Nonalcoholic fatty liver disease in Hong Kong Chinese

A Thesis for the Degree of Doctor of Medicine

The Chinese University of Hong Kong

By

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DECLARATION OF ORIGINALITY

The work contained in this thesis is completely original and has not been submitted for any other degrees. All studies were performed at the Prince of Wales Hospital, Hong Kong under the guidance of Prof. Henry Lik-Yuen Chan, Professor, Department of Medicine and Therapeutics, The Chinese University of Hong Kong. I was primarily responsible for the study design, patient recruitment and liver biopsies, monitoring of experiments, data entry and analysis, and manuscript preparation.

I would like to thank my coworkers for the support of the studies. Dr. Paul Choi, Dr. Anthony Chan, Dr. K.F. Chan and Dr. W.F. Ng performed histological assessment and scoring. Miss Ada Tse, Miss H.Y. Chan and Miss C.Y. Leung performed biochemical assays and genotyping. Dr. W.Y. So provided expert advice on study design and arranged oral glucose tolerance test for the study subjects.

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Précis

Background

Nonalcoholic fatty liver disease (NAFLD) is the most common chronic liver disease in affluent countries. It may progress to nonalcoholic steatohepatitis (NASH), cirrhosis and hepatocellular carcinoma. It is also strongly associated with metabolic syndrome as well as cardiovascular and cerebrovascular diseases. Although many Chinese people are adopting Western lifestyle, they are still generally less obese than Caucasians. It is thus important to investigate the clinical features, natural history, and pathogenesis of NAFLD in Chinese.

Aims

1. To study the severity and progression of NAFLD in Hong Kong Chinese
2. To study the metabolic and adipokine profile of NAFLD patients
3. To validate clinical models to predict advanced fibrosis in NAFLD patients

Hypothesis

1. Significant necroinflammation and fibrosis occur in Chinese NAFLD patients.
2. Histological features of NAFLD may progress with time.
3. Metabolic syndrome is common in NAFLD patients. Ethnicity-specific definition of metabolic syndrome is useful to characterize Chinese NAFLD patients.

4. Post-challenge hyperglycemia after oral glucose tolerance test is common in NAFLD patients and is associated with advanced disease.
5. Abnormal levels of serum adipokines are associated with NAFLD and its severity.
6. Gene polymorphisms of adipokines are associated with NAFLD and its severity.
7. The NAFLD fibrosis score can predict advanced fibrosis in Chinese NAFLD patients.

Laboratory tests

1. Insulin and adipokines

Insulin level was measured by commercial ELISA kits (Dako, UK). Adiponectin, resistin and tumor necrosis factor-alpha were measured by the Quantikine® immunoassay (R&D System, USA).

2. Histology

Brunt scoring system was used to report the steatosis grade, necroinflammatory grade and fibrosis stage of the liver histology.

Research models and results

1. Histological severity of Chinese NAFLD patients

A retrospective study was conducted to study the histological severity of Chinese NAFLD patients. Forty-two patients with histology-proven NAFLD were identified

by reviewing the records of 247 liver biopsies performed between 1996 and 2003.[Wong 2004] Thirty-six (86%) patients had steatohepatitis and 11 (26%) also had fibrosis. The presence of diabetes mellitus predicted higher grade steatohepatitis and fibrosis (p=0.02) whereas alanine aminotransferase (ALT) level had no correlation with histological severity.

Based on these results, we conducted a two-center prospective study.[Wong 2006] Among 80 consecutive patients with histology-proven NAFLD, 16 (20%) had grade 2 or 3 necroinflammation, and 7 (9%) had stage 3 or 4 fibrosis. Fifty-two (65%) patients had NASH.

These data suggest that severe NAFLD exists in the Chinese population, though the proportion of advanced disease is lower than that of the Western population.

2. Histological progression of NAFLD

Seventeen NAFLD patients underwent paired liver biopsies at a median interval of 6 years (range 4 to 8 years).[Hui 2005] Nine (53%) patients had progressive disease with worsening of fibrosis stage. Changes in body mass indices, plasma glucose, glycosylated hemoglobin, total cholesterol and triglycerides had no statistical correlation with changes in necroinflammation or fibrosis. Seven (41%) patients developed new onset of diabetes mellitus or hypertension during follow-up.

These data suggest that NAFLD in Chinese commonly progress with time. The diagnosis of NAFLD may predate the development of components of metabolic syndrome.

3. Metabolic syndrome and adipokines in NAFLD

Eighty prospectively recruited patients with histology-proven NAFLD and 40 age- and gender-matched controls had comprehensive metabolic assessment.[Wong 2006] Fasting Adiponectin, tumor necrosis factor-alpha, leptin, and resistin levels were measured on the day of liver biopsy. Twenty-eight (35%) patients had simple steatosis, and 52 (65%) patients had NASH. According to metabolic syndrome criteria of the National Cholesterol Education Program Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel III [NCEP-ATPIII]), metabolic syndrome was present in 62% of NASH patients, 46% of patients with simple steatosis, and 5% of controls. The ethnicity-specific metabolic syndrome definition by the International Diabetes Federation (IDF) could better differentiate patients with simple steatosis from those with NASH. According to the IDF criteria, metabolic syndrome was present in 79% of NASH patients, 54% of patients with simple steatosis, and 7% of controls.

Adipokines are bioactive proteins secreted by adipose tissue. They have important actions on insulin resistance and inflammation. In Study 2, we further measured serum adipokine levels in NAFLD subjects and controls. Compared to controls, NAFLD patients had lower adiponectin level and higher leptin level. NASH patients had higher tumor necrosis factor-alpha level than patients with simple steatosis. On multivariate analysis, low adiponectin level, increased leptin level, and diabetes mellitus were associated with NAFLD. High tumor necrosis factor-alpha level and high body mass index were independent factors associated with NASH. The level of tumor necrosis factor-alpha had positive correlation with necroinflammatory grade ($R=0.35$, $p=0.002$) and fibrosis stage ($R=0.31$, $p=0.005$).

To further elucidate the role of adipokines, nucleoside polymorphisms at adiponectin -11391, -11377, +45 and +276 and tumor necrosis factor-alpha promoter -863, -308, and -238 were tested.[Wong 2008] There was no significant deviation in the adiponectin and tumor necrosis factor-alpha gene polymorphisms between NAFLD patients and controls, or between patients with simple steatosis and those with stage 2 to 4 fibrosis. NAFLD patients with -11377G and +45G at the adiponectin gene were more likely to have hypertriglyceridemia.

These data suggest that metabolic syndrome is closely related to NAFLD. Ethnicity-specific criteria of metabolic syndrome are more useful in characterizing NAFLD patients. Adiponectin and tumor necrosis factor-alpha may have important roles in the pathogenesis of NASH, but polymorphisms of these genes have little influence on disease severity.

4. Post-challenge hyperglycemia in NAFLD

Since NAFLD is closely associated with metabolic syndrome and diabetes, one important question is whether oral glucose tolerance test is necessary for NAFLD patients. To answer this question, 73 consecutive patients with histology-proven NAFLD and no history of diabetes underwent oral glucose tolerance test.[Wong 2006-2] Diagnosis of diabetes and impaired glucose regulation was based on the 2006 American Diabetes Association criteria.

The prevalence of undiagnosed diabetes and impaired glucose tolerance was 33% and 29%, respectively. Among patients with 2-hour plasma glucose above 7.8 mmol/l, 47% had normal fasting glucose (below 5.6 mmol/l). If oral glucose tolerance test was only performed in patients with fasting glucose above 5.6 mmol/l, the sensitivity, specificity, positive and negative predictive values in detecting undiagnosed diabetes were 79%, 74%, 59%, and 88%, respectively.

Impaired glucose tolerance was more common in patients with NASH than those with simple steatosis (25% vs. 6%, p=0.04). 2-hour plasma glucose had positive correlation with fibrosis stage (R=0.25, p=0.046). The presence of abnormal oral glucose tolerance test was also associated with advanced liver fibrosis. None of the patients with normal glucose regulation had stage 3 or 4 fibrosis, compared to 4% of patients with impaired glucose tolerance and 17% of patients with diabetes.

These data suggest that undiagnosed diabetes and post-challenge hyperglycemia are common in NAFLD patients. Post-challenge hyperglycemia is also associated with advanced NAFLD. Most of these cases would be missed if only fasting glucose was checked. These results were included in guidelines by the Asia-Pacific Working Party on NAFLD (J Gastroenterol Hepatol 2007;22:801-8), which suggested consideration of oral glucose tolerance test as part of the evaluation of NAFLD patients.

5. Clinical models to predict advanced fibrosis in NAFLD patients

NAFLD patients with simple steatosis have good prognosis, while those with significant liver injury tend to run a progressive course and have higher long-term mortality. Therefore, it is important to assess the severity of liver disease. Liver biopsy is considered the gold standard for the assessment of liver injury.

However, it is invasive and expensive, and may not be acceptable to some patients. Non-invasive tests for liver fibrosis in NAFLD patients are needed.

The NAFLD fibrosis score was derived from 6 clinical parameters: age, body mass index, impaired fasting glucose or diabetes, aspartate aminotransferase-to-alanine aminotransferase ratio, platelet count and albumin.[Angulo 2007] This score had an overall accuracy of 80% to 90% in predicting stage 3 or 4 fibrosis. However, most of the patients used to derive and validate the NAFLD fibrosis score were Caucasians. Compared to Chinese, Caucasian NAFLD patients have higher body mass index and a higher prevalence of advanced fibrosis.

In a prospective cohort of 162 biopsy-proven NAFLD patients, we validated the NAFLD fibrosis score in Chinese.[Wong 2008-2] Eighteen (11%) patients had advanced fibrosis. Using the proposed low cutoff point of -1.455, 117 of 128 (91%) patients without advanced fibrosis were correctly staged, while 11 (9%) patients were under-staged. The negative predictive value of this cutoff point was 91%. If liver biopsies were only performed in patients with NAFLD fibrosis score above -1.455, 128 (79%) of 162 liver biopsies could be avoided.

These data confirm that the NAFLD fibrosis score has high negative predictive value in the Chinese population with low prevalence of advanced fibrosis.

Implementation of the score as a screening test may relieve the burden of liver biopsies in the vast majority of cases.

Conclusions

These studies confirm that significant necroinflammation and fibrosis occur in Hong Kong Chinese patients with NAFLD. Progression of liver fibrosis is common. The diagnosis of NAFLD may predate the diagnosis of hypertension and diabetes. Using the ethnicity-specific criteria by the International Diabetes Federation, the vast majority of NASH patients and around half of the patients with simple steatosis suffer from metabolic syndrome. Hypoadiponectinemia is associated with NAFLD and NASH patients have significantly higher level of tumor necrosis factor-alpha. These adipokines may have important role in the pathogenesis of NAFLD. On the other hand, gene polymorphisms of adiponectin and tumor necrosis factor-alpha were not associated with the development of NAFLD or NASH. Moreover, post-challenge hyperglycemia is common in NAFLD patients and is associated with advanced fibrosis. Oral glucose tolerance test is useful in NAFLD patients for detecting undiagnosed diabetes and predicting advanced NAFLD. Though liver biopsy remains the gold standard in assessing the degree of liver fibrosis, the NAFLD fibrosis score has good negative predictive value in excluding Chinese NAFLD patients with advanced fibrosis.

Captions for tables

Caption for figures

List of abbreviations

ALT	Alanine aminotransferase
APRI	Aspartate aminotransferase-to-platelet ratio index
AST	Aspartate aminotransferase
AUROC	Area under the receiver operating characteristics curve
BMI	Body mass index
CI	Confidence interval
HDL	High density lipoprotein
HOMA-IR	Homeostasis model assessment of insulin resistance
IDF	International Diabetes Federation
IKK	Inhibitor of nuclear factor kappa-B kinase
JNK	Jun N-terminal kinase
LDL	Low density lipoprotein
NAFLD	Nonalcoholic fatty liver disease
NASH	Nonalcoholic steatohepatitis
NCEP-ATPIII	National Cholesterol Education Program: Adult Treatment Panel III
NF-κB	Nuclear factor kappa-B
PPAR	Peroxisome proliferators-activated receptor
TNF-α	Tumor necrosis factor-alpha
WHO	World Health Organization
WHR	Waist-hip ratio

PART I

LITERATURE REVIEW

CHAPTER ONE

EPIDEMIOLOGY OF NONALCOHOLIC FATTY LIVER DISEASE

1.1 Global epidemiology

Nonalcoholic fatty liver disease (NAFLD) is the most common chronic liver disease in affluent countries. [Farrell 2005] It may progress to liver cirrhosis and hepatocellular carcinoma. [Bugianesi 2002] Although the proportion of NAFLD patients dying from liver-related complications is lower than that of patients with other chronic liver diseases such as chronic hepatitis B and C, it is anticipated that NAFLD will still emerge as a global health burden because the total number of sufferers is increasing.[Adams 2005, Ekstedt 2006] It is estimated that patients with fatty liver had 26% higher overall health care costs than the general population.[Baumeister 2008]

Unlike other chronic liver diseases, the diagnosis of NAFLD is based on histology instead of serological or biochemical markers. Therefore, the prevalence of NAFLD in the community can only be estimated by indirect measures. The most common method is by population screening using ultrasound scan. The reported prevalence of NAFLD was around 23-45% in the United States and 16-20% in Italy (Table 1.1). [Browning 2004, Lonardo 1997, Bellantini 2000] Nevertheless, ultrasound scan has limited sensitivity for mild hepatic steatosis. The typical features of bright liver echotexture, attenuation of ultrasound signal, and blurring of vascular structures are apparent when 30% or more of hepatocytes are

steatotic.[Saadeh 2002, Chan 2007] Therefore, the true prevalence of NAFLD is likely to be even higher.

Another method to estimate the prevalence of NAFLD is to perform autopsies on patients dying from accidents. The main advantage of this method is that histology is the gold standard for the diagnosis of NAFLD. Milder steatosis can be detected. Besides, nonalcoholic steatohepatitis (NASH), a more severe and active form of NAFLD, can also be detected. Using this method, the prevalence of NAFLD in the community is estimated to be 16-24%, and the prevalence of NASH is 2.1% (Table 1.1).[Hilden 1977, Ground 1982] However, these studies often lack comprehensive assessment of metabolic profile. The history on alcohol consumption is also less reliable.

Indirect evidence suggests that NAFLD is also affecting adolescents. Among 5586 American adolescents aged 12 to 19 years, elevated alanine aminotransferase (ALT) above 30 U/l was found in 7.4% of white adolescents, 11.5% of Mexican American adolescents, and 6.0% of black adolescents.[Fraser 2007] Waist circumference and fasting insulin level were independent factors associated with elevated ALT level.

In selected high-risk groups, the prevalence of NAFLD is considerably higher. Among 97 patients with body mass index (BMI) above 40 kg/m^2 who had liver

biopsy during Roux-en-Y gastric bypass surgery, hepatic steatosis and NASH were detected in 89% and 36%, respectively.[Gholam 2007] Even in hypertensive patients with normal liver enzymes, 31% had NAFLD, compared to 13% among controls without high blood pressure.[Donati 2004]

Recently, women with polycystic ovary syndrome, a condition characterized by marked insulin resistance, were also found to have a high prevalence of NAFLD. In a retrospective study of 88 pre-menopausal women with polycystic ovary syndrome, 48 (55%) had NAFLD.[Gambarin-Gelwan 2007] Although higher BMI, insulin resistance, low HDL-cholesterol and impaired fasting glucose were associated with NAFLD, 39% of the women with both NAFLD and polycystic ovary syndrome had BMI below 25 kg/m^2. In another prospective study of 41 women of polycystic ovary syndrome in Chile, 17 (42%) had NAFLD.[Cerda 2007]

NAFLD appears to be more common in children of people with metabolic syndrome. Among 1732 participants of the Framingham Offspring Study, offsprings with paternal early-onset obesity were more likely to have elevated ALT levels compared to those without paternal obesity (odds ratio 1.75; 95% CI 1.06-2.89; p=0.03).[Loomba 2008]

Table 1.1 Prevalence of NAFLD in Western studies

Author/year	Population	N	Prevalence
Ultrasound studies			
Lonardo 1997	Italian patients	363	19.8%
Bellentani 2000	Northern Italy	257	76% in obese persons, 16% in controls
Proton magnetic resonant spectroscopy			
Browning 2004	Urban US	2287	45% in Hispanic men, 45% in Hispanic women, 42% in white men, 24% in white women, 23% in black men, 24% in black women
Autopsy studies			
Hilden 1977			24%
Ground 1982			16% had NAFLD. 2.1% had NASH.

1.2 Asian epidemiology

Traditionally, the Asian population is believed to be leaner and carries a lower risk of metabolic syndrome and its complications. However, with the adoption of western lifestyle, metabolic syndrome is on the rise in most Asian countries. In a cross-sectional survey of 15540 Chinese adults, the prevalence of metabolic syndrome was 9.8% in men and 17.8% in women, according to the guidelines from the US National Cholesterol Education Program.[Gu 2005] The prevalence of type 2 diabetes in India, Japan, Korea, and Hong Kong is equal to or higher than that in the United States and some European countries.[Yoon 2006]

It is important to note that China is a big country with significant regional variation in disease prevalence. In early 1990s, a study was conducted on 1513 subjects from a public utility company and a regional hospital in Hong Kong.[Cockram 1993] The overall prevalence of diabetes and impaired glucose tolerance was 4.5% and 7.3%, respectively. The prevalence of diabetes in Hong Kong was similar to that in Korea, Thailand and Singapore, but higher than that in Mainland China.[Asia Pacific Cohort Studies Collaboration 2007] A high prevalence of metabolic syndrome was also found in Hong Kong Chinese.[Ko 2005] According to the World Health Organization criteria, the overall prevalence of metabolic syndrome was 13.4%. The crude prevalence of metabolic syndrome by the International Diabetes Federation criteria was 7.4%.[Ko 2006]

Although the prevalence of diabetes and metabolic syndrome is generally higher in affluent regions, glucose intolerance is more commonly observed in subjects with low socio-economic status. In a study of 2847 Chinese subjects with known risk factors for glucose intolerance, age-adjusted odds ratio of having diabetes was 4.5 in female with the lowest socio-economic status compared to those with the highest socio-economic status.[Ko 2001] The findings echo Caucasian studies showing that low socio-economic status is associated with obesity, diabetes and coronary heart disease.[Rose 1981, Marmot 1986, Millar 1986, Woodward 1992] Educated people are more health-conscious and lead a more healthy lifestyle.

Similar to metabolic syndrome, the prevalence of NAFLD is increasing in Asia (Table 1.2). According to a recent review by the Asia-Pacific Working Party on NAFLD, the prevalence of NAFLD ranges from 5% in Singapore to 30% in Japan and Indonesia (Figure 1.1).[Amarapurkar 2007] In 2001, 4401 apparently healthy Japanese adults underwent medical health checkup. Eight hundred and twelve (18%) subjects were found to have NAFLD. Importantly, after a mean follow-up of 414 days, 308 of 3147 subjects (10%) who did not have fatty liver at baseline developed incident NAFLD.[Hamaguchi 2005]

Another Japanese study suggests that the prevalence of NAFLD is rising. Among 39151 subjects attending the Tokai University Hospital Health Checkup Center,

the prevalence rose from 12.6% in 1989 to 30.3% in 1998.[Kojima 2003] BMI was the parameter most correlated with the onset of fatty liver.

Although the prevalence of metabolic syndrome is lower in Mainland China than other affluent Asian countries such as Japan and Korea, a significant proportion of the Chinese population also suffers from NAFLD. Among 3175 adults in Shanghai, China, 17% had fatty liver and 15% had NAFLD.[Fan 2005] Obesity increased the risk of fatty liver by 4.8 fold.

There are few histological studies in Asia. Among 60 NAFLD patients in Hong Kong, most had features of metabolic syndrome.[Tsang 2006] Ninety percent had BMI above 25 kg/m^2, 70% had hypertriglyceridemia, 68% had hypercholesterolemia, 58% had metabolic syndrome, 53% had hypertension, 47% had diabetes mellitus, and 18% had obstructive sleep apnea. Thirty percent had septal fibrosis. Significant necroinflammation and diabetes were independent factors associated with septal fibrosis. Among 75 NAFLD patients in Malaysia who had liver biopsies, 59 (84%) and 8 (11%) had NASH and cirrhosis, respectively.[Malik 2007] Male gender and Indian race were independent factors associated with liver fibrosis.

Patients with metabolic syndrome are at increased risk of NAFLD (Table 1.3). In patients with type 2 diabetes, 30-90% have NAFLD. [Jimba 2005, Fan 2005, Fan

2007, Park 2006, Gupte 2004, Amarapurkar 2002] Similarly, 10-80% and 26-58% of patients with obesity and dyslipidemia suffer from NAFLD.[Jimba 2005, Fan 2005, Fan 2007, Park 2006, Chen 2006]

Table 1.2 Prevalence of NAFLD in Asian studies

Author/year	Population	N	Prevalence
Ultrasound studies			
Omagari 2002	Japanese	3432	Overall 22% had fatty liver. Among non-alcoholics, 9% had fatty liver
Kojima 2004	Japanese	39151	13% in 1989, 30% in 1998 (did not exclude alcohol users)
Fan 2005	Shanghai Chinese	3175	17% had fatty liver, 15% had NAFLD
Hamaguchi 2005	Japanese	4401	18%

Table 1.3 Prevalence of NAFLD in high-risk groups (modified from Amarapurkar 2007)

Country	Diabetes (%)	Obesity (%)	Dyslipidemia (%)
Japan	40-50	50-80	42-58
China	35	70-80	57
Korea	35	10-50	26-35
India	30-90	15-20	Not reported
Indonesia	52	47	56

Figure 1.1 Prevalence of NAFLD in Asia (modified from Amarapurkar 2007)

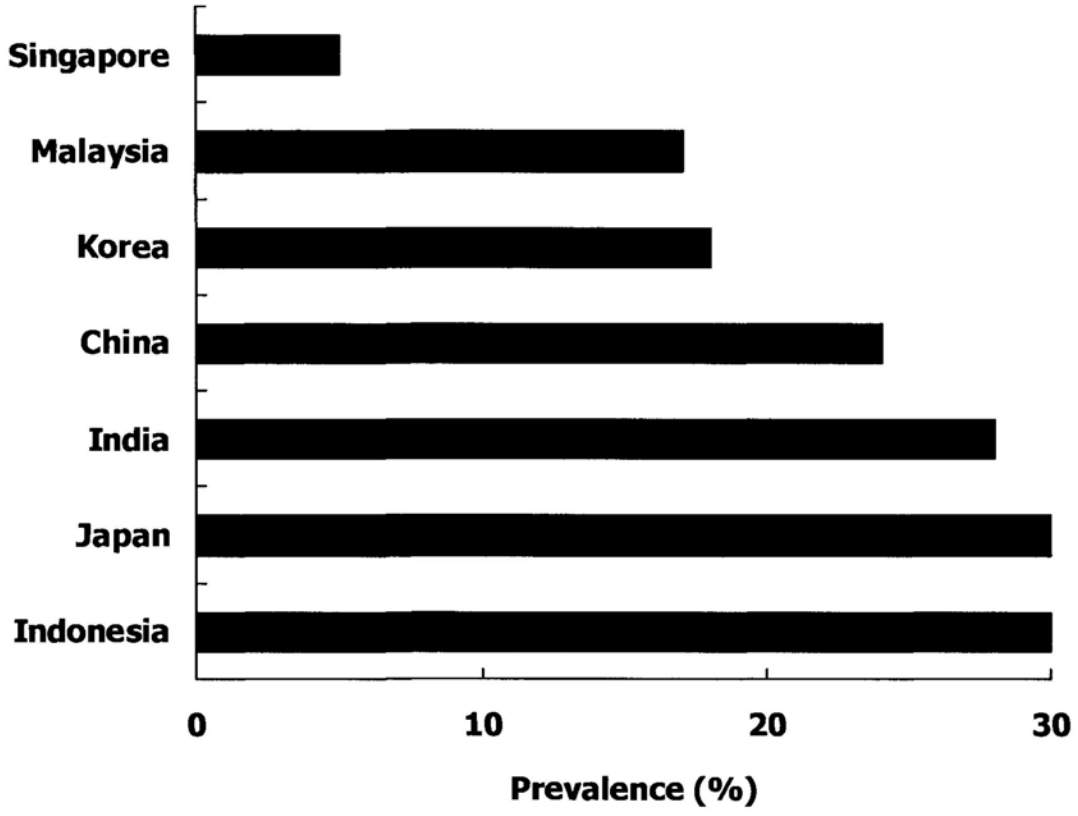
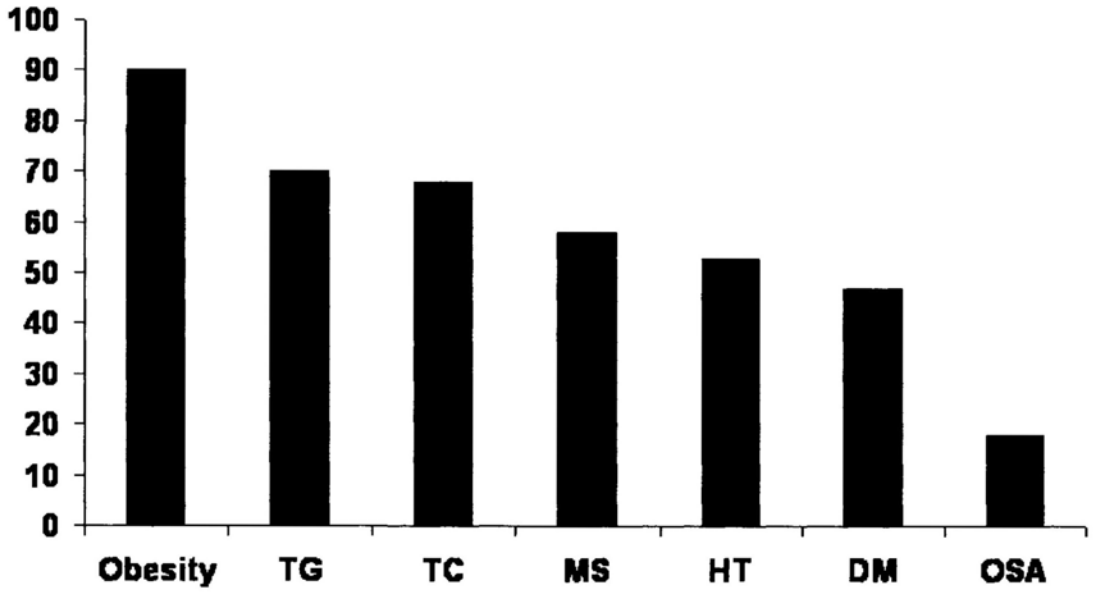


Figure 1.2 Comorbid illnesses among Hong Kong NAFLD patients [Tsang 2006]



TG: hypertriglyceridemia; TC: hypercholesterolemia; MS: metabolic syndrome; HT: hypertension; DM: diabetes; OSA: obstructive sleep apnea

CHAPTER TWO

NATURAL HISTORY OF NONALCOHOLIC FATTY LIVER DISEASE

2.1 Clinical studies

NAFLD patients have increased mortality compared to the general population. They are at risk of cardiovascular complications and liver-related morbidities. In the Rochester Epidemiology Project, 420 NAFLD patients were identified in Olmsted County, Minnesota, between 1980 and 2000.[Adams 2005] The mean follow-up was 7.6 years (0.1 to 23.5 years). Compared to the general population, the survival of NAFLD patients was lower (standardized mortality ratio 1.34; 95% confidence interval [CI], 1.003-1.76; p=0.03). Cirrhosis was an independent factor associated with mortality (hazard ratio 3.1, 95% CI, 1.2-7.8). Thirteen (3.1%) patients developed liver-related complications. One patient required liver transplantation and two developed hepatocellular carcinoma. As a result, liver disease was the third leading cause of death in NAFLD patients, after malignancy and cardiovascular disease. In comparison, liver disease was only the thirteenth leading cause of death in the general population.

In another study at the Oskarshamn County Hospital, 129 NAFLD patients were identified between 1988 and 1993.[Ekstedt 2006] At a mean follow-up of 13.7 years, 26 NAFLD patients died. Similar to the finding of the Rochester Epidemiology Project, liver disease was the third leading cause of death in this cohort. One patient died of metastatic hepatocellular carcinoma, and one died of variceal bleeding. Five other patients developed cirrhotic complications (ascites in 2 patients, ascites and hepatocellular carcinoma in 2 patients, and hepatocellular

carcinoma in 1 patient). Overall, 5.4% of the study cohort developed cirrhotic complications during follow-up. Another important finding is that patients with simple hepatic steatosis did not have increased mortality compared to the general population. All 7 patients who developed cirrhotic complications had NASH at baseline. The absence of periportal fibrosis at baseline had 100% negative predictive value in predicting liver-related complications.

Another French group performed liver stiffness measurements by transient elastography (Fibroscan) in 429 consecutive apparently healthy subjects.[Roulot 2008] Liver stiffness values were higher in subjects with BMI above 30 kg/m^2 (6.3±1.9 vs. 5.4±1.5 kPa, p=0.0003). After adjustment for gender and BMI, metabolic syndrome was associated with higher liver stiffness values. Although this study was limited by the lack of histological or radiological evaluation of the liver, this suggests that liver fibrosis and cirrhosis may be more common in NAFLD subjects with the associated features of obesity and metabolic syndrome.

2.2 Histological studies

The histological progression of NAFLD is not clearly understood. Few histological series with paired biopsies after long-term follow-up exist in the literature. Most patients who had paired liver biopsies were high-risk individuals with advanced disease at baseline.

One of the largest cohorts included 103 NAFLD patients who underwent serial liver biopsies at a mean interval of 3.2 years (range 0.7 to 21.3 years).[Adams 2005-2] Fibrosis stage progressed in 37%, remained stable in 34%, and regressed in 29%. Diabetes and high BMI were associated with higher rate of fibrosis progression.

Among 106 NAFLD patients at the Professor Alejandro Posadas Hospital, Argentina, 22 underwent a second liver biopsy 4.3 years later (range 3.0-14.3 years).[Fassio 2004] Overall, 7 (32%) patients had progression of liver fibrosis. High BMI at baseline was associated with fibrosis progression.

In another retrospective study of 22 patients having paired liver biopsies at a mean of 5.7 years (1.4 to 15.7 years), 32% had increases in fibrosis score, and 18% had decreases in fibrosis score.[Harrison 2003] At baseline, 9% had stage 3 or 4 fibrosis. The percentage rose to 18% at the follow-up biopsies.

In another series of 42 NAFLD patients followed for a median of 4.5 years (range 1.5 to 21.5 years), one patients progressed from fibrosis to cirrhosis over 5 years and died from hepatocellular carcinoma.[Powell 1990] However, follow-up liver biopsies were not arranged routinely at designated time intervals, precluding a detailed analysis of the rate of histological progression.

Summarizing the reported data, histological progression appears to occur in up to one-third of NAFLD patients.

2.3 Other complications

NAFLD causes a full spectrum of liver disease from simple steatosis, NASH, to cirrhosis and hepatocellular carcinoma. Since the progression of NAFLD to end-stage liver disease likely develops over decades, most of our understanding is based on indirect evidence.

In three case series, patients with cryptogenic cirrhosis were more likely to suffer from diabetes and obesity than those with cirrhosis due to viral hepatitis.[Caldwell 1999, Poonawala 2000, Sakugawa 2003] In one study, 70 patients with cryptogenic cirrhosis were compared with 50 NASH patients, 39 patients with hepatitis C-related cirrhosis, and 33 patients with primary biliary cirrhosis.[Caldwell 1999] Diabetes or obesity was present in 73% of cryptogenic cirrhosis subjects and 70% of NASH subjects. The prevalence of diabetes or obesity was significantly higher than that of hepatitis C-related cirrhosis (28%) and primary biliary cirrhosis (33%) patients. Besides, hepatocellular carcinoma has been reported in cases of NASH.[Shimada 2002, Zen 2001] These data all suggest that cryptogenic cirrhosis may be a manifestation of burnt-out NASH, and hepatocellular carcinoma may develop.

Since NAFLD is closely related to obesity and metabolic syndrome, it is not surprising that it is also associated with cardiovascular diseases. In 85 healthy volunteers, the presence of hepatic steatosis by imaging studies was associated

with subclinical atherosclerosis.[Targher 2004] Carotid intima-media thickness was 1.18±0.14 mm in subjects with hepatic steatosis and 0.94±0.12 mm in subjects without hepatic steatosis ($p<0.001$).

Using the Framingham equation and the scores derived from the PROCAM study and the US National Cholesterol Education Program: Adult Treatment Panel III (NCEP-ATPIII) proposals, NAFLD patients were estimated to have higher 10-year risk of coronary events.[Villanova 2005] The risk was even higher in NASH patients than those with simple steatosis. In the same study, NAFLD patients were also found to have endothelial dysfunction when flow-mediated vasodilatation during reactive hyperemia was measured.

In the Valpolicella Heart Diabetes Study, 2103 out-patients with type 2 diabetes were followed prospectively.[Targher 2007] During 6.5 years of follow-up (range 5 to 84 months), 384 (18.3%) participants developed cardiovascular events (myocardial infarction, ischemic stroke, coronary revascularization, or cardiovascular death). Patients developing cardiovascular events were more likely to have NAFLD and elevated ALT level. In multivariate regression analysis, NAFLD remained an independent factor associated with incident cardiovascular event (hazard ratio 2.0, 95% CI 1.4-2.7).

Even in young non-diabetic adults, fatty liver was associated with abnormal left ventricular energy metabolism, as measured by the phosphocreatine-to-adenosine triphosphate ratio by magnetic resonance spectroscopy.[Perseghin 2008] In addition, one study showed that NAFLD subjects had lower peak oxygen consumption during treadmill exercise than control subjects.[Krasnoff 2008] The health-related fitness was also lower in NASH patients than those with simple steatosis.

CHAPTER THREE

PATHOGENESIS OF NONALCOHOLIC FATTY LIVER DISEASE

3.1 Two-hit hypothesis

The two-hit hypothesis was first proposed by Christopher Day in 1998 (Figure 3.1.[Day 1998] Since then, this has remained a popular model to describe the pathogenesis of NAFLD. According to this model, the first hit is characterized by the development of steatosis. A number of mechanisms are involved in the second hit, which finally results in necroinflammation and fibrosis. At present, the pathogenesis of NAFLD is not completely understood. Oxidative stress and lipid peroxidation have important roles in liver injury. Abnormal production of various cytokines leads to inflammation, fibrosis and insulin resistance. Endoplasmic reticulum stress, apoptosis and innate immunity are also involved in the development of NASH. These mechanisms will be discussed in this chapter. The relationship between adipokines and NAFLD is further discussed in section 4.2. Figures 3.2 to 3.4 demonstrate histological features of different spectrums of NAFLD in local patients.

Figure 3.1 The two-hit model describes the pathogenesis of NASH

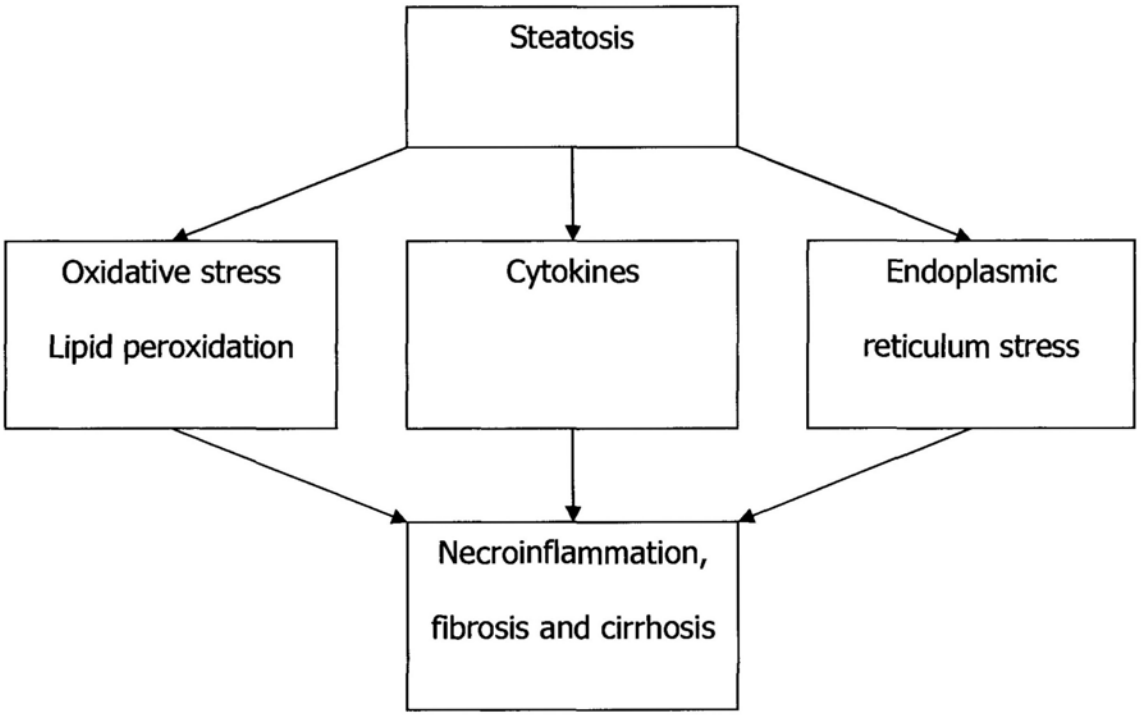


Figure 3.2 Histological feature of a patient with simple steatosis. Macrovesicular steatosis is noted in over two-thirds of hepatocytes. (Courtesy of Dr Anthony Chan)

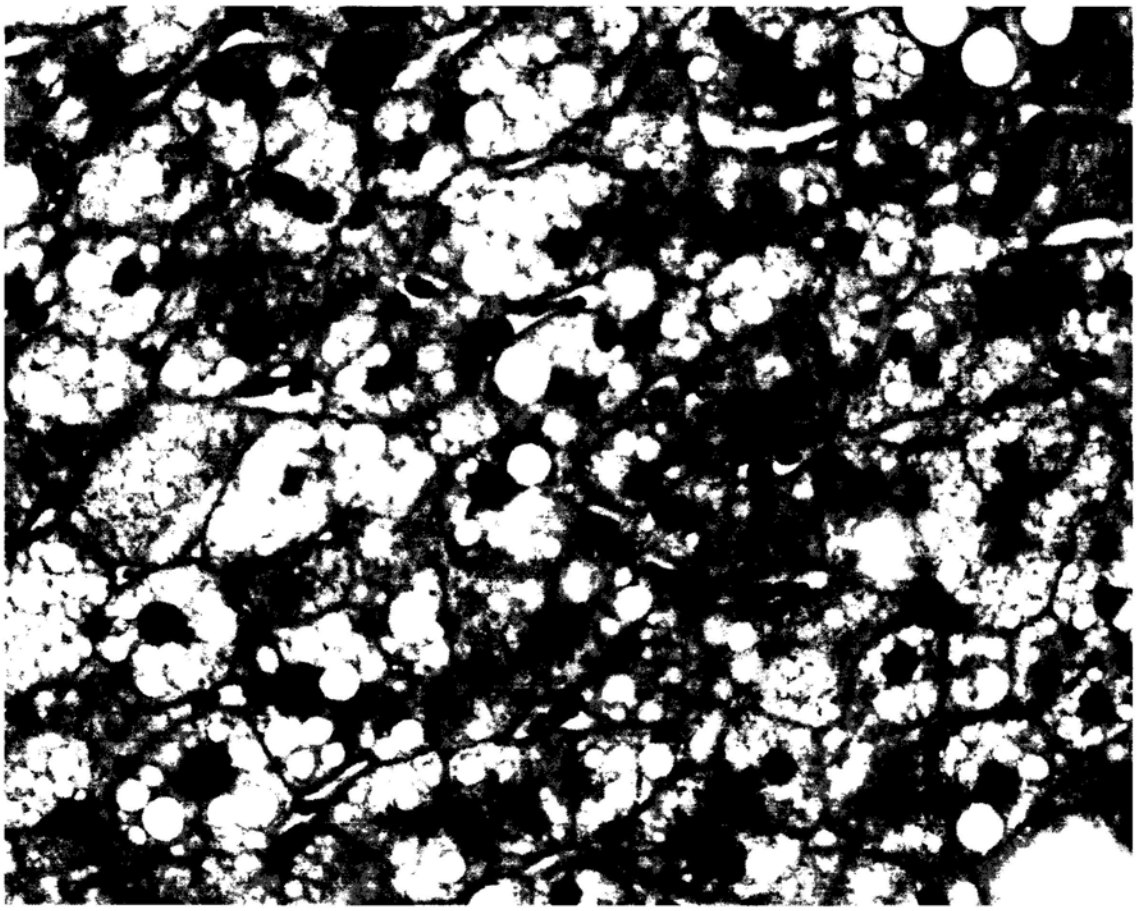


Figure 3.3 Histological features of a patient with nonalcoholic steatohepatitis. Lobular inflammation, ballooning, and Mallory's hyaline are common features. (Courtesy of Dr Anthony Chan)

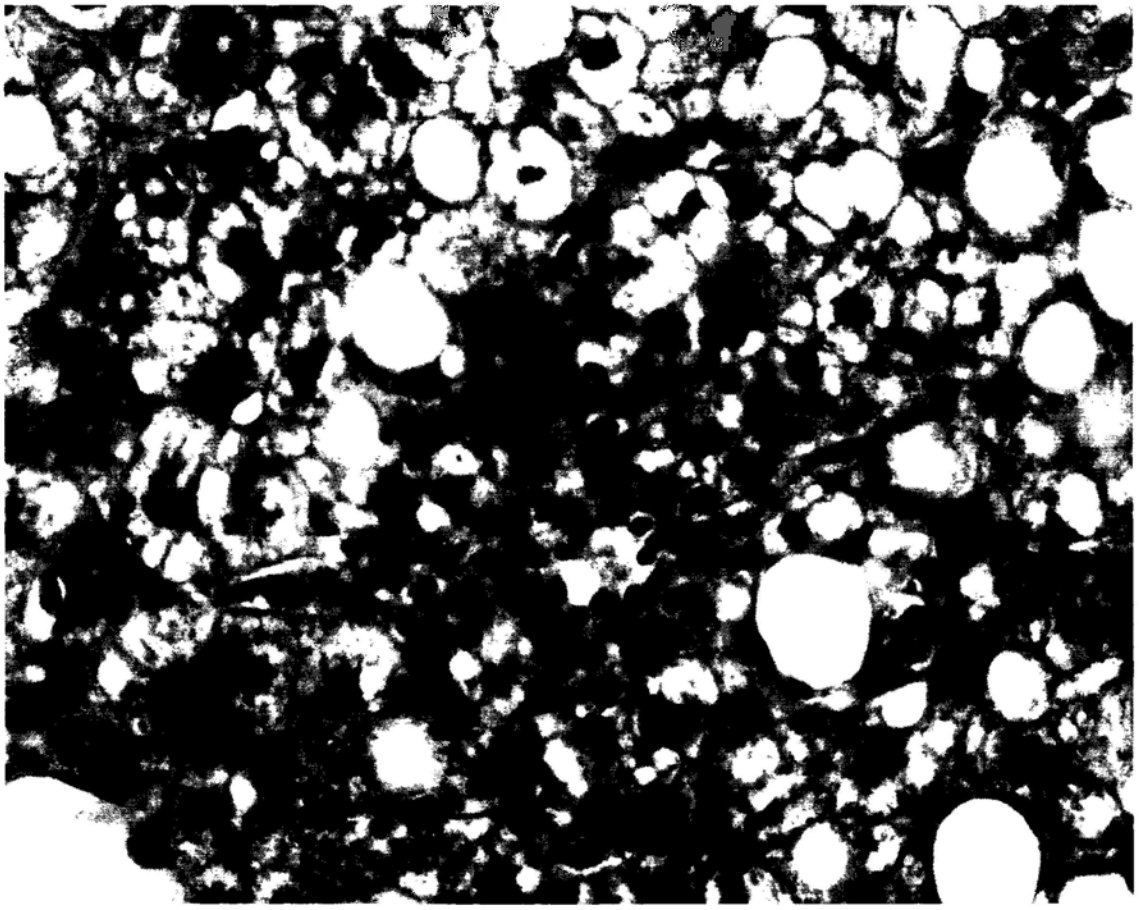
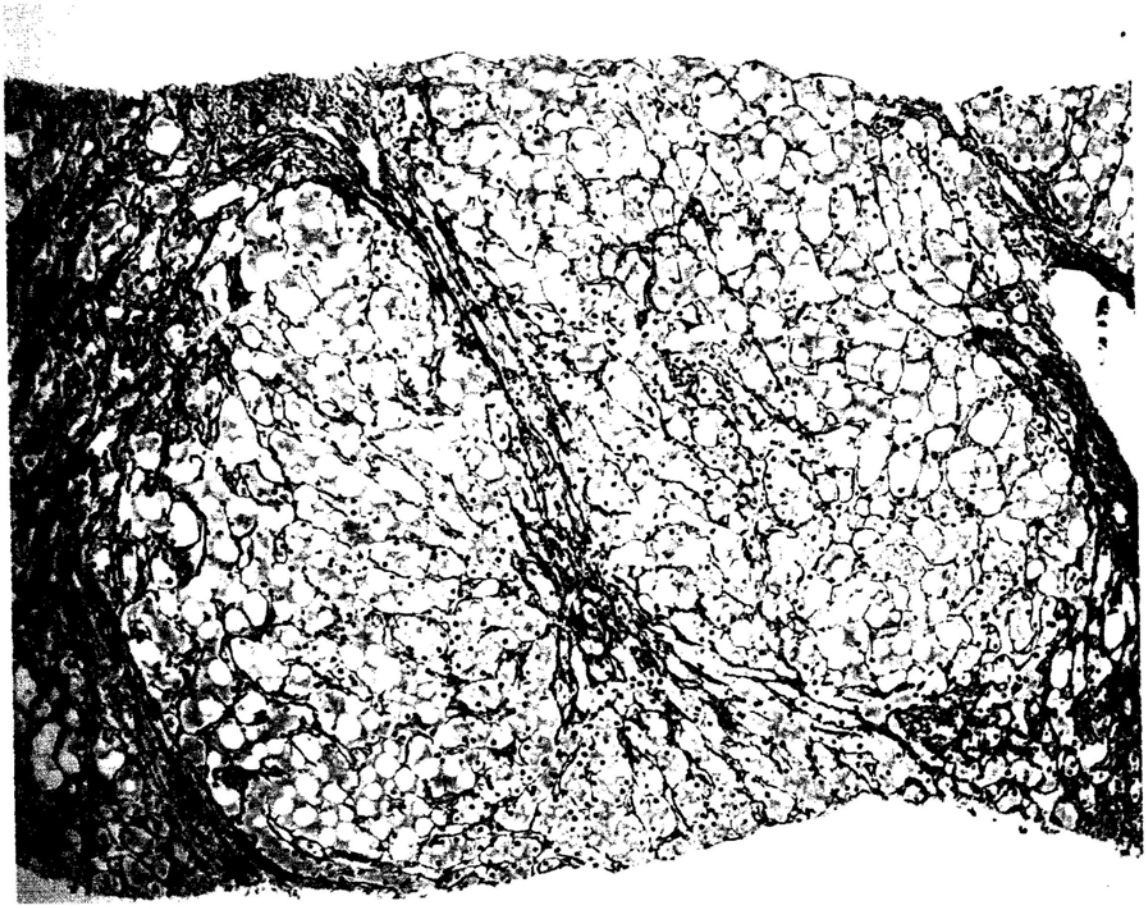


Figure 3.4 Histological features of a patient with NAFLD-related cirrhosis. Thick fibrous septa and nodule formation are evident. (Courtesy of Dr Anthony Chan)



3.2 Lipid peroxidation and oxidative stress

Lipid peroxidation and oxidative stress have been unequivocally demonstrated in animal models of NASH and human subjects.[Letteron 1996, Leclercq 2000, Sanyal 2001, Garcia-Monzon 2000, Seki 2002] The severity of NASH is closely associated with the degree of oxidative stress.[Feldstein 2003, Seki 2002] Knockout mice deficient in hepatic S-adenosylmethionine spontaneously develop steatohepatitis and cirrhosis.[Lu 2001] Subsequent functional proteomics study on this model confirm that the liver injury occurs chiefly because several proteins involved in mitochondrial function (prohibitin 1, cytochrome c oxidase I and II, and ATPase beta-subunit) are targets of S-adenosylmethionine.[Santamaria 2003] Using a similar mechanism, mice receiving methionine choline deficient diet also develop steatohepatitis without induction of insulin resistance.[Yu 2006, Shen 2008]

Peroxidation of plasma and mitochondrial membrane may induce apoptosis. Apoptosis and caspase activation are prominent features of NASH.[Feldstein 2003] Besides, the degree of apoptosis is associated with the severity of NASH.[Feldstein 2004, Ribeiro 2004, Ramalho 2006] In fact, plasma level of caspase 3-generated cytokeratin-18 fragments has been tested as a marker of NASH.[Wieckowska 2006] 4-hydroxynonenal, a product of oxidative stress, stimulates extracellular matrix deposition by activated human hepatic stellate

cells.[Zamara 2004] This supports the involvement of oxidative stress in accelerated liver fibrosis.

Lipid peroxidation and the subsequent immune response have been implicated in the progression of NAFLD. In a study involving 79 patients with simple steatosis, 74 patients with NASH, 14 patients with NAFLD-related cirrhosis, and 59 control subjects, titers of immunoglobulin G against human serum albumin adducted with malondialdehyde or arachidonic acid hydroperoxide and against oxidized cardiolipin were increased in NAFLD subjects.[Albano 2005] While there was strong association between oxidative stress dependent immune response and NAFLD, the same relationship was not observed for obesity, type 2 diabetes, or serum cholesterol.

The source of reactive oxygen species includes inflammatory cells after NASH sets in, hepatocyte itself, and adipose tissue. Peripheral insulin resistance results in unopposed lipolysis and increased flux of free fatty acid into the liver.[Nielsen 2004] Accumulation of fat in the liver leads to hepatic insulin resistance, which further promotes the entry of free fatty acid in mitochondria, where oxidation occurs.[Samuel 2004, Cai 2005] Moreover, free fatty acids and their metabolites are ligands for peroxisomal proliferator-activated receptor alpha (PPARα). Upregulation of PPARα promotes mitochondrial, peroxisomal and microsomal fat oxidation, and results in generation of reactive oxygen species.[Reddy 2001]

Outside the liver, reactive oxygen species are also produced in adipose tissue of obese animals and humans. Augmented expression of NADPH oxidase and decreased expression of anti-oxidative enzymes are observed.[Furukawa 2004] Oxidative stress also leads to dysregulated production of adipokines, including adiponectin, plasminogen activator inhibitor-1, interleukin-6, and monocyte chemotactic protein-1.

Recently, we demonstrated that a herbal compound, *Phyllanthus urinaria*, ameliorated the severity of NASH.[Shen 2008] In two cell line models and two animal models, *P. urinaria* suppressed lipid peroxidation and inflammation.

3.3 Endoplasmic reticulum stress

Endoplasmic reticulum stress was first implicated in the pathogenesis of alcoholic liver disease.[Ji 2003] When there is endoplasmic reticulum stress, cell death pathways are activated, and translocation of client proteins is reduced.[Ron 2002] Activation of transcription factors leads to up-regulated lipid synthesis via sterol regulatory element-binding proteins. In nutritional models of NASH, endoplasmic reticulum stress pathway is activated, as evidenced by phosphorylation of the pancreatic endoplasmic reticulum kinase (PERK), nuclear factor kappa-B and C-jun N-terminal kinase.[Rahman 2007] In the livers of apolipoprotein E knockout mice, inflammatory stress increased the expression of LDL receptor, sterol regulatory element-binding protein cleavage-activating protein, and sterol regulatory element-binding protein.[Ma 2008] This increases cholesterol influx and progression of NAFLD.

3.4 Innate immunity

Leukocytes, soluble mediators and receptors are components of the innate immune system. Fat-induced proinflammatory pathways can increase insulin resistance. Besides, adipose tissue inflammation not only increases hepatic steatosis but also stimulates innate immune response in the liver.

Fat accumulation stimulates a number of serine kinases in adipose tissue, skeletal muscle, and the liver.[Gao 2004, Kim 2004, Yu 2002, Solinas 2006, Samuel 2007] The serine kinases phosphorylate regulatory serine residues on the insulin receptor substrates IRS-1 and IRS-2, resulting in reduced tyrosine phosphorylation and insulin response. Among the serine kinases, Jun N-terminal kinase (JNK), inhibitor of nuclear factor kappa-B kinase (IKK), and novel isoforms of protein kinase C are most implicated in NAFLD. Apart from insulin resistance, these serine kinases are also proinflammatory signal molecules.[Shoelson 2006] As discussed in sections 3.2 and 3.3, proinflammatory pathways can be activated by lipid peroxidation and endoplasmic reticulum stress. Recently, Toll-like receptors are found to be activated by various non-microbial molecules.[Lee 2006] For example, Toll-like receptor-4 can be activated by fibronectin, fibrinogen, heparin sulfate and taxol. Similarly, both Toll-like receptors 2 and 4 can be activated by saturated fatty acids.

Fatty acids and high fat diet can activate serine kinases even in the absence of obesity and peripheral insulin resistance.[Samuel 2004] JNK and IKK further induce inflammation by activating activator protein-1 and nuclear factor kappa-B. Activation of JNK and IKK in hepatocytes also stimulates the expression of cytokines and cell-adhesion molecules.[Arkan 2005, Cai 2005, Schattenberg 2006, Tuncman 2006] In nutritional models of steatohepatitis, blockade of JNK or IKK reduces liver injury.[Schattenberg 2006, Dela Pena 2005]

Hepatic steatosis also upregulates Fas and stimulates apoptosis.[Feldstein 2003, Ribeiro 2004] Recently, the hepatocyte growth factor receptor Met was found to be dissociated from Fas in human NAFLD.[Zou 2007] Using structure-function studies, a YLGA amino acid motif of the Met α-subunit was found to be sufficient to bind to Fas and act as a potent Fas ligand antagonist. In *ob/ob* mice, synthetic YLGA peptide tempered hepatocyte apoptosis and liver damage.

Inflammation of adipose tissue was first demonstrated in 2003.[Wellen 2003] Inflammation in adipose tissue is mainly mediated by macrophages. Macrophage exists in at least two polarization states, M1 and M2. M1 represents activated macrophages having high antigen-presenting capacity, producing pro-inflammatory cytokines, and activating Th1 responses.[Verreck 2004] M2 represents macrophages expressing high levels of anti-inflammatory cytokines.

The link between macrophage polarization and NAFLD is mediated by the peroxisome proliferators-activated receptor-gamma (PPARγ). PPARγ is required for the M2 polarization.[Odegaard 2007] PPARγ agonists inhibit macrophage cytokine production by antagonizing activator protein-1, signal transducer and activator of transcription (STAT) and nuclear factor kappa-B.[Ricote 1998] In pilot studies in NASH patients, pioglitazone ameliorates histological features and improves insulin resistance.[Belfort 2006]

CHAPTER FOUR

METABOLIC SYNDROME AND NONALCOHOLIC FATTY LIVER DISEASE

4.1 Metabolic syndrome

Metabolic syndrome represents a group of closely linked disorders – central obesity, type 2 diabetes, hypertension and dyslipidemia. A number of metabolic syndrome criteria have been proposed (Table 4.1). The World Health Organization (WHO) first proposed diagnostic criteria of metabolic syndrome in 1998.[Alberti 1998] However, the criteria require demonstration of insulin resistance, which is not routinely assessed in clinical practice. The NCEP-ATPIII criteria included common clinical parameters and are more frequently used.[NCEP-ATPIII 2002]

Recently, new criteria of metabolic syndrome were issued by the International Diabetes Federation (IDF).[Alberti 2005] Although the IDF criteria are similar to the NCEP-ATPIII criteria, there are a few important differences. Firstly, the need for ethnic specific definition of central obesity was emphasized. This is based on studies linking waist circumference with other components of metabolic syndrome in different ethnic groups.[Tan 2004, Snehalatha 2003] As a result, cutoff values of normal waist circumference in men and women were issued (Table 4.2). Secondly, the cutoff of fasting glucose was lowered from 6.1 mmol/l to 5.6 mmol/l according to the new definition of impaired fasting glucose.[American Diabetes Association 2006] Thirdly, central obesity was considered an essential component of metabolic syndrome. The diagnosis of metabolic syndrome is made only if the waist circumference criteria are fulfilled.

The IDF criteria emphasize simplicity and usefulness in primary care for screening purpose. The definition also emphasizes the ethnic difference for central obesity. In Hong Kong Chinese, a waist circumference at or above 94 cm in men and 80 cm in women correlated well with a body mass index of 25 kg/m^2 or above.[Ko 1996] Similar cutoffs also correlated well with traditional cardiovascular risk factors such as diabetes, hypertension, hyperinsulinemia and albuminuria.[Ko 1997]

On the other hand, the WHO criteria attempt to predict risk, in particular cardiovascular risk. Among 5202 Chinese patients with type 2 diabetes, 4.3% with metabolic syndrome by the WHO criteria and 2.4% without metabolic syndrome died at a median follow-up of 2.1 years ($p=0.002$).[Ko 2006] When the same cohort was followed up to 7.1 years, the NCEP-APTIII, but not the IDF criteria, predicted the development of coronary heart disease and cardiovascular deaths.[Tong 2007]

Recently, a joint statement from the American Diabetes Association and the European Association for the Study of Diabetes challenged the significance of metabolic syndrome.[Kahn 2005] The choice of criteria and the rationale of thresholds were considered ill-defined. The value of including diabetes in the definition is questionable and it is unclear if insulin resistance is the unifying

cause. More importantly, there is yet convincing evidence to show that the cardiovascular risk associated with metabolic syndrome is higher than the sum of its parts. The treatment of metabolic syndrome is also not different from treatment for the individual components. In line with this argument, a case-control study showed that although patients with early-onset coronary heart disease were more likely to have metabolic syndrome, the syndrome was not an independent factor associated with coronary heart disease in multivariate analysis.[Iribarren 2006] Two large prospective studies including 4812 non-diabetic elderly also only showed modest association between metabolic syndrome and incident cardiovascular disease.[Sattar 2008]

Metabolic syndrome has close relationship with NAFLD. In a study involving 46 patients with histology-proven NAFLD, BMI was associated with hepatic steatosis and inflammation.[Ryan 2005] Subjects with metabolic syndrome had a higher degree of liver fibrosis than those without metabolic syndrome. Patients with more components of metabolic syndrome according to the NCEP-ATPIII criteria had more severe liver fibrosis in a dose-dependent manner.

In another study of 19 NASH patients and 19 age- and gender-matched controls, insulin sensitivity was lower and insulin secretion was higher in the NASH group.[Pagano 2002] Nine (47%) patients had metabolic syndrome according to the European Group for the Study of Insulin Resistance.

Obesity is also closely linked to metabolic syndrome and NAFLD. Nevertheless, it is important to note that Western definitions of obesity may not apply to the local population. Firstly, Asians may have lower BMI for the same age and percentage of body fat.[Wang 1994, Ko 1999-2, He 2001, WHO Expert Consultation 2004] Secondly, Asians tend to develop diabetes and complications at a lower BMI. In 27 cohort studies from Asia, New Zealand and Australia involving 154,989 participants, each 2 kg/m^2 lower BMI was associated with a 27% lower risk of diabetes.[Asia Pacific Cohort Studies Collaboration 2006] Increased risk was already evident in the group with BMI 22.5-24.9 kg/m^2. Similarly, each 2 kg/m^2 decrease in the body mass index is associated with a 12% lower risk of ischemic stroke, 8% lower risk in hemorrhagic stroke, and 11% lower risk of ischemic heart disease.[Asia Pacific Cohort Studies Collaboration 2004]

Table 4.1 Diagnostic criteria of metabolic syndrome

	WHO	NCEP-ATPIII	IDF
Diagnostic requirement	Hyperinsulinemia, high blood glucose with ≥2 other criteria	≥3/5 criteria	Central obesity plus ≥2 other criteria
Central obesity	Waist-hip ratio >0.9, BMI ≥30 kg/m2, or waist circumference >94 cm	Waist circumference >102 cm	Ethnic specific (see Table 3.2)
High triglycerides	≥1.7 mmol/l	≥1.7 mmol/l	≥1.7 mmol/l
Low HDL-cholesterol	<0.9 mmol/l	<1.0 mmol/l	<1.0 mmol/l in men, <1.3 mmol/l in women
High fasting glucose		≥6.1 mmol/l	≥5.6 mmol/l
High blood pressure	≥140/90 mmHg	≥130/85 mmHg	≥130/85 mmHg
Microalbuminuria	Urinary albumin excretion rate >20 μg/min or albumin-to-creatinine ratio ≥30 mg/g		

WHO: World Health Organization; NCEP-ATPIII: National Cholesterol Education Program: Adult Treatment Panel III; IDF: International Diabetes Federation

Table 4.2 Ethnic specific values for waist circumference

Ethnic group	Waist circumference (as measure of central obesity)
Europids	
Men	≥94 cm
Women	≥80 cm
South Asians	
Men	≥90 cm
Women	≥80 cm
Chinese	
Men	≥90 cm
Women	≥80 cm
Japanese	
Men	≥85 cm
Women	≥90 cm
Ethnic south and central Americans	Use south Asian recommendations until more specific data available
Sub-Saharan Africans	Use European data until more specific data available
Eastern Mediterranean and middle east (Arab) populations	Use European data until more specific data available

4.2 Adipokines

Adipose tissue is not only a storage organ for lipids but also an endocrine organ. It secretes a number of bioactive proteins collectively known as adipokines or adipocytokines, including leptin, adiponectin, resistin, tumor necrosis factor-alpha (TNF-α) and interleukin-6. These adipokines have many actions on insulin resistance and inflammation, and may have important roles in the pathogenesis of NAFLD.

In murine models of alcoholic and nonalcoholic fatty liver disease, delivery of recombinant adiponectin ameliorated hepatomegaly and steatosis, and reduced the inflammation and ALT level.[Xu 2003] Adiponectin enhances hepatic free acid oxidation and increases carnitine palmitoyltransferase I activity. Besides, adiponectin decreases the activities of acetyl-CoA carboxylase and fatty acid synthase, both involved in fatty acid synthesis. Adiponectin also suppresses the hepatic production of TNF-α. In human studies, reduced level of serum adiponectin is associated with obesity, insulin resistance, type 2 diabetes, and dyslipidemia, all closely related to the development of NAFLD.[Arita 1999, Hotta 2000, Weyer 2001, Hotta 2001, Matsubara 2002] In human leukocytes, adiponectin has been shown to induce anti-inflammatory mediators interleukin-10 and interleukin-1 receptor antagonist but inhibit proinflammatory cytokine interferon-gamma.[Wolf 2004] In 109 Australian NAFLD patients and 82 healthy volunteers, hypoadiponectinemia was associated with NAFLD and NASH.[Hui

2004] Subsequently, the association between reduced level of adiponectin and NAFLD has been confirmed in several Caucasian studies, both in adults and adolescents.[Bugianesi 2005, Pagano 2005, Aygun 2006, Louthan 2005, Zou 2005, Musso 2005, Sargin 2005]

Among Asians, only indirect evidence for the association between NAFLD and adiponectin exists. In 791 male Japanese workers, adiponectin level was inversely proportional to the ALT, aspartate aminotransferase (AST), and γ-glutamyltransferase levels.[Yokoyama 2004] Thirty-eight Koreans with ultrasound features of fatty liver had lower adiponectin level than controls.[Yoon 2005] Moreover, hypoadiponectinemia was also found in conditions related with hepatic steatosis, such as chronic hepatitis C and alcoholic liver disease.[Petit 2005]

The association between TNF-α and NAFLD is more controversial. Intrahepatic TNF-α mRNA expression was increased in NAFLD patients, more so among patients with liver fibrosis.[Crespo 2001] Administration of pentoxifylline, a TNF-α inhibitor, might lead to normalization of ALT among NAFLD patients.[Adams 2004-2, Satapathy 2004] In a murine model, free fatty acid-mediated lipotoxicity was shown to be mediated through lysosomal destabilization and nuclear factor kappa B-dependent TNF-α expression.[Feldstein 2004] In contrast, anti-TNF antibodies protected *ob/ob* mice against liver injury.[Li 2003] Whereas the above

findings suggest that TNF-α has significant contribution in the pathogenesis of NASH, other experimental data also suggest that it might not be essential. *Ob/ob* mice lacking both TNF receptors have similar hepatic lipid content and serum aminotransferase level as mice without TNF receptor knockout.[Memon 2001] Similarly, in mice receiving intragastric overfeeding, steatohepatitis develops even after knockout of TNF type 1 receptor.[Deng 2005] In mice receiving methionine- and choline-deficient diet, TNF and TNF type 1 receptor gene deleted mice had similar degree of steatohepatitis.[Dela Pena 2005]

4.3 Gene polymorphisms of adipokines

Although adiponectin and TNF-α may be implicated in different stages of NAFLD development, the association may be causal or secondary. On one hand, patients with particular genetic make-up may have unfavorable adipokine profiles and are more prone to develop NAFLD. On the other hand, the adipokine profile may be just a global manifestation of insulin resistance without any causal role.

Genetic polymorphisms of the adiponectin gene have not been extensively studied in NAFLD patients. The -11391A, +45G and +276T alleles were shown to be associated with hypoadiponectinemia in diabetic patients.[Woo 2006, Jang 2005, Mackevics 2006] In patients without diabetes, the +45G allele was also associated with central obesity and insulin resistance.[Menzaghi 2002] Besides, the +276T allele was more prevalent in young individuals with coronary artery disease.[Filippi 2005]

A recent report from Japan showed that the TNF-α -1031C and -863A polymorphisms were associated with NASH and increased serum level of soluble TNF-α receptor-2.[Tokushige 2007] Using concanavalin A-activated peripheral blood mononuclear cells, TNF-α production was also increased by around 2-fold in blood donors with the -1031C/-863A allele.[Higuchi 1998] The -238A allele was also more prevalent in Italian NAFLD patients than controls, and was associated with insulin resistance.[Valenti 2002] These genetic relationship

provides indirect evidence suggesting a causal relationship between TNF-α production and the pathogenesis of NAFLD.

4.4 Oral glucose tolerance test and post-challenge hyperglycemia

Since NAFLD is closely related to type 2 diabetes, it is important to evaluate how impaired glucose tolerance and type 2 diabetes are diagnosed. Impaired glucose tolerance was first defined by the WHO and the National Diabetes Data Group in 1979.[World Health Organization 1985, National Diabetes Data Group 1979] This was to identify patients with increased cardiovascular risk and subsequent development of type 2 diabetes.

Type 2 diabetes is diagnosed if the fasting glucose is at or above 7.0 mmol/l, or the 2-hour post-challenge plasma glucose is at or above 11.1 mmol/l after a standard 75 g oral glucose tolerance test (Table 4.3).[World Health Organization 1999, American Diabetes Association 2006] However, the American Diabetes Association discourages clinicians to perform oral glucose tolerance test because of the greater costs and inconvenience. In contrast, the WHO Consultation retained oral glucose tolerance test as an integral part of the diagnosis of diabetes.

Compared to the older diagnostic criteria, the fasting glucose cutoff value has been lowered from 7.8 mmol/l to 7.0 mmol/l for diabetes, and 6.1 mmol/l to 5.6 mmol/l for impaired fasting glucose.[American Diabetes Association 2006] The lowered cutoffs are supposed to correlate better with post-challenge plasma glucose level. Among 680 oral glucose tolerance tests performed in Chinese

subjects, a fasting glucose cutoff of 5.6 mmol/l correlated best with a post-challenge glucose level of 11.1 mmol/l, with sensitivity and specificity of 87%.[Cockram 1992] Besides, the cutoff for normal fasting glucose is based on the level associated with increased risk of developing diabetic retinopathy. However, in three cross-sectional studies with standardized retinal photograph examination (the Blue Mountains Eye Study, n=3162; the Australian Diabetes, Obesity and Lifestyle Study, n=2182; the Multi-Ethnic Study of Atherosclerosis, n=6079), no clear threshold effect of the fasting glucose was found.[Wong 2008] Using fasting glucose above 7.0 mmol/l as the cutoff, the sensitivity in detecting diabetic retinopathy was less than 40%. In 17 cohort studies in the Asia-Pacific region involving 237,468 participants, continuous positive associations between fasting glucose and cardiovascular risk were observed at a glucose level down to 4.9 mmol/l.[Asia Pacific Cohort Studies Collaboration 2004-2]

Local data also support the relevance of post-challenge hyperglycemia. In Northern China, 11.3% of patients with impaired glucose tolerance progress to diabetes per year.[Pan 1997] In Hong Kong, half of the patients with impaired glucose tolerance developed diabetes in 4 years.[Ko 1999-3] The 2-hour plasma glucose is also an independent factor associated with increased blood pressure in Chinese women.[Ko 1999-4]

Moreover, some studies suggest that fasting and post-challenge plasma glucose criteria may diagnose two different groups of diabetic patients. The DECODA Study Group re-analyzed data of Asian people between 30 and 89 years of age from 11 population-based studies (n=17666), 6 pre-selected hyperglycemic cohorts (n=12221), and one suspected diabetic cohort (n=8382).[Qiao 2000] The concordance of the fasting and post-challenge plasma glucose criteria for diabetes was only 37%. Among 1215 subjects diagnosed to have diabetes by either criterion, only 449 met both criteria. Among 14718 patients with fasting glucose below 6.1 mmol/l, 1984 (13%) patients had impaired glucose tolerance and 291 (2%) patients had type 2 diabetes. Patients who were diagnosed to have diabetes by the fasting glucose criteria were younger than those diagnosed by the post-challenge criteria. In men, the mean age of patients who fulfilled only the fasting criteria and only the post-challenge criteria was 53 and 59 years, respectively. Corresponding age in women was 56 and 60 years, respectively.

Similarly, the DECODE Study Group included data from 13 studies from nine European countries with 7680 men and 9251 women aged 30 to 89 years.[The DECODE Study Group 2003] Mean 2-hour plasma glucose concentration increased linearly with age, but fasting glucose did not. Older patients with diabetes were more likely to have isolated post-challenge hyperglycemia.

Since the prevalence of diabetes in NAFLD patients is high, isolated post-challenge hyperglycemia is likely to be common. Among nineteen Italian patients with histological evidence of NASH, 5 had impaired glucose tolerance and 1 had impaired fasting glucose.[Pagano 2002] Even when NASH patients with normal glucose regulation were included, this cohort had lower insulin sensitivity and higher total insulin secretion than control subjects. In 114 Turkish patients with elevated ALT levels and bright liver on ultrasonography, 50 had impaired glucose tolerance or diabetes according to the post-challenge glucose level.[Sargin 2003]

Another study included 1950 subjects attending a general health examination program in Japan.[Jimba 2005] NAFLD was diagnosed in 566 (29%) patients. NAFLD occurred in 27% of patients with normal fasting glucose, 43% of patients with impaired fasting glucose, and 62% of patients with newly diagnosed diabetes. NAFLD was independently associated with increasing fasting glucose by multivariate regression analysis. Oral glucose tolerance test was not performed in this study.

Even in NAFLD patients with normal lipid profile who were normotensive and non-diabetic, peripheral glucose disposal was markedly decreased due to impaired glucose oxidation and glycogen synthesis, as demonstrated by a two-step euglycemic insulin clamp coupled with tracer infusion and indirect calorimetry.[Bugianesi 2005] Lipid oxidation was significantly related to

endogenous glucose production, glucose disposal, hepatic steatosis, and the oxidation of low density lipoprotein (LDL) cholesterol particles.

Further studies suggest that isolated post-challenge hyperglycemia can have serious clinical consequence. Among 134 Japanese patients with acute coronary syndrome, 50 (37%) and 13 (10%) had impaired glucose tolerance and diabetes, respectively.[Hashimoto 2005] Fifty-three (40%) had isolated post-challenge hyperglycemia (fasting glucose below 6.1 mmol/l). Isolated post-challenge hyperglycemia is also associated with higher cardiovascular mortality.[Barrett-Connor 1998, Hashimoto 2005, Shaw 1999]

In the Rancho Bernardo Study, 769 men and 1089 women aged 50 to 89 years had oral glucose tolerance test.[Barrett-Connor 1998] Isolated hyperglycemia was defined as 2-hour post-challenge plasma glucose at or above 11.1 mmol/l with fasting glucose less than 7.0 mmol/l. Seven years after the baseline visit, women with isolated post-challenge hyperglycemia had increased risk of fatal cardiovascular disease and heart disease. After adjusting for age, hypertension, central obesity, cigarette smoking, HDL-cholesterol, and triglycerides, the adjusted hazard ratios for fatal cardiovascular disease and heart disease were 2.6 (95% CI 1.4, 4.7) and 2.9 (95% CI 1.3, 6.4), respectively.

In three population based longitudinal studies in Mauritius, Fiji and Nauru, 9179 people were followed for 5 to 12 years, 243 having isolated post-challenge hyperglycemia.[Shaw 1999] Compared to people with normoglycemia, patients with isolated post-challenge hyperglycemia had increased risk of all-cause mortality (men: hazard ratio 2.7, 95% CI 1.8, 3.9; women: hazard ratio 2.0, 95% CI 1.3, 3.3) and cardiovascular mortality (men: hazard ratio 2.3, 95% CI 1.2, 4.2; women: hazard ratio 2.6, 95% CI 1.3, 5.1). Men with isolated post-challenge hyperglycemia also had increased risk of cancer deaths (hazard ratio 8.0, 95% CI 3.6, 17.9).

Table 4.3 Diagnostic criteria of type 2 diabetes [World Health Organization 1999, American Diabetes Association 2006]

World Health Organization 1999 criteria			
Fasting glucose (mmol/l)	<6.1 Normal fasting glucose	6.1-6.9 Impaired fasting glycemia	≥7.0 Diabetes
Post-challenge plasma glucose* (mmol/l)	<7.8 Normal glucose tolerance	7.8-11.0 Impaired glucose tolerance	≥11.1 Diabetes
American Diabetes Association 2006 criteria			
Fasting glucose (mmol/l)	<5.6 Normal fasting glucose	5.6-6.9 Impaired fasting glucose	≥7.0 Diabetes
Post-challenge plasma glucose* (mmol/l)	<7.8 Normal glucose tolerance	7.8-11.0 Impaired glucose tolerance	≥11.1 Diabetes

* Measured 2 hours after a standard 75 g oral glucose tolerance test

CHAPTER FIVE

NON-INVASIVE TEST FOR LIVER FIBROSIS IN NONALCOHOLIC FATTY LIVER DISEASE

5.1 Limitations of liver biopsy

Traditionally, liver biopsy is considered the gold standard for the assessment of liver fibrosis. Besides, it provides other important information such as the degree of hepatic steatosis and the degree and pattern of inflammation. Liver biopsy is also useful when the etiology of liver disease is uncertain, or when mixed etiologies are possible.

Although the risk is small, post-biopsy pain, bleeding and deaths can occur.[Piccinino 1986, Janes 1993, Van Thiel 1993] This would be a major limitation as the majority of patients are asymptomatic and many may harbor mild disease only. Moreover, liver biopsy is an expensive procedure. It may not be acceptable to all patients. Frequent assessment of disease progress is not practical.

Furthermore, the validity of liver biopsy as the gold standard is questioned recently. Firstly, a usual piece of liver tissue obtained from needle biopsy represents only 1/50000 of the whole liver mass. There may be sampling bias if the disease process affects different parts of the liver unevenly. One study attempted to assess the variability by performing biopsies of both the right and left lobes of the liver during bariatric surgery.[Merriman 2006] Forty-one patients had acceptable liver specimen quality. The differentiation between patients with or without NASH is satisfactory when the right and left lobe specimens were

assessed separately, with kappa coefficient of 0.82 (95% CI 0.62-1.0). However, there was more discrepancy in the assessment of fibrosis, with kappa coefficient of 0.53 (95% CI 0.34-0.72). Secondly, histological assessment is subjective and may suffer from both intra-observer and inter-observer variability. In the same study involving patients with morbid obesity, the kappa coefficients of intra-observer variability for NASH and fibrosis were 0.90 (95% CI 0.81-0.99) and 0.68 (95% CI 0.51-0.86), respectively.

Apart from the above limitations, it is impossible to perform liver biopsies on every NAFLD patient when the prevalence of NAFLD in the general population is between 15% and 30%.[Amarapurkar 2007] To the least, it would be useful to screen out patients unlikely to have significant fibrosis, so that the burden of liver biopsies can be reduced. According to the Asia-Pacific Working Party on NAFLD, liver biopsy should be considered in patients at high risk of having cirrhosis.[Farrell 2007, Chan 2007] Thrombocytopenia, low serum albumin, elevated serum bilirubin and prolonged prothrombin time are features of cirrhosis, but none of these are sensitive enough to detect early cirrhosis. Therefore, accurate non-invasive tests for liver fibrosis in NAFLD patients are urgently needed.

5.2 Liver biochemistry

Alanine aminotransferase (ALT) is an enzyme that catalyzes the transfer of amino groups to form oxaloacetate. Since ALT test is relatively inexpensive and readily available, it has become a routine investigation for the evaluation and monitoring of patients with chronic liver diseases. Traditionally, patients with normal ALT are considered to have inactive disease and little liver injury.[Kim 2008]

Nevertheless, recent data challenge the utility of ALT. First, the normal range of ALT is derived from community subjects with presumably normal liver. However, community subjects are not necessarily healthy – a significant proportion would harbor undiagnosed viral hepatitis and NAFLD. An Italian group attempted to answer this question by analyzing 6835 first-time blood donors with negative anti-hepatitis C virus antibody and 209 persons with chronic hepatitis C.[Prati 2002] By excluding patients with overweight or obesity, dyslipidemia, hyperglycemia, and concurrent medication use, 3927 persons (1995 men and 1932 women) were selected to represent a group with low risk of liver disease. Using these healthy individuals as reference, the suggested ALT cutoffs were lowered to 30 U/l in men and 19 U/l in women.

In support of Prati's findings, other groups also confirmed that people with high-normal ALT levels were at increased risk of adverse outcomes. In the Korea

Medical Insurance Corporation study, 94533 men and 47522 women aged 35 to 59 years were followed for 8 years.[Kim 2004] Subjects with high-normal ALT were also at risk of mortality from liver disease. Compared with subjects with ALT below 20 IU/l, the adjusted relative risks of liver-related mortality in subjects with ALT 20-29 IU/l and 30-39 IU/l were 2.9 (95% CI 2.4-3.5) and 9.5 (95% CI 7.9-11.5) in men, and 3.8 (95% CI 1.9-7.7) and 6.6 (95% CI 1.5-25.6) in women, respectively. Similar to Prati's findings, an ALT level of 30 IU/l was found to be the best cutoff for identifying men who were at risk of dying from liver disease. Echoing the results, chronic hepatitis B patients with high-normal ALT at baseline were also found to have increased risk of death and hepatocellular carcinoma.[Yuen 2005]

Following these observations, the proposed new ALT cutoff was evaluated in 233 women who underwent bariatric surgery.[Kunde 2005] Liver biopsies were performed during surgery. Thirty-seven percent and 73% of the women were classified as having normal ALT at cutoff values of 19 IU/l and 30 IU/l, respectively. Using the new cutoff, the sensitivity in detecting NASH increased from 42% to 74%, but the specificity dropped from 80% to 42%.

In summary, although ALT levels correlate with the activity of liver disease and the risk of liver-related mortality, people with high-normal ALT are still at risk. On the other hand, drastic lowering of the upper limit of normal of ALT would

reduce the specificity and probably would increase the burden on health care resources by increasing the number of subjects with false positive results for further diagnostic evaluation. ALT should not be the sole test to exclude advanced NAFLD.

While ALT is mainly a cytosolic enzyme, aspartate aminotransferase (AST) is mainly concentrated in mitochondria.[Wieckowska 2007] Elevation in serum AST level is commonly seen in liver disease caused by mitochondrial toxin such as alcohol.[Zakhari 2007] AST is markedly elevated in severe liver injury such as massive hepatic necrosis and hypoxic hepatitis.[Henrion 1999] AST is also raised in patients with liver cirrhosis, and AST/ALT ratio has been proposed as a marker of liver cirrhosis (see section 5.3).

5.3 Clinical models

Clinical prediction models are developed by identifying independent factors associated with significant fibrosis. Using these variables, a clinical model or equation is built. The performance of the original model may be over-estimated because it is built from the most significant discriminating factors in the same training cohort. Therefore, it is important to validate the model in a different group of patients before the model is ready for use.

The main advantage of such kinds of clinical models is their low cost. Most clinical models are derived from parameters that are commonly measured. No additional tests or equipments are required. However, these parameters are just factors associated with liver fibrosis but not direct measurements of fibrogenesis or fibrinolysis. Besides, the accuracy of these models is assessed using histology as the gold standard. As discussed in chapter 4.1, liver biopsy is actually limited by sampling bias, intra-observer and inter-observer variability. Since neither the clinical model nor liver biopsy is perfect, it is unlikely that studies can show that clinical models are very accurate.

The AST/ALT ratio is one of the simplest serum markers to predict the presence of advanced fibrosis. It has been mostly validated in patients with chronic hepatitis C. In a retrospective study of 139 patients with chronic hepatitis C, the AST/ALT ratio was higher in cirrhotic patients than non-cirrhotic patients

(1.06±0.06 vs. 0.60±0.09, $p<0.001$).[Sheth 1998] At a cutoff of 1, the sensitivity, specificity, positive and negative predictive values of the AST/ALT ratio to predict cirrhosis were 53%, 100%, 100%, and 81%, respectively. The AST/ALT ratio was confirmed to have moderate accuracy for the diagnosis of liver cirrhosis in another retrospective Australian cohort and a prospective Italian cohort of chronic hepatitis C patients.[Park 2000, Giannini 2003]

The AST to platelet ratio index (APRI) is another simple marker of cirrhosis. In a prospective cohort of 270 chronic hepatitis C patients, cirrhosis could be predicted accurately in 81% using the optimal cutoff.[Wai 2003] On the other hand, APRI only had 51% accuracy in predicting the presence of significant fibrosis (Ishak stage 3 or above). As thrombocytopenia is a feature of hypersplenism secondary to cirrhosis, it is not surprising that APRI is less useful in patients without cirrhosis. However, these models have not been thoroughly evaluated in NAFLD patients.

In 2007, a NAFLD fibrosis score was derived using data from a number of major centers in the United States, Europe and Australia.[Angulo 2007] With a formula including age, BMI, impaired fasting glucose or diabetes, AST/ALT ratio, platelet count and albumin, the score had an overall accuracy of 80 percent to 90 percent in predicting stage 3 or 4 liver fibrosis. Compared to previously reported serum markers, the main advantage of this score is that it involves parameters

that are widely available and almost routinely measured. Besides, relatively few patients were in the indeterminate zone, in which the score would be unable to predict whether there was significant fibrosis.

5.4 Serum tests of fibrosis

Unlike clinical models, serum tests also include measurements of markers of fibrogenesis and fibrolysis. Although these are not tests commonly performed in clinical practice, it is hoped that they may be more accurate markers of liver fibrosis.

Before new biomarkers can be used clinically, they must fulfill a few criteria. Firstly, the tests should be simple and easy to handle. They should be accurate and provide additional information to the clinical tests that are already commonly used. The accuracy should be validated in large cohorts across different population groups. Lastly, the tests should be cost-effective. Again, serum tests have been mostly validated in patients with chronic hepatitis C.

One of the first serum tests was the 'PGA index', which comprises prothrombin time, gamma-glutamyltransferase, and apolipoprotein A1.[Teare 1993] In a cohort of 104 patients with alcoholic liver disease, 38 patients with primary biliary cirrhosis, 27 patients with chronic hepatitis B, and 30 age-matched controls, the PGA index had 91% sensitivity and 81% specificity in detecting cirrhosis.

In another study, the performance of 11 markers was tested in 339 patients with chronic hepatitis C.[Imbert-Bismut 2001] The most informative markers included

α_2 macroglobulin, α_2 globulin, γ globulin, apolipoprotein A_1, γ glutamyltranspeptidase, and total bilirubin. The area under the receiver operating characteristics curve (AUROC) of 6 markers in predicting METAVIR stage 2 fibrosis or more was 0.84 and 0.87 in the training and validation cohorts, respectively. This work subsequently led to commercialization of the FibroTest, which has been validated in patients with chronic hepatitis C and B.[Rossi 2003, Myers 2003] Nevertheless, the test is limited by false-positive results. Bilirubin may be raised in patients with intravascular hemolysis, Gilbert's syndrome and cholestasis. This is particularly relevant in chronic hepatitis C patients on ribavirin, which is a common cause of hemolytic anemia. Besides, acute inflammation also causes non-specific increases in α-2 macroglobulin and haptoglobin. The same group also attempted to generate the ActiTest by adding ALT and SteatoTest by adding BMI, serum cholesterol, triglycerides, and glucose in the model. Although the chosen lower and higher cutoffs of the SteatoTest had good sensitivity and specificity in diagnosing grade 2 to 4 steatosis, a significant proportion of patients were in the gray zone between the two chosen cutoffs.[Poynard 2005]

Another commercially available panel, the Fibrospect, used a panel comprising hyaluronic acid, TIMP1, and α-2 macroglobulin. The test had AUROC of 0.83 for METAVIR stage 2 to 4 fibrosis, but could not accurately differentiate individual fibrosis stages.[Patel 2004]

In a recent study involving 75 patients with chronic hepatitis C, the diagnostic accuracies of hyaluronic acid, FIBRO*Spect* II and YKL-40 (chondrex, human cartilage glycoprotein-39) were compared.[Mehta 2008] Hyaluronic acid was more effective in discriminating Ishak stages 0-1 and stages 2-3. The false positive rate of hyaluronic acid was 33%.

Overall, these tests are more accurate in diagnosing liver cirrhosis than earlier stages of fibrosis. This may be explained by the wide overlapping of the serum test results in earlier stages of fibrosis. Furthermore, the intra-observer and inter-observer variability are also greater for earlier stages of fibrosis even using histology. This makes validation of these markers for early fibrosis difficult.

5.5 Transient elastography

Transient elastography by Fibroscan (Echosens, Paris, France) is another promising non-invasive method for liver fibrosis detection (Figure 5.1). An ultrasound transducer probe is mounted on the axis of a vibrator. When vibrations are transmitted to the liver tissue, an elastic shear wave is induced. Pulse-echo ultrasound can detect the velocity of the shear wave. The stiffness of the liver tissue correlates with higher shear wave velocity.[Sandrin 2003]

Transient elastography has been validated mainly in chronic hepatitis C. In 40 treatment-naïve chronic hepatitis C patients, inter-observer agreement was excellent for Fibroscan (weighted kappa = 1.0), and poor for FibroTest (weighted kappa = -0.041).[Colletta 2005] The sensitivity, specificity, positive and negative predictive values to predict stage 2 fibrosis or above were all 100%. Corresponding values for FibroTest were 64%, 31%, 33% and 62%, respectively. Like most pilot studies, the estimation of the accuracy by Colletta and colleagues tends to be over-optimistic. Subsequent validation studies confirmed a lower but still satisfactory accuracy of transient elastography. According to a recent meta-analysis, the pooled estimates of sensitivity and specificity were 87% and 91%, respectively for stage 4 fibrosis and 70% and 84%, respectively for stage 2-4 fibrosis.[Talwalkar 2007]

In contrast, data on transient elastography in NAFLD are scarce. Among 67 Japanese NAFLD patients, the AUROC for F1, F2, F3, and F4 were 0.88, 0.88, 0.91, and 1.00, respectively.[Yoneda 2007] At a cutoff of 8.0 kPa, the sensitivity, specificity, positive and negative predictive values of transient elastography in predicting F3 disease were 87.5%, 84.3%, 63.6%, and 95.6%, respectively.

There are notable advantages of transient elastography. Firstly, it is non-invasive and is likely to be acceptable to most patients. Secondly, the core of liver tissue accessible by transient elastography is 1 cm wide and 4 cm long. Since this is 100 times the volume of a routine liver biopsy specimen, the problem of sampling bias is much reduced. Moreover, transient elastography does not require complicated training and is less operator-dependent. Among operators who have performed 50 procedures or more, the intra-observer and inter-observer variability is very low.[Fraquelli 2007]

On the other hand, transient elastography has a number of limitations. The success rate is lower in patients with morbid obesity and narrow intercostals spaces.[Sandrin 2003] It is also virtually impossible to perform transient elastography in patients with ascites. In a prospective study of 935 chronic hepatitis C patients, BMI was an independent factor associated with successful liver stiffness measurement.[Kettaneh 2007] In patients with BMI above 30 kg/m^2, the odds ratios of successful liver stiffness measurement were 0.11 and

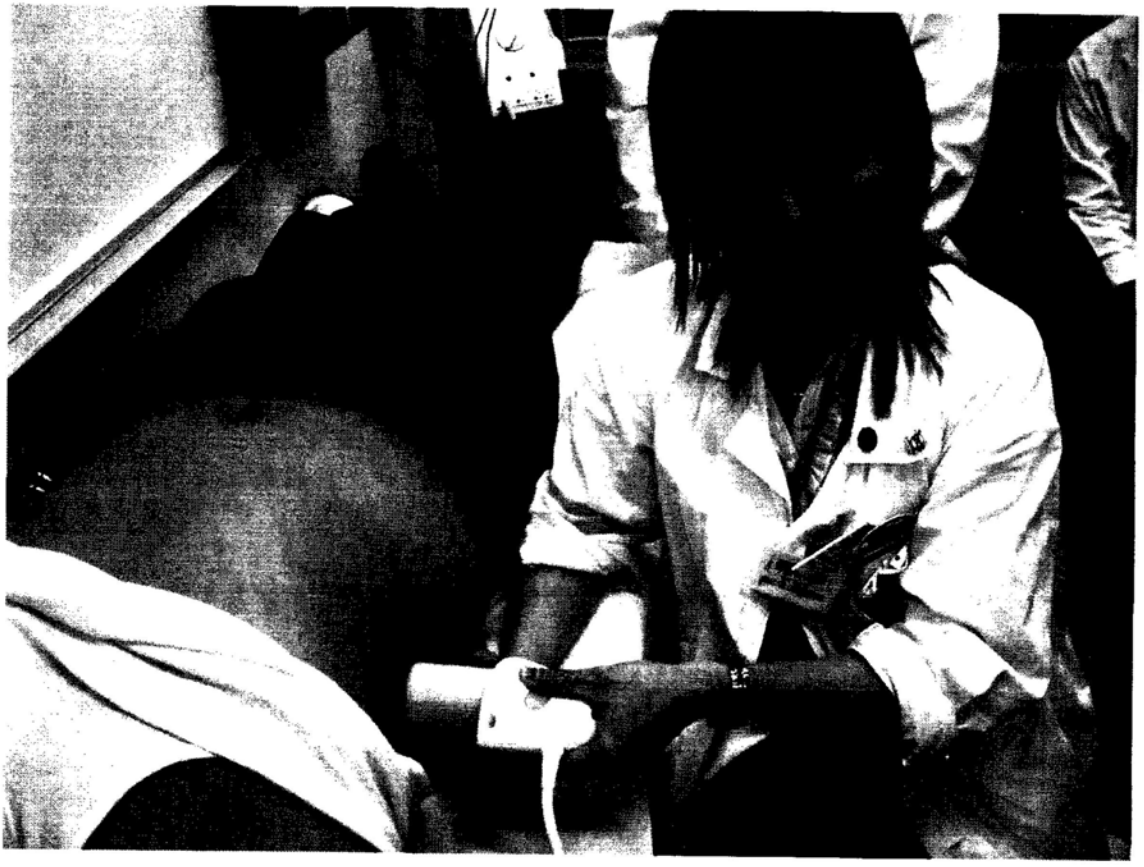
0.16 in men and women, respectively. Since NAFLD is associated with central obesity, it is doubtful if transient elastography is useful in this patient group.

In 2008, two separate groups reported false positive results of transient elastography in patients with high ALT. The first study included 18 consecutive patients with acute viral hepatitis (7 had hepatitis A, 8 had hepatitis B, and 3 had hepatitis C).[Arena 2008] At the time when ALT peaked, all patients had liver stiffness measurements exceeding 7 kPa, a cutoff suggestive of significant fibrosis or cirrhosis. The liver stiffness measurement dropped from 12.7±5.7 kPa during peak ALT, to 9.4±3.6 kPa when ALT dropped to half the peak level, and 6.2±1.2 kPa when ALT was less than 2 times the upper limit of normal. Clearly, the final liver stiffness measurements suggested that these patients did not have significant fibrosis or cirrhosis, but might be misdiagnosed when the measurements were done during active hepatitis. Similarly, another group reported the liver stiffness measurements of 20 patients with heterogeneous causes of acute hepatitis (viral hepatitis, drug-induced liver injury, and autoimmune hepatitis).[Sagir 2008] Fifteen (75%) patients had liver stiffness measurements suggestive of cirrhosis. Eleven patients had liver biopsies, and all showed F1 or F2 disease only. These data support the notion that liver stiffness is not entirely due to fibrosis. Other factors such as inflammatory infiltrates also contribute to the liver stiffness. Currently, it is still uncertain if hepatic steatosis or subcutaneous fat may affect the liver stiffness measurement. Both factors

would have important implication if transient elastography is to be used in NAFLD patients. In fact, when 429 consecutive apparently healthy individuals underwent transient elastography, female gender, higher BMI and metabolic syndrome were independent factors associated with higher liver stiffness measurements.[Roulot 2008] While it is likely that the results reflect the presence of NAFLD and/or significant fibrosis in patients with obesity and metabolic syndrome, whether the results can be explained by increased hepatic steatosis or subcutaneous fat alone needs to be clarified.

Recently, magnetic resonance elastography has also been developed by combining magnetic resonance imaging and a transmit/receive body coil.[Yin 2007] When performed in 35 healthy volunteers and 50 patients with chronic liver disease, magnetic resonance elastography had 98% sensitivity and 99% specificity for differentiating any stage of liver fibrosis from normal liver. The sensitivity and specificity to detect stage 2 to 4 fibrosis were 86% and 85%, respectively.

Figure 5.1 Transient elastography by Fibroscan is a non-invasive and reproducible method to estimate liver fibrosis.



Courtesy of Dr. Grace Wong

PART II

HYPOTHESIS AND CLINICAL STUDIES

CHAPTER SIX

AIMS AND HYPOTHESIS

While there is much evidence of NAFLD being a common health problem worldwide, histological studies in Asia are scarce. It is uncertain if advanced NAFLD, namely NASH and NAFLD with severe fibrosis or cirrhosis, also occurs in the Chinese population. It is also important to investigate if NAFLD is a progressive disease. Moreover, factors associated with advanced disease and non-invasive methods to detect advanced disease need to be elucidated.

To answer these questions, we first conducted a retrospective study on patients with biopsy-proven NAFLD. The proportion of patients with NASH and liver fibrosis was studied. Since retrospective studies are limited by selection bias and missing data, we subsequently conducted a cross-sectional study to investigate the histological severity of NAFLD patients. We further conducted a longitudinal study with paired liver biopsies to assess disease progression. Based on the cross-sectional study, we studied insulin resistance using the homeostasis model. Adiponectin, TNF-α, resistin and leptin were studied because of their effects on insulin resistance and inflammation. As the serum levels of adiponectin and TNF-α were associated with NAFLD and NASH, gene polymorphisms of these two adipokines were studied. TNF-α-238, TNF-α-308, TNF-α-863, ACDC-11391, ACDC-11377, ACDC+45 and ACDC+276 were genotyped because of previous reports on their possible association with inflammation, obesity, insulin resistance and coronary artery disease. Since NAFLD is closely related to metabolic syndrome and insulin resistance, we reported the prevalence of impaired glucose

tolerance and undiagnosed diabetes by performing oral glucose tolerance test in NAFLD subjects. Finally, we validated various clinical models to predict liver fibrosis in NAFLD.

The aims and hypotheses of the project are summarized as follows:

Aims

1. To study the severity and progression of NAFLD in Hong Kong Chinese
2. To study the metabolic and adipokine profile of NAFLD patients
3. To validate clinical models to predict advanced fibrosis in NAFLD patients

Hypothesis

1. Significant necroinflammation and fibrosis occur in Chinese NAFLD patients.
2. Histological features of NAFLD may progress with time.
3. Metabolic syndrome is common in NAFLD patients. Ethnicity-specific definition of metabolic syndrome is useful to characterize Chinese NAFLD patients.
4. Post-challenge hyperglycemia after oral glucose tolerance test is common in NAFLD patients and is associated with advanced disease.
5. Abnormal levels of serum adipokines are associated with NAFLD and its severity.
6. Gene polymorphisms of adipokines are associated with NAFLD and its severity.

7. The NAFLD fibrosis score can predict advanced fibrosis in Chinese NAFLD patients.

CHAPTER SEVEN

STUDY METHODS

7.1 Clinical assessment

Blood pressure was measured using the Omron T8 Intellisense Blood Pressure Monitor (OMRON Electronics, Hong Kong). After resting quietly for 15 minutes, blood pressure was measured with feet on the floor and arm supported at heart level. Caffeine, exercise, and smoking were avoided for at least 30 minutes prior to measurement. Two measurements were made and the average was recorded. Hypertension was defined as blood pressure at or above 140/90 mmHg.[Chobanian 2003]

Body weight and body height were measured in all subjects using the same scale. Waist circumference and hip circumference were measured using a tape ruler. Waist circumference was measured at a level midway between the lower rib margin and iliac crest with the tape all around the body in the horizontal position.

BMI was calculated as body weight (kg) divided by height (m) squared. Using the WHO Asia Pacific criteria, obesity was defined as BMI $\geq$25 kg/m^2, and overweight was defined as BMI $\geq$23 kg/m^2.[World Health Organization 2000] Waist-hip ratio (WHR) was defined as waist circumference (cm) divided by hip circumference (cm).

7.2 Metabolic profile and adipokine assays

On the day of liver biopsy, a fasting venous blood sample was taken for albumin, bilirubin, ALT, glucose, glycosylated hemoglobin (HbA_{1c}), total cholesterol, HDL-cholesterol, LDL-cholesterol, triglycerides, adiponectin, resistin, TNF-α, leptin and insulin. The patients were instructed to have fasting for at least 8 hours before blood sampling. Serum was stored at -80°C until further use. Adiponectin, resistin and TNF-α were measured by the Quantikine® immunoassay (R&D System, USA) according to manufacturer's instructions. In brief, 100 µl of assay diluent RD1W was added to each well, followed by the addition of 50 µl of standard, control, or sample. The plate was incubated for 2 hours at room temperature. Afterwards, each well was aspirated and washed for four times with 400 µl of wash buffer. The plate was then inverted and blotted against clean paper towels. 200 µl of assay conjugates were added to each well, and the plate was incubated for 2 hours at room temperature. After aspiration and washing again, 200 µl of substrate solution was added to each well. After further incubation for 30 minutes, 50 µl stop solution was added to each well. The optical density was determined using a microplate reader set. Insulin level (Dako, UK) and leptin (Diagnostic Systems Laboratory, USA) were measured by commercial ELISA kits. The range of insulin concentration detectable by the ELISA kit was 3 to 300 U/ml.

Adipokines and insulin were measured in duplicate. Figure 7.1 showed a representative standard curve of insulin measurement. Figure 7.2 showed two paired absorbance results for insulin measurement. The intra-assay and inter-assay coefficients of variation for both adipokines and insulin were below 3% and 10%, respectively.

Insulin resistance was calculated by the homeostasis model (HOMA-IR), which was equal to fasting insulin (mU/L) × fasting glucose (mmol/l)/22.5.[Levy 1998]

Patients with fasting plasma glucose below 7.0 mmol/l underwent a 75 gram oral glucose tolerance test. A patient was diagnosed to have diabetes mellitus if the fasting glucose was above 7.0 mmol/l or 2-hour post-glucose load plasma glucose was above 11.1 mmol/l.[World Health Organization 1999, American Diabetic Association 2006]

The definitions of metabolic syndrome were according to the NCEP-ATPIII and IDF criteria (Table 4.1).[NCEP 2002, Alberti 2005]

Figure 7.1 A representative standard curve of serum insulin measurement by ELISA (Dako, UK).

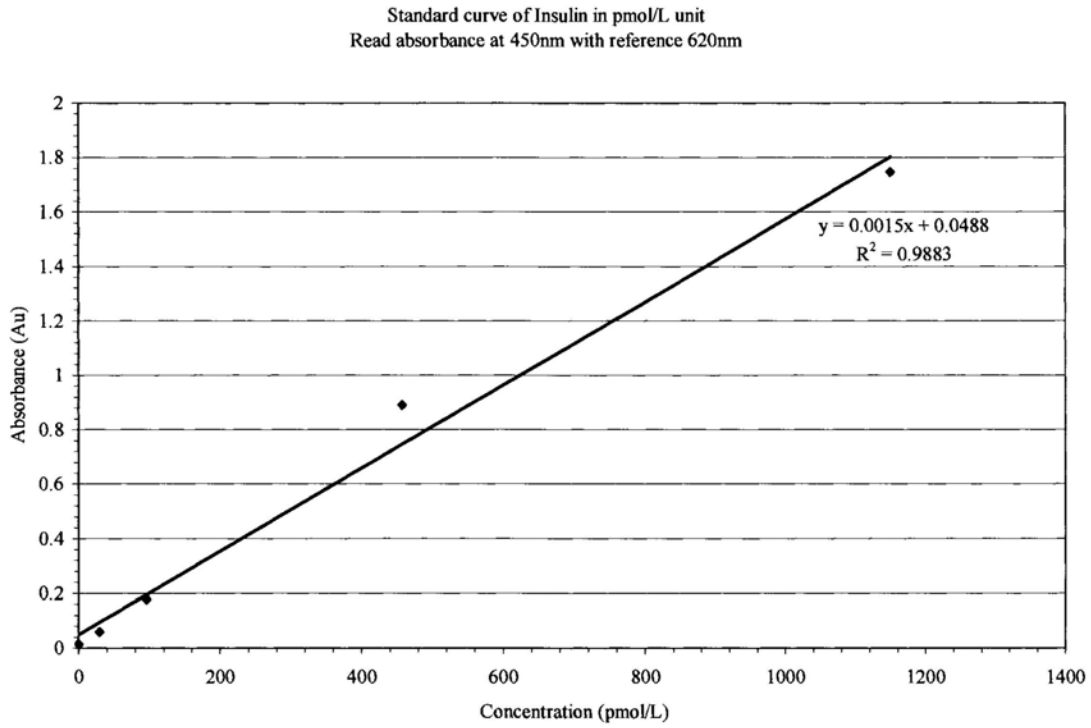
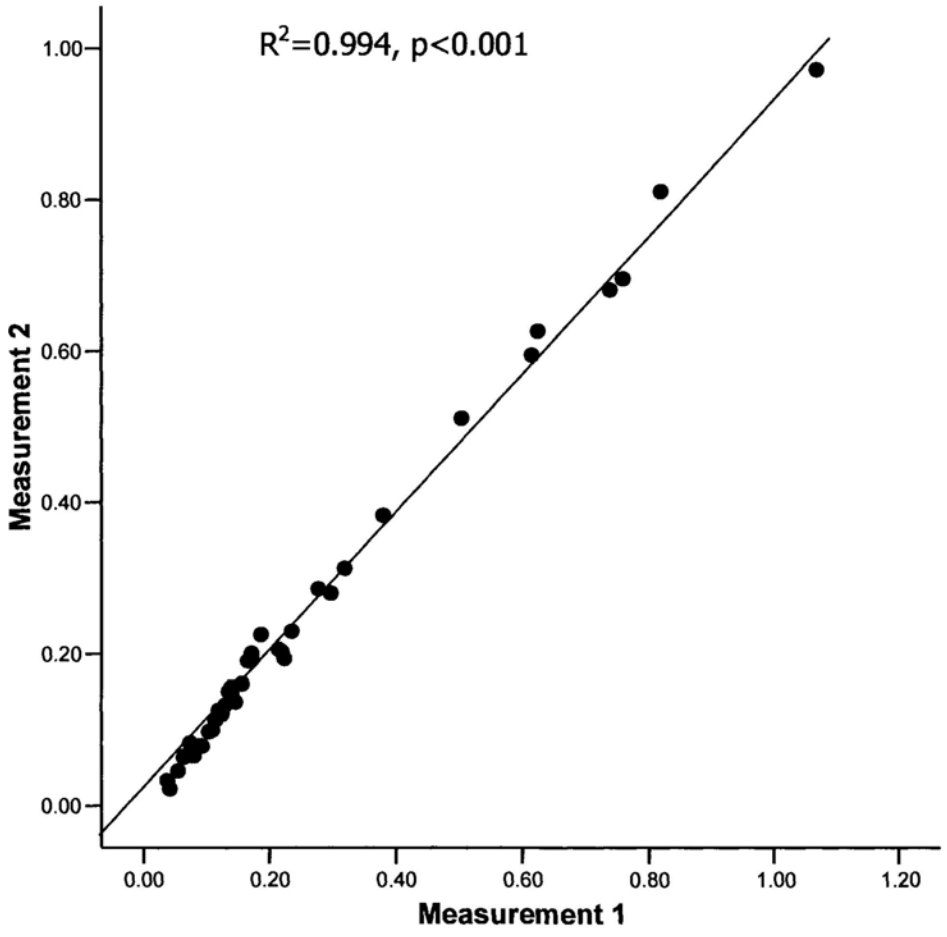


Figure 7.2 Correlation of duplicate measurement of serum insulin.



7.3 Gene polymorphisms

Genomic DNA was extracted from peripheral blood leukocytes using the QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany). All genotyping was performed by real time polymerase chain reaction based assays. Both amplification and detection were performed in iCycler iQ Real Time PCR Detection System (Bio-Rad, Hercules, CA). Thermal cycling conditions and primer and probe sequences were shown in Table 7.1. Genotype of TNFα-863 (C/A) was determined by validated MGB Eclipse ™ Assay - Assay number 002_2147 (Nanogen, San Diego, CA) in which two probes each labeled with MGB Eclipse Dark Quencher (EDQ) at 5' end and FAM or TET at 3' end were used. Reactions were set up in a volume of 25ul containing 1X MGB Eclipse™ PCR Reagent Kit (Sigma-Aldrich, St. Louis, MO), 0.1uM of forward primer, 2uM of reverse primer, (Nanogen), 0.2uM of each probe (Nanogen) and 25ng genomic DNA. After amplification, a dissociation curve was generated. Upon dissociation, the two fluorescein-labeled probes gave out different signals and their melting temperatures were compared for allelic discrimination. Two polymorphisms of TNFα-308 (G/A) were genotyped by TaqMan® Assay (Applied Biosystems, Foster City, CA) as described previously.[El-Omar 2001, Chan 2007-2] Polymorphisms of TNFα-238 (G/A) were analyzed by 5'nuclease assay using TaqMan® probes.[Chan 2007-2] Amplification was performed in a volume of 25ul containing 1X Taqman® Universal PCR Master Mix (Applied Biosystems), 900nM of each primer (Invitrogen, Carlsbad, CA), 200nM of each probe (Biosearch Technologies,

Novato, CA) and 50ng genomic DNA. Genotypes of ACDC-11391 (G/A), +45 (T/G) & +276 (G/T) was analyzed by 5'nuclease assay using TaqMan® probes.[Woo 2006] ACDC-11377 (G/C) was genotyped by validated TaqMan® SNP Genotyping Assay - Assay number C_2412786_10 (Applied Biosystems). Assay was performed in a volume of 25ul containing 1X Taqman® Universal PCR Master Mix (Applied Biosystems), 1X Assays-On-Demand™ SNP Genotyping Assay Mix (Applied Biosystems) and 50ng genomic DNA. Positive controls were included in each assay for assay verification. Direct sequencing was performed for randomly selected samples to reconfirm genotype results.

Table 7.1 Primer and probe sequences for adiponectin and TNF-α genotyping

Adiponectin		
-11391	ACDC11391G	5′ *FAM* – CAA GAA CCG GCT CAG AT – *MGB* 3′
	ACDC11391A	5′ *VIC* – CAA GAA CCA GCT CAG AT – *MGB* 3′
	ACDC11391F	5′ – GCA TCC TAA GCC CTT GCT G – 3′
	ACDC11391R	5′ – TGG CAC GCT CAT GTT TTG TTT T – 3′
+45	ACDC45T	5′ *FAM* – CCC GGT CAT GAC C – *MGB* 3′
	ACDC45G	5′ *VIC* – CCC GGG CAT GAC C – *MGB* 3′
	ACDC45F	5′ – GGG AGC TGT TCT ACT GCT ATT AGC – 3′
	ACDC45R	5′ – CCC TTG AGT CGT GGT TTC CT – 3′
+276	ACDC276G	5′ *FAM* – ACT ATA TGA AGG CAT TCA T – *MGB* 3′
	ACDC276T	5′ *VIC* – AAA CTA TAT GAA GTC ATT CAT – *MGB* 3′
	ACDC276F	5′ – TTC ATC ACA GAC CTC CTA CAC TGA – 3′
	ACDC276R	5′ – TCC CTG TGT CTA GGC CTT AGT TAA T – 3′

TNF-α		
-238	TNFα-238G	5′ *FAM* – CCT CCC TGC TCC GAT TCC GA – *BHQ* 3′
	TNFα-238A	5′ *JOE*–ATC CTC CCT GCT CTG ATT CCG AG–*BHQ* 3′
	TNFα-238F	5′ – GGT CCT ACA CAC AAA TCA GTC A – 3′
	TNFα-238R	5′ – GGA CAC ACA AGC ATC AAG GA – 3′
-308	TNFα-308G	5′ *FAM* – AGG GGC ATG GGG ACG GG – *BHQ* 3′
	TNFα-308A	5′ *JOE* – CCT TGA GGG GCA TGA GGA CGG G – *BHQ* 3′
	TNFα-308F	5′ – CCC CAA AAG AAA TGG AGG C – 3′
	TNFα-308R	5′– GGT TCT TCT GGG CCA CTG ACT GAT – 3′
-863	TNFα-863A	5′ *MGBEDQ* – TGT GACCCCCACTTA – *FAM* 3′
	TNFα-863C	5′ *MGBEDQ* – GACCCCCCCTTA – *TET* 3′
	TNFα-863F	5′ – TGG*GGAGATGTGACCACAGCAAT – 3′
	TNFα-863R	5′ – GGCCCTCTACATGGCCCTGTCTT – 3′

MGB Eclipse probes and primers are designed and synthesized using modified bases where A*=super A, G*=super G and T*=super T (Nanogen, San Diego, CA).

7.4 Histology

Percutaneous liver biopsy was performed using the 16G Temno needle. The site of liver biopsy was identified by bedside ultrasound scanning and percussion. The usual site of biopsy was at the 7^{th} to 9^{th} intercostal space, over the right anterior axillary line. Lignocaine 2% was injected to provide local anesthesia at the puncture site.

Liver histology was assessed by two pathologists specialized in liver diseases who were blinded to the clinical data. A sample was considered adequate if it was longer than 1.5 cm and contained 6 portal tracts or more. Liver biopsy specimens were prepared with hematoxylin and eosin stain, Masson trichrome stain, Prussian blue stain, reticulin stain, orcein stain and periodic acid Schiff stain. The histological grading and staging of NAFLD followed the Brunt's criteria (Table 7.2).[Brunt 1999]

NASH was defined as grade 2 or 3 necroinflammation and/or fibrosis. Simple steatosis was defined as NAFLD with grade 0 or 1 necroinflammation and no fibrosis. Significant fibrosis was defined as stage 2 to 4 fibrosis. Advanced fibrosis was defined as stage 3 to 4 fibrosis.

Thirty liver specimens were randomly chosen to be scored by two pathologists together. Table 7.3 showed the kappa coefficients of various histological parameters.

Table 7.2 Histological grading and staging according to the Brunt system [Brunt 1999]

Grading and staging of NAFLD	
Grading NAFLD	
1. Macrovesicular steatosis	
Grade 0: None	
Grade 1: Up to 33%	
Grade 2: 33%–66%	
Grade 3: > 66%	
2. Necroinflammatory activity	
Grade 1 (mild)	Steatosis up to 66%, occasional ballooned hepatocyte (mainly zone 3), scattered intra-acinar neutrophils (PMN) ± lymphocytes, no or mild portal inflammation
Grade 2 (moderate)	Steatosis of any degree, obvious zone III ballooning degeneration, intra-acinar PMNs, zone III perisinusoidal fibrosis may be present, mild to moderate, portal and intra-acinar inflammation
Grade 3 (severe)	Panacinar steatosis, widespread ballooning, intra-acinar inflammation, PMNs associated with ballooned hepatocytes, mild to moderate portal inflammation

Staging NAFLD	
Stage 1	Zone III perisinusoidal/pericellular fibrosis; focally or extensively present
Stage 2	Zone III perisinusoidal/pericellular fibrosis with focal or extensive periportal fibrosis
Stage 3	Zone III perisinusoidal/pericellular fibrosis and portal fibrosis with focal or extensive bridging fibrosis
Stage 4	Cirrhosis

Table 7.3 Interobserver variability of major histological features. One-fifth of the liver specimens were randomly chosen to be scored by two pathologists together.

	Kappa coefficient
Steatosis grade	0.92
Necroinflammatory grade	0.55
Fibrosis stage	0.90
NASH	0.93
Advanced fibrosis	0.93
Cirrhosis	1.0

7.5 Clinical models to predict liver fibrosis

The NAFLD fibrosis score was calculated according to the following formula: -1.675 + 0.037 × age (years) + 0.094 × BMI (kg/m^2) + 1.13 × IFG/diabetes (yes = 1, no = 0) + 0.99 × AST/ALT ratio − 0.013 × platelet (×10^9/l) − 0.66 × albumin (g/dl).[Angulo 2007] This score was constructed from 480 NAFLD patients, mostly Caucasians, and validated in 253 patients. At a low cutoff point of -1.455, the sensitivity, specificity, positive and negative predictive values for advanced fibrosis were 82%, 77%, 56% and 93%, respectively. At a high cutoff point of 0.676, the sensitivity, specificity, positive and negative predictive values for advanced fibrosis were 51%, 98%, 90%, and 85%, respectively.

AST/ALT ratio was calculated as AST (IU/l) divided by ALT (IU/l). The AST to platelet ratio index (APRI) was calculated as AST (IU/l) ÷ upper limit of normal for AST × 100 / platelet (×10^9/l).[Wai 2003] Both the AST/ALT ratio and APRI were validated in patients with chronic hepatitis C but not in NAFLD. The HAIR score was calculated by summation of the scores of hypertension = 1, ALT > 40 IU/l = 1, and insulin resistance (IR) index > 5 = 1. IR index was calculated as log insulin (μU/ml) + log fasting plasma glucose (mg/dl).[Dixon 2001] The HAIR score was derived from 105 consecutive patients undergoing laparoscopic obesity surgery. The score had 80% sensitivity and 89% specificity for NASH.

7.6 Statistics

Statistical analysis was performed by Statistical Package for Social Science (SPSS) version 11.5 (Chicago, IL, USA). Continuous variables were expressed in mean ± standard deviation or median (interquartile range). Continuous variables between patients with and without NAFLD, simple steatosis and NASH, and with and without advanced fibrosis were compared using independent *t* test or Mann-Whitney U test. Categorical variables were compared using chi-square test or Fisher exact test as appropriate. Correlation was assessed using the Spearman test.

Multivariate analysis with binary logistic regression model was used to identify independent factors associated with NAFLD, NASH and advanced fibrosis.

Statistics analysis of the genotype data was performed using PowerMarker software version 3.23 (http://statgen.ncsu.edu/powermarker/index.html). Hardy-Weinberg equilibrium was assessed by chi-square test or Fisher's exact test. D' and r^2 were calculated to evaluate linkage disequilibrium (LD) for all pairwise single nucleotide polymorphism combinations. At a 5% level of significance, 40 patients in each of the NAFLD and control groups would achieve 70% power in detecting a 30% difference in allele frequencies.

The overall accuracy of the NAFLD fibrosis score in determining different stages of liver fibrosis was calculated using the area under the receiver operating characteristics (ROC) curve and its 95 percent confidence intervals. Delong test was used to compare the area under ROC curve of different prediction models.

All statistical tests were two-sided. Statistical significance was taken as $p<0.05$.

CHAPTER EIGHT

HISTOLOGICAL SEVERITY OF NONALCOHOLIC FATTY LIVER DISEASE IN HONG KONG CHINESE

8.1 Retrospective study

To study the severity of NAFLD in Hong Kong Chinese, we first conducted a retrospective study on patients who underwent liver biopsies at the Prince of Wales Hospital, Hong Kong from 1996 to 2003.[Wong 2004] All Chinese patients who had liver biopsy performed during the study period were identified by computer search of the hospital medical registry using the key word 'liver biopsy'. Two investigators (VWSW and HLYC) reviewed all case notes of the patients. Patients included for analysis must have histological evidence of steatosis with or without the presence of necroinflammation and fibrosis. Patients with known etiologies of liver diseases including chronic hepatitis B, chronic hepatitis C, autoimmune hepatitis, Wilson's disease, hemochromatosis and drug-related hepatitis were excluded from analysis. We excluded 'social drinkers' and 'regular drinkers' as documented in case records.

Clinical characteristics

Forty-two patients with biopsy-proven NAFLD were identified. Thirty-four patients had liver biopsy to investigate the cause of abnormal liver function tests. Two patients had liver biopsy because of suspected cirrhosis on ultrasound scan, and six patients requested liver biopsy to assess the severity of NAFLD because ultrasound imaging showed heavy fatty changes.

The median age was 47 years (range 23-69). Twenty-two (52.4%) patients were male. Ten (23.8%) patients had hypertension (blood pressure ≥140/90 or on anti-hypertensive medications), 11 (26.2%) patients had diabetes mellitus, and 1 (2.4%) patient had impaired glucose tolerance. Thirty-four (81%) had BMI above 23 kg/m^2, and 23 (55%) patients had BMI above 25 kg/m^2. Only 1 (2.4%) patient did not have any feature of metabolic syndrome. Using stricter criteria according to the National Cholesterol Education Program, 29 (69.0%) patients had metabolic syndrome at the time of liver biopsy.[NCEP-ATPIII 2002]

No patient took thiazolidinediones or vitamin E. Three (7.1%) patients took statins for hyperlipidemia, and no patient took fibrates. Among patients with diabetes mellitus and non-alcoholic fatty liver disease, 6 (54.5%) were on metformin, 6 (54.5%) were on sulfonylurea, and 1 (9.1%) was using insulin. Median glycosylated hemoglobin (HbA_{1c}) was 7.4% (range 5.2-10.5%).

The majority of patients was asymptomatic and presented with incidental finding of abnormal liver function tests. Seven (14.7%) patients had generalized malaise, 2 (4.8%) had right upper quadrant pain, 1 (2.4%) had pruritus, and 1 (2.4%) had tea-colored urine. The presence of symptoms was not associated with higher ALT or higher histological grading or staging.

The patients were followed up for a median of 42 months (range 9-87). No patient developed liver decompensation, cirrhotic complications or hepatocellular carcinoma. The median serum bilirubin was 9 mol/l (range 2-26), albumin level was 41 g/dl (range 31-48), and prothrombin time was 10.1 s (range 9.1-11.4). The median ALT was 93 IU/l (range 24-270) (Table 8.1). At the time of liver biopsy, 34 (81%) patients had ALT above the upper limit of normal.

Histology

Approximately one-third of patients had grade 1, grade 2 and grade 3 steatosis respectively by Brunt's criteria (Table 8.2). Thirty-six (85.7%) patients had necroinflammation, and 11 (26.2%) had fibrosis. Only one (2.4%) patient had stage 3 fibrosis. Both steatosis (Spearman's coefficient 0.51, p=0.001) and necroinflammatory activity (Spearman's coefficient 0.49, p=0.001) grading were correlated with the development of fibrosis.

Thirteen patients had more severe steatohepatitis i.e. grade 2-3 necroinflammation and/or fibrosis. Diabetic patients were more likely to develop severe steatohepatitis (hazard ratio 7.3, 95 percent confidence interval 1.6 to 33.3) (Table 8.1). The type of diabetic treatment (metformin, sulfonylurea or insulin) did not affect histological severity. Including both diabetic and non-diabetic patients, patients with severe steatohepatitis had higher fasting glucose level (median glucose 6.4 mmol/l versus 5.0 mmol/l, p = 0.004).

Hypertension, serum cholesterol level, triglyceride level and BMI did not correlate with severe steatohepatitis. Using the Asian cutoff, obesity did not predict severe steatohepatitis (33% versus 30%, p=1.0). The level of ALT also could not predict the development of severe steatohepatitis (p=0.40). Among the eight patients with persistently normal ALT, 2 (25%) had severe steatohepatitis, and 6 (75%) had grade 1 steatohepatitis according to Brunt's criteria.

Table 8.1 Clinical features of 42 NAFLD patients in the retrospective study.

Clinical features	Simple steatosis (n = 29)	NASH (n = 13)	All (n = 42)	P-value
Male gender	18 (62%)	4 (31%)	22 (52%)	0.10
Age	45 (23-69)	51 (34-62)	47 (23-69)	0.13
Diabetes mellitus	4 (14%)	7 (54%)	11 (26%)	0.019
Hypertension	5 (17%)	5 (38%)	10 (24%)	0.14
Body mass index (kg/m^2)	27 (20-36)	26 (22-35)	27 (20-36)	0.81
Serum albumin (mmol/l)	41 (31-48)	40 (34-46)	41 (31-48)	0.77
Total bilirubin (mmol/l)	8 (4-26)	10 (2-20)	9 (2-26)	0.57
ALT (IU/l)	88 (24-270)	101 (50-196)	93 (24-270)	0.40
Fasting glucose (mmol/l) *	5.0 (4.2-7.9)	6.4 (5.1-12.5)	5.3 (4.2-12.5)	0.004
Total cholesterol (mmol/l) *	6.2 (4.4-7.5)	5.6 (4.7-8.3)	5.9 (4.4-8.3)	0.43
LDL-cholesterol (mmol/l) *	3.6 (2.0-5.0)	3.6 (3.0-6.0)	3.6 (2.0-6.0)	0.98
HDL-cholesterol (mmol/l) *	1.2 (0.7-2.1)	1.2 (1.0-2.1)	1.2 (0.7-2.1)	0.80
Triglycerides (mmol/l) *	2.1 (0.7-4.1)	1.8 (0.9-4.1)	2.0 (0.7-4.1)	0.71

* Missing data: fasting glucose 19, total cholesterol 6, LDL-cholesterol 18, HDL-cholesterol 17, triglycerides 6. Missing data were not included in the comparison between patients with simple steatosis and NASH by Mann-Whitney U test.

Table 8.2 Histological features of 42 NAFLD patients in the retrospective study.

Steatosis grade	1	13 (31%)
	2	15 (36%)
	3	14 (33%)
Necroinflammatory grade	0	6 (14%)
	1	29 (69%)
	2	7 (17%)
	3	0
Fibrosis stage	0	31 (74%)
	1	10 (24%)
	2	0
	3	1 (2%)
	4	0

8.2 Cross-sectional study

Since significant necroinflammation and fibrosis were found in Chinese NAFLD subjects in the retrospective study, we decided to confirm the findings in a cross-sectional study. We prospectively recruited patients at the liver and general clinics of the Prince of Wales Hospital and Tseung Kwan O Hospital, Hong Kong.[Wong 2006] Consecutive patients with biopsy-proven NAFLD were included in this study. Liver biopsy was performed for Chinese patients aged 18 to 65 with ALT above 58 IU/l on 2 separate occasions at least 6 months apart. We excluded male patients who consumed more than 30 grams of alcohol per day and females who consumed more than 20 grams per day. Patients with co-existing liver disease, namely chronic viral hepatitis, autoimmune hepatitis, primary biliary cirrhosis, primary sclerosing cholangitis, Wilson's disease, hemochromatosis, α1-antitrypsin deficiency, biliary obstruction and drug-induced liver disease were excluded. Secondary causes (e.g. corticosteroid use, gastric bypass surgery) of liver steatosis were also excluded. The study was approved by the Joint CUHK-NTEC Clinical Research Ethics Committee. All patients gave informed written consent.

From January 2004 to June 2005, 80 ethnic Chinese (52 men and 28 women, age 44.5±9.1 years) with biopsy-proven NAFLD were recruited in this study. Fifty-five (69%) patients were asymptomatic while the remaining 25 (31%) patients had non-specific symptoms including malaise and right-upper quadrant

discomfort. No patient had liver decompensation. Table 8.3 shows their histological severity. Seventy-seven (96%) of the biopsy samples were adequate as defined. Sixteen (20%) patients had grade 2 or 3 necroinflammation. Fifty-two (65%) patients had liver fibrosis, and one patient had cirrhosis (fibrosis stage 4). Fifty-two (65%) patients had NASH according to pre-defined criteria.

Table 8.3 Histological features of 80 NAFLD patients in the cross-sectional study.

	Simple steatosis (N=28)	**NASH (N=52)**	**All NAFLD (N=80)**
Steatosis grade			
1	17 (61%)	21 (40%)	38 (48%)
2	9 (32%)	20 (39%)	29 (36%)
3	2 (7%)	11 (21%)	13 (16%)
Necroinflammatory grade			
0	14 (50%)	1 (2%)	15 (19%)
1	14 (50%)	35 (67%)	49 (61%)
2	NA	13 (25%)	13 (16%)
3	NA	3 (6%)	3 (4%)
Fibrosis stage			
0	28 (100%)	0	28 (35%)
1	NA	34 (65%)	34 (43%)
2	NA	11 (21%)	11 (14%)
3	NA	6 (12%)	6 (8%)
4	NA	1 (2%)	1 (1%)

8.3 Longitudinal study

In cross-sectional studies, NAFLD patients in Hong Kong had low prevalence of advanced fibrosis and cirrhosis. To study the natural history of NAFLD, we conducted a longitudinal study to investigate the histological progression. Patients in Study 1 with more than 3 years of follow-up were invited to have follow-up liver biopsies.[Hui 2005] Factors associated with histological progression were explored. The study was approved by the Joint CUHK-NTEC Clinical Research Ethics Committee. All patients provided informed written consent.

Patient characteristics

The demographic, clinical and laboratory data of the patients at the time of liver biopsies are shown in Table 8.4. The mean (±SD) age of the cohort at the time of second liver biopsy was 41.8±2.6 years and 11 (65%) were male. The median interval between the first and second biopsies was 6.1 (3.8-8.0) years. Though there was a trend for patients' body weight to decrease over time, this did not reach statistical significance. Insignificant decrease was also noted in the levels of ALT, total cholesterol and triglyceride.

Histological progression of disease

Three out of the 17 patients had only steatosis but no inflammation or fibrosis in the first biopsy (Table 8.5). Among the remaining 14 patients, 6 had evidence of

fibrosis at that time. No statistically significant change was noted in histological scores between the two biopsies in all three categories – macrovesicular steatosis, necroinflammation and fibrosis (Table 8.4). There was a trend for the fibrosis score to increase but this was not statistically significant (P=0.07).

Since previous studies indicated that initial histological features may predict disease progression, we determined the correlation between the scores of the first biopsy and the second biopsy (Table 8.6). No significant correlation was found between the two biopsies. Analysis of data from individual patients revealed that the scores for macrovesicular steatosis and necroinflammation remained unchanged or improved in 16 and 15 patients respectively. However, there was increase of 1-2 points in fibrosis score in 8 (47%) patients (Table 8.5). Among these 8 patients who deteriorated histologically with development or worsening of fibrosis, 7 had necroinflammation in the first biopsy (Table 8.5). In other words, half of the patients with steatohepatitis developed more advanced disease over time. Only one patient (out of 3) with pure steatosis developed fibrosis in the second biopsy.

It should be noted that not all patients with steatohepatitis worsened with time. Four patients (Patient Nos.11-14) who had grade 1 necroinflammation in the first biopsy were found to have only steatosis in the second biopsy. Patient 14 was unique in having resolution of fibrosis (decrease from stage 2 to 0). This patient

had no evidence of diabetes and hypertension and his BMI was reduced by 12% from 31.8 to 28 kg/m^2 during the follow-up period.

Progression of metabolic syndrome

All patients had at least one component of the metabolic syndrome at the time of the first biopsy. Four patients had type 2 diabetes mellitus and all were overweight or obese (Table 8.5). Twelve patients had hypercholesterolemia and 9 patients had hypertriglyceridemia. During the period between the two biopsies, four patients developed hypertension and three became diabetic. There was nevertheless no significant change in the median cholesterol and triglyceride levels between the two biopsies.

Relationship between histological progression and metabolic syndrome

To elucidate whether histological progression of NAFLD occurs in synchrony with evolution in metabolic syndrome, we determined the correlation between the change in histological scores and the change in metabolic parameters including BMI, blood glucose, HbA_{1c}, total cholesterol and triglyceride, as well as age and duration between biopsies (Table 8.7). No significant correlation was identified between these variables.

Table 8.4 Clinical and laboratory characteristics of 17 NAFLD patients with paired liver biopsies. Variables are expressed as median (range) except histological scores which are expressed as mean ± SE.

	First biopsy	**Second biopsy**	**P value**
Body weight (kg)	77.5 (51.3-102.7)	71.4 (53.7-103.4)	0.173
Body mass index (kg/m^2)	29.3 (23-35.5)	27.6 (22.9-35.8)	0.173
Hip circumference (cm)	-	103 (89-116)	
Waist circumference (cm)	-	81 (70-116.5)	
Waist/hip ratio	-	0.88 (0.79-1.0)	
Total protein (g/l)	80 (72-89)	80 (73-85)	0.979
Albumin (g/l)	42 (37-46)	45 (40-49)	0.019
Total bilirubin (μmol/l)	9 (1-24)	12 (5-18)	0.176
ALP (IU/l)	104 (57-367)	101 (59-275)	0.758
ALT (IU/l)	104 (32-193)	50 (24-194)	0.055
AST (IU/l)	-	30.5 (17-88)	
GGT (IU/l)	-	57 (16-569)	
Total cholesterol	6.1 (4.0-7.3)	5.1 (3.0-7.3)	0.187
HDL cholesterol	1.3 (1.0-2.2)	1.3 (1.0-2.3)	0.917
LDL cholesterol	3.95 (3.1-5.0)	3.5 (1.6-5.0)	0.207
Triglyceride	2.2 (0.65-4.14)	1.41 (0.41-4.30)	0.074
Fasting glucose	6.2 (4.8-13.2)	5.8 (4.6-19.1)	0.918
HbA1 (%)	5.8 (4.7-8.3)	6.3 (9.1-13.2)	0.398
Platelet count ($10^9/l$)	234 (158-347)	250 (170-345)	0.705
Prothrombin time (sec)	10.1 (9.4-11.4)	10.3 (9.6-12.8)	0.107
Macrovesicular steatosis	1.88 ± 0.19	1.53 ± 0.19	0.165

Necroinflammation	0.82 ± 0.10	0.65 ± 0.12	0.257
Fibrosis	0.41 ± 0.15	0.82 ± 0.18	0.074

Table 8.5 Histological progression of NAFLD patients. Age at time of second biopsy is presented. Data collected at the time of first (Bx1) and second (Bx2) biopsies are represented in separate columns. Presence of diagnosis of hypertension and diabetes mellitus is indicated by 'Y'. Histological scoring was performed using Brunt's criteria and presented in the order of macrovesicular steatosis, necroinflammation, fibrosis. The change in fibrosis score or stage of disease is summarized in the last column.

Patient No.	Sex	Age (yr)	Body mass index		Hypertension		Diabetes mellitus		Histology		Duration between biopsies (yr)	Disease stage progression
			Bx1	Bx2	Bx1	Bx2	Bx1	Bx2	Bx1	Bx2		
1	M	27	29.7	29.0	-	Y	-	-	2,1,0	2,1,1	6.4	Worse
2	M	50	35.5	35.8	-	Y	-	-	1,1,0	3,1,1	4.2	Worse
3	M	37	28.3	28.7	-	-	-	-	2,1,0	2,1,1	4.2	Worse
4	F	54	24.6	26.3	-	-	Y	Y	3,1,1	1,1,2	4.6	Worse
5	M	32	29.6	23.3	-	-	-	-	3,0,0	1,1,1	4.2	Worse
6	M	33	28.9	27.6	-	-	-	-	1,0,0	1,0,0	6.6	No change
7	F	46	30.2	28.5	-	Y	Y	Y	1,1,1	1,1,2	7.7	Worse
8	M	55	23.4	23.4	-	-	-	Y	1,0,0	1,1,0	6.8	No change
9	F	59	28.5	22.9	-	-	-	Y	2,1,0	1,1,2	6.5	Worse
10	M	36	30.0	31.6	-	-	-	-	3,1,1	3,1,1	6.5	No change
11	M	36	27.8	25.5	-	-	-	Y	2,1,0	1,0,0	3.8	No change
12	F	43	23.0	25.7	-	Y	-	-	1,1,0	1,0,0	5.1	No change
13	M	33	25.7	26.5	-	-	-	-	3,1,0	3,0,0	4.2	No change
14	M	38	31.8	28.0	-	-	-	-	1,1,2	1,0,0	8.0	Better
15	F	26	33.9	30.5	-	-	Y	Y	2,1,1	1,1,1	7.2	No change
16	F	56	30.5	30.2	-	-	-	-	2,1,0	2,1,1	6.1	Worse
17	M	50	25.1	25.0	-	-	Y	Y	2,1,1	1,0,1	5.7	No change

Table 8.6 Correlation coefficients (Spearman's) of histological scores of the first biopsy and the second biopsy and the change in the scores.

		First biopsy		
		Steatosis	Necro-inflammation	Fibrosis
Second biopsy	Steatosis	0.296 P=0.248	0.215 P=0.408	0.320 P=0.211
	Necro-inflammation	0.334 P=0.190	-0.019 P=0.942	0.325 P=0.204
	Fibrosis	-0.251 P=0.331	-0.030 P=0.909	0.277 P=0.282

Table 8.7 Correlation coefficients (Spearman's) of change in clinical and metabolic parameters and change in histological scores between the two biopsies.

	Age	Duration between biopsies	Δ BMI	Δ Glucose	Δ HbA_{1c}	Δ Total cholesterol	Δ Triglyceride
Δ Macrovesicular steatosis	0.062 P=0.814	0.102 P=0.696	0.397 P=0.127	-0.411 P=0.114	-0.418 P=0.350	-0.147 P=0.588	-0.190 P=0.481
Δ Necro-inflammation	0.052 P=0.842	0.226 P=0.383	-0.195 P=0.470	-0.038 P=890	0.259 P=0.575	0.281 P=0.293	0.003 P=0.990
Δ Fibrosis	0.319 P=0.212	-0.206 P=0.428	-0.103 P=0.704	0.196 P=0.466	-0.154 P=0.741	0.172 P=0.523	0.151 P=0.576

8.4 Conclusions

In both the retrospective and cross-sectional studies, necroinflammation and fibrosis were seen among NAFLD patients. However, the majority of the NAFLD patients in Hong Kong had mild disease. Only around 10% had advanced fibrosis or cirrhosis. Grade 2 or 3 necroinflammation only occurred in around 20% of subjects. Chinese patients with lower BMI also developed NAFLD. ALT had poor correlation with histological severity.

Upon long-term follow-up, NAFLD patients may have progressive disease. The diagnosis of NAFLD may also predate the development of new components of metabolic syndrome. Alternatively, the phenomenon may be due to under-diagnosis of diabetes and metabolic syndrome. In either case, these data suggest that proper evaluation and follow-up of NAFLD patients are important.

CHAPTER NINE

METABOLIC SYNDROME AND ADIPOKINES IN NONALCOHOLIC FATTY LIVER DISEASE

9.1 Metabolic syndrome and nonalcoholic fatty liver disease

In study 2, we prospectively recruited 80 patients with biopsy-proven NAFLD (See section 8.2).[Wong 2006] Healthy volunteers who had normal ALT levels were recruited as controls. They included medical staff and their family members as well as patients from general medical clinics with minor symptoms such as headache and dyspepsia. No control subject had history of hypertension, diabetes mellitus or cardiovascular diseases. All subjects in the control group underwent abdominal ultrasound examination to screen for fatty liver. In this study, 41 control subjects were recruited.

NAFLD versus controls

Compared to controls, NAFLD patients were more obese (Table 9.1). Twenty-seven (34%) patients had BMI above 30 kg/m^2, and 41 (51%) had BMI between 25 and 30 kg/m^2. Using the Asian cutoff, 76 (95%) patients were overweight or obese, as defined as BMI above 23 kg/m^2. The proportion of patients with NAFLD started to increase as the BMI exceeded 23 kg/m^2 ($P<0.0001$ for trend). Central obesity was more common among NAFLD patients. Both male and female NAFLD patients had greater waist circumference as compared to controls (98±10 cm vs. 87±9 cm, $P<0.0001$ in males; 93±10 cm vs. 78±7 cm, $P<0.0001$ in females). Female NAFLD patients also had greater waist-hip ratio than controls (0.90±0.05 vs. 0.85±0.08, $P=0.014$).

NAFLD patients were more likely to have dyslipidemia (Table 9.1). Fifty-four (68%) patients had LDL-cholesterol above 2.6 mmol/l. HDL-cholesterol was low in 15 male and 14 female NAFLD patients. Fifty-three (66%) patients had triglycerides above 1.7 mmol/l. Seventy (88%) patients had one or more dyslipidemia components.

Forty-six (58%) NAFLD patients had type 2 diabetes mellitus. Their fasting plasma glucose was 7.5±3.2 mmol/l, and HbA_{1c} was 7.0±1.5%. Twenty-nine (63%) patients had HbA_{1c} below 7%. Only nine (20%) patients had HbA_{1c} above 8%. No patient in this cohort used thiazolidinediones or insulin. Fifteen patients were treated with metformin, and 8 patients used a sulfonylurea.

Forty-five (56%) patients fulfilled the NCEP-ATPIII criteria of metabolic syndrome (Table 9.1). The number of patients with 1, 2, 3, 4 and 5 NCEP-ATPIII criteria was 10 (13%), 20 (25%), 21 (26%), 17 (21%) and 7 (9%), respectively. Using the IDF criteria for Chinese patients, 56 (70%) had metabolic syndrome ($P<0.0001$ compared to the NCEP-ATP III criteria). The number of patients with 1, 2, 3, 4 and 5 IDF criteria was 8 (10%), 14 (18%), 22 (28%), 20 (25%) and 16 (20%), respectively. All patients had at least one component of the IDF criteria, while 5 (6%) patients did not have any component of the NCEP-ATP III criteria.

Hyperinsulinemia was more common in NAFLD patients. The fasting insulin level of NAFLD patients was 236±310 pmol/l, versus 57±42 pmol/l among controls (P<0.0001). This was also paralleled with higher insulin resistance among NAFLD patients. The HOMA-IR of NAFLD patients was 2.9-fold higher than that of controls (2.9±2.3% vs. 1.0±0.7%; P<0.0001).

NASH versus simple steatosis

Among patients with NAFLD, fifty-two (65%) patients had NASH. NASH patients were heavier and had greater waist circumference than patients with simple steatosis (98±10 cm vs. 93±10 cm; P=0.031; Table 9.2). Forty-eight (92%) NASH patients had BMI more than 25 kg/m^2, compared to 20 (71%) patients with simple steatosis (P=0.02). The proportion of patients with NASH started to increase as their BMI exceeded 23 kg/m^2 (P<0.0001 for trend). Sixty-eight (85%) patients had central obesity according to the IDF criteria for Chinese.

As NASH patients were more likely to have central obesity and hyperglycemia, metabolic syndrome occurred more commonly among NASH patients than those with simple steatosis according to the IDF criteria (79% vs. 54%; P=0.019). On the other hand, the difference in the proportion of patients with metabolic syndrome was not statistically significant when the ATP III criteria were used (62% of NASH patients vs. 46% of patients with simple steatosis; P=0.19). The

number of IDF criteria correlated with fibrosis stage (Spearman correlation coefficient 0.24; P=0.031).

Furthermore, NASH patients in this cohort had lower ALT level than patients with simple steatosis (62±47 vs. 38±40; P=0.018). Indeed, ALT had a negative correlation with fibrosis stage (Spearman correlation coefficient -0.31; P=0.006). On the other hand, AST-to-ALT ratio was similar between the two groups.

Table 9.1 Clinical and metabolic profile of NAFLD patients and controls in the cross-sectional study

	NAFLD patients (N=80)	**Controls (N=41)**	**P-value**
Age	45±9	42±10	0.18
Gender (Male:Female)	52:28	17:24	0.013
Body weight (kg)	79±15	65±22	<0.0001
Body mass index (kg/m^2)	29.0±4.8	24.1±6.8	<0.0001
Male	28.9±4.4	26.7±9.8	0.19
≥23	49 (94%)	14 (82%)	0.15
≥25	45 (87%)	10 (59%)	0.014
≥30	17 (33%)	2 (12%)	0.12
Female	29.1±5.7	22.3±2.2	<0.0001
≥23	27 (96%)	10 (42%)	<0.0001
≥25	23 (82%)	3 (13%)	<0.0001
≥30	10 (36%)	0	0.001
Waist circumference (cm)	95±10	82±9	<0.0001
Male	98±10	87±9	<0.0001
Female	93±10	78±7	<0.0001
Waist-hip ratio	0.93±0.07	0.88±0.14	0.008
Male	0.95±0.07	0.93±0.19	0.39

Female	0.90±0.05	0.85±0.08	0.014
ALT (IU/L)	47±44	24±9	<0.0001
AST (IU/L)	33±20	24±4	<0.0001
GGT (IU/L)	64±90	26±16	<0.0001
Fasting glucose (mmol/l)	6.7±2.6	5.0±0.6	<0.0001
Hb_{A1c} (%)	6.6±1.5	5.2±0.3	<0.0001
Total cholesterol (mmol/l)	5.5±1.1	5.2±0.9	0.09
HDL-cholesterol (mmol/l)	1.23±0.30	1.65±0.46	<0.0001
LDL-cholesterol (mmol/l)	3.1±1.0	2.9±0.7	0.28
Triglycerides (mmol/l)	2.14 (1.37-2.86)	1.00 (0.64-1.87)	<0.0001
Insulin (pmol/l)	236±310	57±42	<0.0001
HOMA-IR¶	2.9±2.3	1.0±0.7	<0.0001
Comorbidities			
Diabetes mellitus	46 (58%)	1 (2%)	<0.0001
Hypertension	30 (38%)	0	<0.0001
Ischemic heart disease	1 (1.3%)	0	1
Metabolic syndrome	45 (56%)	2 (5%)	<0.0001
(ATP III criteria)			
Abdominal obesity *	31 (39%)	2 (5%)	<0.0001
Triglycerides ≥1.7 mmol/l	53 (66%)	11 (27%)	<0.0001
Low HDL-cholesterol #	29 (36%)	4 (10%)	0.002

Blood pressure ≥130/85 mmHg	49 (61%)	0	<0.0001
Fasting glucose ≥6.1 mmol/l	54 (68%)	2 (5%)	<0.0001
Metabolic syndrome (IDF criteria for Chinese)	56 (70%)	3 (7%)	<0.0001
Abdominal obesity **	68 (85%)	18 (44%)	<0.0001
Fasting glucose ≥5.6 mmol/l	58 (73%)	6 (15%)	<0.0001

Continuous variables were expressed as mean ± standard deviation or median (interquartile range).

* Waist circumference ≥102 cm in men or ≥88 cm in women

** Waist circumference ≥90 cm in men or ≥80 cm in women

HDL-cholesterol <1.03 mmol/l in men and <1.29 mmol/l in women

¶ Excluded patients with established diabetes

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; GGT, gamma-glutamyl transferase; ATP, Adult Treatment Panel; IDF, International Diabetes Federation

Table 9.2 Clinical and metabolic profile of patients with simple steatosis and NASH

	Simple steatosis (N=28)	**NASH (N=52)**	**P-value**
Age	44±9	45±9	0.49
Gender (Male:Female)	19:9	33:19	0.69
Body weight (kg)	74±14	81±15	0.057
Body mass index (kg/m^2)	27.1±4.0	30.0±5.0	0.01
Male	27.3±4.6	29.8±4.0	0.047
≥23	16 (84%)	33 (100%)	0.044
≥25	13 (68%)	32 (97%)	0.007
≥30	5 (26%)	12 (36%)	0.46
Female	26.6±2.5	30.2±6.4	0.11
≥23	9 (100%)	18 (95%)	1
≥25	7 (78%)	16 (84%)	1
≥30	1 (11%)	9 (47%)	0.10
Waist circumference (cm)	93±10	98±10	0.031
Male	94±10	100±9	0.059
Female	89±7	95±11	0.21
Waist-hip ratio	0.92±0.07	0.94±0.07	0.16
Male	0.93±0.08	0.97±0.07	0.13

Female	0.89±0.03	0.90±0.06	0.52
ALT (IU/L)	62±47	38±40	0.018
AST (IU/L)	37±25	31±18	0.16
GGT (IU/L)	94±140	47±43	0.083
Fasting glucose (mmol/l)	6.4±1.5	6.9±3.1	0.36
Hb_{A1c} (%)	6.1±1.1	6.9±1.6	0.026
Total cholesterol (mmol/l)	5.6±1.3	5.4±1.1	0.43
HDL-cholesterol (mmol/l)	1.30±0.31	1.20±0.29	0.15
LDL-cholesterol (mmol/l)	3.3±0.9	3.0±1.1	0.20
Triglycerides (mmol/l)	2.01 (0.88-2.43)	2.37 (1.45-2.91)	0.23
Insulin (pmol/l)	163±214	275±347	0.12
HOMA-IR¶	2.8±2.9	3.0±1.9	0.84
Comorbidities			
Diabetes mellitus	12 (43%)	34 (65%)	0.052
Hypertension	8 (29%)	22 (42%)	0.23
Ischemic heart disease	0	1 (2%)	1
Metabolic syndrome	13 (46%)	32 (62%)	0.19
(ATP III criteria)			
Abdominal obesity *	6 (21%)	25 (48%)	0.02
Triglycerides ≥1.7 mmol/l	18 (64%)	35 (67%)	0.78
Low HDL-cholesterol #	7 (25%)	22 (42%)	0.13

Blood pressure ≥130/85 mmHg	16 (57%)	33 (64%)	0.58
Fasting glucose ≥6.1 mmol/l	16 (57%)	38 (73%)	0.15
Metabolic syndrome (IDF criteria for Chinese)	15 (54%)	41 (79%)	0.019
Abdominal obesity **	21 (75%)	47 (90%)	0.066
Fasting glucose ≥5.6 mmol/l	18 (64%)	40 (77%)	0.23

Continuous variables were expressed as mean ± standard deviation or median (interquartile range).

* Waist circumference ≥102 cm in men or ≥88 cm in women

** Waist circumference ≥90 cm in men or ≥80 cm in women

HDL-cholesterol <1.03 mmol/l in men and <1.29 mmol/l in women

¶ Excluded patients with established diabetes

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; GGT, gamma-glutamyl transferase; ATP, Adult Treatment Panel; IDF, International Diabetes Federation

9.2 Adipokines and nonalcoholic fatty liver disease

In Study 2, we also tested the serum adipokine profile of NAFLD patients and control subjects to evaluate their association with NAFLD and its severity.[Wong 2006]

NAFLD versus controls

NAFLD patients had lower adiponectin level than controls (4.3±2.4 μg/ml vs. 7.9±4.5 μg/ml; P<0.0001; Figure 9.1). They had higher leptin level (25±19 ng/ml vs. 17±20 ng/ml; P=0.009). On the other hand, TNF-α level (2.4±1.3 pg/ml vs. 2.3±2.1 pg/ml; P=0.85) and resistin level (15±13 ng/ml vs. 15±8 ng/ml; P=0.87) were similar between NAFLD patients and controls.

On multivariate analysis (covariates including age, gender, BMI, waist-hip ratio, diabetes mellitus, leptin, adiponectin, TNF-α and resistin to adjust for potential metabolic confounders), diabetes mellitus, hypoadiponectinemia and raised leptin levels were independent factors associated with NAFLD (Table 9.3).

NASH versus simple steatosis

Among NAFLD patients, NASH patients had higher TNF-α level than patients with simple steatosis (2.7±1.4 pg/ml vs. 1.9±1.0 pg/ml; P=0.012; Figure 9.2). On the other hand, leptin level (26±20 ng/ml vs. 22±17 ng/ml; P=0.38), adiponectin level (4.2±2.6 μg/ml vs. 4.5±2.1 μg/ml; P=0.62) and resistin level (14±13 ng/ml

vs. 18±14 ng/ml; P=0.21) were similar between NASH and simple steatosis patients. Although TNF-α level did not correlate with steatosis score, it had positive correlation with necroinflammation grade (Spearman correlation coefficient 0.35; P=0.002) and fibrosis stage (Spearman correlation coefficient 0.31; P=0.005). Figures 9.3 and 9.4 showed the TNF-α levels according to different necroinflammatory grades and fibrosis stages.

On multivariate analysis (covariates including age, gender, BMI, waist-hip ratio, waist circumference, diabetes mellitus, leptin, adiponectin, TNF-α and resistin to adjust for potential metabolic confounders), elevated TNF-α level and high BMI were independent factors associated with NASH (Table 9.4).

Figure 9.1 Adipokine levels of NAFLD patients and control subjects

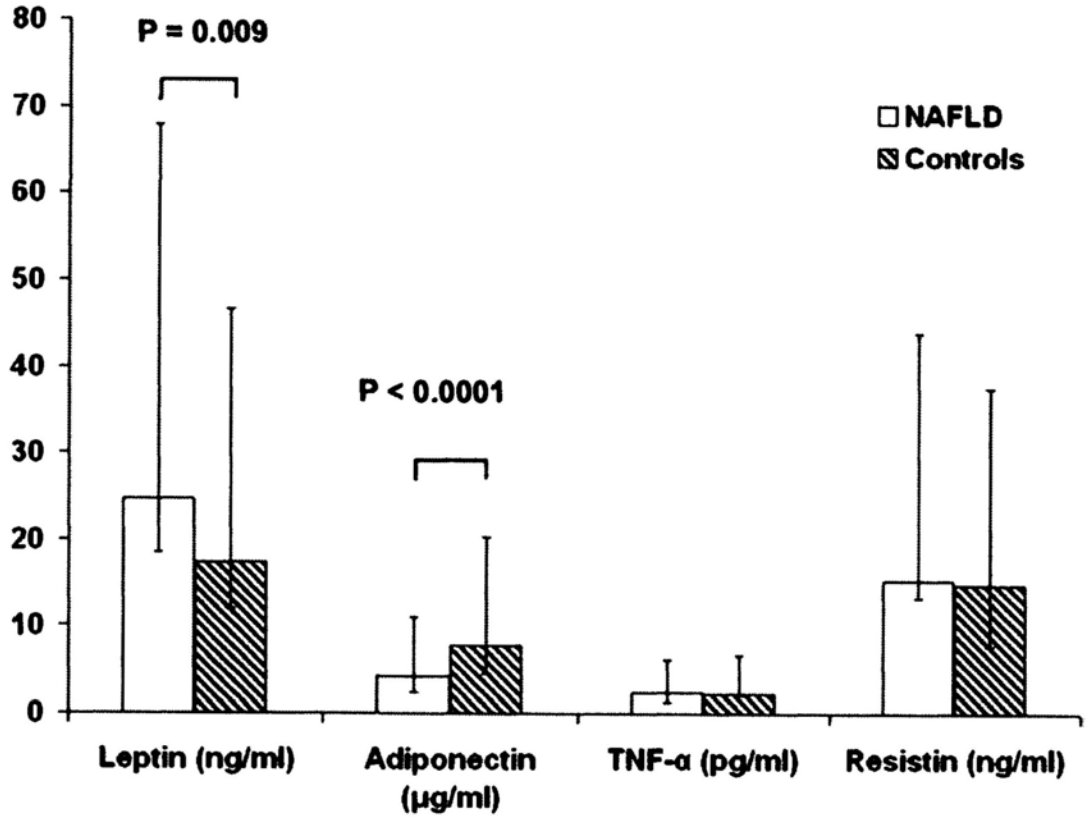


Figure 9.2 Adipokine levels of patients with simple steatosis and NASH

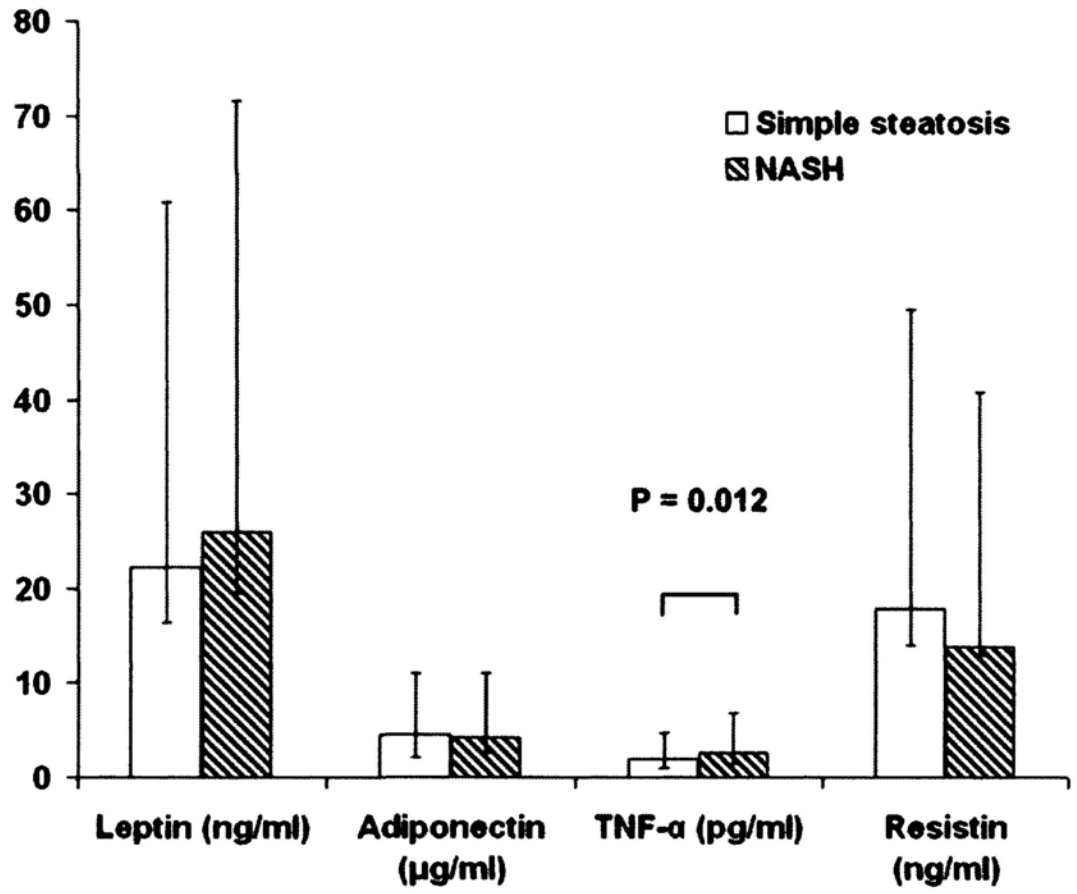


Figure 9.3 TNF-α levels of NAFLD patients with different necroinflammatory grades

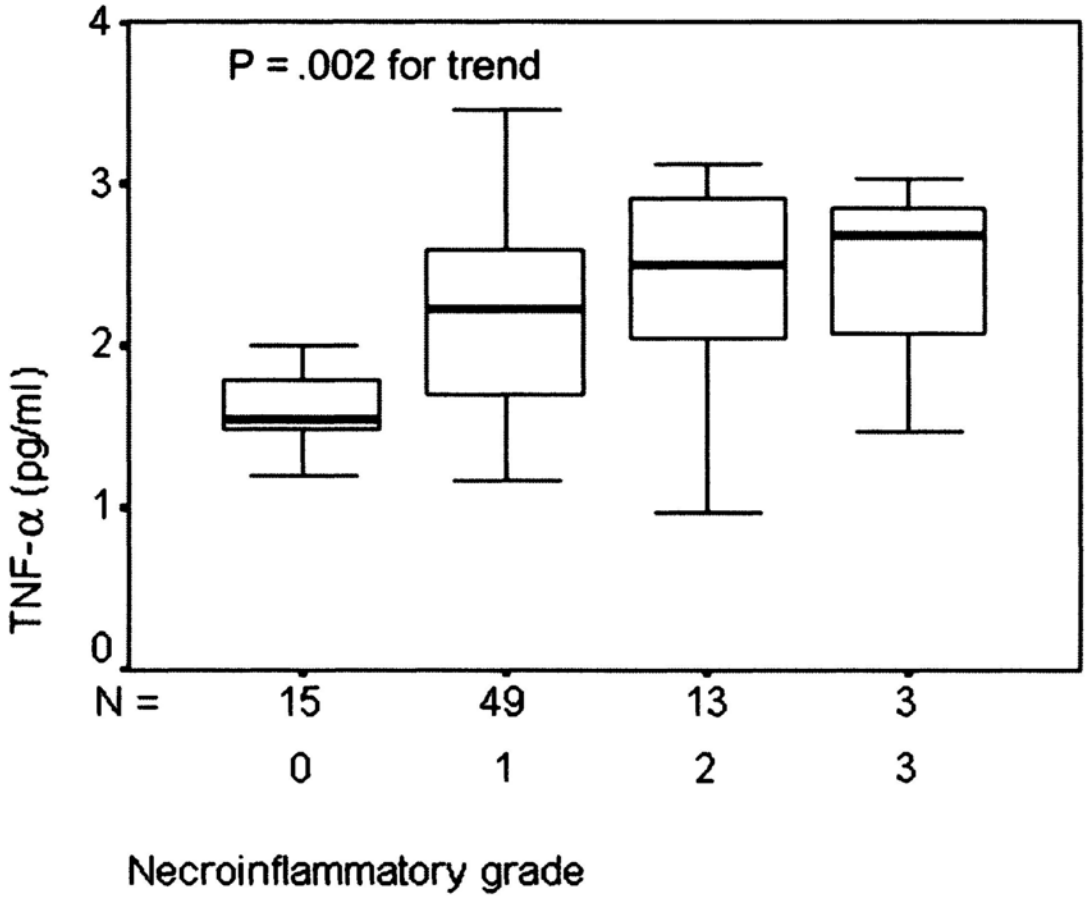


Figure 9.4 TNF-α levels of NAFLD patients with different fibrosis stages

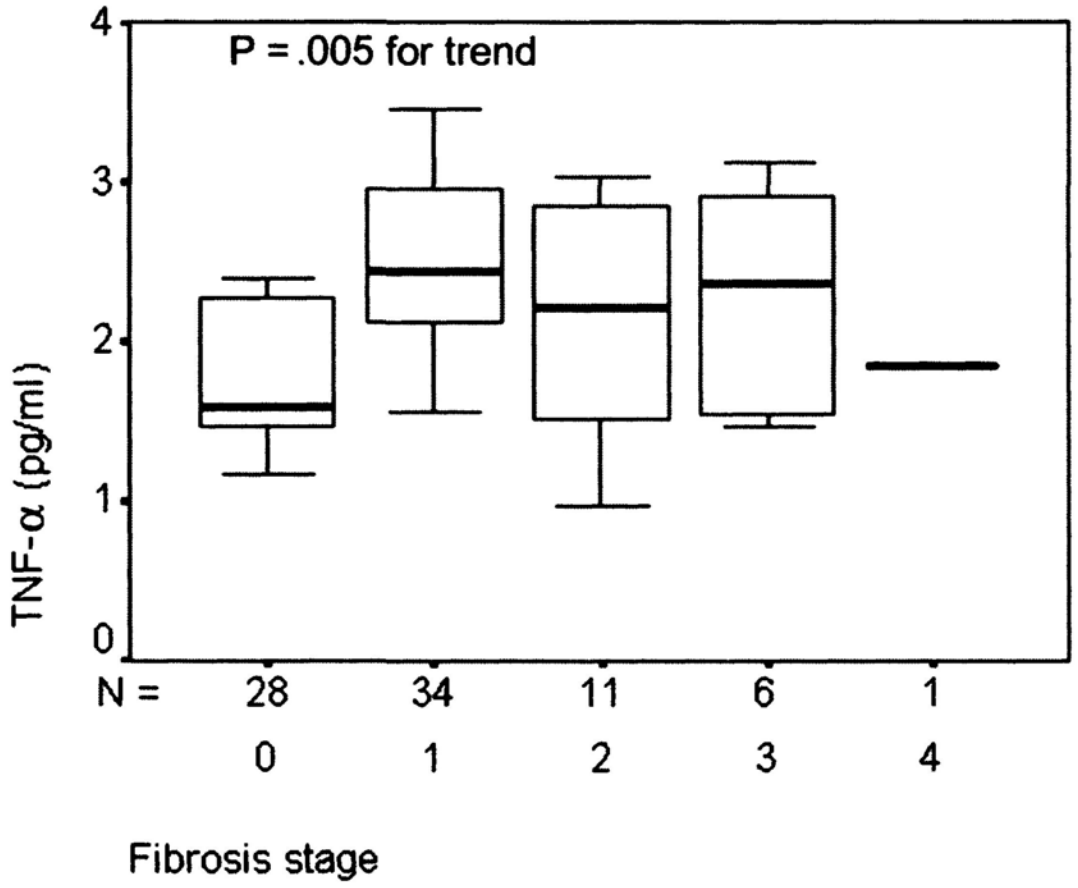


Table 9.3 Multivariate analysis of factors associated with NAFLD

	Odds ratio	**95% confidence interval**	**P-value**
Diabetes mellitus	53	6-470	<0.0001
Adiponectin (per μg/ml rise)	0.78	0.64-0.96	0.02
Leptin (per ng/ml rise)	1.1	1.0-1.2	0.009

Covariates include age, gender, body-mass index, waist-hip ratio, diabetes mellitus, adiponectin, resistin, TNF-α and leptin.

Table 9.4 Multivariate analysis of factors associated with NASH

	Odds ratio	95% confidence interval	P-value
Body mass index (per kg/m^2 increase)	1.4	1.0-2.0	0.05
TNF-α (per pg/ml increase)	2.5	1.1-5.7	0.03

Covariates include age, gender, body-mass index, waist-hip ratio, waist circumference, diabetes mellitus, adiponectin, resistin, TNF-α and leptin.

9.3 Gene polymorphisms of adipokines

Since the serum levels of adiponectin and TNF-α were found to be associated with NAFLD and NASH, we further tested the genetic polymorphisms of these two adipokines and investigated their association with the disease.[Wong 2008]

Adiponectin gene polymorphisms

The median (interquartile range) adiponectin level was 3.48 (2.63-5.12) μg/ml in NAFLD patients and 7.49 (4.32-11.5) μg /ml in controls ($p<0.001$). All the gene polymorphisms of adiponectin were in Hardy-Weinberg equilibrium. Moderate linkage disequilibrium was observed between ACDC+45 and ACDC+276 (linkage disequilibrium coefficient 0.36, $p=0.002$). There was no significant difference in allelic frequencies among all 4 adiponectin gene polymorphisms between NAFLD patients and controls. All NAFLD patients and controls had ACDC-11391G. ACDC-11377G was present in 28 of 79 (35%) NAFLD patients and 12 of 40 (30%) controls ($p=0.55$). ACDC+45G was present in 49 of 79 (62%) NAFLD patients and 25 of 40 (63%) controls ($p=0.96$). ACDC+276T was present in 33 of 79 (42%) NAFLD patients and 16 of 40 (40%) controls ($p=0.85$).

The median (interquartile range) adiponectin level did not differ between patients with or without significant necroinflammation (2.98 [2.66-3.73] μg /ml vs. 3.78 [2.51-5.46] μg /ml, $p=0.21$), with or without significant fibrosis (3.11 [2.57-4.68] μg /ml vs. 3.49 [2.62-5.46] μg /ml, $p=0.65$, and with or without

NASH (3.25 [2.48-5.10] μg /ml vs. 4.39 [2.84-5.45] μg /ml, p=0.42). There was also no significant difference in adiponectin allelic frequencies between NAFLD patients with and without significant necroinflammation, significant fibrosis or NASH. ACDC-11377G was present in 23 of 61 (38%) patients without significant fibrosis and 5 of 18 (28%) patients with significant fibrosis (p=0.44). ACDC+45G was present in 40 of 61 (66%) patients without significant fibrosis and 9 of 18 (50%) patients with significant fibrosis (p=0.23). ACDC+276T was present in 25 of 61 (41%) patients without significant fibrosis and 8 of 18 (44%) patients with significant fibrosis (p=0.79). Besides, none of the 4 adiponectin gene polymorphisms was associated with elevated or suppressed serum adiponectin level (Table 9.5).

On the other hand, NAFLD patients with the -11377G allele had higher plasma triglyceride level than those without this allele (p=0.03, Table 8.5). NAFLD patients with the +45G allele tend to have lower plasma triglyceride level than those without this allele (p=0.08). Less patients with the +45G allele had plasma triglyceride above 1.7 mmol/l (odds ratio 0.3; 95% CI 0.1, 1.0; p=0.04). The other adiponectin gene polymorphisms did not have significant association with other metabolic syndrome features including central obesity, HDL-cholesterol level, hypertension, and glucose level. In addition, the adiponectin gene polymorphisms were not associated with the degree of hepatic steatosis or fasting insulin level. ACDC-11377G was present in 13 of 38 (34%) patients with

grade 0 or 1 steatosis and 15 of 41 (37%) patients with grade 2 or 3 steatosis (p=0.83). +45G was present in 25 or 38 (66%) patients with grade 0 or 1 steatosis and 24 of 41 (59%) patients with grade 2 or 3 steatosis (p=0.51). The median fasting insulin in NAFLD patients with or without -11377G was 124 (81-195) pmol/l and 135 (62-283) pmol/l, respectively (p=0.91). The median fasting insulin in patients with or without +45G was 115 (68-218) pmol/l and 139 (76-269) pmol/l, respectively (p=0.59).

Tumor necrosis factor-alfa gene polymorphisms

The median (interquartile range) TNF-α level was higher in NAFLD patients (2.13 [1.55-2.67] pg/ml) than controls (1.75 [1.27-2.29] pg/ml, p=0.01). In men, the median TNF-α level was 2.16 (1.56-2.65) pg/ml in NAFLD patients and 1.76 (1.38-2.14) pg/ml in controls (p=0.067). In women, the median TNF-α level was 2.09 (1.2-2.81) pg/ml in NAFLD patients and 1.73 (1.25-2.33) pg/ml in controls (p=0.16). All the gene polymorphisms of TNF-α were in Hardy-Weinberg equilibrium. There was no significant difference in allelic frequencies among all 3 TNF-α gene polymorphisms between NAFLD patients and controls. TNFα-238A was present in 5 of 79 (6%) NAFLD patients and 4 of 40 (10%) controls (p=0.48). TNFα -308A was present in 13 of 79 (17%) NAFLD patients and 10 of 40 (25%) controls (p=0.27). TNFα -863A was present in 28 of 79 (35%) NAFLD patients and 13 of 27 (33%) controls (p=0.75).

The median (interquartile range) TNF-α level was 1.55 (1.45-2.03) pg/ml in patients with simple steatosis and 2.42 (1.94-2.91) pg/ml in NASH patients ($p<0.001$). However, there was also no significant difference in TNF-α allelic frequencies between NAFLD patients with and without significant necroinflammation, significant fibrosis and NASH. TNFα-238A was present in 5 of 61 (8%) patients without significant fibrosis and none of the patients with significant fibrosis ($p=0.58$). TNFα-308A was present in 10 of 61 (16%) patients without significant fibrosis and 3 of 18 (17%) patients with significant fibrosis ($p=1.0$). TNFα-863A was present in 22 of 61 (36%) patients without significant fibrosis and 6 of 18 (33%) patients with significant fibrosis ($p=0.83$). Besides, none of the 3 TNF-α gene polymorphisms was associated with changes in serum TNF-α or soluble TNF-α receptor-2 levels (Table 9.6).

NAFLD patients with the -238A allele had lower fasting plasma glucose than patients without the allele (Table 9.6). They were also less likely to have impaired fasting glucose or diabetes (odds ratio 0.1; 95% CI 0, 0.9; $p=0.05$). The other TNF-α gene polymorphisms had no significant association with other metabolic syndrome features.

Factors associated with significant fibrosis

On univariate analysis, NAFLD patients with significant fibrosis had older age, greater waist circumference, higher fasting glucose and HOMA-IR (Table 9.7).

On the other hand, none of the adiponectin or TNF-α gene polymorphisms was associated with significant fibrosis. On multivariate analysis, older age, higher BMI and higher fasting glucose were independent factors associated with significant fibrosis (Table 9.8). Inclusion of gender in the multivariate analysis did not alter the results.

Table 9.5 Adiponectin genetic polymorphisms and metabolic profile of NAFLD patients. Continuous variables were expressed as median (interquartile range).

Parameters	-11377		P-value	+45		P-value	+276		P-value
	C/C (N=51)	C/G or G/G (N=28)		T/T (N=30)	T/G or G/G (N=49)		G/G (N=46)	G/T or T/T (N=33)	
Central obesity (%)	44 (86)	24 (86)	1	27 (90)	41 (84)	0.52	40 (87)	28 (85)	0.79
Triglycerides (mmol/l)	1.92 (1.21-2.63)	2.50 (1.62-4.15)	0.03	2.37 (1.84-3.02)	1.92 (1.19-2.65)	0.08	2.17 (1.53-2.93)	1.99 (1.19-2.78)	0.54
HDL-cholesterol (mmol/l)	1.20 (1.04-1.32)	1.14 (1.04-1.30)	0.53	1.16 (1.00-1.30)	1.21 (1.08-1.36)	0.36	1.20 (1.06-1.32)	1.20 (1.02-1.32)	0.93
Fasting glucose	6.0 (5.4-6.9)	6.4 (5.4-7.4)	0.33	6.2 (5.6-7.2)	6.3 (5.2-7.1)	0.51	6.3 (5.6-7.0)	6.2 (5.3-7.2)	0.74
Hypertension (%)	36 (71)	21 (75)	0.68	23 (77)	34 (69)	0.48	34 (74)	23 (70)	0.68
Adiponectin level (μg/ml)	3.35 (2.61-4.97)	4.00 (2.53-5.63)	0.69	3.02 (2.25-4.95)	3.87 (2.91-5.46)	0.15	3.52 (2.75-4.99)	3.23 (2.31-5.46)	0.91

Table 9.6 Tumor necrosis factor-alpha genetic polymorphisms and metabolic profile of NAFLD patients. Continuous variables were expressed as median (interquartile range).

Parameters	-238		P-value	-308		P-value	-863		P-value
	G/G (N=74)	G/A (N=5)		G/G (N=66)	G/A (N=13)		C/C (N=51)	C/A or A/A (N=28)	
Central obesity (%)	65 (88)	3 (60)	0.14	55 (83)	13 (100)	0.20	43 (84)	25 (89)	0.74
Triglycerides (mmol/l)	2.15 (1.35-2.90)	1.46 (0.63-4.68)	0.33	2.06 (1.29-2.93)	2.26 (1.45-2.73)	0.92	2.26 (1.21-2.82)	2.01 (1.46-3.43)	0.48
HDL-cholesterol (mmol/l)	1.20 (1.06-1.31)	1.25 (0.94-1.45)	0.98	1.20 (1.02-1.30)	1.10 (1.10-1.51)	0.43	1.20 (1.09-1.32)	1.18 (1.00-1.30)	0.29
Fasting glucose	6.3 (5.6-7.3)	4.9 (4.9-5.7)	0.01	6.2 (5.3-7.3)	6.5 (5.9-6.9)	0.29	6.2 (5.4-6.9)	6.4 (5.3-7.9)	0.44
Hypertension (%)	55 (74)	2 (40)	0.13	48 (73)	9 (69)	0.75	35 (69)	22 (79)	0.35
Tumor necrosis factor-alpha level (pg/ml)	2.14 (1.55-2.71)	1.76 (1.37-2.42)	0.39	2.18 (1.57-2.67)	2.03 (1.47-2.66)	0.46	2.09 (1.53-2.77)	2.18 (1.66-2.54)	0.86
Soluble TNF-α receptor-2 level (μg/ml)	2.03 (1.67-2.44)	2.07 (1.77-2.43)	0.99	2.05 (1.74-2.48)	1.80 (1.63-2.33)	0.30	1.94 (1.72-2.33)	2.12 (1.64-2.69)	0.46

Table 9.7 Univariate analysis of factors associated with significant fibrosis in NAFLD patients.

Factors	Simple steatosis (N=61)	Significant fibrosis (N=18)	P-value
Age	42 (37-50)	53 (41-56)	0.02
Male gender (%)	43 (71)	9 (50)	0.11
Waist circumference (cm)	94 (89-101)	99 (95-111)	0.03
Body mass index (kg/m^2)	28.1 (25.5-30.4)	29.6 (27.0-37.2)	0.08
Triglycerides (mmol/l)	2.08 (1.33-2.91)	2.17 (1.23-2.81)	0.96
HDL-cholesterol (mmol/l)	1.20 (1.08-1.32)	1.14 (1.00-1.35)	0.39
Fasting glucose (mmol/l)	6.0 (5.3-6.7)	7.1 (6.1-8.7)	0.007
HOMA-IR (%)	2.2 (1.3-3.6)	3.3 (2.1-6.2)	0.05
Systolic blood pressure (mmHg)	135 (124-148)	132 (120-151)	0.70
Diastolic blood pressure (mmHg)	80 (73-90)	78 (71-82)	0.12

Table 9.8 Multivariate analysis of factors associated with significant fibrosis in NAFLD patients

Factor	Odds ratio	95% CI	p-value
Age (per year)	1.2	1.1-1.3	0.001
Body mass index (per kg/m2)	1.5	1.1-2.0	0.01
Waist circumference (per cm)	0.92	0.81-1.05	0.18
Fasting glucose (per mmol/l)	1.4	1.1-1.8	0.008
HOMA-IR (per unit rise)	1.2	1.0-1.5	0.06

9.4 Conclusions

Our data showed that NAFLD in Chinese is probably associated with hypoadiponectinemia, increased leptin level, and diabetes. NASH may be associated with raised TNF-α level and high BMI. Ethnic-specific definitions of obesity and metabolic syndrome are more useful in the assessment of NAFLD patients.

On the other hand, adiponectin and TNF-α gene polymorphisms were not shown to be associated with NAFLD or significant fibrosis in Chinese. The adiponectin -11377G and +45G alleles were associated with hypertriglyceridemia in NAFLD patients. Since the current study is not adequately powered to detect smaller differences in allele frequencies, larger sized studies in different ethnic groups are required.

CHAPTER TEN

POST-CHALLENGE HYPERGLYCEMIA IN NONALCOHOLIC FATTY LIVER DISEASE

10.1 Prevalence of post-challenge hyperglycemia

Since type 2 diabetes is closely related to NAFLD and its severity, it is important to evaluate the role of oral glucose tolerance test in NAFLD patients. In this study, we performed 75 gram oral glucose tolerance test on NAFLD patients without known history of type 2 diabetes.[Wong 2006-2] For comparison, we also recruited community controls at a ratio of 2 to 1. The control subjects had no history of diabetes, hypertension, dyslipidemia, or chronic liver disease.

From January 2004 to December 2005, 124 ethnic Chinese had liver biopsy-confirmed NAFLD. Fifty-one patients had pre-existing diabetes on diet or pharmacological treatment. As expected, patients with pre-existing diabetes had higher fasting glucose and HbA_{1c} (Table 10.1). However, their insulin resistance, as depicted by fasting insulin and HOMA-IR, was similar to patients without pre-existing diabetes. Patients with pre-existing diabetes had similar necroinflammatory grades to patients in the current cohort, but were more likely to have liver fibrosis (86% vs. 60%; P=0.002).

Oral glucose tolerance test

Among 73 NAFLD patients without pre-existing diabetes, fasting glucose was 5.8±1.3 mmol/l. Nine (12%) patients had fasting glucose above 7.0 mmol/l, and were diagnosed to have diabetes. Sixty-four NAFLD patients with fasting

glucose below 7.0 mmol/l underwent oral glucose tolerance test. The 2-hour plasma glucose of this cohort was 9.1±2.9 mmol/l (Table 10.2). Fifteen (21%) patients were diagnosed to have diabetes with post-load plasma glucose above 11.1 mmol/l, among them 10 also had impaired fasting glucose (fasting glucose between 5.6 and 6.9 mmol/l) (Figure 10.1). Twenty-one (29%) patients did not reach the criteria of diabetes but had impaired glucose tolerance (2-hour plasma glucose between 7.8 and 11.0 mmol/l), among them nine also had impaired fasting glucose. Among the 36 patients with post-load glucose above 7.8 mmol/l, 17 (47%) had normal fasting glucose. On the other hand, only four (5%) patients had isolated impaired fasting glucose. Twenty-four (33%) patients had normal oral glucose tolerance test results.

NAFLD and insulin resistance

NAFLD patients were more likely to have impaired glucose tolerance (21 of 73 NAFLD patients vs. 21 of 146 controls had impaired glucose tolerance, $P<0.0001$) and diabetes (24 of 73 NAFLD patients vs. 10 of 146 controls had diabetes, $P<0.0001$) than controls. The level of post-challenge hyperglycemia among NAFLD patients paralleled that of insulin resistance. NAFLD patients had significantly higher fasting insulin level than controls (251±377 pmol/l versus 57±43 pmol/l, respectively; mean difference 194 pmol/l; 95 percent

confidence interval 127 to 260 pmol/l) and HOMA-IR (4.6±2.9 percent versus 1.1±0.8 percent, respectively; mean difference 3.5 percent; 95% CI 3.0 to 4.0 percent).

Predictors of undiagnosed diabetes among NAFLD patients

NAFLD patients with diabetes had higher fasting glucose than non-diabetic counterparts (Table 10.3). They also had lower HDL-cholesterol level and higher serum triglycerides. There was also a trend that NAFLD patients with diabetes were older. In a binary logistic regression model (covariates included age, fasting glucose, HDL-cholesterol and triglycerides), fasting glucose and HDL-cholesterol remained as independent factors predicting the presence of diabetes (Table 10.3).

Figure 10.2 shows the ROC curve of fasting glucose to predict diabetes in NAFLD patients without pre-existing diabetes. If oral glucose tolerance test was only performed in patients with IFG according to the 1997 ADA criteria (fasting glucose at or above 6.1 mmol/l), nearly 40% of diabetes cases would be missed (Table 10.4).[American Diabetes Association 1997] Even if the normal fasting glucose cutoff was lowered to 5.6 mmol/l according to the 2006 ADA criteria, the sensitivity in detecting diabetes was only 79%.[American Diabetes Association 2006]

Table 10.1 Demographic, biochemical and histological data of NAFLD patients with and without pre-existing diabetes.

Characteristics	Patients with pre-existing diabetes (N=51)	Patients without pre-existing diabetes (N=73)	P-value
Age	46 ± 13	45 ± 8	0.51
Male gender (%)	23 (45%)	52 (71%)	0.003
Body weight (kg)	76 ± 15	78 ± 14	0.36
Body mass index (kg/m^2)	29 ± 4	29 ± 5	0.84
Waist circumference (cm)	95 ± 10	96 ± 10	0.49
Hip circumference (cm)	102 ± 9	102 ± 9	0.66
Waist-hip ratio	0.94 ± 0.07	0.94 ± 0.07	0.83
Systolic blood pressure (mmHg)	137 ± 22	134 ± 14	0.43
Diastolic blood pressure (mmHg)	78 ± 11	80 ± 11	0.46
ALT (IU/l)	53 ± 39	69 ± 58	0.088
Fasting glucose (mmol/l)	7.7 ± 3.2	5.8 ± 1.3	<0.0001
HbA_{1c} (%)	7.5 ± 1.6	6.1 ± 1.0	<0.0001
Total cholesterol (mmol/l)	5.5 ± 1.3	5.7 ± 1.1	0.39
HDL-cholesterol (mmol/l)	1.22 ± 0.29	1.27 ± 0.36	0.40
LDL-cholesterol (mmol/l)	3.0 ± 1.2	3.3 ± 1.1	0.14
Triglycerides (mmol/l)	2.64 ± 2.41	2.48 ± 1.90	0.70
Fasting insulin (pmol/l)	237 ± 245	249 ± 364	0.86
HOMA-IR (%)	4.3 ± 4.0	3.3 ± 2.9	0.20

Brunt's score				
Steatosis grade	1	19 (37%)	32 (44%)	0.55
	2	26 (51%)	30 (41%)	
	3	6 (12%)	11 (15%)	
Necroinflammatory grade	0	2 (4%)	14 (19%)	0.079
	1	35 (69%)	43 (59%)	
	2	12 (24%)	15 (21%)	
	3	2 (4%)	1 (1%)	
Fibrosis stage	0	7 (14%)	29 (40%)	0.015
	1	27 (53%)	31 (43%)	
	2	13 (26%)	8 (11%)	
	3	4 (8%)	4 (6%)	
	4	0	1 (1%)	

Continuous variables were expressed in mean ± standard deviation. ALT: alanine aminotransferase; HDL: high density lipoprotein; LDL: low density lipoprotein; HOMA-IR: homeostasis model for insulin resistance.

Table 10.2 Metabolic profile and oral glucose tolerance test results of NAFLD patients without pre-existing diabetes and controls.

		NAFLD patients (N=73)	Controls (N=146)	P-value
Age		45 ± 8	45 ± 8	0.90
Gender		52 (71%)	104 (71%)	1
Body weight (kg)		78 ± 14	65 ± 11	<0.0001
Body mass index (kg/m^2)		29 ± 5	24 ± 3	<0.0001
Waist circumference (cm)		96 ± 10	82 ± 8	<0.0001
Hip circumference (cm)		102 ± 9	94 ± 6	<0.0001
Waist-hip ratio		0.94 ± 0.07	0.86 ± 0.06	<0.0001
Oral glucose tolerance test				
0-h plasma glucose (mmol/l)		5.8 ± 1.3	5.1 ± 0.7	<0.0001
1-h plasma glucose* (mmol/l)		10.8 ± 2.9	8.7 ± 2.9	0.001
2-h plasma glucose* (mmol/l)		9.1 ± 2.9	6.6 ± 2.8	<0.0001
0-h glucose	≥5.6 mmol/l	32 (44%)	20 (14%)	<0.0001
	≥6.1 mmol/l	20 (27%)	9 (6%)	<0.0001
	≥7.0 mmol/l	9 (12%)	3 (2%)	0.003
2-h glucose[a]	≥7.8 mmol/l	37 (57%)	31 (21%)	<0.0001
	≥11.1 mmol/l	16 (25%)	10 (7%)	<0.0001
Fasting insulin (pmol/l)		249 ± 364	53 ± 36	<0.0001
HOMA-IR (%)		3.3 ± 2.9	1.0 ± 0.7	<0.0001

[a] Oral glucose tolerance test was performed in 64 NAFLD patients with fasting glucose <7.0 mmol/l and all controls. HOMA-IR: homeostasis model for insulin resistance.

Table 10.3 Factors associated with diabetes among NAFLD patients without pre-existing diabetes.

Univariate analysis			
Factors	Diabetes (N=24)	No diabetes (N=49)	P-value
Age	47 ± 7	44 ± 9	0.12
Male gender (%)	18 (75%)	34 (69%)	0.62
Body weight (kg)	77 ± 12	79 ± 15	0.48
Body mass index (kg/m^2)	29 ± 4	29 ± 5	0.79
Waist circumference (cm)	97 ± 8	96 ± 10	0.72
Hip circumference (cm)	103 ± 9	102 ± 10	0.89
Waist-hip ratio	0.95 ± 0.06	0.94 ± 0.08	0.56
Systolic blood pressure (mmHg)	136 ± 17	132 ± 11	0.42
Diastolic blood pressure (mmHg)	82 ± 12	79 ± 10	0.45
ALT (IU/l)	73 ± 54	68 ± 60	0.72
Fasting glucose (mmol/l)	6.9 ± 1.7	5.2 ± 0.6	<0.0001
HbA_{1c} (%)	6.5 ± 1.2	6.1 ± 0.7	0.14
Total cholesterol (mmol/l)	5.6 ± 0.8	5.7 ± 1.2	0.52
HDL-cholesterol (mmol/l)	1.14 ± 0.27	1.33 ± 0.38	0.015
LDL-cholesterol (mmol/l)	3.2 ± 0.9	3.4 ± 1.1	0.56
Triglycerides (mmol/l)	3.16 ± 2.87	2.15 ± 1.05	0.032
Fasting insulin (pmol/l)	262 ± 323	243 ± 293	0.86
HOMA-IR (%)	3.6 ± 3.6	3.4 ± 1.9	0.67
Presence of NASH (%)	16 (67%)	29 (59%)	0.54

Multivariate analysis

Factors	Adjusted odds ratio	95% confidence interval	P-value
Age (per year rise)	1.0	0.9-1.1	0.82
Fasting glucose (per mmol/l rise)	21	4-115	0.001
HDL-cholesterol (per mmol/l rise)	0.007	0-0.42	0.017
Triglycerides (per mmol/l rise)	2.1	1.0-4.4	0.057

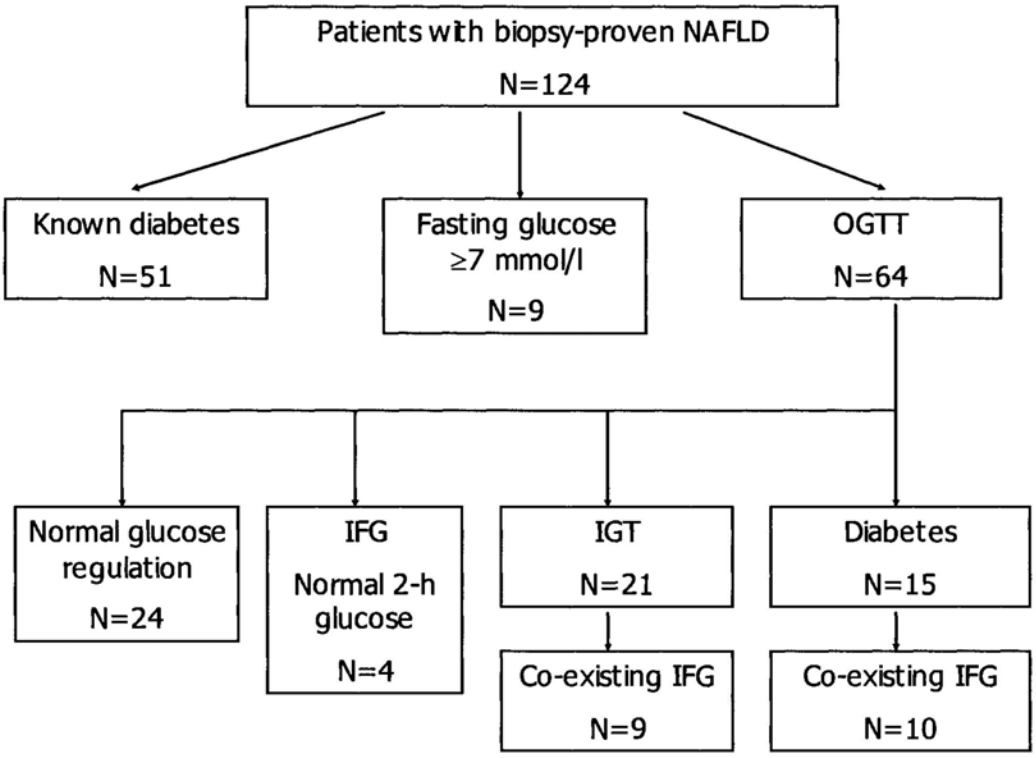
Co-variates in the multivariate analysis include age, fasting glucose, HDL-cholesterol and triglycerides. ALT: alanine aminotransferase; HDL: high density lipoprotein; LDL: low density lipoprotein; HOMA-IR: homeostasis model for insulin resistance

Table 10.4 Accuracy of different fasting glucose cutoffs in detecting undiagnosed diabetes in 73 NAFLD patients. Diabetes was defined as fasting glucose at or above 7.0 mmol/l and/or post-load plasma glucose at or above 11.1 mmol/l.

	Fasting glucose ≥5.6 mmol/l	Fasting glucose ≥6.1 mmol/l
Sensitivity (%)	79.2 (62.9 – 95.4)	62.5 (43.1 – 81.9)
Specificity (%)	73.5 (61.1 – 85.8)	89.8 (81.3 – 98.3)
Positive predictive value (%)	59.4 (42.4 – 76.4)	75.0 (56.0 – 94.0)
Negative predictive value (%)	87.8 (77.8 – 97.8)	83.0 (72.9 – 93.1)

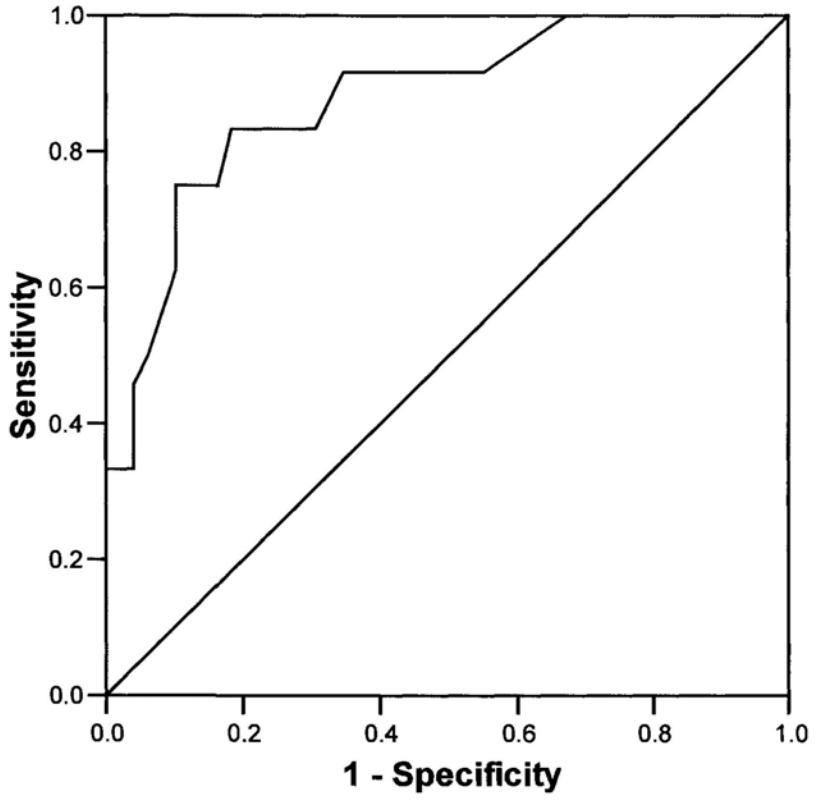
Figures in brackets indicate 95% confidence intervals.

Figure 10.1 Study population of Study 5



IFG: impaired fasting glucose; IGT: impaired glucose tolerance; OGTT: oral glucose tolerance test

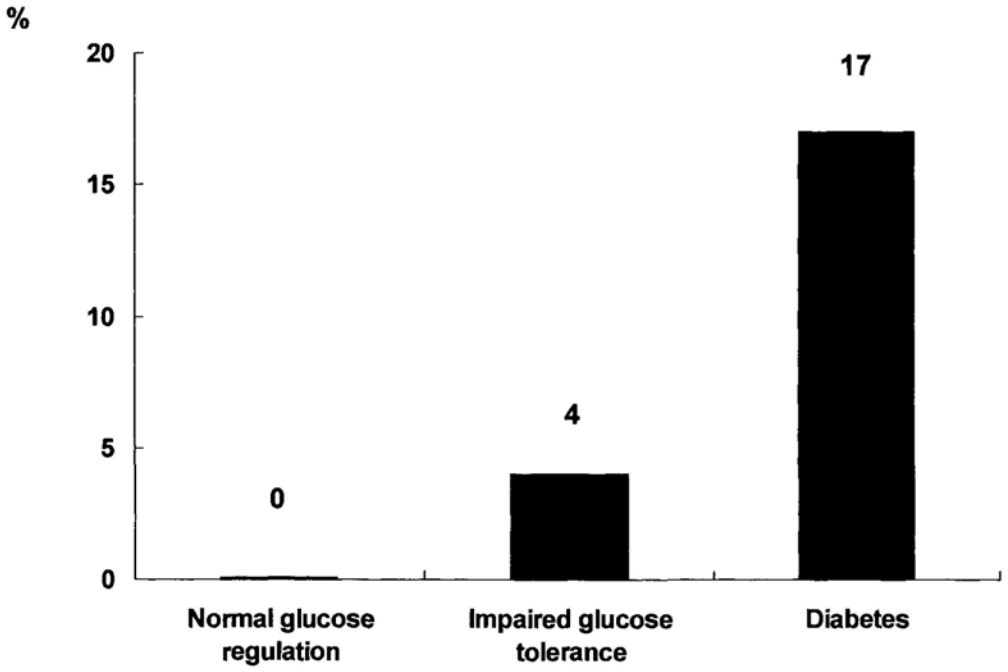
Figure 10.2 Receiver operating characteristics curve of fasting glucose to predict undiagnosed diabetes in NAFLD patients. Area under the ROC curve was 0.88 (95% CI 0.79, 0.96).



10.2 Correlation between post-challenge hyperglycemia and histologic severity

Sixteen of 45 (36%) NASH patients and eight of 28 (29%) patients with simple steatosis had diabetes (P=0.54). Among NAFLD patients not fulfilling the criteria of diabetes, NASH patients were more likely to have impaired glucose tolerance than patients with simple steatosis (16 of 29 NASH patients vs. 5 of 20 patients with simple steatosis had impaired glucose tolerance; P=0.036). Moreover, 2-hour plasma glucose had positive correlation with fibrosis stage (Spearman coefficient 0.25, P=0.046). While none of the patients with normal glucose regulation had advanced fibrosis, advanced disease was prevalent in patients with impaired glucose tolerance and diabetes (Figure 10.3).

Figure 10.3 Prevalence of advanced fibrosis (F3-F4) according to glucose tolerance



10.3 Conclusions

Isolated post-challenge hyperglycemia is common among Chinese NAFLD patients without history of diabetes. It is associated with histological severe disease. However, the association between post-challenge hyperglycemia and histological severity was based on patients without known diabetes and fasting glucose at or above 7.0 mmol/l. The relative importance of fasting and post-challenge glucose warrants further investigation.

CHAPTER ELEVEN

PREDICTION OF LIVER FIBROSIS IN NONALCOHOLIC FATTY LIVER DISEASE

11.1 NAFLD fibrosis score

The majority of Chinese NAFLD patients had mild disease histologically, but advanced fibrosis does occur and may progress with time. Therefore, a non-invasive test with high negative predictive value to exclude advanced fibrosis is welcomed. In this study, we validated the NAFLD fibrosis score in a prospective cohort of Chinese patients with histology-proven NAFLD. NAFLD fibrosis score was calculated according to the formula in Chapter 7.5. The study was approved by the Joint CUHK-NTEC Clinical Research Ethics Committee. All patients provided informed written consent.

From December 2004 to May 2007, 162 patients with biopsy-proven NAFLD and adequate liver specimens (biopsy sample longer than 1.5 cm and containing 6 or more portal tracts) were included in the current analysis. Ninety-six (59%) patients were male, and the age was 46±10 years (Table 11.1). The BMI was 28.5±4.4 kg/m^2. One hundred and fifty-two (94%) patients had BMI above 23 kg/m^2 and 136 (84%) patients had BMI above 25 kg/m^2. Seventy-eight (48%) patients suffered from hypertension (blood pressure ≥140/90 mmHg or on anti-hypertensive medications), 23 (14%) patients had IFG, and 92 (57%) patients had type 2 diabetes. The number of patients taking metformin, sulfonylurea, thiazolidinedione and insulin was 45 (28%), 31 (19%), 1 (1%) and 7 (4%), respectively. Ninety-three (57%) and

24 (15%) patients had ALT and AST above the upper limit of normal, respectively.

The mean (±SD) length of the liver biopsy was 18±3 mm (median 17 mm; interquartile range 16, 20). The number of portal tracts was 7±2 (median 6; interquartile range 5, 8). Fifty-five (34%) patients did not have liver fibrosis. The number of patients with stage 1, 2, 3 and 4 fibrosis was 66 (41%), 23 (14%), 11 (7%) and 7 (4%), respectively. The interobserver agreement was 0.93 for fibrosis staging and 1.0 for the presence of stage 3 fibrosis or above.

There was a trend that patients with stage 3 to 4 fibrosis had older age and higher AST level than those with stage 0 to 2 fibrosis (Table 11.1). Patients in this cohort had relatively low AST levels (43±27 IU/l). Only 8 (5%) patients had AST/ALT ratio above 1. Liver decompensation and hypersplenism were rare. Only 5 (3%) patients had serum bilirubin above 30 μmol/l. No patient had albumin level below 35 g/l or international normalized ration (INR) above 1.3. Three (2%) patients had platelet count below 150×10^9/l.

NAFLD fibrosis score

The NAFLD fibrosis score had moderate correlation with the fibrosis stage (R=0.25, p=0.001). The median (interquartile range) NAFLD fibrosis score was -2.448 (-3.301, -1.728) in patients with stage 0 to 2 fibrosis and -1.738 (-

2.625, -0.862) in patients with stage 3 to 4 fibrosis (p=0.05) (Figure 10.1). Similarly, the NAFLD fibrosis score was significantly lower in patients with stage 0 to 1 fibrosis than in patients with stage 2 to 4 fibrosis (-2.571 [-3.522, -1.935] vs. -1.933 [-2.736, -0.995], p=0.002). The area under ROC of the NAFLD fibrosis score was 0.64 (95% CI 0.49, 0.79) for the prediction of stage 3 to 4 fibrosis and 0.67 (95% CI 0.57, 0.76) for stage 2 to 4 fibrosis (Figures 11.2 and 11.3).

Using the low cutoff point proposed by Angulo and colleagues (less than -1.455), 117 of 128 (91%) patients without stage 3 or 4 fibrosis were correctly staged, while 11 (9%) were under-staged (Table 11.2). Among the 11 patients with advanced fibrosis but NAFLD fibrosis score below the low cutoff point, 8 had stage 3 fibrosis and only 3 had stage 4 fibrosis. The negative predictive value of this cutoff for stage 3 or 4 fibrosis was 91%. When this cutoff was applied to predict significant fibrosis, 102 of 128 (80%) patients without stage 2 to 4 fibrosis were correctly staged, while 26 of 128 (20%) patients were under-staged (Table 11.2). The negative predictive value for stage 2 to 4 fibrosis was 80%.

On the other hand, only two patients from the entire cohort had NAFLD fibrosis score above the proposed high cutoff point (0.676) by Angulo and colleagues. Neither patient had advanced fibrosis. One patient had no

detectable fibrosis, and the other patient had only stage 1 fibrosis. The patient with no fibrosis was a 45-year old man with type 2 diabetes and dyslipidemia on diet control. His BMI was 27.3 kg/m^2, AST was 198 IU/l, ALT was 54 IU/l, platelet count was 252×10^9/l, and albumin was 5.0 g/dl. Although he had persistently raised AST/ALT ratio, a repeated liver biopsy 3 years later also showed no fibrosis. The other patient with stage 1 fibrosis was a 75-year old gentleman with type 2 diabetes, hypertension, stroke, peptic ulcer disease and benign prostatic hyperplasia. His BMI was 22.2 kg/m^2, AST was 38 IU/l, ALT was 53 IU/l, platelet count was 94×10^9/l, and albumin was 4.0 g/dl. The cause of thrombocytopenia was uncertain, though blood film did not identify any abnormal platelets or platelet clumping. However, his subsequent platelet count on repeated testing 3 and 6 months later was back in the normal range. The liver biopsy specimens of both patients were 1.5 cm in length, and contained 5 and 10 portal tracts, respectively. Correspondingly, the positive predictive value of this cutoff to predict the presence of advanced fibrosis was 0. This calculation was limited by the fact that the prevalence of advanced fibrosis in this cohort was low (11%).

If liver biopsies were only performed in patients with NAFLD fibrosis score above the low cutoff point (-1.455), 128 (79%) of 162 biopsies could be avoided.

Table 11.1 Baseline characteristics of 162 Chinese patients with NAFLD in the NAFLD fibrosis score validation study (Study 6).

Variable	All patients (n = 162)	Stage 0 to 2 fibrosis (n = 144)	Stage 3 to 4 fibrosis (n = 18)	P-value
Age	46±10	46±10	50±10	0.06
Male gender	96 (59%)	84 (58%)	12 (67%)	0.50
BMI (kg/m^2)	28.5±4.4	28.5±4.4	28.2±4.8	0.77
Waist circumference (cm)	95±9	95±9	95±10	0.95
Waist-hip ratio	0.94±0.06	0.93±0.07	0.95±0.05	0.30
Central obesity	139 (86%)	124 (87%)	15 (83%)	0.72
ALT (IU/l)	75±45	73±44	95±50	0.09
AST (IU/l)	43±27	41±26	57±32	0.053
AST/ALT ratio	0.63±0.34	0.63±0.36	0.63±0.18	0.95
Albumin (g/l)	45±4	45±4	45±3	0.47
Bilirubin (μmol/l)	12±7	12±7	14±9	0.29
Platelet count ($\times 10^9$/l)	274±75	277±73	257±88	0.36
APRI	0.27±0.21	0.25±0.19	0.39±0.26	0.007
Fasting glucose (mmol/l)	6.6±2.3	6.5±2.4	7.0±2.4	0.45
HbA_{1c} (%)	6.6±1.4	6.6±1.4	6.9±1.5	0.38
Fasting insulin (pmol/l)	198±254	197±262	206±191	0.88
HOMA-IR	3.5±3.3	3.4±3.3	3.9±3.3	0.63
Total cholesterol (mmol/l)	5.5±1.3	5.6±1.3	5.0±1.2	0.08
HDL-cholesterol (mmol/l)	1.3±0.3	1.3±0.4	1.2±0.2	0.08

LDL-cholesterol (mmol/l)	3.2±1.0	3.2±1.0	2.9±1.1	0.22
Triglycerides (mmol/l)	2.6±3.6	2.6±3.6	2.0±0.8	0.08
Diabetes	92 (57%)	78 (54%)	14 (78%)	0.08
IFG/diabetes	115 (71%)	101 (70%)	14 (78%)	0.59
Hypertension	78 (48%)	70 (49%)	8 (44%)	0.74

Continuous variables were expressed in mean ± SD and categorical variables in number (percentage). P-values represented comparisons between patients with stage 0 to 1 fibrosis and those with stage 2 to 4 fibrosis. BMI: body mass index; ALT: alanine aminotransferase; AST: aspartate aminotransferase; HOMA-IR: homeostasis model for insulin resistance; IFG: impaired fasting glucose

Table 11.2 Accuracy of the NAFLD fibrosis score in predicting advanced fibrosis (stage 3 to 4) and significant fibrosis (stage 2 to 4).

	Predicting advanced fibrosis				Predicting significant fibrosis			
	Low cutoff point (< -1.455)	Indeterminate (-1.455-0.676)	High cutoff point (> 0.676)	Total	Low cutoff point (< -1.455)	Indeterminate (-1.455-0.676)	High cutoff point (> 0.676)	Total
Total	128	32	2	162	128	32	2	162
Fibrosis stage 0 to 2	117	25	2	144				
Fibrosis stage 3 to 4	11	7	0	18				
Fibrosis stage 0 to 1					102	17	2	121
Fibrosis stage 2 to 4					26	15	0	41
Sensitivity (%)	39 (16, 61)		0		37 (22, 51)		0	
Specificity (%)	81 (75, 88)		99 (97, 100)		84 (78, 91)		98 (96, 100)	
Positive predictive value (%)	21 (7, 34)		0		44 (27, 61)		0	
Negative predictive value (%)	91 (87, 96)		89 (84, 94)		80 (73, 87)		74 (68, 81)	
Likelihood ratio (+)	2.05		0		2.31		0	
Likelihood ratio (-)	0.75		1.01		0.75		1.02	

Figures in brackets represent 95% confidence interval

Figure 11.1 Box-plots of the NAFLD fibrosis score according to the fibrosis stage. The bottom and top of each box represent the 25th and 75th percentiles, giving the interquartile range. The line through the box indicates the median, and the error bars indicate the 10th and 90th percentiles.

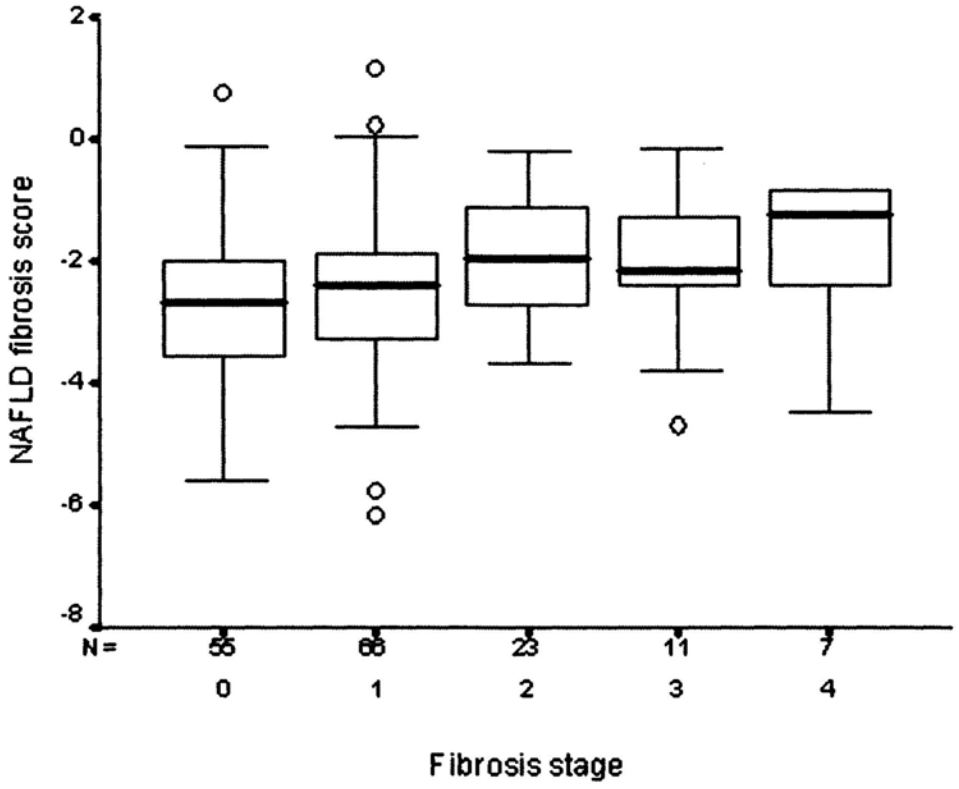


Figure 11.2 Receiver operating characteristics curve of the NAFLD fibrosis score to predict stage 3 to 4 fibrosis. The area under the receiver operating characteristics curve was 0.64 (95% CI 0.50, 0.79).

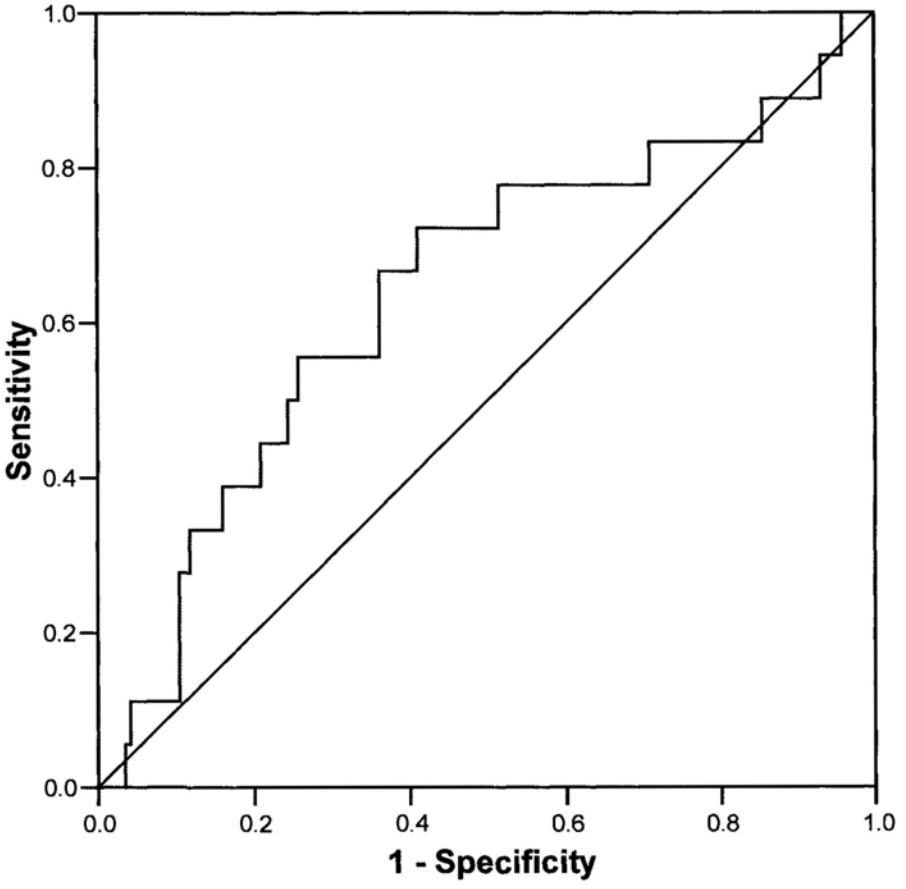
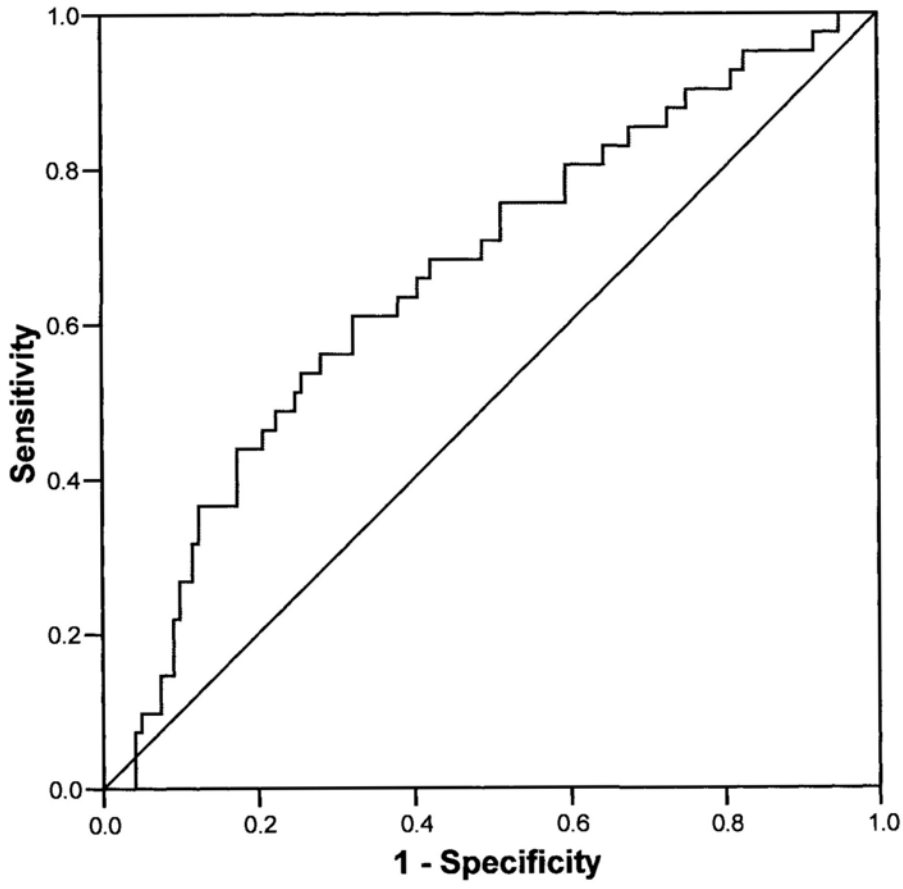


Table 11.3 Receiver operating characteristics curve of the NAFLD fibrosis score to predict stage 2 to 4 fibrosis. The area under the receiver operating characteristics curve was 0.66 (95% CI 0.57, 0.76).



11.2 Other clinical models

The APRI was 0.32 (interquartile range 0.23, 0.48) in patients with advanced fibrosis and 0.19 (0.14, 0.31) in patients without advanced fibrosis (p=0.007). On the other hand, the AST/ALT ratio did not differ significantly between patients with or without advanced fibrosis (0.62 [0.49, 0.76] vs. 0.56 [0.45, 0.70], p=0.31). There was also no significant difference in the HAIR score in patients with or without advanced fibrosis (median score 1 in both groups, p=0.98). Table 11.3 showed the areas under the ROC curves of the AST/ALT ratio, APRI and HAIR in comparison to that of the NAFLD fibrosis score. Among the four prediction models, the NAFLD fibrosis score was superior to the HAIR score in predicting stage 2 to 4 fibrosis (p=0.009).

Table 11.3 Area under ROC curve of the NAFLD fibrosis score, AST/ALT ratio, APRI and HAIR score to predict advanced (stage 3 to 4) and significant (stage 2 to 4) fibrosis. P-values represent comparisons with the NAFLD fibrosis score using the Delong test.

Test	Area under ROC curve (95% CI) for advanced fibrosis	P-value	Area under ROC curve (95% CI) for significant fibrosis	P-value
NAFLD fibrosis score	0.64 (0.49, 0.79)		0.67 (0.57, 0.76)	
AST/ALT ratio	0.58 (0.44, 0.71)	0.26	0.63 (0.53, 0.73)	0.37
APRI	0.70 (0.55, 0.84)	0.25	0.62 (0.51, 0.72)	0.22
HAIR score	0.50 (0.36, 0.65)	0.09	0.51 (0.41, 0.61)	0.009

11.3 Conclusions

The NAFLD fibrosis score has good negative predictive value in excluding advanced fibrosis in Chinese NAFLD patients and can reduce the burden of liver biopsies. A larger Asian study is required to validate the high cutoff point of the NAFLD fibrosis score.

CHAPTER TWELVE

DISCUSSIONS

12.1 Disease severity of nonalcoholic fatty liver disease in Hong Kong

NAFLD exists in the Chinese population. More severe forms of NAFLD, namely NASH and fibrosis, are also common in our population. Although the BMI of our patients in Studies 1 and 2 was lower than that reported in most Western series, almost all NAFLD patients had features of insulin resistance and metabolic syndrome.

On the other hand, the histological grading and staging NAFLD among our patients were generally milder than that of the Caucasian patients. Among Western series, steatohepatitis was found in 60% to 70%, fibrosis in 40% to 75% and cirrhosis in 4% to 17% of studied patients.[Angulo 1999, Matteoni 1999, Loguercio 2001, Dixon 2001] In our retrospective study (study 1), almost all patients with fibrosis had stage 1 disease only. In the prospective study (study 3), only 22% of the population had advanced fibrosis. The higher proportion of obese patients among the Western population may be part of the reasons for the discrepancy. The difference in histological severity in different reports may also be explained by the different inclusion criteria. In our study, all patients with histology confirmed NAFLD were included. In contrast, some investigators only targeted high risk individuals. For example, Dixon and colleagues only included severely obese patients.[Dixon 2001] The mean body mass index of their NAFLD patients was 47 ± 7 kg/m^2 as compared to 27 ± 4 kg/m^2 in Study 1. In addition, patients in our studies were younger than NAFLD

patients in studies from North American, Europe and Australia. The average age of patients in the Hong Kong cohorts was 40 to 50 years, compared to 50 to 60 years in Caucasian studies.[Wong 2004, Wong 2006, Angulo 1999, Matteoni 1999, Loguercio 2001, Dixon 2001] Since liver injury and progression of fibrosis is time-dependent, it is possible that the difference in histological severity may be explained by the difference in patients' age among studies.

Moreover, many patients with clinical or radiological evidence of cirrhosis may not undergo liver biopsy despite the presence of metabolic syndrome. This might underestimate the severity of NAFLD in our population.

In our study, patients with normal ALT also had NASH and fibrosis. Traditionally, the normal range of ALT is derived from the levels in the general population. Recently, this concept is challenged. Firstly, the supposedly healthy subjects may include asymptomatic patients with chronic hepatitis C and NAFLD. Prati and colleagues examined the ALT level of a large cohort of blood donors.[Prati 2002] After excluding subjects with viral hepatitis and features of metabolic syndrome, new cutoffs of normal ALT were suggested – 30 IU/l in men and 19 IU/l in women. Secondly, patients with ALT within the upper half of the normal range are also at risk of liver-related morbidity and mortality.[Kim 2004] Young adults with high-normal ALT also have a higher prevalence of metabolic syndrome and cardiovascular risk factors.[Patel 2007] Similarly, in another retrospective study

of 51 patients with fatty liver and normal ALT, 12 had bridging fibrosis and 6 had cirrhosis.[Mofrad 2003] These data suggest that the assessment of the severity of NAFLD should not rely on the ALT level, especially if it is in the high-normal range. Other risk factors of advanced disease should be considered.

The natural history of NAFLD is not well defined. There have been scanty longitudinal or prospective studies on the disease. A summary of five series included 54 of 257 patients with the disease who underwent liver biopsy during an average follow-up of 3.5 to 11 years.[Angulo 2002] Of these patients, 28% had progression of liver damage and 59% had no change histologically. Patients with steatosis alone in the initial biopsy seemed to have best prognosis, whereas presence of steatohepatitis or fibrosis predicted worse outcome. Nevertheless, it is known that even patients with pure steatosis may progress to cirrhosis.[Neuschwander-Tetri 2003] Since all of these studies were performed in the Western countries, it was unclear if the conclusions were equally applicable to Asian populations which have different life style, body habitus and prevalence of metabolic syndrome.

In Study 3, we performed serial liver biopsy in 17 Chinese NAFLD patients after a median follow up of 6.1 years. We found that nearly half of them had more advanced disease compared to the first biopsy. This could be due to the fact that majority (82%) of the cohort had steatohepatitis in the first biopsy and were

hence more likely to have progressive disease. The deterioration in stage of disease occurred in the background of static or unchanged status in degree of steatosis and necroinflammation. Meanwhile, 4 patients with steatohepatitis (one with fibrosis) had spontaneous resolution in necroinflammation.

Our finding is consistent with results from similar studies on Caucasian patients. In the report by Powell et al, 13 patients with NASH underwent serial liver biopsies after follow-up of 1 to 9 years.[Powell 1990] Five patients were found to have progressive histological disease, 6 remained unchanged and two showed improvement. In a study by Teli et al, 12 patients with only steatosis and no steatohepatitis or fibrosis had a repeat liver biopsy after an interval of 7.6 to 16 years.[Teli 1995] Only one patient developed fibrosis; none progressed to steatohepatitis.

Metabolic syndrome is an important predisposing factor of NAFLD.[Pagano 2002, Eriksson 1986, Chitturi 2002] An important question is whether the converse is also true, that is, if NAFLD predicts development of metabolic syndrome or the components of metabolic syndrome. Since the diagnosis of metabolic syndrome requires complete workup of its individual components, the finding of NAFLD before metabolic syndrome may reflect the difficulties in diagnosing metabolic syndrome rather than a genuine chronological order. A prospective study is needed to answer this question though our current retrospective data showed

that significant proportions (41%) of patients developed either hypertension or diabetes mellitus during the period of follow-up. The small sample size in the longitudinal study precluded detailed analysis on the changes in metabolic profile over time and its relationship with histological progression.

Increasing affluence may have contributed to recent increase in the prevalence of cardiovascular disease and diabetes mellitus in Chinese populations.[Cockram 1993, Ko 1999, Woo 2003] The proportions of people who are overweight or obese in Asia are also similar to that in the West, using the ethnicity-adjusted cut-off values of BMI as defined by the WHO.[Ko 2001-2, Ge 1997] With this in mind, it is anticipated that NAFLD will gradually become an increasing health problem to the Asian populations. Our study provides new evidence that just like their Western counterparts, Chinese NAFLD patients may have progressive disease over time. The diagnosis of NAFLD may also predate the development of new components of metabolic syndrome. NAFLD in Chinese can no longer be treated as a benign disease and regular follow up as well as appropriate intervention is needed to reduce their risk of long-term cardiovascular and hepatic complications.

12.2 Metabolic syndrome and adipokines

In this cross-sectional study on biopsy-proven NAFLD in Chinese, we found that the levels of some adipokines correlated with the histologic severity of liver disease. Hypoadiponectinemia was an independent factor associated with NAFLD as compared to healthy subjects, but it was not associated with histologic disease activity. High TNF-α level, a proinflammatory adipokine, was associated with more severe histologic necroinflammation and fibrosis among patients with NAFLD.

Our findings support the notion that adiponectin is protective against the development of NAFLD. Adiponectin improves insulin resistance and may have anti-inflammatory activities. In human leucocytes, adiponectin has been shown to induce anti-inflammatory mediators interleukin-10 and interleukin-1 receptor antagonist, but inhibit pro-inflammatory cytokine interferon-gamma.[Wolf 2004] Hypoadiponectinemia is also involved in other hepatic conditions closely related to steatosis, such as chronic hepatitis C and alcoholic liver disease.[Petit 2005] Among Asians, only indirect evidence for the association between NAFLD and adiponectin exists. In 791 male Japanese workers, adiponectin level was inversely proportional to the ALT, AST and GGT levels.[Yokoyama 2004] Thirty-eight Koreans with ultrasound features of fatty liver had lower adiponectin level than controls.[Yoon 2005] Since sonography have limited accuracy and does not assess disease severity of NAFLD,[Saadeh 2002] the current study provides the

first major Asian liver biopsy series to demonstrate the association of hypoadiponectinemia and NAFLD independent of adiposity and other features of metabolic syndrome.

In contrast to the findings by Hui et al. and Musso et al.,[Hui 2004, Musso 2005] adiponectin level did not correlate with disease severity in our population. Similarly, another study in Italy failed to demonstrate the correlation.[Bugianesi 2005] One possible explanation is that different series included patients of different disease severity. For example, 20 percent of their NASH patients had stage 4 fibrosis (i.e. cirrhosis), compared to 2 percent in our series. Another possible explanation is that the triggering events for NASH may be different among different ethnic groups. This hypothesis can be tested by investigating polymorphism of adiponectin and its receptor among different populations. A study on 13 NAFLD patients showed that adiponectin receptor RII mRNA in liver samples had negative correlation with serum aminotransferase and liver fibrosis,[Kaser 2005] suggesting that both adiponectin and its receptor are important in the pathogenesis of NASH.

The -11391A, +45G, and +276T alleles were shown to be associated with hypoadiponectinemia in diabetic patients.[Woo 2006, Jang 2005, Mackevics 2006] The +45G allele was also associated with central obesity and insulin resistance.[Menzaghi 2002] In our study, both the -11391A and +45G alleles

were associated with hypertriglyceridemia in NAFLD patients. However, the genetic polymorphisms did not significantly affect serum adiponectin levels, and were not associated with histological severity.

The serum TNF-α level is higher among patients with NASH than those with simple steatosis. TNF-α is a pro-inflammatory cytokine with a pivotal role in alcoholic liver disease. On the other hand, whether TNF-α is essential for the development of NASH is controversial. For example, the prevalence of high TNF-α production genotype at the polymorphisms of TNF-α-238 was increased among patients with insulin resistance and NAFLD.[Valenti 2002] This genotype-phenotype association suggests a possible causal relationship. Intrahepatic TNF-α mRNA expression was also increased in NAFLD patients, more so among patients with liver fibrosis.[Crespo 2001] Administration of pentotoxifylline, a TNF-α inhibitor, may lead to normalization of ALT among NAFLD patients.[Adams 2004, Satapathy 2004] In a murine model, free fatty acid-mediated lipotoxicity was shown to be mediated through lysosomal destabilization and nuclear factor kappa B-dependent TNF-α expression.[Feldstein 2004] In contrast, anti-TNF antibodies protected *ob/ob* mice against liver injury.[Li 2003] While the above findings suggest that TNF-α has significant contribution in the pathogenesis of NASH, other experimental data also suggest that it may not be essential. *Ob/ob* mice lacking both TNF receptors have similar hepatic lipid content and serum aminotransferase level as mice without TNF receptor knockout.[Memon 2001]

Similarly, in mice receiving intragastric overfeeding, steatohepatitis develops even after knockout of TNF type 1 receptor.[Deng 2005] In mice receiving methionine- and choline-deficient diet, TNF and TNF type 1 receptor gene deleted mice had similar degree of steatohepatitis.[Dela Pena 2005]

In Chinese diabetic patients, TNF-α-308A was associated with higher HbA_{1c} level.[Ko 2003] However, the same polymorphism was not associated with type 2 or type 1 diabetes in Caucasians and Chinese.[Hamann 1995, Deng 1996] This might be explained by the fact that only 5 patients in the report by Ko et al. had homozygosity for the A allele. Individual variation in glycemic control might have heavy influence on the analysis. Similarly, low prevalence of the A allele was found in our current study.

TNF-α-238A was associated with NAFLD and insulin resistance in a study of 99 Italian NAFLD subjects, among which 53 had liver biopsy.[Valenti 2002] This contrasts with our findings that -238A appeared to be associated with better glycemic control. However, cautious interpretation of the data from both studies is required because the number of patients with -238A was small (5 in our study and 24 in the Italian cohort).

Our study confirms that conventional metabolic risk factors, including diabetes mellitus and obesity, are associated with increased risk of NAFLD and NASH in

Chinese.[Braillon 1985, Nomura 1986, Assy 2000, Marchesini 1999, Marceau 1999, Cortez-Pinto 1999, Laine 2004] Diabetes mellitus is independently associated with NAFLD, and higher body mass index is independently associated with more severe histologic damage among patients with NAFLD. Interestingly, the IDF criteria of metabolic syndrome for Chinese, but not the ATP III criteria, predicted patients with NASH. The main difference between the two criteria is the cutoff values for waist circumference.[NCEP-ATPIII 2002, Alberti 2005] Although Asian patients are generally leaner, it has also been shown that features of metabolic syndrome and complications like proteinuria start to emerge in patients with lower body-mass index.[Tan 2004] Our study shows that the same phenomenon also holds true for NASH. The proportion of patients developing NAFLD and NASH increased dramatically as their BMI exceeded 23 kg/m^2, i.e. the cutoff value of overweight for Asians.[Anonymous Lancet 2004] Ethnic group-specific cutoff points of waist circumference and BMI would be important for future studies on metabolic syndrome and NAFLD.

In our cohort, NAFLD patients were more likely to have all components of metabolic syndrome than control subjects. On the other hand, NASH patients were only more likely to have central obesity than patients with simple steatosis, while the proportion of patients with other components of metabolic syndrome was similar. In fact, diabetes mellitus was an independent factor associated with NAFLD, central obesity was an independent factor associated with NASH, while

fasting glucose and waist circumference were independent factors associated with significant liver fibrosis. Among the different components of metabolic syndrome, obesity appears to be central to the development of NAFLD and NASH.

Moreover, the prevalence of diabetes in our cohort is relatively high. A number of reasons may explain the phenomenon. First, diabetes is common in Chinese. Among several population based studies, the prevalence of diabetes in Chinese is up to 15%, which is even higher than some of the Western countries.[Qiao 2000] Second, this finding may be partly due to the referral pattern. Physicians taking care of diabetic patients may be more aware of the problem of NAFLD, and diabetic patients in general have more frequent blood tests. Nevertheless, thirteen of our NAFLD patients actually had diabetes newly diagnosed after formal evaluation at the liver clinic. Therefore, our finding is not entirely the result of referral bias.

In this study, we noted that more NAFLD patients were males. Male predominance in NAFLD was also found in the Alameda County Chronic Liver Disease Surveillance Study.[Weston 2005] However, hypoadiponectinemia remained an independent factor associated with NAFLD after adjustment for gender and other potential confounders.

Another unexpected finding is that NASH patients had lower ALT level than

patients with simple steatosis. This suggests that ALT might actually go down as the disease progresses, and is supported by the negative correlation between ALT and fibrosis stage. Besides, this also reflects that ALT is a poor surrogate marker for NASH. Previous reports specifically looking at NAFLD patients with normal ALT found significant fibrosis in one-quarter to one-third of cases.[Mofrad 2003]

12.3 Post-challenge hyperglycemia and nonalcoholic fatty liver disease

In Study 5, we set out to assess the prevalence of undiagnosed diabetes in Chinese NAFLD patients. The response to oral glucose challenge was abnormal in a significant proportion of this cohort. Thirty-three percent of the patients had diabetes and another 29% had impaired glucose tolerance. Notably, isolated post-challenge hyperglycemia was common in this cohort. Twelve percent of the patients had isolated IGT but normal fasting glucose, and 25% of the diabetic NAFLD patients had normal fasting glucose.

Abnormal glucose metabolism has been found in different NAFLD series. For example, among 19 Italian patients with biopsy-proven NAFLD, 5 had IGT and 1 had IFG.[Pagano 2002] The higher prevalence of IGT than IFG echoed our findings. The overall prevalence of abnormal glucose metabolism is lower than that of our series probably because the Italian cohort contained patients with less severe disease (only 3 patients had grade 3 necroinflammation and no patient had stage 4 fibrosis). In 114 Turkish patients with elevated serum aminotransferase levels and bright liver on ultrasonography, 50 had IGT or diabetes according to the post-load glucose level, suggesting the need to perform OGTT in NAFLD patients.[Sargin 2003] However, the investigators combined IGT and diabetes in the analysis and did not take account of the fasting glucose level, making it difficult to assess the prevalence of isolated post-challenge hyperglycemia. In another large population screening in Japan, the

prevalence of ultrasonography-diagnosed NAFLD rose from 27% in people with normal fasting glucose, 43% in people with IFG, to 62% in people with newly-diagnosed diabetes.[Jimba 2005]

Recently, an Italian group performed OGTT in 90 chronic hepatitis C and 90 NAFLD patients.[Svegliati-Baroni 2007] The prevalence of basal insulin resistance was 23% in chronic hepatitis C patients and 58% in NAFLD patients. The prevalence of post-load insulin resistance rose to 29% and 68%, respectively. In a multivariate model, post-load insulin resistance was associated with increased risk of severe fibrosis.

Isolated post-challenge hyperglycemia also exists in the general population. Summarizing 13 European series, around 2 to 6% of people with fasting glucose below 7.0 mmol/l had 2-hour plasma glucose above 11.1 mmol/l.[The DECODE Study Group 2003] The corresponding percentage in the Asian DECODA series was 3%.[Qiao 2000] Among 680 Chinese patients referred for oral glucose tolerance tests, 218 had 2-hour plasma glucose at or above 11.1 mmol/l, of which only 86 had fasting plasma glucose at or above 7.8 mmol/l.[Cockram 1992] As our series represent a group of patients with high risk of having metabolic syndrome, the prevalence of post-challenge hyperglycemia was considerably higher. Our findings suggest strong insulin resistance among NAFLD patients. Using insulin clamp technique, it has been demonstrated that glucose disposal is

reduced almost by half even among non-diabetic NAFLD patients.[Pagano 2002, Marchesini 2001, Bugianesi 2005] The defect is more severe among NASH patients than those with simple steatosis.[Sanyal 2001] On the other hand, hepatic glucose output is less suppressed after administration of insulin, indicating that hepatic sensitivity to insulin is reduced in NAFLD patients.[Marchesini 2001, Sanyal 2001]

Although fasting glucose was an independent predictor of diabetes, using fasting glucose alone to screen NAFLD patients was limited by its low sensitivity. When normal fasting glucose was set at 5.6 mmol/l according to the latest ADA criteria, the sensitivity in detecting diabetes was only 79%. This is comparable to another large study in Hong Kong, which reported a sensitivity of 87% to detect diabetes using this fasting glucose cutoff.[Cockram 2002] In other words, OGTT can be considered a routine assessment for NAFLD patients in view of the high prevalence of post-challenge hyperglycemia.

Does it matter to miss post-challenge hyperglycemia? In our study, IGT and diabetes patients were more likely to have significant necroinflammation or liver fibrosis. Since patients with pre-existing diabetes and fasting glucose above 7 mmol/l did not undergo oral glucose tolerance test, we cannot conclude if the 2-hour plasma glucose level predicts liver fibrosis independent of fasting glucose in the whole NAFLD population. Among 181 Swedish patients with acute myocardial

infarction, previous undiagnosed IGT and diabetes occurred in 40% and 25%, respectively.[Norhammar 2002] Moreover, isolated post-challenge hyperglycemia doubles the risk of cardiovascular events and mortality.[Barrett-Connor 1998, Hashimoto 2005, Shaw 1999] In a prospective study on more than 2500 Japanese, IGT, but not IFG, increased the risk of dying from cardiovascular disease by 2.2 fold.[Tominaga 1999] These data suggest that isolated post-challenge hyperglycemia not only is common in high-risk individuals, but also predicts poor outcome.

12.4 Non-invasive tests of liver fibrosis

Despite the limited sensitivity of the NAFLD fibrosis score in a population with low prevalence of advanced fibrosis, the score was useful in ruling out severe disease. In the cohort of Study 6, 79% of the liver biopsies could be avoided if the procedure was not performed in patients with NAFLD fibrosis score below -1.455. The tradeoff would be missing just 8 (6%) cases of stage 3 fibrosis and 3 (2%) cases of stage 4 fibrosis among 128 patients who would not undergo liver biopsies based on the NAFLD fibrosis score. Therefore, the score would be particularly useful to reduce unnecessary liver biopsies and costs of managing NAFLD patients in Asia where advanced fibrosis is uncommon.

Similar to other Asian NAFLD series, the prevalence of stage 0 to 2 fibrosis was substantially higher than the prevalence of stage 3 to 4 fibrosis (89% vs. 11% in the current study).[Tsang 2006, Malik 2007] The high negative predictive value (91%) of the low cutoff point (< -1.455) is of particular relevance. All but 9% of the patients (11 of 128 cases) below the low cutoff point were correctly identified. Although the NAFLD fibrosis score was initially developed to predict stage 3 to 4 fibrosis, the negative predictive value of the low cutoff remained high (80%) in correctly identifying patients without significant (stage 2 to 4) fibrosis.

Our results contrast with that reported by Angulo and colleagues, in which a high cutoff NAFLD fibrosis score of 0.676 had an 80 percent to 90 percent overall accuracy and an 82% positive predictive value in predicting stage 3 or 4 fibrosis.[Angulo 2007] However, the prevalence of advanced fibrosis in our study was only 11%, compared to 27% in Angulo's study. Therefore, our study cannot reliably validate the high cutoff point, and a larger Asian study is warranted.

We also attempted to test the performance of other simple serum markers to predict advanced fibrosis. The AST/ALT ratio and APRI were shown to have moderate accuracy in predicting cirrhosis in patients with chronic hepatitis C.[Wai 2003, Sheth 1998, Park 2000, Giannini 2003] However, the AST/ALT ratio alone did not discriminate patients with advanced fibrosis from those without. This supports the observation that NAFLD patients generally have low AST levels until late stage of disease. Moreover, the HAIR score had moderate accuracy in predicting nonalcoholic steatohepatitis in patients with morbid obesity undergoing bariatric surgery.[Dixon 2001] The score does not appear useful in a less obese population.

Recently, transient elastography has emerged as another promising non-invasive test for the measurement of liver fibrosis. In 1257 patients with different chronic liver diseases, transient elastography had an overall accuracy of 95 percent in detecting cirrhosis.[Ganne-Carrie 2006] The accuracy remained high in patients

with NAFLD and alcoholic liver disease. However, only 47 patients in that study had NAFLD and 3 had cirrhosis secondary to nonalcoholic steatohepatitis. In another study involving 67 NAFLD subjects, the positive and negative predictive values of transient elastography to detect stage 2 fibrosis or above were 90% and 84%, respectively.[Yoneda 2007] Nevertheless, obesity, a common feature in NAFLD patients, is one of the main reasons for failure to obtain fibrosis measurement.[Kettanah 2007] This may affect the feasibility of using transient elastography in NAFLD patients.

CHAPTER THIRTEEN

SUMMARY

Through a series of clinical studies in Chinese NAFLD patients in Hong Kong, we demonstrated that NASH and advanced fibrosis do occur in Chinese, though the proportion of patients with advanced disease is lower than that in Caucasians. This may reflect a milder disease spectrum in Chinese, or may be explained by the younger age of our cohort. In our prospective cohort, around 10% of NAFLD patients had advanced fibrosis, and 80% had necroinflammation. Importantly, up to half of the patients had progression of liver fibrosis upon long-term follow-up.

NAFLD is closely related to metabolic syndrome. Using the ethnic-specific IDF criteria, 70% of NAFLD patients had metabolic syndrome, compared to 7% of the general population. A significant proportion of NAFLD and NASH patients had BMI between 23 and 25 kg/m^2. Since the body composition of Asians is different from that of other ethnic groups, ethnic-specific definitions of metabolic syndrome, overweight and obesity are important in the evaluation of NAFLD patients. The diagnosis of NAFLD may predate the development of different components of metabolic syndrome. Whether NAFLD actually predates metabolic syndrome or the finding is due to under-diagnosis of metabolic syndrome and its components requires further clarification in prospective studies. Alternatively, as NAFLD becomes more easily diagnosed, it may be useful to incorporate NAFLD in the definitions of metabolic syndrome.

NAFLD patients have lower serum adiponectin level than healthy controls. Among NAFLD patients, those with NASH have higher serum TNF-α level than those with simple steatosis. The differences in adipokines remained significant after adjustment for traditional metabolic risk factors. These suggest that abnormalities in adipokines may be involved in the pathogenesis of NAFLD and NASH. On the other hand, genetic polymorphisms of the adiponectin and TNF-α genes are not associated with histological severity. Age, body mass index and fasting glucose were independent factors associated with significant fibrosis. While it is possible that environmental factors may play a bigger role in the development of NAFLD and NASH, it is important to note that the lack of significant associations with the single nucleotide polymorphisms may be explained by inadequate statistical power.

Moreover, isolated post-challenge hyperglycemia is common in NAFLD patients. Among NAFLD patients without known diabetes or high fasting plasma glucose at or above 7.0 mmol/l, 21% had undiagnosed diabetes and 29% had impaired glucose tolerance. In this population, post-challenge hyperglycemia was associated with NASH and liver fibrosis. However, whether the importance of post-glucose load glucose level is independent of fasting glucose is unclear because the patients in this cohort had fasting glucose below 7.0 mmol/l. Oral glucose tolerance test should be considered in the evaluation of NAFLD patients.

Since advanced fibrosis is less common in Chinese NAFLD patients, performing liver biopsies on every NAFLD patient for disease staging does not appear to be essential or cost-effective. However, there are no commonly performed tests that can reliably diagnose or exclude advanced fibrosis. Recently, the NAFLD fibrosis score is developed using 6 clinical parameters including age, BMI, impaired fasting glucose or diabetes, AST/ALT ratio, platelet count and albumin. The score had high negative predictive value of 91% in excluding advanced fibrosis in Chinese NAFLD patients. It may also reduce the burden of liver biopsies in the majority of cases.

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Annex

List of publications used in this thesis

Study 1

Wong VW, Chan HL, Hui AY, Chan KF, Liew CT, Chan FK, Sung JJ. Clinical and histological features of non-alcoholic fatty liver disease in Hong Kong Chinese. Aliment Phar Ther 2004;20:45-9.

Study 2

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Study 3

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Study 4

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necrosis factor-alpha and nonalcoholic fatty liver disease in Chinese. J Gastroenterol Hepatol 2008;23:914-21.

Study 5

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Study 6

Wong VW, Wong GL, Chim AM, Tse AM, Tsang SW, Hui AY, Choi PC, Chan AW, So WY, Chan FK, Sung JJ, Chan HL. Validation of the NAFLD fibrosis score in a Chinese population with low prevalence of advanced fibrosis. Am J Gastroenterol 2008;103:1682-8.

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