• CLINICAL RESEARCH •

Association between thrombotic risk factors and extent of fibrosis in patients with non-alcoholic fatty liver diseases

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Abstract

AIM: To evaluate the prevalence of genetic and acquired prothrombotic risk factors and their association with the extent of fibrosis and fatty infiltration in patients with non-alcoholic fatty liver disease (NAFLD).

METHODS: Forty-four patients with chronic hepatitis (28 men and 16 women, with mean age of 45±11 and 49±12 years, respectively) constituted the patient population of this study. The groups were divided as follows: 15 patients with fatty liver (FL); 15 with non-alcoholic steatohepatitis (NASH); 14 with chronic viral hepatitis (CH) diagnosed by histology and liver technetium scan or ultrasound; and 10 healthy individuals. Thrombophilic, coagulation factors and genetic mutations were diagnosed by standard hemostatic and molecular coagulation assays.

RESULTS: Activated protein C (APC) resistance and protein S were the most prevalent thrombotic risk factors (6% and 10% in NAFLD *vs* 21% and 14% in CH; *P*<0.01 and *P*<0.05, respectively). One thrombotic risk factor was identified in 41% of patients (23% mild fibrosis, 18% severe fibrosis) and two thrombotic risk factors in 6% of patients with NAFLD and severe fibrosis. While no differences in APC ratio, lupus anticoagulant, fibrinogen, factor V Leiden, prothrombin, and MTHFR mutation were found. Protein S levels were significantly lower in NASH patients than in patients with FL alone (92±19 *vs* 106±2, *P*<0.01). Protein C levels were markedly higher in patients with NAFLD and mild or severe fibrosis as compared to the patients with CH, respectively (128±40 *vs* 96±14, *P*<0.001 or 129±36 *vs* 88±13, *P*<0.01).

CONCLUSION: Up to 46% of patients with NAFLD may have thrombotic risk factors, and the presence of thrombotic risk factors is correlated with the extent of hepatic fibrosis, suggesting a crucial role of the coagulation system in the pathogenesis of hepatic fibrosis.

INTRODUCTION

The clinical implications of non-alcoholic fatty liver disease (NAFLD) are derived mostly from its common occurrence in the general population (10-24%) and the potential of the condition to progress to fibrosis (40%) and cirrhosis (30%)[1-3]. Non-alcoholic steatohepatitis (NASH) is the most common cause of cryptogenic cirrhosis and is an increasingly common indication for liver transplantation[3]. Obesity, diabetes mellitus, and hyperlipidemia are conditions frequently associated with NASH[4,5]. Although the pathogenesis of NASH is unknown, it has been suggested that hepatic fatty infiltration may stem from continuous delivery of free fatty acids to the liver after ingestion of fatty foods and from increased splanchnic lipolysis of visceral fat, both of which increase hepatic insulin resistance[6]. Recently, two additional mechanisms were reported: oxidative stress/lipid peroxidation; and TNF-α/endotoxin-mediated injuries[7]. To our knowledge, it is still unclear what causes progression from steatosis to steatohepatitis and from steatohepatitis to bridging fibrosis and cirrhosis. Alcohol consumption, hepatic iron deposition[8], drugs, endotoxin[9,10], and polymorphism in cytochrome p450 enzymes[11] have been proposed as putative mechanisms. However, the definitive mechanism is yet to be determined.

Thrombosis of the intrahepatic veins is frequently observed in cirrhosis and has been associated with its progression[12], while occlusion of small intrahepatic veins and sinusoids has been considered as a potential triggering factor of liver tissue remodeling[13]. More recently, Wanless and Shiota[14] proposed a four-step model in patients with NAFLD, including steatosis facilitated by insulin resistance (first step), necrosis induced by lipid peroxidation (second step), release of lipid from hepatocytes into the interstitium leading to direct and inflammatory injury to small hepatic veins (third step), and the venous obstruction with secondary collapse and ultimately fibrosis (fourth step). The involvement of various genetic and acquired thrombotic risk factors in the pathogenesis of vein thrombosis is well established but their role in

NAFLD progression, to our knowledge, has not yet been investigated. The present study was designed to determine whether genetic and acquired thrombotic risk factors might be observed at some stage of liver disease progression in patients with NAFLD as well as their possible association with the extent of fatty infiltration and the extent of hepatic fibrosis.

MATERIALS AND METHODS

Patient population

Forty-four patients (28 men and 16 women, mean age of 45±11 and 49±12 years, respectively) were divided into three groups for the purpose of this study: 15 patients with fatty liver (FL); 15 with steatohepatitis (NASH); 14 with chronic viral hepatitis (CH) diagnosed by histology and liver technetium scan or ultrasound. Ten healthy controls were also included in this study. Patients with antiviral therapy within the past 6 mo, hepatocellular carcinoma, other forms of chronic liver diseases, positive anti-human immunodeficiency antibodies, previous venous thrombosis, chronic immunosuppression therapy, contraceptives or any current anticoagulation therapy were excluded.

Laboratory studies

All laboratory studies including coagulation assays were done within 6 wk of liver biopsy. Full blood count, platelets count, prothrombin time (PT), or INR, partial thromboplastin time (PTT), fibrinogen, liver enzymes including aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphates, γ-glutamyl transferase (GGT), glucose, insulin, cholesterol, and triglycerides were measured using commercially available assays. The following parameters were considered as thrombotic risk factors: deficiency in anti-thrombin, protein S, protein C, presence of lupus anticoagulant, activated protein C (APC) resistance with factor V Leiden mutation, fibrinogen, G20210A mutation in the prothrombin gene and MTHFR mutations. Deficiency in anti-thrombin III, protein S, and protein C were diagnosed when the protein level was below 80%, 60%, and 70%, respectively. Blood samples were collected into the vacuum tubes containing trisodium citrate. Platelet poor plasma was prepared by double centrifugation at 2 000 r/min for 15 min at room temperature. The assay, performed on fresh plasma or aliquots stored at -70 ℃ until assay, included factor VIII coagulant activity using factor VIII deficient plasma (Dae Behring) and Platelin-LS (Organon Tecknica, Durham, NC, USA). Antithrombin activity using an amidolytic assay (Coamatic antithrombin, Chromogenix, Montpellier, France), protein C activity using a clotting assay (IL Pro Clot, Instrumentation Laboratory, Lexington, MA, USA) and protein C antigen using an immunoenzymatic assay (Asserachrom protein C, Diagnostica Stago, France), free protein S using an immunoenzymatic assay (Asserachrom free protein S, Diagnostica Stago), and the presence of lupus anticoagulant were determined as previously described[15]. Molecular diagnosis of the factor V Leiden mutation was performed as described by Ridker *et al.*[16]. The G20210A mutation in the prothrombin gene was detected as described by Poort *et al.*[17]. Body mass index (BMI) was calculated as weight in kilograms divided by the square of height in meters. Insulin resistance was estimated using the homeostasis model assessment index (HOMA index) derived from the following equation: HOMA index = (fasting plasma glucose level mg%×0.055)×(fasting plasma insulin level μIU/mL)/22.5.

Histological evaluation

Liver biopsy specimens were fixed in Bouin's and embedded in paraffin, and the sections were stained with hematoxylin-eosin and Masson's trichrome. The diagnosis of NASH was established, if abnormal liver enzymes were associated with steatosis more than 10% in the presence of lobular and/or portal inflammation, with or without Mallory bodies, ballooning degeneration or fibrosis and the exclusion of other viral, metabolic or immunologic liver diseases for more than 3 mo[18]. The histological classification of NASH was made according to Brunt classification[19]. Steatosis was graded as follows: mild (5-30% of hepatocytes affected); moderate: (30-60% of hepatocytes affected); and severe (>60% of hepatocytes affected). Fibrosis was scored on a scale from 0 to 4, with 0 denoting no fibrosis, 1 describing focal pericellular fibrosis in zone three, 2 describing perivenular and pericellular fibrosis confined to zones two and three with or without portal/periportal fibrosis, 3 describing bridging fibrosis with architectural distortion but no cirrhosis, and 4 determining cirrhosis. Inflammatory grade was scored on a scale from 0 to 4, with 0 denoting no inflammation, grade 1 = sparse zone three inflammation, grade 2 = mild focal zone three hepatocyte inflammation, grade 3 = moderate zone three hepatocyte inflammation, and grade 4 = severe zone three hepatocyte inflammation. The histological classification of chronic viral hepatitis was made according to the new classification of Desmet *et al.*[20].

Statistical analysis

Data were expressed as mean±SD or as median and range. Kruskall-Wallis test for two-way analysis of variance (ANOVA) was used to evaluate difference between three or four groups. The difference between two groups was assessed by the two-tailed Mann-Whitney *U*-test for continuous variables and by χ^2 test for categorical variables. Bonferroni method was employed to correct for multiple comparisons. Correlations coefficient was evaluated by the Spearman's test and by simple linear or multiple stepwise regression analysis. The Winstat computer program was used for all calculations. Two-tailed *P* values less than 0.05 were considered statistically significant.

RESULTS

Epidemiological, biochemical, and histological characteristics of all patient populations are presented in Table 1. The patients with NAFLD were older and had increased BMI and increased insulin resistive index as compared to the patients with chronic viral hepatitis and to healthy controls (*P*<0.001). Inflammation score and ALT levels were obviously less pronounced in patients with NASH as compared to the patients with chronic viral hepatitis (inflammation score, 8.7±2.4 *vs* 10.4±5.0, *P*<0.01; and ALT, 43±18 *vs* 79±25, *P*<0.001). There was no significant difference in the severity

Table 1 Clinical, biochemical, and demographic characteristics of study population (mean±SD, *n*)

Characteristics	FL n = 15	NASH n = 15	CH n = 14	Healthy controls n = 10	P
Age (yr)	50±11	53±9	40±13	39±7	0.01
Gender (M/F)	10M/5F	7M/8F	11M/3F	6M/4F	NS
BMI	32±5	33.0±6	24.0±3	25.0±4	0.001
IRI	4.0±3.3	4.7±2.4	1.7±0.9	1.8±0.4	0.001
Hb (g/dL)	14±1.0	14±2.0	14±1.0	13.9±1.0	NS
WBC (×10^3 cell/mm^3)	6.2±1.4	7.4±1.9	7.2±2.1	5.9±0.9	NS
Platelets (10^3 cell/mm^3)	245±49	247±57	226±44	279±72	NS
INR	0.9±0.08	0.9±0.08	0.9±0.09	0.9±0.05	NS
PTT (s)	32±5.0	32±4.0	31.0±4.0	33.0±6.0	NS
Fibrinogen (mg/dL)	336±69	326±57	296±49	291±69	NS
ALT (IU/mL)	45±21	43±18	79±25	20±1.0	0.001
AST (IU/mL)	36±16	35±8	83±8	20±1.0	0.001
ALKPH (IU/mL)	87±22	87±26	73±20	70±1.0	NS
GGT (IU/mL)	73±35	56±29	62±45	50±1.0	0.5
Activity grade	2.1±1.3	8.7±2.5 (3.9)[1]	10.4±5.1 (12)[1]	ND	0.01
Fibrosis stage	0.5±0.0	1.4±1.6 (0.5)[1]	1.5±1.6 (1.0)[1]	ND	NS

[1]Numbers in parenthesis represent the median value.

of fibrosis stage between patients with NASH and patients with chronic viral hepatitis (1.4±1.6 *vs* 1.5±1.6).

APC resistance and protein S were the two most prevalent thrombotic risk factors observed in patients with NAFLD and in patients with chronic viral hepatitis (6% and 10% in NAFLD *vs* 21% and 14% in CH, *P*<0.01 and *P*<0.05, respectively, Table 2). While the prevalence of antithrombin III was more frequent in NAFLD as compared to chronic hepatitis (6% *vs* 0%, *P*<0.01), there was no significant difference between the two groups in APC ratio, lupus anticoagulant, and fibrinogen levels. There were also no significant differences in the genetic risk factors including factor V Leiden, prothrombin 20210A mutation, and MTHFR mutation. Finally, one patient in the NAFLD group was heterozygous for MTHFR mutation.

Table 2 Prevalence of thrombotic risk factors in 44 patients of study population (*n*, %)

Thrombotic risk factors	NAFLD (%)	CH (%)	Total (%)
Protein C deficiency	1 (3)	1 (7)	2 (10)
Protein S deficiency	2 (6)	3 (21)	5 (27)
Anti-thrombin III deficiency	2 (6)	0 (0)	2 (6)
Lupus anticoagulant	2 (6)	1 (7)	3 (13)
APC resistance	3 (10)	2 (14)	5 (24)
PT20210A mutation	None	None	None
Factor V Leiden	None	None	None
MTHFR mutation	1 (3)[1]	None	1 (3)

[1]Heterozygous mutation.

One thrombotic risk factor was present in 23% of patients with NAFLD and mild fibrosis as compared to 36% of patients with chronic hepatitis and mild fibrosis. Two thrombotic risk factors were present in 6% of patients with NAFLD. Absence of thrombotic risk factors was seen in 47% of patients with NAFLD and mild fibrosis and 6% with severe fibrosis (Table 3). No healthy individuals showed evidence of thrombotic risk factors.

Table 3 Number of thrombotic risk factors per patient in patients with NAFLD with mild and severe fibrosis score (*n*)

Number of thrombotic risk factors per patient fibrosis (%)	Mild fibrosis(%)	Severe
No risk factors	8 (47)	1 (6)
One risk factor	4 (23)	3 (18)
Two risk factors	1 (6)	0 (0)

Levels of thrombophilic and coagulation factors as well as the prevalence of thrombotic risk factors for the study population are presented in Tables 2 and 4, respectively. The patients with NASH had significantly more decrease in protein S levels as compared to the patients with FL alone (92±19 *vs* 106±20, *P*<0.01). Anti-thrombin III tended to be lower in NASH patients but did not reach statistical significance.

The association of fibrosis staging with patient characteristics and with levels of thrombophilic and coagulation factors is presented in Table 5. The patients with NASH and severe fibrosis had markedly more decrease in protein S levels compared to the patients without fibrosis (84±16 *vs* 100±22, *P*<0.01). An important finding was that protein C levels were higher in NASH patients at mild or severe fibrosis stage as compared to patients with chronic viral hepatitis (for mild fibrosis, 128±40 *vs* 96±14, *P*<0.001; or for severe fibrosis, 129±36 *vs* 88±13, *P*<001, Table 5, Figure 1A) and that protein C levels were correlated strongly with the extent of fatty infiltration (*r* = 0.6, Figure 1B). Table 6 shows the capacity of combined thrombotic risk factors, including protein S, antithrombin III, and fibrinogen to predict correctly the diagnosis of simple FL *vs* NASH. They classified the outcome correctly in 70% of cases. Finally, we observed a significant correlation between fibrotic score and proteins S level in patients with NAFLD (*r* = 0.4, *P*<0.05, Figure 2).

DISCUSSION

Our study has evaluated the association between thrombotic risk factors and the extent of hepatic fibrosis and fatty

Table 4 Levels of thrombophilic and coagulation factors in all study population (mean±SD)

Factors	FL *n* = 15	NASH *n* = 15	CH *n* = 14	Healthy controls *n* = 10	*P* ANOVA
Protein C (%)[a] (normal 70-130)	150±34	130±37	101±24	121±12	0.001
Protein S (%)[b] (normal 60-140)	106±20	92±19	95±37	90±17	0.01
Anti-thrombin III (%) (normal 80-120)	115±11	108±17	110±10	116±10	NS
APC ratio (normal >2.0)	2.2±0.1	2.2±0.9	2.2±0.3	2.2±0.1	NS
Lupus anticoagulant (normal <1.2 U/mL)	1.0±0.1	1.05±0.1	1.0±0.1	1.0±0.1	NS
Fibrinogen (normal 200-400 mg/dL)	336±69	326±57	296±49	291±69	NS

[1]*P* = 0.07, FL *vs* NASH; [b]*P*<0.01, FL *vs* NASH.

Table 5 Clinical and biochemical characteristics in all study population in relation to their fibrosis staging (mean±SD)

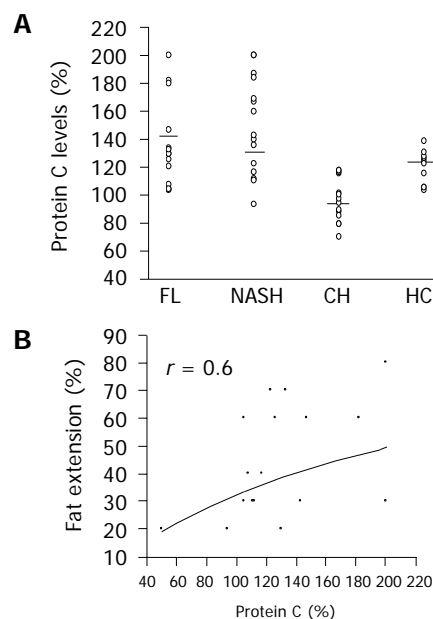| Characteristics	NAFLD		CH	
	Normal-mild fibrosis	Moderate-severe fibrosis	Normal-mild fibrosis	Moderate-severe fibrosis
IRI	3.9±3.0	3.9±1.6	1.2±0.7	1.9±0.8
BMI	31±5.0	34±6.7	23.6±1.5	24±3.9
Protein C (%)	128±40	129±36	96±14[b]	88±13
Protein S (%)	100±22[d]	84±16	96±19	77±16
Antithrombin III	110±17	116±8.9	111±3.7	101±7.4
APC ratio	2.3±0.1	2.2±0.1	2.01±0.3	2.3±0.3
Lupus anticoagulant	1.0±0.09	1.0±0.09	0.99±0.4	0.99±0.8
PT (%)	105±13	107±13	103±22	97±10
PTT (s)	32±5	29.5±2.0	32±4.6	30±4.3
Fibrinogen, mg/dL	306±57	337±57	290±29	314±64
PT mutation 20210A	Normal	Normal	Normal	Normal
Factor V Leiden	Normal	Normal	Normal	Normal
MTHFR (*n*)	One mutation	Normal	Normal	Normal
Platelets	244±43	237±47	242±36	225±44
Activity score	5.5±4.2	7.5±2.3	8.0±5.0	12.8±4.3

[a]*P*<0.01, CH mild fibrosis *vs* CH severe fibrosis; [b]*P*<0.01, NAFLD mild fibrosis *vs* NAFLD severe fibrosis.

Table 6 Results of capacity of combined risk factors for thrombosis (protein S, anti-thrombin III, and fibrinogen) in predicting the presence of FL or NASH patients (outcome)[1]

Results/ LNB	Actual number of patients	Predicted group	
		NASH (%)	FL(%)
NASH	15	11 (73)	4 (27)
FL	15	5 (33)	10 (67)

[1]70% of the cases were classified correctly.

Figure 1 A: Protein C levels (normal 70-130%) in all study population (- median levels). **B:** Correlation between fat extension and protein C levels (*r* = 0.6, *P*<0.001).

infiltration in patients with NAFLD. Thrombotic risk factors were detected in 46% of patients with NAFLD with APC resistance and protein S as the most common. While the patients with NASH and advanced fibrosis demonstrated a greater decrease in protein S levels compared to the patients with FL alone, the patients with NAFLD had a greater increase in protein C levels as compared to the patients with chronic viral hepatitis and healthy individuals.

The mechanism by which the patients with NAFLD display a decrease in some hemostatic parameters remains to be established. Since protein C, protein S, and antithrombin
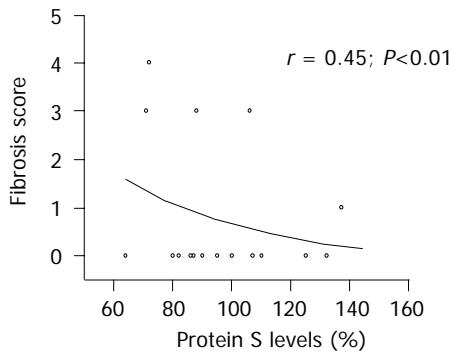
Figure 2 Correlation between fibrotic score and protein S levels in patients with NAFLD ($r = 0.45$, $P < 0.01$).

III are produced by the liver, their levels may be decreased in patients with advanced chronic liver diseases[21]. This is considered to be the case in patients with established cirrhosis. However, patients with cirrhosis were excluded from our study and only four patients with NAFLD had severe bridging fibrosis. More important was the finding that protein C levels were obviously correlated with the extent of fatty infiltration and were higher in the patients with NAFLD when compared to the patients with chronic viral hepatitis and to healthy individuals (Figure 1A). This increase in protein C levels is related either to increased hepatic synthesis or impaired clearance of plasma protein C[22]. Bruckert et al.[23], has suggested that steatosis may be one of the factors leading to an increase in protein C levels resulting from accelerated turnover of triglycerides. Moreover, an increased level of protein C has been reported in patients with diabetes, hypertriglyceridemia, nephrotic syndrome, abuse of anabolic steroids, and alcoholism[22]. Diabetes and hypertriglyceridemia are predisposing conditions to fatty infiltration of the liver[4] and were present in 23% and 73% of our cases, respectively. The remaining conditions were excluded by clinical and biochemical findings.

Thrombotic risk factors have been found to be independently associated with the extent of fibrosis in patients with chronic viral hepatitis C and hepatitis B[21]. In particular, anti-thrombin III deficiency was associated with more extensive fibrosis. On contrary, in our study, anti-thrombin III deficiency was not associated with increased fibrosis, rather, protein S deficiency showed the greatest decrease in NASH with advanced fibrosis. The presence of combined thrombotic risk factors as the only independent variable associated with advanced fibrosis supports the hypothesis of vascular obstruction for the histological progression of NAFLD but does not indicate a cause and effect relationship.

Obliteration of small portal and hepatic veins due to thrombosis and phlebitis has been proposed as an important factor for the progression of chronic liver diseases[12-14]. Thus, changes in the composition of blood towards a hypercoagulability state in combination with changes in the endothelium of intrahepatic vessels and intrahepatic blood flow[24-26] certainly favor the development of thrombosis in intrahepatic veins. More recently, Wanless and Shiota[26] suggested a four-step model for the progression of FL towards NASH with fibrosis in which the fourth step is venous obstruction with secondary collapse and ultimately

fibrous septation and cirrhosis. We have also demonstrated the beneficial effect of aspirin and enoxaparin on fibrosis progression in a rat model of cirrhosis, which supports the hypothesis of vascular thrombosis in hepatic fibrosis[27]. Again, a cause and effect relationship remains to be determined since more than half (54%) of the patients with NAFLD do not have thrombotic risk factors.

While APC, factor V Leiden, prothrombin mutation 20210A and MTHFR mutations had no association with advanced fibrosis, protein S deficiency was the only thrombotic risk factor associated with advanced staging (Table 5). Sixty percent of protein S plasma concentration is bound to a complement binding protein, which increases in inflammatory conditions (acute phase reactant), and only 40% of the concentration represent the active free form of protein S, which is a cofactor of APC but not a direct anticoagulant[28,29]. Thus, laboratory findings of protein S deficiency may not always reflect abnormal anticoagulation activity.

A link between thrombotic risk factors and hepatic fibrosis has been demonstrated. Liver inflammation resulting from NASH leads to activation of the coagulation system. In patients with protein S deficiency, the degree of activation is enhanced leading to increased thrombin activity and fibrin production. Thrombin is a stellate cell mitogen, and therefore activation of the coagulation cascade may stimulate stellate cell activation and fibrosis[30,31]. Recently, treatment with thrombin antagonist has been shown to reduce liver fibrogenesis in the rat via downregulation of TIMP-1 mRNA levels[32].

Increased levels of anticardiolipin or the presence of lupus anticoagulant were not associated with histological staging in our study. Anticardiolipin antibody has been reported to be present in up to 44% of patients with chronic hepatitis C, but the clinical significance remains controversial[33]. Positive anticardiolipin antibodies were detected in only 6% and 7% of our NAFLD and CH patients, respectively. None of our patients with positive anticardiolipin antibody had a history of thrombotic episodes or clinical signs of portal hypertension. Only one patient had advanced histological staging and one with low platelet count. Taken together, our data are in agreement with previous reports that anticardiolipin antibodies are epiphenomenona without any clinical relevance[34].

The deficiency in protein S is likely to result from an acquired defect, since in the healthy population, the prevalence of heterozygous deficiency has been estimated at 0.2%[35]. Decreased liver synthesis appears as the most plausible mechanism for the high prevalence of protein S deficiency in patients with NASH and advanced fibrosis. The 6% prevalence of protein S deficiency found in the group with NAFLD and the 21% in the group with CH strengthens this hypothesis. On the other hand, increase in protein C levels may explain the non-progression of fibrosis in some patients with NAFLD via its anti-coagulant and anti-inflammatory effects[36].

In conclusion, our data suggest that thrombotic risk factors are detected in approximately half of the patients with NAFLD and that the presence of one or more risk factors is associated with more fibrosis. This does not indicate a cause and effect relationship, but has a significant clinical implication. Whether it is a primary or secondary phenomenon needs further evaluation.

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