

Serum High Mobility Group Box1 Protein and Toll-like Receptor 4 Correlation with Lipid Profile in Patients with Non-alcoholic Fatty Liver Disease

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Abstract Background: HMGB1 (high mobility group box1 protein) extracellularly acts as a cytokine in response to free fatty acids (FFA) infusion to the liver in which TLR4 (toll-like receptor4) is a receptor for HMGB1 to generate the inflammatory response and it is responsible for the emergence of Non-alcoholic fatty liver disease (NAFLD). This study aimed to correlate the HMGB1 and TLR4 with lipid profile in NAFLD patients with dyslipidemia. **Methods:** This prospective study included NAFLD patients with dyslipidemia (n=87) with a mean age of 50.5±6.25, of which 54 were males and 33 were females. Data for the lipid profile and liver enzyme parameters except HMGB1 and TLR4 were collected from the data management system of the central laboratory. HMGB1 and TLR4 values were estimated by enzyme-linked immunosorbent assay (ELISA) method, statistically Karl Pearson's, Spearman's correlation and Independent t-test, Mann-Whitney U-test were applied to find the correlation and comparison of HMGB1 and TLR4 with lipid profile and liver enzymes based on the age group and gender respectively. **Results:** No correlation was observed with the HMGB1 and TLR4 with lipid profile in NAFLD patients. Additionally, it was noted that the comparison study of AST/ALT ratio with gender showed a significant difference but not with HMGB1 and TLR4.

Conclusion: Males were affected earlier than females. The study group had significantly higher levels of LDL-cholesterol as against the treatment goals. Levels of extracellular HMGB1, which is a damage-associated molecular pattern (DAMP) were found to be elevated, similar to the findings with TLR4 levels which act as a receptor for HMGB1. However, we did not find a correlation of HMGB1 and TLR4 with lipid profile in non-alcoholic fatty liver disease.

Keywords Aspartate Transaminase/Alanine Transaminase Ratio, High Mobility Group Box1 Protein, Non-alcoholic Fatty Liver Disease, Toll-like Receptor 4, Triglycerides, Low-density Lipoprotein-cholesterol

1. Introduction

Globally, non-alcoholic fatty liver disease (NAFLD) encompasses chronic liver diseases with the redundant accumulation of triglycerides (TG). The abnormalities that comprise NAFLD vary from an evident increase in hepatocellular lipid content (steatosis) to non-alcoholic

steatohepatitis (NASH), which has distinct levels of necrotic inflammation, hepatocyte enlargement, collagen build-up, and eventually cirrhosis [1].

A condition known as Non-Alcoholic Steatohepatitis (NASH), in which steatosis exists along with hepatic damage and inflammation, can lead to cirrhosis, fibrosis, and necrosis of the liver as well as a markedly elevated risk of hepatocellular carcinoma [2]. The prevalence of NAFLD is increasing year by year so it's indeed necessary to more precisely characterize the current and prospective effects of liver disease caused by NAFLD [3].

The current prediction of the prevalence of NAFLD worldwide in 2022 is between 25.2% and 29.8% of the world's population. The occurrence of NAFLD in the Indian population varies from 9% to 53% and the incidence rate in Haryana's rural regions is currently expected to be 30.7% [4]. Based on predictions, 2.6% to 17.3% of children in the globe have been reported to have NAFLD, whereas 32% are men and 21% are women [5]. However, the incidence of NAFLD is rising in patients having obesity, type2 diabetes mellitus, metabolic dysfunction, insulin resistance, hyperlipidemia and it has a significant correlation to metabolic risk variables [6].

In the progression of NAFLD, free fatty acids (FFA) liberated from lipolysis play a contributory role in the hepatocytes. These FFA are either retained in the form of hepatic lipid droplets or released from the liver as very low- density lipoprotein (VLDL) [7].

High mobility group box1 protein (HMGB1), a DAMP (damage-associated molecular patterns) has two roles to play extracellularly acting as a cytokine to induce inflammation while intracellularly involved in the regulation of transcription, DNA replication, and repair process of DNA. HMGB1 activates the innate immune system and acts as a ligand for toll-like receptors [8].

Expression of HMGB1 is widespread in both parenchymal and non-parenchymal liver cells. In the early phases of NAFLD, plasma HMGB1 hastens liver injury and inflammation due to a high-fat diet which is undetermined. HMGB1 released from hepatocytes results in liver necro-inflammation and fibrosis which is crucial for the emergence of NAFLD. So, uncertainty exists regarding the contribution of loss of cytoplasmic HMGB1 in hepatocytes to the etiology of NAFLD [9].

Several receptors regulate the HMGB1 function like toll-like receptors (TLR), which act as protective receptors expressed in different hepatic cells, monocytes, Kupffer cells, and hepatic stellate cells and shows a connection between injury to the liver, endotoxemia, and intestinal flora [10]. The transport of endotoxin from gut microbiota, results in the activation of TLR4 signaling that triggers the release of cytokines [11][12]. TLR4 is a receptor for lipopolysaccharides (LPS) which is a component of gram-negative bacteria and LPS is linked to the formation of NAFLD making TLR4 signaling a critical pathway in the progression of NAFLD [13]. So,

the correlation of HMGB1 and TLR4 with lipid profile, in patients with NAFLD still has not been completely assessed. Therefore, the correlation of HMGB1 and TLR4 with lipid profiles in NAFLD patients was studied.

2. Materials and Methods

2.1. Study Setting

This cross-sectional study was conducted in Kasturba Medical College, Hospital, Ambedkar Circle, (KMCHAC) and Department of Biochemistry, Centre for Basic Sciences, Bejai, Mangalore, for the period Nov 2022-March 2023. The study protocol was approved by the Institutional Ethical Committee [IEC KMC MLR 10/2022/428]. Patients were enrolled with informed consent.

2.2. Study Design

The study participants included eighty-seven (87) patients (54 males and 33 females), with NAFLD and dyslipidemia diagnosed by upper abdominal ultrasonography. The mean age of the participants was 50.5 ± 6.25 years, of these, 86.2% had Grade I fatty liver, and 13.8% had Grade II fatty liver.

2.3. Inclusion Criteria

Individuals included were those with non-alcoholic fatty liver disease aged 39 -60 years. The non-alcoholic individuals were defined as either total abstainers or individuals who consumed less than 20 g of alcohol per day. The history of alcohol consumption was confirmed by two family members of the patient. Patients with deranged lipid profile values and on treatment with lipid-lowering drugs like statins were included.

2.4. Exclusion Criteria

Patients with alcoholic fatty liver disease, viral hepatitis, hepatobiliary disease, malignancies, inflammatory bowel disease, chronic cardiac, renal, and respiratory diseases were excluded.

2.5. Sample Collection Methodology

Details of fatty liver and history of drug intake were obtained from patients. Data for lipid profile and AST & ALT were collected from the data management system of the central laboratory after consent from the authorized personnel. Leftover serum samples were collected on the day after the analysis of the selected subjects from the clinical laboratory, stored at -20°C , and used for the estimation of HMGB1 and TLR4 by ELISA method using LISA PLUS ELISA reader.

2.6. Statistical Analysis

Statistical analysis was done using Jamovi software version 2.3.21.0. Results were summarized using mean \pm SD and median (IQR). The Shapiro-Wilk test was used to assess the normality of the data. The correlation of HMGB1 and TLR4 with Lipid Profile (LP) and liver enzymes was done by Karl Pearson's correlation for normally distributed data and Spearman's correlation coefficient for not normally distributed data. Independent t-test was used for normally distributed data and Mann-Whitney U test was used for not normally distributed data to find the association of LP, liver enzymes, HMGB1, and TLR4 within the age group and gender. A p-value <0.05 is considered statistically significant.

2.7. Age Group

The age of the included patients was between 39-60 years. The patients were divided into Group 1 - (39-49) years of age and Group 2 - (50-60) years of age.

3. Results

The demographic and biochemical characteristics of NAFLD patients are shown in Table 1.

No correlation was observed between the lipid parameters, liver enzymes with the HMGB1 and TLR4 in NAFLD patients as shown in Table 2.

According to statistical analysis, HMGB1 & TLR4 showed no correlation with age. AST/ALT ratio is significantly correlated with the age (p value-0.018) as shown in Table 3.

There was a significant difference in terms of the mean value of HMGB1 and AST/ALT ratio among Group 1 and Group 2 age groups ($p \leq 0.022$) and ($p \leq 0.042$) and other variables did not show a significant difference between the age groups as shown in Table 4.

There was a significant difference in terms of the mean value of TG ($p < 0.030$), VLDL-C ($p < 0.039$), AST ($p < 0.004$), ALT ($p < 0.001$) and AST/ALT ($p < 0.001$) among males and females. Other variables did not show significant difference between males and females as shown in Table 5.

Table 1. Baseline clinical characteristics and biochemical assessments of study population

Parameters (Reference Range)	Baseline Characteristics (n=87)	Range of Values
Males: females	54:33	-
※Fatty liver grades	75:12	-
‡Age(Years)	50.5 \pm 6.25	39-60
‡Male (Years)	48.03 \pm 5.90	-
‡Female (Years)	51.03 \pm 6.61	-
‡TC (Up to 200 mg/dl)	205 \pm 45.5	101-371
*TG (Up to 150 mg/dl)	141 (90.5)	47.0-530
*HDL-C (40-60 mg/dl)	43.1 (11.9)	19.5-90.2
‡LDL-C (Up to 100 mg/dl)	143 \pm 40.5	58.0-299
*TC/HDL-C (2.5 – 5.0)	4.60 (1.25)	2.50-10.1
*VLDL-C (Up to 40 mg/dl)	28.2 (17.7)	9.40-106
‡Non-HDL-C	159 \pm 42.8	76.3-216
‡LDL-C/HDL-C	3.26 \pm 1.08	1.50-7.44
‡Non-HDL-C/HDL-C	3.64 \pm 1.23	1.54-9.11
‡TG/HDL-C	3.94 \pm 2.76	0.776-16.9
*AST (5-40 U/L)	25 (13.0)	10-97
*ALT (5-40 U/L)	33 (25.5)	10-229
*AST/ALT	0.768 (0.365)	0.347-1.65
‡ALT/AST	1.35 \pm 0.458	0.605-2.88
‡HMGB1 (pg/ml)	1438 \pm 320	570-2132
*TLR4 (pg/ml)	1606 (650)	185-2289

n = total number of patients. ‡= indicates data with normal distribution presented as mean \pm SD. *= indicates data with non-normal distribution presented as Median (Interquartile range). ※= indicates the grades of fatty liver (grade I and grade II)

Abbreviations: high mobility group box1 protein (HMGB1), toll-like receptor4 (TLR4), total cholesterol (TC), triglycerides (TG), high density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), very low-density lipoprotein cholesterol (VLDL-C), Aspartate transaminases (AST), Alanine transaminases (ALT).

Table 2. Correlation of HMGB1 and TLR4 with lipid parameters, liver enzymes

Individual Parameters	HMGB1(pg./ml)		TLR4(pg/ml)	
	Correlation Value (r)	Statistical significance Value (p)	Correlation Value (r)	Statistical significance Value (p)
Age (Yrs.)	-0.150	0.164	-0.176	0.104
TC (mg/dl)	0.142	0.190	-0.001	0.995
‡TG (mg/dl)	0.065	0.551	-0.122	0.259
‡HDL-C (mg/dl)	0.138	0.203	0.087	0.422
LDL-C (mg/dl)	0.091	0.403	0.039	0.719
TC/HDL-C	-0.049	0.652	-0.134	0.218
‡VLDL-C (mg/dl)	0.054	0.617	-0.118	0.277
Non-HDL-C (mg/dl)	0.122	0.261	0.002	0.982
LDL-C/HDL-C	0.010	0.925	0.034	0.755
Non-HDL-C/HDL-C	0.025	0.816	-0.002	0.988
TG/HDL-C	0.027	0.807	-0.110	0.312
AST(U/L)	-0.031	0.774	-0.123	0.257
ALT(U/L)	0.043	0.694	-0.047	0.663
AST/ALT	-0.122	0.260	0.072	0.509
ALT/AST	0.107	0.324	0.041	0.706

Parametric Pearson's correlation, ‡ Non-parametric Spearman's rank correlation *p<0.05

Table 3. Correlation of HMGB1, TLR4, and AST/ALT ratio with Age

Correlation Coefficient	Age (Yrs)	
	Correlation value (r)	Statistical Significance (p-value)
*HMGB1	-0.150	0.164
‡TLR4	-0.150	0.167
AST/ALT	0.253	0.018※

* Indicates Pearson's correlation, ‡ indicates Spearman's correlation ※p≤0.05 statistically significant

Table 4. Comparison of HMGB1, TLR4, lipid parameters & liver Enzymes between age groups

	Age group	n	Mean \pm SD	Statistical test value	p-value
↓HMGB1	Group 1	40	1522.89 \pm 290.01	2.326	0.022※
	Group 2	47	1366.51 \pm 330.30		
*TLR4	Group 1	40	1571.031 \pm 458.69	813	0.281
	Group 2	47	1434.509 \pm 490.37		
↓TC	Group 1	40	212.50 \pm 40.4	1.522	0.132
	Group 2	47	197.70 \pm 48.90		
*TG	Group 1	40	165.17 \pm 104.94	860	0.498
	Group 2	47	162.76 \pm 73.32		
*HDL-C	Group 1	40	46.21 \pm 10.8	854	0.464
	Group 2	47	44.70 \pm 10.06		
↓LDL-C	Group 1	40	148.82 \pm 35.55	1.261	0.211
	Group 2	47	137.87 \pm 44.05		
↓TC/HDL-C	Group 1	40	4.80 \pm 1.40	1.368	0.175
	Group 2	47	4.45 \pm 0.96		
*VLDL-C	Group 1	40	33.04 \pm 20.98	875	0.583
	Group 2	47	32.03 \pm 14.32		
↓Non-HDL-C	Group 1	40	166.29 \pm 40.68	1.565	0.121
	Group 2	47	152.0 \pm 43.871		
*AST	Group 1	40	26.90 \pm 13.22	782	0.179
	Group 2	47	31.14 \pm 17.71		
*ALT	Group 1	40	43.37 \pm 37.37	874	0.577
	Group 2	47	38.91 \pm 27.78		
↓AST/ALT	Group 1	40	0.76 \pm 0.29	-2.062	0.042※
	Group 2	47	0.88 \pm 0.27		

↓ indicates the Independent t-test, * indicates the Mann Whitney U test, ※ p \leq 0.05 is statistically significant, Age groups (yrs): Group 1 indicates Quartile (39-49) and Group 2 indicates Quartile (50-60)

Table 5. Comparison of HMGB1, TLR4 lipid parameter & liver enzymes between Gender

	Gender	n	Mean±SD	Statistical test value	p-value
↓HMGB1	Males	54	1484.867 ±275.10	1.7507	0.084
	Females	33	1362.40 ±375.37		
*TLR4	Males	54	1548.681 ±488.34	715	0.124
	Females	33	1413.16 ±456.01		
↓TC	Males	54	204.167 ±43.50	-0.0883	0.930
	Females	33	205.06 ±49.40		
*TG	Males	54	180.905 ±100.27	642	0.030※
	Females	33	136.0 ±56.66		
*HDL-C	Males	54	44.272 ±9.49	720	0.136
	Females	33	47.23 ±11.59		
↓LDL-C	Males	54	140.886 ±38.53	-0.5931	0.555
	Females	33	146.22 ±43.97		
↓TC/HDL-C	Males	54	4.728 ±1.353	1.1178	0.267
	Females	33	4.43 ±0.863		
*VLDL-C	Males	54	35.733 ±19.920	655	0.039※
	Females	33	27.21 ±11.329		
↓Non-HDL-C	Males	54	159.022 ±42.403	0.1253	0.901
	Females	33	157.83 ±44.085		
*AST	Males	54	32.722 ±18.479	563	0.004※
	Females	33	23.42 ±7.467		
*ALT	Males	54	50.648 ±37.164	348	<0.001 ※
	Females	33	25.12 ±10.959		
↓AST/ALT	Males	54	0.723 ±0.249	-4.9931	<0.001 ※
	Females	33	1.01 ±0.270		

↓indicates the Independent t-test, * indicates the Mann Whitney U test, ※ indicates statistically significant p≤0.05.

4. Discussion

The overall prevalence of NAFLD in Asia is higher compared to Western populations. NAFLD is characterized by a significant lipid buildup, due to the increased breakdown of adipose tissue leading to the release of FFA which gets deposited in the liver. The present cross-sectional study was conducted in 39–60-year-old NAFLD patients with dyslipidemia. High mobility group box1 protein (HMGB1) and toll-like receptor4 (TLR4) were determined in NAFLD subjects with dyslipidemia who were diagnosed with abdominal ultrasonography and based on the history of alcohol intake. Of the 87 participants in the study who were selected from the health care lounge of our tertiary care hospital from Nov 2022 to March 2023 following the inclusion and exclusion criteria 54 were males and 33 were females. Among the study participants, 86.2% had grade I fatty liver and 13.8% had grade II fatty liver [14].

In a study reported by Khanal et al., [15] where 109 fatty liver patients above the age of 16 years without a history of alcohol consumption were studied at Tribhuvan University, Kathmandu, G-I fatty liver was found in 68.78%, G-II fatty liver was 28% and G-III fatty liver was 3.22%. In apparently healthy individuals, fatty liver is a common occurrence. The individuals who participated in the current study had dyslipidemia and were on treatment. Despite the effective treatment status, as seen by the mean values of the lipid profile of the study participants where the dyslipidemia was well controlled, patients showed early steatosis of G-I and G-II.

Several factors are associated with the development of NAFLD, such as increasing age, obesity, dyslipidemia, diabetes mellitus, metabolic syndrome, cardiovascular diseases, ingestion of drugs and toxins, etc. In the current study, we observed an increase in the occurrence of NAFLD with age. A recent review of NAFLD among older adults found that the risk increases with advancing age and

declines after the age of 80. It was hypothesized that the decline in very elderly adults may be because of fibrosis which does not allow fat accumulation. A gender difference is also noted with females developing NAFLD much later than their male counterparts, explained as the protective impact of estrogens in women of reproductive age [15]. No difference was found between males and females beyond post-menopausal age. Prevalence in males was reported to increase from younger to middle-aged group of people, and it begins to drop around the age of 50 or 60 [16]. In the present study, the mean age of males with fatty liver was 48.03 ± 5.90 and females was 51.03 ± 6.61 years (Table 1), which was similar to that reported by Khanal et al., [15] in which the mean age of males was found to be 44.3 years and in females 51.9 years.

In the study done by Singla et al. [17], it has been noted that the serum total cholesterol, triglycerides, LDL, and VLDL levels were significantly increased, and HDL levels were decreased in subjects with NAFLD. In the present study, it was observed that serum LDL-C levels were significantly higher in the subjects with NAFLD compared to the study by Singla et al. [18], in which $LDL < 70$ mg/dl is considered as a high-risk factor as the selected patients were on lipid-lowering drugs like statins. It is a known fact that other risk factors such as obesity, dyslipidemia, oxidative stress, mitochondrial dysfunction, and cardiovascular diseases which are directly related to lipid accumulation lead to NAFLD.

As mentioned in the study by Jin et al. [19] serum HMGB1 levels are increased in patients with hyperlipidemia. They found a decrease in the values of HMGB1 on treating with atorvastatin drugs which was proportional to the reduction in serum lipids. Study participants of the current study were all under treatment for dyslipidemia and HMGB1 values found in them concurred with the values reported by Jin et al. [19], after the 3-month treatment period with atorvastatin in their study.

NAFLD has a multi-hit pathogenesis, in which insulin resistance is one of the well-established pathologies in the development and progression of NAFLD. Mahaling et al., stressed the role of insulin resistance in the progression of NAFLD. Several studies have reported that the ALT/AST ratio is a surrogate marker of insulin resistance and showed a good correlation with HOMA-IR (homeostatic model assessment for insulin resistance) [20]. A value > 1.0 signified insulin resistance. The mean value of the ALT/AST ratio in the present study was 1.35 denoting the presence of insulin resistance in the study participants. Recent reports have linked statin use with the risk of developing insulin resistance and diabetes mellitus [20]. In this context, it may be worthwhile to closely monitor the patients for adverse outcomes for NAFLD.

AST/ALT ratio has traditionally been used as a biochemical marker to differentiate between NAFLD & alcoholic fatty liver disease (AFLD) and to evaluate the degree of hepatic fat infiltration & hepatic steatosis. A

value < 1 indicates NAFLD, and a value > 1 is suggestive of AFLD. According to the studies by Mahaling et al., [14] they reported that AST & ALT values are shown to be associated with NAFLD. In the present study, the absolute values of AST and ALT were within the normal reference range and the ratio of AST/ALT further ascertained the NAFLD status. However, this reliable biomarker did not correlate with HMGB1 or TLR4. A significant correlation was noted with age of the NAFLD patients ($p < 0.018$), highlighting that advancing age aggravates NAFLD.

The current study estimated HMGB1, an indicator of DAMP, which extracellularly acts as a cytokine to induce innate immune response in patients with NAFLD [21]. According to the previous studies by Khambu et al. [21], HMGB1 is significantly expressed in liver diseases. In NAFLD, HMGB1 plays an important role in lipid metabolism. Huang et al. [18], reported that a decrease in HMGB1 resulted in oxidative stress and a reduction in oxidation of fatty acids which culminated in the accumulation of fat and injury to the liver. Haraba et al., [22] reported that hyperlipidemia increases the release of HMGB1 from the nucleus, whereas hypolipidemia inhibits HMGB1 expression. This proved that raised HMGB1 levels may be linked to hyperlipidemia. In the present study, the participants were known as dyslipidemia patients on treatment. Despite the treatment, the serum HMGB1 levels were as per the values reported in the literature in NAFLD patients with no treatment. Jin et al., [19] reported that the high serum HMGB1 level in patients with dyslipidemia before treatment with the statin drugs reduced significantly post-treatment.

Another effect of the rise in HMGB1 levels explained by Chandrashekar et al., [23] is the trigger of the inflammatory pathways in the intestine as HMGB1 is associated with the pattern recognition receptors of the intestine followed by liver injury. The deposition of fat in the liver leads to liver injury by the generation of cytokine, endotoxin, and reactive oxygen species. Chen et al. [24] suggested that HMGB1-TLR4 signaling is involved early in the progression of NAFLD. The gut-liver axis created significant interest in the etiology of NAFLD, in which the balance between nutritional absorption and energy storage is disrupted [10]. The derived pro-inflammatory metabolites from the gut-microbiota are carried to the liver through the portal vein. TLR4 is a principal receptor for LPS (lipopolysaccharides) an endotoxin & major component of the cell membrane of gram-negative bacteria which activates the host's innate immune system [25]. Over-activation of immune cells results in inflammation, severe liver damage & fibrosis. Activation of TLR4 triggers the production of pro-inflammatory cytokines by inducing oxidative stress and further accelerating the progression of NAFLD [26]. Sharifnia et al. [27] studies report that a high-fat diet increases the circulating levels of LPS by altering gut-microbiota & epithelial permeability. Furthermore, studies support that HMGB1 release due to an increase in FFA level mediates the activation of TLR4

signaling in hepatocytes [24]. Li et al. [28] showed that in the early stages of NAFLD, HMGB1 levels are increased in hepatocellular expression which triggers the TLR4-mediated inflammatory response due to free fatty acid infusion. Our results showed that TLR4 levels were in the previously reported range in NAFLD in spite of the treatment status. Thus, the interdependence of HMGB1 and TLR4 may be said to be bidirectional, connecting the liver- gut axis.

5. Conclusions

In this hospital-based cross-sectional study of 87 patients with dyslipidemia and NAFLD, NAFLD was found more frequently in higher age groups. Males were affected earlier than females. Even with treatment, the study group had significantly higher levels of LDL-cholesterol than the treatment goals. Levels of extracellular HMGB1, which is a DAMP were found to be elevated, similar to the findings with TLR4 levels which act as a receptor for HMGB1. Both the molecules also are activated in gut dysbiosis associated with dyslipidemia, which is another contributor to altered lipid accumulation in NAFLD. However, we did not find a correlation of HMGB1 and TLR4 with lipid profile in non-alcoholic fatty liver disease. AST/ALT ratio, a biochemical marker has been used to differentiate alcoholic liver disease and non-alcoholic liver disease correlated with age supporting the finding that NAFLD increases with age. ALT/AST ratio, an indirect measure of insulin resistance was also found to be high stressing the role of insulin resistance in the pathogenesis of NAFLD. Strategies may be developed to target this signaling pathway to prevent the activation of the downstream inflammatory pathway and its subsequent ramifications.

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