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REVIEW

Nonalcoholic fatty liver disease and nonalcoholic steatohepatitis in Japan

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The authors do not have any conflicts of interest to disclose.

Abstract

During the past 20 to 30 years, the frequency of patients presenting with nonalcoholic fatty liver diseases (NAFLD) has increased gradually in Japan in proportion to the increase in the population with life-style related diseases. We describe here the current status of the clinical and basic aspects of research into NAFLD in Japan.

The increase in the incidence of life-style-related diseases has resulted in an increase in NAFLD throughout the past 20 to 30 years. The rate of obesity in the population is not high compared to western countries but the incidence of NAFLD is similar to those countries. In 2008 we started a nationwide study of NAFLD which has been supported by the Ministry of Labor and Welfare Japan. In this project, we planned to investigate the epidemiology, genetic backgrounds and biochemical markers, and liver injury in patients with diabetes mellitus (DM) and hepatocellular carcinoma in NASH, and treatment of NASH. Approximately 20 to 25% of DM patients showed NAFLD in which the prevalence of NASH might be more than 30 to 40%. Fortunately, we have been able to obtain very interesting results from our group studies, including single necleotide polymorphisms (SNPs) which will be published in the near future.

Introduction

In 1980, Ludwig *et al.* proposed a new disease concept called nonalcoholic steatohepatitis (NASH), a condition which may progress to cirrhosis and hepatocellular carcinoma (HCC). In Japan, much attention has been paid over the past few decades to patients infected with hepatitis B virus (HBV) and hepatitis C virus (HCV), because the rates of carriage of these viruses are high and most cases of cirrhosis and hepatocellular carcinoma (HCC) in Japan are associated with persistent HBV and HCV infection. In recent years, however, with the westernization of the Japanese lifestyle, public interest in lifestyle-related diseases has increased rapidly metabolic syndrome has attracted attention for its association with underlying insulin resistance, and the risk of dyslipidemia, and hypertension even in non-obese Japanese. In 2007, the Japan Society of Diabetes Mellitus reported that, among the causes of death for 18 385 individuals with diabetes, liver cancer was the leading cause (8.6%), while death from liver cirrhosis also was very common (4.7%). Altogether, 13.3% of death among diabetes patients were attributable to liver disease (Fig. 1);[1] however, the prevalence of hepatitis virus infections and heavy alcohol drinking were not analyzed in that paper.

Seventy to seventy-five percent of HCC in Japan is associated with HCV infection, approximately 15% of patients are positive for hepatitis B surface B antigen (HBsAg), and the remaining 10–15% are so-called non-B non-C HCC. The proportion of non-B non-C HCC increased from 6.8% in 1992 to almost twice that during the subsequent ten years. Total alcohol consumption in Japan has not increased in the past 15 years, the possibility arises that NASH is responsible for this apparent increase in non-B non-C HCC (Fig. 2).

Most Japanese are not obese but nonalcoholic fatty liver disease (NAFLD) is becoming more common. At present, NASH is one of the most important liver diseases in Japan. In 2008, the Japan NASH Study Group (of which Takeshi Okanoue is a member) was founded, supported by the Ministry of Labor and Welfare, Japan. The purpose was to elucidate the epidemiology, pathophysiology, genetic backgrounds and long-term prognosis of NAFLD. Other objectives are to establish biochemical markers for differential diagnosis between simple steatosis (SS) and NASH, and devise treatment guidelines based on the individual pathophysiology of NASH.

Epidemiology

In the last two decades, patients diagnosed with fatty liver by image analysis and with elevated serum alanine aminotransferase (ALT) increased in number in proportion to the increase in

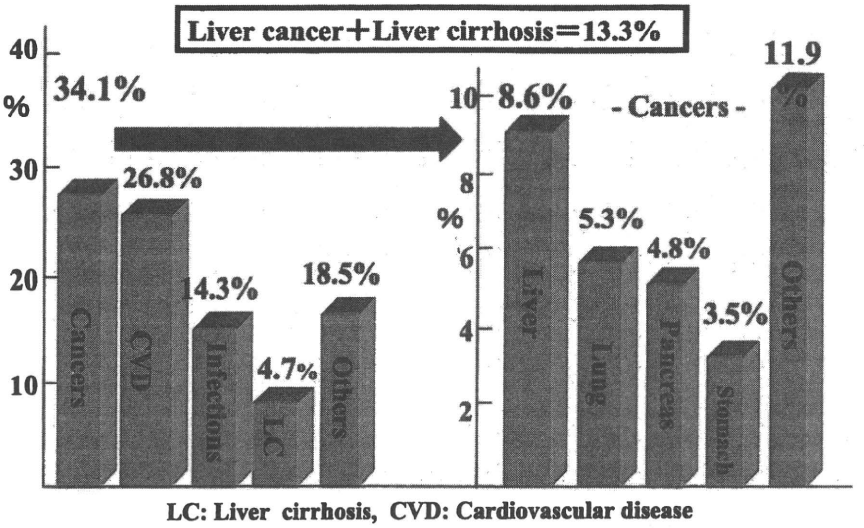

Figure 1 Cause of death in diabetes mellitus patients in Japan. The leading cause of death in 18 385 diabetes mellitus patients, who died from 1991 to 2000, was cancer, the second was cardiovascular diseases, the third was infection and the fourth was liver cirrhosis. Among the cancer deaths, the highest rate was of hepatocellular carcinoma (8.6%; males: 10.5% and females: 5.2%). In the general Japanese population, the primary cause of cancer death is lung cancer, the next is gastric cancer, the third is colon cancer and the fourth is HCC in males.

lifestyle-related diseases, such as obesity, diabetes and dyslipidemia. The Japan Society of Ningen Dock (health check-up organization) reported in 2008[2] that the prevalence of liver dysfunction, including fatty liver, was 31.9% in men and 17.1% in women, based on a study carried out on 1 814 864 adult men and 1 136 903 adult women. The prevalence of obesity, liver dysfunction, and high levels of cholesterol and triglyceride showed no significant differences in distribution with age in men, but the prevalence increased with age in women; for those in their 60s, it reached a high level comparable to that in men. Glucose intolerance and high blood pressure increased with age in both men and women (Fig. 3a/3b). A comparison of annual variations showed increase of all these factors, but the increase was especially marked in the incidence of liver dysfunction, obesity, and hypercholesteremia, and these became prominent in the late 1990s (Fig. 4).

Kojima *et al.* reported that the prevalence of fatty liver detected by medical health checks increased year after year, from 12.6% in 1989 to 30.3% in 1998.[3] According to the report by the Japan Society of Ningen Dock in 2008, 26.2% of subjects who underwent health check-ups showed fatty liver by abdominal ultrasonography.[2]

The majority of fatty liver disease comprises alcoholic fatty liver and NAFLD, including NASH. Tanaka *et al.* reported that approximately 25% of the health check-up examinees had fatty liver.[4] Hamaguchi *et al.* reported that the prevalence of NAFLD was 23.3% in Japanese adults.[5] There is a gender difference in the incidence of NAFLD; men are more likely to develop fatty liver. There is also a gender difference in the age distribution; in men, the incidence of fatty liver is about 25% and remains unchanged from the 30s to the 60s, whereas in women, the prevalence of fatty liver increases gradually with age and, in the 60s and beyond, reaches nearly the same level as in men. According to previous reports, the number of NAFLD patients is estimated to be 10 million (the population in Japan is around 130 million), and, from recent studies around 2% of them are considered to have NASH.

In 2008, a fact-finding survey was conducted on the causes of cirrhosis at the 44th Annual Meeting of the Japan Society of Hepatology; 33 379 cirrhotic patients were enrolled in 58 hospitals, and 2.1% were diagnosed with NASH-induced cirrhosis (Fig. 5). According to that survey, the proportion of NASH cirrhosis is 1.4% in males and 3.4% in females, and there is a significant gender difference ($P < 0.005$). In that study, obese subjects were few and, at that time, the concept of NASH was not yet commonly accepted by many Japanese doctors. Furthermore, many cases of advanced stage NASH show no fatty deposit, so-called "burn-out NASH", resulting in the diagnosis of cryptogenic liver cirrhosis. Therefore, the actual incidence of NASH-related cirrhosis might be higher than was reported.

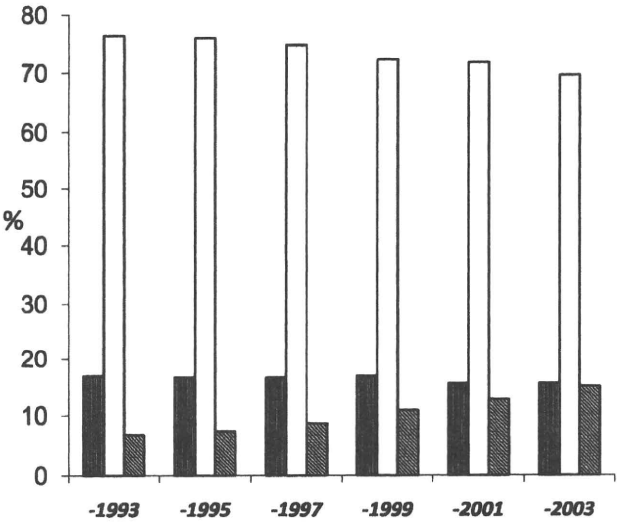

Figure 2 Change of the etiology of hepatocellular carcinoma (HCC) in Japan from 1993 to 2003. Among the causes of HCC, the HCV infection rate was decreasing gradually and HBV infection showed no significant change; however, the prevalence of non B, non C HCC has doubled over the past 10 years. HBV, hepatitis B virus; HCV, hepatitis C virus; NBNC, non B, non C. ▥, HBV; □, HCV; ▧, NBNC.

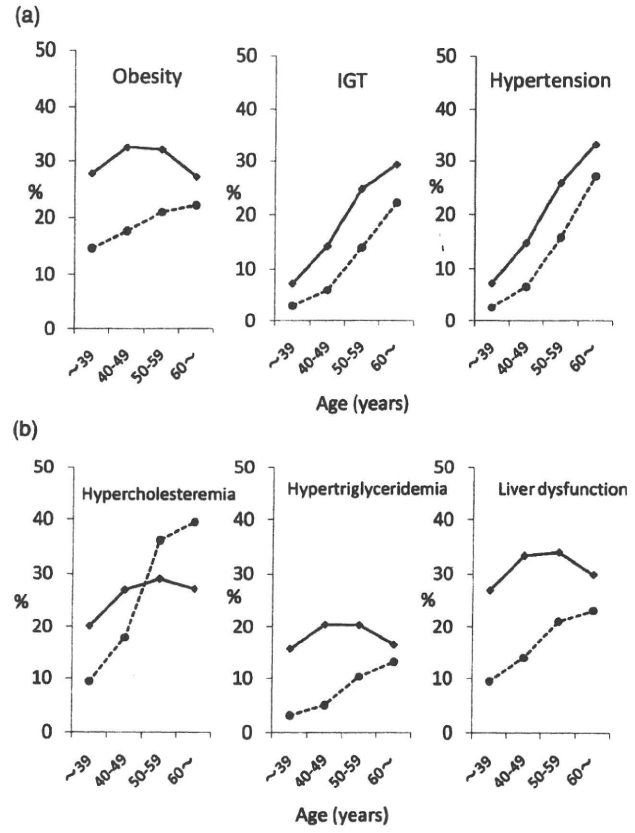

Figure 3a/3b The complication rates of life-style related diseases in the general population receiving health check-ups in 2008. In women, the prevalence of obesity, hypercholesteremia, hypertriglyceridemia, and liver dysfunction increased with age and, for women in their 60s, these reached a high level comparable to those in men. Glucose intolerance and high blood pressure increased with age in both sexes. IGT, impaired glucose tolerance. —◆—, Male; --●--, Female.

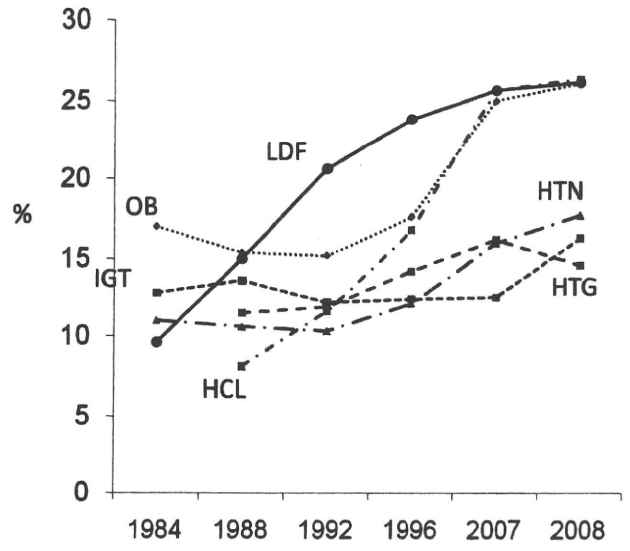

Figure 4 Annual variation of life-style related diseases in the general population. During the past 20 years all diseases significantly increased year by year. ···◆···, Obesity(OB); --■--, Impaired Glucose Tolerance (IGT); —▲—, Hypertension(HTN); —■—, Hypercholesteremia(HCL); —■—, Hypertriglyceridemia(HTG); —●—, Liver dysfunction(LDF).

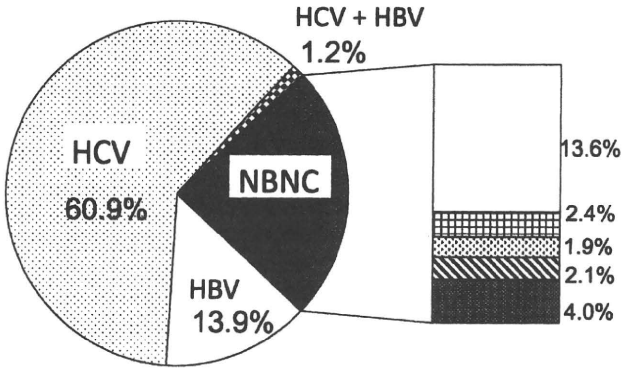

Figure 5 Etiology of liver cirrhosis in Japan. A nationwide survey was carried out in 2008 at the 44th Annual Meeting of the Japan Society of Hepatology. The non B, non C group occupied 24%, of which 2.1% were diagnosed as definite NASH. HBV, hepatitis B virus; HCV, hepatitis C virus; NBNC, non B, non C; PBC, primary biliary cirrhosis; AIH, autoimmune hepatitis; NASH, nonalcoholic steatohepatitis. □, Alcohol; ▦, PBC; ▨, AIH; ▧, NASH; ■, Others.

Metabolic syndrome

Patients with metabolic syndrome are increasing in number in Japan (Figs. 4,6). Visceral fat accumulation and insulin resistance are usual in these patients. The enhanced insulin resistance caused by the excessive accumulation of body fat (especially visceral fat) is considered to be important in the pathogenesis of fatty liver.

The diagnostic criteria for metabolic syndrome established by the Japanese Society of Internal Medicine are as follows:[6] an umbilical abdominal circumference (men: 85 cm or more; women: 90 cm or more) which reflects visceral fat accumulation (a visceral fat area of 100 cm^2 or more), and any two of the following four criteria: (i) elevated serum triglyceride level; (ii) reduced HDL cholesterol; (iii) elevated blood pressure; and (iv) elevated fasting plasma glucose. According to the National Health and Nutrition Examination Survey conducted in Japan in 2008, the prevalence of patients afflicted by metabolic syndrome was 25.3% among men and 10.6% among women, whereas patients with pre-metabolic syndrome (patients with an abdominal circumference of ≥85 cm in men and 90 cm in women, and who fulfill one other criterion) accounted for 21.9% of the men and 8.3% of the women. Therefore, approximately half of Japanese men and about 20% of Japanese women might have metabolic syndrome or be predisposed to metabolic syndrome.[7]

The criteria for metabolic syndrome are useful for the screening of NAFLD. The previous report by Ishibashi *et al.* stated that abdominal circumference was well correlated with NAFLD in men, but not in women.[8] Waist circumference has been reported to be smaller in men than women and there has been considerable debate regarding whether this criterion is appropriate or not.[9] There is the possibility that the amount of visceral fat might be underestimated and that the estimate may detect fewer than the actual number of

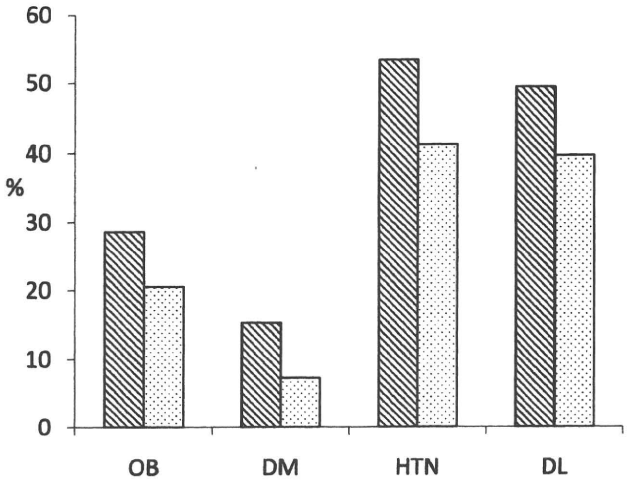

Figure 6 The complication rate of lifestyle-related diseases in Japan in 2007 to 2008. OB, obesity; DM, diabetes mellitus; HTN, hypertension; DL, dyslipidemia. ▨, Male; ▩, Female.

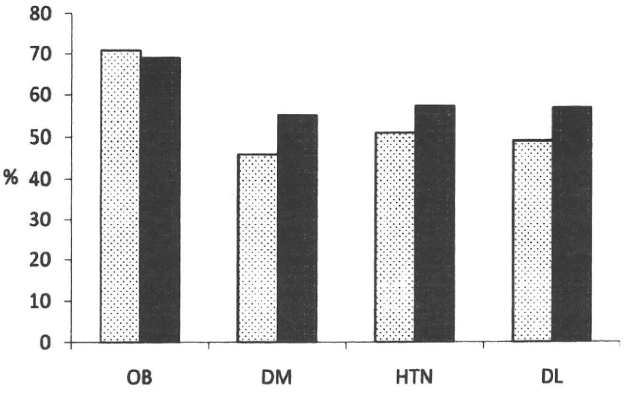

Figure 7 The complication rates of life-style related diseases among 283 NAFLD patients subjected to liver biopsy at Saiseikai Suita Hosptital from 2008 to 2010. SS, simple steatosis; NASH, nonalcoholic steatohepatitis; OB, obesity; DM, diabetes mellitus; HTN, hypertension; DL, dyslipidemia. ▨, SS n=96; ▩, NASH n=187.

women with NAFLD. In women, caution is required when the abdominal circumference is used instead of the visceral fat area.

Epidemiologically, it is clear that the risk of cardiovascular diseases is increased markedly in people with multiple risk factors for life-style related diseases. In addition, Hamaguchi *et al.* showed that NAFLD patients were at high risk of cardiovascular diseases and NAFLD, but not metabolic syndrome (MS), and showed a statistically significant correlation with cardiovascular disease in a multivariate model (OR = 4.12; 95% CI, 1.58–10.75).[10] Having multiple life-style related diseases is considered to be a risk factor for developing NASH.

The incidence of complications of life-style related diseases among 283 biopsy-proven NAFLD patients in Saiseikai Suita Hospital from April 2007 to March 2010 was high. However, no significant difference was seen in the incidence of individual factors between 187 NASH patients and 96 SS patients; obesity: NASH 69.0% *versus* SS 70.8%, diabetes: NASH 55.1% *versus* SS 45.8%, hypertension: NASH 57.2% *versus* SS 51.0%, and dyslipidemia: NASH 56.7% *versus* SS 49.0% (Fig. 7). Most NASH-cirrhotic patients have been reported to be obese with an average BMI of 27.6 ± 4.5 kg/m^2; the prevalence of the complications of diabetes and hypertension were 66.6% and 50.2%, respectively.[11]

Hamaguchi *et al.* also showed that the presence of metabolic syndrome was related to the new onset of NAFLD, with a 4-fold increase in men and an 11-fold increase in women compared to non-metabolic syndrome subjects.[5] Mitsumune *et al.* reported that obesity (6.3 fold), dyslipidemia (2.4 fold), hyperglycemia (1.8 fold), and hypertension (1.4 fold) all increased the odds of having NAFLD.

Obesity

The National Health and Nutrition Examination Survey[7] conducted in 2008 showed that the proportion of obese subjects with a BMI of 25 kg/m^2 or more was 28.6% of men and 20.6% of women. Classified by age, this category accounted for over 29% of men aged 30–69 years, whereas, for women, the obesity rate increased with age: 11.8% in their 30s, 18.0% in their 40s, 21.1% in their 50s, 24.4% in their 60s, and 26.8% in their 70s. The prevalence of fatty liver according to age shows the same tendency as obesity. Fatty liver was noted in only 2.7% of non-obese subjects with a BMI less than 23 kg/m^2 and was 10.5% in those with a BMI ≥ 23 but < 25 kg/m^2, 34.6% in those with a BMI ≥ 25 but < 30 kg/m^2, and 77.6% in highly obese subjects (BMI > 30 kg/m^2).[12] Kojima *et al.* carried out an analysis of 39 151 people who underwent a health check-up over a period of 12 years from 1989 to 2000. They reported that the grade of obesity correlated with the development of fatty liver. The prevalence was 12.8% in non-obese subjects (BMI < 25 kg/m^2), 51.4% in subjects with BMI ≥ 25 but < 30 kg/m^2, and 80.4% in highly obese subjects (BMI > 30 kg/m^2). In subjects with BMI values of 25 kg/m^2, the odds ratio for fatty liver was 6.3 compared with of non-obese subjects.[3]

Hamaguchi *et al.* reported that, in a group receiving a health check-up, 18% showed NAFLD at the time of the initial health check-up, and 10% (14% of men and 5% of women) developed NAFLD during the follow-up period of an average 414-days.[5] Patients with newly developed NAFLD showed weight gain of 1.7 ± 1.7 kg for men and 1.3 ± 1.4 kg for women. Logistic regression analysis showed that weight gain was an independent risk factor for the newly developed NAFLD, with an OR = 1.51 (95% CI, 1.40–1.63) for men and OR = 1.62 (95% CI, 1.39–1.89) for women.

Diabetes/impaired glucose tolerance

In the National Health and Nutrition Examination Survey conducted in Japan in 2007, 8 900 000 people were strongly suspected of diabetes (HbA1c ≥ 6.1%, or currently under treatment); the number of people with an undeniable possibility of diabetes (HbA1c ≥ 5.6% but < 6.1%) was 13 200 000, in total, the number of people possibly with diabetes was 22 100 000, which was 1.6-fold higher than 10 years earlier.[13] Kojima *et al.* reported that the prevalence of fatty liver was 18.6% in subjects with normal glucose metabolism (FBS < 110 mg/dL), 43.7% in borderline subjects (FBS ≥ 110 but < 126 mg/dL), and 53.3% in diabetic

patients (FBS ≥ 126 mg/dL). FBS ≥ 110 mg/dL was an independent risk factor for fatty liver (OR = 3.1).[3] Likewise, Jimba *et al.* reported that the overall prevalence of NAFLD was 29% among 1950 Japanese people receiving a health check-up; the prevalence was 27% in the normal glucose metabolism group (FBG < 6.1 mmol/L) and rose to 43% for the borderline type (FPG ≥ 6.1 but < 7.0 mmol/L) and 62% for the diabetic type (FBG ≥ 7.0 mmol/L or a medical history of diabetes). In addition, the incidence of complications with abnormal glucose metabolism (borderline type and diabetic type) was 19.1% in NAFLD patients, which was higher than the 5.6% of patients without NAFLD ($P < 0.001$).[14]

Miyaaki *et al.* examined the relationship between the stage of hepatic fibrosis and the prevalence of diabetes in Japanese patients. In the mild fibrosis group, 42% were complicated with diabetes, whereas in the severe fibrosis (bridging fibrosis or cirrhosis) group, the prevalence was as high as 71% ($P = 0.020$). Diabetes might be a factor responsible for the development of hepatic fibrosis in NAFLD.[15] Shiga *et al.* performed a 75-g oral glucose tolerance test on the participants of a health check-up. They found that blood glucose levels at one and two hours after glucose load showed a closer relationship with NAFLD than the fasting blood glucose level. Therefore, they stated the importance of the evaluation of impaired glucose tolerance (IGT) in detecting NAFLD.[16]

Hypertension

According to the criteria of the Japanese Society of Hypertension, systolic blood pressure under 130 mm Hg/diastolic blood pressure under 85 mm Hg is normal, pressure higher than 140/90 mm Hg is diagnosed as hypertension, and pressure 130–139/85–89 mm Hg is high-normal blood pressure.

In the National Health and Nutrition Examination Survey conducted in 2007, the prevalence of subjects with hypertension (including 24.0% currently under treatment) was 46.2%, the prevalence of high-normal blood pressure was 13.8%, and the normal pressure group was 40.0%.

Hypertension is frequently seen in NASH/NAFLD patients, but there are no reports describing the prevalence of NAFLD among hypertensive patients in Japan. Reports from overseas stated that systolic and diastolic blood pressures were correlated with liver fat content, and that the risk of developing NAFLD was 2-fold higher in patients with systolic hypertension, and 1.7-fold higher in patients with diastolic hypertension.[17,18] In addition, Donati *et al.* reported that even in non-obese, non-diabetic high blood pressure patients, the prevalence of fatty liver was three times higher than in healthy individuals. Further such patients showed high levels of HOMA-IR, indicating insulin resistance.[19] The pathogenesis of hypertension is influenced by various factors, such as salt intake, and also is associated with insulin resistance. It is important to know that even non-obese high blood pressure patients with no other lifestyle-related diseases are likely to develop NAFLD if they have insulin resistance. In Japan, large-scale studies on hypertension and NAFLD are currently underway, including among subjects with chronic kidney disease (CKD).

Recently, we reported the prevalence of CKD in 174 NAFLD patients. The prevalence of CKD was significantly higher in NASH patients (19 of 92; 21%) than SS patients (5 of 82; 6%), and associated with a higher body mass index and the presence of hypertension.[20]

Dyslipidemia

Dyslipidemia is a generic term describing a clinical condition in which the levels of cholesterol esters or triglycerides increase in the blood: high levels of triglycerides (150 mg/dL or higher) and LDL cholesterol (140 mg/dL or higher), with decreased levels of HDL cholesterol (less than 40 mg/dL) are each risk factors for other diseases. In the National Health and Nutrition Examination Survey conducted in 2007, the percentage of subjects suspected of dyslipidemia (including 9.7% currently under treatment) was 44.1%, and that of normal subjects was 55.9%.

Dyslipidemia in NAFLD often involves hypertriglyceridemia and decreased blood levels of HDL cholesterol. This is due to the insufficient effects of lipoprotein lipase (LPL), which leads to a decreased metabolism of triglyceride-rich lipoproteins into HDL cholesterol. In addition, there is also an increased synthesis of very-low density lipoprotein (VLDL).

Pediatric NASH/NAFLD

The incidence of pediatric NAFLD in Japan is increasing in proportion to the increase in the prevalence of childhood obesity. In a previous study conducted on children aged 6–15 years, Tominaga *et al.* reported that the prevalence of NAFLD was 3.4% in children aged 6–10 years and 5.2% in those aged 11–15 years.[21] In addition, the prevalence of NAFLD in children who met the diagnostic criteria for pediatric metabolic syndrome was 40.0% in those diagnosed with pre-metabolic syndrome, and 76.8% in those who fulfilled the criteria for metabolic syndrome. Tsuruta *et al.* also reported that, in a similar study conducted in 2007 on 288 junior high school students (13–15 years old), 5.9% were obese, the prevalence of NAFLD was 4.5%, and obesity and ALT levels of 30 IU/L or higher were independent risk factors for NAFLD in children.[22] In addition, the prevalence of complications with obesity (degree of obesity ((body weight—standard body weight)/standard body weight × 100) ≥ 20%) showed a higher frequency in NAFLD patients (58.3%) than those without NAFLD (5.7%, $P < 0.001$).

Pathophysiology and genetic background

Racial differences might affect the onset and pathophysiology of NAFLD. Weston *et al.* reported that the prevalence of obesity, dyslipidemia, and diabetes in NAFLD was similar among racial and ethnic groups, except that body mass index was lower in Asians compared to Whites, Hispanics, and African Americans ($P < 0.001$). Compared with the base population, Hispanics with NAFLD were overrepresented and Whites were underrepresented.[23] In addition, Mohanty *et al.* reported that African Americans showed a lower degree of steatosis than Whites. In contrast, it has been considered that Asians showed higher grades of ballooning and Hispanics showed higher grades of Mallory-Denk bodies, than Whites and other ethnicities combined.[24] These findings indicate the importance of racial differences for the development and progression of NAFLD.

There are many reports concerning the genetic predisposition to the development of NASH and NAFLD, and most of them refer to functional genetic polymorphisms. Tumor necrosis factor-alpha (TNF-α) is known to be produced by adipocytes in visceral fat and Kupffer cells in the liver. It inhibits insulin receptor substrate-1 (IRS-1) of target cells, and insulin receptor kinase in skeletal muscles and adipocytes, thereby cause or exacerbating insulin resistance. Increased blood levels of TNF-α have been reported in NAFLD and NASH patients whose BMI and insulin resistance were matched, thereby suggesting a relationship between increased levels of TNF-α and the development of NAFLD or the progression of NASH.[25] It has been reported in Japanese subjects that functional genetic polymorphisms of TNF-α are present at positions T-1031C and C-856A in the promoter region, and these were more frequent in patients with NASH, potentially mediating progression of the disease.[26]

Adiponectin has an insulin sensitivity effect by opposing fatty acid accumulation which causes insulin-resistance, an anti-atheriosclerotic effect, and an anti-inflammatory effect. Therefore, hypoadiponectinemia associated with obesity has been considered to play a crucial role in the development of metabolic syndromes. In addition, the serum adiponectin level has been shown to be lower in NASH patients than in healthy groups and simple fatty liver groups.[27] The presence of functional polymorphisms G45T and G276T is the adiponectin gene have been reported to be associated with diabetes.[28,29] Regarding Japanese subjects with NASH, it has been reported that the G/G homo-allele at the 45th base of the exon of adiponectin was more frequent in NASH with advanced fibrosis than that in mild fibrosis, and that insulin resistance was distinctly more prominent.[30] Yoneda *et al.* reported that genetic variations in angiotensin II type1 receptor (ATGR1) may influence the risk of NAFLD and liver fibrosis in NAFLD.[31]

Functional polymorphisms in the β3-adrenergic receptor gene, microsomal triglyceride transfer protein (MTP), phosphatidylethanolamine *N*-methyltransferase (PEMT), interleukin-1β (IL-1β), and manganese superoxide dismutase (MnSOD) have also been reported in Japan.[32–34] MTP and PEMT are important factors for the metabolism in triglyceride.

In addition, sex hormones are involved in gender differences in the incidence of NAFLD, and in postmenopausal women the decreased level of estrogen results in the accumulation of visceral fat and insulin resistance.[35] This may explain why postmenopausal women appear to be at a higher risk for the development of NAFLD.

Pathological diagnosis

NAFLD can be diagnosed in patients from whom hepatitis virus infection, alcoholic liver disease and autoimmune hepatitis have been excluded when over 5% of hepatocytes contain fatty droplets.

NAFLD encompasses a histological spectrum ranging from simple steatosis (SS) to NASH, the latter showing hepatocyte degeneration (ballooning hepatocyte), necrosis, inflammation and fibrosis.[36]

Recently, Matteoni *et al.* categorized NAFLD into four types; type 1 (simple fatty liver), type 2 (steatohepatitis), type 3 (steatonecrosis) and type 4 (steatonecrosis + Mallory-Denk body (MDB) or fibrosis). They proposed that types 1 and 2 should be categorized as SS, and types 3 and 4 as NASH, according to the prognosis based on their follow-up study.[37] Actually we sometimes encounter difficulty in the differential diagnosis between type 2 and type 3 NAFLD, and between type 3 and type 4 NAFLD. This is because the criteria of ballooning hepatocytes and presence of pericentral and pericelluar fibrosis are unclear when these morphological changes are very mild.

In 2005, Kleiner *et al.* proposed a new scoring system, the so-called NAFLD activity score (NAS), according to the extent of the three features: steatosis, hepatocellular ballooning and lobular inflammation. By the NAS, NASH is defined as having a score of five or more.[38] This score is based on disease activity and the evaluation of fibrosis is excluded; this might be not suitable for the diagnosis of advanced staged NASH. Brunt and others proposed a grading and staging system according to the grade of inflammation and fibrosis,[39] and this method is widely accepted in Japan.

Ten to 30% of NASH cases have the potential to develop to cirrhosis within 10 years. However, much attention should be paid to so-called "burn-out NASH", in which fatty droplets have disappeared during the progression of hepatic fibrosis, resulting in difficulty making a precise diagnosis of NASH. In such a case, we must make an effort to collect the detailed background and previous patient history. This difficulty could lead to an underestimation of the prevalence of NASH-cirrhosis the Mallory-Denk bodies (MDB) are one of the morphological hallmarks for the diagnosis of type 4 NAFLD: they are an abnormal flocculent producter in degenerated hepatocytes and are comprised of intermediate filaments (IF).[40] We consider that the frequency of MDB in Japanese NASH is lower than in western countries.

Biochemical markers: non-invasive score assessment

Liver histology is the gold standard for the diagnosis of NASH; however, it is invasive and there is a risk of sampling errors in some cases. It has been anticipated that it should be possible to use serum biochemical markers to diagnose NASH, and various parameters reflecting oxidative stress, insulin resistance, inflammation, apoptosis, and fibrosis have been proposed to discriminate between SS and NASH. A NASH test that allows prediction on the basis of 13 parameters has been reported in Europe but, in recent years, Gholam *et al.* designed a more convenient differential formula based on only two criteria: the AST level and the presence or absence of diabetes mellitus (DM).[41] Campos *et al.* proposed a clinical scoring system for NASH[42] in which the scored criteria consist of hypertension (HTN), type 2 DM, AST, ALT, sleep apnea syndrome, and race (exception for blacks). However, these reports are from Europe and the USA. Recently, it was reported that the serum level of soluble fraction in cytokeratin 18 (soluble CK-18) was able to discriminate between SS and NASH,[43] and this has been adopted for our Japanese patients (unpublished data).

We reported previously the importance of serum ferritin and thioredoxin levels, reflecting status of oxidative stress, in the differential diagnosis between SS and NASH.[44,45] Recently, Sumida *et al.* proposed the NAFIC (NASH, Ferritin, Insulin, Collagen) scoring using Japanese patients. This comprises three measurements: serum ferritin, insulin, and type-4 collagen 7s.[46] To determine the utility of this score, we conducted a validation study in collaboration with ten centers all over Japan (Japan Study Group of NAFLD; JSG-NAFLD).[46]

Various indicators have been proposed for the evaluation of the degree of fibrosis in NASH. From a study based on the analysis of 50 NASH patients including nine with cirrhosis, Fujii *et al.* reported that the cirrhosis determinant score (CDS) and the hepatitis C antiviral long-term treatment against cirrhosis (HALT-C) model were valuable for the differentiation of cirrhosis induced by NASH and HCV infection.[47] A French group proposed the BAAT score[48] and Fibrotest,[49] which assign one point to each of the following items: BMI, ALT, age, and triglycerides. Angulo *et al.* proposed the NAFLD fibrosis score which can be calculated from parameters such as age, platelet count, albumin, AST/ALT ratio, fasting hyperglycemia/DM, and BMI.[50] The NAFLD fibrosis score is simple and has advantages. However, the major problem is that liver biopsy cannot be avoided in around 25% cases, which are classified as intermediate because of scores halfway between the high cut-off level and the low cut-off level. Harrison *et al.* proposed the simple and easy BARD score based on BMI $\geq$ 28 kg/m^2, AST/ALT ratio, and DM; and reported that the odds ratio increased 17-fold for cases with scores of two points or higher, associated with F3 or higher stages of fibrosis.[51] However, Fujii *et al.* suggested that the BARD score was not useful in Japanese patients because the average BMI is significantly lower than western people.[52] The N (Nippon) score[15] is very simple; it can be calculated on the basis of only gender, age, and the presence or absence of type 2 DM and HTN, and has been evaluated by a multicenter study in Japan.[16] Recently we showed that senescence marker protein 30 (SMP-30), which has an antiapoptotic activity and an effect on Ca^{++} efflux, was significantly decreased in NASH compared to SS. Thus, SMP-30 is a useful marker for the differential diagnosis between SS and NASH. However, at present we cannot detect it in serum.[53]

Prognosis

It has been reported that cardiovascular-related death and liver-related death are significantly higher in NAFLD patients than with the general population.[54] A cohort study conducted in 2006, reported a development of cancers among 97 771 individuals in the general Japanese population; 6.7% of men and 3.1% of women had DM, in diabetes patients, the hazard ratio of developing liver cancer was 2.24 (95% CI, 1.64–3.04) in men, and 1.94 (95% CI, 1.00–3.73) in women during an average follow-up period of 10.7-years.[55]

In a comparative study between HCV and NASH cirrhosis matched by gender and age, obesity, diabetes, and dyslipidemia were significantly more frequent in NASH cirrhosis. The 5-year cancer rate was 11.3% in NASH cirrhosis and 30.5% in HCV cirrhosis.[55] The leading cause of death in these two types of cirrhosis was HCC, 47% in NASH and 68% in HCV, and the second cause was hepatic failure, 32% in NASH and 25% in HCV.[56,57] The annual incidence of HCC in Japan is 2.2% in NASH cirrhosis and 6.1% in HCV cirrhosis. Meanwhile, Ascha *et al.* reported that the annual incidence of HCC was 2.6% in patients with NASH cirrhosis, compared to 4.0% in HCV cirrhosis in the USA.[58]

Treatment

Diet and exercise therapy

Weight loss achieved by diet and exercise is the most important aspect of treatment in obese patients with NAFLD, including NASH. In those treated weight, blood biochemical data such as ALT, albumin, cholinesterase, total cholesterol and fasting blood glucose values, and steatosis decreased significantly after significant weight loss.[59] The recommended daily energy intake is 25–35 kcal/kg, daily protein intake is 1.0–1.5 g/kg and fat should be less than 20% of total calories.

Antihyperlipidemic drugs

Saibara *et al.* showed that bezafibrate for tamoxifen-induced NASH resulted in biochemical and histological improvement.[60] Dohmen *et al.* reported that administration of fenofibrate for fatty liver complicated with dyslipidemia improved dyslipidemia and led to a decrease in the levels of ALP, whereas the levels of ALT showed no significant change.[61] Hyogo *et al.* reported that atorvastatin led to an improvement in liver function, fibrosis marker, adipocytokine, and improvement of fatty liver and hepatic inflammation.[62] Nozaki *et al.* reported the utility of ezetimibe and acarbose in mouse models of NAFLD.[63] Recently, we also demonstrated the histological improvement using 96 weeks of ezetimibe monotherapy in the 45 biopsy-proven NAFLD patients.[64]

Angiotensin II type-1 receptor blockers (ARB)

There also have been a number of reports from Japan regarding the utility of angiotensin II type-1 blockers (ARB) in NASH. This application is derived from basic studies which showed the inhibitory effect of ARB on the progression of fibrosis via inhibition of the activation of hepatic stellate cells.[65–67]

Antidiabetic drugs

Morita *et al.* demonstrated the effect of nateglinide on glucose metabolism, liver function, and liver histology in NASH patients with type 2 diabetes.[68] The effects of metformin and thiazolidine derivatives such as pioglitazone and rosiglitazone on NASH were reported in Japan, however, the numbers were small and the trials were uncontrolled.

Other drugs

There is the possibility that combination therapies using pantethine and probucol,[69] colestimide[70] and α-tocopherol[71] are useful for NASH; however, the subjects were in small numbers and there was no histological analysis after treatment. Recently, Sanyal *et al.* reported that administration of vitamin E for 96 weeks administration for non-DM NASH patients significantly improved liver histology compared to placebo, this result being more promising than pioglitazone administration.[72] Phlebotomy might be effective in NASH with excessive iron deposition in the liver.[73]

Group survey in NASH in Japan

As mentioned above, the Japan NASH Study Group founded in April 2008 (the representative: Takeshi Okanoue, Table 1), has started the following research projects: (i) nationwide study of 5000 cases of diabetes mellitus; (ii) SNP study of 1000 cases of SS and NASH; (iii) long-term follow-up study of 1000 cases of SS

Table 1 The members of Japan NASH study group

The representative of this study group:
Takeshi Okanoue MD, PhD., Hepatology Unit, Saiseikai Suita Hospital
The members of this study group:
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and NASH; (iv) collection of 100 cases of NASH-HCC; (v) biochemical markers of differential diagnosis between SS and NASH; and (vi) therapeutic guidelines based on the individual pathophysiology. Projects i, ii, iii, and iv are going well and we are expecting to present these results, including SNPs, in the near future.

Conclusion

Recently, much attention has been paid to NAFLD in Japan because the number of NAFLD patients has been increasing, while non-B, non-C HCC also is increasing gradually. We suspect that NASH might be responsible for this increase in HCC in Japan; however, the precise cause of the increased non B, non C HCC has not yet been established. In this review, we have described the epidemiology and the present status of clinical and basic aspects of NASH/NAFLD in Japan.

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Efficacy of long-term ezetimibe therapy in patients with nonalcoholic fatty liver disease

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Abstract
Background Hyperlipidemia, insulin resistance, and oxidative stress can heavily contribute to the initiation and progression of nonalcoholic fatty liver disease (NAFLD). Currently, there is no established treatment for this disease. Recently, several studies have shown that ezetimibe (EZ), a lipid-lowering drug, attenuates liver steatosis in an experimental NAFLD model. This study was designed to assess the efficacy of long-term EZ monotherapy in patients with NAFLD.
Methods A total of 45 patients with newly diagnosed liver biopsy-proven NAFLD were treated with EZ (10 mg/day) for 24 months. NAFLD-related biochemical parameters, imaging by computerized tomography, and liver biopsy were studied before and after treatment.
Results Ezetimibe therapy significantly improved NAFLD-related metabolic parameters including visceral fat area, fasting insulin, homeostasis model assessment of insulin resistance (HOMA-R), triglycerides, total cholesterol, low-density lipoprotein cholesterol (LDL-Ch), oxidative-LDL, the net electronegative charge modified-LDL, profiles of lipoprotein particle size and fatty acids component, and estimated desaturase activity. EZ therapy also significantly lowered serum alanine aminotransferase and high-sensitivity C-reactive protein levels, whereas no significant changes were found in serum type IV collagen 7S, adiponectin, leptin, and resistin levels. Histological features of steatosis grade ($P = 0.0003$), necroinflammatory grade ($P = 0.0456$), ballooning score ($P = 0.0253$), and NAFLD activity score (NAS) ($P = 0.0007$) were significantly improved from baseline. However, the fibrosis stage was not significantly ($P = 0.6547$) changed.
Conclusion The results in this study suggest that the long-term EZ therapy can lead to improvement in metabolic, biochemical, and histological abnormalities of NAFLD. Therefore, EZ may be a promising agent for treatment of NAFLD.

Keywords Ezetimibe · NAFLD · Insulin resistance · Lipid metabolism · Fatty acid metabolism

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Introduction

Nonalcoholic fatty liver disease (NAFLD) is one of the most common causes of chronic liver injury in the world [1–3]. NAFLD is a metabolic condition which encompasses a wide spectrum of liver disease ranging from

simple steatosis to nonalcoholic steatohepatitis (NASH). Although the exact intricacies of the molecular and cellular mechanisms responsible for progression from simple steatosis to NASH have not been fully elucidated, hyperlipidemia, insulin resistance, and oxidative stress are major contributors to the initiation and progression of NAFLD [4–6]. A two-hit hypothesis has been proposed, whereby steatosis (first hit) sensitizes the liver to a variety of metabolic injuries (second hit) that lead to necrosis, inflammation, and fibrosis [6]. Several investigators have suggested that NASH is the hepatic manifestation of the metabolic syndrome [2–7]. While there are few proven beneficial therapies for NASH, its association with insulin resistance has provided the rationale for evaluation of medical therapies that increase insulin sensitivity. Indeed, several pilot studies have shown that treatment with the biguanides and the thiazolidinediones, two classes of insulin-sensitizing drugs, can lead to improvements in biochemical and histological features of NASH [8–16].

Ezetimibe (EZ) is a useful lipid-lowering agent that inhibits the absorption of dietary and biliary cholesterol by selectively binding to the intestinal cholesterol transporter Niemann–Pick C1-like 1 [17, 18]. Several recent studies in an experimental NAFLD model have shown that EZ monotherapy not only protects against diet-induced hyperlipidemia, but also attenuates liver steatosis in an experimental NAFLD model [19–21].

In the present study, we investigated the efficacy of long-term EZ monotherapy in patients with NAFLD.

Patients and methods

Patients

The study protocol was approved by the ethical committee of Saiseikai Suita Hospital and the Kyoto Prefectural University of Medicine, and informed consent was obtained from all subjects prior to enrollment in the study. A total of 45 patients who had been newly diagnosed histologically as having NAFLD at Saiseikai Suita Hospital and Kyoto Prefectural University Hospital between 2007 and 2009 were evaluated in this study.

All liver biopsy specimens were examined by two experienced pathologists blinded to the patients' clinical or laboratory data or liver biopsy sequence. Histological features of samples were interpreted as outlined by Brunt et al. [22]. The stage of fibrosis was classified as follows: stage 0 = no fibrosis, stage 1 = zone 3 predominant pericellular fibrosis, stage 2 = zone 3 fibrosis plus periportal fibrosis, stage 3 = bridging fibrosis, stage 4 = cirrhosis. Necroinflammation was graded 0 (absent) to 3 (1, occasional ballooned hepatocytes and no or very mild inflammation; 2, ballooning of hepatocytes and mild-to-moderate portal inflammation; 3, intra-acinar inflammation and portal inflammation). The grade of steatosis was defined as mild (≤33%), moderate (34–65%), and advanced (≥66%). The NAFLD activity score (NAS) was calculated as the unweighted sum of the scores for steatosis (0–3), lobular inflammation (0–3), and ballooning (0–2) as reported by Kleiner et al. [23], and as shown in Table 1 was used to classify NAFLD into "not NASH" (NAS ≤2), "borderline NASH" (NAS = 3–4), and "definite NASH" (NAS ≥5).

Prior to evaluation of liver histology we excluded patients with an alcohol intake exceeding 20 g/day and those who reported any sign, symptom, and/or history of known liver disease including viral, genetic, autoimmune, and drug-induced liver disease, previous use of anti-diabetic medication including insulin-sensitizing agents such as metformin and pioglitazone. All patients received EZ (10 mg/day) for 24 months.

Estimation of energy and nutrient intake

All patients were asked to adhere to a dietary plan tailored to their energy requirements and metabolic control by a registered dietitian and/or physician, using the current Japan Diabetes Society recommendations. The patients

Table 1 Baseline anthropometrics and demographics

Male/female	24/21
Age (years)	50.2 ± 9.4
Body mass index (kg/m^2)	26.9 ± 3.3
Waist circumference (cm)	92.3 ± 5.7
Visceral fat area (cm^2)	155.9 ± 38.9
Subcutaneous fat area (cm^2)	170.9 ± 51.3
Obesity[a] (%)	41 (91.1)
Hyperlipidemia[b] (%)	45 (100)
Hypertension[c] (%)	23 (48.9)
75 g oral glucose tolerance test	
NGT (normal glucose tolerance; %)	7 (15.6)
IGT (impaired glucose tolerance; %)	28 (62.2)
Diabetes (%)	10 (22.2)
NAFLD activity score (NAS)	
NAS ≤2	4 (11.1)
NAS 3–4	3 (8.9)
NAS ≥5	38 (80.0)

NAFLD nonalcoholic fatty liver disease

[a] Obesity was defined as a body mass index of ≥25.1

[b] Hyperlipidemia diagnosed if serum total cholesterol level was ≥220 mg/dl and/or serum triglyceride level was ≥160 mg/dl on at least two occasions

[c] Hypertension was diagnosed if the patient was taking antihypertensive medication and/or had a resting recumbent blood pressure ≥140/90 mmHg on at least two occasions

recorded their daily dietary intake in a diary by using the calorie and lipid list in the Japan Diabetes Society recommendations guidebook. The dietary diary was collected every month, and the results were reported back to the subjects the following month. In addition, daily activity and physical condition were recorded every month using a checklist; and depending on the report, the physician checked the patient's condition and provided appropriate advice.

Clinical and laboratory investigations

The intra-abdominal visceral (VSA) and subcutaneous fat areas (SFA) were determined at the umbilical level by a computed tomography (CT) scanning technique (TSX-012A, X-Vigor, Toshiba Co. Ltd, Tokyo, Japan) using a method described previously [24].

Blood samples were obtained in the morning after an overnight fast. Plasma glucose (PG) was measured by the glucose oxidase method and HbA1c was determined by high-performance liquid chromatography (HPLC: Arkray Inc., Kyoto, Japan). Plasma insulin immunoreactive insulin (IRI) concentrations were measured by an immunoradiometric assay (Insulin-RIAbead II, Abbott, Japan). The homeostasis model assessment of insulin resistance (HOMA-R) was calculated from fasting insulin and plasma glucose levels by the following equation: HOMA-R = fasting IRI (μU/ml) × fasting PG (mg/dl)/405. Serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), total cholesterol (T-Ch), high-density lipoprotein cholesterol (HDL-Ch), low-density lipoprotein cholesterol (LDL-Ch), and triglyceride (TG) were measured by enzymatic methods using a chemical autoanalyzer (Hitachi Co., Tokyo, Japan). Serum type IV collagen 7S was measured by a radioimmunoassay kit (Mitsubishi Chemical Group, Tokyo, Japan). Serum high-sensitivity C-reactive protein (hs-CRP) was measured by nephelometry using a latex particle-enhanced immunoassay (Dade Behering, Tokyo, Japan). Serum oxidized LDL (oxLDL) was measured by an enzyme-linked immunoassay (ELISA) kit (Kyowa Medex Co., Ltd., Tokyo, Japan). The net electronegative charge modified-LDL (emLDL) was analyzed by using an agarose gel electrophoresis lipoprotein fraction system, according to the manufacturer's instructions (Chol/Trig Combo System™; Helena Labs, Saitama, Japan). The percentage frequency of emLDL was calculated on a computer from the migration distance (b) of the LDL fraction in the test samples and the migration distance (a) of normal control sera according to the following formula: emLDL density = $[b - a/a] \times 100\%$.

Serum lipoproteins were also analyzed by an HPLC system according to the procedure described by Okazaki et al. [25], while lipoprotein particle size was determined based on individual elution times that corresponded to peaks on the chromatographic pattern of cholesterol fractions. In this study, we defined 3 VLDL, 4 LDL, and 5 HDL subclasses according to lipoprotein particle size, expressed as diameter.

Analysis of fatty acid composition in plasma cholesterol esters (CEs) was as follows: total lipid was extracted from plasma by using the method of Bligh and Dyer [26], followed by separation of the CEs by thin-layer chromatography using silica gel plates (Silica Gel 60, Merck, Darmstadt, Germany) and a solvent system of petroleum ether/ethyl ether/acetic acid (80:20:1, v/v/v). The spot corresponding to CEs was scraped from the plate and transmethylated with 2 ml of acetyl chloride/methanol (5:50, v/v) at 90°C for 2 h. Heptadecanoic acid (17:0) was used as an internal standard. Fatty acid methyl esters were quantified by using a model GC14A gas chromatograph (Shimadzu, Kyoto, Japan) equipped with a 25-m × 0.5-mm capillary column (HR-SS-10, Shinwa Chemical Industries, Ltd., Kyoto, Japan). Desaturase and elongase activities were estimated as the ratio product to the precursor of individual fatty acids in plasma CEs according to the following: D9-16D = 16:1n-7/16:1, D9-18D = 18:1n-9/18:1, D6D = 18:3n-6/18:2n-6, and D5D = 20:4n-6/20:3n-6.

Statistical analysis

All statistical analyses were performed using Statview version 5.0 (Abacus Concepts, Berkeley, CA, USA). Data were summarized by frequencies and percentages for categorical variables, and means ± SD for continuous variables. Comparison of pre- and posttreatment of EZ data was carried out using nonparametric Wilcoxon signed rank test. A P value less than 0.05 was considered statistically significant.

Results

Effect of ezetimibe on clinical and laboratory parameters

Compared to baseline, VFA level reduced significantly from 155.9 ± 38.9 to 146.5 ± 34.8 ($P < 0.05$) at the end of the study (Table 2). There were no significant changes in body mass index (BMI), waist circumference, and SFA at the end of the study.

Mean ALT level decreased significantly by the end of the study from 62 ± 25 to 49 ± 23 ($P < 0.01$), whereas the AST level did not. The mean level of fasting insulin level and HOMA-R decreased significantly (both $P < 0.05$), although mean HbA1c and fasting glucose levels remained unchanged at the end of the study. Regarding lipid metabolism, the mean levels of TG, T-Ch, LDL-Ch, oxLDL, and

Table 2 Clinical and laboratory parameters of baseline and after ezetimibe treatment

	Baseline	At 12 months	At 24 months
Body mass index (kg/m^2)	26.9 ± 3.3	26.0 ± 3.5	26.1 ± 3.2
Waist circumference (cm)	92.3 ± 5.7	90.5 ± 5.8	90.9 ± 6.0
Visceral fat area (cm^2)	155.9 ± 38.9	150.8 ± 33.6	146.5 ± 34.8*
Subcutaneous fat area (cm^2)	170.9 ± 51.3	166.4 ± 41.5	167.1 ± 41.5
HbA1c (%)	6.3 ± 0.8	6.5 ± 0.7	6.4 ± 0.9
Fasting glucose (mg/dl)	113 ± 24	112 ± 27	112 ± 28
Fasting insulin (μU/ml)	10.9 ± 5.6	9.2 ± 5.8*	9.4 ± 5.1*
HOMA-R	3.04 ± 1.17	2.60 ± 1.33*	2.62 ± 1.24*
Aspartate aminotransferase (IU/l)	40 ± 22	36 ± 16	36 ± 16
Alanine aminotransferase (IU/l)	62 ± 25	48 ± 25**	49 ± 23**
Triglycerides (mg/dl)	168 ± 94	136 ± 90*	138 ± 88*
Total cholesterol (mg/dl)	228 ± 44	193 ± 36**	194 ± 36**
HDL cholesterol (mg/dl)	49 ± 13	53 ± 15	52 ± 14
LDL cholesterol (mg/dl)	136 ± 33	117 ± 34*	114 ± 31*
Oxidative LDL (U/ml)	14.1 ± 6.9	13.6 ± 7.1	11.8 ± 5.5*
Electronegative charge modified-LDL (ecd)	6.4 ± 3.5	3.5 ± 3.6#	3.4 ± 3.2#
Type IV collagen 7S (ng/dl)	5.1 ± 2.9	4.7 ± 2.5	4.7 ± 2.5
Adiponectin (μg/ml)	5.8 ± 3.1	6.1 ± 3.4	6.1 ± 3.4
Leptin (ng/l)	4.0 ± 2.9	3.8 ± 3.1	3.8 ± 3.1
Resistin (ng/ml)	7.7 ± 3.1	7.4 ± 3.4	7.4 ± 3.4
High-sensitivity C-reactive protein (ng/ml)	883 ± 408	677 ± 392*	685 ± 377*

Data are the mean ± SD

ecd electronegative charge density

* $P < 0.05$, ** $P < 0.01$, and # $P < 0.005$ versus baseline

emLDL were decreased significantly at the end of study ($P < 0.05$, $P < 0.01$, $P < 0.05$, $P < 0.05$, and $P < 0.005$, respectively). However, there was no significant change in serum HDL-Ch levels during the study. Serum hs-CRP was decreased significantly ($P < 0.05$), whereas no significant changes were found in the levels of serum adiponectin, leptin, resistin, and type IV collagen 7S.

Effect of ezetimibe on lipoprotein subclass according to particle size

As shown in Fig. 1, the levels of large VLDL (44.5–64.0 nm), corresponding to VLDL1 (Sf 60–400), decreased significantly compared with baseline from 6.6 ± 1.2 to 4.2 ± 1.4 mg/dl ($P < 0.005$). At the end of the study, the mean levels of small LDL (23 nm) and very small LDL (16.7–20.7 nm) were also significantly decreased compared with baseline (from 37.9 ± 5.4 to 33.2 ± 5.1 mg/dl, $P < 0.05$ and from 23.8 ± 4.8 to 18.6 ± 2.8 mg/dl, $P < 0.01$, respectively). No significant changes from baseline levels were observed in any of the HDL-Ch subclass at the end of the study.

Effect of ezetimibe on fatty acid composition in plasma CEs

The changes in plasma CEs fatty acid composition and estimated desaturase activities are shown in Table 3. Compared with baseline, myristic acid (C14:0), palmitic acid (C16:0), palmitoleic acid (16:1n-7), oleic acid (C18:1n-9), and dihomo-γ-linoleic acid (C18:3n-6) were decreased significantly at the end of the study, while linoleic acid (C18:2n-6) was significantly increased. At the end of the study, activity of D9-16D, a major de no lipogenesis enzyme (known alternatively as stearoyl-CoA desaturase 1; SCD1), was increased significantly ($P < 0.005$). Activity of D5D was also increased significantly ($P < 0.01$) compared with baseline.

Histological responses

Follow-up liver biopsies were performed on 33 patients at the end of the 24 months of EZ treatment. Table 4 shows the histologic changes before and after treatment. The mean level of steatosis grade (from 2.3 ± 0.7 to 1.9 ± 0.8, $P = 0.0003$), necroinflammatory grade (from 1.9 ± 0.7 to 1.8 ± 0.7, $P = 0.0456$), ballooning score (from 1.4 ± 0.5 to 1.3 ± 0.5, $P = 0.0253$), and NAS score (from 5.6 ± 1.6 to 5.1 ± 1.8, $P = 0.0007$) improved significantly during the study. Of 33 patients, 24 had a one or more point improvement in the NAS score, 8 had no change, and one had a one-point increase. In contrast, the mean level of fibrosis stage level did not change significantly (from 2.0 ± 0.8 to 2.1 ± 0.9, $P = 0.6547$). Overall, one patient had a one-point improvement, 29 had no change, and 3 had a one-point deterioration.

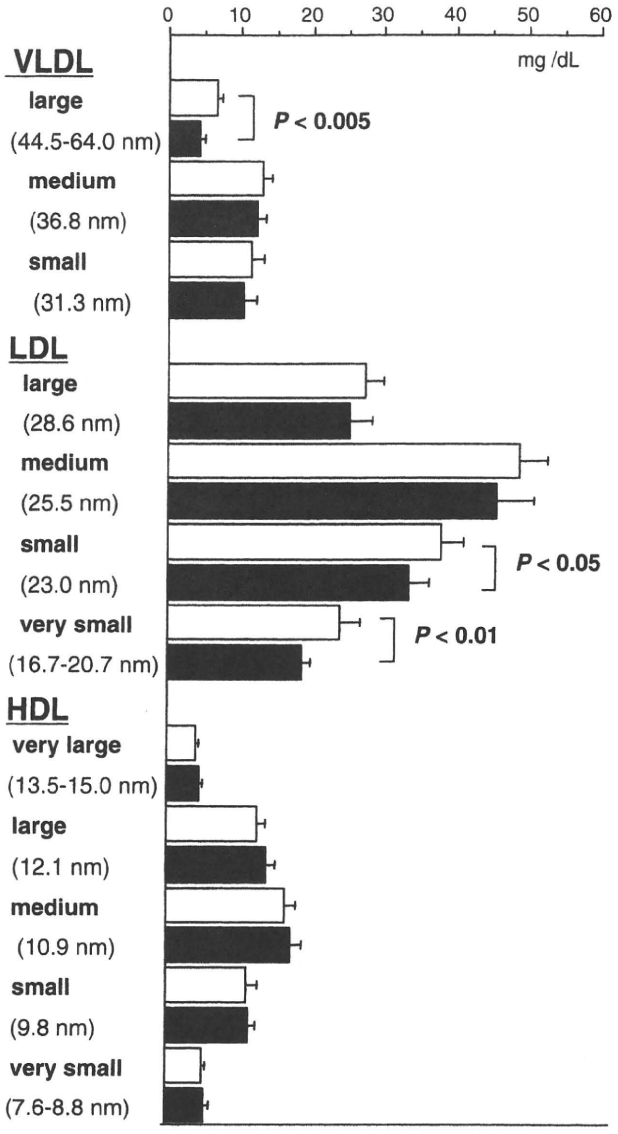

Fig. 1 Changes in the cholesterol concentration of each lipoprotein subclass, grouped according to particle size, following ezetimibe administration. The values are shown as mean ± SD. The *open columns* represent baseline data and the *filled columns* represent data collected after 24 months of ezetimibe treatment

Discussion

This study involved a 24-month follow-up of histological and metabolic syndrome-related parameters. In this study, we demonstrated that a 24-month course of EZ in doses of 10 mg/day improved liver histology and several metabolic syndrome-related parameters, and serum ALT levels. While we were preparing this manuscript, Yoneda et al. [27] also reported that NAFLD-related clinical parameters and histological observations were significantly improved by the treatment with EZ for 6 months, although the number of patients was small and treatment period was short. Although we did not observe significant improvement in the serum type IV collagen 7S level, our findings are essentially consistent with their report.

A noteworthy finding of this study was that EZ decreased not only the levels of T-Ch and LDL-Ch but also the levels of oxLDL and emLDL, which are the most

Table 3 Changes in plasma cholesterol esters fatty acid composition and estimated desaturase activities

Fatty acids (% of total fatty acids)	Baseline	After treatment
C14:0 (myristic acid)	0.56 ± 0.27	0.54 ± 0.31*
C16:0 (palmitic acid)	12.58 ± 1.03	11.36 ± 1.09**
C16:1n-7 (palmitoleic acid)	3.13 ± 1.02	2.81 ± 1.02**
C18:0 (stearic acid)	1.09 ± 0.30	1.07 ± 0.31
C18:1n-9 (oleic acid)	19.30 ± 2.41	18.52 ± 2.41*
C18:2n-6 (linoleic acid)	48.67 ± 4.32	50.83 ± 4.42**
C18:3n-6 (γ-linoleic acid)	0.82 ± 0.28	0.82 ± 0.24
C18:3n-3 (α-linoleic acid)	0.77 ± 0.35	0.74 ± 0.34
C20:3n-6 (dihomo-γ-linoleic acid)	0.74 ± 0.23	0.69 ± 0.28*
C20:4n-6 (arachidonic acid)	5.63 ± 1.17	5.65 ± 1.23
C20:5n-3 (eicosapentaenoic acid)	2.13 ± 0.75	2.15 ± 0.70
C22:6n-3 (docosahexaenoic acid)	0.84 ± 0.31	0.85 ± 0.30
Estimated desaturase index		
D9–16D (16:1n-7/16:0)	0.26 ± 0.08	0.22 ± 0.09#
D9–18D (18:1n-9/18:0)	17.71 ± 4.05	17.31 ± 4.02
D6D (18:3n-6/18:2n-6)	0.016 ± 0.009	0.014 ± 0.010
D5D (20:4n-6/20:3n-6)	7.60 ± 2.38	8.18 ± 2.50**

* $P < 0.05$, ** $P < 0.01$, and # $P < 0.005$ versus baseline

Table 4 Histological changes in 33 patients with NAFLD

	Baseline	After treatment	*P* value
Steatosis grade	2.3 ± 0.7	1.9 ± 0.8	0.0003
0	0 (0)	1 (3)	
1	5 (16)	9 (31)	
2	14 (44)	16 (44)	
3	14 (41)	7 (22)	
Necroinflammatory grade	1.9 ± 0.7	1.8 ± 0.7	0.0456
1	10 (30)	12 (36)	
2	16 (48)	16 (45)	
3	7 (21)	5 (18)	
Fibrosis stage	2.0 ± 0.8	2.1 ± 0.9	0.6547
0	1 (3)	1 (33)	
1	6 (18)	8 (24)	
2	17 (52)	12 (42)	
3	9 (27)	12 (30)	
4	0 (0)	0 (0)	
Ballooning score	1.4 ± 0.5	1.3 ± 0.5	0.0253
0	0 (0)	1 (3)	
1	19 (64)	22 (70)	
2	14 (36)	10 (27)	
NAS score	5.5 ± 1.6	5.0 ± 1.8	0.0007

atherogenic forms of lipoproteins. Analysis of lipoprotein subclass by HPLC also revealed that EZ treatment caused significant decrease in large VLDL (VLDL1), small LDL, and very small LDL. An increased serum level of large VLDL (VLDL1) is a common characteristic of the dyslipidemia associated with insulin resistance and type 2 diabetes mellitus [28–30]. Griffin and Packard [31] demonstrated that large VLDL1 is a precursor of small dense LDL, and that it is produced preferentially in the liver during development of insulin resistance. Adiels et al. reported that overproduction of large VLDL particles is driven by increased liver fat content [32] and that acute suppression of VLDL1 secretion by insulin is associated with hepatic fat content and insulin resistance [33]. In the present study, the change in large VLDL levels associated with EZ therapy correlated positively with changes in very small LDL ($r = 0.706$, $P < 0.001$), emLDL ($r = 0.412$, $P < 0.01$), and HOMA-R ($r = 0.565$, $P < 0.01$). Taken together these previous data and the findings of the present study suggest strongly that EZ ameliorates both hepatic and systemic vascular insulin resistance.

Another noteworthy finding of this study was that the long-term EZ therapy was associated with significant decrease in the levels of myristic acid, palmitic acid, palmitoleic acid, oleic acid, dihomo-γ-linoleic acid, and D9-16D activity, and significant increase in linoleic acid and D5D activity. It has been reported that EZ causes significant reduction in the absorption of several saturated fatty acids in diet-induced obese and diabetic mice [34]. Joshi-Barve et al. [35] reported that palmitic acid induces interleukin-8 from hepatocytes and consequent liver injury. Several recent studies have demonstrated that palmitic acid induces apoptosis in liver cells [36–38]. In the present study, palmitic acid levels were significantly and positively correlated with ALT levels ($P < 0.05$). Therefore, the reducing of palmitic acid levels may inhibit liver injury. Stearoyl-CoA desaturase 1(SCD1; known alternatively as D9D) is the final step in de novo lipogenesis and converts saturated fatty acid to monounsaturated fatty acid, whereas Δ5- and Δ6-desaturases participate in the metabolism of polyunsaturated fatty acids. Miyazaki et al. [39] have shown that the biosynthesis of hepatic CEs and triglycerides is highly dependent on the expression of the SCD1 gene. On the other hand, it has been reported that the activities of both D5D and D6D are linked with insulin sensitivity [40–43]. These previous data and the findings of the present study, including histological examinations, imply that EZ inhibits the development and progression of liver steatosis and insulin resistance.

Previous studies of the natural history of serial liver biopsies revealed that fibrosis stage progressed in 30–40%, remained stable in 30–40%, and regressed in 20–30% of cases, and that severity of steatosis, inflammation, hepatocytes ballooning, and Mallory's hyaline usually improved [44, 45]. In the present study, fibrosis stage progressed in 9%, remained in 88%, and regressed in 3% of cases over the 2-year period. Liver histologic findings were also improved in steatosis grade, necroinflammatory grade, ballooning score, and NAS score in this study. However, 3 of 33 patients had progression of fibrosis stage over the 2-year period. It is unclear whether this divergent response represents sampling error or heterogeneity in the population. Further studies are needed in order to clarify this point.

In conclusion, the results of this study suggest that the long-term EZ therapy can lead to improvement in metabolic, biochemical, and histological abnormalities of NAFLD through both improvement of insulin resistance and reduction in absorption of monosaturated fatty acids, especially palmitic acid. Therefore, EZ may be a promising agent for treatment of NAFLD. To confirm our finding, an appropriately designed, large-scale, controlled trial is need.

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ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

A simple clinical scoring system using ferritin, fasting insulin, and type IV collagen 7S for predicting steatohepatitis in nonalcoholic fatty liver disease

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Abstract

Background Liver histology is the gold standard for the diagnosis of nonalcoholic steatohepatitis (NASH). Noninvasive, simple, reproducible, and reliable biomarkers are greatly needed to differentiate NASH from nonalcoholic fatty liver disease (NAFLD).

Methods To construct a scoring system for predicting NASH, 177 Japanese patients with biopsy-proven NAFLD were enrolled. To validate the scoring system, 442 biopsy-proven NAFLD patients from eight hepatology centers in Japan were also enrolled.

Results In the estimation group, 98 (55%) patients had NASH. Serum ferritin [≥200 ng/ml (female) or ≥300 ng/ml (male)], fasting insulin (≥10 μU/ml), and type IV collagen 7S (≥5.0 ng/ml) were selected as independent variables associated with NASH, by multilogistic regression analysis. These three variables were combined in a weighted sum [serum ferritin ≥200 ng/ml (female) or ≥300 ng/ml (male) = 1 point, fasting insulin ≥10 μU/ml = 1 point, and type IV collagen 7S ≥5.0 ng/ml = 2 points] to form an easily calculated composite score for predicting NASH, called the NAFIC score. The area under the receiver operating characteristic (AUROC) curve for predicting NASH was 0.851 in the estimation group and 0.782 in the validation group. The NAFIC AUROC was the greatest among several previously established scoring systems for detecting NASH, but also for predicting severe fibrosis.

Conclusions NAFIC score can predict NASH in Japanese NAFLD patients with sufficient accuracy and simplicity to be considered for clinical use.

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Introduction

Nonalcoholic fatty liver disease (NAFLD) is the most common chronic liver disease (CLD) in many developed countries and is a serious public health problem worldwide. NAFLD includes a wide spectrum of liver diseases that range from simple steatosis, which is usually a benign and nonprogressive condition, to nonalcoholic steatohepatitis (NASH), which can progress to liver cirrhosis (LC) and hepatocellular carcinoma (HCC) despite the absence of significant alcohol consumption [1–5]. Liver biopsy remains a reliable tool for the diagnosis of NASH [1, 6], and the most sensitive and specific method of providing prognostic information. Practically speaking, however, it is difficult to perform liver biopsy for every patient with NAFLD to ascertain the presence of NASH [7]. Moreover, biopsy itself has significant limitations such as pain, risk of severe complications, sampling error [8, 9], cost, and patient unwillingness to undergo invasive testing. Therefore, there is an urgent need to develop and validate simple, reproducible, noninvasive tests that accurately distinguish NASH from NAFLD and determine the stage of the disease [7]. Noninvasive approaches for this purpose have included a combination of clinical features and routine laboratory investigations, as well as some readily available serum markers of fibrosis [6, 7, 10, 11]. Most of these noninvasive approaches have consisted of small sample sizes and have lacked rigorous external validation.

The purposes of this study were (1) to develop a simple noninvasive scoring system aimed at differentiating NASH from NAFLD patients by using easily available clinical and biochemical variables and (2) to validate the results in a separate cohort of patients.

Methods

Patients

A total of 177 patients with well-characterized and liver-biopsy-confirmed NAFLD were included in this study to establish a simple method to detect NASH. They were consecutively biopsied patients who were seen at the Center for Digestive and Liver Diseases, Nara City Hospital from 2002 to 2008. To validate the model, 442 patients with biopsy-proven NAFLD from 2002 to 2008 were enrolled from eight Hepatology Centers in Japan: Division of Gastroenterology, Yokohama City University Graduate School of Medicine; Department of Medicine and Molecular Science, Graduate School of Biomedical Sciences, Hiroshima University; Department of Gastroenterology and Hepatology, Kochi Medical School; Department of Internal Medicine, Saga Medical School, Saga University; Department of Hepatology, Graduate School of Medicine, Osaka City University; Department of Gastroenterology and Hepatology, Kyoto Prefectural University of Medicine; Division of Gastroenterology and Hematology/Oncology, Department of Medicine, Asahikawa Medical College; and Hepatology Center, Saiseikai Suita Hospital.

The diagnosis of NAFLD was based on the following criteria: (1) liver biopsy showing steatosis in at least 5% of hepatocytes [12] and (2) appropriate exclusion of liver diseases of other etiology including viral hepatitis, autoimmune hepatitis, drug-induced liver disease, primary biliary cirrhosis, biliary obstruction, hemochromatosis, Wilson's disease, and α-1-antitrypsin-deficiency-associated liver disease. Patients who consumed >20 g alcohol per day and patients with evidence of decompensated LC or HCC were excluded. Diabetic patients treated with exogenous insulin or insulin sensitizers (metformin or pioglitazone) were also excluded. Written informed consent was obtained from all patients at the time of their liver biopsy, and the study was conducted in accordance with the Helsinki Declaration.

Anthropometric and laboratory evaluation

Venous blood samples were taken in the morning after a 12-h overnight fast. The laboratory evaluation in all patients included a blood cell count and the measurement of aspartate aminotransferase (AST), alanine aminotransferase (ALT), γ-glutamyl transpeptidase, cholinesterase (ChE), total cholesterol, triglyceride, albumin, fasting plasma glucose (FPG), immunoreactive insulin (IRI), ferritin, hyaluronic acid (HA), and type IV collagen 7S. These parameters were measured using the standard techniques of clinical chemistry laboratories. Body mass index (BMI) was also calculated. Obesity was defined as BMI >25, according to the criteria of the Japan Society for the Study of Obesity [13]. Patients were assigned a diagnosis of diabetes mellitus (DM) if they had documented use of oral hypoglycemic medication, a random glucose level >200 mg/dl, or FPG >126 mg/dl [14]. Dyslipidemia was diagnosed if the cholesterol level was >220 mg/dl and/or triglyceride level was >160 mg/dl. Hypertension was diagnosed if the patient was taking antihypertensive medication and/or had a resting recumbent blood pressure ≥140/90 mmHg on at least two occasions.

The HAIR score [15] was calculated by summation of the scores of hypertension (1 point), ALT >40 IU/l (1 point), and insulin resistance (IR) index >5 (1 point). IR

index was calculated using the formula: 1/quantitative insulin sensitivity check index (QUICKI) [16] = log fasting IRI (μU/ml) + log FPG (mg/dl). Palekar's score [17] was calculated by summing the risk factor of age ≥50 years, female sex, AST ≥45 IU/l, BMI ≥30 kg/m^2, AST/ALT ratio (AAR) ≥0.80, and HA ≥55 ng/ml. Gholam's score [18] was calculated by the formula: 2.627 × ln AST + 2.13 for DM. The BAAT score [19] was calculated by summing the risk factor of BMI ≥28, age ≥50 years, ALT levels measuring twice normal or higher, and triglyceride level ≥1.7 mmol/l (150 mg/dl). The BARD score [20] is a weighted sum of three easily available variables [BMI ≥28 kg/m^2 (1 point), AAR ≥0.8 (2 points), and DM (1 point)]. Modified scores (with cutoff values of BMI changed to 25 kg/m^2) of Palekar's, BAAT, and BARD were also calculated. The NAFLD fibrosis score (NFS) [21] was calculated according to the following formula: −1.675 + 0.037 × age (years) + 0.094 × BMI + 1.13 × impaired fasting glycemia (IFG)/DM (yes = 1, no = 0) + 0.99 × AAR − 0.013 × platelet (×10^9/l) − 0.66 × albumin (g/dl). The N (Nippon) score [22] was calculated as the total number of the following risk factors: female sex, older age (>60 years), type 2 DM (T2DM), and hypertension.

Histological evaluation

All patients enrolled in this study underwent a percutaneous liver biopsy under ultrasonic guidance. The liver specimens were embedded in paraffin and stained with hematoxylin and eosin, Masson-trichrome, and reticulin silver stain. Two hepatopathologists (T.O. and Y.S.) who were blinded to the clinical data reviewed the liver biopsy specimens. Adequate liver biopsy samples were defined as >1.5 cm long and/or having more than six portal tracts. NASH was defined as steatosis with lobular inflammation and ballooning degeneration, with or without Mallory-Denk body or fibrosis [2, 3]. Patients whose liver biopsy specimens showed simple steatosis or steatosis with nonspecific inflammation were identified as the nonNASH cohort [2, 3]. The presence or absence of hepatocyte ballooning degeneration is influenced by the variability in pathologists' interpretation. The NAFLD Activity Score (NAS) proposed by Kleiner et al. [12] was the unweighted sum of the scores for steatosis (0–3), lobular inflammation (0–3), and ballooning degeneration (0–2). If liver histology was too atypical to make a judgment, cases with an NAS of ≥5 were considered to be NASH. The severity of hepatic fibrosis (stage) was defined as follows: stage 1, zone 3 perisinusoidal fibrosis; stage 2, zone 3 perisinusoidal fibrosis with portal fibrosis; stage 3, zone 3 perisinusoidal fibrosis and portal fibrosis with bridging fibrosis; and stage 4, cirrhosis [23].

Statistical analysis

Results are presented as the means and standard deviation (SD) for quantitative data, or as numbers with percentages in parentheses for qualitative data. Statistical differences in quantitative data were determined using the t test. Fisher's exact probability test or χ^2 analysis was used for qualitative data. Multivariate analysis was performed by logistic regression analysis to identify variables independently associated with the presence of NASH. Those variables with $P < 0.05$ by multivariate analysis were used to construct a scoring system to predict NASH. The scoring system was a weighted sum of significant variables on the basis of odds ratio (OR) obtained from logistic regression analysis. To assess the accuracy of the clinical scoring system in differentiating NASH from NAFLD, we calculated the sensitivity (Se) and specificity (Sp) for each value of each test, and then constructed receiver operating characteristic (ROC) curves by plotting the Se against (1 − Sp) at each value. The diagnostic performance of the scoring systems was assessed by analysis of ROC curves. The most commonly used index of accuracy was the area under the ROC curve (AUROC), with values close to 1.0 indicating high diagnostic accuracy. To evaluate the overall accuracy of our score and NFS in detecting significant or advanced fibrosis, the sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) were calculated. Differences were considered statistically significant at $P < 0.05$.

Results

Characteristics of the patient population in the estimation group

Table 1 summarizes the clinical, laboratory, and liver biopsy data of the patient population in the estimation group. Eighty-six (49%) patients were female, and 120 (68%) were obese. Of 177 NAFLD patients involved in this estimation group, 98 (55%) were histologically diagnosed with NASH, and 79 (45%) had nonNASH NAFLD. NASH patients were significantly older, predominantly female, heavier, hypertensive, and more likely to have T2DM; had lower hemoglobin (Hb), platelet count and ChE; and had higher levels of AST, ALT, AAR, ferritin, FPG, IRI, HA, and type IV collagen 7S.

Predictors of NASH

Table 2 shows the univariate comparison and the results of the multivariate analysis performed in the 177 patients in the estimation group. Univariate analysis showed that age