

Serum anandamide level as a potential indicator for nonalcoholic fatty liver disease severity

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Introduction and aim Nonalcoholic fatty liver disease (NAFLD) is a very common disease, ranging from simple steatosis to nonalcoholic steatohepatitis (NASH) and is considered the hepatic expression of metabolic syndrome. Liver biopsy is currently considered the gold standard in diagnosis of NAFLD; however, it is an invasive technique and carries many risks. The serum anandamide level is recently discovered to play an important role as the potential indicator for NAFLD severity. The purpose of the study is to determine the association of endocannabinoid metabolite anandamide and NAFLD severity and to investigate its association with anthropometric and metabolic features in NAFLD patients.

Methodology A case-control study on 36 NAFLD biopsy-proven NAFLD patients and 15 healthy volunteers. They were subjected to full clinical history and examination, laboratory tests, abdominal ultrasound and serological testing of anandamide.

Results The anandamide level was significantly higher among NAFLD subgroups (simple steatosis and NASH) vs. the normal group (1.1, 0.29 vs. 0.2 P value=0.00085), with cutoff 0.58 in the NASH group (accuracy 89%; sensitivity 66% and specificity 100%) (P value<0.01).

Conclusion Anandamide could be a specific serum marker for NASH and can be used to detect NAFLD severity. Eur J Gastroenterol Hepatol XXX: 00–00

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Introduction

Nonalcoholic fatty liver disease (NAFLD) is a very common disease, ranging from simple hepatic steatosis to nonalcoholic steatohepatitis (NASH). NAFLD is considered the hepatic presentation of metabolic syndrome, being usually associated with obesity, insulin resistance and dyslipidemia. Thus, its prevalence rises in parallel with the worldwide metabolic disease epidemic, frequently developing on the background of obesity [1].

Steatosis is defined as the presence of hepatic steatosis with no evidence of hepatocellular injury in the form of ballooning of the hepatocytes, whereas NASH is defined as the presence of hepatic steatosis and inflammation with hepatocyte injury (ballooning) with or without fibrosis; 30% of NAFLD patients may progress to NASH [2].

Progression of NAFLD to that of NASH dramatically increases the risks of cirrhosis and hepatocellular carcinoma. Although the morbidity and mortality from liver causes are greatly increased in patients with NASH, they

correlate even more strongly with the morbidity and mortality from cardiovascular disease. NAFLD pathophysiology is related to multiple factors, involving ecological, genetic and metabolic, limited physical activity, high energy intake and a dysbalanced diet [3]. Together with epigenetic factors, this promotes insulin resistance and hepatic fat accumulation [4].

The endocannabinoid system has been implicated in the pathogenesis of fatty liver diseases. endocannabinoids are endogenous arachidonic acid-derived mediators synthesized from membrane phospholipids ‘on demand’ and are released from cells immediately after production to activate the cannabinoid receptor to elicit a biological response, after which they are inactivated through reuptake [5].

The first of endocannabinoids was identified in 1992 and designated as 2 arachidonylethanolamine (anandamide) [6].

Anandamide shows higher affinity for cannabinoid1 over cannabinoid2 and also binds the vanilloid VR1 receptor and is generated on demand via phospholipid-dependent distinct pathways in response to a rise in intracellular calcium or metabotropic receptor activation [7]. Once released, they remain largely membrane-associated because of their hydrophobic nature. Clearance of endocannabinoids relies on cellular uptake and enzymatic degradation. Anandamide was shown to be involved as the biomarker in diagnosis of NASH [8].

Aim of the work

This article aims to evaluate the serum level of anandamide and its correlation with metabolic

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Keyword:anandamide level, liver biopsy, nonalcoholic steatohepatitis, nonalcoholic fatty liver disease

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Table 1. Demographic features among nonalcoholic fatty liver disease subgroups

Variable	NASH group (18)		Simple steatosis group (18)		Control group (15)		P value
	Median (IQR)		Median (IQR)		Median (IQR)		
Age (years)	45.8 (27–60)		43.05 (31–55)		28.1 (19–24)		0.000001**
BMI	37 (29.6–55.8)		36.9 (24.3–45.9)		22.5 (20–24.3)		0.000001**
Waist circumference (cm)	Female	Male	Female	Male	Female	Male	0.000001**
	(107_145)	(120_123)	(110_144)	(111_120)	(85_90)	(100_110)	
	123.4	121.5	128.3	117	87.5	107.8	
DM	+ve	7 (38.9%)	3 (16.7%)		0 (0%)		0.018*
HTN	+ve	4 (22.2%)	4 (22.2%)		0 (0%)		0.1385
Gender	Female	16 (88.9%)	12 (66.7%)		4 (26.7%)		0.001**
	Male	2 (11.1%)	6 (33.3%)		11 (73.3%)		

DM, diabetes mellitus; HTN, hypertension; NASH, nonalcoholic steatohepatitis.

*Moderately significant P value.

**Highly significant P value.

Table 2. Laboratory features among nonalcoholic fatty liver disease subgroups

Variable	NASH group (18)	Simple steatosis group (18)	Control group (15)	P value
	Median (IQR)	Median (IQR)	Median (IQR)	
Hb (g/dL)	13.3 (13–14.6)	12.9 (12.5–14)	14.2 (13–14.7)	0.2478
PLT (10 ³ /μL)	258 (214–335)	257 (227–327)	309 (257–384)	0.6034
TLC (10 ³ /μL)	8.1 (7–9.3)	7.4 (6.2–8.5)	8.2 (7–9.2)	0.3917
Total bilirubin (mg/dL)	0.38 (0.3–0.5)	0.4 (0.35–0.74)	0.46 (0.32–0.6)	0.3006
Alb. (g/dL)	4.2 (3.9–4.6)	4.2 (3.9–4.5)	4.6 (4.2–4.8)	0.1384
AST (U/L)	30 (19–40)	26.5 (20–38)	18 (15.2–20)	0.0019**
ALT (U/L)	29 (21–32)	22.5 (18–27)	15 (12–18)	0.0012**
Total cholesterol (mg/dL)	204 (175–232)	194 (163–249)	163 (150–178)	0.0074**
TGs (mg/dL)	174 (133–192)	173 (125–239)	78 (69–119)	0.00089**
HDL (mg/dL)	41.7 (28–48)	38 (35–46)	38 (35–50)	0.8045
LDL (mg/dL)	149 (120–175)	138 (110–167)	139 (113–174)	0.5507
FBS (mg/dL)	110 (99–121)	111 (96–115)	82 (76–93)	0.00018**
PP (mg/dL)	145 (128–174)	154.5 (129–204)	134 (129–138)	0.3284
Anandamide level (ng/ml)	1.1 (0.5–10.2)	0.29 (0.19–3.1)	0.2 (0.09–0.43)	0.00085**

ALB, albumin; ALT, alanine aminotransferase; AST, aspartate aminotransferase; DM, diabetes mellitus; Hb, hemoglobin; HDL, high density lipoprotein; HTN, hypertension; LDL, low density lipoprotein; NASH, nonalcoholic steatohepatitis; PLT, platelets; PP, post prandial; TGs, triglycerides; TLC, total leucocytic count.

**Highly significant P value.

parameters and disease severity in NAFLD patients vs. healthy control.

Patients and methodology

This is a case–control study was conducted at the hepatology outpatient clinic of Kasr Al Ainy Hospital (from May 2018 to May 2019). The subjects were 36 Egyptian patients with NAFLD confirmed by liver biopsy in addition to 15 healthy control volunteers (they did not perform biopsy). Patients with the following diseases were excluded: chronic viral hepatitis B or C, autoimmune disorders, Wilson's disease, hemochromatosis, drug-induced hepatitis, alcoholic hepatitis or those with any clinical evidence of hepatic decompensation or a contraindication for liver biopsy. All procedures followed were conducted with appropriate approval by the ethics committee of Cairo University in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2008. Informed consent was obtained from all patients for being included in the study. All cases (36 patients and 15 healthy normal control subjects) were subjected to the following: medical history for risk factors of NAFLD (diabetes, hypertension, dyslipidemia and bypass surgeries) and BMI. Blood samples were obtained for lipid profile, fasting plasma glucose and serum levels of alanine aminotransferase (ALT) and

aspartate aminotransferase (AST), using Toshiba Applio 400 (TUS-A400) ultrasound machine serial number W5C1612503 with a 3.5 MHz convex linear transducer; all patients were examined to determine size and grade of brightness of the liver, visceral adipose tissue (VAT) and subcutaneous adipose tissue (SAT).

Assessment of grade of liver brightness was by determining the visibility of the diaphragm and hepatic veins interface, based on these parameters, steatosis was further classified into three grades: grade 0, no steatosis (liver and renal cortex of the same echogenicity); grade 1, mild steatosis: slightly brighter liver as compared to the renal cortex, clear visualization of diaphragm and interface of hepatic veins with sharp contours; grade 2, moderate steatosis: brighter liver with attenuated ultrasound (US) beam at deeper parts of the liver, diaphragm and hepatic veins still visible but with blunted contours; grade 3, severe steatosis: very bright liver, severe US beam attenuation, diaphragm or hepatic veins not visible. This classification was adopted and already tested by other investigators [9].

Additionally, assessment of SAT and VAT by putting ultrasound probe on the location where the xiphoid line intercepted the waist circumference was performed. The visceral thickness was defined as the depth from the peritoneal boundary to the corpus of the lumbar vertebra on longitudinal scanning at the end of a quiet expiration to avoid tensing and distorting the abdominal cavity [10]. Subcutaneous abdominal fat thickness was measured on

the same location, but on a transverse plane, and was defined as the depth from the cutaneous boundary to the linea alba [10].

Ultrasound-guided liver biopsy was conducted for patients only (according to the guidelines of liver biopsy in NAFLD in 2012 by the American Association for the Study of Liver Diseases) [11] and was examined for histopathological diagnosis and scoring using the NAFLD activity score (NAS) scoring system to allow categorization into NASH, borderline NASH or simple steatosis [12]. Fibrosis staging was evaluated using masson trichrome stain from 0 to 4 scales [11].

Measurement of serum anandamide concentration level

A 5 ml of blood is withdrawn from each participant, added to an empty nonheparinized tube and centrifuged on the same day of sample collection, and then added in Eppendorf tubes and stored at temperature of -20°C .

The serum anandamide concentration level is measured by using human anandamide Elisa kits (BT LAP, Shanghai, China, Cat. No E3875Hu).

Table 3. Analysis for basic clinical/Laboratory/radiological factors associated with anandamide level

Associated factor	Anandamide level	
	rho	P value
Clinical		
Age	0.481	0.0004**
BMI	0.321	0.021*
Waist circumference	0.223	0.1154
Laboratory		
Hb (g/dL)	0.158	0.2667
PLT ($10^3/\mu\text{L}$)	-0.0918	0.5218
TLC ($10^3/\mu\text{L}$)	-0.0407	0.7768
Total bilirubin (mg/dL)	0.268	0.0568
Alb. (g/dL)	-0.274	0.0515
AST (U/L)	0.275	0.0512
ALT (U/L)	0.200	0.1594
Total cholesterol (mg/dL)	0.302	0.031*
TGs (mg/dL)	0.259	0.0665
HDL (mg/dL)	0.0763	0.5944
LDL (mg/dL)	0.160	0.2615
FBS (mg/dL)	0.339	0.014*
PP (mg/dL)	-0.0732	0.6098
Radiological		
SAT	-0.0620	0.7196
VAT	0.0617	0.7209
Steatosis	0.161	0.3482
Histopathological		
Inflammation	0.396	0.016*
Ballooning	0.429	0.009**
Fibrosis	-0.0255	0.8825

ALB, albumin; ALT, alanine aminotransferase; AST, aspartate aminotransferase; DM, diabetes mellitus; Hb, hemoglobin; HDL, high density lipoprotein; HTN, hypertension; LDL, low density lipoprotein; PLT, platelets; PP, post prandial; SAT, subcutaneous adipose tissue; TGs, triglycerides; TLC, total leucocytic count; VAT, visceral adipose tissue.

*Moderately significant P value.

**Highly significant P value.

Table 4. Anandamide level among nonalcoholic steatohepatitis

Variable	AUC	SE	Best cutoff point (criterion)	Sensitivity (%)	Specificity (%)	P value
Anandamide level	0.893	0.0535	>0.58	66.67	100	<0.0001**

AUC, area under curve; SE, standard error.

**Highly significant P value.

Anandamide is added to the wells precoated with anandamide mAb. After incubation, a biotin-conjugated anti-human anandamide antibody is added and binds to human anandamide. After washing away unbound biotin-conjugated anti-human anandamide antibody, streptavidin-horseradish peroxidase is added and binds to the biotin-conjugated antihuman anandamide antibody. After the washing step, the substrate solution is added and color develops in proportion to the amount of human anandamide. The reaction is terminated by the addition of acidic stop solution and absorbance is measured at 450 nm.

Precautions

- (1) Prior to use, the kit and sample should be warmed naturally to room temperature 30 min.
- (2) Once the desired number of strips has been removed, immediately reseal the bag to protect the remain from deterioration. Cover all reagents when not in use.
- (3) Make sure pipetting order and rate of addition from well to well when pipetting reagents.
- (4) Avoid using the reagents from different batches together.
- (5) Substrate solution B is sensitive to light; do not expose substrate solution B to light for a long time.
- (6) Stop solution contains acid. Please wear eye, hand and skin protection when using this material. Avoid contact of skin or mucous membranes with kit reagent.
- (7) The kit should not be used beyond the expiration date.

Preparation

- (1) All reagents should be brought to room temperature before use.
- (2) *Standard.* It is strongly recommended that all standards and samples be run in duplicate. If the standard has not been run out, keep the remain at -20°C . Diluted standard cannot be reused.

Calculations of results

Construct a standard curve by plotting the average optical density for each standard on the vertical (Y) axis against the concentration on the horizontal (X) axis and draw a best fit curve through the points on the graph. These calculations can be best performed with computer-based curve-fitting software and the best fit line can be determined by regression analysis. If the standard has been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.

Statistical analysis

Data entry, processing and statistical analysis were carried out using MedCalc ver. 18.2.1 (MedCalc, Ostend,

Belgium). Tests of significance (Mann–Whitney's, Kruskal–Wallis, chi-square test, multiple regression analysis, Spearman's correlation and receiver operating characteristic curve analysis) were used. Data were presented and suitable analysis was done according to the type of data (parametric and nonparametric) obtained for each variable. P values less than 0.05 was considered to be statistically significant.

Results

Our case–control study was subclassified according to results of liver biopsy into NAS (18 patients; 50%) and simple steatosis (18 patients; 50%) in addition to the control group (15 normal persons) (Table 1). We found that age and BMI were significantly higher in NAFLD patients and specifically in the NASH group. As regards biochemical profile (Table 2), ALT, AST and low density lipoprotein were significantly lower among simple steatosis patients. The anandamide level was significantly higher among the NASH group.

Correlation between independent predictors (clinical, laboratory, radiological and histopathological variables) and the anandamide level was conducted with multiple, logistic regression analysis and Spearman's correlation coefficient (as suitable) as shown in Table 3.

Age, BMI, total cholesterol, fasting blood sugar, inflammation, ballooning and total NAS score had a highly significant positive correlation with the anandamide level ($P < 0.05$ respectively). The anandamide level at a cutoff point (>0.58) predicted patients with NASH, with 'good' 89% accuracy, sensitivity = 66% and specificity = 100% ($P < 0.01$) as shown in Table 4, Fig. 1.

The anandamide level showed nonsignificant predictive values in discrimination of patients with simple steatosis

from patients without ($P > 0.05$) as shown in Table 5, Fig. 2.

Discussion

In the recent years, light has been shed on the importance of noninvasive markers as indicator for NAFLD severity. So, in this study, we were identified anandamide as a candidate biomarker linking obesity and hepatic steatosis and also, as a predictor marker for severity on NAFLD.

Although studies showed that the prevalence of NAFLD is higher in males [13], most of our patients were females (77.8%), it could be due to the fact that the prevalence of central obesity is higher among Egyptian females than males [14]. But in our study, we cannot apply this concept due to lack of epidemiological aspect as the small number of our study patients.

Regarding age, it was significantly higher in our NAFLD patients; the age was higher in the NASH group when compared to simple steatosis. This matches with Penninx *et al.* who revealed the significance of age as a precipitating factor for NAFLD [15].

The BMI and waist circumference in our study were proved to be significantly higher in NAFLD especially in NASH cases than in normal control participants. These findings were compatible with [16,17].

Other studies however concluded that Lean-NAFLD (nonobese) is a new unrecognized clinic pathological entity, a frequent cause of cryptogenic liver disease [18,19].

Hepatic steatosis is the manifestation of excessive triglyceride accumulation in the liver. This can occur from the excessive transport and uptake of free fatty acids (FFA) from adipose tissue or from impaired beta-oxidation of FFA. The major sources of triglycerides are from stored fatty acids in adipose tissue and the fatty acids newly synthesized within the liver through de-novo lipogenesis [20]. But our study showed higher cholesterol level than triglycerides among our NAFLD patient which was compatible with Enjoji *et al.* who concluded that excess cholesterol intake, in particular, is a major stimulant for the development of fatty liver. The accumulation of cholesterol rather than triglycerides may play a critical role in the progression from simple steatosis to steatohepatitis [21].

Among our studied patients, there was highly significant increase in the anandamide level in NASH and simple steatosis groups compared to normal control group, with highly significant statistical difference ($P = 0.00085$). Also we found that inflammation, ballooning and total NAS score had a highly significant positive correlation with the anandamide level ($P < 0.05$).

Kimberly *et al.* concluded that anandamide was significantly higher in the NASH group compared with the control group ($P < 0.002$). anandamide was also associated with increased severity of NASH, as assessed by the NAFLD activity score ($P = 0.005$) and the presence of fibrosis ($P = 0.040$). These findings suggested that

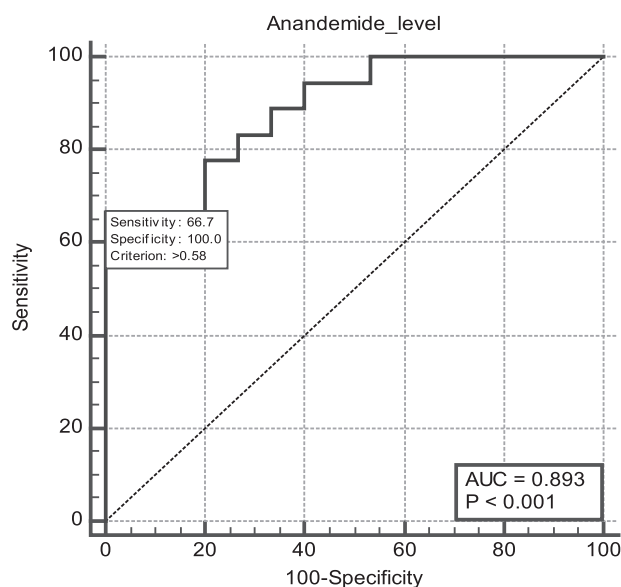


Fig. 1. Anandamide level among nonalcoholic steatohepatitis (NASH).

Table 5. Anandamide level among simple steatosis

Variable	AUC	SE	Best cutoff point (criterion)	Sensitivity (%)	Specificity (%)	P value
Anandamide level	0.652	0.0965	>0.55	38.89	93.33	0.1154

AUC, area under curve; SE, standard error.

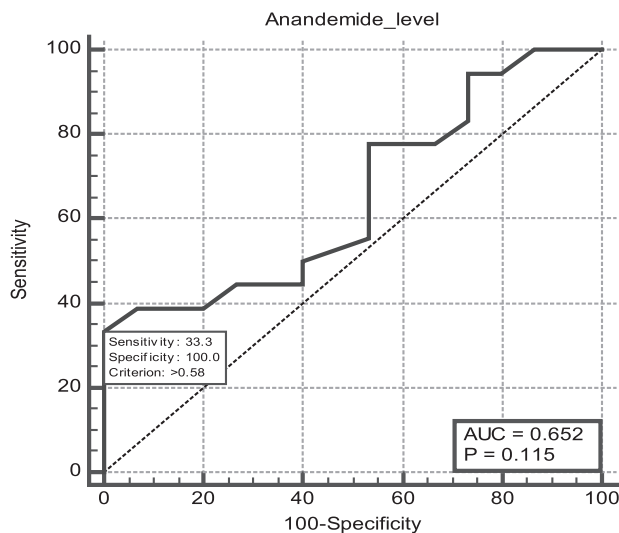


Fig. 2. Anandamide level among simple steatosis. AUC, area under curve.

anandamide is not only associated with biopsy-proven NASH, but also associated with advanced liver histopathology, and that it may serve as a biomarker for NASH [22].

We should point out the following drawbacks of our study. The main limitation of this work is an adjusted sample size, all subjects were ethnic Egyptians and the findings cannot be directly extrapolated to other populations.

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Conflicts of interest

There are no conflicts of interest.

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