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HEPATIC HEMODYNAMICS AND SERUM MARKERS OF FIBROSIS IN NON-ALCOHOLIC FATTY LIVER DISEASE AND NON-ALCOHOLIC STEATOHEPATITIS

Mohamed A. Bedewi[1*] and Sanaa M. Kamal[2,3]

[1]Department of Diagnostic Radiology, Prince Sattam Bin Abdulaziz University, College of Medicine, Kingdom of Saudi.

[2]Department of Internal Medicine, Prince Sattam Bin Abdulaziz University, College of Medicine, Kingdom of Saudi Arabia.

[3]Department of Gastroenetrology, Ain Shams Faculty of Medicine, Cairo, Egypt, Arabia.

***Corresponding Author: Mohamed A. Bedewi**

Department of Diagnostic Radiology, Prince Sattam Bin Abdulaziz University, College of Medicine, Kingdom of Saudi.

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ABSTRACT

Nonalcoholic fatty liver disease (NAFLD) is one of the most important precursors of chronic liver disease all over the world. The current study assessed the ability of several panels to influence prognosis and diagnosis for non-invasive serum markers and Duplex Doppler ultrasound in monitoring NAFLD and non-alcoholic steatohepatitis (NASH).Our study enrolled patients at different stages of NAFLD and NASH. NAFLD liver fat score (NAFLD-LFS)", "Fatty Liver Index (FLI)" "Hepatic Steatosis Index (HSI), the risk score ox NASH, NASHT est, Tumor necrosis factor-alpha (TNF-a), tumor growth factor beta 1 (TGF beta) caspase-generated CK18 fragment levels (CK-18), and YKL-40 (YKL-40) were measured in patients and control subjects. Doppler indices were estimated for the following arteries were assessed: hepatic artery, superior mesenteric artery, and the splenic artery. **Results:** TNF alpha, CK-18, TGF-b and YKL-40 showed statistically significant higher results in patients with NAFLD compared to control subjects. CK-18, TGF-b and YKL-40 were highest among NASH patients with liver fibrosis. TGF-beta, CK-18, and YKL-40 had the highest sensitivity, specificity, PPV and NPV in predicting of liver disease. Adirect correlation was observed between each of TGF-beta, CK-18, and YKL-40 with the NAFLD fibrosis score (r = 0.87, r = 0.63, r=0.69 respectively). Significantly higher SMA RI, HA PSV and SA RI values was noted in NAFLD patients compared to control group ((P<0.001). No significant statistical difference was found between the mean PV velocity in NAFLD patients and the control group. **Conclusion**, TGF-beta, TNF-alpha and cytokeratin-8 can be reliable non-invasive markers for detection of NAFLD and monitoring of NASH. NAFLD correlated positively with the following parameters, liver span, portal vein diameter, SA RI and SMA RI, PSV of the HA, SMA, and SA.

Abbreviations*:* NAFLD: Nonalcoholic Fatty Liver Disease; NASH: Non-alcoholic steatohepatitis; NAFLD liver fat score: NAFLD-LFS; Fatty Liver Index: FLI; Hepatic Steatosis Index: HIS; TNF-□: tumor necrosis factor alpha; TGF-□: tumor growth factor beta; HA, hepatic artery. SA: splenic artery, SMA, superior mesenteric artery, RI: resistive index; HARI: hepatic artery resistive index

KEYWORDS: non-alcoholic fatty liver disease, ultrasound, Doppler.

INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is considered an important cause of chronic liver disease worldwide.[1,2] with prevalence rates reaching 40 %.[3,4,5,6,7] NAFLD is characterized by the presence of hepatic steatosis with no evidence of hepatocellular injury while the main feature of non-alcoholic steatohepatitis (NASH) is the presence of hepatic steatosis and inflammation with hepatocyte injury with or without fibrosis.[5,6] NAFLD includes hepatic steatosis, nonalcoholic steatohepatitis, advanced liver fibrosis and cirrhosis.[7] The pathogenesis of NAFLD comprises intertwining metabolic, genetic, inflammatory and environmental factors.[5,8,9,10,11] NAFLD is associated with high rate of risk factors such as increased body mass index, diabetes mellitus, and patients with disturbed lipid metabolism.[12,13,14,15]

Liver biopsy is the gold standard for characterizing NAFLD hepatic histology. However, liver biopsy is an invasive expensive procedure that carries which may be associated with adverse events such as bleeding, bile duct injury, and diaphragmatic irritation.[16,17] Therefore, active research is being conducted to identify non-

invasive methods for detection of fibrosis in patients with NAFLD. Non invasive biomarkers and indices such as NAFLD Fibrosis Score, Enhanced Liver Fibrosis (ELF) have been evaluated. Abdominal ultrasound contributes in identification and quantitation of hepatic steatosis.[18] Transient electrography has been shown to play a critical role in assessment of liver fibrosis. To date, the hepatic hemodynamic changes with NAFLD have not been adequately characterized in NAFLD.[19,20,21] Some studies reported that assessment of indices of hepatic vasculature detected by Duplex Doppler improved the diagnostic performance of ultrasonography.[22,23] In this study, we assessed several hepatic vascular parameters such portal venous velocity, superior mesenteric artery velocity and splenic artery velocity in NAFLD patients and correlated such parameters with non-invasive markers of liver fibrosis in patients with different stages of NAFLD/NASH.

METHODS

Study design and patient population

The current study is a cross-sectional case control study conducted at a university Hospital from January 2015 to December 2016. The study protocol and patients' informed consent were approved by the institutional review boards. The study was conducted in compliance with the Declaration of Helsinki and was consistent with the International Conference on Harmonization and Good Clinical Practice. A written informed consent was signed by each patient before enrolment and before any study-related procedure.

Diagnosis of NAFLD

Patients with hepatic steatosis (mild, moderate or severe)[23,24] detected by ultrasound (Philips EPIQ 7G ultrasound machine; Philips, Reedsville, PA, USA) suggestive of NAFLD were initially subjected to specific investigations to exclude alcoholic liver disease, viral hepatitis (HBV and HCV), Wilson disease, haemochromatosis, autoimmune hepatitis and drug related steatosis. Patients were further tested by "NAFLD liver fat score (NAFLD-LFS)"[19], Fatty Liver Index (FLI)[20] and Hepatic Steatosis Index (HSI)[21] were then calculated according to the previously described formulas.

- NAFLD-LFS=2.89 +1.18xMS (yes=1/no=0) +0.45xT2DM (yes=2/no=0)+0.15xI_0+ 0.04 xAST-0.94 x AST/ALT.I_0 (μU/ml) represents fasting insulin and AST represents fasting AST levels (U/l). Values ≤−0.640 rule out, while values >−0.640 rule in NAFLD.[19]
- FLI = Logistic (0.953x*In* (TG) +0.139 x BMI+o.718 + In (gGT) x waist -15.745 x100. Values <30 rule out and values ≥60 rules in steatosis. logistic(x)=1/(1+e^{-x}) denotes the logistic function and *ln* the natural logarithm.[20]
- HSI: 8xALT/AST ratio +BMI+2 (if diabetic) +2 (if female). Values <30 rule out steatosis and values >36 ruling in steatosis.[21]

Patients were enrolled in the study if they fulfilled the following criteria: i) presence of hepatic steatosis; ii) NAFLD-LFS values >−0.640, FLI values > 60 and HSI values >36 iii) absence of any evidence of other chronic liver diseases and other causes of hepatic steatosis, iii) no history of significant alcohol consumption, iv) elevated aminotransferase levels found in one of three situations.

Diagnosis of NASH

The risk of NAFLD patients to develop NASH was assessed by the risk score oxNASH which was calculated from age, BMI, AST level, and the ratio of 13-hydroxy octadecadienoic acid to linoleic acid.[22] Patients with oxNASH scores over 72 were 10 times more likely to have NASH than patients with oxNASH scores less than 47. Non-alcoholic steatohepatitis was predicted by the NASH Test which combines 13 clinical and biochemical variables, age; gender; weight; height; and serum levels of cholesterol, triglycerides, α2 macroglobulin, apolipoprotein A1, haptoglobin, gamma glutamyl-transferase (GGT), ALT, AST, and bilirubin.[23] The nonalcoholic Steatohepatitis Clinical Research Network Model (NASH CRN) has been used to non-invasively diagnose NASH. The model is based on AST level, ALT level, AST/ALT ratio, demographic factors, co-morbidities, and other laboratory test results.[24]

Assessment of hepatic fibrosis

Liver fibrosis was evaluated by the NAFLD fibrosis score which includes serum glucose, platelet count, albumin, AST/ALT ratio) and readily available patient characteristics (age, BMI, and diabetes status).[25] Healthy volunteers with no evidence of NAFLD/ NASH or any liver disease were enrolled in the study as control subjects.

Cytokines assessment

Tumor necrosis factor-alpha (TNF-□ELISA kit; BioSource, San Diego, CA, USA), tumor growth factor beta 1 (human TGF beta 1 ELISA kit, BioSource, San Diego, CA, USA), caspase-generated CK18 fragment levels (CK-18 ELISA Kit; BioSource, San Diego, CA, USA) and YKL-40 (human YKL-40 ELISA kit, Quidel, San Diego, CA, USA) were measuredin patients and control subjects according to the manufacturers' instructions.

Ultrasound and Duplex Doppler assessment

All ultrasound scans were performed by an experienced radiologist (M.B) using Philips healthcare, diagnostic ultrasound system [USA], with a C 9-2 MHZ curved. Each subject was fasting for 6-8 hours, and was examined in supine position by a convex probe, 3.5 MHz, liver span was measured in midclavicular line.

The grading of NAFLD in ultrasound was used as follows.[26]

Grade 0: Normal liver echogenicity.

Grade I: Mildly increased liver echogenicity [bright echopattern], with clear visualization of the borders of the intrahepatic vessels and diaphragm.
Grade II: Moderately increased liver echogenicity, with mildly impaired visualization of the diaphragm and intrahepatic vessels.
Grade III: Marked increase in the liver echogenicity, with poor visualization of the diaphragm and the intrahepatic vessels, in addition to the posterior portion of the right lobe. (Table 1).

The portal vein and hepatic artery examinations were performed in a left decubitus position with raised arms above thehead and through an intercostal approach. The sample volume was positioned in the mainportal vein, proximal to the bifurcation to cover two-thirds of the vessel diameter, and the Doppler angle and color flow were adjusted. The portal vein diameter was measured and the direction of flow was recorded, together with the hepatic artery flow. For examination of the superior mesenteric artery, patients were examined in the supine position by an anterior approach. The following measurements were obtained: the peak systolic velocity and resistive index of the hepatic artery, peak systolic velocity and resistive index of the superior mesenteric artery, peak systolic velocity and resistive index of the splenic artery as previously described.[26]

Statistical analysis
Baseline demographic and clinical characteristics were analyzed descriptively for all patients using Student t-tests, ANOVA or Kruskall–Wallis test as appropriate for continuous variables and Chi-square or Fisher's exact tests were used for categorical variables. Cytokine levels were examined in box-plots as continuous variables. A Kruskal–Wallis one-way analysis of variance test tested for a significant overall shift in cytokine levels in cases and controls and the Mann–Whitney *U*-test examined identified sample pairs. Comparison of cytokine levels and Doppler ultrasound findings was assessed by Paired T test. Pearson r correlation test was used to assess the relation between cytokines levels and fibrosis scores. Ninety- five per cent confidence intervals (95% CIs) of the sensitivity, specificity, positive predictive value and negative predictive value were calculated with the Wilson method. Logistic regression was used to predict celiac disease among NAFLD patients. Results are expressed as mean values ±S.D. Statistical analysis was performed using SPSS version 22 (SPSS, IBM Inc., NC, USA), Graph Pad Prism software (GraphPad Software Inc., CA, USA) and Med Calc Statistical software (MedCalc Software, Ostend, Belgium).

RESULTS
Of the patients with ultrasound detected hepatic steatosis, 34 (65.38%) patients (31 men and 21 women; mean age: 43.287±12.38) fulfilled the criteria of NAFLD and 18 (43.62) had NASH. Liver fibrosis was demonstrated by the NAFLD fibrosis score in 14/18 patients with NASH (77.78%). Twenty age, gender and BMI matched healthy control subjects were also enrolled in the study. OxNASH scores exceeding 72 were detected in 28 (82.75%) patients with NAFLD implying that those patients were at high risk of developing NASH. More women had NASH than men (P= 0.0322). Patients with NASH had significantly higher BMI when compared to those with NAFLD and control subjects (P <0.0001). Patients with NASH tended to have higher hemoglobin levels compared to those observed in patients with NAFLD (Table 2). Serum bilirubin, ALT and AST, cholesterol and triglyceride levels and HOMA-IR were significantly higher in NASH patients. Diabetes was detected in 9 patients with NAFLD and 12 patients with NASH.

Serum cytokines and fibrosis mediators in NAFLD and NASH patients
TNF alpha, CK-18, TGF-□□and YKL-40 were significantly higher in patients with NAFLD compared to control subjects. CK-18, TGF-□ and YKL-40 were highest among NASH patients with liver fibrosis. We assessed the diagnostic performance of CK-18, TGF-□□and YKL-40 in predicting hepatic fibrosis. TGF-beta, CK-18, and YKL-40 had the highest sensitivity, specificity, PPV and NPV in predicting of liver disease. Direct correlation was observed between each of TGF-beta, CK-18, and YKL-40 with the NAFLD fibrosis score (r = 0.87, r = 0.63, r=0.69 respectively).

Doppler ultrasound in patients with NAFLD, NASH and control group
No significant statistical difference was found between HA RI in NAFLD and control group. Significantly higher SMA RI values was noted in NAFLD patients (0.7885±0.0785), compared to control group (0.4957±0.1509), (P<0.001). Significantly higher SA RI values was noted in NAFLD patients (0.6277±0.821), compared to control group (0.3833±0.06), (P<0.0027). No significant statistical difference was found between the mean PV velocity in NAFLD patients (20.37 ± 5.142), and the control group (20.1± 5.683), (P= 0.7623). Significantly higher HA PSV was noted in NAFLD patients (40.878 ± 12.833) compared to the control group (12.700 ± 3.129), (P<0.0001). (Figures 1-2).

Significantly higher SMA PSV was noted in NAFLD patients (78.426± 20.101) compared to the control group (29.875±16.694), (P<0.0001). Significantly higher SA PSV was noted in NAFLD patients (53.988±15.389) compared to the control group (44.217±12.155), (P= 0.0501).

The liver span and the portal vein diameter were significantly higher in NAFLD patients compared to controls.

Table 1 Grading of fatty infiltration of the liver by ultrasound

Grade 0	0	0	20	P value
Grade 1	9	0	0	<0.0001
Grade 2	20	3	0	<0.0001
Grade 3	5	7	0	<0.05
Grade 4	0	5	0	<0.0001

p = p-value (Mann-Whitney *U*-test for continuous variables and Fisher's Exact test for categorical variables).* Significant; non alcoholic steatohepatitis.

Table 2: Demographics and baseline clinical characteristics and laboratory data in patients with NAFLD, NASH and healthy controls.

Parameter	Patients with N=34	Patients with NASH; N=18	P value between patients with concomitant NAFLD& celiac vs. NAFLD control group	Control group N=10	P value between patients with NAFLD or NASH *vs.* healthy controls
Age (years); mean±SD	42.391±7.374	41.968±10.017	0.2066	41.241±11.286	0. 24
Male: Female	26:11	8:10	0.0322*	6: 4	0.1643
BMI (mean±SD)	26.473±4.207	29.718±4.519	<0.0001**	22.768±2.137	0.0640
Diabetes; (n, %)	9(26.47)	12 (66.67)	0.0076*	0	<0.0001**
Total bilirubin (mg/dl) (Normal: 0.3 to 1.0 mg/dL)	1.04±1.85	2.97±1.06	0.0002**	0.89±0.72	<0.0001 **
ALT (U/L) ; (Normal: 10 to 40 U/L)	68.3±31.27	72.2±26.14	<0.0001**	36.31±9.47	<0.0001**
AST (U/L); (Normal: 10 to 40U/L)	64.36±29.46	74.92±21.74	0.0038*	31.18±10.15	<0.0001**
Serum albumin (g/dL); (Normal: 3.5 to 5.5 g/dL)	3.8±0.81	3.2±0.98	0.0221*	3.835±0.88	0.2035
HOMA-IR score	3. 31 ±2.43	5.615±3.241	0.0042*	2.305±2.363	<0.0001**
Triglycerides (mg/dL) (Normal: <150 mg/dL)	138.753±12.18	151.83±29.38	0.0277*	124.51±20.16	<0.0001**
Cholesterol (mg/dL) (Normal:< 200 mg/dL)	188.23±17.68	214.065±21.78	< 0.0001214.065±21.78	135.64±26.18	<0.0001**
Hemoglobin (Range: Men-13.5 to 17.5 gm/dL; women, 12.0 to 15.5 gm/dl)	11.48±2.13	13.52±2.17	0.4621	14.103±2.607	0.049 *
RBCs ($1x10^6$cells/mcl) (Range: Men-4.7 to 6.1 million cells/mcl; women, 4.2 to 5.4 million cells/mcl)	4.202±0.52	3.93±0.86	0.1609	4.004±0.859	0.2805
Total leucocytic count; (Range: 4,500 and 10,000 cells/mcl)	6,674±2,730	7,621±3.86	0.8469	6,293±3,104	0.1430
Platelets (Range: 150,000 to 450,000 /mcl	193,194±48,937	179,271±71,201	0.0202*	195,107±73,286	0.7112

p = p-value (Mann-Whitney *U*-test for continuous variables and Fisher's Exact test for categorical variables). * Significant; ** highly significant; NASH: non alcoholoic steatohepatitis; NAFLD: non-alcoholic fatty liver disease; BMI: body mass index.

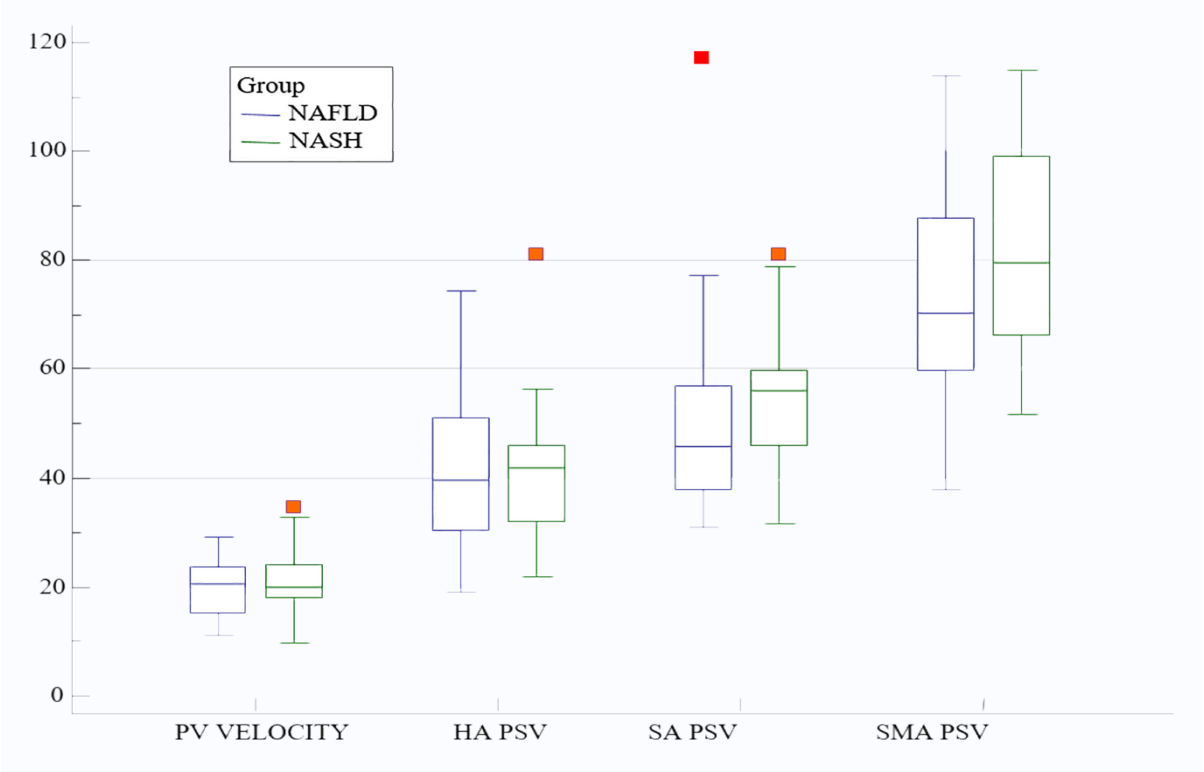

Figure 1: Velocities of the portal vein,hepatic artery (HA), splenic artery (SA) and superior mesenteric artery in NAFDL and NASH patients.

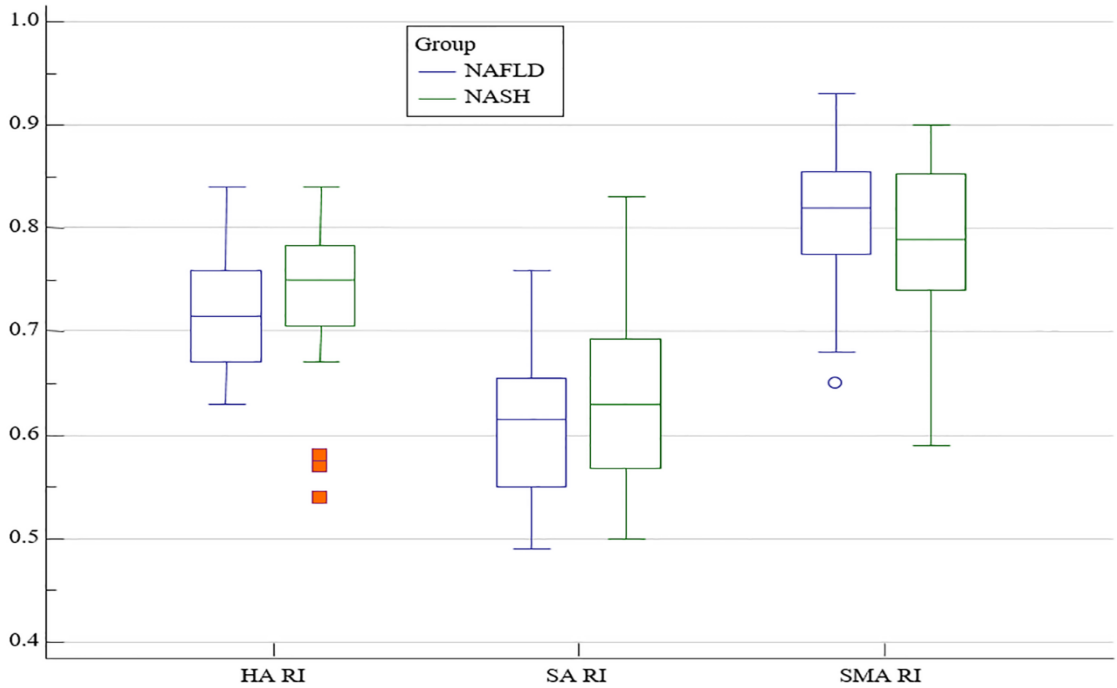

Figure 2: The resistive index of hepatic artery (HA), splenic artery (SA) and superior mesenteric artery in NAFDL and NASH patients.

DISCUSSION

This cross-sectional study investigated non-invasive serologic and imaging parameters in NAFLD and NASH patients. The enrolled subjects were more likely to suffer from hypertension, T2DM, MetS, cardiovascular disease and impaired renal function. Laboratory tests revealed significant increased glucose, ALT and ALP levels and decreased HDL cholesterol. NAFLD and NASH are prevalent in the Kingdom of Saudi Arabia due to the high prevalence of metabolic syndrome, obesity and Type 2 diabetes. A study conducted in Riyadh, KSA,[13] estimated a NAFLD prevalence of NAFLD of 20%.

Furthermore, 70% of enrolled patients were obese with BMI values over 30.

In the current study, a significant association was observed between hemoglobin, inflammatory markers (e.g. CRP and total leucocyte count) and NASH. Patients with NASH tended to have higher hemoglobin level and NAFLD. A similar observation was previously reported.[33,34] and has been related to progression of NAFLD to NASH and fibrosis. Potential interpretation of increased hemoglobin levels maybehepatic hypoxia, oxidative stress, formation of reactive oxygen species and lipid peroxidation. The elevated inflammatory markers be attributed to increased visceral adipose tissue conferring a pro-inflammatory state.[29]

Non-alcoholic fatty liver disease (NAFLD) represents a group of conditions ranging from simple liver steatosis, usually asymptomatic, to nonalcoholic steatohepatitis (NASH), which is characterized by the presence of apoptosis/ inflammation and fibrosis, and also by a progressive course.[6] Diagnostic procedures is aim to identify the patients with higher risk of developing NASH before the onset of advanced fibrosis. Liver biopsy is considered the "gold standard" for the assessment of liver fibrosis. However, liver biopsy is an invasive procedure with certain unavoidable risks and complication.[8] Due to the high prevalence of NAFLD and its progressive nature, there has been an urgent need to develop reliable noninvasive tests that can accurately predict the presence of advanced disease without the need for liver biopsy.[30] The current study has been designed to investigate the Duplex Doppler profile in NAFLD and to correlate this pattern with NAFLD indices.

In the pathogenesis of NASH, the cytokines represent as central mediators which promote injury and inflammation that may finally lead to end-stage liver diseases.[9] The balance between pro- and anti-inflammatory cytokines plays a major role in reducing the progression of NASH to cirrhosis.[10,11]

The current study observed significantly elevated levels of TG-F beta, TNF-α, and CK-8 in patients with NAFLD compared to the control group and even higher levels in patients with NASH compared to NAFLD patients and the control group. The elevation in such cytokines may reflect a systemic inflammation in patients with NASH. Similar findings were previously reported by previous studies. published data.[31,32] Zahran et al. showed that TNF-α was increased in all patients with NASH, including subgroup of patients with fibrosis, compared to the control one. But Zahran et alalso noticed a reduction of IL-10 in the serum, observation that has been confirmed in the present study.[32] However, we did notassessed IL-10 levels in relation to the rates of disease progression or staging the patients according to the degree of fibrosis.

The present study showed the high diagnostic and prognostic performance of the cytokines: TGF-beta, TNF alpha and CK-8 which can serve and reliable markers for NAFLD and NASH as previously reported.[31-34]

In our study, we found no correlation between the PV velocity and the degree of fatty infiltration this suggest that the mean PV velocity is not useful in differentiating NAFLD patients from healthy subjects. Balci et al, found that the degree of severity of fatty infiltration is inversely proportional to the portal vein velocity, however our results are similar to the results in the study done by Ulusan et a.[35,36] Also some studies like Barakat et al,[37] demonstrated a relation the portal vein shape wave and chronic diseases, however, we did not demonstrate such relationship coinciding with Tarzamni et al.[26]

In contrast to Tana et al and Balasubramanian et al , HA RI was not significantly different in NAFLD patients compared to controls in our study, however, SMA RI and SA RI showed significant difference was noted between NAFLD patients and controls. These changes could be attributed to the pathophysiological changes associated with NAFLD.

Coinciding with Tana et al the SA RI was significantly higher in NAFLD patients compared to controls**.** Considering the PV diameter our study showed significantly higher values in NAFLD patients compared to controls. Also, the liver span was significantly higher in NAFLD patients compared to controls, coinciding with the results of Tana et al and Basalubramanian et al.[38,39]

The study has some limitations that should be addressed. The cross-sectional nature of the study did not monitor the progression of NAFLD to NASH. Although the non-invasive NAFLD indicesare accepted diagnostic tool for NAFLD, it is not an absolute measure of hepatic fat accumulation and thus over- and underestimation of NAFLD could have occurred. Finally, liver biopsies were not performed. However, the use of several indices and serologic tests provided good diagnosis of NAFLD/NASH. Overall, these limitations do not materially affect the interpretation of the presented results.

In conclusion, TGF-beta and cytokeratin-8 can be reliable non-invasive markers for detection of NAFLD and monitoring of NASH. Our findings also demonstrated that NAFLD correlated positively with the following parameters, liver span, portal vein diameter, SA RI and SMA RI, PSV of the HA, SMA, and SA. No significant correlation could be seen between NAFLD and HA RI, or portal vein flow pattern.

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