

Peripheral Blood Expression of Angiotensin II type 1- Receptor (AT₁- R) as A Non Invasive Marker of Liver Disease Progression in Patients with Non Alcoholic Fatty Liver Disease

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Abstract: Angiotensin-II (AT-II) has been suggested to play an important role in liver fibrogenesis. It induces hepatic stellate cell (HSC) proliferation and up-regulates the transforming growth factor beta expression via AT-II type 1 receptor (AT₁-R) *in vitro*. There is accumulating evidence that renin-angiotensin system (RAS) does not only play an important role in the regulation of systemic hemodynamics but is also involved in hepatic inflammation and fibrogenesis. **Aim of the study:** is to evaluate the efficacy of AT₁-R as a non-invasive marker of liver disease progression in patients with non alcoholic fatty liver disease (NAFLD). **Patients and methods:** This study was conducted on 62 NAFLD patients (33 with pure steatosis and 29 with non alcoholic steatohepatitis (NASH). All evidenced by the liver biopsy histopathological features of NAFLD. Twenty potential liver transplant donors were selected as a control group. All patients and control group were subjected to the following:- fasting insulin, HOMA index of insulin resistance, and serum Angiotensin II (AG II). Serum AT₁R mRNA AT receptor was measured by Real-Time PCR. **Results:** a positive correlation was found between AT₁R mRNA with fasting insulin, HOMA index of insulin resistance and serum AG-II levels in NAFLD patients. While a negative correlation was found between AT₁R mRNA and ALT or AST in NAFLD patients. No significant differences between the two subgroups of steatosis and NASH for the all tested parameters except for AG-II and AT₁ R mRNA that show a significant increase in NASH patients (p<0.01) for each. **Conclusion:** Increased expression of AT₁R on circulating leukocyte subsets of NAFLD patients, suggesting its possible role in disease progression, and it could be used as a non invasive marker for disease diagnosis and prognosis. In the future, AT₁R expression may be used as a follow up marker for monitoring of therapeutic efficacy of currently available agents.

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Key words: NAFLD, NASH, Angiotensin II type I receptor, antifibrotic therapy.

1. Introduction

The high prevalence and chronic nature NAFLD subsequently translates to a significant health burden for the general community. Non-alcoholic fatty liver disease (NAFLD) is considered the hepatic manifestation of metabolic syndrome (Montecucco and Mach, 2008; Preiss and Sattar, 2008). This disease refers to a spectrum of hepatic manifestations ranging from steatosis (fat accumulation in the liver), through non-alcoholic steatohepatitis (NASH) (hepatic steatosis and inflammation) and sometimes fibrosis towards advanced and irreversible fibrosis (hepatic cirrhosis) (Adams *et al.*, 2005 ; Ratziu *et al.*, 2007). NAFLD may be progressive resulting in cirrhosis that may be complicated by hepatocellular carcinoma and liver failure. Overall, about 5% of patients with NAFLD develop cirrhosis over an average of a seven year period with 1.7% dying from complications of liver cirrhosis (Adams *et al.*, 2005).

The American Association for the study of Liver Diseases has defined NAFLD as a fat accumulation in the liver over 5-10% of the organ weight

(Neuschwander-Tetri and Caldwell, 2003). The prevalence of NAFLD in the developed countries ranges from 20 to 30% (Jimba *et al.*, 2005).

NASH has been recognized as a major cause of liver fibrosis and it is included in a disease spectrum ranging from a simple steatosis to advanced fibrosis and cirrhosis (Browning and Horton, 2004; Bataller and Brenner, 2005). NASH is associated with both increased cardiovascular and liver related mortality (Fan, 2008; Angulo, 2010).

Although the pathogenesis of NASH is not well understood, the most important theory proposed to explain the development of this disease is the "two-hit hypothesis" (Day and James, 1998). Consistent with this theory, the first "hit" is the fat accumulation within the liver; this is associated with an "insulin resistance state." Then, the hepatic steatosis development into NASH is related to injury caused by oxidative stress and inflammatory cytokines (the second hit) (Browning and Horton, 2004). As a result, abnormal cytokine production within the liver may be playing an essential role in the pathogenesis of NASH.

There is accumulating evidence that renin-angiotensin system (RAS) does not only play an important role in the regulation of systemic hemodynamics but is also involved in hepatic inflammation and fibrogenesis (**Yokohama et al., 2004; Bataller et al., 2005**).

Angiotensin II (AG-II), which is mainly generated by angiotensin converting enzyme (ACE) and chymase, is a peptide that plays a crucial role in regulating blood pressure and sodium homeostasis via specific receptors including angiotensin II type 1 receptor (AT₁ R) (**Murphy et al., 1992**). Angiotensin II is recognized to induce hepatic inflammation and to stimulate a range of fibrogenic action, including cell migration, cell proliferation, secretion of proinflammatory cytokines, and collagen synthesis predominantly throughout AT₁ receptor (**Bataller et al., 2005**).

Furthermore, several reports indicate that AG-II is able to be generated by different cells in a variety of tissues, and its production is activated in certain pathological states associated with tissue repair (**Sun et al., 2000**). The mechanism postulated for fibrotic actions of AG-II seems to involve an important mediator of fibrous tissue formation such as transforming growth factor- β 1 (TGF- β 1) (**Yoshiji et al., 2001; Bataller et al., 2005**). Other Previous studies have showed that the inhibition of RAS significantly attenuates TGF- β 1 expression and fibrosis in heart (**Toblli et al., 2003**), kidney (**Sun et al., 2000**), and liver (**Toblli et al., 2002**).

Muñoz et al. (2006) demonstrated that obese Zucker rat (OZR) show excessive fat accumulation in the liver together with an increased expression of AG-II, suggesting that local AG-II generation may contribute to the pathogenesis of NASH. Therefore, due to its biological properties, RAS is an important target to prevent fibrosis in chronic inflammatory states.

The better understanding of the underlying mechanisms involved in liver fibrosis makes effective antifibrotic therapy an imminent reality. However, treating this disease remains a challenge and, up to this moment, no antifibrotic agent has been approved for routine human use.

Aim of the study:

The aim of this study was to clarify the relationship between the expression of AT₁ R in blood samples and progression of NAFLD from simple steatosis to NASH reaching up to cirrhosis.

2. Patients and Methods:

This study was conducted on 62 NAFLD patients (35 males and 27 females, age range 30- 52 years) attending the outpatient clinic of the National Liver Institute, from March, 2009 till September 2010.

NAFLD diagnosis was based on the NAFLD histopathological features in liver biopsy, absence of hepatitis B and C viral markers, absence of autoantibodies indicative of autoimmune hepatitis, absent alcohol consumption. All patients had a bright liver at ultrasound scanning. NAFLD patients were further classified as 33 cases of pure steatosis and 29 cases as NASH by the criteria proposed by **Brunt et al. (1999)**.

Exclusion criteria: Hypertensive patients, patients with renal impairment, those with features of early cirrhosis and /or portal hypertension, patient with diabetes mellitus were excluded from both the study and control group.

All participants were subjected to complete medical and anthropometric examination. Body mass index (BMI) was calculated as weight (in kilograms) divided by height squared (meters squared). Waist circumference (at the nearest half centimetre) was measured at the midpoint between the lower rib margin and the iliac crest.

Twenty potential liver transplant donors matching age and sex of the patients were selected as a control group. They were negative for serological markers of hepatitis B and C, with normal ultrasonographic evaluation of their liver as well as histopathology of their liver biopsy.

The following investigations were done for patients and control subjects:

Venous blood samples were obtained after a fasting night, by use of heparinized tubes, and circulating leukocytes were isolated for subsequent studies. Samples of blood were collected in pyrogen free tubes, then centrifuged and the resulting serum was divided, aliquoted and kept at -70°C until assayed.

An aliquot of blood was used to monitor lipid profile including: total cholesterol, low-density lipoprotein cholesterol (LDL-c), high-density lipoprotein cholesterol (HDL-c), triglycerides, fasting blood sugar, and to perform routine laboratory examinations (serum albumin, aspartate aminotransferase, alanine aminotransferase, gamma glutamyl transpeptidase and alkaline phosphatase). These tests were measured using COBAS Integra-400 autoanalyzer (Roche- Germany).

The determination of fasting insulin level was done using Diagnostic System Laboratories incorporation kits (DSL-10-1600 ACTIVE[®] insulin, enzyme linked immunosorbent assay (ELISA) kit, Texas- USA). It is an enzymatically amplified one step sandwich type immunoassay. The minimum detection limit is 0.26 uIU/ml, the intra- and inter-assay coefficient variations were 2.6% and 6.2% respectively. Standards, controls and samples were incubated with Horseradish Peroxidase (HRP) labeled

anti-insulin antibody in microtitration wells which were coated with another anti-insulin antibody, the assay was performed according to the manufacturer's instructions (Rasmussen *et al.*, 1990).

The degree of insulin resistance was calculated from the homeostasis model assessment (HOMA). The HOMA index was calculated by the formula (Emoto *et al.*, 1999):

$$\frac{\text{Fasting plasma insulin (uIU/ml)} \times \text{fasting plasma glucose (millimoles / liter)}}{22.5}$$

HOMA index >3 is a criterion of insulin resistance (Machado and Cortez-Pinto, 2005).

Measurement of serum AG- II:

AG-II was measured using an ELISA kit from Usen Life Science Inc. Wuhan. Briefly, The microtiter plate provided in this kit has been pre-coated with an antibody specific to AG-II. Standards or samples are then added to the appropriate microtiter plate wells with a biotin-conjugated polyclonal antibody preparation specific for AG-II. Next, Avidin conjugated to Horseradish Peroxidase (HRP) is added to each microplate well and incubated. Then a TMB substrate solution is added to each well. Only those wells that contain AG-II, biotin-conjugated antibody and enzyme-conjugated Avidin will exhibit a change in color. The enzyme-substrate reaction is terminated by the addition of a sulphuric acid solution and the color change is measured spectrophotometrically at a wavelength of 450 nm. The concentration of AG-II in the samples is then determined by comparing the O.D. of the samples to the standard curve.

Measurement of AT1R mRNA AT receptor by Real-Time PCR:

1- Isolation of Polymorphnuclear Leukocytes:

Whole blood was allowed to sediment on dextran at 37°C for 30 minutes. Supernatant was recovered and PMNs were separated from peripheral blood mononuclear cells (PBMCs) by Ficoll-Paque Plus density-gradient centrifugation. Contaminating erythrocytes were eliminated by 10 minute hypotonic lysis in distilled water with added (g/L) NH₄Cl 8.25, KHCO₃ 1.00, and ethylenediamine tetraacetic acid (EDTA) 0.04. Cells were then washed 3 times in NaCl 0.15 M and resuspended in 1 mL phosphate buffered saline with added bovine serum albumin 0.1% (Guasti *et al.*, 2006).

2- RNA Isolation and Real-Time PCR Analysis of AT₁R mRNA:

Total mRNA was extracted from 1×10^6 cells by a Total RNA Isolation Kit (Roche -Mannheim, Germany) and the amount of extracted RNA was estimated by spectrophotometry at 260 nm. Reverse transcription reactions were performed using the high-

capacity cDNA Archive Kit (Applied Biosystems, Foster City, California, USA). Briefly, 1 µg of total RNA, 1 µL of oligo dT-primer, and 1 µL of dNTPs were incubated at 65°C for 5 min, then 10 µL of a cDNA synthesis mixture was added and the mixture was incubated at 50°C for 50 min. The reaction was terminated by adding 1 µL of RNaseH and incubating the mixture at 37°C for 20 minute kept at -80°C until RNA extraction (Marino *et al.*, 2007).

Real-Time PCR was performed by Roche LightCycler-2.0 TM (Mannheim, Germany) using the assay on demand kit for human AT₁Rs (Applied Biosystems). Cycles included one for 2 minute hold (50°C) and one for 10 minute and 45 for 15 second cycles of denaturation (95°C). Raw data were analyzed by the machine software. Threshold cycle value (Ct) for human AT₁Rs was used to calculate a linear regression line generated by performing serial dilutions (1:10; 1:50; 1:500; 1:1,000; 1:10,000) of the total mRNA obtained from human PMN. The values were then normalized for Ct values of 18S ribosomal RNA (Guasti *et al.*, 2008).

Statistical analysis

The data were statistically analyzed using SPSS computer program version 11, data were expressed as mean ± SD and differences between groups were analyzed by Mann-Whitney or ANOVA tests, while chi square or Fisher's exact test were used to compare categorical variables. Pearson's correlation coefficient was used to test the relationship between various variables.

3. Results:

All data were calculated as mean ±SD and compared in tables 1-4. Table (1) shows a significant increase of BMI, waist circumference, fasting glucose, fasting insulin and HOMA index (insulin resistance) in the NAFLD group compared to the control group, while the age showed no significant difference.

Table (2) shows a statistically significant increase in AST, ALT, ALP and GGT. Also, a highly significant increase in the serum levels of AG-II and AT₁ R mRNA expression was detected in the NAFLD group compared to the control group (Figure 1).

Table (3) shows no significant differences between the two subgroups for the all tested parameters except for AG-II and AT₁ R mRNA that show a significant increase in NASH patients (p<0.01) for each (Figure 1).

Table (4) shows a positive correlation between AT₁ R mRNA and fasting insulin, HOMA index of insulin resistance and serum AG-II levels in NAFLD patients. But, AT₁ R mRNA not correlated either to ALT or AST in NAFLD patients.

Table (1): Comparison between NAFLD patients and control group

Variables	NAFLD (n=62) Mean \pm SD	Control (n=20) Mean \pm SD	t- Test	p-value
Age (years)	41.2 \pm 3.5	39.4 \pm 4.2	3.01	>0.05
BMI (kg/m ²)	32.6 \pm 5.4	22.5 \pm 3.7	4.12	<0.05
Waist circum. (cm)	96.2 \pm 6.8	86.2 \pm 5.4	4.08	<0.05
Fasting glucose (mg/dl)	92.7 \pm 10.2	85.8 \pm 8.7	3.98	<0.05
Fasting insulin (uIU/ml)	9.8 \pm 2.4	6.9 \pm 1.7	5.12	<0.05
HOMA index	4.06 \pm 1.13	2.6 \pm 1.3	6.23	<0.01

P>0.05 is non significant, p value <0.05 and p value <0.01 is significant

Table (2): Comparison between NAFLD patients and control group

Variables	NAFLD (n=62) Mean \pm SD	Controls (n=20) Mean \pm SD	t- Test	p-value
AST (U/L)	49.3 \pm 12.6	22.7 \pm 9.5	5.66	<0.05
ALT (U/L)	57.6 \pm 19.8	26.7 \pm 11.22	4.67	<0.05
ALP U/L)	63.2 \pm 12.4	35.2 \pm 8.6	4.63	<0.05
GGT (U/L)	58.4 \pm 10.2	39.5 \pm 7.6	5.01	<0.05
Albumin (g/dl)	3.97 \pm 0.72	4.17 \pm 0.62	3.22	>0.05
Triglycerides (mg/dl)	156.3 \pm 45.6	134.1 \pm 18.3	1.98	>0.05
Total cholesterol (mg/dl)	198.2 \pm 33.4	177.4 \pm 13.2	2.17	>0.05
HDL-Cholesterol (mg/dl)	36.5 \pm 5.6	40.2 \pm 6.7	3.23	>0.05
LDL-Cholesterol (mg/dl)	129.2 \pm 31.4	102.6 \pm 19.2	1.25	>0.05
AG-II (pg/ml)	23.6 \pm 9.4	8.1 \pm 1.7	7.56	<0.01
AT ₁ R mRNA	9.84 \pm 1.25	4.25 \pm 1.67	5.81	<0.01

P>0.05 is non significant, p value <0.05 and p value <0.01 is significant

Table (3) Comparison between steatosis and NASH patient subgroups

Variables	Steatosis (n=33) Mean \pm SD	NASH (n=29) Mean \pm SD	t- Test	p-value
BMI (kg/m ²)	29.4 \pm 4.1	34.5 \pm 5.6	2.13	>0.05
Fasting Glucose (mg/dl)	92.8 \pm 10.2	96.1 \pm 6.54	2.03	>0.05
Fasting Insulin (uIU/ml)	9.1 \pm 2.3	9.6 \pm 2.4	1.68	>0.05
HOMA index	3.71 \pm 0.98	3.92 \pm 1.62	1.27	>0.05
AST (U/L)	39.8 \pm 5.2	45.2 \pm 12.4	3.06	>0.05
ALT (U/L)	51.3 \pm 12.8	55.2 \pm 10.2	2.62	>0.05
ALP (U/L)	56.5 \pm 7.9	64.7 \pm 10.8	2.26	>0.05
GGT (U/L)	50.2 \pm 4.6	56.7 \pm 9.5	1.67	>0.05
Albumin (g/dl)	3.65 \pm 0.42	3.47 \pm 0.23	1.12	>0.05
Triglycerides (mg/dl)	136.6 \pm 25.9	144.1 \pm 46.1	2.23	>0.05
Total cholesterol (mg/dl)	182.1 \pm 21.5	209.1 \pm 21.3	2.17	>0.05
HDL-Cholesterol (mg/dl)	31.5 \pm 3.2	34.4 \pm 6.4	1.03	>0.05
LDL-Cholesterol (mg/dl)	112.5 \pm 21.3	132.3 \pm 27.1	2.25	>0.05
AG-II (pg/ml)	18.5 \pm 6.2	27.6 \pm 4.3	5.13	<0.01
AT ₁ R mRNA	7.42 \pm 0.52	10.25 \pm 1.31	5.12	<0.01

P>0.05 is non significant, p value <0.05 and p value <0.01 is significant

Table (4) Correlation between AT₁ R mRNA and some parameters in NAFLD patients

Parameters	NAFLD (N=62)	
	r	p-value
AST (U/L)	0.234	>0.05
ALT (U/L)	0.211	>0.05
Fasting Insulin (uIU/ml)	0.485	<0.05
HOMA index	0.692	<0.01
AG-II (pg/ml)	0.601	<0.01

P>0.05 is non significant, p value <0.05 and p value <0.01 is significant

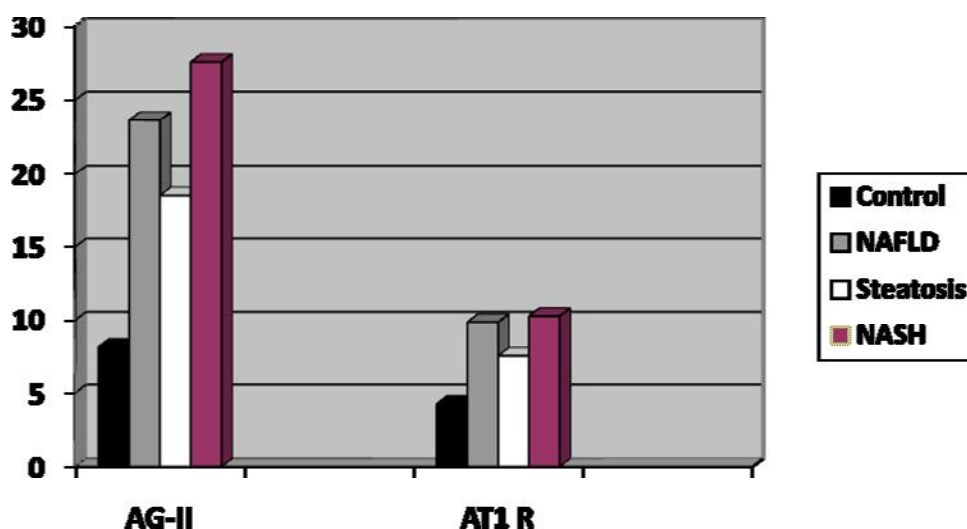


Figure (1): Shows the levels of Ang-II and AT₁ R mRNA in controls, NAFLD, steatosis and NASH patients.

4. Discussion:

Non-alcoholic fatty liver disease (NAFLD) and its severe clinical form, non-alcoholic steatohepatitis (NASH), are becoming increasingly prevalent in industrialised countries, along with the epidemic of obesity. The prevalence of NAFLD is estimated to be 10%-25% in the western world, while the corresponding prevalence of NASH ranges from 2%-7%. Insulin resistance and metabolic syndrome have been implicated both in the pathogenesis and disease progression of NAFLD (Tsochatzis and Papatheodoridis, 2011).11) pen

There is accumulating evidence that renin-angiotensin system (RAS) does not only play an important role in the regulation of systemic hemodynamics but is also involved in hepatic inflammation and fibrogenesis. Angiotensin II (AG-II) is recognized to induce hepatic inflammation and to stimulate a range of fibrogenic action, including cell migration, cell proliferation, secretion of proinflammatory cytokines, and collagen synthesis predominantly throughout AT₁ receptor (Toblli *et al.*, 2008).

The current study showed that a significant increase in BMI, waist circumference in NAFLD patients, a finding corresponds with two facts that NAFLD is strongly associated with metabolic syndrome and people with NAFLD have higher waist circumference (WC) or BMI than those without NAFLD (Jakobsen *et al.*, 2007 & Almeda-Valdes *et al.*, 2009).

Fasting blood sugar, fasting insulin and HOMA index of insulin resistance were found to be elevated in NAFLD patients in comparison with the control group. These are in agreement with the finding of Yoon *et al.*

(2005), who reported that NAFLD is associated with hyperinsulinaemia and insulin resistance in even non obese subjects. They suggested that insulin resistance is the most pathognomonic condition responsible for NAFLD. Also, Hui *et al.* (2003) stated that Insulin may injure the liver both directly and indirectly due to insulin's ability to generate oxidative stress or up-regulation of the lipogenic protein, sterol regulatory element-binding protein. It also seems to have direct profibrogenic effects by stimulating connective tissue growth factor, especially in the presence of hyperglycemia (Arthur & McCullough, 2007).

As serum aminotransferases are markers of liver injury (despite their relative lack of sensitivity and specificity), they are often used as surrogate markers of disease activity (Promrat *et al.*, 2010). Angulo *et al.* (1999) reported that, mildly to moderately elevated serum levels of AST, ALT, or both are the most common and often the only laboratory abnormality found in patients with NAFLD. The ratio of AST to ALT is usually less than 1, but this ratio increases as fibrosis advances, leading to a loss of its diagnostic accuracy in patients with cirrhotic NAFLD (Pinto *et al.*, 1996).

The current study comes in accordance with this showing a significant increase in the levels of AST, ALT in NAFLD patients compared to the control group. On the other hand, comparing patients with simple steatosis and those with NASH in their transaminases levels, a non significant difference between them was found with still relatively elevated levels in NASH patients than those with only steatosis. This comes in accordance with the fact that normal serum aminotransferases or with intermittent elevations

on repeated studies, do not exclude NASH or advanced stages of disease (Mofrad *et al.*, 2003).

Detection of serum levels of GGT in patients of our study revealed significant elevations of this enzyme levels in NAFLD patients than controls. This comes in accordance with many studies revealing that an elevated GGT was the commonest biochemical abnormality in NAFLD patients and, unlike transaminases; this was associated with the presence of cirrhosis. Biochemical profiles that omit GGT will be inadequate in screening for NAFLD (Masterton & Hayes, 2010).

Interestingly, in our study, alkaline phosphatase was found to be significantly elevated in NAFLD patients than controls. Despite the absence of any bile duct damage or proliferation, elevated alkaline phosphatase was associated with activity, fibrosis and overall NAFLD severity. Possible sources of elevated alkaline phosphatase could be through modulation of bile acid transporters by inflammatory cytokines as in cholestasis, or simply by intrahepatic or peripheral neutrophils as part of the inflammatory response (Fisher *et al.*, 1989). Alternatively, a small percentage of patients, possibly as many as 10%, may present with an isolated elevated alkaline phosphatase. These patients tend to be older women with auto-antibodies, such as antinuclear antibodies, may be found in up to one third of patients (Mofrad *et al.*, 2003).

As regards serum albumin levels which were found within normal levels is agreed with Baldrige *et al.* (1995), who reported that, hypoalbuminemia is only expected in NAFLD patients with cirrhosis.

In this study, elevated triglycerides, total cholesterol, LDL with lowered HDL cholesterol were detected in NAFLD patients however, the difference between the NAFLD group and the control group was not statistically significant. This could be explained by the postulation that, hypertriglyceridemia, hypercholesterolemia, or both were present in only 20-80% of patients with NASH. Most patients with NASH have multiple risk factors; including obesity, type 2 DM, and hyperlipidemia (Tarantino *et al.*, 2007). Taken together with the fact that some of our healthy controls have levels of dyslipidemias on the high normal states, this condition may be accepted.

The serum biochemistries, including serum AST, ALT, alkaline phosphatase, cholesterol, triglyceride, and the grade of fatty changes at ultrasound did not show significant differences between the steatosis and NASH subgroups. Several studies of hepatology clinic patients undergoing liver biopsy and morbidly obese individuals undergoing bariatric surgery, have found ALT levels to be higher in the presence of NASH compared with simple steatosis, although this has not been universally observed. (Harrison *et al.*, 2000). Till now there is no reliable marker could be counted upon

in differentiation between simple steatosis and NASH except for liver biopsy.

The RAS is frequently activated in patients with chronic liver diseases. AT-II has been suggested to play an important role in liver fibrogenesis. It induces hepatic stellate cell (HSC) proliferation and up-regulates the transforming growth factor-beta 1 (TGF-beta 1) expression via AT₁ R. These results suggested that the RAS, especially AT-II and AT₁ R interaction plays a pivotal role in liver fibrosis development through HSC activation (Yoshiji *et al.*, 2001).

In spite of many studies regarding the development of fibrosis, the understanding of the pathogenesis remains obscure. The hepatic tissue remodeling process is highly complex, resulting from the balance between collagen degradation and synthesis. Among them any mediators that take part in this process, the components of the RAS have progressively assumed an important role. Angiotensin acts as a profibrotic mediator and Ang-(1-7), the newly recognized RAS component, appears to play a role in fibrogenesis (Pereira *et al.*, 2009).

In our study, with respect to the postulated role of the RAS system in liver inflammation and fibrosis, serum AG-II and AT₁ R mRNA were found to be significantly elevated in NAFLD patients than control group which opens the way to accept the postulated role of this system in the pathogenesis of NAFLD. Also a significant difference of serum AG-II and AT₁ R mRNA was found between the two subgroups of NAFLD and there was a progressive increase of serum AG-II and AT₁ R mRNA from patients of simple steatosis to NASH, the higher grade of NAFLD. An observation suggesting a big role of AG-II and AT₁ R mRNA in progression of NAFLD mainly incriminated in both inflammation and fibrosis development.

These results reinforce the previous information suggesting that Ang- II causes development and progression of NAFLD in the transgenic rat model by increasing hepatic respiratory oxidative stress (ROS). A finding which supports a potential role of RAS in prevention and treatment of NAFLD. This hypothesized role may be explained by increased hepatic oxidative stress due to increased RAS activity causes NAFLD to occur and progress (Yongzhong *et al.*, 2008). The RAS reportedly plays an important role in insulin resistance, and suppression of AT-II ameliorates insulin resistance (Yoshiji *et al.*, 2009). Comes with this postulation the positive correlation between AT₁ R mRNA and fasting insulin and HOMA index of insulin resistance which was detected in NAFLD patients of our study.

As many previous reports showing that AG II plays a major role in liver fibrogenesis (Yoshiji *et al.*, 2009), a further step using RAS blockade either by ACE inhibition or AT₁ receptor blockade preserves liver biochemical parameters together with a substantial

reduction in the liver injury. It is well known that both IL-6 and TNF- α are inflammatory cytokines that play a crucial role in the regulation of inflammatory responses. On this basis, the anti-inflammatory effects of anti-RAS therapy may be associated with a reduction in the local expression of these cytokines (Muñoz *et al.*, 2006). Consequently, blockade of RAS could be a new approach to prevent or to treat patients with NASH (Toblli *et al.*, 2007).

In spite what is mentioned by the previous studies that AG II induces hepatic inflammation suggesting an association between AG II signalling and hepatic inflammation, in the current study no correlation was found between serum levels of AG-II and AT₁ R mRNA and AST, ALT serum levels. These results could be explained as mentioned before that normal serum aminotransferases or with intermittent elevations on repeated studies, do not exclude ongoing NASH or advanced stages of disease (Mofrad *et al.*, 2003), and that serum aminotransferases are markers of liver injury despite their relative lack of sensitivity and specificity (Promrat *et al.*, 2010).

Angiotensin affects insulin signaling, and promotes inflammation, fibrosis and endothelial dysfunction. Suppression of AT-II by the clinically used angiotensin-converting enzyme inhibitor (ACE-I) and AT-II type I receptor blocker significantly attenuated the liver fibrosis development along with inhibition of the activated hepatic stellate cells (HSC) (Georgescu *et al.*, 2009 & Yoshiji *et al.*, 2009).

Angiotensin-II type I receptor blocker and iron chelator reportedly exert suppressive effects on non-alcoholic steatohepatitis (NASH) progression, including liver fibrosis and hepatocarcinogenesis. This combination treatment exerted a stronger inhibitory effect in comparison with treatment with a single agent. These inhibitory effects occurred almost concurrently with the suppression of oxidative stress, neovascularization, and HSC activation. The dual inhibition by combined treatment of AT₁ R blocker and iron chelator attenuated the progression of NASH. Since both agents are widely used in clinical practice, this combination therapy may represent a potential new strategy against NASH in the near future (A positive correlation between AT₁ R mRNA and fasting insulin and HOMA index of insulin resistance in NAFLD patients was detected (Kaoru *et al.*, 2008).

Conclusion: serum levels of AT₁ and AT₁ receptors were found to be progressively elevated in NAFLD patients starting from simple steatosis up to NASH reaching to liver cirrhosis. These postulations suggest a new role of AT₁ receptor as a diagnostic and prognostic reliable serum marker for NAFLD. More controlled randomized trials for treatment of NAFLD using new drugs should be done depending on the serum levels of AT₁ and AT₁ receptors as a diagnostic

and prognostic serum markers of NAFLD from steatosis cirrhosis.

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