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ORIGINAL RESEARCH

Hepatic Expression of Endocannabinoid Receptors (CB1 and CB2) in Patients with Non-Alcoholic Steatohepatitis and Its Relationship with Metabolic Syndrome*

Ayça EROĞLU HAKTANIR[1], Fatma ÖZ ATALAY[2], Talat AYYILDIZ[3], Şaduman BALABAN ADIM[2], Enver DOLAR[4]

[1] Kocaeli University Faculty of Medicine, Department of Gastroenterology, Kocaeli, Türkiye.
[2] Bursa Uludağ University Faculty of Medicine, Department of Pathology, Bursa, Türkiye.
[3] Ondokuz Mayıs University Faculty of Medicine, Department of Gastroenterology, Samsun, Türkiye.
[4] Bursa Uludağ University Faculty of Medicine, Department of Gastroenterology, Bursa, Türkiye.

ABSTRACT

The pathogenetic pathways in the progression from steatosis to NASH have not yet been fully elucidated. Recent studies reported that the endocannabinoid system has a role in a variety of steps during chronic liver disease. Endocannabinoids are endogenous lipid mediators with a mechanism of action through activating cannabinoid receptor 1 (CB1) and cannabinoid receptor 2 (CB2). Stimulation of the CB1 receptors increases hepatic fatty acid synthesis. Expression of CB2 receptors in fatty liver may be indicative of their association with metabolic syndrome. The fact that the endocannabinoid system activates various metabolic functions in peripheral tissues suggests that this system may play a role in the treatment of various diseases. In this study, we examined whether metabolic syndrome and non-alcoholic fatty liver disease (NAFLD) are associated with expression of cannabinoid receptors. Fifty-four individuals with nonalcoholic steatohepatitis (NASH) and 17 with steatosis based on pathology reports of liver biopsies were included in the patient group. Forty individuals whose liver tissue samples revealed hemangioma, focal nodules and/or simple cyst, and liver areas and liver tissues that were normal in pathology reports were selected as the control group. The association between cannabinoid receptor-1 (CB-1R) and -2 (CB-2R) expression, determined immunohistochemically, and metabolic syndrome criteria and NAS score were determined. A comparison of control (n = 40), steatosis (n = 17) and NASH groups (n = 54) revealed a significant difference in CB-2R expression between patients with steatosis and patients with NASH. The expression of CB-2 receptor in the steatosis group was statistically significantly higher than in the NASH group (p = 0.017). But showed no significant difference in CB-2R expression between NAFLD and control groups (p = 0.924). In our study, it was determined that Cannabinoid receptors-2 (CB-2Rs) were expressed in the liver cells of all patients and the control groups. In addition, the expression of these receptors was found to be associated with various parameters such as arterial hypertension, obesity, hyperlipidemia and lobular inflammation.

Keywords: NAFLD. CB-2 receptors. Metabolic syndrome.

Non-Alkolik Steatohepatitli Hastalarda Hepatik Endokannabinoid Reseptör CB1 ve CB2 ekspresyonu ve Metabolik Sendromla İlişkisi

ÖZET

Steatozdan NASH'a progresyona sebep olan patojenik metabolik yolaklar tam olarak açıklığa kavuşmamıştır. Son dönemdeki çalışmalarda endokannabinoid sistemin kronik karaciğer hastalığı sürecinde pek çok basamakta rol oynayabileceği belirtilmiştir. Endokannabinoidler canlılarda, Cannabinoid reseptör 1 (CB1) ve Cannabinoid reseptör 2'yi (CB2) aktive ederek etkinliklerini gösteren endojen lipid mediyatörlerdir. Endokannabinoid sistemin kronik karaciğer hastalığı sürecinde pek çok basamakta rol oynayabileceği belirtilmiştir. CB1 reseptörlerinin uyarılması hepatik yağ asidi sentezini arttırdığı ve yağlı karaciğerde CB2 reseptörlerinin eksprese olduğunun gösterilmesi bu reseptörlerin metabolik sendromla ilişkili olabileceğinin göstergesi olabilir. Endokannabinoid sistemin periferik dokularda çeşitli metabolik fonksiyonları aktive etmesi, bu sistemin çeşitli hastalıkların tedavisinde rol oynayabileceğini düşündürmektedir. Bizim çalışmamızda metabolik sendrom ve Nonalkolik yağlı karaciğer hastalığıyla cannabinoid reseptörlerinin ekspresyonu arasında ilişki olup olmadığı belirlenmiştir. Karaciğer biyopsilerinin patolojisi raporu NASH olan 54; Steatoz olan 17 kişi hasta grubuna dahil edildi. Karaciğer doku örneklerinde hemanjiom, fokal nodüler hiperplazi, basit kist saptananların patoloji preperatlarındaki normal karaciğer alanları ve normal karaciğer dokusu olarak raporlanan 40 kişi kontrol grubu olarak seçildi. İmmunhistokiyasal olarak CB1 ve CB2 Reseptörleri ile boyanma olup olmadığı, boyanmanın metabolik sendrom kriterleri ve NAS skoru ile olan ilişkisi belirlendi. Kontrol (n=40), Steatoz (n=17) ve NASH grubu (n=54) CB2-R ekspresyonu açısından karşılaştırıldıklarında, Steatozlu hastalar ve NASH'li hastaların CB2-R ekspresyonu aralarında anlamlı farklılık saptandı. Steatoz grubunda CB-2 reseptörünün ekspresyonu, NASH grubuna göre istatistiksel olarak anlamlı şekilde daha fazlaydı (p= 0.017). Fakat NAYKH ve kontrol grubundaki hastaların CB2-R ekspresyonu yüzdeleri karşılaştırıldığında iki grup arasında CB2-R ekspresyonu arasında anlamlı farklılık yoktu (p= 0.924). Çalışmamızda Kannabinoid reseptörleri-2'nin (CB-2R'ler), tüm hastaların ve kontrol grubunun karaciğer hücrelerinde eksprese edildiği belirlenmiştir.Ayrıca bu reseptörlerin ekspresyonu, arteriyel hipertansiyon, obezite, hiperlipidemi ve lobüler inflamasyon gibi çeşitli parametrelerle de ilişkili saptanmıştır.

Anahtar Kelimeler: NAYKH; CB-2 reseptörleri. Metabolik sendrom.

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Dr. Ayça EROĞLU HAKTANIR
Kocaeli University Faculty of Medicine,
Department of Gastroenterology,
Kocaeli, Türkiye.
Phone: 0536 349 85 23
E-mail: draycaaycaeroglu@gmail.com

Authors' ORCID Information:
Ayça EROĞLU HAKTANIR: 0000-0002-6174-0817
Fatma ÖZ ATALAY: 0000-0002-7188-6115
Talat AYYILDIZ: 0000-0003-1075-7499
Saduman BALABAN ADIM: 0000-0002-5039-164X
Mahmut Enver DOLAR: 0000-0001-8944-2793

The prevalence of metabolic syndrome is high, and this large population of metabolic syndrome patients is at risk for developing non-alcoholic fatty liver disease (NAFLD)[1]. Endocannabinoids are endogenous lipid mediators that function through activation of cannabinoid receptor 1 (CB-1R) and cannabinoid receptor 2 (CB2-R)[2-5]. Recent studies have indicated that the endocannabinoid system (ECS) is involved in a variety of steps in the path leading to chronic liver disease, including inflammation, regeneration and fibrogenesis of the liver and associated with a lower insulin resistance risk[5-8]. ECS regulates energy intake through central and peripheral metabolic pathways. Activation of ECS leads to increased food intake and affect lipolysis and glucose metabolism[9]. ECS affects homeostatic pathways through modification of anorexigenic (such as leptin) and orexigenic hormones (such as ghrelin). There is a decrease in the inhibition of endocannabinoid levels of obese individuals which causes insulin resistance[10,11]. Therefore, inhibition of endocannabinoids may be effective for reducing the prevalence of the metabolic syndrome[2,4,10-12].

As shown in the studies of animal models, activation of CB-1R in hepatocytes causes hepatic steatosis by increasing de novo fatty acid synthesis and expression of lipogenic enzymes, conversely was showed on CB-1 knockout mice that the hepatic steatosis did not develop despite the high-fat diet[12].

Rimonabant (SR141716) is a selective CB-1R antagonist, with an affinity to centrally acting CB-1R. In a human study to determine the role of CB-1R in NAFLD development, liver fat content was measured in patients who were using rimonabant by magnetic resonance spectroscopy[13]. In obese individuals, rimonabant has been shown to reduce liver fat and to cause loss of weight in humans[13,14]. However, there were negative side effects of rimonabant including impairment of cognition, motor functions and a predisposition to psychoses, depression. As a result, Rimonabant was withdrawn from the markets to avoid side effects, researchers evaluated peripherally restricted CB-1 antagonists. Dronabinol, a synthetic tetrahydrocannabinol (THC), is a CB-1R and CB-2R agonist, increases body mass index probably through appetite stimulation[15]. Compared with rimonabant, the second generation reverse agonist / antagonist of CB-1R, TM-38837, reduced CNS penetration with peripheral selectivity[15].

It was shown that CB-2R antagonist and cannabis inhibit cytokines and increase anti-inflammatory action, prevent fibrosis progression and NAFLD[3,5,6,16]. Namacizumab (RI-018) is a first in class functional antagonist of CB-1R, and currently evaluated in treatment of NASH, liver fibrosis, metabolic disease[17].

CB-2 agonist stimulation causes conversion of white adipose tissue (WAT) to brown, which stimulates thermogenesis, resulting in calorie expenditure during rest and exercise[10]. CB-2 antagonists affects energy homeostasis by increasing food intake[10]. The role of CB-2 in NAFLD and its relationship with CB-1 are not fully understood. Da Gottardi et al have shown that on human hepatocytes incubated with oleic acid, addition of CB-1 and CB-2 agonists increased the steatosis[18]. Furthermore, CB-2 agonists increased CB-1 expression, steatosis occurred in hepatocytes result of lipid accumulation.

A significant increase in both CB-1 and CB-2 expression was detected in liver biopsies from patients with acute and chronic liver damage, correlating with the stage of inflammation.[3,14,19]. There are contradictory data in studies on animal models that cannabis contributes to fibrosis or preserving from fibrosis[16,20]. More research is required on this issue. It was shown that CB-1 was expressed in activated hepatic stellate cells (HSCs)[7].

There are conflicting data on how cannabis and its derivatives affect fibrogenesis in patients with chronic liver disease in animal models studies, which is why further research is needed on this subject.

In this study, we determined the expression of CB-2R and CB-1R as immunohistochemically in the patients who have non-alcoholic fatty liver disease and in the people who have normal liver and we investigated the association between these receptors and obesity, metabolic syndrome, type 2 diabetes, hepatic steatosis, steatohepatitis, insulin resistance hyperlipidemia, and other biochemical parameters.

Material and Method

Study Design and Case Selection

Fifty-four individuals with nonalcoholic steatohepatitis (NASH) and 17 with steatosis based on

pathology reports of liver biopsies in the School of Medicine, Uludag University, were included in the patient group. Pathology reports of patients who underwent liver partial resection or wedge biopsy by detecting a solitary lesion in the imaging performed for any reason in the liver were scanned from electronic files. Forty individuals whose liver tissue samples revealed hemangioma (n = 21), focal nodules (n = 3), simple cyst (n = 4), or normal livers/liver areas (n = 12) in pathological preparations were chosen as the control group. Signed informed consents were taken from the subjects and their height, weight, circumferences of waist and hips and systolic and diastolic blood pressures were measured. Individuals with a BMI (body mass index) between 18.-25 kg/m2 were considered normal weight, with a BMI between 25-29.5 kg/m2 overweight and with a BMI ≥ 30 kg/m2 obese. Anthropometric measurements and routine biochemical examinations of the control group were achieved through retrospective scanning of electronic and archived medical records. Hepatic steatosis was graded on a score of 1 to 3 as follows: Grade 1, mild; Grade 2, moderate; Grade 3, severe. Metabolic syndrome (MS) was diagnosed based on NCEP-ATP III criteria. MS was diagnosed if three or more of these factors were present[21]. Exclusion criteria were less than 18 years of age, daily alcohol use (females, >20 g/day; males, >30 g/day), presence of acute or chronic viral hepatitis in serological and pathological examinations, positive serological results for autoimmune hepatitis or biliary cirrhosis, presence of inflammatory bowel disease, presence of hereditary metabolic disease, presence of acute or chronic diseases, continuous drug use, and pregnancy. Insulin resistance was defined as a Homeostasis Model Assessment (HOMA) index ≥ 2.5.

Ethical considerations

Methods and principles of the present study were approved by Ethics Committee of Uludag University School of Medicine (Decision number: 2010-2/14).

Immunohistochemical Analysis

Pathological samples from patients with NAFLD (n = 71) and pathological samples from control group individuals (n = 40) were selected from the pathology archive and stained immunohistochemically with antibodies against CB-1R and CB-2R using suitable techniques. NASH was scored based on the criteria of The Pathology Committee of the NASH Clinical Research Network (Network scoring system)[22]. Non-alcoholic fatty liver disease activity score (NAS); It consisted of the sum of steatosis, lobular inflammation and hepatocyte ballooning scores, and the total score was between 0-8. Steatosis, lobular inflammation, and ballooning degeneration were scored histologically and assigned a grade of 0-3 and a stage of 0-4 (fibrosis = stage). A NASH activity score (NAS) ≥ 5 was regarded as NASH, NAS = 3-4 as borderline, and NAS < 3 as no NASH. Patients were divided into two groups: a NASH group, with NAS ≥ 5 (n = 54), and a Steatosis group, with NAS ≤ 4 (n = 17). Steatosis and NASH groups together composed NAFLD group (n=71).

Hematoxylin/eosin staining for histological evaluation of paraffin-embedded liver sections (3-4 micrometer) was performed using standard methods. Paraffin-embedded liver sections were deparaffinized and rehydrated, incubated in hydrogen peroxide than washed in PBS buffer. Super block was applied, and samples were incubated for 5 minutes to block nonspecific background staining. The samples were treated for 1 hour with anti-CB-1R (ab23703, 200 µg at 0.4 mg/ml; Abcam, Cambridge, England) and rabbit polyclonal anti-CB-2R (1:100; Abcam, Cambridge, England) antibodies. After washing, Anti-polyvalent and Histostain-Plus were sequentially applied, and samples were incubated for 10 minutes. Diaminobenzidine (DAB) was applied to the tissue as a chromogen. Staining was assessed under a light microscope, with intense and homogenous brown staining of hepatocyte nuclei considered to indicate positive antibody staining. The percentages of hepatocytes with nuclear or cytoplasmic staining were calculated by examining specimens under high magnification.

Statistical analysis

Statistical analyses of the data were performed using the SPSS13.0 statistical software package. The Shapiro-Wilk test was used to investigate whether the data were normally distributed. For non-normally distributed data, the Mann-Whitney U test was used for comparisons between two groups, whereas the Kruskal Wallis test was used for comparisons among more than two groups. Descriptive statistical data were expressed as median (minimum - maximum) for continuous variables. Categorical variables were expressed as frequency and percentages and were analyzed using Pearson Chi-square test and Fisher's exact Chi-square test. Correlations between variables were investigated using Pearson correlation and Spearman correlation coefficients. Differences with a p-value < 0.05 were considered significant.

Results

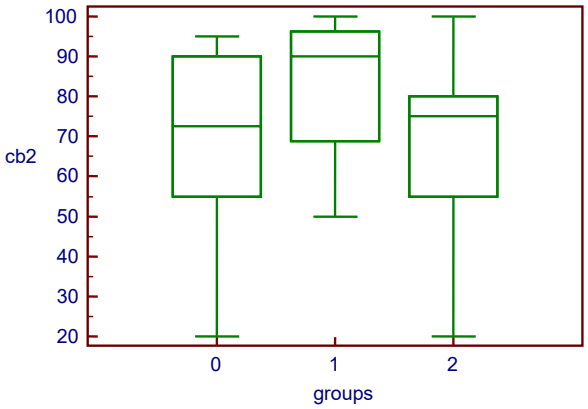
Pathological findings review of the liver biopsies of the patients revealed that the most common type of histological steatosis in steatosis group was grade 1 steatosis with 64.7% (n = 11) while grade 2 steatosis was the most common type in NASH and NAFLD groups with 53.7% and 45% (n = 32), respectively. No grade 3 lobular inflammation was detected in our patients with steatosis, whereas lobular inflammation

and ballooning were present in all patients in NASH. An analysis of the distribution of patients in the steatosis group according to NAS score showed that the highest percentage of patients (58.8%) had a score of 4; 33.3% (n = 18) of patients had a score of 5, 33.3% (n = 18) had a score of 6, 31.5% (n =17) had a score of 7, and 1.9% (n = 1) had a score of 8. No correlation was detected between CB-2R expression percentages of patients and NAS score in both steatosis group and NASH group (respectively, p = 0.941 and p = 0.889).

A comparison of CB-2R expression among control (n = 40), steatosis (n = 17), and NASH groups (n = 54) revealed a significant difference in CB-2R expression between patients with steatosis and patients with NASH. The expression of CB-2 receptor in the steatosis group was statistically significantly higher than in the NASH group (p = 0.017) [Table I and Figure 1]. However, showed no significant difference in CB-2R expression between patients in NAFLD and control groups (p = 0.924).

Table I. Percentage of cells immunohistochemically stained with anti-CB-2R antibodies

	% CB--R expression Median (min -max)	*p*- value	Inter-group significance (*p* values)	
Control (n = 40)	72.5 (20-95)	0.043	Control-Steatosis	0.084
Steatosis (n = 17)	90 (50-100)		Control-NASH	0.243
NASH (n = 54)	75 (20-100)		Steatosis-NASH	0.017
Min, minimum; max, maximum; n, number of patients.				



(0: Control, 1: Steatosis, 2: NASH groups)

Figure 1:
Box plot of patients' immunohistochemical staining percentages of cell nuclei with CB-2R antibodies

The percentages of immunohistochemically stained hepatocyte nuclei and correlations between histopathological findings and CB-2R expression are shown in Tables I, II and III. The percentages of hepatocytes with nuclear or cytoplasmic staining are shown in Figure 2.

Table II. Correlation between CB-2R expression and pathological findings in the NAFLD group (n =71)

	R	*p*-value
Histological steatosis	*-0.182*	0.129
Lobular inflammation	-0.243	0.041
Ballooning	*-0.055*	0.648
Portal inflammation	*-0.075*	0.533
Fibrosis stage	*-0.041*	0.732

Table III. Correlation between histopathological findings and CB-2R expression

	Steatosis		NASH	
	R	*p*- value	*r*	*p*- value
Histological steatosis	0.202	0.438	-0.067	0.632
Lobular inflammation	-0.171	0.512	-0.083	0.550
Ballooning	-0.055	0.835	0.127	0.359
Portal inflammation	-0.226	0.383	-0.056	0.687
Fibrosis stage	-0.093	0.723	-0.070	0.613
NAS score	0.020	0.941	0.019	0.889

Detailed demographic, clinical and laboratory findings relating to Steatosis, NASH and Control groups are shown in Table IV. CB-2R expression in patients with hyperlipidemia was significantly different between steatosis and NASH groups (p = 0.019, Table V). In the assessment of all the patients with steatosis and NASH (NAFLD group), 40.84% (n = 29) of the patients had hyperlipidemia while 57.74% (n = 41) did not. No significant difference was detected between CB-2R expressions of patients with and without hyperlipidemia in NAFLD group (p=0.650).

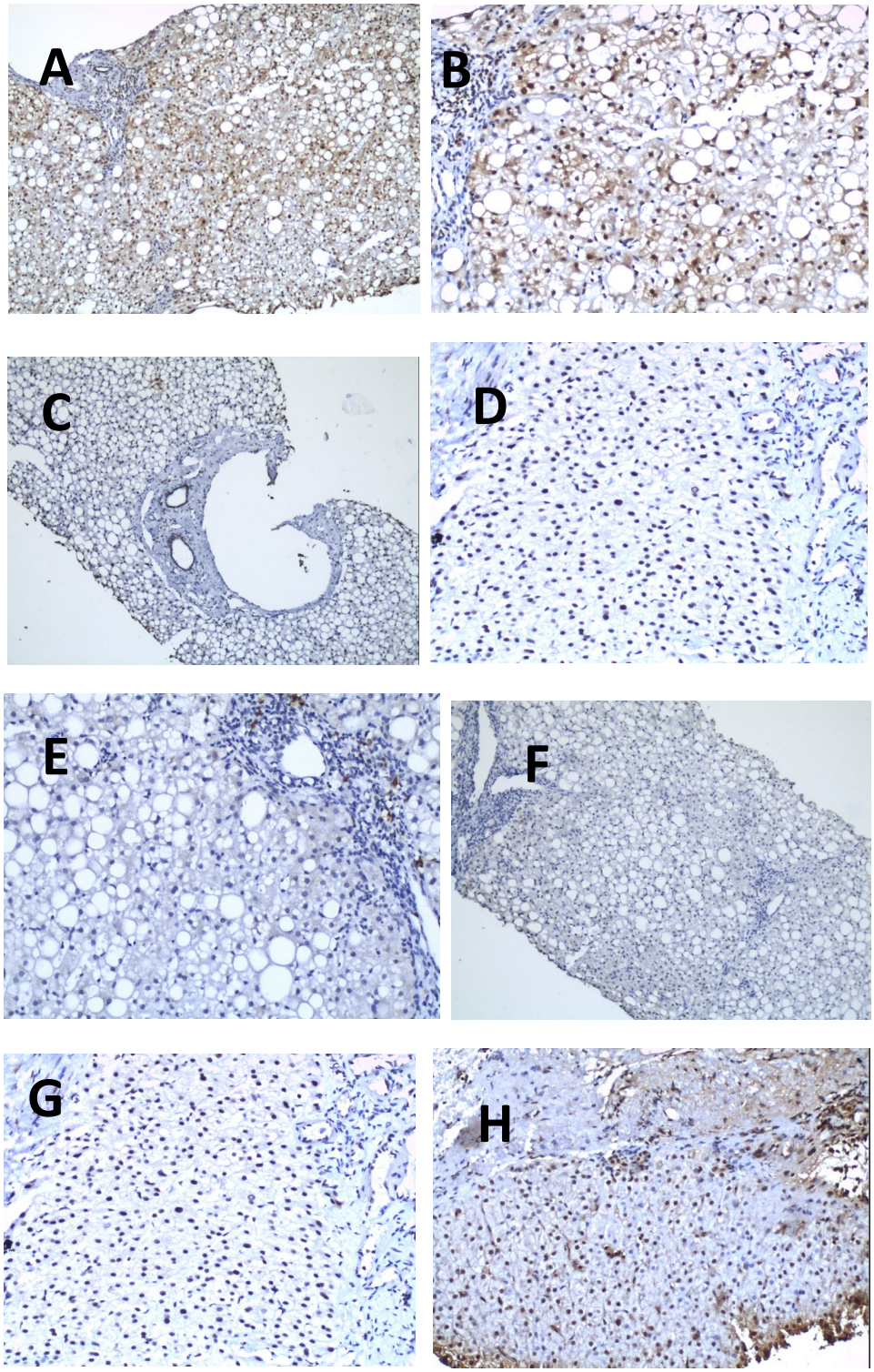


Figure 2.
The percentages of hepatocytes with nuclear or cytoplasmic staining

A-B: Hepatocytes showing 90% nuclear and cytoplasmic staining for CB-2R (NAS score, 6) Original magnification: ×100 (A) or ×200 (B).
C-D: Hepatocytes and bile ducts in portal area showing 50% nuclear staining for CB-2R (NAS score, 7) Original magnification: ×100 (C) or ×200 (D).
E: Negative staining for CB-1R in hepatocytes and positive staining for CB-1R in lymphocytes in portal area (NAS score, 6). Original magnification: ×200.
F: Negative staining for CB-1R in hepatocytes. Original magnification: ×100.

Table IV. Demographic, clinical and laboratory findings

Variables	Steatosis (*n*=17) Median (min-max)	NASH (*n*=54) Median (min-max)	Control (*n*=40) Median (min-max)	*p*- value	Inter-group significance		
AST (IU/L)	53.0 (25.0-159.0)	56.0 (20.0-181.0)	20.5 (7.0-58.0)	**<0.001**	**c-s <0.001**	**c-n 0.001**	s-n 0.731
ALT (IU/L)	78.0 (14.0-296.0)	98.0 (30.0-271.0)	17.0 (7.0-82.0)	**<0.001**	**c-s <0.001**	**c-n 0.001**	s-n 0.345
ALP (IU/L)	74.0 (47.0-175.0)	85.5 (47.0-625.0)	66.5 (30.0-323.0)	**0.007**	c-s 0.365	**c-n 0.002**	s-n 0.159
GGT (IU/L)	58.0 (16.0-100.0)	68.5 (21.0-559.0)	46.0 (12.0-168.0)	**0.002**	c-s 0.188	**c-n 0.001**	s-n 0.138
T.bilirubin (mg/dL)	0.59 (0.22-1.34)	0.64 (0.27-2.9)	0.735 (0.16-2.38)	0.800			
D.bilirubin (mg/dL)	0.25 (0.1-0.9)	0.260 (0.01-0.94)	0.355 (0.07-1.0)	0.181			
Albumin (g/dL)	4.8 (3.6-5.58)	4.5 (2.3-5.6)	4.2 (2.8-5.1)	**0.041**	**c-s 0.032**	**c-n 0.036**	s-n 0.521
Triglyceride (mg/dL)	144.0 (50.0-503.0)	191.0 (49.0-553.0)	105.0 (37.0-641.0)	**<0.001**	**c-s 0.003**	**c-n 0.001**	s-n 0.544
HDL-C (mg/dL)	42.0 (27.0-65.0)	42.0 (26.0-62.0)	49.5 (14.8-86.0)	**0.006**	c-s 0.114	**c-n 0.001**	s-n 0.450
LDL-C (mg/dL)	110.0 (84.0-220.0)	150.5 (72.0-242.0)	99.5 (65.0-186.0)	**<0.001**	c-s 0.05	**c-n 0.001**	**s-n 0.006**
FBG (mg/dL)	103.0 (85.0-316.0)	100.0 (66.0-287.0)	99.0 (96.0-100.0)	**<0.001**	**c-s 0.001**	**c-n 0.001**	s-n 0.941
Insulin (µIU/mL)	18.6 (7.6-10.8)	14.8 (0.3-78.6)	-	0.655			
Diabetes (%)	41.2	37.0	-	**<0.001**	**c-s 0.001**	**c-n 0.001**	**s-n 0.759**
Hypertension (%)	29.4	33.3	25.0	0.682			
Hyperlipidemia (%)	35.3	42.6	2.5	**<0.001**	**c-s 0.002**	**c-n 0.001**	**s-n 0.593**
Age (years)	50.0 (29.0-62.0)	46.0 (20.0-59.0)	43.0 (18.0-80)	0.467			
Sex (Female/Male)	10/7	28/26	25/15				
BMI (kg/m^2)	32.7 (25.0-35.6)	32.25 (21.0-49.0)	24.39 (19.7-29.0)	**<0.001**	**c-s 0.001**	**c-n 0.001**	s-n 0.590
WC (cm)	105.0 (90.0-114.0)	106.0 (80.0-132.0)		0.594			
USG steatosis grade	2.0 (0.0-3.0)	2.0 (1.0-3.0)		0.670			

ALT, alanine aminotransferase; AST, aspartate aminotransferase; ALP, alkaline phosphatase; BMI, body mass index; FBG, fasting blood glucose; GGT, gamma-glutamyl transpeptidase; HDL-C, high density lipoprotein cholesterol; LDL-C, low density lipoprotein cholesterol; WC, waist circumference

c, control group; **s,** steatosis group; **n,** NASH group

Table V. Percentage of cells immunohistochemically stained with anti-CB-2R antibodies in patients with hyperlipidemia

	% CB-2R expression Median (min-max)	*p*- value
Steatosis (*n* = 17)	90 (65-100)	0.019
NASH (*n* = 54)	80 (50-100)	

When association between CB-2R and age, height, circumferences of waist, hips and neck, BMI, weight, systolic tension and diastolic tension values were independently investigated among the patients in steatosis group, no correlation was detected ($p > 0.05$).

There was a negative correlation between CB-2R expression and systolic arterial tension, diastolic arterial tension, and HDL values of patients in the NASH group. CB-2R expression was also negatively correlated with systolic tension in the NAFLD group (respectively, $r = 0.254$; $p = 0.032$ and $r = 0.272$; $p = 0.023$). There was no correlation between CB-2R expression and other biochemical parameters and demographical data.

An examination of the association between CB-2R expression and histological steatosis, lobular inflammation, ballooning, portal inflammation and fibrosis stage in the NAFLD group showed a significant correlation only for inflammation ($r= 0.243$; $p = 0.041$). In this case, the percentage of CB-2R expression decreased as lobular inflammation increased (Table IV).

HOMA IR was ≥ 2.5 in 70% ($n = 12$) of the steatosis group and 83% ($n = 46$) of the NASH group. No statistically significant difference was detected between CB-2R expressions of patients with HOMA IR ≥2.5 and patients with HOMA IR<2.5 both in the steatosis and NASH groups ($p = 0.442$, $p = 0.765$, respectively).

A comparison of CB-2R expression percentages in obese patients in both steatosis and NASH groups showed that CB-2R was detected at higher levels in obese patients in the steatosis group ($p = 0.09$). In the comparison between CB-2R expressions of patients

without metabolic syndrome in steatosis group and patients without metabolic syndrome in the NASH group, receptor expression was detected to be higher in the steatosis group (p = 0.002). No statistical significance was determined in terms of CB-2R expression between existence or non-existence of metabolic syndrome in NAFLD group (p = 0.940)

When the percentage of stained nuclei was identified, CB-2R expression median value was found to be 90 (50-100) in the steatosis group, 75 (20-100) in the NASH group and 72.5 (20-95) in the control group (p = 0.043). In 14% of patients in the NAFLD group, nuclear CB1-R staining was observed only in some lymphocytes in the portal area. CB-2Rs showed nuclear and cytoplasmic staining in 62.5% (n = 25) of individuals in the control group, 17.6% (n = 3) of patients in the steatosis group, and 40.7% (n = 22) of patients in the NASH group. In 73% (n = 52) of cases in the NAFLD group, epithelial cell nuclei of bile ducts in portal areas showed positive CB-2R staining. Histological and immunochemical evaluations showed no nuclear staining and very slight cytoplasmic staining for CB-1R in patients in the NAFLD and control groups.

Discussion and Conclusion

Despite the numerous recent studies which were conducted with animals, the therapeutic effects of cannabinoids have been evaluated in a limited number of human studies and have been indicated an association between cannabinoids and the development of fatty liver. Animal studies have shown an association between the cannabinoid system and the development of fatty liver secondarily to various other factors, such as obesity, a high-fat diet, and ethanol consumption[11,12,23]. In our study, a comparison of patients with steatosis and NASH patients with hyperlipidemia showed that CB-2R expression was significantly higher in the steatosis group (p = 0.019). Detection of CB-2R in fatty liver in a previous study was taken as evidence of an association with metabolic syndrome[3,5,11,24]. In our study, we found no difference between CB-2R expression with and without metabolic syndrome or in insulin-resistant patients either steatosis (p = 0.200) or NASH (p = 0.124) groups. It has been reported that, in NAFLD, CB-2 receptors are expressed in hepatocytes, cholangiocytes, and liver stellate cells[12,25-27]. Unlike this latter study, we found that CB-2Rs are expressed not only in patients with steatosis and NASH, but also in hepatocytes of normal liver tissue. We further found that the expression ratio in the steatosis group was higher than that in the NASH group (p = 0.017). It is likely that a clear result on this point could not be obtained in these previous studies because of their very small sample size of the control group with normal liver areas. The reason for the small number of patients with steatosis in our study was that the biopsy was performed on patients who were thought to be at an advanced stage.

CB-R stimulation increases development of diet-induced obesity and fatty liver disease in liver[10,19,27-29]. Glycemic control, insulin resistance and dyslipidemina of the patients has been improved with the treatment with rimonabant[23]. Even though the rimonabant was sold for the treatment of obesity, it was withdrawn from market because of significant psychiatric side effects, especially depression, anxiety and suicidal ideation[30-31]. An immunohistochemical analysis of normal liver tissue for CB-1R expression detected occasional CB-1R immunoreactivity in the sinusoidal wall, but marked immunoreactivity in samples from cirrhosis patients, predominantly in spindle cells in fibrotic septa, inflammatory cells, and ductus epithelial cells[7,14,21]. Our immunohistochemical analyses showed no CB-1R staining of hepatocyte nuclei in any cases, whereas some lymphocytes in the portal area displayed positive nuclear staining. Our findings are consistent with the hypothesis that CB1-Rs are expressed at levels too low to detect immunohistochemically in both normal and NAFLD tissues.

Experimental studies have demonstrated that CB-2R stimulation decreased IL-6 production and enhanced the anti-inflammatory cytokine IL-10 so that serum transaminases decreased owing to a reduction in inflammatory cell infiltration and improved hepatic inflammatory response[32,33]. Conversely, CB-1R has caused hepatic fibrosis. According to these findings the liver EC system can be the target of treatment in various liver disease.

In our study, we found that, in the NAFLD group, CB-2R expression was negatively correlated with only one pathological finding, namely lobular inflammation (r = 0.243, p = 0.041).

Attempts to form a coherent synthesis of all these results highlight the incompleteness of our understanding of the *in vivo* effects of CB-2R agonists. Further research is needed as to whether these agonists (CB-1 and CB-2) have effects and benefits in the treatment of various stage liver diseases.

The current study, in which we investigated the roles of the endocannabinoid system in NAFLD by studying clinical, demographic, biochemical and histopathological parameters of patients, is perhaps the most comprehensive and methodical of the admittedly few previous human studies on the subject in the literature. In contrast to the limited information in the literature, although we found that the absence of CB-1R expression in liver cells and the presence of CB-2R in the liver cells of all patients as well as those of control individuals were associated with several

parameters, including arterial tension, obesity, hyperlipidemia and lobular inflammation, we are unable to form a clear and definitive conclusion.

Ultimately, there is a need for more data and additional studies to determine the importance of the endocannabinoid system in the pathogenesis of NAFLD and metabolic disorders and its role in treatment options.

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References

1. Chalasani N, Younossi Z, Lavine JE, Charlton M, Cusi K, Rinella M, et al. The diagnosis and management of nonalcoholic fatty liver disease: Practice guidance from the American Association for the Study of Liver Diseases. Hepatology. 2018; 67:328-357.
2. Kunos G, Osei-Hyiaman D. Endocannabinoids and liver disease. IV. Endocannabinoid involvement in obesity and hepatic steatosis. Am J Physiol Gastrointest Liver Physiol 2008; 294: 1101-4.
3. Teixeira-Clerc F, Belot MP, Manin S, Deveaux V, Cadoudal T, Chobert MN, et al. Beneficial paracrine effects of cannabinoid receptor 2 on liver injury and regeneration. Hepatology 2010;52:1046-59.
4. Osei-Hyiaman D, DePetrillo M, Pacher P, Liu J, Radaeva S, Bátkai S, et al. Endocannabinoid activation at hepatic CB1 receptors stimulates fatty acid synthesis and contributes to diet-induced obesity. J Clin Invest 2005;115:1298-305
5. Julien B, Grenard P, Teixeira-Clerc F, Van Nhieu JT, Li L, Karsak M, et al. Antifibrogenic role of the cannabinoid receptor CB2 in the liver. Gastroenterology 2005;128:742-55.
6. Siegmund SV, Schwabe RF. Endocannabinoids and liver disease. II. Endocannabinoids in the pathogenesis and treatment of liver fibrosis. Am J Physiol Gastrointest Liver Physiol 2008;294:G357-62.
7. Dai E, Zhang L, Ye L, Wan S, Feng L, Qi Q et al. Hepatic expression of cannabinoid receptors CB1 and CB2 correlate with fibrogenesis in patients with chronic hepatitis B. Int J Infect Dis 2017;59:124-130.
8. Mallat A, Teixeira-Clerc F, Lotersztajn S. Cannabinoid signaling and liver therapeutics. J Hepatol 2013;59:891-6.
9. Chanda D, Neumann D, Glatz JFC. The endocannabinoid system: Overview of an emerging multi-faceted therapeutic target. Prostaglandins Leukot Essent Fatty Acids 2019;140:51-56.
10. Rossi F, Bellini G, Luongo L, Manzo I, Tolone S, Tortora C, et al. Cannabinoid Receptor 2 as Antiobesity Target: Inflammation, Fat Storage, and Browning Modulation. J Clin Endocrinol Metab 2016;101(9):3469-78.
11. Deveaux V, Cadoudal T, Ichigotani Y, Teixeira-Clerc F, Louvet A, Manin S, et al. Cannabinoid CB2 receptor potentiates obesity-associated inflammation, insulin resistance and hepatic steatosis. PLoS One 2009;4:e5844.
12. Osei-Hyiaman D, Liu J, Zhou L, Godlewski G, Harvey-White J, Jeong WI, et al. Hepatic CB1 receptor is required for development of diet-induced steatosis, dyslipidemia, and insulin and leptin resistance in mice. J Clin Invest 2008;118:3160-9.
13. Bergholm R, Sevastianova K, Santos A, Kotronen A, Urjansson M, Hakkarainen A, et al. CB(1) blockade-induced weight loss over 48 weeks decreases liver fat in proportion to weight loss in humans. Int J Obes 2013;37:699-703.
14. González-Mariscal I, Krzysik-Walker SM, Doyle ME, Liu QR, Cimbro R, Santa-Cruz Calvo S, et al. Human CB1 Receptor Isoforms, present in Hepatocytes and β-cells, are Involved in Regulating Metabolism. Sci Rep 2016;6:33302.
15. Amato G, Khan NS, Maitra R. A patent update on cannabinoid receptor 1 antagonists (2015-2018). Expert Opin Ther Pat 2019;29:261-69.
16. Farooqui MT, Khan MA, Cholankeril G, Khan Z, Mohammed Abdul MK, Li AA, et al. Marijuana is not associated with progression of hepatic fibrosis in liver disease: a systematic review and meta-analysis. Eur J Gastroenterol Hepatol. 2019;31:149-156.
17. Gatta-Cherifi B, Cota D. New insights on the role of the endocannabinoid system in the regulation of energy balance. Int J Obes 2016;40(2):210-9.
18. Gottardi A, Spahr L, Ravier-Dall'Antonia F, Hadengue A. Cannabinoid receptor 1 and 2 agonists increase lipid accumulation in hepatocytes. Liver Int 2010;30:1482-9.
19. Auguet T, Berlanga A, Guiu-Jurado E, Terra X, Martinez S, Aguilar C, et al. Endocannabinoid receptors gene expression in morbidly obese women with nonalcoholic fatty liver disease. Biomed Res Int 2014;2014:502542.
20. Baldassarre M, Giannone FA, Napoli L, Tovoli A, Ricci CS, Tufoni M, et al. The endocannabinoid system in advanced liver cirrhosis: pathophysiological implication and future perspectives. Liver Int 2013;33:1298-308
21. Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults. Executive Summary of The Third Report of The National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation, And Treatment of High Blood Cholesterol In Adults (Adult Treatment Panel III). JAMA 2001;285:2486-97.
22. Kleiner DE, Brunt EM, Van Natta M, Behling C, Contos MJ, Cummings OW et al; Nonalcoholic Steatohepatitis Clinical Research Network. Design and validation of a histological scoring system for nonalcoholic fatty liver disease. Hepatology. 2005;41:1313-21.
23. Gary-Bobo M, Elachouri G, Gallas JF, Janiak P, Marini P, Ravinet-Trillou C, et al. Rimonabant reduces obesity-associated hepatic steatosis and features of metabolic syndrome in obese Zucker fa/fa rats. Hepatology. 2007;46:122-9.
24. Silvestri C, Paris D, Martella A, Melck D, Guadagnino I, Cawthorne M, et al. Two non-psychoactive cannabinoids reduce intracellular lipid levels and inhibit hepatosteatosis. J Hepatol 2015;62:1382-90.
25. Mallat A, Teixeira-Clerc F, Deveaux V, Manin S, Lotersztajn S. The endocannabinoid system as a key mediator during liver diseases: new insights and therapeutic openings. Br J Pharmacol 2011;163:1432-40.
26. DeLeve LD, Wang X, Kanel GC, Atkinson RD, McCuskey RS. Prevention of hepatic fibrosis in a murine model of metabolic syndrome with nonalcoholic steatohepatitis. Am J Pathol. 2008;173:993-1001.
27. Rajesh M, Pan H, Mukhopadhyay P, Bátkai S, Osei-Hyiaman D, Haskó G, et al. Cannabinoid-2 receptor agonist HU-308 protects against hepatic ischemia/reperfusion injury by

attenuating oxidative stress, inflammatory response, and apoptosis. J Leukoc Biol 2007;82:1382-9.

28. Bergholm R, Sevastianova K, Santos A, Kotronen A, Urjansson M, Hakkarainen A, et al. CB(1) blockade-induced weight loss over 48 weeks decreases liver fat in proportion to weight loss in humans. Int J Obes 2013;37:699-703.
29. Schneeberger M, Everard A, Gómez-Valadés AG, Matamoros S, Ramírez S, Delzenne NM, et al. Akkermansia muciniphila inversely correlates with the onset of inflammation, altered adipose tissue metabolism and metabolic disorders during obesity in mice. Sci Rep 2015;5:16643.
30. Després JP, Golay A, Sjöström L; Rimonabant in Obesity-Lipids Study Group. Effects of rimonabant on metabolic risk factors in overweight patients with dyslipidemia. N Engl J Med 2005;353:2121-34.
31. Dhopeshwarkar A, Mackie K. CB2 Cannabinoid receptors as a therapeutic target-what does the future hold? Mol Pharmacol. 2014;86:430-7.
32. Louvet A, Teixeira-Clerc F, Chobert MN, Deveaux V, Pavoine C, Zimmer A, et al. Cannabinoid CB2 receptors protect against alcoholic liver disease by regulating Kupffer cell polarization in mice. Hepatology. 2011;54:1217-26.
33. Mahmoud HM, Osman M, Elshabrawy O, Abdallah HMI, Khairallah A. AM-1241 CB2 Receptor Agonist Attenuates Inflammation, Apoptosis and Stimulate Progenitor Cells in Bile Duct Ligated Rats. Open Access Maced J Med Sci 2019;7:925-936.