



## Are Serum Levels of Nuclear Factor Kappa B and Forkhead Box Protein P3 in Patients with Non-Alcoholic Fatty Liver Disease Related to Severity of Fibrosis?

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### ABSTRACT

#### BACKGROUND

Inflammation has a significant impact on the development and progression of fatty liver diseases. In this study, we aimed to investigate the relation between serum levels of nuclear factor kappa B (NFkB) and Forkhead box protein P3 (FOXP3) with fibrosis severity among patients with non-alcoholic fatty liver disease (NAFLD).

#### METHODS

In a prospective study, the patients suspicious of having fatty liver were enrolled. The exclusion criteria lack of viral hepatitis, autoimmune hepatitis, Wilson's or other known liver diseases, history of liver or biliary surgery, bariatric surgery, and medications that influence liver metabolism. The participants underwent liver fibroscan. According to liver fibrosis, the patients were divided into two groups; 1) fibrosis less than 7.2 KP, 2) advanced NAFLD, fibrosis  $\geq 7.3$  KP. A 10 cc fasting blood sample was taken from each patient for laboratory assessments. The variables between the two groups were compared using Chi-square or Fisher's exact test. The independence of cytokines was assessed by a logistic regression test.

#### RESULTS

Totally 90 patients were enrolled. The mean age was  $42.21 \pm 11$  years. Of them, 50 and 47 participants were allocated to groups 1 and 2, respectively. In the univariate analysis, we revealed a significant difference between age, body mass index (BMI), fasting blood glucose, liver enzymes, total cholesterol, and triglyceride levels. Also, there was a significant difference between the levels of NFkB and FOXP3 in group one compared with group two of the participants, as FOXP3 ( $9.17 \pm 10.0$  vs.  $18.63 \pm 12.9$ ;  $p < 0.001$ ) and NFkB ( $1.70 \pm 1.70$ ;  $p < 0.01$ ). After excluding the confounding factors, we observed a significant association between fibrosis level and cytokine levels in logistic regression.

#### CONCLUSION

Serum levels of NFkB and FOXP3 increased by advancing liver fibrosis in patients with NAFLD. This is an independent association. The identification of intermediary regulatory factors would be necessary

#### KEYWORDS:

NFkB, FOXP3, Liver, NAFLD, Fibrosis, Steatosis

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## INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is a global health issue due to its vast morbidity and complications. In a large multiethnic cohort study, Setiawan and colleagues concluded that NAFLD was the main etiology of chronic liver disease across all ethnic groups and affected about 52% of participants.<sup>1</sup>

The pathophysiology of NAFLD, despite many attempts, remains unclear. It may be due to an imbalance of lifestyle as well as inflammation. It is considered that the pathogenesis of this disease follows multiple hit theories. In this theory, fat accumulation and inflammation are strongly involved in NAFLD progression from simple steatosis to steatohepatitis, cirrhosis, and consequently liver cancer.<sup>2,3</sup> The inflammation process that causes cell injury is developed by chronic.<sup>4,5,6,7</sup>

Cytokines and chemokines regulate the function of inflammatory cells. Moreover, their production is strongly affected by the activation of transcription factors, including the nuclear factor kappa B (NFκB) and Forkhead box protein P3 (FOXP3).<sup>8-10</sup>

Studies showed that NFκB was primarily a cytoplasmic factor expressed by almost all types of cells and was a major inductive transcription factor that regulated a series of events at the molecular level that might be critical targets for treating inflammation.<sup>10-12</sup> In normal and primary conditions, NFκB forms a complex in the cytoplasm of the unstimulated cells with its inhibitor, IκB.<sup>12,13</sup> NFκB activation can be induced upon physical (UV- or γ-irradiation), physiological (ischemia and hyperosmotic shock), or oxidative stresses.<sup>14</sup> In pathological states such as the oxidant agents and the presence of viral proteins, NFκB separates from IκB, is transferred to the nucleus, and induces activation of various enzymes and proinflammatory molecules, including IL6 and TNF-α.<sup>3,11</sup>

FOXP3 is also a key regulator of regulatory T (Treg) cell gene expression. Regulatory T cells are heterogeneous T cells that play a crucial function in preserving peripheral immunological tolerance and control of immune responses toward pathogens and tumors.<sup>15</sup> Also, former studies in humans illustrated that lack or muta-

tions in the FOXP3 gene lead to autoimmune disorder and immune dysregulation.<sup>16,17</sup>

Studies in humans regarding the association between inflammatory factors and steatosis and fibrosis levels are limited. Indeed, non-invasive liver status evaluation can help us diagnose and assess the disease progression more simply. In a recent study by Monserrat and colleagues, intrahepatic fat content (IFC) measured by magnetic resonance imaging (MRI) was used to describe the fibrosis state and assessed the inflammatory factors according to IFC levels. They reported that the severity of NAFLD was associated with an increase in oxidative stress and proinflammatory status.<sup>18</sup> Therefore according to the high prevalence of NAFLD and crucial roles of NFκB and FOXP3 in the development and progression of the disease, we designed a study to evaluate the association between NFκB and FOXP3 serum levels and liver fibrosis in a group of patients with NAFLD for future diagnostic and therapeutic purposes.

## MATERIALS AND METHODS

### The study population

This cross-sectional study was done among patients who had been referred to the liver clinic in Firoozgar Hospital from June 2017 to January 2019 for fatty liver assessment. According to laboratory scales, the inclusion criteria were adult patients aged more than 20 years with fatty liver in ultrasonography with or without elevated liver enzyme levels. Furthermore, subjects with normal to mild fatty liver in ultrasonography and normal liver enzymes were enrolled as the control group.

Exclusion criteria were viral hepatitis, autoimmune hepatitis, hepatic metabolic diseases, post-treatment of chronic hepatitis C infection, diabetes mellitus, bariatric surgery, taking medication with effects on liver status, or FOXP3 and NFκB levels such as silymarin or oral antidiabetics, anti-inflammatory medications, and alcohol consumption more than 30g/day in men or more than 20 g/day in women. In the end, NAFLD was approved by an experienced gastroenterologist.

**Definition of fatty liver****Ultrasonography**

The patients underwent ultrasonography by an expert radiologist. On ultrasonography, fatty liver is defined as normal, mild, and moderate to severe. A normal liver is defined when the consistency is homogeneous, with fine level echoes, minimally hyperechoic, or even isoechoic compared with the regular renal cortex.<sup>19,20</sup>

**Fibroscan**

In the next step, the patients with approved fatty liver underwent fibroscan evaluation. In fact, fibroscan is a non-invasive method applying for assessment of liver stiffness measurement (LSM) and steatosis level-control attenuated parameter (CAP). The fibroscan was performed by an expert physician in Firoozgar Hospital by using a fibroscan device (FibroScan; Echosens, Paris, France) with probes M and L. The examination was performed according to the standard protocol.<sup>15</sup>

**Laboratory assessments**

A 10 ml of fasting venous blood was taken from each patient for laboratory assessment. An auto-analyzer BS200 (Mindray, Shenzhen, China) was used. In this context, biochemical examination including fasting blood sugar (FBS), total cholesterol (Chol), high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), triglycerides (TG), alanine aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (ALP) were done by using Pars Azmoon Company (Pars Azmoon Co., Tehran, Iran) commercial diagnostic kits.

Human FOXP3 ELISA Kit (MBS2503897, MyBioSource Co., USA) was used for in vitro quantitative determination of human FOXP3 concentrations in serum by using a quantitative sandwich method according to the manufacturer's protocol. According to the protocol, ELISA kit for NFkB (MyBioSource, San Diego, California, United States) was used.

**Ethics**

In following Helsinki's declaration of medical ethics, this study was approved by the Ethics Committee

of Iran University of Medical Sciences (ethical code IR.IUMS1397.32992). Written informed consent was obtained from each participant before enrollment.

**Data analysis**

Participants were categorized into two following groups according to fibrosis: 1) group one; fibrosis less than  $< 7.2$  KP, 2) group 2 with advanced fibrosis  $\geq 7.2$  KP and steatosis  $\geq 290$  dbm.

The descriptive data are presented as mean  $\pm$  SD. Chi-square or Fisher's exact test was used to evaluate differences between the groups. Univariate analysis and logistic regression model were finally performed to evaluate the independent effects of NFkB and FOXP3 on liver fibrosis (age, BMI, liver enzymes, lipid profile, and FBS were considered as confounding factors). The data were analyzed using SPSS software version 20.0 (IBM-SPSS, IL, USA). P values lower than 0.05 were considered statistically significant.

**RESULTS**

Totally 97 patients were enrolled. 50 patients were categorized in group one with normal fibrosis, and 47 patients were categorized as having advanced fibrosis. The mean age was  $42.0 \pm 11.30$  years. 60.0% of them were female. Table 1 shows the descriptive characteristics of the participants in the two groups of patients, including descriptive laboratory results. BMI, LDL, and HDL were not significantly different among the groups.

In the univariate analysis, we observed that the serum NFkB was significantly lower in group 1 than group 2 ( $1.70 \pm 1.70$  vs.  $3.52 \pm 2.39$ ,  $p < 0.001$  [95%CI = 1.22-1.84]). Regarding the FOXP3 level, we observed a significant difference in FOXP3 concentrations in group 1 vs. group 2 ( $9.10 \pm 9.90$  vs.  $18.62 \pm 12.9$ ,  $p < 0.001$  [95%CI = 1.03- 1.11]) (table 2).

In the next step, a multiple logistic regression model was applied. After adjustment of age, sex, BMI, liver enzymes, and lipid profile, we observed that the levels of NFkB and FOXP3 were not independently associated with advanced liver fibrosis (table 3).

**DISCUSSION**

In the present study, we showed that the serum levels of NFkB and FOXP3 had significantly positive associations

**Table 1: Basic characteristic of participants according to fibrosis stages**

Variables	Fibrosis status		Total (N = 97)	p-value
	Normal (N= 50)	Abnormal (N= 47)		
Age(year)	37.2 ± 10.0	47.51± 10.3	42.2 ± 11.3	0.05
Sex (M/F)(N)	22/28	16/31	38/59	0.04
BMI	26.7 ± 6.12	31.8 ± 4.76	32.70	0.08
WC	97.6 ± 14.3	103.2 ± 24.2	103.3 ± 19	0.06
Wrist C	18 ± 5.1	18.2 ± 2.2	18.01 ± 4.0	0.02
FBS	95.4 ± 16.9	123.5 ± 44.2	107.9 ± 34.8	0.001
Total Chol	168.3 ± 46.6	216.1 ± 46.5	189.6 ± 52.1	0.02
LDL	114.7 ± 33.4	127.1 ± 32.8	120.2 ± 35.5	0.23
HDL	43.01 ± 9.2	44.06 ± 8.6	43.7 ± 8.9	0.54
TG	133.30 ± 70.84	194.7 ± 99.2	161.8 ± 89.7	0.01
AST	22.40 ± 10.4	60.35 ± 33.80	40.7 ± 31.1	0.01
ALT	25.5 ± 25.30	74.60 ± 39.31	48.8 ± 41.2	0.01
ALP	160.30 ± 54.70	228.30 ± 48.50	193.2 ± 61.8	0.01
FOXP3	9.56 ± 9.40	17.5 ± 13.0	13.55 ± 12.31	0.001
NFkB	1.84 ± 1.7	3.45 ± 2.41	2.54 ± 2.23	0.001

BMI: Body mass index; WC: Waist circumference; FBS: Fasting blood sugar; Chol: Cholesterol; LDL: Low-density lipoprotein; HDL: High-density lipoprotein; TG: Triglyceride; AST: Aspartate transaminase; ALT: alanine aminotransferase; ALP: Alkaline phosphatase

**Table 2: Univariate analysis of the association between studied factors and fibrosis**

Variables	OR (95CI)	Standard error	p-value
Age(year)	1.100(1.05-1.15)	0.025	< 0.001
Sex (M/F)(N)	1.11(0.50-2.52)	0.056	< 0.001
BMI	1.21(1.12-1.33)	0.056	< 0.001
FBS	1.08(1.03- 1.13)	0.002	< 0.001
CHOL Total	1.01(1.00-1.02)	0.005	0.002
LDL	1.00(0.99-1.03)	0.007	0.243
HDL	0.98(0.94-1.04)	0.023	0.656
TG	1.00(1.00-1.02)	0.003	0.005
AST	1.09(1.05- 1.13)	0.020	< 0.001
ALT	1.10(1.05-1.13)	0.018	< 0.001
ALP	1.02(1.01- 1.03)	0.005	< 0.001
FOXP3	1.08(1.03- 1.11)	0.020	< 0.001
NFkB	1.50(1.22- 1.85)	0.160	< 0.001

OR: Odds ratio; CI: Confidence interval; BMI: Body mass index; WC: Waist circumference; FBS: Fasting blood sugar; Chol: Cholesterol; LDL: Low-density lipoprotein; HDL: High-density lipoprotein; TG: Triglyceride; AST: Aspartate transaminase; ALT: alanine aminotransferase; ALP: Alkaline phosphatase

**Table 3: Multiple analysis of the association between FOXP3 and NFkB levels and fibrosis**

Variables	OR (95% CI)	Standard error	p-value
FOXP3	1.37(0.730-2.620)	0.450	0.320
NFkB	13.07(0.075- 22960.82)	49.83	0.500

OR: Odds ratio; CI: Confidence interval

with the liver fibrosis stage along with liver enzymes and lipid profiles. Furthermore, in the multiple regression model, we did not observe independent relation of FOXP3 with liver fibrosis. However, NFkB with  $p=0.50$  may have a weak independent role in the progress of fibrosis.

The pathogenesis of NAFLD is multifaceted. In NAFLD, oxidative stress and lipotoxicity were seen secondary to lipid accumulation that consequently cause inflammatory responses, leading to alterations in inflammatory cytokines and eventually hepatocyte damage, including fibrosis development and its complications.<sup>21,22</sup> Hence, inflammation has been a notable concern in liver diseases. It might be a good research issue in preventing and treating this disease. In this regard, NFkB and FOXP3 may play essential roles. We observed that levels of NFkB and FOXP3 are associated with the fibrosis stage. Hence, we can consider that the inflammation process initiation may occur in the primary stage of NAFLD and liver fibrosis. Indeed, the progression of NAFLD seems to be dependent on different factors.

The NFkB has a significant role in the expression and regulation of proinflammatory substances, including chemokines and cytokines, and the regulator of immune development and immune responses.<sup>10-12</sup> In the present study, in regression analysis, we observed a significant association between NFkB and severe fibrosis (table 2). NFkB is activated by different stress factors and has many liver pathologies, including NAFLD, alcoholic liver damage, and hepatic cancers, but the clinical implications were not elucidated.<sup>23</sup> In the present study, we found that NFkB level is associated with fibrosis stages, but the independent effect of NFkB needs more studies. Hence other confounding factors such as TG and cholesterol can play their roles independently or synergically with NFkB. Therefore we can consider that triggering the NFkB pathway may be complex and be initiated by different substances or circumstances. In fact, activation of NFkB is the first event in viral and non-viral liver diseases. Former studies demonstrated that this substance had dual functions; proinflammatory and antiapoptotic; therefore, imbalance of its concentration may lead to an inappropriate

response that may cause expression of inflammation reactions.<sup>9,24</sup> According to NFkB's vital role in hepatic injury and fibrosis, it should be considered for a new therapeutic protocol in liver diseases.<sup>25,26</sup>

In the present study, results showed a positive association between FOXP3 serum level and liver fibrosis progression comparable with former reports. Matthaïos and colleagues, in a study on liver specimens of patients with chronic liver diseases, revealed that expression of FOXP3 increased in the liver tissue, which was positively associated with inflammation severity independent of its primary etiology.<sup>27</sup> Amorasa and Sand co-workers, in their study on patients with chronic liver diseases, reported a high expression of FOXP3 along with advancing of diseases.<sup>28</sup> In this context, Wie and others, in a study on regulatory T cell components indicated that FOXP3 positive cells might have an important function in limiting liver injury.<sup>29</sup> Regulatory T-cells are a subtype of CD4 cells that work as inhibitors of effectors T cell, NK cell, and consequently helping to maintain the inflammatory homeostasis. These cells, despite different types, express FOXP3, which is the major marker and functional balancing of regulatory T cell. The balance between effector and regulatory T cells causes the elimination of viral hepatitis.<sup>30-32</sup> Whether the role of FOXP3 in patients with NAFLD is the same has not been understood well. Furthermore, we illustrated the increasing level of FOXP3 by advancing fibrosis. It was observed that mRNA levels of the transcription factor FOXP3 were lower in groups of patients without fibrosis (stage F0) and increased by advancing fibrosis and inflammation regardless of the cause.<sup>28</sup> Therefore, according to these studies, we can offer that our findings were in line with previous studies. In fact, we observed an imbalance of inflammatory regulation along with steatosis and advanced stages of fibrosis. As mentioned above, NFkB has a regulatory function, and FOXP3 is expressed as an anti-inflammatory substance. It can be considered that steatosis, as a milestone of NAFLD, can induce an inflammatory process that may be presented by elevated liver enzymes and developing fibrosis. How the inflammatory regulatory system goes off balance and fails to protect the cell



needs more studies.

## CONCLUSION

The current study indicates that NFκB and FOXP3 serum levels significantly increase with the progress of NAFLD fibrosis levels. The increasing level of these factors indicates their key role, particularly the NFκB, in the progression of fibrosis. These effects may be applied in different pathways. Therefore other co-factors or intermittent substances would be considered.

## ETHICAL APPROVAL

There is nothing to be declared.

## CONFLICT OF INTEREST

The authors declare no conflict of interest related to this work.

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