

Age is a Negative, and Visceral Fat Accumulation is a Positive, Contributor to Hepatic Steatosis, Regardless of the Fibrosis Progression in Non-alcoholic Fatty Liver Disease

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contributor. Fat is distributed less in the liver, relative to the visceral compartment, with increasing age.

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ABSTRACT

AIM: Because aging affects fat distribution, we evaluated age-related changes in the deposition of fat in the liver, compared to the visceral and intramuscular fat compartments, in non-alcoholic fatty liver disease (NAFLD). A “burn-out” phenomenon of hepatic steatosis is associated with progression of fibrosis in NAFLD. We clarified the effect of progression of fibrosis on steatosis.

METHODS: Sixty patients clinically diagnosed as NAFLD, of whom 28 were confirmed histologically, and 26 normal subjects were examined. Platelet counts and serum concentrations of alanine aminotransferase (ALT), ferritin and hyaluronic acid (HA) were measured. Hepatic steatosis, visceral fat area (VFA), subcutaneous fat area (SFA) and the multifidus muscle/subcutaneous fat attenuation (MM/F) ratio were evaluated by computed tomography. The ratio of hepatic fat deposition to VFA, defined as the liver-viscera (LV) index, quantified the ratio of hepatic steatosis to visceral adiposity.

RESULTS: In NAFLD patients, univariate analysis showed the degree of steatosis was correlated positively with VFA, SFA and ALT, and negatively with age, MM/F ratio and HA. Age and VFA retained significance in multivariate analysis. Age was negatively correlated with the LV index. Age was negatively correlated with steatosis and the LV index in 17 patients with fibrosis stage 0 or 1 but not in normal subjects.

CONCLUSION: Age is a negative, and visceral fat accumulation is a positive, independent contributor to steatosis. Hepatic fibrosis is negatively correlated with steatosis but not an independent

INTRODUCTION

An important initial process in the pathogenesis of metabolic syndrome is the development of insulin resistance, induced by the secretion of various adipocytokines from accumulated visceral fat^[1]. These cytokines can cause diabetes, hyperlipidemia, hypertension and obesity. In the liver, metabolic syndrome manifests as non-alcoholic fatty liver disease (NAFLD), a wide spectrum of pathologic conditions ranging from simple steatosis to steatosis with necroinflammation or fibrosis, the condition termed non-alcoholic steatohepatitis (NASH)^[2].

Triglycerides are stored in three forms: as subcutaneous fat, visceral fat and ectopic fat, the fat accumulated in various organs outside the adipose tissues, including the liver and the skeletal muscles^[3]. The liver is unique as a site of ectopic fat deposition because triglycerides stored as visceral fat are transported in the form of free fatty acids directly into this large organ through the portal venous system. Because of this close anatomical connection, hepatic steatosis is considered to be the result of excessive accumulation of visceral fat, which is regarded as a first hit in the pathogenesis of NASH^[4]. However, there are conflicting reports of the adipometric analysis of this association; some studies have suggested a quantitative relationship between visceral fat and hepatic fat accumulation^[5,6], whilst others have reported excessive hepatic fat accumulation in obese individuals, regardless of visceral fat,^[7,8] or no correlation between hepatic steatosis and the visceral fat volume

adjusted for body surface area^[9].

In one report, intrahepatic triglycerides were shown to be increased in elderly healthy volunteers^[10], whereas in another report there was no significant correlation between aging and hepatic steatosis in healthy, nonobese volunteers^[11]. With respect to NAFLD, there are conflicting reports on the relationship between hepatic steatosis and age. Some researchers have shown an age-dependent increase in intramuscular fat but a lack of this change in hepatic fat content^[12] but others have shown that the distribution of NAFLD peaks in middle-age and declines with more advanced age, suggesting the disappearance of liver fat with aging^[13,14].

Another issue is the association of hepatic steatosis with the stage of fibrosis. It has been reported that the deposition of triglycerides in hepatocytes decreases with the progression of fibrosis in NASH, defined as burned-out NASH^[15,16]. Much attention is paid to this phenomenon because it is thought to be the major cause of cryptogenic cirrhosis. Because the progression of fibrosis in NASH usually is gradual, older patients may have a greater likelihood of advanced fibrosis. It should be determined whether reduction of steatosis really is associated with the progression of fibrosis or whether it is simply an age-dependent process throughout the long-term clinical course of NAFLD.

The first aim of this study is to clarify the age-related change in the degree of hepatic steatosis and in the distribution of triglycerides in the visceral and ectopic fat compartments in NAFLD. Furthermore, we elucidated whether the progression of fibrosis really is associated with hepatic steatosis.

MATERIALS AND METHODS

Patients

Among the patients who visited the Department of Medicine, Saiseikai Kyoto Hospital between 2006 and 2010 and underwent computed tomography (CT), 60 were clinically diagnosed as NAFLD and recruited retrospectively and consecutively. Of these patients, 27 consented in a writing to undergo liver biopsy and were confirmed histologically as NAFLD. NAFLD was defined using the following criteria^[17,18]: (1) imaging findings of an apparently bright liver on ultrasonography or an apparent decrease of the CT number in the liver or (2) histological features of steatosis (fat droplets in more than 10% of hepatocytes) or (3) a body mass index (BMI) >25 in the past or present and with histological diagnosis of NAFLD at another center and (4) the absence of significant alcohol consumption (daily alcohol consumption <20 g) and (5) no evidence of alternative causes of chronic liver disease, including chronic viral hepatitis, autoimmune hepatitis, hemochromatosis, Wilson's disease, primary biliary cirrhosis and drug-induced liver disease. Informed consent for the use of clinical and histological data for this study was obtained in writing from all patients. Thirteen male and 13 female normal subjects were selected randomly from patients who underwent CT, had normal liver function tests, did not meet the criteria of NAFLD and other chronic liver diseases, and consented to the use of clinical and radiographic data for this study.

Biochemical analysis

Venous blood samples were taken from all patients and platelet counts [PLT ($\times 10^4/\mu\text{L}$)] and the serum concentrations of alanine aminotransferase [ALT (IU/L)], ferritin (ng/mL) and hyaluronic acid [HA (ng/mL)] were measured.

Radiological analysis

The visceral fat area (VFA) and subcutaneous fat area (SFA) in cm^2

were measured at the umbilical level and calculated using a computer software program (Fat Scan; N2 System, Osaka, Japan).

To evaluate the correlation between the amount of hepatic steatosis estimated radiologically and that assessed histologically, CT numbers (in Hounsfield units) were measured in the right lobe of the liver, from which the biopsy specimen was obtained, and at two locations in the spleen. The Lr/Sa ratio was defined as the CT number of the right lobe divided by the average CT number of the spleen. To estimate the average amount of hepatic steatosis, the CT numbers were measured at three locations (right, middle and left lobes) in the liver and at two locations in the spleen and the mean numbers were used to determine the average liver-spleen (La/Sa) ratio.

To quantify intramuscular fat, we assessed the multifidus muscle/fat ratio (MM/F ratio), using the protocol proposed by Kitajima *et al.*^[12]. Specifically, subfascial muscular tissue in the multifidus muscle in an umbilical-level CT cross-sectional image was traced precisely and the CT number and area (cm^2) were determined. CT numbers of regions of interest of 60 mm^2 in the subcutaneous fat, away from major vessels, were measured at five points and the mean numbers were used to determine the MM/F ratio.

Histological analysis

For the patients who underwent liver biopsy, a $5 \mu\text{m}$ -thick tissue section was cut from each paraffin block of the biopsied tissues for HE staining. The stage of fibrosis was scored as 0-4, based on the standards proposed by Brunt *et al.*^[19]. Hepatic steatosis was assessed semi-quantitatively in low- to middle-power fields. We calculated the average hepatic steatosis in all cases by applying the La/Sa ratio to the correlation formula between the Lr/Sa ratio and histologically-assessed hepatic steatosis.

Calculation of the liver-viscera (LV) index

To quantify the hepatic fat deposition relative to visceral fat, the liver-viscera (LV) index was defined as the estimated average hepatic steatosis (%) divided by the VFA (cm^2).

Statistical analysis

Data are expressed as means \pm standard deviations. Backward stepwise linear regression analysis was used to determine the effects of age, gender, VFA, SFA, MM/F ratio, platelet cell counts and the serum concentrations of ALT, ferritin and hyaluronic acid. For this analysis, when the F value was larger than 2.0, the variable was selected. Values of $P < 0.05$ were considered statistically significant. All statistical analyses were performed using Excel 2010 software (Microsoft Japan Co., Tokyo, Japan).

RESULTS

The profiles of the NAFLD patients and normal subjects are summarized in Table 1. In the NAFLD patients who underwent liver biopsy, the degree of hepatic steatosis assessed histologically was negatively correlated with the Lr/Sa ratio (Figure 1-a, $y = -86.24x + 113.03$, $r = -0.903$, $n = 27$, $P < 0.0001$). Using this formula, we estimated the average percentage of hepatic steatosis of all patients using the La/Sa ratio. The estimated values of hepatic steatosis as percentages were used for the following analyses.

Univariate analysis clarified that the degree of hepatic steatosis was correlated positively with VFA ($r = 0.321$, $P < 0.05$) and SFA ($r = 0.349$, $P < 0.01$) and negatively with the age of the patient ($r = -0.509$, $P < 0.0001$), MM/F ratio ($r = -0.426$, $P < 0.01$) and HA ($r = -0.347$, $P < 0.01$) (Table 2).

According to backward stepwise linear regression analysis, age

Table 1 The profiles of NAFLD patients and normal subjects.

	NAFLD patients	Normal subjects
Age	58±16	51±22
Male	32 (53.3%)	13 (50.0%)
VFA (cm ²)	157.9±74.4	82.3±45.5
SFA (cm ²)	203.6±87.8	145.0±62.1
MM/F ratio	-0.240±0.186	-0.683±2.077
ALT (IU/L)	61±54	19±9
PLT (×10 ⁴ /mL)	23.3±7.7	23.0±4.8
Ferritin (ng/mL)	175±147	nd
Hyaluronic acid (ng/mL)	124±179	nd
Hepatic steatosis (%)	40.6±22.3	4.4±3.6

Data are shown as means±standard deviations. The number of male patients is shown with the percentage in parentheses. Hepatic steatosis was estimated by using the formula described in Results section. nd: not done.

Table 2 Univariate analysis for determinants of hepatic steatosis.

	standardized partial regression coefficient	P value
Age	-0.509	<0.0001
Male	0.245	0.059
VFA (cm ²)	0.321	0.012
SFA (cm ²)	0.349	0.006
MM/F ratio	-0.426	0.0007
ALT (IU/L)	0.219	0.093
PLT (×10 ⁴ /mL)	0.218	0.095
Ferritin (ng/mL)	0.091	0.491
Hyaluronic acid (ng/mL)	-0.347	0.007

Table 3 Multivariate analysis for independent determinants of hepatic steatosis (stepwise backward regression).

	standardized partial regression coefficient	F value	P value	VIF
age	-0.58	32.20	<0.0001	1.03
visceral fat area	0.42	16.71	0.0001	1.03
R value	0.65			
R ² value	0.43			
F value	21.23			
P value	<0.0001			

was a significantly negative ($P<0.0001$), and VFA was a positive ($P=0.0001$), contributor to hepatic steatosis but SFA, the MM/F ratio and HA were not significant (Table 3).

The LV index ranged from 0.057 to 0.947 and was negatively correlated with age (Figure 1-b, $r=-0.646$, $n=60$, $P<0.0001$). Of the 27 patients who underwent liver biopsy, 17 showed no hepatic fibrosis (F0) or mild fibrosis (F1). When these 17 patients were assessed, age remained negatively correlated with hepatic steatosis (Figure 1-c, $r=-0.498$, $n=17$, $P<0.05$) and LV index (Figure 1-d, $r=-0.750$, $n=17$, $P=0.005$). However, there was no negative correlation between hepatic steatosis and the age of the normal subjects (Figure 2-a). In addition, the LV index ranged from 0 to 0.23 and did not show a negative correlation with age (Figure 2-b).

With respect to the relationship between age and subcutaneous or visceral fat in NAFLD patients, SFA showed a significant age-dependent decrease (Figure 3-a), while visceral fat did not show this trend (Figure 3-b).

DISCUSSION

It has been reported that, during a three year observation period, 62% of patients showed histological changes from simple steatosis to borderline NASH or NASH, and that 27% of NASH patients showed progression of fibrosis^[20]. Therefore, elderly patients with NAFLD might have a greater chance of developing advanced fibrosis. In addition, there is a general observation that the progression of fibrosis is associated with a reduction of steatosis, which is known as a burn-out phenomenon^[15,16]. Multivariate analysis was carried

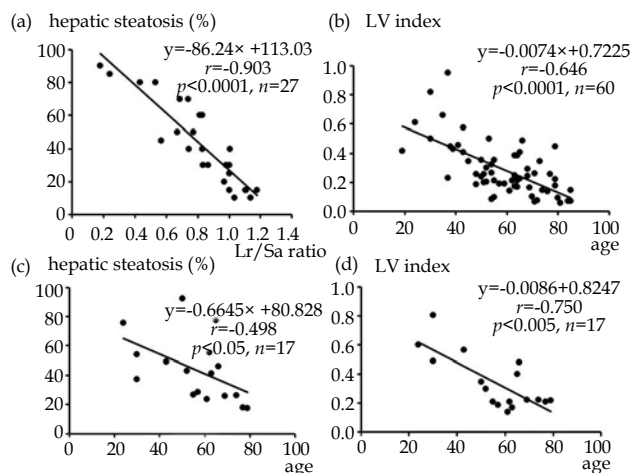


Figure 1 (a) The degree of hepatic steatosis was negatively correlated with the Lr/Sa ratio in the patients who underwent liver biopsy. The Lr/Sa ratio was defined as the CT number of the right lobe of the liver divided by the average CT number of the spleen. (b) The liver-viscera (LV) index and age were negatively correlated. The LV index was defined as the ratio of the amount of hepatic steatosis (%) to the visceral fat area (cm²), which was proposed to quantify the tendency for hepatic fat deposition relative to visceral fat. By assessing the patients whose stage of fibrosis was histologically diagnosed as F0 or F1, age was negatively correlated with (c) hepatic steatosis and (d) the LV index.

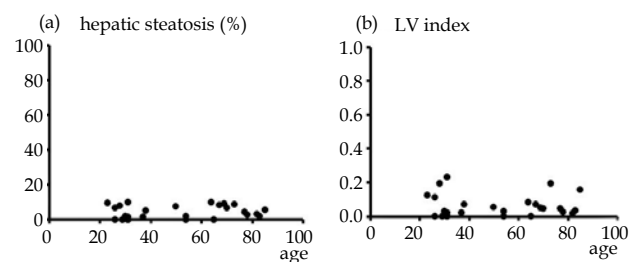


Figure 2 Normal subjects did not show a negative correlation between hepatic steatosis and age (a). In addition, the LV index ranged from 0 to 0.23 and did not show a negative correlation with age (b).

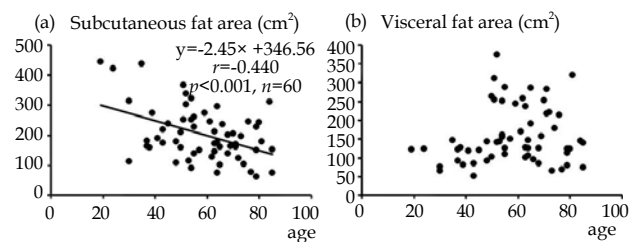


Figure 3 The subcutaneous fat area showed a significant age-dependent decrease (a), while the visceral fat area did not show this trend (b).

out to clarify whether a reduction of the amount of steatosis really is induced by the progression of fibrosis or whether it is simply an age-dependent process during the clinical course of NAFLD. We showed that the progression of fibrosis was not a significantly negative contributor to hepatic steatosis. This finding is supported by an age-dependent reduction of steatosis in the cases with no or mild fibrosis.

Our study showed that visceral fat accumulation is a positive, and age is a negative, contributor to hepatic steatosis in NAFLD. This is consistent with previous reports that the severity of hepatic steatosis is associated with the degree of visceral fat^[5,6] but, in addition, the degree of steatosis decreases in an age-dependent manner.

We suggest three possible reasons for this age-dependent decrease in steatosis: 1) a decrease in the portal influx of free fatty acids due to decreased visceral fat in the elderly, 2) an age-dependent increase in the mortality of patients with severe steatosis and,

consequently, selective survival of elderly NAFLD patients with mild steatosis and 3) an age-dependent change in the kinetics of free fatty acids, leading to increased visceral adiposity relative to hepatic steatosis. Our study showed a significant age-dependent decrease in subcutaneous fat, while visceral fat, which is directly associated with hepatic steatosis via the portal vein, did not show an age-dependent decrease. Therefore, our results did not support the first hypothesis. If the second hypothesis is true and hepatic steatosis is correlated with visceral fat regardless of age, only those NAFLD patients who lose visceral fat or maintain a low visceral fat volume can survive to a more advanced age. However, again the absence of an age-dependent decrease in visceral fat did not support this hypothesis. The age-dependent decrease in the LV index supports the third hypothesis strongly. Moreover, this age-dependent decrease in the LV index was specifically observed in NAFLD patients and not in normal subjects.

We showed an age-dependent decrease in the LV index and this trend is also seen in the cases with no or mild hepatic fibrosis. As discussed above, this result suggests that triglycerides are distributed less in the liver relative to the visceral compartment during the aging process, regardless of the progression of fibrosis. Therefore, for the assessment of NAFLD as a hepatic manifestation of metabolic syndrome in the elderly, we should not underestimate the degree of hepatic steatosis and should consider the effect of aging and visceral fat accumulation. To confirm this point, a follow-up study is needed with repeated liver biopsies to determine the relationship between hepatic fibrosis and steatosis in a cohort of NAFLD patients.

Chronic exposure to lipid in the hepatocytes might cause hepatocellular injury and possibly increase the risk of hepatocarcinogenesis through direct lipotoxicity, oxidative stress and lipid peroxidation^[21,22]. Moreover, in our previous studies, we suggested that chronic lipid peroxidation in NAFLD can accelerate hepatocellular aging^[23] and this may constitute a risk of hepatocarcinogenesis, based on chronic liver injury^[24]. Consequently, even if hepatic steatosis is mild in elderly patients, we should consider the age-dependent decrease in steatosis during the clinical course of NAFLD and the cumulative effect of long-term steatosis on hepatocarcinogenesis^[26,27].

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