



Yu.M. Stepanov, N.V. Nedzvetskaya, V.B. Yagmur, I.A. Klenina, N.Yu. Oshmyanskaya
State Institution "Institute of Gastroenterology of the National Academy of Medical Sciences of Ukraine", Dnipro, Ukraine

Development of a non-invasive model to improve the accuracy of determining liver fibrosis stage in nonalcoholic fatty liver disease

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Abstract. Background. The differentiation of mild (F1-F2) and advanced fibrosis (F3-F4), as well as the exclusion of fibrosis in patients with nonalcoholic fatty liver disease (NAFLD), are extremely important for prediction of the disease course. Integrative analyses of serum markers have been proposed as promising alternatives to biopsy method. Our study was targeted to develop a new model for determining the stage of fibrosis based on a more efficient combination of serological markers and to compare it with well-established algorithms.

Materials and methods. Sixty patients with biopsy-proven NAFLD, including 26 (43 %) men and 34 (57 %) women, with average age of 37.10 ± 12.4 and 44.30 ± 7.25 years, respectively, were recruited for the study. Particularly, advanced fibrosis was diagnosed in 8 patients, 28 had mild fibrosis and 24 didn't have any fibrosis according to morphological study. The following fibrosis markers were calculated: aspartate aminotransferase and alanine aminotransferase ratio (AAR), aspartate aminotransferase to platelet ratio index (APRI), fibrosis index based on the 4 factor (FIB-4). Among many variables, hyaluronic acid, α_2 -macroglobulin, apolipoprotein A1, fibronectin, and haptoglobin were included in comprehensive study. Integrative model have been built up to determine the stage of fibrosis. The models were compared with the area under the receiver operating characteristic (AUROC) curves. **Results.** The ROC analysis showed that the FIB-4 demonstrated the largest AUROC, for the F2 – 0.72, F3 – 0.8, F4 – 0.82, respectively. Obtained results of the APRI were significantly higher for mild and advanced fibrosis (F2 – 0.74, F3 – 0.82). The AAR values were reliable only for liver cirrhosis (AUROC 0.89). A strong direct correlation was determined between the stage of fibrosis and the level of hyaluronic acid, α_2 -macroglobulin and fibronectin ($r = 0.72, 0.93$ and $0.71, p < 0.05$, respectively). Whereas, we observed a moderate negative linear correlation between fibrosis stage and the indices of both apolipoprotein A1 and haptoglobin ($r = -0.61; r = -0.35$, respectively, $p < 0.05$). The positive correlation was determined between activity of the inflammatory process and the content of hyaluronic acid, α_2 -macroglobulin and fibronectin ($r = 0.54, 0.67$ and 0.55 at $p < 0.05$), while the reverse moderate relation observed for apolipoprotein A1 and haptoglobin ($r = -0.56$ and $-0.33, p < 0.05$). **Conclusions.** The analysis of obtained results showed that α_2 -macroglobulin, apolipoprotein A1, hyaluronic acid, and fibronectin had the greatest diagnostic validity among non-invasive markers of fibrosis. Every of them get the AUROC level higher than 0.75 for minimal fibrosis and, moreover, for moderate, significant fibrosis and cirrhosis they had an area more than 0.9.

Keywords: nonalcoholic fatty liver disease; liver fibrosis; liver biopsy; non-invasive diagnostic methods

Introduction

Nonalcoholic fatty liver disease (NAFLD) is the most prevalent liver disease in the Western world and a common reason for clinical evaluation due to advance the liver function tests [1]. The histopathological features of NAFLD include a wide spectrum of changes, ranging from simple steatosis to steatohepatitis and cirrhosis with risk of developing hepatocellular carcinoma [1]. Moreover, NAFLD has been

established as a risk factor for cardiovascular morbidity and it is closely associated with an increased risk of metabolic disease, including diabetes [2]. Several follow-up studies have demonstrated increased mortality among patients with NAFLD. The main reason of the excess mortality belong to cardiovascular diseases, however, liver-related mortality is also greatly overrepresented [3, 4]. There is no mutual consent on the item of the differences between NAFLD patients that need

(12 ± 32) mm. Biopsies were graded and staged according to the Brunt classification.

In the morphological study, the absence of fibrosis was found in 24 (40 %) patients, mild fibrosis (F1) occurred in 23 (38.3 %), moderate (F2) — 5 (8.3 %), severe (F3) — 4 (6.7 %), cirrhosis (F4) was detected in 4 (6.7 %) patients.

Non-invasive fibrosis algorithms. For each patient, the **AST/ALT (AAR)** ratio was determined. The **APRI** and **FIB-4** indices were calculated as follows:

$$FIB-4 = (age (years) \times AST (U/L)) / (platelet count (G/L) \times \sqrt{ALT (U/L)}).$$

$$APRI = (AST (U/L) \times 100) / (AST (ULN) \times platelet count (G/L)).$$

All relevant primary patient-level data are shown in table 2.

Statistical analysis. Statistical analyses were performed using SPSS (Statistica for Windows 10.0.) unless otherwise specified. The Shapiro-Wilk test was used to test for normal distribution. Median (range) was calculated for continuous variables, frequencies for categorical variables. Continuous variables were compared using the Student's t test or the Mann — Whitney U test when ap-

propriate. The χ^2 test or Fischer's exact test were used to compare categorical variables. A two-sided p-value < 0.05 was considered statistically significant if not specified otherwise. Receiver-operating characteristics (ROC) curves of the tested scores were constructed for each stage of fibrosis as well as the area under the ROC curve (AUROC) was calculated to assess the overall diagnostic accuracy of serum fibrosis algorithms and to identify optimal cutoffs. The sensitivity, specificity were calculated according to standard methods.

Results

60 patients with NAFLD were examined (26 (42.3 %) male and 34 (56.7 %) female), the average age of the subjects was (37.1 ± 12.4) and (44.3 ± 7.3) years in accordance. Anthropometric data of patients are presented in table 3.

The ROC analysis showed that FIB-4 index had the largest AUROC level for F 2 — 0.72, F3 — 0.8, F4 — 0.82, p < 0.05. We revealed APRI index was significantly higher for moderate and severe fibrosis (F2 — 0.74, F3 — 0.82, p < 0.05). And the AAR values were reliable only for liver cirrhosis (AUROC 0.89, p < 0.05).

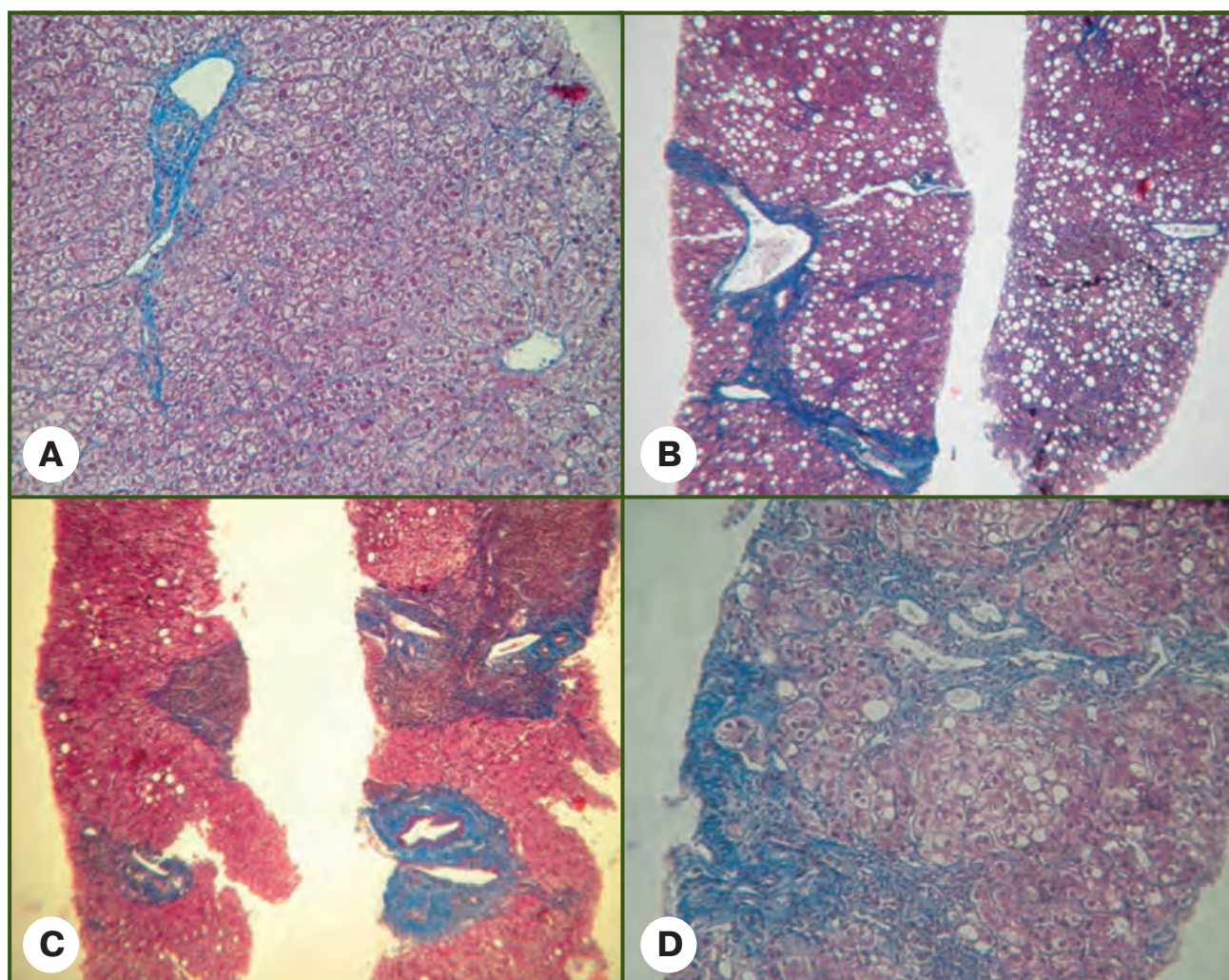


Figure 1 — A — Liver biopsy specimen of the patient A. with F1 fibrosis; B — Liver biopsy specimen of the patient B. with F2 fibrosis; C — Liver biopsy specimen of the patient G. with F3 fibrosis; D — Liver biopsy specimen of the patient with F4 fibrosis

The data of the ROC analysis, depending on the stage of fibrosis, as well as the reliability of the differences in the indicators between the groups are shown in table 4.

Correlation analysis showed a strong direct correlation between the stage of fibrosis and the level of hyaluronic acid, α_2 -macroglobulin and fibronectin ($r = 0.72, 0.93$ and 0.71 at $p < 0.05$ for Spearman correlation, respectively). The moderate negative linear correlation ($r = -0.61$ and -0.35 , respectively, $p < 0.05$ for Spearman correlation) was found between the indices of apolipoprotein A1, haptoglobin and the fibrosis stage. We determined moderate positive correlation between the degree of inflammatory process activity and the indices of hyaluronic acid, α_2 -macroglobulin and fibronectin ($r = 0.54, 0.67$ and 0.55 , respectively, $p < 0.05$ for the Spearman correlation) and the inverse correlation for apolipoprotein A1 and haptoglobin ($r = -0.56$ and -0.33 , respectively, $p < 0.05$ for the Spearman correlation).

To determine the diagnostic value of the investigated serum markers of fibrosis, an ROC analysis was performed, which resulted in a high sensitivity of the protein-bound oxyproline for fibroblastic stages from F2 to F4 and sufficient sensitivity for minimal fibrosis and cirrhosis of the liver (75 %).

The largest AUROC level (0.699, CI 0.466–0.931; the optimal threshold — 167) was observed in a case of liver cirrhosis. But these results were not statistically significant ($p > 0.05$). On the contrary, the sensitivity and specificity of hyaluronic acid for F3 fibrosis were statistically significant ($p < 0.005$), and had the largest AUROC level (0.915, CI 0.837–0.992; the optimal threshold — 1.82).

The analysis of the results on the apolipoprotein A1 indices showed the largest AUROC level (0.997, CI 0.987–1.0;

Table 2 — Laboratory and non-invasive test data

Parameter	Fibrosis stages				
	F0	F1	F2	F3	F4
	n = 24	n = 23	n = 5	n = 4	n = 4
ALT, U/L	67.7 ± 77.5	54.8 ± 35.1	109.2 ± 111.1	94.9 ± 65.4	66.8 ± 52.1
AST, U/L	42.7 ± 47.8	35.3 ± 16.2	53.0 ± 36.9	56.0 ± 13.0	90.7 ± 76.1
Hyaluronic acid, g/l	1.14 ± 0.30	1.49 ± 0.35	1.81 ± 0.19	1.88 ± 0.04	2.03 ± 0.34
Apolipoprotein A1, g/l	1.91 ± 0.22	1.79 ± 0.24	1.33 ± 0.32	1.09 ± 0.05	0.98 ± 0.08
α_2 -macroglobulin, g/l	1.48 ± 0.15	1.99 ± 0.10	2.57 ± 0.18	2.87 ± 0.07	3.48 ± 0.41
Haptoglobin, g/l	1.22 ± 0.42	1.12 ± 0.28	0.998 ± 0.12	0.93 ± 0.14	0.14 ± 0.03
Fibronectin, g/l	0.27 ± 0.03	0.30 ± 0.03	0.35 ± 0.02	0.37 ± 0.08	0.45 ± 0.02
Protein-bound oxyproline, μ mol/l	203.3 ± 50.3	229.3 ± 65.0	231.95 ± 28.95	219.3 ± 67.7	197.0 ± 109.3
FIB-4	0.82 ± 0.52	0.79 ± 0.35	0.80 ± 0.35	1.05 ± 0.28	1.46 ± 0.66
APRI	0.40 ± 0.43	0.32 ± 0.14	0.45±0.30	0.70±0.34	0.84 ± 0.53
AAR	0.76 ± 0.25	0.74 ± 0.24	0.62±0.21	0.74±0.31	1.32 ± 0.65

Table 3 — Anthropometric data of patients

Gender	Weight, kg	BMI, kg/m ²
Men	91.4 ± 12.9	28.2 ± 3.6
Women	84.4 ± 12.7	29.0 ± 4.5

Table 4 — The data of the ROC analysis of the non-invasive tests

Non-invasive index	Fibrosis stage				
		F1	F2	F3	F4
AAR	sensitivity, %	79.2	48.9	80.7	80.4
	specificity, %	38.9	76.9	62.5	100
	AUC	0.51	0.56	0.7	0.89
	p	0.45	0.3	0.08	0.008
APRI	sensitivity, %	95.8	91.5	90.4	91.1
	specificity, %	27.8	53.8	75	75
	AUC	0.58	0.74	0.82	0.77
	p	0.24	0.02	0.007	0.06
FIB-4	sensitivity, %	50	63.8	61.5	51.1
	specificity, %	75	84,6	100	100
	AUC	0.59	0.72	0.8	0.82
	p	0.21	0.031	0.01	0.03

the optimal threshold — 1.13) was established for severe fibrosis (F3). The sensitivity of the method for all fibrosis stages was quite high (96.4–100 %), but the specificity for minimal fibrosis (F1) was low (38.9 %).

The determining of α_2 -macroglobulin was almost the ideal method in our study, for which AUROC level reached 1 for fibroblasts from F2 to F4 with the optimum threshold — 2.17 for F2, 2.75 — for F3 and 2.96 — for F4.

Concerning haptoglobin, high level of specificity was determined in the case of F1 and F2 (94.4 and 100 % respectively) and had a rather low sensitivity level (33.0 and 40.4 % respectively) in patients with minimal fibrosis and was not statistically significant. The largest AUROC level (1; CI 1–1; the optimal threshold — 0.67) was defined for liver cirrhosis (F4).

The fibronectin indices with a high level of sensitivity and specificity at all stages of fibrosis were statistically significant. The largest AUROC level (curve) (0.996, CI 0.981–1.01; the optimal threshold — 0.411) was defined for liver cirrhosis (F4).

Consequently, based on the data of non-invasive fibrosis markers in patients with NAFLD, the highest diagnostic value was shown for α_2 -macroglobulin, apolipoprotein A1, hyaluronic acid and fibronectin, for which the AUROC level for minimal fibrosis was higher than 0.75, and for moderate, severe fibrosis and cirrhosis was more than 0.9.

Based on presented results and correlation analysis data, as well as the sensitivity and specificity of non-invasive markers, a model for diagnosing liver fibrosis was developed. The linear regression analysis was used for validation of the model.

The model of multiple linear regression for the indicator of the stage of liver fibrosis in patients with NAFLD had the following form:

$$Y = -2.0323 - 0.0011 \times X1 + 0.1173 \times X2 - 0.3090 \times X3 + 1.5905 \times X4 + 0.0283 \times X5 + 1.44 \times X6,$$

where Y — stage of fibrosis; X1 — the level of oxyproline protein-bound, X2 — the level of hyaluronic acid, X3 — the level of apolipoprotein A1, X4 — the level of α_2 -macroglobulin, X5 — the level of haptoglobin, X6 — the level of fibronectin.

Discussion

According to the results of our study, APRI was the most sensitive of the calculated indices, but its sensitivity was sufficient only with developed fibrosis (F3–4). These data correspond with foreign studies.

Recent study of F.C. Kruger et al. [34], presented comparative analysis of the sensitivity and specificity of APRI, NFS and the ratio of ALT/AST. The analysis showed that for the diagnosis of severe fibrosis, APRI was more accurate than NFS and ALT/AST. Indicators of NFS in steatohepatitis were significantly lower in the group with severe fibrosis. Positive prognostic value for APRI was 54 %, while for NFS it was 34 %. Negative predictive value was 93 % for APRI and 94 % for NFS.

Study of Chinese researchers [35] included 131 patients, 41 (31.3 %), 20 (15.3 %) and 10 (7.6 %) of patients had moderate fibrosis, severe fibrosis and cirrhosis, respectively.

The highest accuracy of APRI with severe fibrosis (AUROC 0.77) and liver cirrhosis (AUROC 0.86) was observed.

The results of our study of serum liver fibrosis markers showed the high correlation several of them with the stage of fibrosis and the degree of inflammation activity. Using a combination of these indicators can greatly improve the diagnosis of NAFLD. Thus, according to data of Indian researchers, [36] it was found the presence of strong positive correlation between the platelet count, gamma-glutamyltranspeptidase and apolipoprotein A and the absence of liver fibrosis in patients with NAFLD (negative predictive value — 94.3 %). The results of study Sakugawa and coauthors, showed that level of hyaluronic acid can be used to reliably distinguish the initial (F0–2) and severe fibrosis (F3–4) (AUC = 0.97) [37]. Kaneda et al. [38] determined that hyaluronic acid was independent of prognostic factors of severe fibrosis (F3–4) (AUC = 0.97).

Conclusions

Consequently, the results of the study indicate a high diagnostic accuracy of the combination of serum markers for the diagnosis of both fibrosis and its stage in patients with NAFLD. A few intuition, accessibility and the possibility to control these markers will allow developing more effective treatment tactics and screening for the dynamics changes in this category of patients.

The characterized markers may be useful in primary care to «rule out» NAFLD patients with advanced fibrosis that should be referred for monitoring of liver-related complications. In secondary care they may be useful to «rule in» NAFLD patients with advanced fibrosis thereby reducing need for liver biopsies. However, our results lack external validation and need to be evaluated in future studies.

Conflicts of interests. Authors declare the absence of any conflicts of interests that might be construed to influence the results or interpretation of their manuscript.

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Степанов Ю.М., Недзвецька Н.В., Ягмур В.Б., Кленіна І.А., Ошмянська Н.Ю.
ДУ «Інститут гастроентерології НАМН України», м. Дніпро, Україна

Розробка неінвазивної моделі для підвищення точності діагностики стадії фіброзу печінки при неалкогольній жировій хворобі печінки

Резюме. Актуальність. Відмінність між м'яким (F1–2) і розвинутим фіброзом (F3–4), як і відсутність фіброзу у хворих на неалкогольну жирову хворобу печінки (НАЖХП), є важливим питанням для з'ясування прогнозу. Сироваткові маркери були запропоновані як альтернатива біопсії. **Мета:** розробити нову модель для визначення стадії фіброзу на основі більш ефективної комбінації серологічних маркерів та порівняння її з встановленими алгоритмами. **Матеріали та методи.** У дослідження включені 60 пацієнтів із НАЖХП, які проходили обстеження і лікування у відділенні захворювань печінки та підшлункової залози ДУ «Інститут гастроентерології НАМН України». Серед досліджених 26 (42,3 %) чоловіків та 34 (56,7 %) жінки, середній вік яких $(37,1 \pm 12,4)$ та $(44,3 \pm 7,3)$ року відповідно. Всім пацієнтам була проведена черешчкова пункційна трепан-біопсія печінки з подальшим морфологічним дослідженням пунктату; виконано визначення вмісту гіалуронової кислоти, α_2 -макроглобуліну, аполіпопротеїну A1, фібронектину, гаптоглобіну в сироватці крові та був проведений розрахунок неінвазивних маркерів фіброзу печінки: AAR, APRI, FIB-4. **Результати.** Проведений ROC-аналіз показав, що найбільші показники площі під AUROC-кривою були для індексу FIB-4 для F2 — 0,72, F3 — 0,8, F4 — 0,82 при $p < 0,05$. Результати індексу APRI

були вірогідно високими для помірного та вираженого фіброзу (F2 — 0,74, F3 — 0,82, $p < 0,05$). А показники AAR були вірогідними лише для цирозу печінки (площа під AUROC-кривою 0,89, $p < 0,05$). Установлено сильний прямий зв'язок між стадією фіброзу та рівнем гіалуронової кислоти, α_2 -макроглобуліну і фібронектину ($r = 0,72, 0,93$ та $0,71$ відповідно при $p < 0,05$) та обернений зв'язок помірної сили між показниками аполіпопротеїну A1, гаптоглобіну ($r = -0,61$ і $-0,35$ при $p < 0,05$). Визначено прямий зв'язок помірної сили між ступенем активності запального процесу і показниками гіалуронової кислоти, α_2 -макроглобуліну і фібронектину ($r = 0,54, 0,67$ та $0,55$ при $p < 0,05$) і обернений помірний зв'язок — аполіпопротеїну A1 та гаптоглобіну ($r = -0,56$ і $-0,33$ при $p < 0,05$). **Висновки.** Установлено, що найбільшу діагностичну цінність із неінвазивних маркерів фіброзу мають показники α_2 -макроглобуліну, аполіпопротеїну A1, гіалуронової кислоти та фібронектину, для яких площа під AUROC-кривою для мінімального фіброзу не була нижчою ніж 0,75, а для помірного, вираженого фіброзу та цирозу становила більше 0,9.

Ключові слова: неалкогольна жирова хвороба печінки; фіброз печінки; біопсія печінки; неінвазивні методи діагностики

Степанов Ю.М., Недзвецкая Н.В., Ягмур В.Б., Кленина И.А., Ошмянская Н.Ю.
ГУ «Институт гастроэнтерологии НАМН Украины», г. Днепр, Украина

Разработка неинвазивной модели для повышения точности диагностики стадии фиброза печени при неалкогольной жировой болезни печени

Резюме. Актуальность. Отличие между мягким (F1–2) и развитым фиброзом (F3–4), как и отсутствие фиброза у больных неалкогольной жировой болезнью печени (НАЖБП), является важным вопросом для выяснения прогноза. Сывороточные маркеры были предложены как альтернатива биопсии. **Цель:** разработать новую модель для определения стадии фиброза на основе более эффективной комбинации серологических маркеров и сравнения ее с установленными методами. **Материалы и методы.** В исследование включены 60 пациентов с НАЖБП, которые проходили обследование и лечение в отделении заболеваний печени и поджелудочной железы ГУ «Институт гастроэнтерологии НАМН Украины». Среди исследованных 26 (42,3 %) мужчин и 34 (56,7 %) женщины, средний возраст $(37,1 \pm 12,4)$ и $(44,3 \pm 7,3)$ года соответственно. Всем пациентам была проведена чрескожная пункционная трепан-биопсия печени с последующим морфологическим исследованием пунктата; выполнено определение содержания гиалуроновой кислоты, α_2 -макроглобулина, аполипротеина A1, фибронектина, гаптоглобина в сыворотке крови и был проведен расчет неинвазивных маркеров фиброза печени: AAR, APRI, FIB-4. **Результаты.** Проведенный ROC-анализ показал, что наибольшие показатели площади под AUROC-кривой были для индекса FIB-4 для F2 — 0,72, F3 — 0,8, F4 — 0,82 при $p < 0,05$.

Результаты индекса APRI были достоверно высокими для умеренного и выраженного фиброза (F2 — 0,74, F3 — 0,82, $p < 0,05$). А показатели AAR были достоверными только для цирроза печени (площадь под AUROC-кривой 0,89, $p < 0,05$). Установлены сильная прямая связь между стадией фиброза и уровнем гиалуроновой кислоты, α_2 -макроглобулина и фибронектина ($r = 0,72, 0,93$ и $0,71$ при $p < 0,05$ соответственно) и обратная связь умеренной силы между показателями аполипротеина A1, гаптоглобина ($r = -0,61$ и $-0,35$ при $p < 0,05$). Определена прямая связь умеренной силы между степенью активности воспалительного процесса и показателями гиалуроновой кислоты, α_2 -макроглобулина и фибронектина ($r = 0,54, 0,67$ и $0,55$ при $p < 0,05$) и обратная умеренная связь — аполипротеина A1 и гаптоглобина ($r = -0,56$ и $-0,33$ при $p < 0,05$). **Выводы.** Установлено, что наибольшую диагностическую ценность из неинвазивных маркеров фиброза имеют показатели α_2 -макроглобулина, аполипротеина A1, гиалуроновой кислоты и фибронектина, для которых площадь под AUROC-кривой для минимального фиброза не была ниже 0,75, а для умеренного, выраженного фиброза и цирроза составляла больше 0,9.

Ключевые слова: неалкогольная жировая болезнь печени; фиброз печени; биопсия печени; неинвазивные методы диагностики