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Serum Zonulin as a Potential Biomarker in Nonalcoholic Fatty Liver Disease (NAFLD): A Case-Control Study

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ABSTRACT

Background: The buildup of excess fat in the liver is a defining characteristic of non-alcoholic fatty liver disease (NAFLD), the most common chronic liver condition globally. NAFLD is also closely linked to metabolic syndrome. New research points to increased intestinal permeability as a possible mechanism by which NAFLD-related liver inflammation and injury persist, with increased serum zonulin levels serving as the primary driver.

Objective: This study aimed to investigate serum zonulin levels and liver enzyme activities (ALT, AST, and GGT) in NAFLD patients and assess the diagnostic potential of zonulin as a biomarker for the disease.

Methods: Ninety people, 60 with NAFLD and 30 healthy controls, ranging in age from 30 to 60 years old, were involved in the clinical case-control study. In order to assess the amounts of serum zonulin and liver enzymes, blood samples were taken following an overnight fast. In order to find noteworthy distinctions and correlations, statistical tests were run.

Results: Serum zonulin concentrations were markedly elevated in NAFLD patients relative to healthy controls (patients: 72.50 ± 8.24 ng/ml vs. controls: 57.17 ± 8.26 ng/ml, $p < 0.01$). Levels of liver enzymes (ALT, AST, and GGT) were also markedly elevated in NAFLD patients (all $p < 0.01$). A strong positive correlation was found between serum zonulin levels and liver enzyme concentrations (GGT, ALT, AST), suggesting that increased intestinal permeability contributes to the development of NAFLD. Receiver Operating Characteristic (ROC) analysis demonstrated excellent diagnostic accuracy for zonulin, with an AUC of 0.903, supporting its potential as a biomarker for NAFLD.

Conclusion:

Increased intestinal permeability and liver dysfunction are directly associated with higher blood zonulin concentrations in NAFLD patients. A potential diagnostic technique for better clinical management of NAFLD and earlier disease identification could be measuring zonulin levels.

INTRODUCTION

All stages of NAFLD are collectively referred to as NAFLD. This illness affects individuals when there is a random accumulation of fat in at least 5% of their liver cells, which can occur due to factors such as certain drugs, malnutrition, or genetics. People who don't drink much at all (men should consume fewer than 30 grams per day, while women should limit their intake to 20 grams per day) are the ones that are affected [(1)]. Approximately 25% of the global population is afflicted by NAFLD, and for many, it can cause severe liver damage [(2)] **.** There is a wide variety of NAFLD conditions, including simple steatosis without inflammation (NAFL) and non-alcoholic steatohepatitis (NASH), which is characterized by inflammatory steatosis of the liver and enlargement of the liver cells (hepatocytes), this could eventually lead to fibrosis, cirrhosis, and cancer of the liver[(3)]**.** The increasing incidence of diabetes and obesity throughout the world is making the detrimental effects of NAFLD a concern for public health. NAFLD predominates in Western countries. Metabolic syndrome, which may include systemic hypertension, dyslipidemia, insulin resistance, and overt diabetes, is often seen in patients with NAFLD. Visceral obesity is becoming acknowledged as a risk factor for NAFLD [(4)].

Zonulin, a protein released by enterocytes, was one of the earliest proteins found to have a significant role in controlling intestinal permeability. Several investigations in animals have demonstrated that the 47-kDa protein zonulin increases intestinal epithelial permeability[(5)].Although the majority of zonulin secretion happens in the liver, it can also happen in various other tissues such as enterocytes, adipose tissue, the brain, the heart, the lungs, the kidneys, the skin, and immune cells [(6)].

Aim and objective: This study aims to evaluate serum zonulin concentrations and liver enzyme levels (ALT, AST, GGT) in NAFLD patients. The objective is to determine whether serum zonulin can serve as a reliable diagnostic biomarker for NAFLD and assess its correlation with liver dysfunction markers**.**

MATERIALS AND METHODES

Study Setting

A total of ninety individuals, including both ill and healthy controls, participated in the case-control study. Research at Kirkuk Teaching Hospital in Kirkuk City, Iraq, and the University of Tikrit's College of Medicine's Scientific Research Committee both gave their consent to this study.

Duration of Research

The participants in this study were recruited between 15 September 2024 and 18 January 2025.

Study Groups

- **Patients Group**

We included patients with NAFLD found by upper abdomen ultra-sonography in this study. Sixty patients, 30 male and 30 female, spanning the ages of 30 to 60, were examined in this research. The study was completed in the main accommodations of Kirkuk Teaching Hospital.

- **Controls Group**

We used an upper abdominal ultrasonogram to choose 30 people who seemed to be in excellent condition for the purposes of standardization and comparison. All of the participants were matched with the patient groups according to age, weight, and sex. There were 15 men and 15 women, spanning the ages of 30 to 60. It was determined that this control group was healthy and not medicated.

Biochemical analysis

Each person had around 6 milliliters of venous blood drawn after an 8–12 hour fast. The serum was separated by centrifugation after the blood was promptly allowed to coagulate. We used sandwich enzyme-linked immunosorbent assay (ELISA) kits to evaluate the amounts of zonulin in the serum. The Clinical Biochemistry Laboratory also used a fully automated analyzer to detect serum AST, ALT, and GGT using commercially available kits.

All of our statistical analyses were conducted using GraphPad Prism 9.0 and IBM SPSS Statistics 26.0, software from GraphPad Software and IBM Corp, respectively, located in San Diego, CA, USA and Armonk, NY, USA. The ROC curve analysis was conducted using MedCalc Statistical Software version 20.115, (MedCalc Software Ltd of Ostend, Belgium). To check for properly distributed data, we employed the Shapiro-Wilk test. Displaying continuous variables is done by taking the mean and either adding or subtracting the standard deviation (SD). When comparing groups, we utilized independent t-tests for data that normally distributed and the Mann-Whitney U test for data that did not. We used Spearman's rank correlation coefficient to look at how the variables were related to one another. All tests were two-tailed, and statistical significance was defined as a p-value below 0.05.

RESULTS

This study reported that the Zonulin levels differed significantly ($p<0.01$) between the patient group (72.50 ± 8.24 ng/mL) and the control group (57.17 ± 8.26 ng/mL). As seen in Figure 1. This research reveals significant differences in liver enzyme levels between patients and the control group. Gamma-Glutamyl Transferase (GGT) levels were markedly elevated in patients (64.52 ± 15.11 U/L) in comparison to controls (32.79 ± 6.79 U/L), exhibiting a statistically significant difference ($p<0.01$). Similarly, Alanine Aminotransferase (ALT) levels were significantly higher in patients (47.16 ± 5.66 U/L) than in the control group (34.47 ± 3.90 U/L) ($p<0.01$).

Additionally, Aspartate Aminotransferase (AST) levels were markedly elevated in patients (46.38 ± 6.35 U/L) in comparison to controls (31.00 ± 3.52 U/L), also exhibiting a statistically significant difference ($p<0.01$). These findings indicate potential liver dysfunction or increased hepatic stress in NAFLD. As shown in Figure 2.

In our study, we established a positive correlation between the degree of zonulin and the degree of liver enzymes in NAFLD patients, including GGT ($r = 0.546$, $p < 0.001$), ALT ($r = 0.519$, $p < 0.001$), and AST ($r = 0.400$, $p = 0.002$). Such findings indicated that increased intestinal permeability was the cause of the inflammation and injury to the liver, and

this implicated the role played by zonulin in the development of NAFLD. As seen in Table 1.

The ROC curve analysis demonstrated that zonulin is a highly effective marker for the diagnosis of NAFLD, with AUC equal to 0.903 (95% CI: 0.823–0.956). The optimal cut-off point for zonulin (>66.13 ng/ml) was highly sensitive (81.67%) and specific (86.67%), with very high positive predictive value (PPV, 92.5%), so that subjects with zonulin above this cut-off point are very likely to be affected by NAFLD. Moreover, the +LR equal to 6.13 further supports the clinical usefulness of this assay by significantly increasing the probability of correct identification of affected individuals. Thus, zonulin concentration measurement is a reliable and effective method for the identification of NAFLD patients in clinical settings. As shown in figure 3.

DISCUSSION

Figure (1) illustrates the patient group had a noticeably greater serum zonulin concentration (72.50 ± 8.24 ng/ml) ($p < 0.01$) in comparison to the control group (57.17 ± 8.26 ng/ml). Levels of zonulin have increased by 26.8%, which may indicate alterations in intestinal permeability. Because the standard deviations are comparable across groups, we can conclude that zonal elevation is constant throughout the patient population.

Our data shows that zonulin levels in the blood are higher in NAFLD patients than in healthy controls. Our findings align with those of Wang et al, who found that NAFLD is associated with higher blood zonulin levels in children and adolescents than in those without the disease [7].

An excess of hepatic fat is a hallmark of NAFLD, which is associated with low-grade chronic inflammation and elevated interleukin-6 (IL-6). This, in turn, activates STAT3 and miR-18a, leading to the induction of zonulin expression [8,9]. A condition called "leaky gut" occurs when there is an excess of the intestinal permeability modulator zonulin, which allows bacteria to translocate and their byproducts to enter the bloodstream. By increasing inflammation within the liver, this process leads to NASH, a more serious form of the disease than simple steatosis [10]. Compared to healthy controls, people with NAFLD had higher levels of zonulin and IL-6, according to Hendy et al [11]. Previous findings on elevated zonulin levels in NAFLD patients are supported by our research.

Figure (2) shows that there was a substantial increase in alanine aminotransferase (ALT) concentrations in patients (47.16 ± 5.66 U/L) compared to controls (34.47 ± 3.90 U/L) ($p < 0.01$). Consistent hepatic stress across the patient population is suggested by this relatively narrow standard deviation and this 36.8% rise. The results show that the serum ALT enzyme differs significantly between the control and sick groups.

Our results demonstrate that ALT levels in the blood are greater in individuals with NAFLD vs to healthy controls. Our findings corroborate those of Hadinia [12] and Ali [13],who also discovered that the NAFLD group exhibited a higher ALT level than the control group.

Hepatocytes contain high levels of the enzyme ALT in their cytoplasm. Reductions in serum ALT levels are common in healthy populations. After hepatocyte damage and death, serum ALT

levels rise significantly. A common measure of liver function, serum ALT levels reveal hepatic inflammation and liver damage in people with various chronic liver disorders. In the majority of previous research, particularly when combined with NASH, NAFLD risk was shown to be higher in those with elevated ALT levels [14] .

Furthermore, there was a significant difference in the levels of aspartate aminotransferase (AST) between the control group (31.00 ± 3.52 U/L) and the patients (46.38 ± 6.35 U/L) ($p < 0.01$). There is constant but varying hepatic dysfunction throughout this patient population, as shown by the elevated standard deviation and this 49.6% elevation. Serum aspartate aminotransferase enzyme levels were significantly different in the treatment and control groups, as shown by this finding. We found that AST levels in the blood of NAFLD patients were significantly greater than those of healthy controls. Our findings corroborate those of Ou, Hongjie, et al [15] and Allam et al, who previously discovered that the NAFLD group exhibited a higher AST level compared to the control group [16].

Hepatocyte injury can be detected by the sensitive markers aminotransferases ALT (cytoplasm) and AST (both mitochondrial and cellular). Hepatocytes in fatty liver are triacylglycerol saturated. Insulin causes a cascade of events that culminate in hepatocyte injury and enzyme release, including an increase in intracellular fatty acids, lipid peroxidation, mitochondrial enlargement and malfunction, compromised membrane integrity, and strengthened lysosomal fragility [17].

Patients had significantly higher levels of Gamma-Glutamyl Transferase (GGT) (64.52 ± 15.11 U/l) than controls (32.79 ± 6.79 U/l) ($p < 0.01$). This 96.8% increase indicates that the patients are experiencing significant liver stress. Patients show more variable GGT elevation compared to controls, as seen by the significantly greater standard deviation. This finding shows that the serum GGT enzyme differs significantly between the control and sick groups. Our findings align with those of Hassan et al, who also discovered a high GGT level in NAFLD patients [18] and Mukherjee et al, who also discovered a considerably higher GGT level in NAFLD patients [19] .

Study Limitations

Despite the significant findings, this study had several limitations. The sample size was relatively small and limited to a single geographic region, which may affect generalizability. Diagnosis of NAFLD was based on ultrasound imaging rather than liver biopsy, which is considered the gold standard.

CONCLUSION

Non-Alcoholic Fatty Liver Disease (NAFLD) patients had significantly higher levels of serum zonulin and liver function tests (ALT, AST, and GGT) compared to healthy controls. Increased intestinal permeability, popularly known as "leaky gut," is a key component of inflammation and liver damage in NAFLD, as we found when serum zonulin concentrations were positively correlated with liver enzyme concentrations. Zonulin was found to be a very specific and accurate biomarker for the diagnosis of NAFLD according to the ROC curve study. A clinically relevant, efficient, and effective diagnostic tool for

the early diagnosis of NAFLD can thus be the assessment of serum zonulin levels. Improving patient outcomes and decreasing illness complications may be possible with the use of zonulin as a diagnostic biomarker, which can greatly improve patient care and allow for early treatment interventions.

RECOMMENDATIONS

While this study suggests a potential role for serum zonulin as a biomarker in NAFLD, it is recommended that further research be conducted on larger, diverse populations to confirm its diagnostic value. Longitudinal and interventional studies using gold-standard diagnostic tools (e.g., liver biopsy or elastography) are needed to establish causality and clinical utility.

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TABLES

Table (1): Correlation analysis of clinical parameters in patients' group.

		Zonulin ng/ml	GGT U/l	ALT U/L	AST U/L
Zonulin ng/ml	r	1.00	0.546	0.519	0.400
	p		<0.001	<0.001	0.002

FIGURES

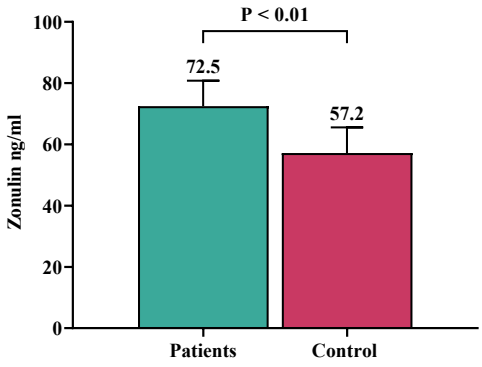

Figure (1): Comparison of Serum Zonulin Levels Between Patients and Controls

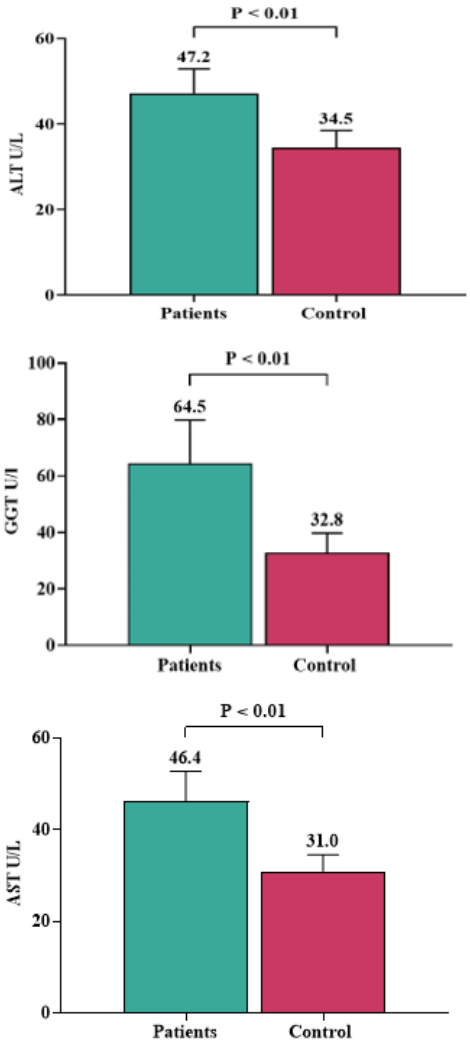

Figure (2): Comparative Analysis of Liver Function Parameters in Control and Patient classes.

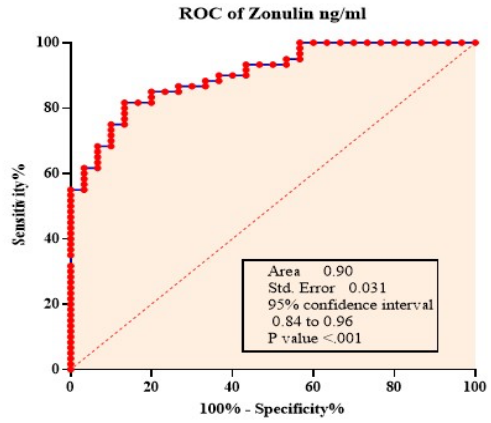

Figure (3): Receiver Operating Characteristic Curve Analysis for Serum Zonulin as a Diagnostic Biomarker

ROC curve analysis demonstrating the diagnostic accuracy of serum Zonulin in distinguishing between patients and control