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Upregulation of serum expression of miR-217 in non alcoholic fatty liver disease

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ABSTRACT

Alongside the rise in obesity and other metabolic dysfunction components, non-alcoholic fatty liver disease (NAFLD) has grown more widespread. Obesity, T2DM and hyperlipidemia are main risk factors. This condition incorporates a wide variety of clinical manifestations, from steatosis, NASH, fibrosis and cirrhosis. MiR-217 exerts a significant influence in numerous biological processes. This study was carried out to examine miR-217 expression and its potential contribution to NAFLD pathophysiology. Thirty patients with NAFLD and thirty healthy controls participated in the current study. The chosen miR-217 expression profile and its possible correlation with clinical and laboratory indicators were assessed using RT-PCR. Our study revealed a significant increase of miR-217 expression in NAFLD patients versus control ones (with a $p < 0.0001$). Furthermore, according to ROC curve analysis, miR-217 showed outstanding diagnostic ability (AUC of 1, a cutoff point of 1.329, sensitivity and specificity of 100%). There was a substantial negative correlation among miR-217 and free T4 without any significantly observed correlation between miR-217 and other measured variables. In conclusion, significantly higher serum miR-217 levels in NAFLD patient compared to healthy volunteers support the notion that miR-217 is a promising biomarker for NAFLD disease and advance our knowledge of the illness.

Keywords: NAFLD; MicroRNAs; Biomarkers; Gene expression

INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) constitutes the most widespread cause of hepatic aliments on a global scale , which is caused by an excessive buildup of hepatic lipid without any secondary triggers or alternative hepatic etiologies. NAFLD includes simple NAFL, non-alcoholic steatohepatitis (NASH), fibrosis, cirrhosis and in many cases, hepatocellular carcinoma (HCC) [1]. NAFLD incidence of is still rising as a result of growing trends in physical inactivity and nutritional habits.

The development of NAFLD is largely dependent on insulin resistance. NAFLD risk factors are also associated with other metabolic syndrome manifestations, as diabetes, obesity, dyslipidemia, and hypertension, indicating a connection between these metabolic characteristics

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and the chance of developing NAFLD and advanced fibrosis [2-4]. Hepatocellular carcinoma (HCC) is also linked to NAFLD [5]. Significantly, compared to other chronic liver disease types, patient exhibiting NAFLD are more likely to have noncirrhotic HCC, according to a recent analysis of US veterans [6].

NAFLD is often diagnosed using a combination of radiological diagnostics, biopsies, laboratory results, and clinical history. When aminotransferases rise or liver imaging shows excessive hepatic fat, the clinical diagnosis of NAFLD is typically taken into consideration [7]. Despite being the most reliable method for diagnosing NAFLD and a useful technique for pathological features, fine details , liver biopsy is invasive related to serious complica -tions [8]. Therefore finding non-invasive tools for NAFLD diagnosis is necessary. In NAFLD, significance of miRNA expression dysregulation has been widely recognized.

The most extensively researched class of non-coding RNAs (ncRNAs) is miRNAs, which are short non-coding RNAs of approximately 22 nucleotides (nt) and can modulate a diverse spectrum of biological pathways and metabolic homeostasis ,encompassing lipid biogenesis, fatty acids oxidation , carbohydrate catabolism, inflammatory responses, necrosis and apoptosis ,all of which epigenetically desregulated in NAFLD [9].

Using miRNAs as prospective biomarker for identifying steatohepatitis, Pirola et al. [10] developed an appealing strategy for improving NAFLD non-invasive assessment. Mi-RNA applications extend to diverse clinical settings for early disease discovery and disease progression monitoring.

MiR-217, situated on human chromosome 2p16.1, is essential for inflammation and fibrosis [11]. MiR-217-5p is highly expressed during endothelial cell senescence course and cardiovascular disease (CVD) dysfunction [12, 13]. It also related to podocyte apoptosis in diabetic nephropathy [14] and apoptosis of macrophage in atherosclerosis [15]. In addition, MiR-217 has been identified in several cancers, as liver cancer [16] and acute myeloid leukemia [17]. Moreover, miR-217 Knockdown was intimately linked to improving manifestations in Parkinson’s disease [18].

Thus, the current study's objective was to assess miR-217 expression in NAFLD patients relative to healthy controls and to elucidate any correlations with clinical traits and laboratory results. Knowing these connections could help find new biomarkers and advance our knowledge of the pathophysiology of NAFLD.

MATERIALS AND METHODS

Participants: Thirty NAFLD patients, aged between 23 and 66, were chosen from Fayoum University Hospital's hematology department for this case-control research. In addition, thirty healthy participants were enrolled in current work. To reduce the possibility of confounding variables, the control participants and patients were chosen to be age and sex matched. To rule out other diagnoses that would sufficiently explain the disease's symptoms, laboratory and clinical assessments were carried out.

Every patient had a thorough clinical assessment. The following predetermined inclusion criteria were used in this study:(1) confirmed diagnosis of NAFLD through clinical evaluation and abdominal ultrasonography, which revealed "bright liver" or enhanced hepatic echogenicity. (2) Age Range: Adult patients aged from 23 and 66, regardless of gender. (3) Laboratory Profile: Individuals who fit the metabolic criteria for fatty liver, with or without having high liver enzymes.

Individuals with any of the following criteria were not allowed to participate in this study: (1) Significant alcohol consumption (2) Viral Hepatitis: Individuals who test positive for HBs-Ag or HCV-Ab, since viral diseases dramatically change miRNA expression. (3) Other progressive hepatic dysfunction: Wilson's disease, Hemochromatosis, immune-mediated chronic hepatopathy, etc. (4) Co-morbidities: Individuals with heart failure, chronic kidney failure, or any known cancer. (5) Pregnancy and Lactation: As hormonal changes can affect metabolic

markers and miRNA profiles. (6) Steatogenic Medications: Individuals using medications (such as amiodarone, methotrexate, or systemic corticosteroids) that are known to cause secondary hepatic steatosis.

Clinical and radiological investigation of patients: Blood pressure was measured after 15 minutes of rest using a standard calibrated sphygmomanometer. Participants were classified as hypertensive if they met the standard diagnostic criteria or were currently receiving anti-hypertensive therapy; otherwise, they were considered non-hypertensive. Fibrosis-4 (FIB-4) index and Aspartate Aminotransferase to platelet Ratio Index (APRI) score are two fibrosis noninvasive tests in NAFLD patients. The following formula is used to determine FIB-4:

FIB-4 = [Age (years) × AST (U/L)] ÷ [PLT (10^9/L) × $ALT^{1/2}$ (U/L)]

Advanced fibrosis>2.67 and mild to moderate fibrosis 1.3–2.6. The APRI score is computed as [AST (U/L) ÷ PLT (10^9/L] × 100, with mild to significant fibrosis scoring < 0.73 and advanced fibrosis scoring ≥ 0.74 [19].

Transient Elastography (TE): Liver stiffness was assessed using FibroScan (Echosens, Paris, France). It calculates determines how quickly an electromagnetic wave passes throughout skin. Skilled operators assessed the stiffness of the liver using M probe (the standard probe). The procedure was facilitated by Ultrasonography time motion and A-mode imaging on the liver right lobe through intercostal gap while the patient was fasting, resting on his back and extending his right arm to its maximum abduction. Ten successful shots resulted in a 100% success rate.. The liver stiffness interquartile range (IQR) to the median (IQR/M) was determined using FibroScan's higher-quality criteria (10 valid measures, 30% IQR/M).

Transient elastography was used to measure liver stiffness at values ranging from 2.5 to 75.0 kPa; the average measurement for healthy individuals is 5.5 kPa. The FibroScan machine calculates the results automatically after manually entering the data. Based on FibroScandata, Transient elastography was used to measure liver stiffness at values ranging from 2.5 to 75.0 kPa; the average measurement for healthy individuals is 5.5 kPa. The degrees of hepatic fibrosis are categorized as follows: F0: 1–6 kPa; no scarring F1: mild liver scarring (6.1–7 kPa), F2: mild liver scarring (7.5–10 kPa). F3 (10–14 Kpa): sever liver damage. Lastly, F4 (14 Kpa or more): severe cirrhosis of the liver [20].

Steatosis grades have been determined using the controlled attenuation parameter (CAP) score. The CAP score is expressed in dB/m, or decibels per meter.The range of this score is 100 to 400 dB/m. If the CAP score (S0 = no steatosis) is less than 237 dB/m. CAP range: 237.0-259.0 dB/m (S1 = moderate steatosis).CAP ranges from 259.0 to 291.0 dB/m (S2 = mild steatosis). Lastly, 291.0 400.0 dB/m (S3 = severe steatosis) [21].

Blood sample processing: A venous blood (5ml) was drawn from participants after 8-12 hr fast. Samples were harvested into dedicated collection tubes with serum separator gel matrix and allowed to coagulate at ambient temperature for 15 minutes. Serum samples were separated by centrifugation at 4000×g for 10 minutes. and stored till analysis at -80°C. An extra blood sample was withdrawn into fluoride containing tubes 2 h after a meal (2 h post-prandial, 2hPP).

Laboratory investigations: The International Federation of Clinical Chemistry's kinetic method was utilized to measure alanine aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (ALP) enzymes [22, 23]. The Szasz method's kinetic colorimetric method was used to measure glutamyltransferase (GGT) [24].

Enzymatic colorimetric methods were used to measure cholesterol (Liquizyme CHOD-PAP), triglycerides (GPO-PAP Liquizyme TR), LDL (Cholesterol Direct Reagent (LDL), and fasting and postprandial blood glucose (Liquizyme GOD-PAP) [25-28] and HDL cholesterol (Precipitant) [29]. Thyroid Stimulating Hormone (TSH) and Free Thyroxine (FT4)levels were evaluated using ELISA method [30, 31].

Extraction of miRNA and subsequent reverse transcription into complementary DNA: Serum samples were processed for total RNA extraction including small RNA, via MiRNeasy purification kits (Qiagen, Valencia, CA, USA) in compliance with the manufacturer's catalog. A Nano Drop® (ND)-1000 spectrophotometer (Nano Drop Technologies, Inc., Wilmington, USA) was used to measure RNA purity and quantity. Following manufacturer's instructions, microRNA was enzymatically converted into cDNA via reverse transcription in a final volume of 10 µl using the miRCURY LNA RT Kit (Qiagen, MD, USA).

MiR-217 detection by quantitative Real-Time PCR (RT qPCR): RT-qPCR was used to determine miR-217 levels in serum. MiRCURY LNA miRNA PCR Assays (Qiagen, MD, USA), miRCURYLNA SYBR® Green Master Mix (Qiagen, MD, USA), and cDNA synthesis reaction were used to create a PCR reaction mix for a 10-µl per well reaction volume. The following procedures were carried out by the real-time cycler (Piko Real 24TM Real-Time PCR System; Thermo Scientific, Finland): two minutes of initial heat activation at 95°C, forty cycles of denaturation at 95°C for ten seconds, and combined annealing/extension at 56°C for sixty seconds.

For assessing the amplified specificity of products, melting curve analysis was conducted among 60 and 95 degrees Celsius. Using miR-16-5p as a reference gene, mir-217 expression values were normalized [32, 33]. Predesigned primers from Qiagen, Valencia, CA, USA, were used targeting both miR-217 and miR-16-5p. Additionally used were primer assays for miR-217 (catalogue no. YP00204010) and miR-16-5p (catalogue no. YP00205702 and its Lot number was 201,910,040,131-3). The $2^{-\Delta\Delta Ct}$ formula was applied to calculate serum miR-217 fold change (FC) for each sample [34, 35]. The healthy group's FC was taken to be 1.

Statistical Analysis: The Statistical Package for Social Sciences (SPSS) program v.22 was utilized to arrange, tabulate, and statistically analyze gathered data. Mean, median, standard deviation (SD), and interquartile range (IQR) were computed for quantitative data. Categorical variables were compared using the Chi-square test. Continuous variables were compared using Student's t-test for normally distributed data or Mann-Whitney test for non-normally distributed ones. Spearman's correlation was used to assess relationships among mir-217 and laboratory and clinical data. Results were considered statistically significant when the *p*-value was less than 0.05.

RESULTS

Regarding the socio-demographic characteristics of the studied groups, the mean age of the patient group was 45.7 ± 11.5 years, compared with 43.2 ± 10.2 years in the control group. No significant difference in age was found between the two groups ($p = 0.372$). The patient group comprised 12 men (40%) and 18 women (60%), while the control group included 14 men (46.7%) and 16 women (53.3%). No significant difference in sex was observed between the groups ($p = 0.602$).

In our study ; there were a significant rise in patient's serum cholesterol, triglyceride and LDL levels (219.30 ± 31.47; 173.23 ± 8.41 and 136.03 ± 35.07 respectively) than control (135.27 ± 13.68 ; 96.33 ± 7.91 and 63.53 ± 14.15 respectively) with $p < 0.0001$. Conversely, there was a significant decrease ($p < 0.0001$) in HDL levels in patient group compared to control one (42.20 ± 7.72 and 71.63 ± 18.69 respectively) (Table 1).

In terms of liver function markers, patient had substantially higher serum activity of ALT, AST, ALP and GGT (67.4 ± 21.4 U/L; 71.03 ± 19.04 U/L; 104.43 ± 29.82 and 73.10 ± 19.3 respectively) than control (23.83 ± 5.58 U/L; 22.90 ± 6.18 U/L; 67.8 ± 18.1 U/L and 19.97 ± 6.38 U/L) with a $p < 0.0001$ (Table 1).

Thyroid and glycemic profile differed significantly among the two groups. TSH and free T4 activities were substantially lower in patients, but still within normal ranges (1.55 ± 0.76

mIU/mL and 1.24 ± 0.14 ng/dl) than controls (2.09 ± 0.73 mIU/mL and 1.52 ± 0.41 ng/dl respectively) with $p = 0.008$ and $p = 0.001$, respectively. Alternatively, FBS and PPS levels were significantly raised in patient's serum (117.6 ± 30.9 mg/dl and 210.9 ± 66.1 mg/dl) than controls (75.20 ± 7.20 mg/dl and 100.8 ±7.3 mg/dl) respectively, with *(p* < 0.0001*)* (Table 1).

Table 1: Clinical and laboratory findings of patients and controls

Parameter	**Patient group (*n*=30)**		**Control group (*n*=30)**		***p-value***
	Mean	**SD**	**Mean**	**SD**	
Cholesterol (mg/dl)	219.30	31.47	135.27	13.69	< 0.0001
Triglycerides (mg/dl)	173.23	8.41	96.33	7.91	< 0.0001
HDL (mg/dl)	42.20	7.72	71.63	18.70	< 0.0001
LDL (mg/dl)	136.03	35.07	63.53	14.15	< 0.0001
ALT (U/L)	67.4	21.4	23.83	5.58	< 0.0001
AST (U/L)	71.03	19.04	22.90	6.18	< 0.0001
ALP (U/L)	104.43	29.82	67.8	18.1	< 0.0001
GGT (U/L)	73.10	19.3	19.97	6.38	< 0.0001
TSH (mIU/mL)	1.56	0.77	2.09	0.73	0.008
Free T4 (ng/dl)	1.24	0.14	1.52	0.41	0.001
FBS (mg/dl)	117.6	30.9	75.20	7.20	< 0.0001
PPS (mg/dl)	210.9	66.1	100.8	7.3	< 0.0001
Hypertension	n (%)		n (%)		
Yes	10 (33.3%)		0 (0%)		< 0.0001
NO	20 (66.70%)		30 (100%)		

Data are expressed as mean ± SD, number (*n*),Statistical significance was evaluated using Chi-square test for categorical variables and student's t-test for continuous variables.

According to data provided in Table 2, average FIB score was 1.62 ± 0.73, average APRI score was 0.74 ± 0.26, and 33.4% of cases had F1 Fibroscan score, and 43.3% had S3 CAP score.

Table 2: Description of different severity scores in cases group

Variables	**Patient group (*n*=30)**
	Mean ±SD
FIB	1.62±0.73
APRI	0.74±0.26
Fibroscan	***n* (%)**
F0	6 (20%)
F1	10 (33.4%)
F2	7 (23.3%)
F3	7 (23.3%)
CAP	
S1	7 (23.3%)
S2	10 (33.4%)
S3	13 (43.3%)

NAFLD patients had a considerably higher median serum levels of miR-217 (3.82, IQR: 1.97–6.94) than controls (1.0, IQR: 1.0–1.0), indicating a strong statistical significance (p < 0.0001) (Table 3).

Table 3: Serum miR- 217 fold change in patients and controls

	Patient group (*n*=30)		**Control group (*n*=30)**		***p- value***
	Median	**IQR**	**Median**	**IQR**	
miR-217	3.82	1.97- 6.94	1	1.0 -1.0	< 0.0001

Data are presented as median (IQR), comparative analysis was performed using Mann-Whitney test.

With the exception of significant negative correlations with free T4 *(p* = 0.034 , as shown in Table 4, the correlation analysis between of miR-217 and all other laboratory biochemical parameters in NAFLD patients displayed no statistically significant associations *(p > 0.05)*. There were no significant link between miR-217 and FIB4, APRI, Fibroscan, and CAP in cases *(p* > 0.05*)*.

Table 4: Correlation between miR- 217 and laboratory and radiological parameters

Parameters	r	*p*-value
Cholesterol	- 0.301	- 0.301
Triglycerides	0.065	0.731
HDL	0.143	0.451
LDL	-0.240	0.202
ALT	0.027	0.889
AST	0.240	0.201
ALP	- 0.240	0.202
GGt	0.157	0.408
TSH	-0.171	0.366
Free T4	- 0.388	0.034
FBS	- 0.044	0.819
PPS	0.006	0.975
FIBA	0.008	0.75
APRI	0.09	0.82
Fibroscan	0.12	0.68
CAP	0.19	0.51

With an excellent discriminatory ability with an AUC of 1, ROC curve analysis for miR-217 showed an exceptional diagnostic performance with cut-off value of 1.329 and sensitivity and specificity of 100% both ($p < 0.0001$) (Fig. 1).

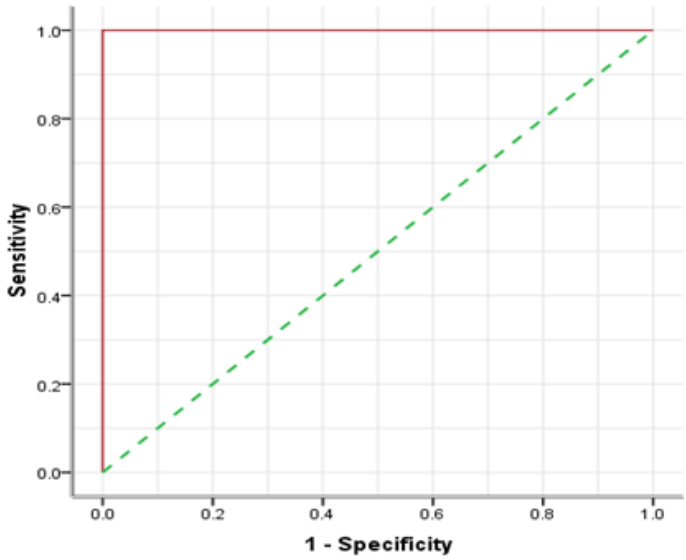

Figure 1: ROC curve demonstrating the role of miR- 217 in diagnosing NAFLD patients.

DISCUSSION

MicroRNAs, modulates gene expression via mRNA hybridization, are pivotal in NAFLD prevention or progression. There is an a pressing need for improving diagnostic tools and efficacious therapeutic agents for NAFLD management, that may progress to cirrhosis and liver cancer. MiRNAs function as regulatory aspects and noninvasive diagnostic tools for NAFLD, facilitating disease staging, prognostic indicators, and therapeutic intervention targets discovery. NAFLD induces fluctuation in hepatocyte miRNAs expression profiles, with some miRNAs

associated with NAFLD progression to NASH [36]. Indeed, extensive research has been conducted to identify a miRNA biomarkers panel that effectively diagnose NAFLD [37].

This study seeks to examine the blood miR-217 levels in NAFLD individuals for the first time. MiR-217 levels were dramatically increased in NAFLD patients relative to healthy participants. The ROC curve of miR-217 indicates excellent diagnostic accuracy for NAFLD (AUC = 1, $p < 0.0001$) with a cutoff value of 1.329 and both sensitivity and specificity of 100%.

Research has consistently shown that people with NAFLD, especially those with the more severe form, NASH, have higher levels of miR-217. For instance, investigating L-carnitine supplementation impact in obese NASH patients revealed markedly elevated miR-217 expression levels in NASH group relative to NAFLD-negative obese controls. This indicates that miR-217 may serve as a prospective non-invasive biomarker for diagnosing and surveilling NASH progression and therapy efficacy [38].

MiR-217 dramatically influences liver disease, encompassing NAFLD and ethanol-induced hepatic damage, by directly interacting with the 3'UTR of Silent Information Regulator T1 (SIRT1) mRNA, reducing its expression. Via Peroxisome Proliferator-Activated Receptor Alpha (PPAR α) regulation, SIRT1 regulates lipid homeostasis and SIRT1 hepatocyte specific repression impairs PPAR α signaling reducing fatty acids beta-oxidation [39]. Furthermore, pro-inflammatory transcription factors as NF-kappa-B and pro-apoptotic markers are released when SIRT1 is depleted. Thus, miR-217-induced SIRT1 inhibition results in both fat buildup and significant hepatic inflammation. Hence, SIRT1 deficiency disrupts lipid metabolism, elevates inflammation, and promotes cellular damage and fat buildup in the liver [40-43].

Another mechanism for miR-217 function has been proposed through its effect on Phosphatase and Tensin Homolog (*PTEN*) gene, one of AKT gene inhibitors, a gene participates in hepatic stellate cells activation, hence higher miRNA-217 levels could stimulate liver fibrosis [44, 45] . Conversely, some studies revealed anti-fibrotic effects of miR-217 where it was found to modulate liver fibrosis by targeting Transforming Growth Factor beta type II receptor (TGFBR2), reducing Angiotensin II (Ang II)-induced Hepatic Stellate Cell activation, and lowering fibrosis in CCL 4 mouse models [46]. Besides, miR-217 could inhibit the malignant progression of HCC by regulating Kruppel-like factor 5 gene (*KLF5* gene) [47].

Despite had no significant correlation with any of biochemically or radiologically assessed parameters, miR-217 exhibited a significant negative association with free T4. Since SIRT1 plays a major role in the hypothalamus-pituitary-thyroid axis regulation, which governs thyroid hormone production and release, MiR-217-mediated suppression of SIRT1 in the liver may indirectly impair the efficiency of thyroid hormone synthesis or regulation, potentially resulting in lower circulating free T4 levels [48]. Research indicates that SIRT1 upregulation can enhance expression of key proteins implicated in thyroid hormone production [49]. Further research is needed to comprehensively clarify direct and indirect molecular pathways linking miR-217, SIRT1, and thyroid hormone regulation in NAFLD progression.

The present study has drawbacks warrant to be addressed: 1) The limited sample size, 2) lacking sufficient evidence to demonstrate important functions of serum miRNA levels in NAFLD pathophysiology and 3) Unmeasured confounding variables may affect miRNA expression levels, those including dietary choices, pharmaceutical usage, and other comorbidities. Mechanistic larger-scale studies with control for confounding variables are required to examine miR-217 exact functions in NAFLD development.

In comparison to age and sex-matched healthy controls, the current study shows that NAFLD patients had considerably higher serum miR-217 levels. These results confirm that miR-217 may be a useful biomarker for NAFLD. Further research is warranted to fully unravel all intricate targets and pathways regulated by miR-217 in NAFLD, which could pave the way for innovative diagnostic tools and innovative targeting this miRNA.

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Conflict of Interest: The authors declare that the research was performed without any commercial or financial relationships that might be interpreted as a potential conflict of interest.

Authors' Contribution: All authors contributed equally to the conception and design of the study, data collection, analysis and interpretation, manuscript drafting, and approved the final version of the manuscript.

Ethics approval and consent to participate: The study was carried out in alignment with the ethical principles of the Helsinki declarations guidelines with ethical approval granted by Fayoum University supreme Committee for Research Ethics and Scientific Integrity (FU-SCRESI) (Code no: EC 2503-a) and documented informed consent was provided by each subject from all enrolled individuals.

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