Bull. Egypt. Soc. Physiol. Sci. *36(1)*, 20-29

Bull. of Egyp. Soc. Physiol. Sci.

(Official Journal of Egyptian Society for Physiological Sciences)

(pISSN: 1110-0842; eISSN: 2356-9514)

Evaluation of circulating zonulin as a potential marker in the pathogenesis of non-alcoholic fatty liver disease

Olfat M. Hendy[1], Maha M Elsabaawy[2], Mona M Aref [3], Fatma M Khalaf [3], Abel Moaty A Oda [2], Helmy M El Shazly [2]

1Department of Clinical Pathology, National Liver Institute, Menoufia University, Egypt.

2 Department of Hepatology, National Liver Institute, Menoufia University, Egypt

3 Department of Clinical Biochemistry[3], National Liver Institute, Menoufia University, Egypt

Received: 16 Oct. 2015
Accepted: 16 Nov. 2015
Available online: 16 Feb 2016

Keywords

- Non-alcoholic fatty liver disease (NAFLD)
- Nonalcoholic steatohepatitis (NASH)
- Homeostasis model assessment of insulin resistance (HOMA-IR)
- IL-6
- Zonuline

Abstract

Nonalcoholic fatty liver disease (NAFLD) is a spectrum of liver disorders ranging from simple fat accumulation in the hepatocytes (hepatic steatosis), to liver inflammation and hepatocytes injury (nonalcoholic steatohepatitis, NASH) with increasing levels of fibrosis to cirrhosis and hepatocellular carcinoma (HCC). So, the current study aimed to determine the role of circulating zonulin in NAFLD and to correlate its level with biochemical parameters, IL-6 and liver histopathology. The study included 56 adults subjects with proved NAFLD by ultrasonography and liver biopsy, as well as 20 healthy subjects were enrolled in the study as a control group. For patients and controls the following were done: Clinical examination, abdominal ultrasonography, routine laboratory investigations, body mass index (BMI) and the homeostasis model assessment of insulin resistance (HOMA-IR) were calculated, the circulating zonulin and IL-6 were measured. The results of the study: HOMA-IR, IL-6 and serum zonulin were significantly increased in NAFLD group compared to controls. Additionally, in NASH group, a significant increase in HOMA-IR and serum zonulin was detected as compared to group of simple steatosis. The serum zonulin level was positively correlated with HOMA-IR, liver histopathology and serum IL-6. **In conclusion**: The increasing serum levels of zonulin in patients with NAFLD and further increase in NASH group denoting its possible role in NAFLD progression. Future large scale studies are recommended to use this novel marker in early detection of NAFLD and to prevent disease progression.

Corresponding author: Fatma A. Khalaf, Department of Clinical Biochemistry, National Liver Institute, Menoufia University, Egypt, **E-mail address**: dr_khalaf268@yahoo.com, **Phone:** +971557277423

Introduction

Nonalcoholic fatty liver disease (NAFLD) comprises a wide range of fat-associated liver injuries with subsequent end-stage liver disease even necessitating liver transplantation (1). The striking increases in its worldwide population prevalence is substantially eminent (4-37%) (2). As NAFLD is the most prevailing cause of chronic liver injury in urbanized countries, a lot of authenticated research had been exerted regarding this issue (3).

Starting from hepatic steatosis; which is a two-sided condition; it can resolve spontaneously and/ or can progress to a necroinflamatory condition named nonalcoholic steatohepatitis (NASH), then to liver fibrosis and cirrhosis with higher susceptibility of hepatocellular carcinoma occurrence (4).

The 3 hit theory is still the most acceptable sequencing of events in NAFLD pathogenesis. Starting with hepatocytes accumulation of triglycerides, boosting to hepatic injury; arbitrated by pro‑inflammatory cytokines induced steatohepatitis and/or fibrosis as a "2nd hit". While the lessened hepatocytes progenitor cells proliferation exemplified the "3rd hit" in NAFLD pathogenesis (5). However the picture is not yet completed and theories are still evolving.

Inspite of its pros and cons, liver biopsy it is still merely the reliable modality for NAFLD diagnosis (6). The noninvasive methods of NAFLD diagnosis had been the preferable research topic for many years. Nevertheless, the compulsory need of developing noninvasive methods for NAFLD diagnosis is still mandated. There is a growing substantiation that gut microbiota is involved in NAFLD development through, among others, obesity induction, endogenous ethanol production, inflammatory response triggering and alterations in choline metabolism (7). NAFLD was mentioned to be significantly associated with increased intestinal permeability and more disrupted tight junctions than healthy individuals (8).

Zonulin as a moderator of intestinal permeability through tempering intracellular tight junctions (9), is implied as an informative marker of intestinal permeability (10). Human zonulin is a 47-kDa protein, representing the eukaryotic counterpart of the Vibrio cholerae zonulaoccludens toxin (11). Increased plasma zonulin levels have been mentioned in celiac disease, type 1diabetes as well as obesity-associated insulin resistance (12, 13).

Wieckowska et al., (14) spotted a highlight on the role of IL-6 production the proinflammatory cytokine and NASH development, as well as in systemic insulin resistance and diabetes. To date the role of circulating zonulin in NAFLD pathogenesis and its relationship to inflammatory cytokines in adults are still a matter of debate.

Aim of the study:

The current study was designed to assess relationship between serum zonulin levels correlated with the proinflammatory cytokine IL6, metabolic, and biochemical parameters, along with the histopathological features of liver injury in NAFLD patients.

Patients and methods:

The study included 56 adults subjects (22 male and 34 females) with age ranged from 29 to 46 years. They attended to the outpatient Clinics of Hepatology Department national Liver Institute -Menofia University with chronically elevated aminotransferase levels (at least 6 months). The diagnosis of NAFLD was based on magnetic resonance imaging (MRI) with high hepatic fat fraction (HFF $\geq$ 5%) (4), which was confirmed by liver biopsy as recommended by the AASLD guidelines of NAFLD diagnosis in addition to absence of competing or co‑existing etiologies for hepatic steatosis (significant alcohol consumption, hepatitis C, medications, parenteral nutrition, Wilson's disease, and severe malnutrition (15).

Exclusion criteria:

The history of diabetes mellitus (type 1 or type 2 diabetes), other causes of chronic liver disease including: hepatic virus infections (hepatitis A, B, C), other viruses can cause viral hepatitis (cytomegalovirus, and Epstein-Barr virus), autoimmune hepatitis, metabolic liver disease, α-1-antitrypsin deficiency, cystic fibrosis, Wilson's disease, hemochromatosis, and celiac disease were excluded. Renal disease, smoking or alcohol consumption, the use of anti-inflammatory drugs, antibiotics or hepatotoxic dugs were from the study.

Twenty apparently healthy subjects (12 females, 8 males), matched for age, gender, BMI the patients were enrolled in the study as control group, they were selected from potentially liver donors (they have also the same exclusion criteria as patient group).

The research protocol was approved by the local Ethics Committee (NLI- Menofia University) and written informed consent was obtained from all subjects participated in the study.

Patients and controls were subjected to:

1- Full history taking along with thorough clinical examination.

2. Abdominal ultrasonography

3. Laboratory investigations:

Blood samples were taken from each subject, after an overnight fast, for estimation of glucose, insulin, C peptide, lipid profile {total cholesterol, high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), triglycerides},liver function tests {alanine aminotransferase (ALT), Aspartate aminotransferase, Gamma-glutamyltransferase (GGT)}. All analytes were measured by COBAS 6000 (Roche Diagnostics). While insulin and C peptide concentrations were measured on COBAS e 601 module (Electro-chemiluminescence Technology, Roche Diagnostics).

4. Wight and height were measured and the body mass index (BMI) was calculated based on the height and body weight: BMI= (kg/m2).

5. The homeostasis model assessment of insulin resistance (HOMA-IR) was calculated as the following equation formula (16):

Fasting plasma insulin (mU/L)X fasting plasma glucose (mmole/L) / 22.5.

HOMA index >3 is a criterion of insulin resistance (17).

6. Serum zonulin concentrations:

Serum zonulin was measured by an ELISA technique used for the quantitative wash to remove any unbound avidin-enzyme reagent, a substrate solution is added to the wells and color develops in proportion to the amount of zonulin bound in the initial step. The color development is stopped and the intensity of the color is measured.

This assay has high sensitivity and excellent specificity for detection of human zonulin. No significant cross-reactivity or interference between human zonulin and analogues was observed. The minimum detectable dose of human zonulin is typically less than 0.156ng/ml. Intra- and inter assay coefficients of variation were 2%-7% and 4%-10%, respectively.

7. IL-6 assay:

IL-6 was assayed by aQuantikine ELISA kit provided by R &D (R&D Systems, Inc., Minneapolis, MN, USA), It used for the quantitative determination of human Interleukin 6 concentrations in cell culture supernates, serum, and plasma.

Principle of the assay: This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for human IL-6 has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any IL-6 present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for human IL-6 is added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added

to the wells and color develops in proportion to the amount of IL-6 bound in the initial step. The color development is stopped and the intensity of the color is measured. Analytical intra-assay sensitivity was 0.7 pg/ml. No cross-reactivity with other cytokines was evident. Intra- and interassay coefficients of variation(CV%) for IL-6 determinations were between2-4.2% and 3.8-6.4% respectively.

The following were done to the patients to diagnose NAFLD:

8. Magnetic resonance imaging (MRI): The diagnosis of NAFLD was based on MRI with high hepatic fat fraction (HFF ≥ 5%)

9. Liver biopsy done by expert pathologists: To confirm the diagnosis of NAFLD and to assess the presence of NASH and degree of fibrosis. The main histologic features of NAFLD were scored according to the scoring system developed by the NASH Clinical Research Network (CNR) **(18):** steatosis [grade 0 (minimal < 5% macrovesicular fat), grade 1 (mild= 5%-33%), grade 2 (moderate = 34%-66%), and grade 3 (severe > 66%)], portal inflammation (0-2), lobular inflammation (0-3), ballooning degeneration (0-2), and fibrosis (stage 0 to 4).

Statistical methods:

The data were statistically analyzed using SPSS computer program version 21 data were expressed as mean ± SD and differences between groups were analyzed by student t or Mann-Whitney tests. Pearson's correlation coefficient was used to test the relationship between various variables. P value is considered significant if <0.05. A multiple regression analysis was used to assess the independent association between circulating zonulin concentrations and some potential clinical and metabolic variables within the NAFD cases after adjustment for age, gender, and other clinical and metabolic variables. A stepwise selection method was used to exclude variables that do not contribute significantly to the independent association.

Results

Among 56 patients with NAFLD, 24 were diagnosed as simple steatosis [18 of them were minimal steatosis (grade 0 = <5% macrovesicular fat), 4 were mild steatosis (grade 1=<10%). The remaining 32 patients have non alcoholic steatohepatitis (NASH), 8 were mild steatosis (grade 1=<10%), 15 were moderate steatosis (grade 2= >33%-66%), 9 cases were grade 3 (severe= > 66%)].

No significant differences between NAFLD and controls regarding age, systolic and diastolic blood pressure, BMI, total cholesterol, LDL-C and Albumin (p>0.05). On the contrary, AST, ALT and γ-GT and triglycerides were significantly increased (p<0.05, <0.01, <0.01, <0.05 respectively), and HDL-C was significantly decreased in NAFLD group compared to control group (p <0.05) (Table 1).

A highly significant increase in fasting insulin, fasting C peptide, HOMA-IR,IL-6 and serum zonulin in the NAFLD group compared to control group (p<0.001) (Figure 1). The fasting and 2-h glucose and HbA_{1c} show no significant difference between two groups (p>0.05) (Table 1).

As comparing between groups of simple steatosis and NASH, a significant increase in BMI, levels of AST, ALT, γ-GT, fasting insulin, fasting C peptide (p<0.05), a significant decrease in HDL-C level (p<0.05) and a high significant increase in HOMA-IR (p<0.01) in the NASH group compared to simple steatosis. In contrast, the age, triglyceride, total cholesterol, LDL-C, serum IL-6 showed no significant difference between two groups (p>0.05) While circulating zonulin levels were found to be significantly higher in the NASH group (p> 0.001) (Table 2).

Serum IL-6 median levels were detected in control, simple steatosis, and NASH groups and were found to be: 0.89, 2.65, 3 ng/ml respectively, with significant progressive increase from control to simple steatosis to NASH groups (figure1).

Circulating zonulin levels in control, simple steatosis and NASH groups were detected and found to be: 2.8, 4.8, and 7.45 ng/ml respectively, with significant progressive increase from control to simple steatosis to NASH groups (Figure2).

Among 56 NAFLD patients, serum zonulin level was positively correlated with BMI, ALT, triglycerides, fasting insulin, HOMA-IR, histopathological features of liver biopsy and serum IL-6. While HDL-C shows an inverse correlation with serum zonulin levels. Regarding Il-6, no significant correlations were detected between it and all studied parameters except for BMI and ALT (Table 3).

Correlation between circulating zonulin and IL-6 was significantly documented in both steatosis and NASH groups (Figure 3).

In the NASH group serum zonulin was found to be significantly positively correlated with BMI, ALT, fasting insulin, HOMA-IR, liver histopathology, and IL6 (P<0.001), while it was negatively correlated with age, triglycerides, total cholesterol, and HDL-C (P<0.001) and no correlation was found between it and gender (p= 0.985). In the steatosis group: a positive correlation between zonulin and BMI, ALT, fasting insulin, HOMA-IR, liver histopathology, and IL6 (P<0.001), while it was negatively correlated with triglycerides, total cholesterol, and HDL-C (p<0.001) and no correlation was proved in this group with sex (p=0.582) and age (p= 0.444) (Table 4).

In the NASH group serum IL6 was found to be significantly positively correlated with BMI, ALT, fasting insulin, HOMA-IR and liver histopathology (P<0.001), while it was negatively correlated with triglycerides, total cholesterol, and HDL-C (p<0.001). No correlation was proved in this group with sex (p=0.816) and age (p= 0.059). In the steatosis group: a positive correlation between IL-6 and BMI, ALT, fasting insulin, HOMA-IR, and liver histopathology (P<0.001), a negative correlation with triglycerides, total cholesterol, and HDL-C (P<0.001) and no correlation was proved in this group with sex (p=0.535) and age (p= 0.884) (Table 5).

A multiple regression analysis was done with stepwise selection method to exclude variables that do not contribute significantly to the independent association. Univariate analysis substantiated the role of grade of liver histopathology, age (P <0.001), ALT(P = 0.008), BMI (P<0.001), triglycerides (P<0.001), HDL-C (P<0.011), HOMA-IR, fasting insulin , IL6, and serum zonulin (P <0.001), while multivariate analysis proved a role only for liver histopathology, serum HDL-C and serum zonulin (P < 0.001) (Table 6).

The cutoff values for NASH occurrence among NAFLD patients was 8.3 ng/ml with 100%Sensitivity, and 100% Specificity (Area under the curve=1.000, P-value=<0.001) (figure 4).

Discussion:

According to the wide world mounting incidence and prevalence rates of NAFLD occurrence, it is alleged to be the plague of the new era. The role of gut-liver axis in NAFLD pathogenesis had been an evolving medical concern attracting most hepatologists. As the liver receives about 70% of its blood supply from the intestine through the portal vein, so it is the first line of defense against gut-derived antigens, and one of the organs most exposed to gut-derived toxic factors, such as bacteria and bacterial products (19). The gut epithelium plays a central role in delineating microbes in the gut from the host immune system. Gut epithelial cells are linked to one another with tight junctions, which play a pivotal role in maintaining the integrity of the intestinal barrier (20).

A novel protein; zonulin, which modulates intestinal permeability by stripping the intercellular tight junctions (TJs) has been rigorously a concern in this axis. The relationship between insulin sensitivity and circulating zonulin might be mediated through the obesity-related circulating IL-6 increase (21)

Table (1): Comparison of demographic data, liver function tests and lipid profile between NAFLD patients versus control group

Parameters	NAFLD (no=56) M±SD	Control (n=20) M±SD	P value
Age (years)	37.22±6.8	34.11±4.35	>0.05
Systolic B.P. (mmHg)	126±11	115±9	>0.05
Diastolic B.P.(mmHg)	83± 9	79±10	>0.05
BMI (kg/m2)	28.2±3.52	26.5±2.78	>0.05
AST (U/L)	44.6 ±19.2	21.5 ±8.7	<0.05*
ALT (U/L)	50.1 ± 20.3	23.6 ± 7.4	< 0.01*
GGT (U/L)	31.8 ±9.2	15.7± 6.5	< 0.01*
Albumin (g/dL)	4.13 ± 0.8	4.85± 0.6	>0.05
Triglycerides (mg/dl)	102±15.4	87.6± 27.3	<0.05*
Total Cholesterol (mg/dl)	161.5±23.1	154.2± 21.4	>0.05
HDL-C (mg/dl)	38.4±6.5	51.3± 8.2	<0.05*
LDL-C (mg/dl)	112.2±24.1	97.3 ±16.7	>0.05
Fasting glucose (mg/dL)	88.3± 5.21	81.22± 7.34	>0.05
2-h glucose (mg/dL)	107.6 ±10.11	98.1± 7.14	>0.05
HbA_{1c} (%)	5.6 ± 0.5	4.9 ± 0.4	>0.05
Fasting Insulin (mIU/L)	17.5±5.9	8.4±2.5	<0.001 **
Fasting C peptide (pmol/L)	988±172	764 ±115	< 0.001* *
HOMA-IR	4.96 ± 1.87	2.23 ± 0.89	<0.001 **
Circulating IL-6 (pg/ml)	2.87 ± 1.04	1.12± 0.79	<0.05
Zonulin (ng/ml)	6.48 ± 2.03	3.65± 1.37	<0.001 **

Table (2): Comparison between demographic and biochemical data of simple steatosis and NASH groups:

Parameters	NASH (no=32) M±SD	Simple steatosis (n=24) M±SD	P value
Age (years)	38.6±4.4	35.3±3.5	>0.05
BMI (kg/m2)	29.5±2.01	25.5±1.6	<0.05*
AST (U/L)	51.1 ±12.5	33.2± 10.1	<0.05*
ALT (U/L)	59.6 ± **10.**3	41.7±8.2	<0.05*
γ-GT (U/L)	36.3 ±5.4	26.8± 6.7	<0.05*
Triglycerides (mg/dl)	106.2±11.8	92.1± 7.3	>0.05
Total cholesterol (mg/dl)	159.6±26.3	146.1±9.5	>0.05
HDL-C (mg/dl)	35.1±4.8	40.2± 5.1	<0.05*
LDL-L (mg/dl)	110.4±19.2	96.2±8.4	>0.05
Fasting Insulin (mU/L)	20.6±3.1	14.7±3.5	<0.05*
Fasting C peptide (pmol/L)	1096±64	844±31	<0.05*
HOMA-IR	5.82 ± 1.02	3.56±0.52	<0.01*
Circulating IL-6 (pg/ml)	3.02 ± 0.89	2.67±0.79	>0.05
Zonulin (ng/ml)	7.60 ± 0.91	4.91±0.46	<0.001**

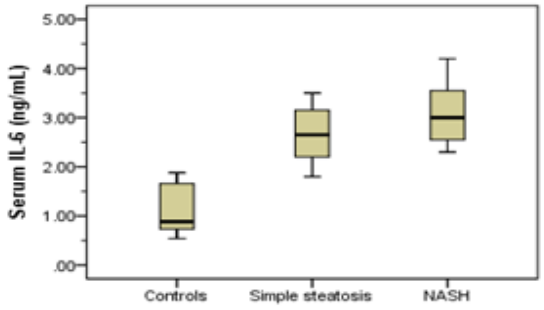

Figure 1: Serum IL-6 level in control, simple steatosis, and NASH groups

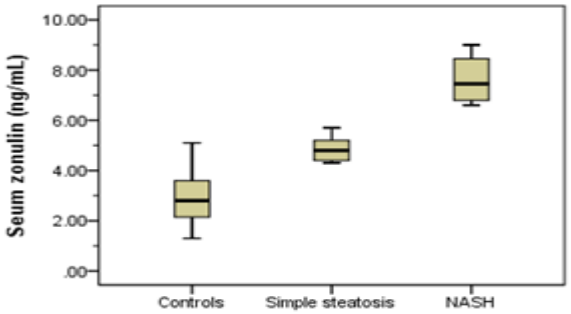

Figure 2: Circulating zonulin level in control, simple steatosis and NASH groups

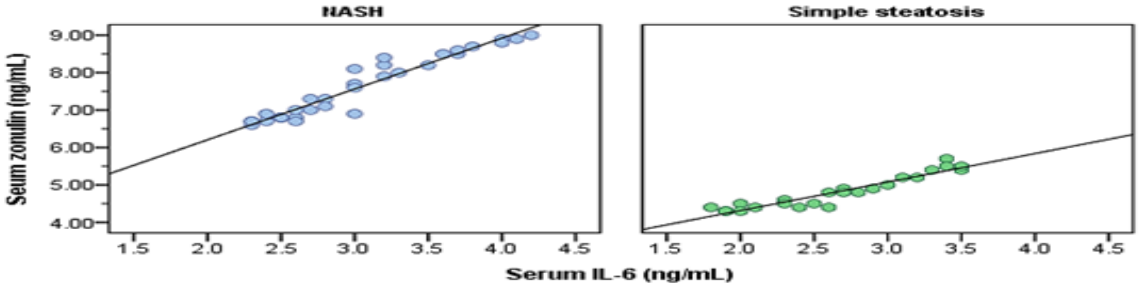

Figure 3: Correlation between serum zonulin level and serum IL-6 level in simple steatosis and NASH groups

Table (3): Correlation between pathogenic parameters in NAFLD patients with serum zonulin and IL6 (n=56):

Parameters	Serum zonulin		Serum IL-6	
	R	P	R	P
BMI	0.378	<0.05*	0.282	<0.05*
ALT	0.312	<0.05*	0.294	<0.05*
Triglycerides	0.296	<0.05*	0.027	>0.05
Total cholesterol	0.175	>0.05	0.165	>0.05
HDL-C	-0.397	<0.01*	-0.134	>0.05
Fasting Insulin	0.305	<0.05*	0.011	>0.05
HOMA-IR	0.413	<0.01*	0.145	>0.05
Liver histopathology	0.518	<0.001*	0.179	>0.05
Serum IL-6	0.288	<0.05*	----------	

Table (4): Correlation study between circulating zonulin and some pathogenic parameters in NASH and simple steatosis patients

Parameters	Circulating Zonulin (ng/mL)			
	NASH (n=32)		Simple steatosis (n=24)	
	r_s	*P*-value	r_s	*P*- value
Age	-0.42	0.017 ^{S}	-0.12	0.582 ^{NS}
Sex	0.00	0.985 ^{NS}	0.16	0.444 ^{NS}
BMI	0.75	<0.001 ^{HS}	0.92	<0.001 ^{HS}
ALT	0.86	<0.001 ^{HS}	0.89	<0.001 ^{HS}
Triglycerides	-0.60	<0.001 ^{HS}	-0.68	<0.001 ^{HS}
Total cholesterol	-0.89	<0.001 ^{HS}	-0.68	<0.001 ^{HS}
HDL-C	-0.95	<0.001 ^{HS}	-0.95	<0.001 ^{HS}
Fasting Insulin	0.98	<0.001 ^{HS}	0.87	<0.001 ^{HS}
HOMA-IR	0.92	<0.001 ^{HS}	0.96	<0.001 ^{HS}
Liver histopathology	0.91	<0.001 ^{HS}	0.64	<0.001 ^{HS}
Serum IL-6	0.96	<0.001 ^{HS}	0.94	<0.001 ^{HS}

- r^s: Spearman correlation coefficient, ^{NS} : Non significant at P-value ≥ 0.05, ^{S} : Significant at P-value < 0.05, ^{HS} : Highly significant at P-value <0.01

Table (5): Correlation study between serum interleukin-6 and some pathogenic parameters in NASH and simple steatosis patients

Parameters	Serum Interleukin-6 (ng/mL)			
	NASH (n=32)		Simple steatosis (n=24)	
	r_s	*P*-value	r_s	*P*- value
Age	-0.34	0.059 ^{NS}	-0.03	0.884 ^{NS}
Sex	-0.04	0.816 ^{NS}	0.13	0.535 ^{NS}
BMI	0.70	<0.001 ^{HS}	0.89	<0.001 ^{HS}
ALT	0.82	<0.001 ^{HS}	0.87	<0.001 ^{HS}
Triglycerides	-0.56	<0.001 ^{HS}	-0.71	<0.001 ^{HS}
Total cholesterol	-0.84	<0.001 ^{HS}	-0.70	<0.001 ^{HS}
HDL-C	-0.93	<0.001 ^{HS}	-0.90	<0.001 ^{HS}
Fasting Insulin	0.93	<0.001 ^{HS}	0.81	<0.001 ^{HS}
HOMA-IR	0.90	<0.001 ^{HS}	0.88	<0.001 ^{HS}
Liver histopathology	0.89	<0.001 ^{HS}	0.59	<0.002 ^{HS}

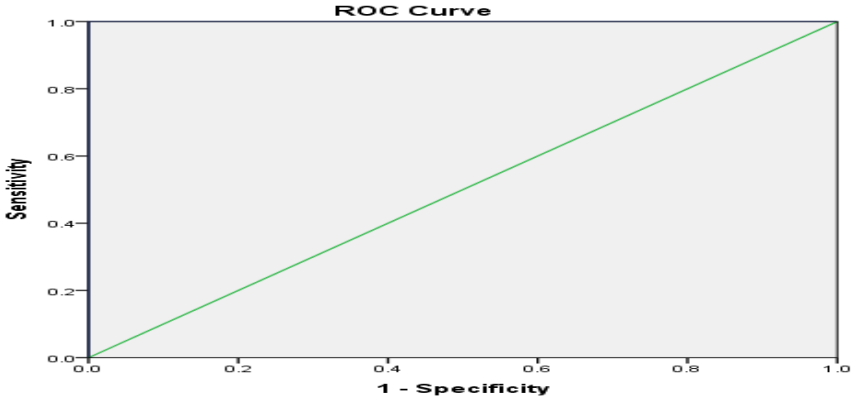

Figure 4: ROC curve for detection of NASH occurrence

Table (6): Shows a binary logistic regression

Variables	Univariable Analysis		Multivariable Analysis	
	OR	***P*-value [a]**	**Adjusted OR**	**Adjusted *P* value[a]**
Liver histopathology	1011615.99	< 0.001 [HS]	1005.496	< 0.001 [HS]
Age (years)	1.22	0.008 [HS]		
Sex; Male (ref.) Female	- 1.62	- 0.385 [NS]		
ALT (U/L)	2.92	< 0.001 [HS]		
BMI (kg/m2)	2114283.51	< 0.001 [HS]		
Triglycerides (mg/dL)	1.19	< 0.001 [HS]		
Cholesterol (mg/dL)	1.03	0.113 [NS]		
HDL-C (mg/dL)	0.65	< 0.001 [HS]	41.246	< 0.001 [HS]
HOMA-IR	1349.89	< 0.001 [HS]		
Fasting insulin (ng/mL)	8.83	< 0.001 [HS]		
Serum IL-6 (ng/mL)	3370.93	0.011 [S]		
Serum Zonulin (ng/mL)	338.72	< 0.001 [HS]	5.316 E+10	< 0.001 [HS]

[a]: Likelihood ratio test OR: odds ratio
- [NS] : Non significant at P-value ≥ 0.05 -[S] : Significant at P-value < 0.05
- [HS] : Highly significant at P-value <0.001

In fact, supporting this observation, zonulin gene has been reported to coincide with pre-haptoglobin- 2 (9), whose promoter is under IL- 6 control through STAT3 activation and miR-18a induction (21), through its collective metabolic activities and host interactions.Ultimately, production of cytokines such as IL-6 and TNFα is one of the earliest events in many types of liver injury.

Zonulin had been merely linked to diabetes mellitus, obesity (12), obesity conjoined to the bacterial burden (22), and lately to NAFLD children (4). Occurrence of small intestinal bacterial overgrowth (SIBO) and increased intestinal permeability due to "leaky" tight junctions has been reported in patients with NAFLD (17).Consequently, the current study was designed to find the possible role of circulating zonulin in NAFLD adult patients either with simple steatosis or with NASH and its relation to liver status, biochemical parameters and inflammatory cytokine, IL-6.

Elevated serum zonulin in NAFLD patients rather than healthy individuals and in those with NASH patients rather than simple steatosis group suggested a great potential role of serum zonulin in NAFLD pathogenesis and progression to NASH.

The documented positive linkage between serum zonulin levels with the clinical, biochemical and histopathological markers of NAFLD: BMI, ALT, triglycerides, fasting insulin, HOMA-IR , IL6, along with the pathological severity of steatosis had authenticated their role in adultery NASH provision as previously conveyed in children by (4). The positive correlation of serum zonulin levels with the severity of steatosis, and lobular inflammation and fibrosis of NASH had been supported by the study of (23) who conveyed their study on children with biopsy-proven NAFLD, with reportedly, significant increase in gut permeability that positively correlated with liver disease severity (24).Of note, the results of (4) *and* (8) had refuted any relation of gut permeability and NAFLD severity or disease progression, exceptionally considering steatohepatitis and NAFLD as two separate disease entities. However the studies previously mentioned had evaluated intestinal permeability relying on different modalities obviously influenced by patients' clinical and metabolic characteristics, along with food variants attributes. Dissimilarly, for avoidance of these pitfalls, in this study, we relied on serum zonulin levels for mirroring the intestinal permeability.

In this study, the documented inverse correlation between serum zonulin levels and HDL-Chad been previously mentioned in **(13)** study. They commented on this negative correlation along with another negative correlation with insulin sensitivity. Counting on this, the assumptive relationship between serum zonulin and obesity-related circulating IL-6 increase was built**.** IL6 had shown magnified serum levels in NAFLD patients when compared to healthy individuals, however no substantial difference was narrated between patients with simple steatosis and those with NASH. A notion freezes any possibility of a speculative share in NASH precognition.

Il-6 connections to all studied parameters except for BMI and triglycerides were negated. Histopathologicaly, the positive linkage was only detected with degree of steatosis, but not with the presence of nonalcoholic steatohepatitis (NASH), lobular inflammation or fibrosis score. Assumptions suggesting that, zonulin might be involved in initiation rather than progression of NAFLD. It could be considered a new confirmation to the new suppositions that simple steatosis and NASH are different and not necessarily inter-related diseases.

The progressive increase of serum circulating zonulin from control to simple steatosis to NASH groups along with its positive correlation with IL6 has substantiated their roles in disease occurrence and progression. The regression analysis had added a value signifying its role in NAFLD pathogenesis and disease progression.

This study was just a preliminary share in setting up the initial pillars of understanding the mechanisms involved in dysbiosis and its functional consequences for human diseases. Ultimately, targeting the development of new therapies directed to restoration of intestinal eubiosis and homeostasis should be the uttermost research concern.

Acknowledgments

We are indebted to all subjects for agreeing to participate in this study.

Declaration of Interest

There is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

This research did not receive any specific grant from any funding agency in the public ,commercial or not-for-profit sector.

Author contributions

All authors listed in this manuscript contributed significantly to the development of the research and writing of the manuscript.

References

1. Angulo, P. Nonalcoholic fatty liver disease. N Engl J Med. 346, 1221-1231, 2002

2. Williams, C.D., Stenge, l. J., Asike, M.I., Torres, D.M., Shaw, J., Contreras, M., Landt, C.L. and Harrison, S.A. Prevalence of nonalcoholic fatty liver disease and nonalcoholic steatohepatitis among a largely middle-aged population utilizing ultrasound and liver biopsy: a prospective study.Gastroenterology. 140 (1), 124-31, 2011

3. Sass, D., Chang, P. and Chopra, K. Nonalcoholic Fatty Liver Disease: A Clinical Review Digestive Diseases and Sciences. 50 (1), 171–180, 2005

4. Pacifico, L., Bonci, E., Marandola, L., Romaggioli, S., Bascetta, S. and Chiesa, C. Increased circulating zonulin in children with biopsy-proven nonalcoholic fatty liver disease. World Journal of Gastroenterology. 2014

5. Pereira, K., Salsamendi, J. and Casillas, J. The Global Nonalcoholic Fatty Liver Disease Epidemic: What a Radiologist Needs to Know. Journal of Clinical Imaging Science. 5 (2), 1-19, 2015

6. Martinez, S. M., Crespo, G., Navasa, M. and Forns, X. Noninvasive assessment of liver fibrosis. Hepatology. 53(1), 325–35, 2011

7. Schwenger, K.J. and Allard, J.P. Clinical approaches to non-alcoholic fatty liver disease. World J. Gastroenterol. 20 (7), 1712–23, 2014

8. Miele, L., Marrone, G., Lauritano, C., Cefalo, C., Gasbarrini, A., Day, C. and Grieco, A. Gut-liver axis and microbiota in NAFLD: insight pathophysiology for novel therapeutic target. Curr Pharm Des. 19, 5314-5324, 2013

9. Tripathi, A., Lammers, K.M., Goldblum, S., Shea-Donohue, T., Netzel-Arnett, S. et al. Identification of human zonulin, a physiological modulator of tight junctions, as prehaptoglobin-2. Proc Natl Acad Sci USA. 106, 16799–16804, 2009

10. Smecuol, E., Sugai, E., Niveloni, S., Vázquez, H., Pedreira, S., Mazure, R., Moreno, M.L., Label, M., Mauriño, E., Fasano, A., Meddings, J. and Bai, J.C. Permeability, zonulin production, and enteropathy in dermatitis herpetiformis. Clin Gastroenterol Hepatol. 3, 335-341, 2005

11. Wang, W., Uzzau, S., Goldblum, S.E. and Fasano, A. Human zonulin, a potential modulator of intestinal tight junctions. J Cell Sci. 113(24), 4435-40, 2000

12. Sapone, A., de Magistris, L., Pietzak, M., Clemente, M.G., Tripathi, A., Cucca, F. et al. Zonulin upregulation is associated with increased gut permeability in subjects with type 1 diabetes and their relatives. Diabetes. 55, 1443-9, 2006

13. Moreno-Navarrete, J.M., Sabater, M., Ortega, F., Ricart, W. and Fernandez-Real, J.M. Circulating zonulin, a marker of intestinal permeability, is increased in association with obesity-associated insulin resistance. PLoS One. 7, e37160, 2012

14. Wieckowska, A.1., Papouchado, B.G., Li, Z., Lopez, R., Zein, N.N. and Feldstein, A.E. Increased hepatic and circulating interleukin-6 levels in human nonalcoholic steatohepatitis. Am J Gastroenterol. 103 (6), 1372-9, 2008.

15. Chalasani, Z.Y.Z., Lavine, J., Diehl, M., Brunt, E., Cusi, K., Charlton, M. and Sanyal, A. Diagnosis and Management of Non-Alcoholic Fatty Liver Disease: Practice Guideline by the American Association for the Study of Liver Diseases, American College of Gastroenterology, and the American Gastroenterological Association. HEPATOLOGY. 2005-2024, 2012

16. Emoto, M., Nishizawa, Y., Maekawa, K., Hiura, Y., Kanda, H., Kawagishi T, Shoji T, Okuno Y. and Morii H. Homeostasis model assessment as a clinical index of insulin resistance in type 2 diabetic patients treated with sulfonylurea. Diabetes Care. 22, 818–822, 1999

17. Machado, M. and Cortez-Pinto. Non-alcoholic fatty liver disease and insulin resistance. Eur J Gastroenterol Hepatol. 17 (8), 823-826, 2005

18. Kleiner, D.E., Brunt, E.M., Van Natta, M., Behling, C., Contos, M.J., Cummings, O.W., Ferrell, L.D., Liu, Y.C., Torbenson, M.S., Unalp-Arida, A., Yeh, M., McCullough, A. J. Design and validation of a historical scoring system for nonalcoholic fatty liver disease. Hepatology. 41, 1313-1321,2005

19. Compare, D., Coccoli, P., Rocco, A., Nardone, O.M., De Maria, S., Cartenì, M. and Nardone, G. Gut--liver axis: the impact of gut microbiota on non alcoholic fatty liver disease. Nutr. Metab. Cardiovasc. Dis. 22, 471-476, 2012

20. Duseja, A. and Chawla, Y.K. Obesity and NAFLD: The role of bacteria and microbiota. Clin Liver Dis. 18, 59-71, 2014

21. Hansen, D., Dendale, P., Beelen, M., Jonkers, R.A, Mullens, A. et al. Plasma adipokine and inflammatory marker concentrations are altered in obese, as opposed to non-obese, type 2 diabetes patients. Eur J Appl Physiol. 109, 397–404, 2010

22. Brock, M., Trenkmann, M., Gay, R.E., Gay, S., Speich, R. et al. MicroRNA- 18a enhances the interleukin-6-mediated production of the acute-phase proteins fibrinogen and haptoglobin in human hepatocytes. J Biol Chem. 286-301, 2011

23. Giorgio, V., Miele, L., Principessa, L., Ferretti, F., Villa, M.P., Negro,V., Grieco, A., Alisi, A. and Nobili, V. Intestinal permeability is increased in Children with non-alcoholic fatty liver disease, and correlates with liver disease severity.Dig Liver Dis. 46, 556-560, 2014

24. Zak-Gołąb, A., Kocełak, P., Aptekorz, M., Zientara, M., Juszczyk, L., Martirosian, G., Chudek, J. and Olszanecka-Glinianowicz, M. Gut microbiota, micro-inflammation, metabolic profile, and zonulin concentration in obese and normal weight subjects. Int J Endocrinol. 674106, 2011