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Assessment of Pro Collagen III as Non-Invasive Biomarker for Diagnosis of Non-Alcoholic Steatohepatitis

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ABSTRACT

Background: One of the most frequent causes of chronic liver disease globally is non-alcoholic fatty liver disease (NAFLD). It is believed that NAFLD is the hepatic expression of metabolic syndrome. Historically, NAFLD was thought to be a rather benign liver condition. Hepatocellular carcinoma, cirrhosis, and liver fibrosis can develop in some NAFLD patients. Consequently, it is crucial from a clinical standpoint to accurately diagnose and stage NAFLD patients.

Objective: This study aimed to evaluate the performance of pro collagen III as a NASH-Fibrosis biomarker within the optimum diagnostic context of usage in comparison with FIB-4.

Patients and Methods: This study was a case-control study that was conducted at Gastroenterology and Hepatology Outpatient Clinic at Damas Centre for Liver and GIT. This study was performed on 56 Egyptian patients who were ≥ 18 years of age and are overweight with Body Mass Index ≥ 25 and diagnosed with NAFLD by US. They were divided into 2 groups: Group 1 included 28 healthy individuals diagnosed with NAFLD by US without any evidence of hepatitis and group II that included 28 patients with NASH diagnosed by elevated liver enzymes.

Results: Patients with non-alcoholic steatohepatitis (NASH) have relatively low platelet count in comparison with NAFLD patients. Patients with NASH had Higher AST & ALT levels than NAFLD patients. FIB-4 was higher in NASH group than NAFLD group. Pro collagen III level was higher in patients with NASH than patients with NAFLD. Pro collagen III was a significant discriminator of NAFLD & NASH. Pro collagen III correlates with the grades of histological steatohepatitis, and stage of fibrosis. Pro collagen III can diagnose steatohepatitis at cut-off 9.57 with sensitivity & specificity was 92.9% & 92.9% respectively.

Conclusion: Pro collagen III is a sensitive biomarker to discriminate between NAFLD & NASH. Pro collagen III level correlate positively with BMI, AST & ALT. Pro collagen III level correlate with grades of histological steatohepatitis and grades of fibrosis.

Keywords: Pro collagen III, Steatohepatitis, NAFLD, NASH.

INTRODUCTION

NAFLD is a spectrum of liver disease that includes cirrhosis, fatty liver with inflammation and hepatocyte ballooning non-alcoholic steatohepatitis (-NASH-), with or without fibrosis, and steatosis affecting more than 5% of hepatocytes [(1)].

The current gold standard for diagnosis and prognosis is a liver biopsy. However, this invasive costly technique has a significant risk of sample mistake and consequences, such as bleeding, discomfort, and in rare cases, death. Furthermore, underestimating the severity of the illness and sampling bias are typical because a biopsy specimen only represents 1/50000 of the liver volume [(2)].Finding more effective non-invasive methods to evaluate NASH as pro collagen III patients is urgently needed. Levels of pro collagen III (Pro-C3) indicate extracellular matrix turnover (ECM). Pro collagen III's terminal peptide and, indirectly, active fibrogenesis are measured using a non-epitope-specific antibody that can attach to the protease-specific cleavage point of collagen fragments. It is possible to distinguish between NASH and plain fatty liver, and it is correlated with the degree of steatohepatitis and the stage of fibrosis [(3)].

The study's objective was to evaluate the performance of pro collagen III as a NASH-fibrosis biomarker within the optimum diagnostic context of usage in comparison with FIB-4.

PATIENTS AND METHODS

This study was a case-control study that was conducted at Gastroenterology and Hepatology Outpatient Clinic at Damas Centre for Liver and GIT. The study included 56 Egyptian patients who were ≥ 18 years of age and are overweight with Body Mass Index ≥ 25 and diagnosed with NAFLD by US.

The study was conducted through the period from August 2022 to January 2023. The patients were divided into 2 groups: Group I included 28 healthy individuals diagnosed with NAFLD by US without any evidence of hepatitis and group II that included 28 patients with NASH diagnosed by elevated liver enzymes.

Exclusion Criteria:

Pregnancy, peoples with liver aetiology other than viral liver disease (HCV and HBV), autoimmune hepatitis, primary biliary cholangitis, primary sclerosing cholangitis, metabolic liver disease, and drug-induced liver disease (Individuals who are on long-term steatosis-inducing medications such as amiodarone, tamoxifen, and corticosteroids and injectable drug abusers, as well as those who have previously or currently consumed large amounts of alcohol.

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Methods (Data collection): All studied patients were subjected to the following:

1. Full history taking: Complaint, present history, past history, drug history and family history.
2. Full clinical examination (General and abdominal examination).
3. Body weight and Height and calculation of BMI: [BMI= $\frac{\text{weight (Kg)}}{\text{height (m}^2\text{)}}$].
4. Calculation of FIB4 ($\frac{\text{Age}\times\text{AST}}{\text{Platelets}\times\sqrt{\text{ALT}}}$.
5. Laboratory investigations: a. Liver enzymes including: serum ALT, serum AST. b. Complete blood picture with differential counting.

6. Pro collagen III (PRO CIII): Sample collection and processing: Participants' venous blood (5 ml) was collected into sterile plain tubes in order to separate the serum. All samples were allowed to clot overnight at 2–8°C or for one hour at room temperature. then were centrifuged at 1000 g for 20 minutes at 2–8°C. Supernatant was collected to carry out the assay and the assay was carried out using Human Procollagen III ELISA Kit, Cat. No: E-EL-H0182, Elabscience Biotechnology Inc. (USA).

Ethical approval:

The Ethics Committee of Ain Shams University's Faculty of Medicine granted the study approval. All participants signed informed written consents after a thorough explanation of the goals of the study. The Helsinki Declaration was followed throughout the study's conduct.

Statistical analysis

IBM SPSS, Version 26.0, was used to analyse the data. The normality of the distribution was confirmed using the Kolmogorov-Smirnov test. For categorical data, numbers and percentage were used, whereas for continuous data, the expressions were mean ± standard deviation, and range. To investigate the relationship between two qualitative variables, we utilised the X^2 test. Student T-test was used for quantitative variables with a normal distribution, to compare the two groups under study. Mann-Whitney test was used for quantitative variables with anomalous distributions, to compare the two groups under study. Assessing the degree of relationship between two quantitative variables was calculated by correlation analysis (using Spearman's approach). Sensitivity and specificity for quantitative diagnostic tests, that divide patients into two groups, were assessed using the ROC Curve. Significance was defined as a P value <0.05.

RESULTS

The mean age in NASH group and NAFLD group were 40.36 ± 10.12 years and 46.68± 13.19 years respectively. The age difference between the two groups was statistically significant (p=0.049), with the NAFLD group being older. Gender distribution showed that 50% of patients in the NAFLD group were females and 50% were males, with non-significant difference between the two groups (p>0.05). Of the cases in the NASH group, 57.1% were males and 42.9% were females. The mean BMI was 30.72 ± 1.87 Kg/m^2 in NASH group, while it was 28.63 ± 2.34 Kg/m^2 in NAFLD group. Given that the NASH group had a much higher BMI than the other group, there was a statistically significant difference in BMI between the two groups (p=0.001) (Table 1).

Table (1): Demographic characteristics and BMI among the studied groups.

		NASH group (No. = 28)		NAFLD group (No. = 28)		Test value	P-value
		No.	%	No.	%		
Gender	**Male**	16	57.1%	14	50.0%	X^2= 0.287	0.592 (NS)
	Female	12	42.9%	14	50.0%		
Age (years)	**Mean± SD**	40.36± 10.12		46.68± 13.19		T= 2.012	**0.049 (S)**
	Range	26.0 - 60.0		25.0 - 68.0			
BMI (Kg/m^2)	**Mean± SD**	30.72± 1.87		28.63± 2.34		T= 3.681	**0.001 (HS)**
	Range	26.0 - 35.0		25.7 - 35.0			

Table (2) showed that the mean hemoglobin level in NASH group and NAFLD group was 13.34 ± 0.85 g/dl and 13.51± 0.83 g/dl respectively. The mean platelets count in NASH group and NAFLD group was 184.57 ± 14.21 and 312.5 ± 59.47 respectively. The mean WBCs in NASH group and NAFLD group was 7.889 ± 1.725 and 8.26 ± 2.051 respectively. When comparing the NASH group to the NAFLD group, the platelet count was significantly decreased ($p<0.001$). Haemoglobin and white blood cell counts did not significantly differ between the two groups under investigation ($p>0.05$).

Table (2): Comparison between the studied groups regarding CBC

	NASH group (No. = 28)	NAFLD group (No. = 28)	Mann-Whitney U Test	
	Mean± SD	Mean± SD	Test value	P-value
Hb (g/dl)	13.34±0.85	13.51±0.83	1.035	0.301 (NS)
Platelets count ($\times10^3$/L)	184.57±14.21	312.5±59.47	6.379	**<0.001 (HS)**
WBCs ($\times10^9$/L)	7.889±1.725	8.26±2.051	1.181	0.238 (NS)

Table (3) showed that the mean AST was 130.93 ± 7.76 in NASH group, while it was 25.71± 4.14 U/l in NAFLD group. The mean ALT was 113.14 ± 18.38 in NASH group, while it was 28.68 ± 4.57 U/l in NAFLD group. There was statistically significant elevation in AST and ALT in NASH group compared to NAFLD group ($p<0.001$). The mean FIB4 in NASH group was 2.67 ± 0.65, and in NAFLD group was 0.78 ± 0.38. Regarding FIB4, there was a statistically significant difference between the two groups ($p<0.001$), with the NASH group having much more FIB4 than the NAFLD group. The mean Pro-collagen III level in NASH group was 11.65 ± 2.74, and in NAFLD group was 3.47 ± 0.87. There was statistically significant difference between the two groups regarding FIB4 ($p<0.001$) as it was significantly higher in NASH group compared to NAFLD group.

Table (3): Comparison between the studied groups regarding AST/ALT ratio, FIB4 and Pro-collagen III

		NASH group (No. = 28)	NAFLD group (No. = 28)	Test value	P-value
AST (U/L)	**Mean± SD**	130.93± 7.76	25.71± 4.14	Z_{MWU}= 6.437	**<0.001 (HS)**
ALT (U/L)	**Mean± SD**	113.14± 18.38	28.68± 4.57	Z_{MWU}= 6.442	**<0.001 (HS)**
FIB4	**Mean± SD**	2.67± 0.65	0.78± 0.38	Z_{MWU}= 6.375	**<0.001**
PRO CIII	**Mean± SD**	11.65± 2.74	3.47± 0.87	Z_{MWU}= 5.999	**<0.001**

In NASH group, there was significant negative correlation between pro-collagen III with platelets count (r=-0.571, p=0.002) (Table 4).

Table (4): Correlation between Pro-collagen III and different variables in NASH group

	Pro-collagen III	
	r	p- value
Age (years)	-0.101-	0.611
BMI	0.207	0.290
Hb	-0.132-	0.504
PLT	-0.571-	**0.002**
WBCs	0.173	0.379
AST	0.157	0.424
ALT	0.136	0.490
FIB4	0.068	0.731

r: Spearman rho

In NAFLD group, there was significant negative correlation between pro-collagen III with platelets count (r=-0.650, p<0.001). While, there was significant positive correlation between pro-collagen III with FIB4 (r=0.739, p<0.001). There was significant positive correlation between pro-collagen III with age (r=0.579, p=0.001) and BMI (r=0.525, p=0.004) (Table 5).

Table (5): Correlation between pro-collagen III and different variables in NAFLD group

	Pro-collagen III	
	r	p- value
Age (years)	0.579	**0.001**
BMI	0.525	**0.004**
Hb	0.121	0.541
PLT	-0.650-	**<0.001**
WBCs	-0.065-	0.743
AST	0.330	0.087
ALT	0.179	0.362
FIB4	0.739	**<0.001**

r: Spearman rho

The FIB4 can diagnose NASH at cutoff 1.54 with area under the curve 0.998 with sensitivity and specificity of 100% and 96.4% respectively (p<0.001). Pro collagen III can diagnose NASH at cutoff of 9.57 with area under the curve of 0.967 with sensitivity and specificity of 92.9% and 92.9% respectively (Table 6).

Table (6): Diagnostic performance of FIB4 and pro collagen III for diagnosis of NASH

Variables	AUC	Best Cutoff	Sensitivity	Specificity	PPV	NPV	P-value
FIB4	0.998	1.54	100%	96.4%	96.5%	100%	<0.001
Procollagen III	0.967	9.57	92.9%	92.9%	92.9%	92.9%	<0.001

DISCUSSION

When > 5% of hepatocytes exhibit large vesicular steatosis without a clear alternative source of steatosis, the population is referred to as having NAFLD, a catch-all term that encompasses all disease grades and stages. NAFL, which is defined by large vesicular hepatic steatosis and may or may not be associated with moderate inflammation. NASH, which is also characterised by inflammation and cellular damage (ballooning) with or without fibrosis, and cirrhosis are in the spectrum of the illness [(4)].

The current gold standard for diagnosis and prognosis is liver biopsy. There is an immediate need for a non-invasive or minimally invasive biomarker due to the low patient acceptability of this invasive standard procedure and the significant sample variability of regular liver biopsy in patients with NAFLD [(3, 5)]. Thus, the growing number of cases of NAFLD requires a move away from histology and towards the creation of non-invasive evaluations. This difficulty affects both the clinical research of possible novel therapeutics to stop the development of fibrotic NASH to cirrhosis and ordinary clinical care [(6)]. Blood-based non-invasive fibrosis tests can be divided into two categories: "direct biomarkers," which gauge collagen deposition or matrix turnover, and "indirect makers," which comprise straightforward non-invasive fibrosis scores derived from clinical and biochemical indices, such as the fibrosis-4 (FIB4) score and the NAFLD fibrosis score (NFS) [(7)].

According to earlier research, determining the development of type III collagen neo-epitopes (PRO-C3) alone or in combination with other diagnostic markers that can yield a diagnostic panel, which is relatively reliable in determining the stage and activity of the illness [(8)].

The average age of the NASH group was 40.36 ± 10.12 years, whereas the NAFLD group's mean age was 46.68 ± 13.19 years. Age showed a statistically significant difference (p=0.049) between the two groups where the NAFLD group was older. This is consistent with the findings of **Gkolfakis *et al.*** [(9)]**,** who discovered that the NAFLD group's mean age was 55 and the NASH group's mean age was 49.8 years. Age differences between the two groups were statistically significant (p value <0.001). Furthermore, this is at odds with the findings of **Allam *et al.*** [(10)] who discovered no statistically significant age difference between the groups under study.

Our study found no statistically significant difference in sex between the studied groups (p>0.05). In the NASH group, there were 57.1% male cases and 42.9% female cases, while in the NAFLD group, there were 50% male cases and 50% female cases. These findings are consistent with those of **Allam *et al.*** [(10)] who also found no statistically significant difference in sex between the studied groups.

According to our research, the NASH group's mean BMI was 30.72 ± 1.87 kg/m^2, but the NAFLD group's mean BMI was 28.63 ± 2.34 kg/m^2. Given that the NASH group had a much higher BMI than the

other group, there was a statistically significant difference in BMI between the two groups (p=0.001). **Boyle *et al.*** [11] concluded that there was no statistically significant difference in BMI between the two groups, which is consistent with our findings. BMI (Kg/m^2) in the NAFLD group was 32.9 ± 7.1, whereas in the NASH group it was 32.4 ± 6.4 (p=0.608). **Allam *et al.*** [10] reported no significant difference in BMI between the examined groups.

In our study, the average platelet count in the NASH and NAFLD groups was 184.57 ± 14.21 and 312.5± 59.47, respectively. Platelet count decreased significantly in NASH compared to NAFLD groups (p<0.001).

As regard liver enzymes, our study concluded that there was statistically significant elevation in AST and ALT in NASH group compared to NAFLD group (p<0.001). The mean AST was 130.93 ± 7.76 in NASH group, while it was 25.71 ± 4.14 in NAFLD group. The mean ALT was 113.14 ± 18.38 in NASH group, while it was 28.68 ± 4.57 in NAFLD group. This is in agreement with **Bril *et al.*** [12] who found higher plasma ALT and AST levels among patients with definitive NASH.

Since NAFLD and NASH are intricate, multifactorial illnesses, it is unlikely that a single surrogate sign will be 100% accurate in predicting treatment outcomes or clinical outcomes. The use of these indicators to forecast data on the course and outcome of the illness is gaining popularity quickly, despite the fact that all biomarkers and scores have their limits [13].

According to our findings, the NASH group's FIB-4 level was considerably greater than in the NAFLD group. The average FIB4 for the NASH and NAFLD groups was 2.67 ± 0.65, and 0.78 ± 0.38 (p<0.001) respectively. These results are consistent with the findings of **McPherson *et al.*** [14] who discovered a statistically significant difference in FIB-4 between the groups under study: FIB-4 was 1.25 ± 0.78 (p<0.001) for the NAFLD group and 2.83 ± 1.71 (p<0.001) for the NASH group.

In our study, the average pro-collagen III level in the NASH group was 11.65 ± 2.74, whereas in the NAFLD group it was 3.47 ± 0.87. PRO-C3 levels were considerably greater in the NASH group compared to the NAFLD group (p<0.001), which is consistent with findings by **Boyle *et al.*** [11] and **Nielsen *et al.*** [15]**.** According to **Boyle *et al.*** [11] there was a correlation between the stage of fibrosis (rs = 0.462, p ≥0.0001) and the grade of histological steatohepatitis (rs = 0.367, p ≥0.0001). Moreover, PRO-C3 levels were found to be relatively linearly correlated with NASH grades by **Piazzolla and Mangia** [3] and to allow for the discrimination of patients with or without a histologic diagnosis of NASH. These findings corroborate the findings of **Nielsen *et al.*** [15] who reported that PRO-C3 increased with increasing grades of ballooning (p<0.001), inflammation (p<0.01), steatosis (p<0.01), and with increasing stages of fibrosis (p<0.001).

Our results showed that in NASH group, pro-collagen III and platelet count showed a strong inverse relationship (r=-0.571, p=0.002). Or results are in agreement with **Boyle *et al.*** [11] who found substantial correlation with progressive fibrosis in the NASH group. With AUROC of 0.68 sensitivity 59%, specificity 69%, accuracy 64%), NASH and a PRO-C3 level > 14.5 ng/ml were identified. The validation cohort (n = 298) showed a replication of this with AUROC = 0.76, sensitivity of 70%, specificity of 68%, and accuracy of 69%. Similarly, NASH-cirrhosis was detected with an AUROC of 0.68 (sensitivity 74%, specificity 67% and accuracy 68%) when the PRO-C3 level was more than 16.5 ng/ml.

Our results showed that In NAFLD group, there was significant negative correlation between pro-collagen III with platelets count (r=-0.650, p<0.001). While, there was significant positive correlation between pro-collagen III with FIB4 (r=0.739, p<0.001). There was significant positive correlation between pro-collagen III with age (r=0.579, p=0.001) and BMI (r=0.525, p=0.004).

In the current study, ROC curve analysis showed that FIB4 can diagnose NASH at cutoff of 1.54 with area under the curve of 0.998 with sensitivity and specificity of 100% and 96.4% respectively (p<0.001). Pro collagen III can diagnose NASH at cutoff 9.57 with area under the curve 0.967 with sensitivity and specificity of 92.9% and 92.9% respectively.

CONCLUSION

Pro collagen III is a sensitive biomarker to discriminate between NAFLD & NASH. Pro collagen III level correlated positively with BMI, AST & ALT. Pro collagen III level correlated with grades of histological steatohepatitis, and grades of fibrosis.

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Conflict of Interest: Nil.

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