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RESEARCH ARTICLE

APRI/P3NP: CAN IT BE AN ACCURATE MARKER FOR NONINVASIVE EVALUATION OF LIVER FIBROSIS IN CHRONIC HCV.

Mervat Essam[1], Ahmed Yamany[1], Asmaa Hanafy [2], Nadia Morcos[3], Dina Seoudi[4] and Mohamed Sherif Mogawer[5].

1. Lecturer of Internal Medicine, Faculty of Medicine, Cairo University, Egypt.
2. Master degree of Biochemistry, Faculty of Science, Ain shams University, Egypt.
3. Professor of Biochemistry, Faculty of Science, Ain shams University, Egypt.
4. Assistant professor of Biochemistry, Faculty of Science, Ain shams University, Egypt.
5. Professor of Internal Medicine and Hepatology, Faculty of Medicine, Cairo University, Egypt.

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Corresponding Author
Mervat Essam.

Abstract

Background and aim:- Hepatitis C virus (HCV) is a major health problem worldwide. Fibrosis is the major complication of HCV infection and has a significant influence on defining the prognosis and indications for therapy**.** Our study evaluated non-invasive markers in testing progression of hepatic fibrosis in patients with chronic HCV.

Material and Methods:- HCV patients (20) of at least 6 months duration with normal kidney function, no ongoing drug or alcohol use, no evidence of malignancy or autoimmune hepatitis. Liver fibrosis was staged according to Sequential algorithm for fibrosis evaluation (SAFE) algorithm. HCV RNA, Alpha fetoprotein(AFP),Antinuclear antibody(ANA), liver and kidney functions, Fibrotest-Actitest , Fibro- quotient(FIBROQ), Four fibrosis indirect biomarker(FIB-4),AST/platelet ratio(APRI)and Amino-terminal type III procollagen (P3NP) were tested.

Results:- APRI / P3NP identified significant fibrosis with 90.1% accuracy (area under the receiver operating characteristic curve 0.987; 0.974- 1). APRI/P3NP& FIB-4 index were superior to FIBROQ &Fibrotest in detecting significant fibrosis.

Conclusion:- APRI/P3NP is a reliable and relatively cheap marker for fibrosis.

Abbreviation:-

NIBMs: Noninvasive biomarkers
ECM: Extracellular matrix
P3NP: Amino-terminal type III procollagen
APRI: AST/platelet ratio
AFP: Alpha feto-protein
ANA: anti nuclear antibody
ALT: alanine aminotransferase
AST: aspartate aminotransferase
ALP: alkaline phosphatase
GGT: gamma-glutamyltranspeptidase
BUN: blood urea nitrogen
SAFE: sequential algorithm for fibrosis evaluation
ROC: receiver operating characteristic curve
PPV: positive predictive values

NPV: negative predictive values
FibroQ: fibro- quotient
FIB4: four fibrosis indirect biomarker
LR: likelihood ratio
SE: standard error

Introduction:-

Hepatitis C is considered as 'viral time bomb'. Over several years, about 20% of those with chronic hepatitis C will proceed into different pathological stages that range from mild liver inflammation without fibrosis to marked hepatic fibrosis and cirrhosis (1).

Evaluation of the stage of liver disease is important for diagnosis, treatment, and follow-up during & after cessation of treatment. Liver biopsy is still the gold standard marker of staging activity of fibrosis.But being invasive, it carries high risks, besides it is unaccepted by many patients. So there is an emerging need for non-invasive tool to assess stage of liver fibrosis and to follow up the effect of therapy (2).

Furthermore, different histological scoring systems have been developed and modified (3-6). Noninvasive biomarkers (NIBMs)NIBMs for liver fibrosis are divided into class 1(direct biomarkers) which directly correlate with the fibrosis stages and they are parts of the liver matrix produced by the hepatic stellate cells during ECM turnover in the fibrosis process (7-10), and class 2(indirect biomarkers) which reflect changes in liver functions and are molecules released into the blood due to liver inflammation, but they do not correlate with ECM turnover (7, 10).

Amino-terminal type III procollagen (P3NP) has been reported to be a non- invasive direct serologic marker of liver fibrosis in liver inflammation and cirrhosis and thereby reduce the need for liver biopsies. (11)

The AST/platelet ratio (APRI) was developed by Wai et al. (12) and is measured as APRI = AST level (ULN) × 100 divided by platelet count.

Many other studies have been conducted to validate the APRI (13, 14). And they demonstrated that APRI is of a great value and has high accuracy in predicting severe fibrosis in different liver diseases (13- 16). In contrast, some studies showed that the APRI is only of moderate accuracy in assessing fibrosis in chronic hepatitis C(17).

So the idea of collaborating both direct and indirect markers may improve the diagnostic accuracy of both. The present study aims to validate the combining markers (dividing APRI value by P3NP) as a new predictive model for detecting significant fibrosis with high degree of accuracy.

Methods:-

Study population:-

A prospective small scale study (pilot study) was conducted on 20 adult male patients aged between 19-65 years, selected from the out patients attending Gastro-intestinal unit of xxx Institute hospital, xxx, xxx. All patients included were serologically confirmed to have Hepatitis C i.e. anti-HCV (+) determined by ELISA assay and confirmed by being HCV-RNA (+), of more than 6 months duration with normal renal function while patients with severe heart disease, psychiatric disorders, autoimmune disease or concordant malignancies, also patients who were receiving steroid / immunosuppressive therapy, positive for HBsAg, ongoing drug abuse or history of alcohol intake , or with decompensated liver cirrhosis were excluded.

The study protocol conformed to the ethical guidelines of 1975 the Hel-sinki declaration and was accepted by the ethical committee. Written informed consents were obtained from participants in this study.

Blood collection and sample preparation:-

- 2 ml of whole venous blood were collected without stasis in Ethylenediamine tetraacetic acid (EDTA) tube (1.2 mg EDTA/ml**)**

- 1.8 ml of venous blood were collected without stasis in 0.2 ml (0.109 M (i.e. 3.2%))tris sodium citrate anticoagulant then mixed well immediately, centrifuged at 3000 round per minute (rpm) for 10 minutes. The plasma was then separated (within two hours) and freezed at -70 °C.
- Six milliliters of venous blood were collected in dry clean centrifuge tubes, left to clot for 30 minutes at 37 °C, then centrifuged at 3000 rpm for 10 minutes. The serum was then separated, divided into several aliquots, and stored at -20°C to be thawed once on demand.

Blood samples (10ml) were collected after an overnight fast. After analyzing cell blood counts (CBC) on aliquots of whole blood in EDTA tube (1.2 mg EDTA/m), on remainder the samples, serum was separated (3000 rpm for 10 minutes) after 30 min of blood collection and stored at-20 C. Hepatitis C antibody was done according to Holmes et al. from Ortho-Clinical Diagnostics Company. Quantitative determination of the HCV b-DNA was according to Choo et al.(18). The kit was supplied from Versant Company and Hepatitis B surface antigen (HBsAg; Axiom Gesellschaft fürDiagnostica und BiochemicambH Company) according Van der Poel et al.(19). AFP and ANA measurements were conducted.

Liver and kidney function tests, including alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), gamma-glutamyltranspeptidase (GGT), total proteins, Serum albumin, total bilirubin, direct bilirubin, prothrombin time, prothrombin concentration , International Normalization Ratio, creatinine and blood urea nitrogen (BUN) were done. Briefly AST and ALT were measured according to Bergmeyer et al. & Saris (20 &21) respectively by the automated device, Peckman. The kit was supplied from DADE BEHRING Company.

The sequential algorithm for fibrosis evaluation (SAFE), which detects significant fibrosis (≥F2 by METAVIR) and cirrhosis (F4) by combining the AST-to-platelet ratio index and Fibrotest was chosen for its validated diagnostic accuracy, combined with its practicability, improved patient acceptance, and reduced risk/cost profile in comparison with the generalized use of liver biopsy (22). Consequently, in the present study patients were not capable to go through liver biopsy, hence the staging of liver fibrosis was done according to this algorithm

The algorithm was done as follows: $APRI^2$ was calculated for all patients through the treatment period. Patients with $APRI^2 \leq 0.5$ were considered to have no significant fibrosis, then Fibrotest was determined for patients with $APRI^2 > 0.5$. If Fibrotest was ≤0.48, these patients were categorized as with no significant fibrosis (No fibrosis). If Fibrotest was > 0.48, therefore these patients were considered to have significant fibrosis (Fibrosis).(23**)**

Serum P3NP was quantitatively assayed using the Orion Diagnostica RIA designed for the in vitro measurement of P3NP concentration in human serum according to the method of Risteli et al.(24).

Combined markers for fibrosis evaluation by using:

- **Fibrotest and Actitest**
- **APRI**= $\frac{\text{AST(/ULN)x100}}{\text{Platelets}(10^9/\text{L})}$
- **FIB-4**: (age [yr] × AST [U/L]) / ((PLT [10^9/L]) × (ALT [U/L])$^{1/2}$**(25)**
- **FibroQ** = [(10 x age x AST x PT INR) / (PLT x ALT)](26)

To assess the diagnostic accuracy of APRI / PIIINP as surrogate marker of liver fibrosis we correlate it with SAFE algorithm and comparing it with other combined markers to validate which of them a reliable tool for evaluation of liver fibrosis in chronic HCV patients.

Statistical analysis:-

Statistical analysis was carried out by the aid of a digital computer, using Excel & SPSS version 15 programs.

Data were expressed as mean ± SD, and P less than 0.05 as considered statistically significant.

Student t-testis suited for assessment of the statistical significance of differences between two sample mean values of quantitative data.

We plotted the receiver operating characteristic curve (ROC). Receiver operating characteristic curves were generated by plotting the relationship of the true positivity (sensitivity) and the false positivity (1- specificity) at various cut-off points of the tests. If AUC = 1, the index is an ideal predictor, and if AUC = 0.5, the index has no predictive value. The greater the AUC the better the test. The diagnostic accuracy, sensitivity, specificity, positive predictive values (PPV) and negative predictive values (NPV) also were calculated.

Result:-

Demographical and laboratory parameters of the patients are demonstrated in table (1**)**

Table 1:- Demographical and laboratory parameters of the patients.

Variables	Mean±SD
Age(years)	49.5±10.55
Platelet count (109/L)	148.25±63.3
ALT(U/L)	99.9±44.55
AST(U/L)	82±39.25
PT(sec)	75.315±16.29
INR	1.23±0.2
T.BIL(mg/dl)	1.05±0.39
D.BIL(mg/dl)	0.375±0.2
P3NP(µg/dl)	10.23±3.05
Fibrotest value	0.83±0.185
Actitest	0.62±0.13
APRI[1]	1.4±0.7
FibroQ	0.83±0.18
FIB4	2.78±1.25
APRI[1]/P3NP ratio	13±6.4

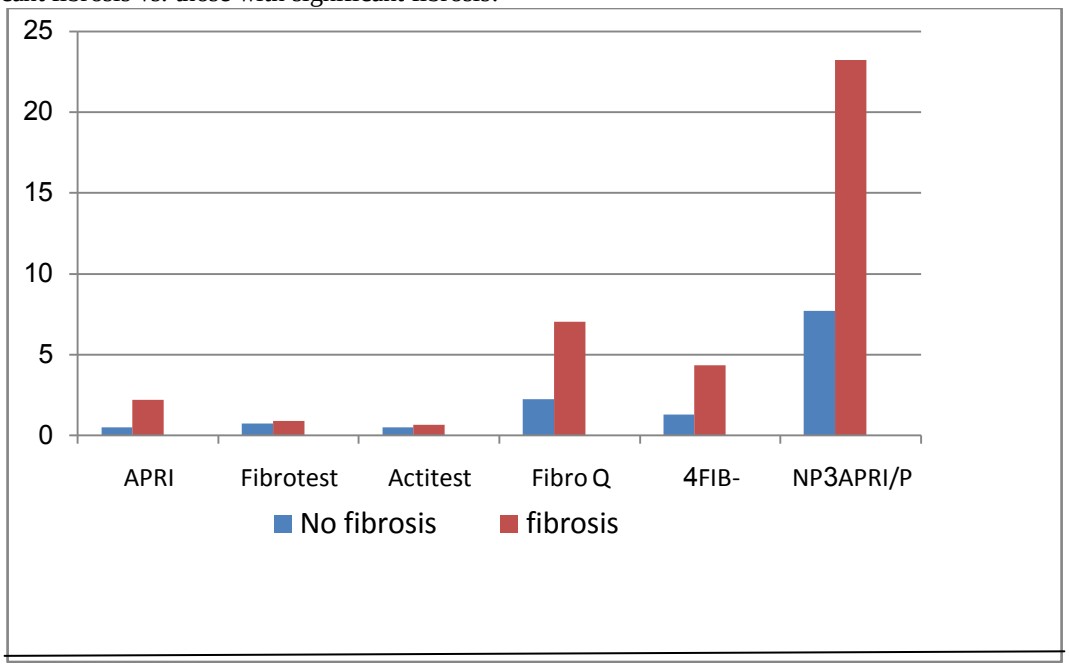
ALT(alanine transaminase), AST (aspartate transaminase),T.Bil(total bilirubin),D.Bil(direct bilirubin), P3NP (procollagen type III N-terminal peptide),APRI(ALT/Platelet), FibroQ(fibro- quotient) , FIB4(four fibrosis indirect biomarker)

Simple parameters for fibrosis are assessed and results revealed increase in the level of platelet number in those with no fibrosis versus those with fibrosis ($226\pm51\times10^3$&$105\pm36\times10^3$ respectively) with p-value 0.001.Also prothrombin concentration in non fibrotic patients(87± 9.4) is higher than in fibrotic patients (67.3± 12)

While AST, ALT & ALP in those with fibrosis are higher (86.6±43.7, 85.5± 48.7 & 104.3± 50.1 respectively) than those with no fibrosis (42.7± 11.2, 55.5± 27.7 & 67.7± 28 .7 respectively) with statistically significance p –value < 0.05

Statistical analysis for differences in combined parameters in patients without significant fibrosis vs. those with significant fibrosis are shown in figure (1)

Figure 1:- Difference in parameters (APRI, Fibrotest, Actitest,FibroQ,FIB-4,APRI/P3NP) in patients without significant fibrosis vs. those with significant fibrosis.



The calculated cut-off values of simple fibrosis markers using Fisher's exact test in patients without significant fibrosis vs. those with significant fibrosis are illustrated in table (2)

Table 2: Cross-tabulation for the calculated cut-off values of simple fibrosis markers using Fisher's exact test in patients without significant fibrosis vs. those with significant fibrosis.

Variable	cuttoff	No Fibrosis	Fibrosis	X^2	LR	PPV	NPV	% Sensitivity	% Specificity
ALT U/L	<63	17	11	0.007	7.5	61	74	68	68
	=>63	8	23						
AST/ALT	<0.87	14	7	0.006	8	67	71	56	79
	=>0.87	11	27						
	<76.5	6	25						
	=>11.65	0	10						
PT %	>78.8	20	5	0.001	27	80	85	80	85
	=<78.8	5	29						
P3NP µg/dL	<6.75	18	6	0.001	18.4	75	80	72	82
	=>6.75	7	28						

PPR:Positive predictive value NPR:Negative predictive value LR:Likelihood ratio

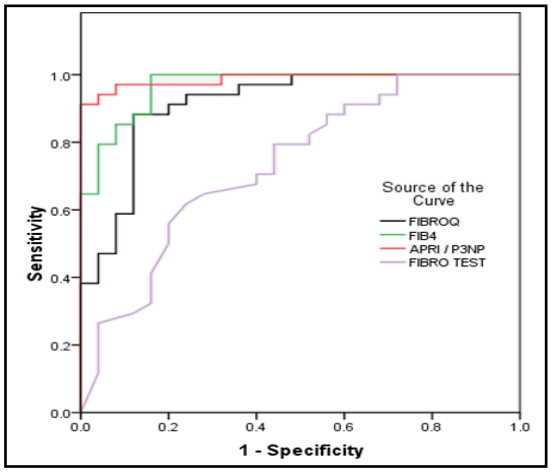
While the calculated cut-off values of Fibro Test, FIB-4, APRI/ P3NP and FibroQ using Fisher's exact test for no fibrosis and significant fibrosis in all treatment periods are shown in table (3).

Table 3:- Cross-tabulation for the calculated cut-off values of Fibro Test, FIB-4, APRI/ P3NP and FibroQ using Fisher's exact test for no fibrosis and significant fibrosis in all treatment periods.

Marker	Cutoff	NO FIBROSIS	FIBROSIS	Area	SE	P	X^2	LR	PPV	NPV	% Sensitivity	% Specificity
FIBRO T.	**<0.895**	18	12	**0.735**	.067	0.002	**0.005**	**8**	**60**	**76**	**72**	**65**
	=>0.895	7	22									
FIB-4	**<1.76**	21	0	.967	.020	0.001	**0.001**	**55**	**100**	**90**	**84**	**100**
	=>1.76	4	34									
APRI[1]/ P3NP %	**<13.73**	25	3	.987	.011	0.001	**0.001**	**61**	**89**	**100**	**100**	**91.2**
	=>13.73	0	31									
FIBROQ	**<3.5**	22	4	.914	.039	0.001	**0.001**	**38**	**85**	**91**	**88**	**88**
	>=3.5	3	30									

PPR:Positive predictive value NPR:Negative predictive value LR: Likelihood ratio SE: Standard error

Figure 2:- ROC curves for Fibro Test, FIB-4, APRI/ P3NP and Fibro Q in significant fibrosis versus non-significant fibrosis.



Results depicted in figure 2 and Table 3 showed that the diagnostic performance of APRI /P3NP for the identification of significant fibrosis, the cutoff (< 13.73) to rule out significant fibrosis showed a NPV of 100% and AUROC of 0.987, while the cutoff (≥13.73) to rule in significant fibrosis. On the other hand, FIB-4 the cutoff (≥1.76) to rule in significant fibrosis showed a PPV of 100% and AUROC of 0.967, while the cutoff (< 1.67) to rule out significant fibrosis. So APRI/P3NP% could be used to exclude the presence of fibrosis, while the FIB-4 marker could be applied to confirm the presence of fibrosis.

Discussion:-

The rapid advances in understanding the pathophysiology of liver fibrosis have generated intense interest in exploiting these insights to develop anti-fibrotic therapies for patients with chronic liver diseases. However, a remaining obstacle is the need to establish effective endpoints of anti-fibrotic drugs that are not reliant on liver biopsy, since changes in extracellular matrix content are likely to evolve more slowly than molecular markers of fibrogenic activity (27 & 28).

Liver biopsy is the most accurate method for diagnosis of liver fibrosis but th**e** following limitations (a)The liver biopsy does not efficiently reflect the fibrotic changes occurring in the whole liver because the biopsy; contains 5–11 complete portal tracts; reflects only 1/50000 the volume of the liver. (b) The process of hepatic fibrosis is not

linear reflecting the presence of different stages of fibrosis concurrently. (c) Several reports have shown that cirrhosis may be missed in 10–30% of patients. (d) A liver biopsy cannot differentiate between early and advanced end stage cirrhosis. (e) There is a risk of complications arising from liver biopsy varying from mild abdominal pain, to severe hemorrhage and injury to the biliary system. (7) Patients require hospital observation for 4–6 hours after liver biopsy. (29)

The rationale of this study was to evaluate some serological markers in testing the progression of hepatic fibrosis in chronic HCV patients.

The staging of liver fibrosis in the present study was done according to the recently described sequential algorithm for fibrosis evaluation (SAFE), which detects significant fibrosis (≥F2 by METAVIR) and cirrhosis (F4) by combining the AST-to-platelet ratio index and Fibrotest-Fibrosure (24). It was chosen for its validated diagnostic accuracy, combined with its practicability, improved patient acceptance, and reduced risk/cost profile in comparison with the generalized use of liver biopsy.

A perfect marker would have 100% sensitivity and 100% specificity; such a marker will identify presence or absence of fibrosis, and differentiate between responders or non-responders (30& 31**).** In practice, however, few markers are perfect, and one has to strike a balance between sensitivity and specificity (31). According to Poynard et al. (32) the receiver operating characteristic (ROC) curve is widely accepted as a method for selecting an optimal cut-off point of a marker for 'normal' or 'abnormal'. The curve may be used also to assess the diagnostic accuracy of a marker(s), and to compare the usefulness of different markers (30 &33**).**Thus by using the cutoff values of different markers, patients could be identified correctly to have or not to have significant fibrosis.

The main outcome of this study could be summarized in that the use of an equation consisting of 3 simple markers, the AST/Platelets (APRI) /P3NP with a cut off value of 13.73%, could be used as a reliable marker for the presence of fibrosis.

It is a good inflammatory score predictor (34 & 35), since it is released into the serum during matrix removal and deposition. P3NP reflects the stage of fibrosis and is known to be elevated in chronic liver disease.

Patients included in the present study were not able to undergo liver biopsy, or receive treatment with the standard pegylated interferon combined with ribavirin regimen. Consequently, the staging of liver fibrosis was done according to the recently described sequential algorithm for fibrosis evaluation (SAFE), which detects significant fibrosis (≥F2 by METAVIR) and cirrhosis (F4) by combining the AST-to-platelet ratio index and Fibrotest-Fibrosure (34). Several serological markers were tested for their reliability as fibrosis markers; they included direct or indirect markers, assessed alone (simple) or combined.

Direct serum markers reflect ECM turnover, balance between hepatic fibrogenesis and fibrolysis, and in the deposition and removal of ECM. Their levels are elevated during disease progression and are independently associated with stage of fibrosis**.** From these markers P3NP was chosen. It is released into the serum during matrix removal and deposition, and reflects the stage of fibrosis where it is elevated in chronic liver disease. It is a good inflammatory score predictor as compared to fibrosis (36)
Results of the current investigation revealed that all of these markers were significantly higher in patients with significant fibrosis except the GGT and AFP. However, further statistical analysis by ROC curves; revealed that only four combined markers showed significant predictive value for presence of fibrosis, these were the known Fibrotest, FIB-4, and FIBROQ tests, and the fourth marker was deduced from the statistical analysis and constituted from APRI/P3NP.

Presently, neither APRI nor P3NP alone showed reliable accuracy in predicting fibrosis in hepatitis patients. However, when combined; by dividing the APRI value by P3NP, a new predictive model was constructed based on these variables, and its area under the ROC curve was 0.987, which demonstrated a high degree of accuracy for predicting significant fibrosis.

The initial study by Borsoi et al (37) reported that APRI [(AST/UNL)/Platelets $x10^9$/L] is a serological marker that has satisfactory sensitivity and specificity together with a high predictive value

The APRI index is usually calculated by dividing the AST level by the upper limit of normal range (ULN) (AST/ULN), divided by the number of platelets $x10^9$/L(38)

From the recorded data, it could be concluded that by combining indirect markers (AST & platelets "APRI") with direct ones (P3NP) we could construct a new predictive model that demonstrates a high degree of accuracy for predicting significant fibrosis.

We should mention some limitations of the present study, first as regard the small sample size; the results of this study will need confirmation in a larger patient population. Secondly no liver biopsy taken tocorrelate fibrosis stages with the marker. Finally no control group to compare with for more validation of the marker.

The study concluded that APRI/P3NP, FIB-4, FIBROQ &Fibrotest tests showed significant predictive value for presence of fibrosis in descending order. APRI/P3NP% could be used to exclude the presence of fibrosis, while the FIB-4 marker could be applied to confirm the presence of fibrosis

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