Al-Azhar University Journal
for Medical and Virus Research
and Studies

Zonulin as a Marker of Intestinal Barrier Integrity in Infants with Cholestasis

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Abstract

Neonatal cholestasis is the consequence of reduced bile synthesis by the hepatocyte or blocked bile flow via the intra- or extrahepatic biliary tree, which leads in the buildup of biliary chemicals in the liver, blood, and extrahepatic tissue. Cholestasis inflicts damage on the patients' gut in addition to their liver, irrespective of the etiology. The sole known physiological regulator of intercellular tight junctions is zonulin, which has been shown to be correlated with intestinal permeability. Consequently, it might be used as a metric for compromised gut barrier function. The aim of this work is to determine whether gut barrier integrity was impaired in infants with biliary atresia in comparison to other cases of cholestasis by measuring serum level of zonulin and intestinal fatty acid binding protein IFABP. This was a case control study. The study population was composed of 30 infants diagnosed as biliary atresia (BA), 27 infants with cholestasis other than BA and 29 apparently healthy infants serving as a control group. During a two-year period, all patients were recruited from the outpatient clinic and the inpatient ward of the pediatric Hepatology, Gastroenterology, and Nutrition department at the National Liver Institute, Menoufia University, in accordance with the inclusion and exclusion criteria (2022-2023). They were matched based on their gender and age. Infants with cholestasis had substantially increased serum zonulin levels and IFAB as compared to healthy controls ($p < 0.001$). Moreover, the serum levels of zonulin were not significantly higher in BA than in cholestasis or any other condition. Serum zonulin and IFABP concentrations were significantly amplified in cholestasis than healthy control as an indicator of intestinal permeability affection in cholestasis.

Keywords: Intestinal barrier; integrity; infants; cholestasis

1. Introduction

The mucosal surface integrity is vital for protecting the body of human contrary to harmful substances for example toxins, antigens or bacteria. The gut function is referred to as the barrier function of intestine. This intestinal epithelial barriers are constituted of a variety of defensive mechanisms .These barriers, which can be categorized as physical and immunological, may be influenced by the lack of bile in the intestinal lumen and by cholestasis [1].

Addition of biliary compounds in the blood, liver, and tissues outside the liver occurs due to diminished bile synthesis by obstruction of bile flow through the intra- or extrahepatic biliary tree or hepatocytes., which result in neonatal cholestasis [2]. Regardless of the etiology, cholestasis results in damage to the patients' intestines in addition to their liver [3].

The immune system acting a critical part in regulating the permeability of intestine by influencing the expression of tight junction (TJ) proteins through the action inflammation-causing substances such as interferon gamma and tumor necrosis factor (TNF) [4].

Without enough bile salts, there is an imbalance in the bacteria that are present in the intestine and the proliferation of gram-negative bacteria, as bile acids can prevent the growth of certain bacteria. Bile also holds immunoglobulin A, that improves the defense of mucosal by either binding to viruses and microbes or sustaining mucosal integrity [5].

Additionally, Research suggests that antibodies in bile, whether specific or nonspecific, can hinder the process where bacteria are taken into cells (endocytosis) by enterocytes, or their attachment to the lining of the intestines (intestinal mucosa), thereby preventing bacterial translocation [1]. There is a bidirectional interaction between the gut flora and bile acids. In turn, intestinal microorganisms have the capacity to modify the bile acid pool, which in turn can influence the gut microbiota community [6].

Moreover, Bile promotes growth and development of the intestinal lining. Promoting hypertrophy of intestinal wall components and increasing villous density. multiple studies have also demonstrated that bile plays a vital role in maintaining bile helps maintain the integrity of enterocyte tight junctions by controlling the expression of crucial proteins associated with these junctions [1].

Zonulin is the main regulator of tight junctions between cells and has been linked to the permeability of intestine. As a result, it could potentially serve as a diagnostic marker for impaired function of gut barrier [7].

Zonulin, a serum biomarker that reflects intestinal permeability, is identical to pre-haptoglobin. Nevertheless, Zonulin is secreted by tissues beyond the digestive tract, including body fat, heart, brain, immune cells, liver, lungs, kidney, and epidermis, in addition to enterocytes. Furthermore, the quantities of zonulin in serum are symptomatic of the release of secretions from organs besides the tract of digestion [8, 9].

An approximately 15 k Da cytosolic protein known as fatty acid binding protein (FABP) is considered accountable for the binding and fatty acids delivery. Depending on the tissue in which they are located, there are several types of FABP with distinct immunological roles. Besides the intestines, Fatty acid binding proteins (FABPs) are found not only in the intestines but also in the heart, liver, muscle, and adipose tissue. In the intestines, enterocytes, especially those in the absorptive part of the villus epithelium, express two types of FABPs: liver-type (L-FABP or FABP1) and intestinal fatty acid binding proteins (I-FABP or FABP2). L-FABP is present in the liver and kidney, while I-FABP is exclusively found in the intestines. Additionally, In the distal ileum, there is a protein known as ileal lipid or bile acid binding protein (ILBP or BABP, also known as FABP6), which is present unique

among gut FABPs for its strong affinity for binding bile acids [10].

2. Patients and Methods

2.1 Study Population

This was a case control study. The study population consisted of 30 infants diagnosed with BA, 27 infants with cholestasis other than BA and 29 apparently healthy infants functioning as a control group. During a two-year period, all Participants were recruited from both the outpatient clinic and the inpatient ward of the Pediatric Hepatology, Gastroenterology, and Nutrition department at the National Liver Institute, Menoufia University, in accordance with the inclusion and exclusion criteria (2022-2023). They were matched based on their gender and age. BA was diagnosed following the criteria outlined by McKiernan et al. and Fischler et al., respectively, after exclusion of alternative causes of newborn cholestasis and confirmation was acquired after laparotomy with an operative cholangiogram [11].

Diagnosing progressive familial intrahepatic cholestasis (PFIC) involves integrating clinical, biochemical, radiographic, and liver histological indicators., as well as specific procedures to rule out other potential causes of infant cholestasis [12].

Congenital CMV infection is diagnosed by detecting the virus in saliva, blood, or urine within the first three weeks after birth [13].

In neonates with pertinent medical histories, inspissated bile syndrome was considered in the event of severe unconjugated hyperbilirubinemia requiring exchange transfusion and ultrasound findings, such as bile sediment and/or bile duct dilatation, and/or improvement with ursodeoxycholic acid (UDCA) therapy. [14]

The patients with a characteristic facial morphology were diagnosed with Alagille syndrome. The jawline is pointed, the eyes are depressed, and the forehead is prominent. The patients exhibit extrahepatic findings in addition to the typical facial morphology, as well as bile tract paucity. Extrahepatic findings consist of skeletal defects, cardiac abnormalities (particularly peripheral pulmonary artery stenosis), and butterfly vertebrae [15].

Cystic fibrosis case was diagnosed by genetic testing.

Galactosemia case was diagnosed by positive GAL profile with positive reducing substance in urine for galactose.

Two groups were established for all patients participating in the investigation. BA group and newborn cholestasis other than BA with comprehensive comparison Groups are categorized based on demographic data, symptoms, signs, and ultrasound findings.

The study has been submitted and has got Approval from the Ethical Committee of the National Liver institute; Menoufia University was obtained. Written consent was also obtained from the parents of entirely study contributors.

2.2 History, Clinical Examination, and Investigations

Full history taking with stress on family history of similar conditions, consanguinity, presenting symptoms (onset, course, duration and associated symptoms), symptoms of hepatocellular decompensation e.g. ascites, hepatic encephalopathy. symptoms of vascular decompensation e.g. hematemesis and melena.

Clinical examination with stress on the following: jaundice, pallor, hepatomegaly, splenomegaly, ascites.

investigations were recruited from patients' files: Routine laboratory parameters, investigations according to suspected etiology, Imaging studies, Liver biopsy.

2.3 Blood Sampling:

Initially, the blood samples from each patient were centrifuged at 1500g for 10 minutes. Furthermore, the serum was centrifuged at 2000g for 3 minutes at 4°C

after being aliquoted. Subsequently, the supernatant was kept at -80°C.

2.4 Measurements of ELISA

A double-antibody sandwich ELISA Kit (produced by Shanghai Korain Biotech Co., Ltd.) was employed to measure serum zonulin levels. The serum zonulin assay was conducted. Serum zonulin concentrations were determined following the instructions provided by the kit manufacturer spectrophotometrically using an ELISA microtiter plate reader (ELX800, Biotech Instruments, Inc, USA, SN: 1502175).

Serum IFABP

The ELISA kit reagent (Glory Science Co., Ltd., Del Rio, TX, USA; catalog number: 96623) was used to measure the concentration of I-FABP at a wavelength of 450 nm.

2.5 Statistical Analysis

Mean ± SD or median values were employed to present data for continuous variables. The frequencies and percentages were employed to present the data for categorical variables. The ANOVA, Kruskal-Wallis, or chi-square test were employed to detect significant differences in clinicopathological parameters among groups, depending on the type of data used. The differences between the categories for normally distributed data were compared using post hoc analyses the ideal diagnostic performance the diagnostic performance of the test was evaluated using receiver operating characteristic (ROC) curve analysis to determine threshold values of zonulin. Statistical significance was set at p-values less than 0.05. Data analysis was performed using SPSS Statistics version 25.0 (SPSS, Inc.).

3. Results

The Patient's Characteristics: As shown in Tables 1 and 2 out of the 57 neonates with cholestasis included in this study, 27 were diagnosed with cholestasis of various origins (47 percent) and 30 with BA (53 percent of the total). A control group of 29 newborns were healthy. Detailed illustrations of the clinical, demographic, and laboratory data are provided.

Table 1: Statistical comparisons regarding demographic data in biliary atresia and cholestasis subgroups and healthy control group

Demographic data	Biliary atresia (GIa, n= 30)	Cholestasis (GIb, n=27)	Healthy controls (GII, n=29)	Significance test	P-value
Age (day)				F= 1.09 [a]	0.343 [NS]
Mean ± SD	69.00 ± 15.13	62.89 ± 17.03	62.17 ± 24.99	0.343 [NS]	
Range (min-max)	37- 100	30 - 90	30 - 120		
Sex, n (%)					
Male	12 (40)	16 (59.3)	17 (58.6)	χ^2=2.81[b]	0.246 [NS]
Female	18 (60)	11 (40.7)	12 (41.4)		
Family history, n (%)					
No	30 (100)	23 (85.2)	29 (100)	χ^2=9.17 [c]	0.044 [S]
Yes	0 (0)	4 (14.8)	0 (0)		
Consanguinity, n (%)					
No	22 (73.3)	18 (66.7)	25 (86.2)	χ^2=3.02 [b]	0.221 [NS]
Yes	8 (26.7)	9 (33.3)	4 (13.8)		

Interquartile range (IQR) (difference between 1st and 3rd quartile). %: Percentage through subgroups of study a: ANOVA test, b: the test of Pearson's chi-square, c: test of Fisher's Exact, NS: Not statistically significant at a P-value of 0.05 or greater.

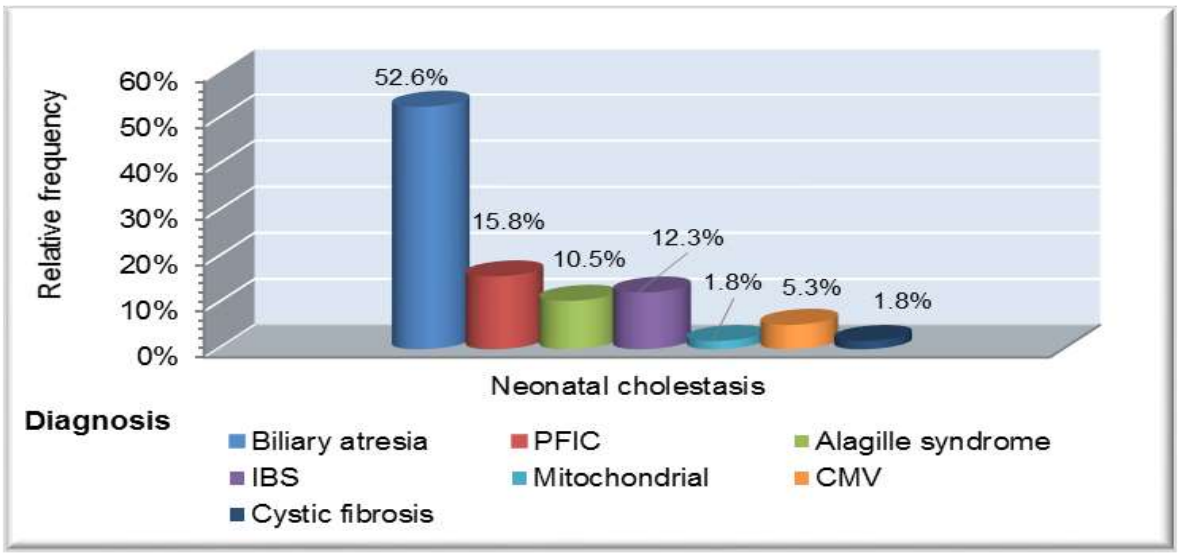

Figure .1: Prevalence of diagnosed diseases in neonatal cholestasis group

Table .2: Statistical comparisons regarding clinical characteristics in biliary atresia and cholestasis subgroups.

Clinical characteristics	Biliary atresia (GIa, n= 30)	Cholestasis (GIb, n=27)	Significance test	P-value
Pruritus	1 (3.3 %)	14 (51.9 %)	χ^2=17.25 [a]	<0.001 [HS]
Stool color	30 (100)	27 (100)	χ^2=16.89 [a]	<0.001 [HS]
Colored	0 (0 %)	12 (44.4 %)		
Clay	30 (100 %)	15 (55.6 %)		
Hepatomegaly	29 (96.7 %)	17 (63 %)	χ^2=10.37 [a]	0.001 [HS]
Liver span (cm)			z= 2.73 [b]	P=0.006 [HS]
Median (IQR)	8.00 (1.00)	7.00 (2.00)		
Range (min-max)	5.00 - 9.00	5.00 - 11.00		
Splenomegaly	16 (53.3 %)	7 (25.9 %)	χ^2=4.44 [a]	0.035 [S]
Splenic length (cm)			z= 3.17 [b]	P=0.002 [HS]
Median (IQR)	5.50 (1.00)	5.00 (1.00)		
Range (min-max)	4.00 - 8.00	4.00 - 7.00		
Ascites	30 (100 %)	27 (100 %)	-	-
Cardiac anomaly, n (%)	4 (13.3 %)	6 (22.2 %)	χ^2=0.78 [c]	0.492 [NS]

a: Pearson chi-square test, b: Mann-Whitney U test, c: Fisher's Exact test, NS: Non-significant at p-value ≥ 0.05 S: Significant at p-value < 0.05, HS: Highly significant at p-value < 0.0.

Table 3: Statistical comparisons regarding liver function tests in biliary atresia and cholestasis subgroups and healthy control groups.

Laboratory data	Biliary atresia (GIa, n= 30)	Cholestasis (GIb, n=27)	Healthy controls (GII, n=29)	Significance test	Pairwise comparisons¥
Range (min-max)	64 – 638	62 – 450	31 – 199		p3=0.003 HS
ALT (U/L)				χ^2= 56.84 a	p1=0.494 NS
Median (IQR)	117.50 (97.75)	100 (135)	17.00 (5)	P<0.001 HS	p2<0.001 HS
Range (min-max)	50 – 500	20 – 550	9 – 23		p3<0.001 HS
AST (U/L)				χ^2= 57.75 a	p1=0.490 NS
Median (IQR)	225 (222.50)	212 (240)	21 (8)	P<0.001 HS	p2<0.001 HS
Range (min-max)	66 – 700	42 – 650	9 – 33		p3<0.001 HS
ALP (U/L)				z= 3.85 b	-
Median (IQR)	700 (317.50)	400 (340)		P<0.001 HS	
Range (min-max)	300 – 1400	166 – 1450			
GGT (U/L)				z= 3.13 b	-
Median (IQR)	457 (460)	190 (410)		P=0.002 HS	
Range (min-max)	120 – 1500	19 – 1700			
Total bilirubin (mg/dL)				χ^2= 57.26 a	p1=0.991 NS
Median (IQR)	9.00 (4.25)	9.00 (6.00)	0.63 (0.29)	P<0.001 HS	p2<0.001 HS
Range (min-max)	6.00 - 17.00	2.50 - 23.00	0.33 - 1.08		p3<0.001 HS
Direct bilirubin (mg/dL)				χ^2= 57.64 a	p1=0.950 NS
Median (IQR)	6 (4)	6 (2)	0.15 (0.06)	P<0.001 HS	p2<0.001 HS
Range (min-max)	3 – 12	10 – 20	0.09 - 0.21		p3<0.001 HS
Albumin (g/dL)				z= 1.94 b	-
Median (IQR)	4 (0.32)	3.7 (0.50)		P=0.052 S	
Range (min-max)	3.3 - 4.5	2.2 – 5			
Total protein (g/dL)				z= 1.71 b	-
Median (IQR)	5.45 (1)	5 (1)		P=0.087 S	
Range (min-max)	4.5 – 7	3.3 – 7			

a: Kruskal-Wallis test, b: Mann-Whitney U test, c: Pearson's chi-square test, ¥: If significant Kruskal-Wallis test, multiple pairwise comparisons were adjusted by Dunn-Sidak post hoc test, p1: p-value for the difference between biliary atresia and cholestasis (GIa vs. GIb), p2: p-value for the difference between biliary atresia and healthy controls (GIa vs. GII), p3: p-value for the difference between cholestasis and healthy controls (GIb vs. GII).

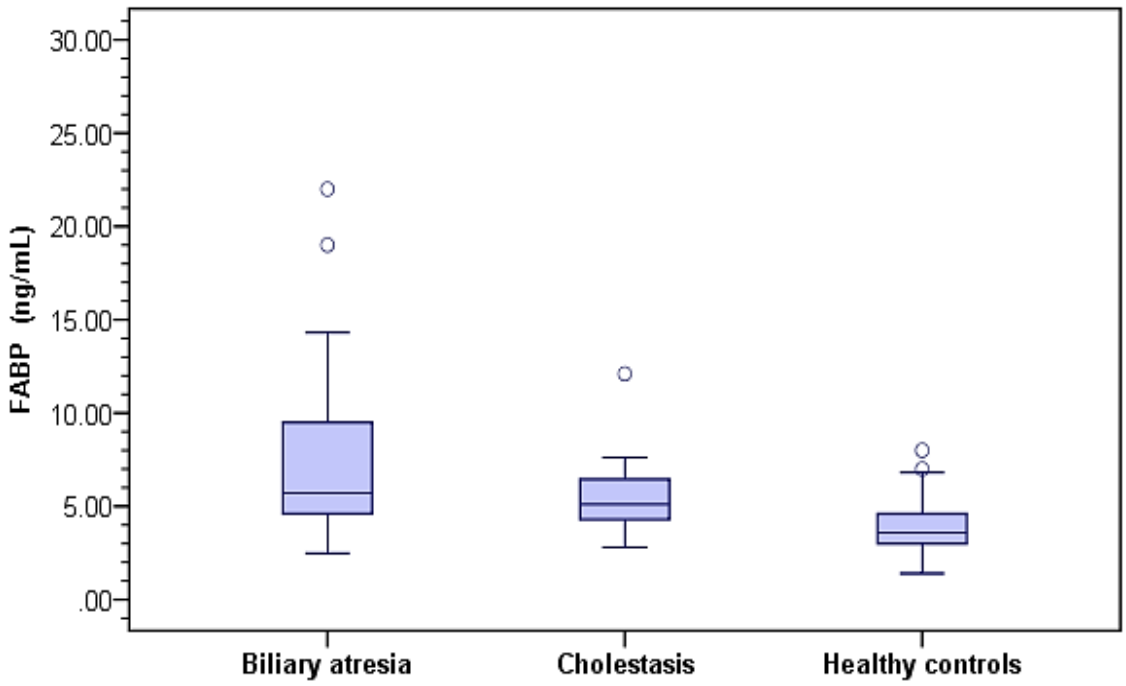

Figure .2: Levels of serum zonulin in neonatal cholestasis and healthy control groups

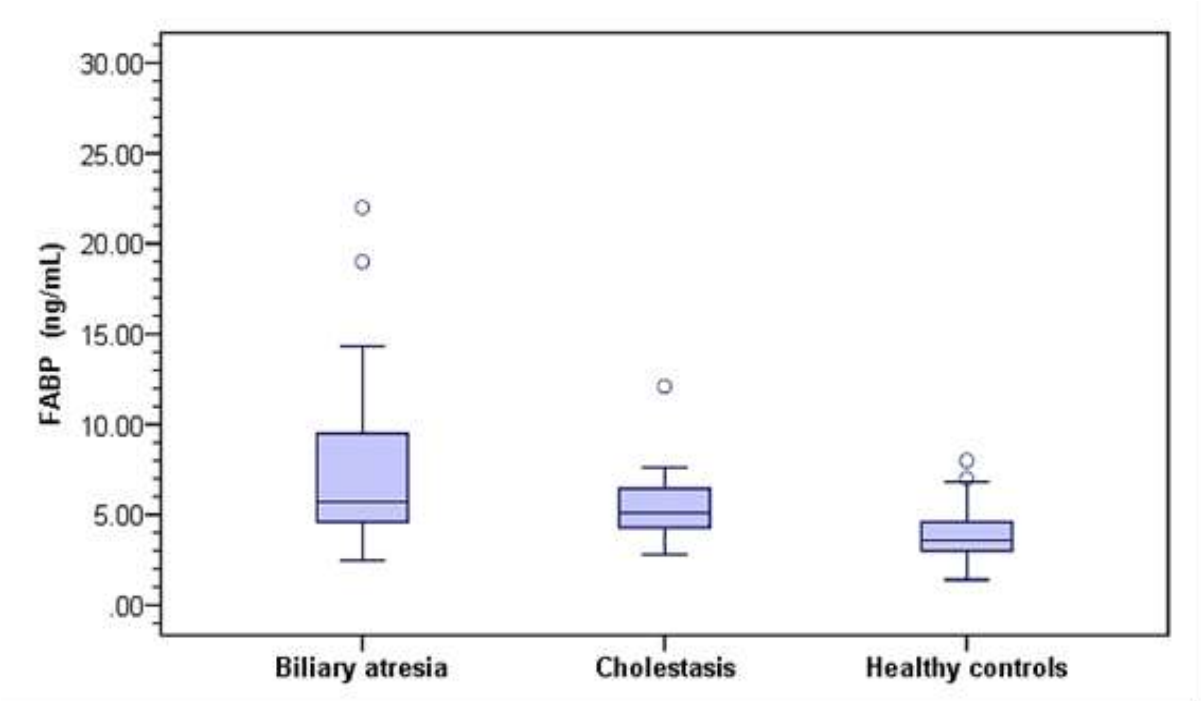

Figure .3: Levels of FABP in neonatal cholestasis and healthy control groups.

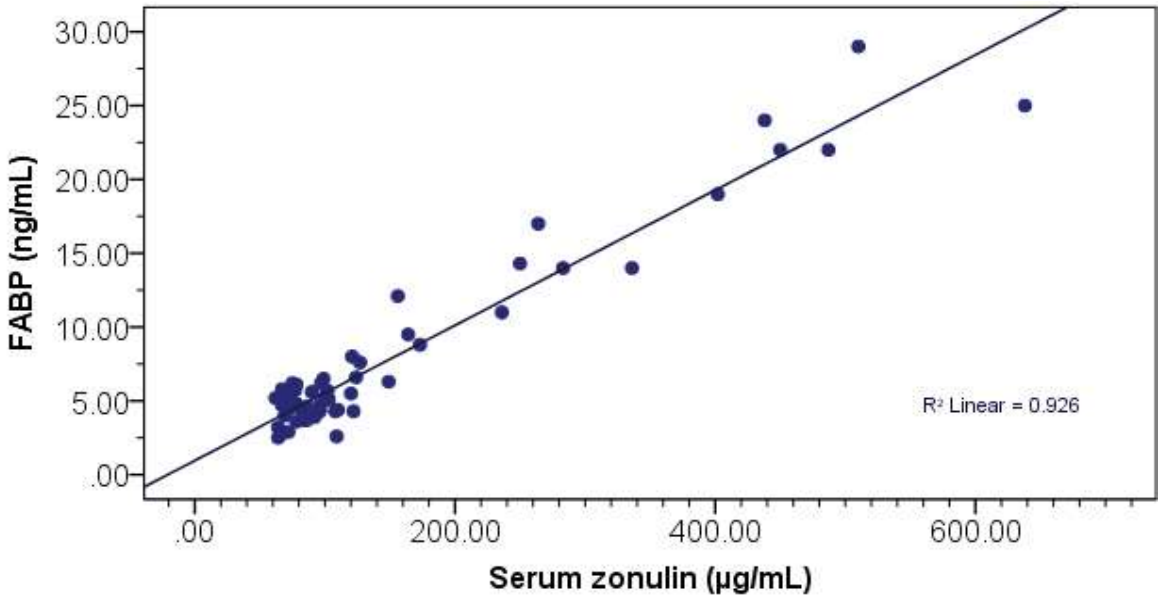

Figure 4: Correlation between FABP and zonulin in neonatal cholestasis group

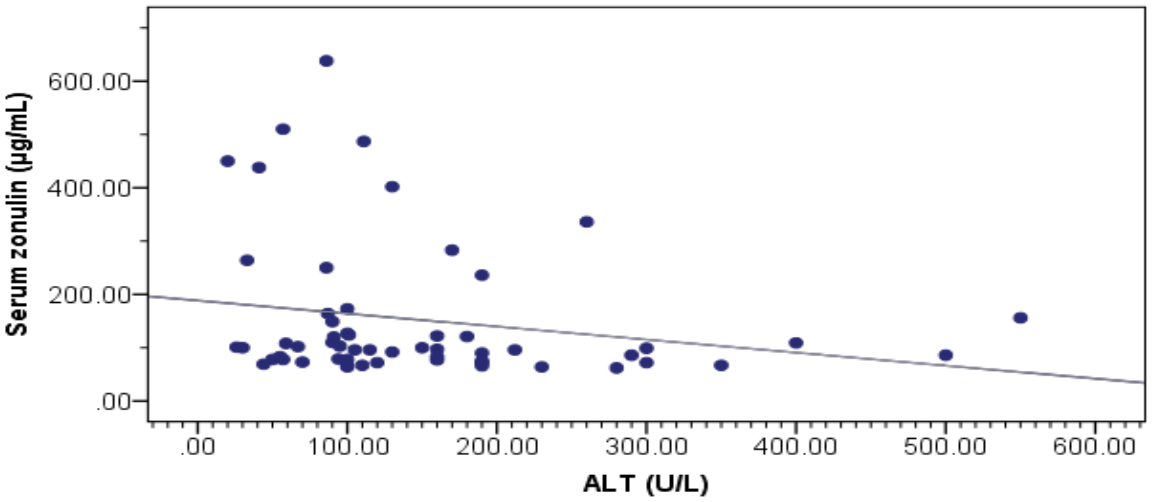

Figure 5: Correlation between serum zonulin and ALT activity in neonatal cholestasis group

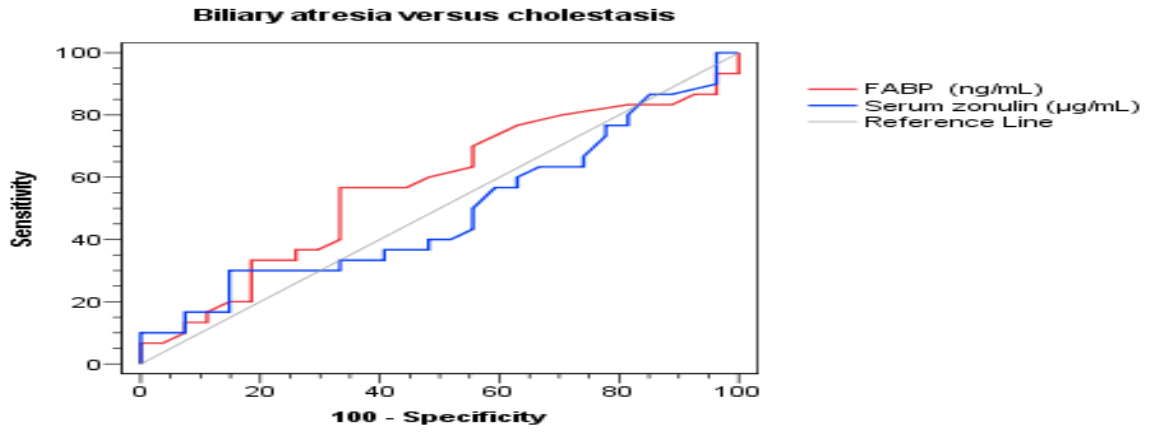

Figure 6: ROC curves for discrimination between biliary atresia and cholestasis subgroups.

4. Discussion

Neonatal cholestasis results from impaired bile formation by the hepatocyte or from obstruction of bile flow through the intra- or extrahepatic biliary tree leading to the accumulation of biliary substances in the liver, blood and extrahepatic tissue [2]. Regardless of the etiology, cholestasis causes injury not only to the patients' liver but also to their intestine [3].

Cholestatic jaundice affects approximately 1 in 2,500 infants and can result from various causes, including infections, anatomic abnormalities of the biliary system, endocrinopathies, genetic disorders, metabolic abnormalities, toxin and drug exposures, parenteral nutrition (PN) administration, cardio-vascular dysfunction, and neoplastic processes [16]. The most common identifiable causes include BA (25%–40%); genetic and metabolic diseases, including α1-antitrypsin (A1AT) deficiency (10%–20%), Alagille syndrome (ALGS; 2%–14%), cystic fibrosis (CF); progressive familial intrahepatic cholestasis (PFIC); hypopituitarism (5%); inspissated bile syndrome; idiopathic neonatal hepatitis (INH) or transient neonatal cholestasis (TNC); and PN-associated cholestasis (PNAC) in preterm infants and those with intestinal failure [17].

Over the past decades, there has been evidence that intestinal barrier integrity loss plays a key role in the development of multiple diseases [18].

Multiple studies investigated the possibility of intestinal barrier integrity loss in adults with sepsis, inflammatory, autoimmune diseases, and obstructive jaundice before and after surgery [19]. This study is to assess the intestinal barrier integrity in infants with cholestasis at our locality.

Zonulin, primarily discovered in 2000 by Fasano, is a 47 kDa protein that increases intestinal permeability in nonhuman primate intestinal epithelia, participates in the development of intestinal innate immunity, and is overexpressed in autoimmune disorders in which TJ dysfunction is central, including celiac disease and type 1 diabetes [21].

In the present study, the functional assessment of intestinal barrier loss was performed by evaluating serum zonulin and FABP level. This test was chosen because it is a simple single blood test.

In our study we found that regarding different etiologies of NC in the studied infants. BA was the most common etiology in group I (52.6%) followed by PFIC disease (15.8%) then Alagile syndrome (10.5%) followed by inspissated bile syndrome (12.3%) then CMV hepatitis (5.3%) and others.

Our results were consistent with, [22] who aimed to determine the various causes of NC that BA was the most common cause of NC (37%) followed by PFIC (12%).

In our study we found that there was no statistically significant difference between group I, group II and group III as regard age and sex.

In the present study, clay-colored stools were significantly more common in infants with BA (100%) than in infants with non-BA (55.6%). However, clay colored stools are not diagnostic for BA because many severe intrahepatic cholestasis may also be associated with persistently pale stools [16, 17]. Therefore, in spite highly significant, clay-colored stools can be present in other etiologies of NC. In the present study, abnormal liver function tests were found in all NC cases. Liver function tests are expected to be deranged nonspecifically in all NC with little yield to the specific diagnosis, out of some exception like normal GGT [21]. There is no biochemical test or imaging method alone or in a combination that can adequately differentiate BA and other NC diseases [22] . In the present study, GGT and ALP were significantly higher in BA than in non-BA cholestasis. GGT is considered to be more specific than ALP, due to the sharing bone origin for this elevatation of ALP [23].

El-Guindi et al., [24] reported that GGT in BA was higher than in other NC disorders with diagnostic performance of 88% sensitivity and 88% specificity. However, it should be kept in mind that very high ALP and GGT levels can also be observed in ductopenias, cholangitic type of CHF, CMV infection, PFIC type III, and IBS [25]. In the present study there was no statistically significant difference between BA and non-BA cases as regards bilirubin, total proteins, ALT, and AST. These results are in agreement with that of McKiernan et al., [26] who reported that the transaminases raised levels were sensitive markers of hepatic inflammation, however, they had little individual diagnostic and prognostic value in BA. In contrast Sarı et al., [25] study revealed that AST, ALT, and bilirubin values were found to be significantly higher in patients with canalicular cholestasis compared to patients with hepatocellular cholestasis. In the present study, the functional assessment of intestinal barrier loss was performed by evaluating serum zonulin and FABP level. This test was chosen because it is a simple single blood test, and various studies. An increased intestinal permeability has been described in various gastrointestinal and non-gastrointestinal disorders. Nevertheless, the concept and definition of intestinal permeability is relatively broad and includes not only an altered paracellular route, regulated by tight junction proteins, but also the transcellular route involving membrane transporters and channels, and endocytic mechanisms. Paracellular intestinal permeability can be assessed in vivo by using different molecules (e.g., sugars, polyethylene glycols, 51Cr-EDTA) and ex vivo in Ussing chambers combining electrophysiology and probes of different molecular sizes. The latter is still the gold standard technique for assessing the epithelial barrier function, whereas in vivo techniques, including putative blood biomarkers such as intestinal fatty acid-binding protein and zonulin, are broadly used despite limitations [33].

This study showed that both I-FABP and zonulin levels were significantly higher in both groups of cholestatic infants (infants with cholestasis other than BA and infants with BA) than healthy controls; however, there were no differences between these two subgroups of cholestasis in serum zonulin and IFABP figure. This indicates that nearly all parts of the small intestine (jejunum and ileum) and to a less extent the colon could be affected in infants with cholestasis regardless of the cause of cholestasis [34].

In acceptance with our study [34] found that FABP was significantly higher in NC than healthy control.

In a comparative analysis by Wang et al. [35] involving 34 patients with BA and 34 healthy controls , a significant disparity in gut microbiota diversity and composition was observed. Patients with biliary atresia exhibited lower diversity and significant structural segregation compared to healthy control.

There is positive correlation between serum zonulin and serum IFABP as a marker of intestinal permeability.

There was no significant relation between serum zonulin and any clinical or laboratory parameters in BA and non-BA cholestasis except for serum ALT level.

Positive correlation between serum zonulin and ALT in all cases of cholestasis that may suggest additive effect of leaky gut on hepatitis and elevated level of ALT [36].

Considering the close physiological relationship between the gut and the liver, intestinal barrier function is crucial for liver homeostasis, and disruption of intestinal barrier integrity may accelerate the pathogenesis of liver disease [37].

Diagnostic performance of serum zonulin for discrimination between biliary atresia and cholestasis subgroups sensitivity 30% and specifically 85%. This is due to an increase in intestinal permeability in all cases of cholestasis regardless of etiology.

Diagnostic performance of serum FABP for discrimination between biliary atresia and cholestasis subgroups sensitivity 56% and specifically 66%. The identification of therapeutic targets for modulating the gut microbiota-bile acid axis represents a promising strategy to ameliorate or perhaps reverse liver fibrosis in cholestatic liver disease [38].

CONCLUSION

We evaluated serum zonulin family peptides levels, as a marker of intestinal mucosal barrier function. We revealed that there was statistically significant difference between cholestasis group and healthy control, but no significance between BA and other causes of NC. Further studies are needed with larger scales needed for confirming our results.

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