤0.001

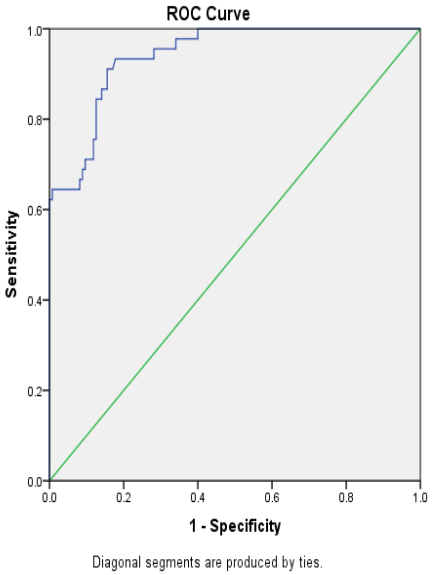

Figure (6): Receiver operating characteristic curve of zonulin in CAD+NAFLD patients.

Table (5): Area under curve, optimal cut-off, sensitivity, and specificity of GDF-15, osteocalcin, and zonulin obtained by the ROC curve for CAD case.

Test Variable	Cut-off points	Sensitivity	Specificity	AUC	CI (95%)	p-value
Zonulin (ng/ml)	65.395	0.75	0.60	0.636	0.554-0.719	0.006

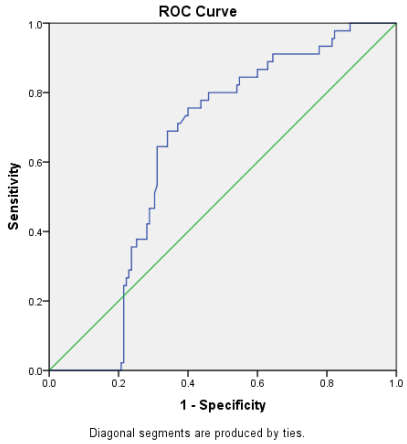

Figure (7): Receiver operating characteristic (ROC) curve of zonulin in CAD patients.

Table (6): Area under curve, optimal threshold, sensitivity, and specificity of GDF-15 and osteocalcin obtained by the ROC curve for NAFLD cases.

Test Variable	Cut-off points	Sensitivity	Specificity	AUC	CI (95%)	p-value
Zonulin (ng/ml)	57.313	0.57	0.3	0.392	0.314-0.501	0.031

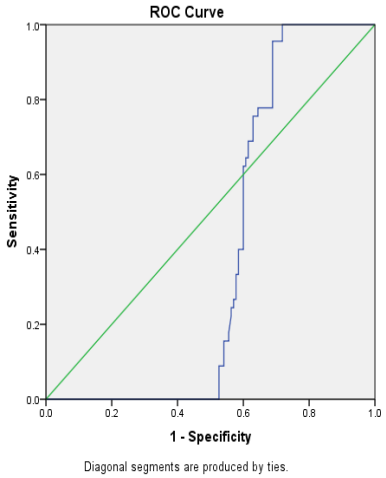

Figure (8): Receiver operating characteristic (ROC) curve of zonulin in NAFLD patients.

Conclusion:

Zonulin played a significant influence in the development of metabolic disorders, including CAD. The relationship between zonulin NAFLD may help medical professionals identify the risk of CVD in NAFLD patients early and reinforce care to improve the poor cardiovascular prognosis. It also has regulatory effects on CVD.

Authors' Contributions

Aliaa Ahmed Jasim, Fadhil Jawad Al-Tu'ma, and Hanaa Addai Ali contributed to this work.

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Declaration of interest:

There are no potential biases or other competing interests that need to be disclosed by the authors.

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