NON-ALCOHOLIC FATTY LIVER DISEASE:

EPIDEMIOLOGY AND NON-INVASIVE ASSESSMENT

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Abstract

This thesis consists ten chapters and presents the findings from four research projects in eight areas of interest concerning the epidemiology and non-invasive assessment of non-alcoholic fatty liver disease (NAFLD). In a cross-sectional study of 399 patients with diabetes mellitus in the Diabetic Clinic, University of Malaya Medical Centre, the prevalence of NAFLD was 49.6% and was highest among the Malays and the Indians compared to the Chinese. Further analysis revealed that low level of physical activity and high percentage calorie intake from fat, high cholesterol food and high saturated fatty acid food was associated with NAFLD in centrally obese but not in lean patients with diabetes mellitus. In addition, NAFLD was not found to be associated with ischemic heart disease in patients with diabetes mellitus. In a separate study on 35 NAFLD patients with paired liver biopsy over a mean interval of 6.4 years, it was found that patients can undergo significant disease progression, and fibrosis is irreversible without specific interventions. From the studies on non-invasive assessment of NAFLD, controlled attenuation parameter was found to be excellent for the detection of significant hepatic steatosis but less useful for distinguishing the different grades of significant hepatic steatosis, while plasma M30 was found to be less useful for the diagnosis of non-alcoholic steatohepatitis among NAFLD patients. The use of liver stiffness measurement for patients with indeterminate and high NAFLD fibrosis scores allowed accurate prediction of advanced fibrosis and reduced the number of patients requiring a liver biopsy. In the study on 472 students at the Faculty of Medicine, University of Malaya, the prevalence of NAFLD was 8.1% and was again highest among the Indians and the Malays compared to the Chinese. This study confirmed that differences in the prevalence of NAFLD among the different ethnic groups in Malaysia can be observed as early as young adulthood.

Abstrak

Tesis ini mengandungi sepuluh bab dan memaparkan hasil-hasil kajian daripada empat projek penyelidikan dalam lapan aspek penting berkenaan epidemiologi and penilaian secara tidak invasif penyakit hati berlemak yang bukan disebabkan alkohol (non-alcoholic fatty liver disease, NAFLD). Dalam kajian 399 pesakit kencing manis di Pusat Perubatan Universiti Malaya, kekerapan penyakit NAFLD adalah 49.6% dan adalah paling tinggi di kalangan Melayu dan India berbanding dengan Cina. Analisa juga menunjukkan bahawa tahap activiti fizikal yang rendah dengan peratus kalori tinggi daripada lemak, makanan tinggi kolesterol dan makanan tinggi asid lemak tepu adalah berkaitan dengan penyakit NAFLD di kalangan pesakit kencing manis yang obes tetapi bukan di kalangan pesakit kencing manis yang tidak obes. Penyakit NAFLD juga didapati tidak berkaitan dengan penyakit jantung di kalangan pesakit kencing manis. Dalam kajian berasingan ke atas 35 pesakit NAFLD dengan biopsi hati berkembar pada purata jangka masa 6.4 tahun, didapati penyakit hati boleh melarat dan fibrosis hati tidak akan bertambah baik tanpa rawatan khusus. Melalui kajian-kajian penilaian secara tidak invasif, didapati controlled attenuation parameter sangat baik untuk mengesan lemak dalam hati tetapi kurang baik untuk membezakan tahap-tahap hati berlemak, manakala M30 plasma tidak begitu berguna untuk diagnosa penyakit hati berlemak dan radang bukan disebabkan alkohol (non-alcoholic steatohepatitis) di kalangan pesakit NAFLD. Pengukuran ketegangan hati untuk pesakit dengan skor fibrosis NAFLD yang tidak tentu dan tinggi membolehkan penentuan tahap fibrosis serius dengan tepat dan mengurangkan pesakit yang memerlukan biopsi hati. Dalam kajian 472 pelajar di Fakulti Perubatan, Universiti Malaya, kekerapan penyakit NAFLD adalah 8.1% dan juga adalah paling tinggi di kalangan India dan Melayu berbanding dengan Cina. Kajian ini mengesahkan bahawa perbezaan kekerapan penyakit NAFLD di kalangan kaum berbeza boleh dilihat pada tahap awal dewasa lagi.

Preface and Acknowledgements

This thesis is the product of several years of work and research in the area of non-alcoholic fatty liver disease (NAFLD). My interest in NAFLD started when I was given the task to conduct a follow-up study on a cohort of NAFLD patients soon after I joined the prestigious Gastroenterology and Hepatology Unit of the University of Malaya and the University of Malaya Medical Centre in 2009. The desire to understand the disease better, particularly in the local setting, has led me to review the literature and to conduct several clinical and epidemiological studies.

My sincere and heartfelt gratitude goes to Professor Dato Dr. Goh Khean Lee, Head of the Gastroenterology and Hepatology Unit, who has accepted me as a member of his wonderful team. I would not have been able to achieve what I have achieved today if not for this great man. He has taught me not only the knowledge and skills in the field of Gastroenterology and Hepatology but also the desired qualities of an academician. Honesty, hard-work and humility were his words and these words will always resound in my mind and serve as my guide. It was also through his encouragement that I embarked on the writing of this thesis in 2013. He has tirelessly supported me throughout my endeavor and I shall always be indebted to him for the successful completion of this thesis.

My special thanks goes to Professor Dr. Sanjiv Mahadeva who has provided me with useful advice and support throughout the period of conducting my research work and writing of this thesis. It was Sanjiv who has first taught me to perform ultrasound-guided percutaneous liver biopsy, a skill which I have since mastered and used, not only in my clinical practice but also in much of the research work that I have subsequently embarked on. Sanjiv was also instrumental in setting up the Fibroscan service and involving me in an investigator-initiated clinical trial, both of which formed the platform for some of the research projects presented in this thesis.

Professor Dr. Goh Khean Jin, Head of the Department of Medicine, has always been supportive of my research work. Professor Dato Dr. Christopher Boey Chiong Meng, Deputy Dean for Postgraduate Program, has been a source of valuable advice and encouragement. To them, I owe my heartfelt gratitude. I would also like to thank my colleagues, Associate Professor Dr. Ida Normiha Hilmi, Dr. Ho Shiaw Hooi, Dr. Chan Weng Kai, Dr. Suresh Sithambaram and Dr. Alex Leow Hwong Ruey who were supportive of my research work.

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List of Abbreviations

ALP	Alkaline phosphatase
ALT	Alanine aminotransferase
AST	Aspartate aminotransferase
BMI	Body mass index
CRN	Clinical Research Network
DBP	Diastolic blood pressure
DNL	De novo lipogenesis
FFAs	Free fatty acids
FFQ	Food-frequency questionnaire
GGT	Gamma glutamyl transpeptidase
GPAQ	Global Physical Activity Questionnaire
HbA1c	Glycated hemoglobin
HDL	High-density lipoprotein
IHD	Ischemic heart disease
LDL	Low-density lipoprotein
NAFLD	Non-alcoholic fatty liver disease
NAS	Non-alcoholic fatty liver disease activity score
NASH	Non-alcoholic steatohepatitis
NEFAs	Non-esterified free fatty acids
NRNM	Nik Raihan, Nik Mustapha
PLC	Phaik-Leng, Cheah
PS	Pavai, Sithaneshwar
PUFAs	Polyunsaturated fatty acids
SBP	Systolic blood pressure
SFA	Saturated fatty acid
SM	Sanjiv, Mahadeva
TC	Total cholesterol
TG	Triglyceride
WC	Waist circumference
WKC	Wah Kheong, Chan

Chapter 1

Introduction and objectives

Non-alcoholic fatty liver disease (NAFLD) has rapidly increased over the years along with obesity and metabolic syndrome and has become one of the most common causes of chronic liver disease worldwide. It is now recognized that the condition is not as benign as previously thought. Patients with NAFLD can progress to cirrhosis and liver cancer. In addition, metabolic syndrome, which is closely related to NAFLD, predisposes patients with NAFLD to cardiovascular diseases. Overall, patients with NAFLD have increased mortality and morbidity compared to the general population. Unfortunately, patients diagnosed with NAFLD have limited options for treatment. Although lifestyle interventions have been shown to be effective, many patients find difficulty in following them. Despite much research, safe and effective treatment for NAFLD is still very limited.

In Malaysia, the prevalence of obesity and metabolic syndrome has increased drastically over the years. The prevalence of NAFLD is expected to be high as well. However, there are limited studies on NAFLD in our local population. Specifically, the prevalence of NAFLD among our patients with diabetes mellitus has never been studied before. Some of the objectives of this thesis are, to determine the prevalence of NAFLD and associated factors, to study the role of diet and physical activity in NAFLD, and to determine if NAFLD is associated with ischemic heart disease, among patients with diabetes mellitus. In addition, there has not been any longitudinal study on NAFLD patients in the local setting. The prevalence of NAFLD among our younger population is also unknown. Other objectives of this thesis are, to elucidate the natural history of NAFLD, and to determine the prevalence of NAFLD and associated factors among our young adults.

Furthermore, at the time that research work for this thesis began, non-invasive methods for assessment of NAFLD were gaining popularity. Hence, another objective of this thesis is to evaluate some of the non-invasive methods for assessment of NAFLD. These objectives were determined to bridge the knowledge gaps in the local setting and to form the foundation for future NAFLD research that could enrich the literature in a more global context.

The outline of this thesis is as follows:

Chapter 2 provides an overview of NAFLD. The content of this chapter includes historical aspects of NAFLD and a description of the disease and its pathogenesis. The relationship between NAFLD and metabolic syndrome, and how the condition may be diagnosed are also described here.

Chapter 3 reports the review of literature on epidemiology of NAFLD in the Asian-Pacific. This review formed the foundation for several of the studies presented in the following chapters of this thesis.

Chapter 4 reports the findings from a cross-sectional study on prevalence of NAFLD among patients with diabetes mellitus at the University of Malaya Medical Centre, Kuala Lumpur. Independent factors associated with NAFLD were determined using multiple logistic regression analysis. The multiethnic composition of the study population has allowed analysis of data according to the different ethnic groups which has helped shed some light into ethnic differences in NAFLD.

Diet and physical activity play important roles in NAFLD. In Chapter 5, interesting findings from a detailed analysis of dietary intake and level of physical activity of diabetic patients with and without NAFLD are presented and discussed.

NAFLD has been associated with cardiovascular disease. The results of a cross-sectional study to determine if ultrasonography-diagnosed NAFLD is associated with

prevalent ischemic heart disease among patients with diabetes mellitus are presented and discussed in Chapter 6.

In Chapter 7, the findings from a follow-up study on NAFLD patients using paired liver biopsy are presented.

Histopathological examination of a liver biopsy specimen is the current best standard for evaluation of NAFLD. However, a liver biopsy is invasive. Chapter 8 focuses on non-invasive methods to evaluate each of the histological components of NAFLD. Findings from studies on the accuracy of some of these non-invasive methods are presented here.

The prevalence of NAFLD in younger populations will reflect the brunt of the disease in the future. Chapter 9 reports the findings of a cross-sectional study on the prevalence of NAFLD among young adults pursuing their tertiary education at the Faculty of Medicine, University of Malaya, Kuala Lumpur. Independent factors associated with NAFLD were determined using multiple logistic regression analysis. The multiethnic composition of the study population has also allowed analysis of data according to the different ethnic groups.

In Chapter 10, the thesis is summarized and conclusions are made.

Chapter 2

Overview of non-alcoholic fatty liver disease

2.1 Historical aspects

In 1980, Ludwig and colleagues described a series of 20 patients with chronic liver disease that had histological findings similar to patients with alcoholic liver disease although the patients denied alcohol intake (Ludwig et al., 1980). The term non-alcoholic steatohepatitis was coined to refer to this condition, the cause of which was unknown then. However, one important observation was that all but one of the patients were overweight or obese. Over the next 3 decades, research has led us to a better understanding of the condition which is now termed non-alcoholic fatty liver disease (NAFLD) and is recognized as the most common cause of chronic liver disease worldwide (Younossi et al., 2011).

2.2 The definition and spectrum of NAFLD

NAFLD encompasses a spectrum of liver conditions that occur in individuals who do not consume alcohol or who consume alcohol but in amounts regarded as insufficient to cause liver damage. Histologically, it is indistinguishable from alcoholic fatty liver disease. At one end of this spectrum is accumulation of fat in the liver or simple steatosis. This is followed by the more severe form of the disease, non-alcoholic steatohepatitis (NASH) which is characterized by the presence of inflammation. While simple steatosis is generally considered benign, NASH may lead to fibrosis and eventually cirrhosis with increased risk of liver-related death and hepatocellular carcinoma (Ekstedt et al., 2006; Sanyal et al., 2006) (**Figure 2.1**).

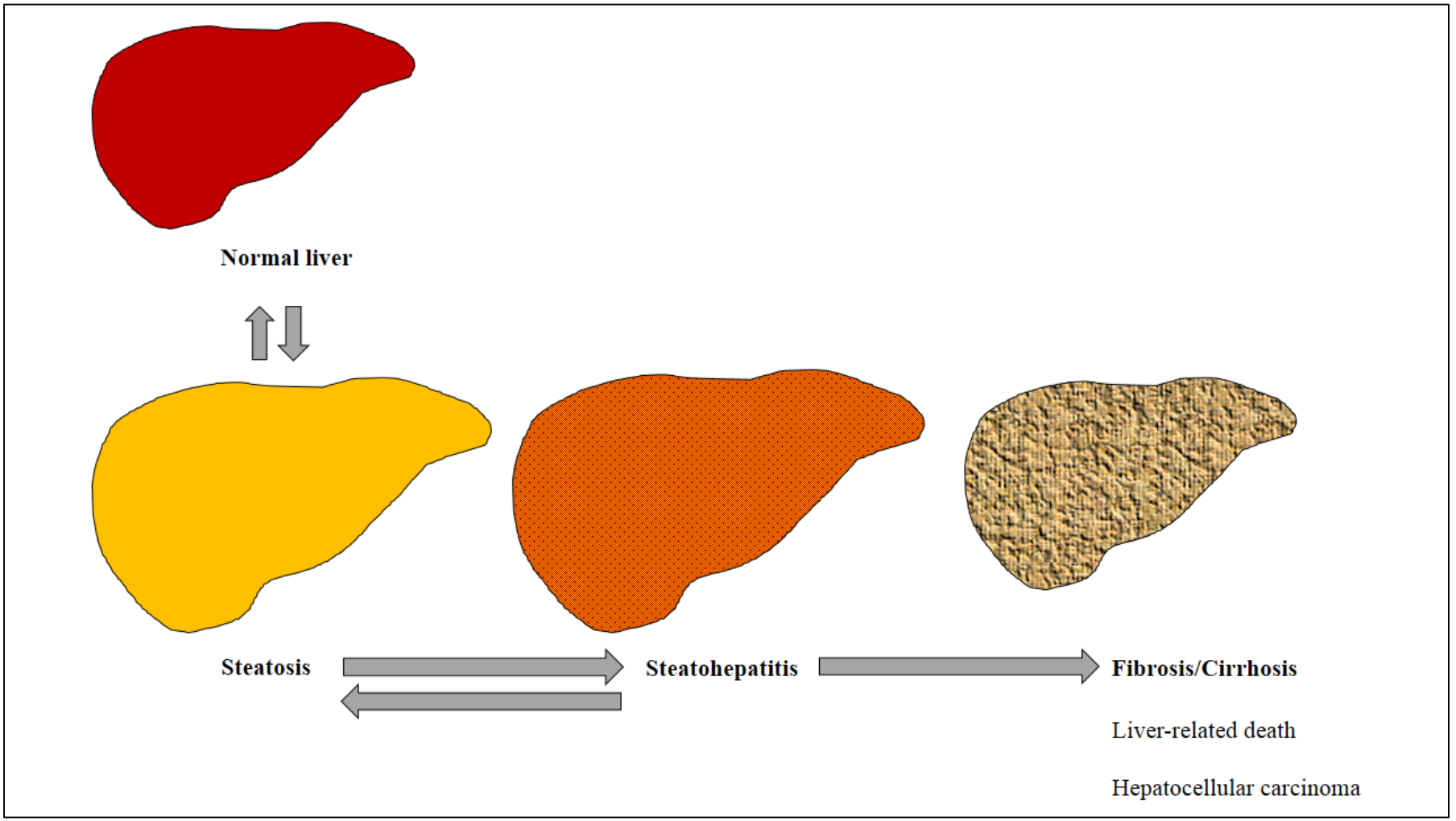

Figure 2.1 The spectrum of NAFLD

2.3 Pathogenesis of NAFLD

Sources of free fatty acids (FFAs) in the liver are serum non-esterified fatty acids (NEFAs) from triglyceride breakdown in adipose tissue, de novo lipogenesis (DNL) and diet. Besides being esterified to triglycerides and stored in the liver, FFAs may undergo β-oxidation or be exported as very low density lipoprotein. Any imbalance that results in excess FFAs in the liver would lead to accumulation of fat in the liver.

NAFLD is the result of a two-hit mechanism (**Figure 2.2**). The first hit is insulin resistance. Insulin resistance results in impaired insulin-mediated suppression of triglyceride breakdown in adipose tissue. This leads to increased serum NEFAs. Insulin resistance also results in impaired insulin-mediated glucose uptake in adipose tissue and skeletal muscle. The resultant increase in blood glucose concentration leads to increased glucose uptake in the liver, a process which is insulin-independent. This promotes DNL. Therefore patients with insulin resistance have excess FFAs in the liver from increased serum NEFAs and DNL and are at risk of NAFLD. In patients with NAFLD, serum NEFAs and FFAs from DNL are main sources of accumulated fat in the liver (Donnelly et al., 2005). In contrast, DNL contributes to less than 5 % of liver fat in healthy individuals (Hudgins et al., 2000; Parks, 2002).

The second hit is oxidative stress. Excessive amounts of FFAs in the liver overwhelms the β-oxidation process within the mitochondria of liver cells. This leads to accumulation of reactive oxygen species that causes mitochondrial damage and activation of inflammatory pathways. Liver inflammation and fibrosis ensues. A detailed description of the two hits can be found elsewhere (Dowman et al., 2010).

The discovery of various other factors in the pathogenesis of NASH subsequently led to the proposal of a more comprehensive multiple parallel hits hypothesis (Tilg et al., 2010).

For example, the gut microbiota is increasingly recognized to play a role in the pathogenesis of NAFLD. High fat diet has been shown to induce changes in the gut microbiota leading to bacterial overgrowth, disruption of intercellular tight junctions, increased intestinal permeability, and increased bacterial DNA and lipopolysaccharide in the portal circulation. This leads to activation of toll-like receptors on Kupffer cells and hepatic stellate cells, generation of inflammatory cytokines, fat accumulation in liver cells, cell death, and fibrogenesis (Wree et al., 2013). Another example is genetic polymorphism in the patatin-like phospholipase domain containing 3 (PNPLA3) or adiponutrin gene which has been associated with increased hepatic fat and inflammation (Romeo et al., 2008). A study on a multi-ethnic population found that the PNPLA3 gene polymorphism is associated with susceptibility to NASH, NASH severity and presence of fibrosis. Interestingly, the study also found that the effect of the gene polymorphism appears to be greater in the Indians followed by the Malays and the Chinese (Zain et al., 2012).

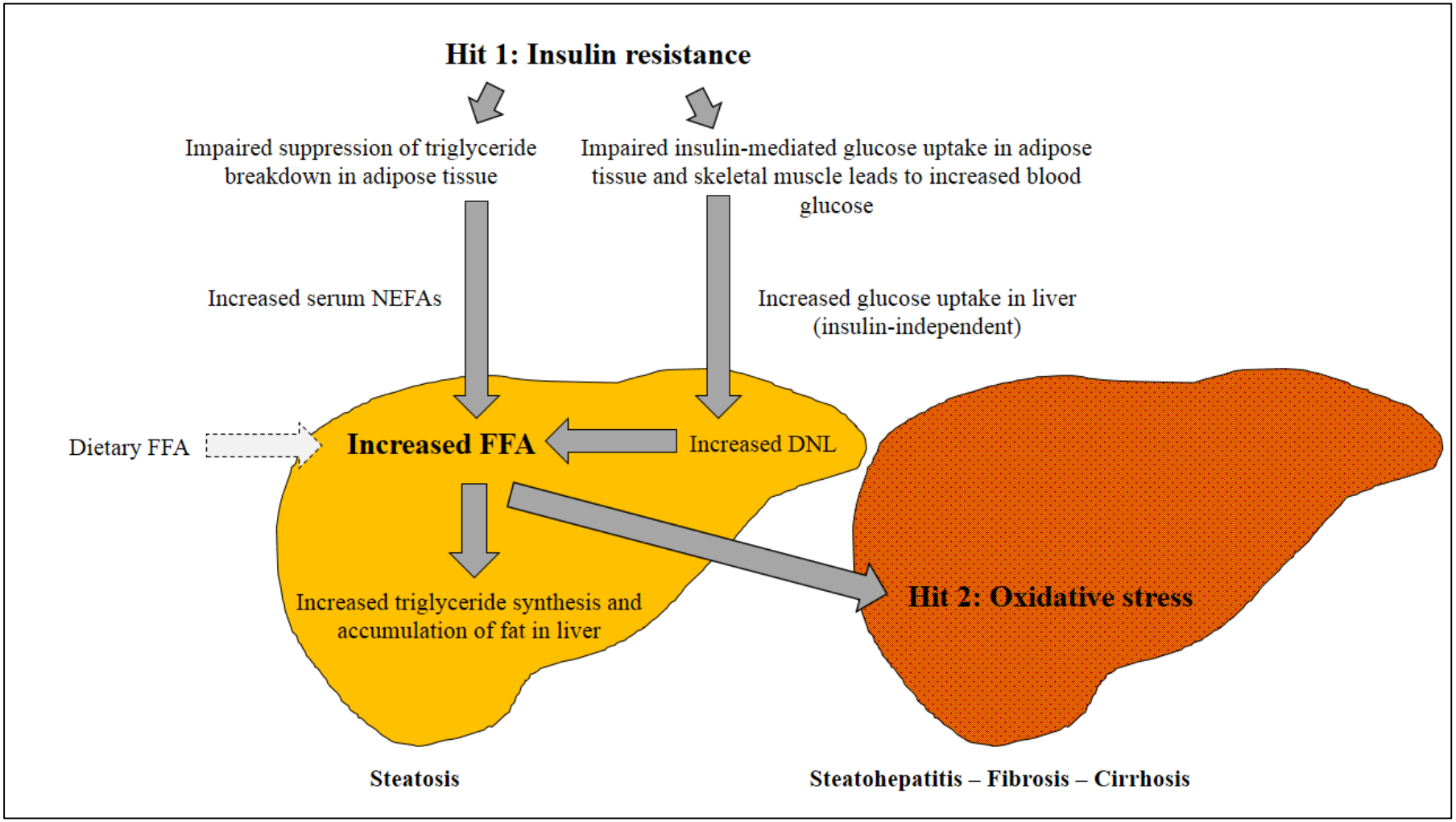

Figure 2.2 The two-hit mechanism of NAFLD

2.4 NAFLD and the metabolic syndrome

The metabolic syndrome is a constellation of closely related cardiovascular risk factors. The International Diabetes Federation defines metabolic syndrome as the presence of central obesity based on ethnic specific cut-off for waist circumference, plus two or more of the following: raised triglycerides, reduced serum high-density lipoprotein (HDL) cholesterol, raised blood pressure and raised fasting plasma glucose (Alberti et al., 2005). Insulin resistance is an important feature of metabolic syndrome. As elucidated in the earlier section, insulin resistance also serves as the first of the two hits in the pathogenesis of NAFLD. Hence, NAFLD is closely related to the metabolic syndrome. In fact, NAFLD has been considered as the liver manifestation of the metabolic syndrome (Kim et al., 2008). The prevalence of NAFLD is high in those with metabolic syndrome and the presence of metabolic syndrome in patients with NAFLD is associated with more severe liver disease (Marchesini et al., 2003).

2.5 Diagnosis of NAFLD

Most patients with NAFLD are asymptomatic. Some may have non-specific symptoms e.g. fatigue. The diagnosis of NAFLD is often suspected from elevated serum aminotransferase level in patients with metabolic syndrome. The diagnosis can be confirmed with ultrasonography and following exclusion of significant alcohol intake and other causes of chronic liver disease e.g. viral hepatitis B and C. NAFLD is also often incidentally diagnosed when patients undergo ultrasonography for unrelated indications. Patients with cirrhosis due to NAFLD present when they decompensate and in ways similar to patients

with decompensated cirrhosis due to other causes of chronic liver disease. Presentations include jaundice, ascites, ankle swelling, bleeding from esophageal varices, hepatic encephalopathy and hepatocellular carcinoma.

Elevated serum aminotransferase level is neither sensitive nor specific for diagnosis of NAFLD. For example, Mofrad and colleagues showed that the entire spectrum of NAFLD can be seen in patients with normal serum alanine aminotransferase (ALT) level (Mofrad et al., 2003). At the same time, elevated serum ALT could be the result of many other liver conditions other than NAFLD.

Ultrasonography is by far the most common method used to diagnose fatty liver in clinical practice and in epidemiological studies. Fatty liver is recognized on ultrasonography based on the following features: increased echogenicity, posterior attenuation and loss of intra-hepatic architectural details (Joy et al., 2003) (**Figures 2.3a and 2.3b**). In a study using a scoring system for ultrasonographic findings of NAFLD, Hamaguchi and colleagues reported excellent sensitivity (91.7 %) and specificity (100 %) of ultrasonography for diagnosis of NAFLD (Hamaguchi et al., 2007). However, a separate study by Saadeh and colleagues showed that ultrasonography is accurate only when fatty liver is moderate to severe (Saadeh et al., 2002). Moreover, ultrasonography is not able to distinguish NASH from simple steatosis and to assess the severity of fibrosis. Both factors carry important prognostic implications in NAFLD patients.

Histopathological examination of a liver biopsy specimen is the current best standard for assessment of NAFLD. It confirms the diagnosis and helps exclude other causes of liver disease in some cases. It also distinguishes NASH from simple steatosis and allows assessment of the severity of fibrosis. The NASH Clinical Research Network (NASH CRN) scoring system provides a standardized manner for reporting of histopathological findings of NAFLD (Kleiner et al., 2005). The scoring system is elaborated in Chapter 8. Although

histopathological examination of a liver biopsy specimen could provide useful information, its use is limited as a liver biopsy is invasive and associated with a small risk of complications. Moreover, technical expertise is required, from obtaining a good specimen to processing and accurately interpreting the result. Histopathological examination of liver biopsy specimen may be further limited by sampling variability (Ratziu et al., 2005) and intra- and inter-observer variability (Younossi et al., 1998). Hence, there is a need for non-invasive tests that can be easily performed to estimate histological severity of NAFLD. These are discussed in Chapter 8.

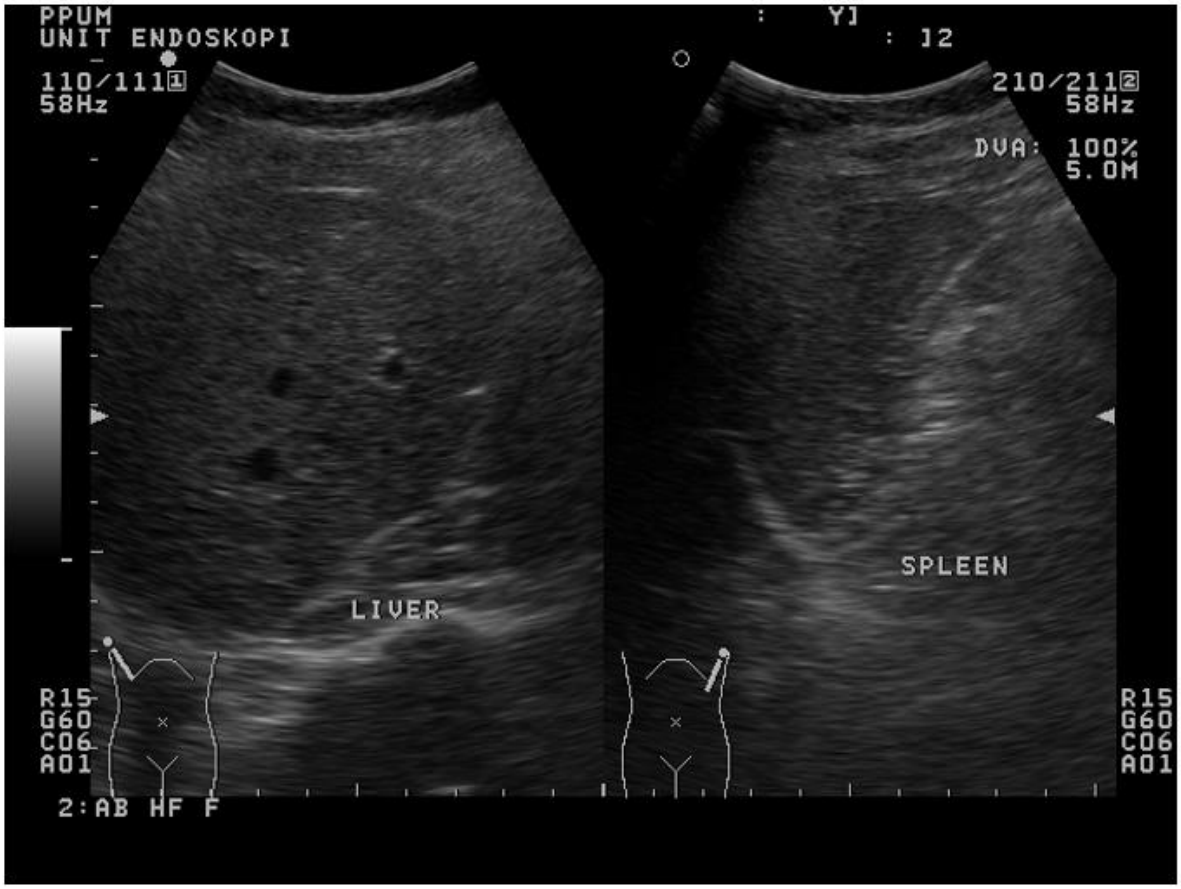

Figure 2.3a Normal liver on ultrasonography

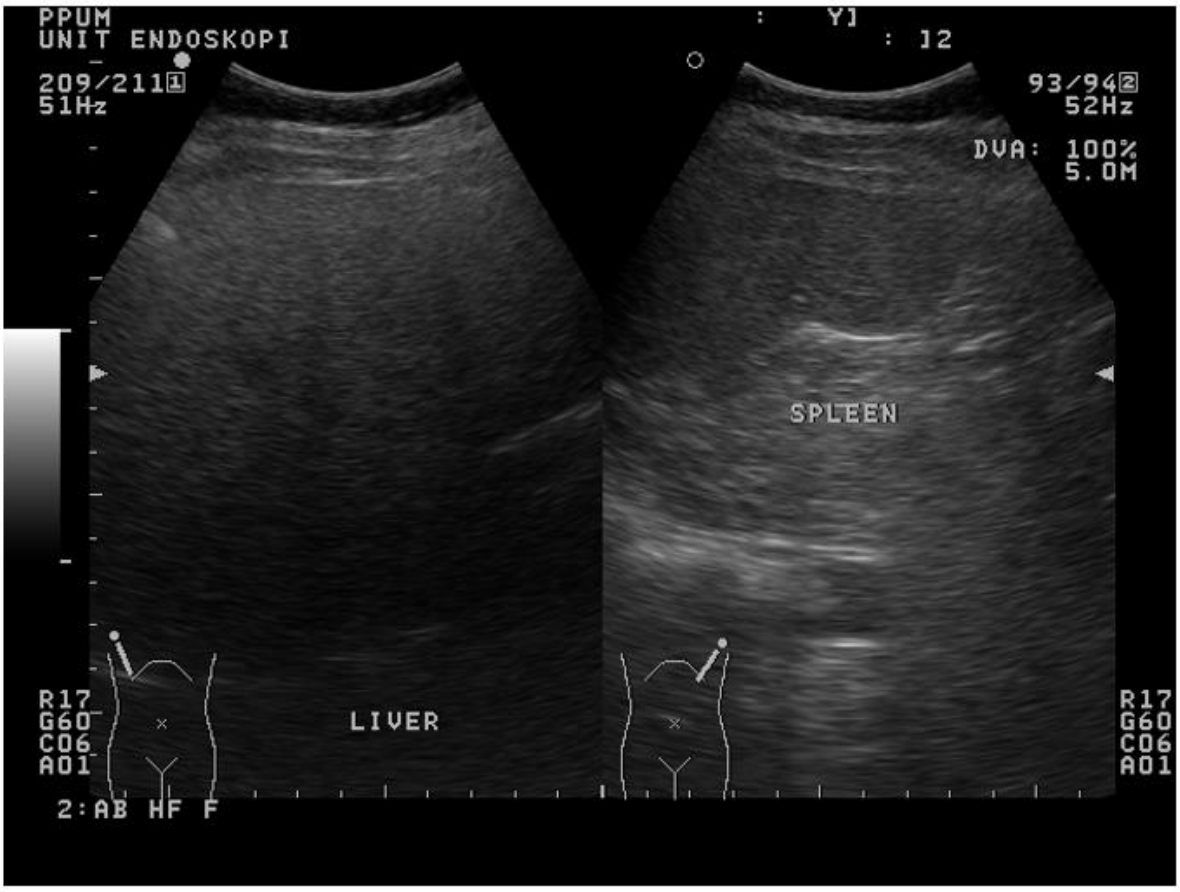

Figure 2.3b Fatty liver on ultrasonography

Fatty liver is characterized by increased echogenicity, posterior attenuation and loss of intra-hepatic architectural details on ultrasonography.

Chapter 3

Review of literature: epidemiology of NAFLD in the Asian-Pacific

3.1 Introduction

At the beginning of my work on non-alcoholic fatty liver disease (NAFLD), I reviewed the literature to get an overview of the epidemiology of the disease, specifically in the Asian-Pacific. The findings are presented in this chapter. The knowledge acquired during the process served as the foundation for some of my subsequent work on NAFLD which are presented in the following chapters of this thesis.

3.2 Method

A search was made on PubMed using the MeSH terms ("Non-Alcoholic Fatty Liver Disease" OR "Fatty Liver") AND ("Epidemiology" OR "Prevalence") in November 2011. The search yielded 495 articles. Out of these, 139 articles were from the Asian-Pacific. The abstracts of the articles were examined and where doubt existed as to the relevance of an article to the review, the full paper was examined. In total, 63 articles were deemed relevant and were included in the review.

3.3 Results and discussion

Prevalence of NAFLD in the general population

Prevalence of ultrasonography-diagnosed NAFLD in the general population in different regions across the Asian-Pacific is shown in **Figure 3.1** and **Table 3.1**. Large studies from China have estimated the prevalence of NAFLD to be in the range of 11.8 % to 24.4 % (Chen et al., 2008; Gao et al., 2008; Hou et al., 2011; Li et al., 2009; Zhou et al., 2007). Only

a study from southwest China demonstrated a remarkably lower prevalence of NAFLD at 6.3 % for which the author attributed to regional differences in age stratification, economic conditions, and dietary habits (Li et al., 2009). As expected, studies on fatty liver disease in general without distinction between alcoholic and non-alcoholic reported higher prevalence rates of between 17.3 % and 40.0 % (Dai et al., 2009; Fan et al., 2005; Kang et al., 2009).

Two retrospective studies from China reported increasing prevalence of fatty liver disease over time. Fan and colleagues reviewed the medical records of employees of a factory in Shanghai and reported that the prevalence of fatty liver disease had increased from 3.9 % between 1995 and 1996 to 14.0 % between 2001 and 2002 (Fan et al., 2007). All the components of metabolic syndrome recorded significant increase during the same period of time while the proportion of subjects with habitual drinking remained unchanged. In a study of subjects who went for health check-up, Wang and colleagues found that the prevalence of fatty liver disease had almost doubled from 12.5 % in 1995 to 24.5 % between 2003 and 2004 (Wang et al., 2007). Although these studies did not make a distinction between alcoholic and non-alcoholic fatty liver disease, the figures probably reflected an increasing prevalence of NAFLD.

In Japan, recent studies on subjects attending health check-up estimated the prevalence of NAFLD to be close to 30 % (Jimba et al., 2005; Oya et al., 2010). This is a remarkable increase as similar studies in the late 1980s reported the prevalence of fatty liver disease in general to be just around 13 % (Kojima et al., 2003; Saito et al., 1989). In a large study of subjects who visited a hospital for health check-up, Kojima and colleagues reported a substantial increase in the prevalence of fatty liver disease, from 12.6 % in 1989 to 30.3 % in 1998 (Kojima et al., 2003). Among the 35 519 subjects who had examinations during the two time points, 14.3 % developed fatty liver while fatty liver resolved in 3.5 %. The development of fatty liver was associated with increase in body mass index (BMI) while

resolution was associated with a decrease in BMI. In a study of elderly subjects of a health check-up program in Nagasaki, the prevalence of NAFLD was estimated to be between 3 % and 4 % among non-obese subjects and between 18 % and 22 % among obese subjects (Akahoshi et al., 2001).

In Korea, a population-based study estimated the prevalence of NAFLD to be 16.1 % (Park et al., 2006). Two other studies on subjects attending health check-up estimated much higher prevalence although one of the studies excluded diabetic patients (Bae et al., 2010) while the other excluded obese and diabetic patients (Kim et al., 2004). Studies from Taiwan have estimated the prevalence of NAFLD to be around 11.5 % (Chen et al., 2006; Kuo et al., 2010). One relatively smaller hospital-based study that focused on metabolic syndrome recorded an unusually high prevalence of NAFLD at 42.6 % (Tsai et al., 2008). As expected, studies that looked at fatty liver disease in general reported higher prevalence of between 29.5 % and 57.8 % (Hsiao et al., 2007; Lai et al., 2002; Lin et al., 2005; Lu et al., 1990).

Studies from India reported high prevalence of NAFLD, between 16.6 % and 32.0 % (Amarapurkar et al., 2007; Mohan et al., 2009; Singh et al., 2004). One study recorded a relatively lower prevalence at 8.7 %, but it is interesting to note that the study was conducted in a rural population where the majority of subjects were young, physically active, less affluent and non-obese. The prevalence is considerably high given the profile of the study population and raised concern that NAFLD will become a significant health burden even in less affluent populations. This study also reported that 12 % of subjects with NAFLD had a BMI of less than 18.5 kg/m^2. The author used the term “third-world non-alcoholic fatty liver” to describe this phenotype where instead of overt obesity, subtle measures of increased adiposity predisposed to NAFLD (Das et al., 2010).

There were limited published studies on prevalence of NAFLD in the general population from other countries in the Asian-Pacific. Population-based studies from Sri

Lanka and Israel estimated NAFLD to be present in approximately 30 % (Dassanayake et al., 2009; Zelber-Sagi et al., 2006). A study on subjects who received health check-up from Thailand reported fatty liver disease in general to be present in 35.9 % (Rungsinaporn et al., 2008). A study on a multi-ethnic health check-up population in Malaysia found the prevalence of NAFLD to be 22.7 % (Goh et al., 2013). Interestingly, the prevalence of NAFLD was remarkably higher among the Indians and Malays compared to the Chinese in this study. This corresponded with the risk of diabetes mellitus and cardiovascular diseases among the different ethnic groups. The observed racial predilection was attributed to differences in genetic and environmental factors, especially the difference in the diet of the different ethnic groups.

Overall, the prevalence of NAFLD in the Asian-Pacific has increased rapidly over the years and is now comparable to that in Western countries, which has been estimated to be between 20 % and 33 % in large population-based studies (Bedogni et al., 2005; Caballeria et al., 2010). If this trend continues, NAFLD will become the most common cause of chronic liver disease in the Asian-Pacific, as is already the case in Western countries.

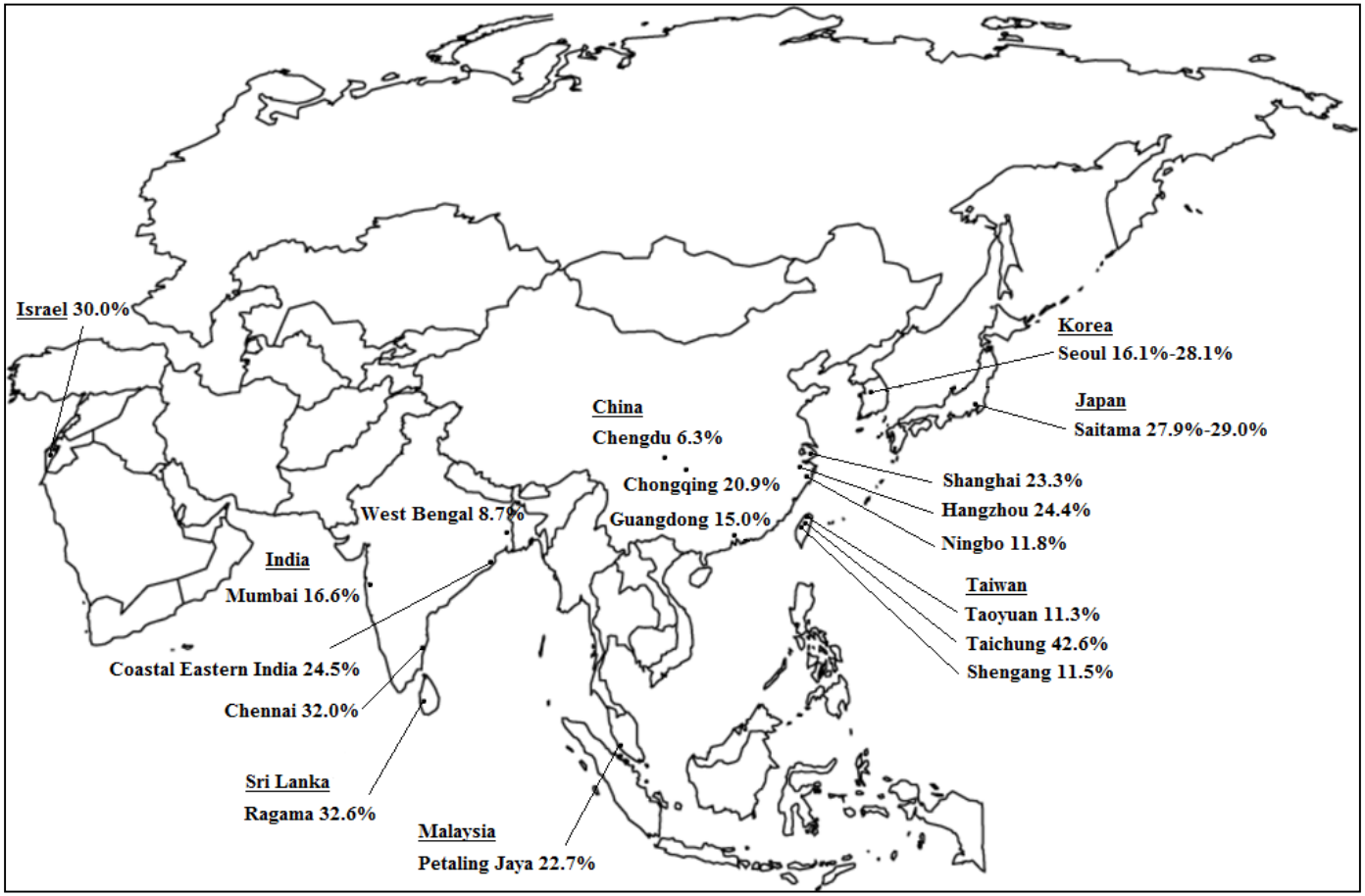

Figure 3.1 Prevalence of ultrasonography-diagnosed NAFLD in the general population in different regions across the Asian-Pacific

All studies were population-based or on subjects attending health check-up, except the studies in Ningbo and Coastal Eastern India. The former was on employees of a company while the latter was on healthy individuals accompanying patients to a Gastroenterology Clinic.

Table 3.1 Description of the studies on the prevalence of ultrasonography-diagnosed NAFLD in the general population in different regions across the Asian-Pacific

Author, Year	Population Studied	n	Prevalence of NAFLD, %
China			
Zhou et al., 2007	Randomized, multi-stage stratified sampling of subjects over 7 years old in Guangdong	3543	15.0
Gao et al., 2008	Randomized, multi-stage stratified sampling of civil servants in Chongqing	2176	20.9
Chen et al., 2008	Adults who received health check-up in a hospital in Hangzhou	26527	24.4
Li et al., 2009	Employees of a company in Ningbo	8925	11.8
Li et al., 2009	Adults who received health check-up in a hospital in Chengdu	9094	6.3
Hou et al., 2011	Randomized, multi-stage stratified sampling of adults in Shanghai	2226	23.3
Japan			
Jimba et al., 2005	Adults who received health check-up in a hospital in Saitama	1950	29.0
Oya et al., 2010	Adults who received health check-up in a hospital in Saitama	796	27.9
Korea			
Kim et al., 2004	Non-obese non-diabetic adults who received health check-up in Seoul	768	23.4
Park et al., 2006	Adults who received health check-up in a hospital in Seoul	6648	16.1
Bae et al., 2010	Non-diabetic adults who received health check-up in a hospital in Seoul	99969	28.1
Taiwan			
Chen et al., 2006	Randomized sampling of adults in a rural area in central Taiwan	3245	11.5
Tsai et al., 2008	Adults who received health check-up in a hospital in Taichung	876	42.6
Kuo et al., 2010	Adults who received health check-up in a hospital in Taoyuan	54325	11.3
India			
Amarapurkar et al., 2007	Residents of two railway colonies in Mumbai	541	16.6

Mohan et al., 2009	Subsample of the Chennai Urban Rural Epidemiology Study	159	32.0
Singh et al., 2004	Healthy individuals accompanying patients to a Gastroenterology Clinic in East India	1168	24.5
Das et al., 2010	Adults in a rural administrative unit in West Bengal	1911	8.7
Other Countries			
Goh et al., 2013	Multi-racial suburban population who received health check-up in a medical centre in Petaling Jaya, Malaysia	1621	22.7
Dassanayake et al., 2009	Randomized stratified sampling of an urban Sri Lankan population	2985	32.6
Zelber-Sagi et al., 2006	Subsample of national health survey in Israel	326	30.0

n = number of subjects

Prevalence of NAFLD in children and adolescents

The prevalence of NAFLD among children has been reported to be between 2.1 % and 4.5 % (Tominaga et al., 1995; Tsuruta et al., 2010; Wan et al., 2007). In one of the largest study on children from two elementary schools in Shanghai, the overall prevalence of NAFLD was estimated to be 2.1 % and was closely associated with body mass index (BMI). Prevalence of NAFLD increased from normal to overweight to obese children at 0.6 %, 2.9 %, and 13.8 %, respectively (Wan et al., 2007). In a study from Japan, Tsuruta and colleagues found reduced daily physical activity to be associated with NAFLD in children, independent of obesity (Tsuruta et al., 2010). In studies that included adolescents, higher prevalence of NAFLD was observed ranging from 7.1 % to 16.9 % (Adibi et al., 2009; Alavian et al., 2009). In a large study of randomly selected children and adolescents from schools in Isfahan, the overall prevalence of NAFLD was 16.9 % and increased from normal to overweight to obese subjects at 1 %, 10.5 % and 54.4 %, respectively (Adibi et al., 2009). In hospital-based studies, the prevalence of NAFLD among overweight or obese children is much higher, ranging from 60.3 % to 80.5 % (Fu et al., 2006; Sagi et al., 2007; Shi et al., 2009). As in adults, NAFLD was found to be closely associated with insulin resistance and features of metabolic syndrome (Shi et al., 2009). Worryingly, fibrosis is common in children with NASH, and cirrhosis due to NASH have been reported in children as young as 10 years old (Molleston et l., 2002).

NAFLD – effect of gender

In younger populations, NAFLD has been consistently shown to be more prevalent among men than women (Amarapurkar et al., 2007; Chen et al., 2006; Fan et al., 2007; Singh et al., 2004; Zelber-Sagi et al., 2006), but such trend was no longer observed in older

populations (Fang et al., 2005; Kang et al., 2009; Zhou et al., 2007), suggesting the potential influence of sex hormones in the development of the disease. For example, in a study of non-diabetic subjects, no significant difference in prevalence of NAFLD was observed in men below and above 50 years old. However, the risk for NAFLD was 3.5 times higher in women above 50 years old (Bae et al., 2010). NAFLD may also have different impact depending on the gender. For example, in an Australian population-based cross-sectional study of 1170 adolescents, male with NAFLD had greater visceral adipose tissue thickness and lower adiponectin levels, and significantly worse metabolic features (higher glucose levels, higher systolic blood pressure, lower high-density lipoprotein cholesterol levels) compared to their female counterparts (Ayonrinde et al., 2011).

NAFLD and serum ALT levels

NAFLD is one of the most common causes of elevated serum alanine aminotransferase (ALT) levels. A cross-sectional study in a rural village of Taiwan identified NAFLD as the single most common cause of elevated serum ALT levels (Chen et al., 2007). NAFLD was also found to be the most common cause of persistently elevated serum ALT levels among the general population in Iran (Jamali et al., 2008). However, not all patients with NAFLD have raised serum ALT levels. In fact, patients across the spectrum of NAFLD may have normal serum ALT levels. Therefore, the use of serum ALT level as a surrogate marker for NAFLD underestimates the prevalence of NAFLD (Zelber-Sagi et al., 2006). In patients with NAFLD, serum ALT levels were found to be significantly higher in those with metabolic syndrome and the mean serum ALT levels increased significantly with increasing number of components of metabolic syndrome (Chen et al., 2008). In another study, raised serum ALT levels in non-NAFLD patients were associated with hypertriglyceridemia while

in NAFLD patients were associated with both hypertriglyceridemia and hyperglycemia (Hou et al., 2011).

NAFLD and metabolic syndrome – new insight

NAFLD has been considered the liver manifestation of metabolic syndrome and is closely related to other features of metabolic syndrome. Recent studies have shown that presence of NAFLD itself increases the risk of developing diabetes mellitus independent of other features of metabolic syndrome. In a retrospective longitudinal study, Yamada and colleagues found that significantly more subjects had newly diagnosed impaired fasting glucose and diabetes mellitus over a 5-year period if they had fatty liver, and fatty liver remained an independent factor after adjusting for other risk factors (Yamada et al., 2010). In an observational cohort study of Japanese male workers over 40 years old, NAFLD was found to be associated with significant increase in the risk of developing diabetes mellitus (Shibata et al., 2007). In an interesting study from Korea, NAFLD was found to be closely associated with metabolic syndrome even in non-obese, non-diabetic subjects, and can be considered an early predictor of metabolic disorders, particularly in the normal-weight population (Kim et al., 2004). Findings in these studies suggest that NAFLD may be a mediator of metabolic syndrome rather than just a manifestation of it.

NAFLD may contribute to insulin resistance and thus metabolic syndrome the way visceral adiposity does. In a large cross-sectional study of non-diabetic subjects, Bae and colleagues found that presence of NAFLD was associated with higher levels of insulin resistance independent of other features of metabolic syndrome including obesity, a well-known determinant of insulin resistance. Furthermore, even subjects in the lowest quartile of HbA1c in the group with NAFLD had significantly higher insulin resistance compared to subjects in the highest quartile of HbA1c in the group without NAFLD. Another interesting

finding was insulin resistance increased with increasing HbA1c levels in the group with NAFLD but not in the group without NAFLD suggesting that HbA1c levels have different meaning on insulin resistance by NAFLD status in non-diabetic subjects (Bae et al., 2010).

In a study of healthy subjects in a health check-up program, the prevalence of NAFLD increased with increasing fasting plasma glucose level: 27% in the subgroup with normal fasting plasma glucose, 43% in the subgroup with impaired fasting glucose and 62% in the subgroup with newly diagnosed diabetes mellitus (Jimba et al., 2005). In a population-based study, Mohan and colleagues demonstrated increasing prevalence of NAFLD with increasing severity of glucose intolerance. NAFLD was diagnosed in 22.5% of subjects with normal glucose tolerance, 33% of subjects with either impaired fasting glucose or impaired glucose tolerance, and 54.5% of subjects with frank diabetes mellitus (Mohan et al., 2009). The reported prevalence of NAFLD among patients with diabetes mellitus is consistent with that of another Indian study which estimated the figure to be 57.2% (Agarwal et al., 2011). Another study on NAFLD in patients with diabetes mellitus from China estimated the prevalence to be 42.1% (J. Zhou et al., 2007).

An interesting study from Hong Kong demonstrated high prevalence of undiagnosed diabetes mellitus and impaired glucose tolerance among patients with NAFLD. This study also showed that nearly half the patients with diabetes mellitus or impaired glucose tolerance had normal fasting glucose and highlighted the importance of oral glucose tolerance test among NAFLD patients with normal fasting glucose (Wong et al., 2006). The reason some diabetic patients have NAFLD but others do not, and the reason some NAFLD patients have diabetes mellitus but others do not remains unclear.

NAFLD – the liver and beyond

NAFLD can progress to liver cirrhosis and patients with liver cirrhosis due to NAFLD are at increased risk of hepatocellular carcinoma. Besides liver-related complications, NAFLD has been associated with several other extra-hepatic conditions, the most important being cardiovascular complications. Progression to significant liver disease occurs over a long period of time, and cardiovascular complications may overshadow liver-related complications in causing morbidity and mortality in patients with NAFLD.

In a cross-sectional study of 124 patients with type 2 diabetes mellitus, NAFLD was identified as an independent predictor of coronary artery disease along with hypertension, elevated LDL cholesterol and microalbuminuria (Agarwal et al., 2011). In other studies, NAFLD was independently associated with ischemic changes on electrocardiography (Lin et al., 2005), significant coronary artery stenosis on computed tomography coronary angiography (Assy et al., 2010), impaired left ventricular systolic and diastolic function (Fotbolcu et al., 2010), and increased carotid artery intima-media thickness (Aygun et al., 2008).

NAFLD has also been associated with hyperuricemia and gout. In a large cross-sectional study of employees of a company in China, Li and colleagues found that the prevalence of NAFLD was significantly higher among patients with hyperuricemia, and that the prevalence increased with increasing level of serum uric acid level, independent of other features of metabolic syndrome (Li et al., 2009). In a large study of subjects attending health check-up in Taiwan, Kuo and colleagues found that the prevalence of NAFLD was significantly higher among patients with gout, independent of other features of metabolic syndrome, and also reported a dose-response relationship between serum uric acid level and the presence of NAFLD (Kuo et al., 2010). Other studies found an association between

NAFLD and chronic kidney disease (Yasui et al., 2011), gallstone disease (Chen et al., 2006), colorectal adenomatous polyps (Hwang et al., 2010), and polycystic ovarian syndrome (Brzozowska et al., 2009).

NAFLD and viral hepatitis

Asia-Pacific carries the major burden of viral hepatitis B and C in the world. NAFLD can affect the progression of other chronic liver diseases and its increasing prevalence may impact on the many patients with chronic liver disease due to viral hepatitis B and C in this region. Several studies from the Asia-Pacific have looked into the association of NAFLD and viral hepatitis B and C with interesting findings.

In a study of liver biopsies of 1915 patients with chronic hepatitis B, it was found that presence of NAFLD was independently associated with metabolic features, namely body mass index, serum triglyceride, serum uric acid and fasting blood glucose. There was no significant difference in HBeAg status and viral load between patients with and without NAFLD. Fibrosis was associated with increasing age and inflammatory grade, the latter associated with viral load (Shi et al., 2008). In another study of liver biopsies of 86 young male patients with chronic hepatitis B, NAFLD was again independently associated with metabolic features, namely insulin resistance and serum triglyceride, but not with significant fibrosis. Significant fibrosis was associated with necro-inflammatory activity and elevated serum gamma-glutamyl transpeptidase (Yun et al., 2009).

Hepatic steatosis is seen in a significant proportion of patients with chronic hepatitis C and substantial evidences support viral hepatitis C, especially genotype 3 as a cause of hepatic steatosis. Term such as “metabolic steatosis” and “viral steatosis” have been used to distinguish the cause of hepatic steatosis in patients with viral hepatitis C, the former referring to patients with features of metabolic syndrome when the hepatic steatosis is attributed to

NAFLD. However, it may be difficult to separate the two causes which may together contribute to hepatic steatosis in the same patient.

In a study of 106 non-diabetic and non-alcoholic patients with chronic hepatitis C, Hwang et al found over half the patients to have hepatic steatosis, and the presence of hepatic steatosis was associated with obesity, but not HCV RNA levels and HCV genotypes. Majority were genotype 1 or 2. Hepatic steatosis was also related to more severe hepatic fibrosis (Hwang et al., 2001). One study with genotype 1 or 2 patients (Liu et al., 2005) and another study (Ahmed et al., 2011) also reported association of hepatic steatosis with features of metabolic syndrome and more severe hepatic fibrosis. The former also reported a trend towards lower response to antiviral therapy in the presence of hepatic steatosis although this was not statistically significant. In another study with large proportion of genotype 3 patients, although metabolic features such as BMI and serum levels of cholesterol, triglyceride and glucose were found to be associated with hepatic steatosis, these were not significant on multivariate analysis. On multivariate analysis, only genotype 3 and viral load were significantly associated with hepatic steatosis. The presence of hepatic steatosis was again associated with significantly higher stage of fibrosis (Minakari et al., 2008).

3.4 Conclusion

The prevalence of NAFLD has been rapidly increasing in the Asia-Pacific. Its prevalence increases with increasing age and is more common in men than women although this trend fades with increasing age. Patients newly diagnosed with NAFLD should undergo a thorough metabolic evaluation and the presence of any features of metabolic syndrome should be addressed accordingly. Patients with NAFLD should be advised for intensified lifestyle modification effort even if non-obese and non-diabetic. Besides liver complications, NAFLD is associated with a wide range of diseases. In patients with chronic hepatitis B, NAFLD seems to be related to host metabolic factors rather than viral factors and does not seem to affect severity of the liver disease. On the other hand, hepatic steatosis may be related to both host metabolic and viral factors in patients with chronic hepatitis C and seems to have adverse impacts in terms of severity of liver disease and possibly response to antiviral therapy.

Note: The full article of this literature review has been published in Hepatology International (Chan et al., 2013)

Chapter 4

NAFLD in diabetics – prevalence and associated factors in a multi-racial hospital clinic population in Malaysia

4.1 Introduction

As elucidated in the earlier chapter, non-alcoholic fatty liver disease (NAFLD) has been rapidly increasing in the Asian-Pacific and is estimated to affect up to 30 % of the general population (Chan et al., 2013). In the only published study on prevalence of NAFLD in the general population in Malaysia, Goh and colleagues reported a prevalence of 22.7 % among individuals attending a health-check in a suburban medical facility (Goh et al., 2013). The study reported an inordinately high prevalence of NAFLD among the Malays and Indians compared to the Chinese.

NAFLD is closely associated with diabetes mellitus and obesity. The prevalence of NAFLD is higher in patients with diabetes mellitus and has been estimated to be between 55 % and 70 % in previous studies from other parts of the world (Leite et al., 2009; Merat et al., 2009; Targher et al., 2006). The prevalence of NAFLD is even higher among the morbidly obese and has been reported to be over 90 % (Machado et al., 2006). In Malaysia, the prevalence of diabetes mellitus and obesity has reached epidemic proportions over the years. The Third National Health and Morbidity Survey (NHMS III) estimated the prevalence of diabetes mellitus among adults aged 30 years old and above to have almost doubled from 8.3 % in 1996 to 14.9 % in 2006 (Institute for Public Health, 2008). Yet to be published, the Fourth National Health and Morbidity Survey found that this figure has increased to 20 % in 2011. The NHMS III also reported that 43.1 % of adults were overweight or obese in 2006, almost double that reported ten years earlier. Moreover, the reports followed the World

Health Organization criteria, which have higher body mass index (BMI) cut-offs for definition of overweight and obese compared to the Western Pacific Regional Office criteria for Asians leading to an underestimation of the true weight of the problem (Anuurad et al., 2003).

This study was carried out to determine the local prevalence of NAFLD and associated factors among patients with diabetes mellitus. There were no published study on this in Malaysia. We also aimed to compare the prevalence of NAFLD among diabetics from the three major ethnic groups in Malaysia, namely the Malays, Chinese and Indians. There were no published study comparing the prevalence of NAFLD among diabetic patients of different ethnicity in the Asian-Pacific.

4.2 Patients and methods

The study was a cross-sectional study on consecutive patients seen at the diabetic clinic of University of Malaya Medical Centre between November 2011 and April 2012. Patients who have been included but returned for follow-up during the study period were identified and not included a second time. Patients with known chronic liver disease other than fatty liver and patients who did not agree to participate were excluded. The study was approved by the University of Malaya Medical Centre's Medical Ethics Committee and informed consent was obtained from all included patients.

Demographic and anthropometric data and relevant clinical and laboratory data were obtained using a standard protocol. Alcohol intake was estimated using the quantity-frequency method (Goddard, 2007). Alcohol intake was estimated based on patient's self-reported frequency and quantity of intake of each of the 3 main types of alcoholic beverages i.e. beer, wine and spirit. Frequency of intake was divided into 7 categories i.e. almost every day, 5 or 6 days a week, 3 or 4 days a week, once or twice a week, once or twice a month,

once every couple of months and once or twice a year. Each of these categories provided a multiplying factor for calculation of alcohol intake per week. Information on average intake during each drinking session was captured using common serving measurements and this was translated into units of alcohol based on the volume consumed and the alcohol by volume for each of the types of alcoholic beverages. Units of alcohol consumed in a week in the form of beer, wine and spirit was calculated separately and summed up to give an estimate of alcohol intake per week for each patient. Significant alcohol intake was defined as more than 21 units per week for men and more than 14 units per week for women (Chalasani et al., 2012).

The Global Physical Activity Questionnaire (GPAQ) was used to measure physical activity (World Health Organization, 2005). The GPAQ categorizes level of physical activity into low, moderate and high according to reported frequency and duration of physical activity in three domains: work, travel and leisure. A semi-quantitative food-frequency questionnaire (FFQ) comprising 200 common Malaysian food items was used to assess dietary intake (Mohd Razif Shahril et al., 2008). This FFQ had similar estimates of dietary intakes when compared to three days 24-hour dietary recall and is an adequate tool for estimation of dietary intakes for epidemiological studies in Malaysia. A copy of the GPAQ and FFQ is enclosed in the Appendix. The dietary composition of each food items was based on a standard reference (Tee et al., 1997). Daily calorie intake, intake of carbohydrate, protein and fat, and percentage calorie intake from carbohydrate, protein and fat were estimated. Some patients who participated in the study did not wish to complete the FFQ as the process required substantial time.

Weight and height were measured using standardized equipment. Body mass index (BMI) was calculated by dividing weight in kilogram by the square of height in meters. Patients were categorized as underweight (BMI < 18.5 kg per m^2), normal (18.5 kg per $m^2 \leq$

BMI < 23.0 kg per m^2), overweight (23.0 kg per m^2 ≤ BMI < 25.0 kg per m^2) or obese (BMI ≥ 25.0 kg per m^2) (Anuurad et al., 2003). Waist circumference (WC) was measured at the mid-point between the lowest margin of the least palpable rib and the top of the iliac crest in the standing position. Central obesity was defined as WC > 90 cm for men and > 80 cm for women (Alberti et al., 2005). Blood pressure was measured in the sitting position using standardized equipment. A patient was considered hypertensive if there was a self-reported history of hypertension, if the patient was on anti-hypertensive medication(s), if the systolic blood pressure (SBP) was ≥ 130 mmHg, or if the diastolic blood pressure (DBP) was ≥ 85 mmHg.

All patients had venous blood drawn after an overnight fast for blood sugar, glycated hemoglobin (HbA1c), lipid profile and liver function test as routine before their appointment at the Diabetic Clinic. Biochemical measurements were performed using standard laboratory procedures. HbA1c ≥ 7.0 % was considered to be reflective of overall suboptimal blood sugar control. A patient was considered to have dyslipidemia if there was a self-reported history of dyslipidemia, if the patient was on lipid-lowering medication(s), if the serum total cholesterol (TC) was ≥ 5.2 mmol/L, if the serum triglyceride (TG) was ≥ 1.7 mmol/L, if the serum high-density lipoprotein (HDL) was < 1.0 mmol/L for men or < 1.3 mmol/L for women, or if the serum low-density lipoprotein (LDL) was ≥ 3.4 mmol/L. A patient was considered to have metabolic syndrome if two or more of the following were present: central obesity, hypertension, hypertriglyceridemia and low serum HDL (according to the aforementioned cut-offs) (Alberti et al., 2009). Our laboratory's upper limit of normal for liver enzymes were as follow: alkaline phosphatase (ALP) 136 IU/L, aspartate aminotransferase (AST) 37 IU/L, alanine aminotransferase (ALT) 65 IU/L and gamma-glutamyl transpeptidase (GGT) 55 IU/L. Serum ALP, AST, ALT and GGT above these levels were considered as elevated. In addition, a more stringent cut-off of 30 IU/L for men

and 19 IU/L for women was used for serum ALT level during data analysis. Additional venous blood was drawn on the day of study for viral hepatitis B and C serology. The Elecsys HBsAg II assay and the Elecsys Anti-HCV II assay (Roche, Mannheim, Germany) were used to test for viral hepatitis B and C infection, respectively.

Diagnosis of NAFLD was by trans-abdominal ultrasonography and following exclusion of significant alcohol intake, use of medications known to cause fatty liver and other causes of chronic liver disease. The following criteria were used for ultrasonographic diagnosis of fatty liver: increased echogenicity, posterior attenuation and loss of intra-hepatic architectural details (Joy et al., 2003). Investigators involved in other parts of the study were blinded to the ultrasonography findings, vice versa.

Statistical analysis

With an estimated prevalence of 65 % based on the average of previous studies (Leite et al., 2009; Merat et al., 2009; Targher et al., 2006), a sample size of 350 patients was needed to estimate the prevalence with 95 % confidence and 5 % precision. Data were analyzed using SPSS 15.0. Continuous variables were expressed as mean ± standard deviation or median (inter-quartile range), and analyzed using student's t-test or Mann-Whitney U test where appropriate. Categorical variables were expressed as percentage and analyzed using chi-square test or Fisher's exact test where appropriate. Independent factors associated with NAFLD were identified using multiple logistic regression analysis. Significance was assumed at $p < 0.05$.

4.3 Results

Patient characteristics

Three hundred and ninety nine patients were included in the analysis (**Figure 4.1**). The mean age of the study population was 62.3 ± 10.5 years old comprising of 43.1 % men. The racial distribution was as follows: Chinese 43.6 %, Indian 33.1 %, Malay 22.3 %, others 1.0 %. Most patients (82.7 %) had at least lower secondary education with a median income of RM 1050 (RM 667 – RM 2000) per household person per month. Mean duration since diagnosis of diabetes mellitus was 16.1 ± 9.7 years. Most patients had co-existing hypertension (91.2 %) and dyslipidemia (97.2 %) while around two thirds (66.4 %) were obese. Central obesity was seen in 73.2 % of the study population and was more prevalent among women than men (79.3 % vs. 65.1 %, p = 0.002). Most patients (95.2 %) had the metabolic syndrome.

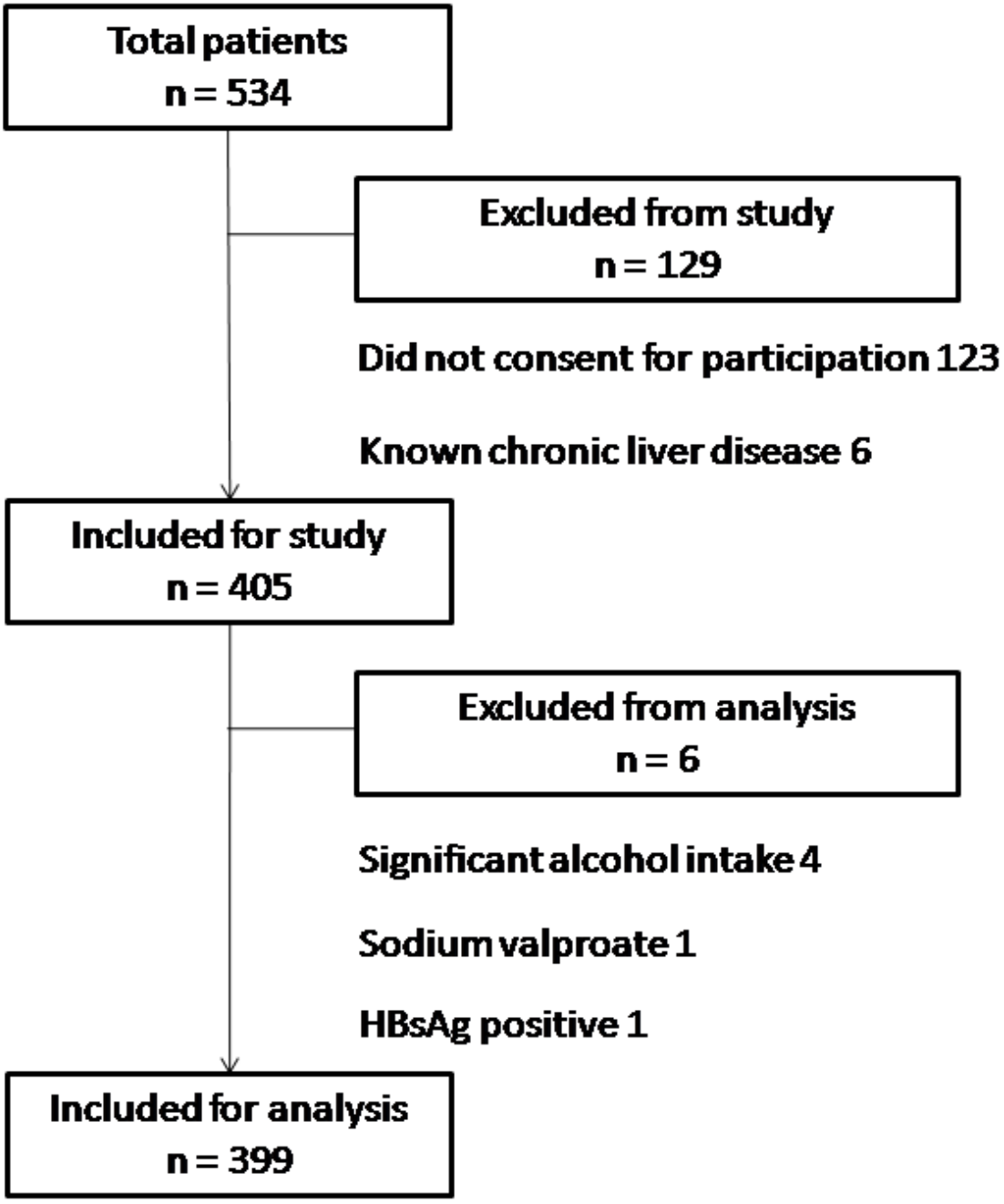

Figure 4.1 Flow chart illustrating the details of patients included/excluded in the analysis

Prevalence of NAFLD and associated factors

The prevalence of NAFLD was 49.6% (198/399). Characteristics of patients with and without NAFLD are summarized in **Table 4.1**. Patients with NAFLD were "younger" with higher BMI and WC, higher DBP, and higher serum TG, HbA1c, AST, ALT and GGT levels compared to patients without NAFLD. The proportion of patients with NAFLD declined with increasing age (**Figure 4.2**). Overall, there was no significant difference in the prevalence of NAFLD between genders. However, there was a trend towards lower prevalence of NAFLD in women compared to men below the age of 50 years old (**Figure 4.3**). This was despite a significantly higher prevalence of central obesity among women compared to men in this age group (92.9 % vs. 42.9 %, $p < 0.001$).

Patients with low level of physical activity were more likely to have NAFLD compared to patients with moderate level of physical activity (OR = 1.67, 95 % CI = 1.06 – 2.63, $p = 0.020$). Daily calorie intake and intake of carbohydrate, protein and fat were not significantly different between patients with and without NAFLD. NAFLD was not associated with duration of diabetes mellitus but was associated with suboptimal control of blood sugar level as reflected by HbA1c level $\geq$ 7.0 %.

Patients with NAFLD had a significantly higher mean serum ALT level (37 ± 18 vs. 28 ± 11, $p < 0.001$). However, only 3.8 % of the study population had elevated serum ALT level using the 65 IU/L cut-off. When the more stringent cut-off of 30 IU/L for men and 19 IU/L for women was used, 71.5 % of the study population had elevated serum ALT level. Patients with elevated serum ALT level using this more stringent cut-off were more likely to have NAFLD (OR = 2.34, 95 % CI = 1.45 – 3.79, $p < 0.001$). However, the sensitivity and specificity of serum ALT level using this more stringent cut-off for the prediction of NAFLD was only 80.0 % and 36.9 %, respectively.

Univariate and multivariate analyses of factors associated with NAFLD

On univariate analysis, factors that were significantly associated with NAFLD were as follows: age < 65 years old, race, obesity, central obesity, HbA1c ≥ 7.0 %, elevated ALT using the more stringent cut-off and elevated GGT. These factors were included in multiple logistic regression analysis. As central obesity is closely related to obesity, a second model of multiple logistic regression analysis was performed without the inclusion of obesity. Independent factors associated with NAFLD were central obesity and elevated ALT in both models. As expected, the effect of central obesity was attenuated in the model that included obesity (**Table 4.2**).

Prevalence of NAFLD according to the different ethnic groups and its association with obesity, central obesity, physical activity and dietary intake

The prevalence of NAFLD was highest among the Malays followed by the Indians and lowest among the Chinese. This paralleled the prevalence of obesity and central obesity in the different ethnic groups. There was no significant difference in daily calorie intake and level of physical activity between the different ethnic groups. However, some differences were seen in the calorie source between the different ethnic groups. The Chinese had significantly higher protein intake compared to the Malays and Indians. Percentage calorie intake from fat was highest among the Malays followed by the Indians and lowest among the Chinese (**Table 4.3**).

Table 4.1 Characteristics of patients with and without NAFLD

	NAFLD		p
	Yes n =198	No n = 201	
Age, years	60.7 ± 11.2	64.8 ± 9.3	< 0.001
Age < 65 years old	63.1 %	49.8 %	0.007
Male	44.9 %	41.3 %	0.461
Race			
Malay	27.3 %	17.4 %	0.022
Chinese	36.9 %	50.3 %	
Indian	34.3 %	31.8 %	
Others	1.5 %	0.5 %	
Education level			
None	5.6 %	10.4 %	0.271
Primary	8.6 %	10.0 %	
Secondary	54.5 %	52.7 %	
Tertiary	31.3 %	26.9 %	
Income per household person per month, RM	1000 (667 – 2000)	1067 (645 – 2000)	0.984
Smoking	5.6 %	2.0 %	0.073
Duration of diabetes mellitus, years	15.5 ± 9.4	16.9 ± 9.9	0.146
Hypertension	89.9 %	92.5 %	0.352
Dyslipidemia	97.0 %	97.5 %	0.741
Body mass index, kg per m^2	29.7 ± 7.9	26.3 ± 5.5	< 0.001
Obesity	75.8 %	57.2 %	< 0.001
Waist circumference, cm	96.0 ± 11.7	89.1 ± 12.6	< 0.001
Central obesity	82.8 %	63.7 %	< 0.001
Systolic blood pressure (SBP), mmHg	135 ± 19	134 ± 21	0.529
SBP ≥ 130 mmHg	67.2 %	62.7 %	0.348
Diastolic blood pressure (DBP), mmHg	75 ± 10	73 ± 11	0.019
DBP ≥ 85 mmHg	14.1 %	14.4 %	0.935

	NAFLD		p
	Yes n =198	No n = 201	
Metformin	81.8 %	77.1 %	0.245
Sulphonylurea	43.9 %	39.8 %	0.402
Insulin	46.5 %	44.3 %	0.661
Statin	85.4 %	85.1 %	0.937
Fibrate	13.1 %	17.4 %	0.235
Fasting blood sugar, mmol/L	8.2 ± 3.0	8.0 ± 3.5	0.530
HbA1c, %	8.31 ± 1.74	7.83 ± 1.79	0.007
HbA1c ≥ 7.0 %	83.0 %	69.0 %	0.001
Total cholesterol (TC), mmol/L	4.25 ± 1.06	4.25 ± 0.94	0.972
TC ≥ 5.2 mmol/L	14.4 %	17.1 %	0.471
High-density lipoprotein (HDL), mmol/L	1.20 ± 0.37	1.28 ± 0.41	0.428
HDL < 1.0 for men and < 1.29 for women	44.3 %	38.7 %	0.257
Low-density lipoprotein (LDL), mmol/L	2.12 (1.74 – 2.60)	2.15 (1.77 – 2.76)	0.506
LDL ≥ 3.4 mmol/L	10.4 %	7.6 %	0.326
Triglyceride (TG), mmol/L	1.50 (1.20 – 2.13)	1.30 (1.00 – 1.80)	0.001
TG ≥ 1.7 mmol/L	37.6 %	33.7 %	0.412
Alkaline phosphatase (ALP), IU/L	68 ± 26	65 ± 22	0.154
ALP ≥ 136 IU/L	2.1 %	0.5 %	0.372
Aspartate aminotransferase (AST), IU/L	23 ± 12	19 ± 9	0.002
AST ≥ 37 IU/L	7.7 %	5.2 %	0.307
Alanine aminotransferase (ALT), IU/L	37 ± 18	28 (11)	< 0.001
Elevated ALT ≥ 65 IU/L	5.1 %	2.6 %	0.188
Elevated ALT ≥ 30 IU/L for men and ≥ 19 IU/L for women	80.0 %	63.1 %	< 0.001
Gamma glutamyl transpeptidase (GGT), IU/L	36 (25 – 57)	25 (17 – 43)	< 0.001
GGT ≥ 55 IU/L	27.3 %	14.4 %	0.002

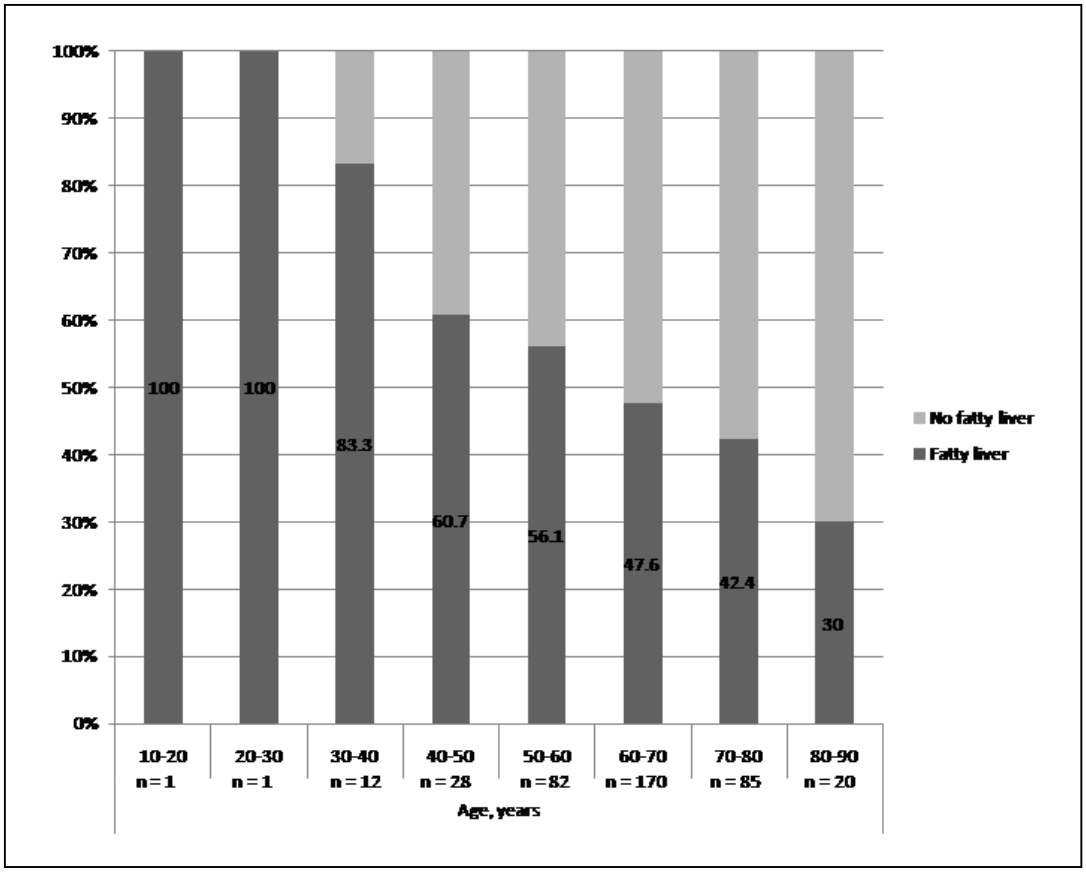

Figure 4.2 Prevalence of NAFLD according to age group

There was a significant decrease in the proportion of patients with NAFLD with increasing age group ($p = 0.031$).

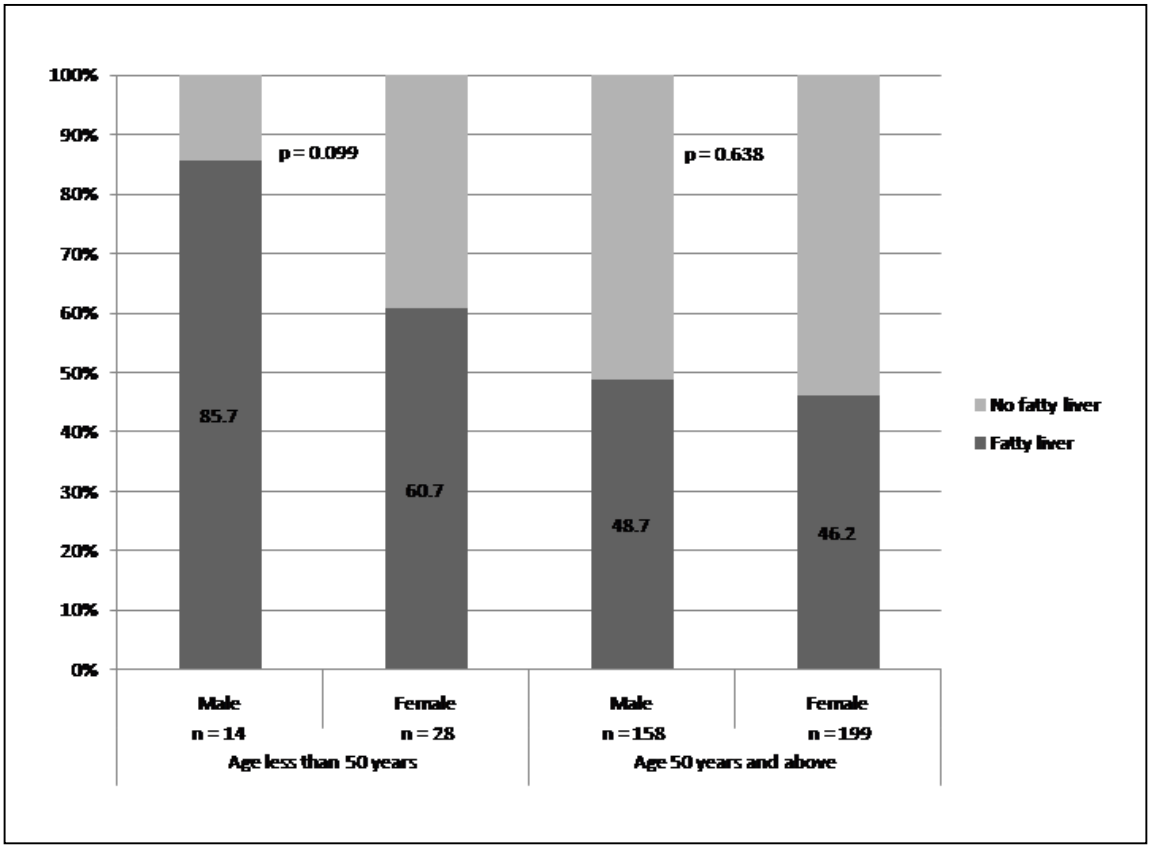

Figure 4.3 Prevalence of NAFLD according to age and gender

There was a trend towards lower prevalence of NAFLD in women compared to men among patients below the age of 50 years old but no such difference was observed among patients 50 years old and above.

Table 4.2 Univariate and multivariate analyses of factors associated with NAFLD

	Univariate analysis			Multivariate analysis Model 1			Multivariate analysis Model 2		
	OR	95 % CI	p	OR	95 % CI	p	OR	95 % CI	p
Age < 65 years	1.73	1.14 – 2.63	0.007	1.48	0.95 – 2.29	0.084	1.52	0.98 – 2.35	0.061
Race									
Chinese	1	–	–	1	–	–	1	–	–
Indian	1.47	0.91 – 2.38	0.097	1.36	0.82 – 2.27	0.240	1.39	0.84 – 2.31	0.205
Malay	2.13	1.23 – 3.72	0.004	1.49	0.83 – 2.68	0.187	1.55	0.87 – 2.78	0.140
Obesity	2.34	1.49 – 3.67	0.000	1.38	0.79 – 2.40	0.262	–	–	–
Central obesity	2.75	1.68 – 4.52	0.000	1.86	1.01 – 3.42	0.045	2.20	1.29 – 3.75	0.004
HbA1c ≥ 7.0 %	2.19	1.32 – 3.64	0.001	1.55	0.91 – 2.62	0.104	1.59	0.94 – 2.68	0.083
Elevated ALT	2.34	1.45 – 3.79	0.000	2.04	1.24 – 3.36	0.005	1.98	1.21 – 3.25	0.007
Elevated GGT	2.23	1.30 – 3.83	0.002	1.69	0.97 – 2.95	0.063	1.73	0.99 – 3.00	0.053

All categorical variables which were significant on univariate analysis were included in multivariate analysis in Model 1.

In Model 2, only central obesity (and not obesity) was included in the multivariate analysis.

ALT = alanine aminotransferase, GGT = gamma glutamyl transpeptidase

Table 4.3 NAFLD prevalence according to different ethnic groups and association with central obesity, physical activity and dietary intake

	Chinese	Malay		Indian	
			p*		p*
NAFLD	42.0 %	60.7 %	0.004	51.5 %	0.097
Central obesity	60.3 %	84.3 %	0.000	82.6 %	0.000
Low physical activity	51.5 %	59.6 %	0.196	53.8 %	0.647
†Calorie intake, kcal/kg/day	19.8 (14.7 – 25.7)	17.0 (13.1 – 24.0)	0.069	18.1 (13.3 – 24.7)	0.231
†Carbohydrate intake, g/kg/day	2.65 (1.97 – 3.33)	2.26 (1.68 – 3.06)	0.064	2.68 (1.80 – 3.44)	0.831
†Protein intake, g/kg/day	0.84 (0.63 – 1.18)	0.76 (0.51 – 0.98)	0.034	0.68 (0.52 – 0.92)	0.003
†Fat intake, g/kg/day	0.56 (0.39 – 0.84)	0.56 (0.37 – 0.81)	0.758	0.57 (0.38 – 0.72)	0.928
†Calorie source, %					
Carbohydrate	54.1 ± 9.7	54.0 ± 9.4	0.955	56.1 ± 9.5	0.110
Protein	17.1 ± 3.5	17.2 ± 2.9	0.372	15.8 ± 3.1	0.000
Fat	26.4 ± 6.7	28.9 ± 7.7	0.018	28.5 ± 7.6	0.072

*p-values were from comparing Malay vs. Chinese, and Indian vs. Chinese, respectively

†For dietary data, n = 140, 61 and 97 for Chinese, Malays and Indians, respectively

4.4 Discussion

Half of the study population has NAFLD. While the prevalence is lower than the reported 55 % – 70 % in previous studies from other parts of the world (Leite et al., 2009; Merat et al., 2009; Targher et al., 2006), NAFLD is poised to be a significant cause of chronic liver disease in Malaysia given the huge and increasing burden of diabetes mellitus in the country. NAFLD should not to be taken lightly as it is not an entirely benign condition (Ekstedt et al., 2006). NAFLD has been recognized as an important cause of cryptogenic cirrhosis (Maheshwari et al., 2006) and is associated with increased risk of hepatocellular carcinoma, even in patients without cirrhosis (Page et al., 2009). In a study on etiology of cirrhosis and association with hepatocellular carcinoma in our center, cryptogenic cause, which is believed to be due to NAFLD contributed to 15.4 % of cases of cirrhosis and was an independent predictor of hepatocellular carcinoma (Qua et al., 2011). NAFLD is also associated with cardiovascular diseases (Bhatia et al., 2012). The implications of NAFLD will be discussed further in Chapter 6 and Chapter 7.

As elucidated in Chapter 3, population-based studies have shown that the prevalence of NAFLD increases with increasing age. Conversely and interestingly, we found that NAFLD is seen less commonly with increasing age in this study population. We hypothesize that NAFLD may be associated with increased co-morbidities in aging diabetic patients and this could have limited their survival and attendance to the clinic and hence their lower representation in the study population. The prevalence of NAFLD was higher among men compared to women below the age of 50 years old despite a significantly higher prevalence of central obesity among women compared to men in this age group. The protective effect

against NAFLD in women of reproductive age has been reported in population-based studies, and seems to apply to diabetic patients as well based on findings from this study.

A recently published study on multiracial health-check subjects from Malaysia reported an inordinately high prevalence of NAFLD among the Malays and Indians compared to the Chinese. Similarly, in our study on diabetic patients, we found that the prevalence of NAFLD was higher among the Malays and Indians compared to the Chinese. The Chinese were significantly less obese (both overall and centrally) compared to their Malay and Indian counterparts. We did not find any differences in the daily calorie intake and the level of physical activity between the different ethnic groups. Obesity is the result of energy intake in excess of expenditure over time and the contribution of differences in dietary intake and level of physical activity to obesity and NAFLD is difficult to demonstrate in cross sectional studies. Nevertheless, we did show that the Malays and Indians had higher percentage of calorie intake from fat compared to the Chinese. Although there is an overlap in the food consumed by the different ethnic groups in Malaysia, there are some distinct differences in the regular choice of food. The Malays and Indians generally consume more curry and deep-fried food while the Chinese often consume stir-fried, steamed or soup-based food. A more detailed analysis of dietary intake and physical activity of diabetic patients with and without NAFLD is presented in the next chapter. Genetic differences between the ethnic groups may also play a role. A study on a multi-ethnic Malaysian population found that the PNPLA3 gene polymorphism is associated with susceptibility to NASH, NASH severity and presence of fibrosis, and that the effect of the gene polymorphism appears to be greater in the Indians followed by the Malays and the Chinese (Zain et al., 2012).

In view of the high prevalence of NAFLD among diabetic patients, it would seem appropriate to look for the condition in diabetic patients. There are currently differing views

on this in the American and European guidelines (Chalasani et al., 2012; Ratziu et al., 2010). Our study echoes previously published studies that serum ALT level is not useful to identify NAFLD patients (see Chapters 2 and 3). Ultrasonography is a simple, non-invasive and relatively inexpensive test for the diagnosis of NAFLD and should be considered in all diabetic patients. The real challenge is to identify diabetic patients with NAFLD who has non-alcoholic steatohepatitis (NASH) or advanced fibrosis. Liver biopsy is invasive and does not seem to be the appropriate test for all diabetic patients with NAFLD. Moreover, it is limited by sampling variability (Ratziu et al., 2005) and inter-observer variability (Younossi et al., 1998). Measurement of cytokeratin-18 fragment levels in the blood has been shown to predict histological NASH (Feldstein et al., 2009). The NAFLD fibrosis score utilizes readily available parameters to identify NAFLD patients with and without advanced fibrosis and limits the need for liver biopsy to only those with indeterminate scores (Angulo et al., 2007). Measurement of liver stiffness using transient elastography has been shown to have high negative predictive value for advanced fibrosis (Wong et al., 2010). All these methods are promising and deserve further studies for use in diabetic patients, not only for initial assessment but also for follow-up purpose. These non-invasive tests are further discussed in Chapter 8.

Despite our effort, this study has several limitations. First, the diagnosis of fatty liver was based on ultrasonography and not histopathological examination of liver biopsy specimen. While the latter is more accurate to diagnose fatty liver, a liver biopsy is invasive and is not feasible in a study of this nature. As discussed in Chapter 2, ultrasonography is by far the most common method to diagnose fatty liver in clinical practice and in epidemiological studies. It has good sensitivity and specificity in moderate and severe fatty liver. However, it lacks sensitivity when fatty liver is mild. Hence, the true prevalence of

NAFLD could have been underestimated as cases of mild fatty liver could have been missed on ultrasonography. Second, measurement of dietary intake (including alcohol intake) and physical activity were self-reported. Strict and continuous measurement of dietary intake and physical activity over time would not be practical in cross-sectional studies involving fairly large number of patients like this and has its own inherent way of causing bias. We did use previously validated questionnaires to capture information on dietary intake and physical activity. To the best of our knowledge, this is the only study from the Asian-Pacific on prevalence of NAFLD among diabetic patients and associated factors that provided information on different ethnic groups and included data on dietary intake and physical activity.

4.5 Conclusion

NAFLD was seen in half of a cohort of diabetic patients and was independently associated with central obesity and elevated serum ALT level. The prevalence of NAFLD was higher among the Malays and Indians compared to the Chinese consistent with higher prevalence of central obesity and higher percentage calorie intake from fat in the former groups of patients.

Note: The findings from this study was presented at the Malaysian Society of Gastroenterology and Hepatology Annual Scientific Meeting in 2012 and won the Best Paper Award. A poster on the findings from this study was also presented at the Asia-Pacific Digestive Week 2012 in Bangkok, Thailand, and the abstract was published in a supplementary issue of the Journal of Gastroenterology and Hepatology (Chan et al., 2012). The full article has been published in the Journal of Gastroenterology and Hepatology (Chan et al., 2013).

Chapter 5

Role of diet and physical activity in NAFLD in diabetics

5.1 Introduction

Diet and physical activity are important factors in non-alcoholic fatty liver disease (NAFLD). A case-control study identified increased fructose consumption as a risk factor for NAFLD (Ouyang et al., 2008). This is consistent with findings from a population-based cross-sectional study that found increased intake of soft drink (which contains fructose) as an independent predictor for NAFLD (Zelber-Sagi et al., 2007). Various other experimental and clinical studies on the role of fructose in the pathogenesis of metabolic syndrome and NAFLD have been reviewed by Yilmaz and colleagues (Yilmaz, 2012).

Higher percentage calorie intake from fat has also been found to be an independent factor for NAFLD in a case-control study (Sathiaraj et al., 2011) and in a randomized study (Westerbacka et al., 2005). Higher carbohydrate intake (Solga et al., 2004), particularly simple carbohydrates (Toshimitsu et al., 2007), and higher fat intake, particularly excessive amount of n-6 polyunsaturated fatty acids (PUFAs) (Cortez-Pinto et al., 2006) have been implicated in non-alcoholic steatohepatitis (NASH), the more severe form of NAFLD.

The importance of diet and physical activity in NAFLD is also reflected by the many interventional trials that have looked at energy restrictions, with and without increased physical activity to reduce liver fat (Cortez-Pinto et al., 2006). We aimed to look specifically at the role of diet and physical activity in NAFLD in diabetics as there were no published data on this.

5.2 Patients and methods

We performed further detailed analysis on diet and physical activity for patients from the study presented in Chapter 4. Only patients who completed the food-frequency questionnaire (FFQ) in that study were included in this analysis.

Food items were further categorized as high sugar, high cholesterol and/or high saturated fatty acid (SFA) where applicable (**Table 5.1**). Percentage calorie intake from high sugar food, high cholesterol food and high SFA food were estimated for each patient. Percentage calorie intake from macronutrients (i.e. carbohydrate, protein and fat), and percentage calorie intake from high sugar food, high cholesterol food and high SFA food were stratified into quartiles. Prevalence of NAFLD was compared across quartiles and between the highest quartile and lower quartiles for each of the variables. Percentage calorie intake from macronutrients, and percentage calorie intake from high sugar food, high cholesterol food and high SFA food were analyzed individually with level of physical activity to look for any association with prevalence of NAFLD. Further analysis was performed for patients who were and were not centrally obese.

Statistical analysis

Data were analyzed using SPSS 15.0. Continuous variables were expressed as mean ± standard deviation or median (inter-quartile range), and analyzed using student's t-test or Mann-Whitney U test where appropriate. Categorical variables were expressed as percentage and analyzed using chi-square test or Fisher's exact test where appropriate. Significance was assumed at $p < 0.05$.

Table 5.1 List of high sugar food, high cholesterol food and high SFA food

High sugar food Sweetened condensed milk, sugar, kaya (coconut jam), fruit jam, chocolate cereal, syrup drink, bandung (syrup-flavored milk), lemonade, iced lemon tea, carbonated drink, doughnut, various types of sweet kuih (bite-sized snack), various types of cake, muffin, chocolate, ice-cream, chocolate biscuits, sweets.
High cholesterol food Full cream milk powder, cheese, sambal (chili-based sauce), deep-fried beef lung, deep-fried chicken liver, other offal, sambal ikan bilis (chili-based sauce with anchovies), squid, prawn, crab, fried egg, boiled egg, salted egg, full cream yogurt, ice-cream.
High SFA food Roti canai (flatbread), roti telur (flatbread with egg), sambal, nasi lemak (rice cooked in coconut milk), fried rice, nasi minyak (ghee rice), biryani rice, chicken rice, fried chicken, curry with coconut milk, kuah masak lemak (creamy coconut sauce), kurma sauce, kuah rendang (a spicy sauce made from coconut milk and mixture of ground spices), deep-fried fish, deep-fried meat, deep-fried beef lung, deep-fried anchovies, sambal ikan bilis, chicken burger, beef burger, sausage/frankfurter, nuggets, French fries, cekodok pisang (bite-sized snack made from banana), banana fritter, shrimp fritter, curry puff, fried spring roll, mung bean fritter, vadai kacang dhal (fritter-type snack made from pulses), various types of cakes, chocolate, ice-cream, chocolate biscuits.

5.3 Results

Patient characteristics

Data for 299 patients were analyzed (**Figure 5.1**). Mean age of the study population was 63.3 ± 10.5 years old with 41.1 % male. Majority (81.9 %) completed at least lower secondary education with median income of RM 1000 (RM 667 – RM 2000) per household person per month. Mean duration since diagnosis of diabetes mellitus was 16.6 ± 9.7 years. Most patients had hypertension (90.3 %) and dyslipidemia (97.7 %). Central obesity was seen in 71.9 %. Majority (95.7 %) had metabolic syndrome. The prevalence of NAFLD was 49.2 %.

The characteristics of patients with and without NAFLD are shown in **Table 5.1**. Similar to the entire cohort of 399 patients presented in Chapter 4, independent factors associated with NAFLD were central obesity and raised serum ALT level (data not shown). Central obesity was associated with NAFLD on multivariate analysis while obesity was not. Hence, central obesity instead of obesity was used during further analysis on dietary intake and physical activity.

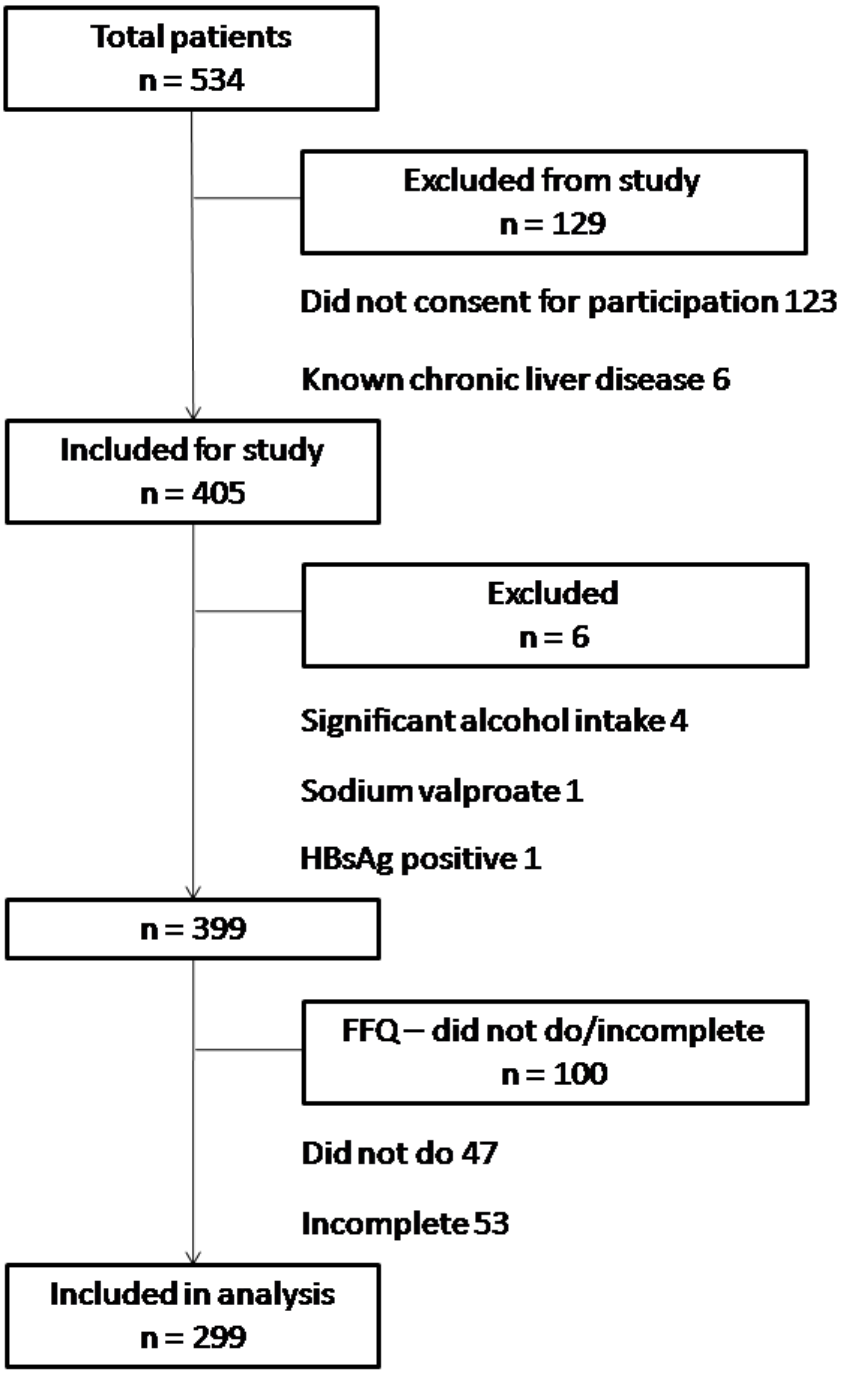

Figure 5.1 Flow chart illustrating the details of patients included/excluded in the analysis

Table 5.2 Characteristics of patients with and without NAFLD

	NAFLD		p
	Yes n = 147	No n = 152	
Age, years	61.5 ± 11.4	65.0 ± 9.3	0.004
Male	42.2 %	40.1 %	0.719
Race			
Malay	25.9 %	15.1 %	0.082
Chinese	42.2 %	51.3 %	
Indian	31.3 %	33.6 %	
Others	0.7 %	0 %	
Education level			
None or primary	17.0 %	19.1 %	0.641
Secondary and above	83.0 %	80.9 %	
Income per household person, RM	1000 (667 – 2000)	1000 (635 – 1969)	0.880
Duration of diabetes mellitus, years	16.1 ± 9.8	16.9 ± 9.6	0.540
Hypertension	88.4 %	92.1 %	0.284
Dyslipidemia	98.0 %	97.4 %	1.000
Body mass index, kg per m^2	28.8 ± 6.3	26.0 ± 5.1	< 0.001
Obesity	72.1 %	54.6 %	0.002
Waist circumference, cm	94.7 ± 11.5	88.8 ± 12.8	< 0.001
Central obesity	81.0 %	63.2 %	0.001
Metabolic syndrome	95.9 %	95.4 %	0.824
Metformin	81.0 %	75.0 %	0.215
Sulphonylurea	46.9 %	44.1 %	0.620
Insulin	45.6 %	44.7 %	0.884
Statin	85.0 %	84.9 %	0.968
Fibrate	10.2 %	17.8 %	0.060

	NAFLD		p
	Yes n = 147	No n = 152	
Fasting blood sugar, mmol/L	7.5 (6.2 – 9.6)	7.3 (5.9 – 9.0)	0.387
HbA1c, %	7.90 (7.03 – 9.08)	7.60 (6.80 – 8.50)	0.018
Total cholesterol, mmol/L	4.2 ± 1.0	4.2 ± 1.0	0.966
High-density lipoprotein, mmol/L	1.2 ± 0.4	1.3 ± 0.4	0.274
Low-density lipoprotein, mmol/L	2.2 ± 0.8	2.3 ± 0.8	0.802
Triglyceride, mmol/L	1.5 (1.2 – 1.9)	1.3 (1.0 – 1.8)	0.011
Alkaline phosphatase, IU/L	65 (54 – 81)	61 (50 – 77)	0.134
Alanine aminotransferase, IU/L	33 (23 – 45)	25 (21 – 34)	< 0.001
Aspartate aminotransferase, IU/L	20 (16 – 28)	18 (15 – 23)	0.010
Gamma glutamyl transpeptidase, IU/L	36 (26 – 58)	26 (18 – 43)	< 0.001

Detailed analysis of level of physical activity, dietary intake and NAFLD

More than half (53.8 %) of patients had low level of physical activity. Percentage of patients with moderate and high level of physical activity was 34.1 % and 12.0 %, respectively. Patients with low level of physical activity were more likely to have NAFLD compared to patients with moderate level of physical activity (OR = 1.75, 95 % CI = 1.03 – 2.99, p = 0.029). There was no significant difference in calorie intake, intake of macronutrient, percentage calorie intake from each macronutrient, and percentage calorie intake from high sugar food, high cholesterol food and high SFA food between patients with and without NAFLD (**Table 5.3**).

When percentage calorie intake from each macronutrient and percentage calorie intake from high sugar food, high cholesterol food and high SFA food was stratified according to quartiles, no significant difference in prevalence of NAFLD was seen across quartiles and between the highest and lower quartiles (**Table 5.4**). The findings were similar when analysis was performed separately for patients who were and were not centrally obese (data not shown).

Table 5.3 Level of physical activity, calorie intake, intake of macronutrients, percentage calorie intake from each macronutrient, and percentage calorie from high sugar food, high cholesterol food and high SFA food in patients with and without NAFLD

	NAFLD	No NAFLD	OR	95 % CI	p
Level of physical activity					
Low	59.2 %	48.7 %	1.75	1.03 – 2.99	0.029
Moderate	27.9 %	40.1 %	1	–	–
High	12.9 %	11.2 %	1.66	0.72 – 3.83	0.190
Calorie intake, kcal/day	1272 (946 – 1600)	1242 (1001 – 1631)	–	–	0.775
Carbohydrate intake, g/day	170.6 (126.7 – 223.9)	165.4 (135.8 – 226.1)	–	–	0.957
Protein intake, g/day	52.0 (39.7 – 70.5)	53.1 (39.9 – 70.5)	–	–	0.883
Fat intake, g/day	37.4 (27.0 – 51.7)	37.4 (27.6 – 54.4)	–	–	0.492
Calorie source, %					
Carbohydrate	55.0 ± 9.6	54.6 ± 9.4	–	–	0.722
Protein	17.1 ± 3.7	16.8 ± 3.0	–	–	0.481
Fat	27.2 ± 6.8	27.5 ± 7.2	–	–	0.690
Percentage calorie from high sugar food, %	2.5 (0.3 – 7.0)	2.8 (0.5 – 5.6)	–	–	0.951
Percentage calorie from high cholesterol food, %	3.0 (0.9 – 7.9)	2.4 (0.8 – 5.9)	–	–	0.317
Percentage calorie from high SFA food, %	12.7 (5.7 -23.5)	11.7 (4.9 – 18.4)	–	–	0.154

Table 5.4 Prevalence of NAFLD across quartiles and between highest and lower quartiles of percentage calorie intake from each macronutrient, and percentage calorie intake from high sugar food, high cholesterol food and high SFA food

	NAFLD	No NAFLD	OR	95 % CI	p
Percentage calorie from carbohydrate					
< 48.2 %	26.5 %	23.0 %	1	–	–
48.2 % – 55.3 %	24.5 %	26.3 %	0.81	0.40 – 1.61	0.514
55.3 % – 61.0 %	23.1 %	27.0 %	0.74	0.37 – 1.49	0.368
≥ 61.0 %	25.9 %	23.7 %	0.95	0.47 – 1.90	0.869
< 61.0 %	74.1 %	76.3 %	1	–	–
≥ 61.0 %	25.9 %	23.7 %	1.12	0.64 – 1.96	0.664
Percentage calorie from protein					
< 14.6 %	25.2 %	23.7 %	1	–	–
14.6 % – 16.9 %	23.1 %	27.0 %	0.81	0.40 – 1.62	0.515
16.9 % – 19.1 %	26.5 %	25.7 %	0.97	0.49 – 1.94	0.933
≥ 19.1 %	25.2 %	23.7 %	1.00	0.50 – 2.02	1.000
< 19.1 %	74.8 %	76.3 %	1	–	–
≥ 19.1 %	25.2 %	23.7 %	1.08	0.62 – 1.90	0.765
Percentage calorie from fat					
< 22.5 %	25.2 %	25.0 %	1	–	–
22.5 % – 27.5 %	25.2 %	23.7 %	1.06	0.53 – 2.12	0.869
27.5 % – 31.5 %	24.5 %	26.3 %	0.92	0.46 – 1.84	0.809
≥ 31.5 %	25.2 %	25.0 %	1	0.50 – 2.00	1.000
< 31.5 %	74.8 %	75.0 %	1	–	–
≥ 31.5 %	25.2 %	25.0 %	1.01	0.58 – 1.76	0.973

	NAFLD	No NAFLD	OR	95 % CI	p
Percentage calorie from high sugar food					
< 0.44 %	26.5 %	23.7 %	1	–	–
0.44 % – 2.68 %	25.2 %	24.3 %	0.92	0.46 – 1.85	0.807
2.68 % – 6.22 %	22.4 %	33.6 %	0.60	0.30 – 1.18	0.108
≥ 6.22%	25.9 %	18.4 %	1.25	0.61 – 2.58	0.507
< 6.22 %	74.1 %	81.6 %	1	–	–
≥ 6.22 %	25.9 %	18.4 %	1.54	0.86 – 2.78	0.122
Percentage calorie from high cholesterol food					
< 0.91 %	23.8 %	25.7 %	1	–	–
0.91 % – 2.68 %	23.8 %	26.3 %	0.98	0.49 – 1.95	0.939
2.68 % – 6.74 %	22.4 %	27.6 %	0.88	0.44 – 1.76	0.686
≥ 6.74 %	29.9 %	20.4 %	1.58	0.79 – 3.19	0.164
< 6.74 %	70.1 %	79.6 %	1	–	–
≥ 6.74 %	29.9 %	20.4 %	1.67	0.95 – 2.93	0.058
Percentage calorie from high SFA food					
< 5.27 %	23.1 %	26.3 %	1	–	–
5.27 % – 11.84 %	23.8 %	26.3 %	1.03	0.51 – 2.06	0.930
11.84 % – 20.23 %	23.8 %	26.3 %	1.03	0.51 – 2.06	0.930
≥ 20.23 %	29.3 %	21.1 %	1.58	0.79 – 3.18	0.164
< 20.23 %	70.7 %	78.9 %	1	–	–
≥ 20.23 %	29.3 %	21.1 %	1.55	0.89 – 2.72	0.102

Among centrally obese patients, patients with low level of physical activity and in the highest quartile of percentage calorie intake from fat were the most likely to have NAFLD (OR = 4.03, 95 % CI = 1.12 – 14.99, p = 0.015). Among patients who were not centrally obese, level of physical activity and percentage calorie intake from fat was not associated with NAFLD (**Table 5.5**). Among centrally obese patients, patients with low level of physical activity and in the highest quartile of percentage calorie intake from high cholesterol food (OR = 3.61, 95 % CI = 1.37 – 9.72, p = 0.004) and high SFA food (OR = 2.67, 95 % CI = 1.08 – 6.67, p = 0.019) were most likely to have NAFLD. Among patients who were not centrally obese, level of physical activity and percentage calorie intake from high cholesterol food and high SFA food was again not associated with NAFLD (**Table 5.6**). These findings were not affected when adjusted for age and gender, and other components of metabolic syndrome (data not shown).

Table 5.5 Level of physical activity and percentage calorie intake from each macronutrient in patients with and without central obesity and the prevalence of NAFLD

	NAFLD	No NAFLD	OR	95 % CI	p
a) Carbohydrate					
All patients					
A1	12.9 %	12.5 %	0.78	0.35 – 1.72	0.509
A2	46.9 %	35.5 %	1	–	–
B1	12.9 %	11.2 %	0.87	0.39 – 1.97	0.725
B2	27.2 %	40.8 %	0.50	0.29 – 0.89	0.012
Patients with central obesity					
A1	14.3 %	14.6 %	0.62	0.25 – 1.54	0.254
A2	49.6 %	31.2 %	1	–	–
B1	13.4 %	12.5 %	0.68	0.26 – 1.76	0.379
B2	22.7 %	41.7 %	0.34	0.17 – 0.70	0.001
Patients without central obesity					
A1	7.1 %	8.9 %	0.96	0.11 – 7.28	1.000
A2	35.7 %	42.9 %	1	–	–
B1	10.7 %	8.9 %	1.44	0.22 – 9.13	0.686
B2	46.4 %	39.3 %	1.42	0.46 – 4.39	0.496
b) Protein					
All patients					
A1	15.6 %	10.5 %	1.88	0.84 – 4.26	0.094
A2	44.2 %	37.5 %	1.50	0.85 – 2.62	0.133
B1	9.5 %	13.2 %	0.92	0.39 – 2.16	0.830
B2	30.6 %	38.8 %	1	–	–
Patients with central obesity					
A1	18.5 %	11.5 %	2.44	0.95 – 6.33	0.040
A2	45.4 %	34.4 %	1.99	1.00 – 3.97	0.033
B1	9.2 %	13.5 %	1.03	0.37 – 2.88	0.948

	NAFLD	No NAFLD	OR	95 % CI	p
B2	26.9 %	40.6 %	1	–	–
Patients without central obesity					
A1	3.6 %	8.9 %	0.31	0.01 – 3.37	0.391
A2	39.3 %	42.9 %	0.71	0.23 – 2.14	0.492
B1	10.7 %	12.5 %	0.66	0.11 – 3.66	0.719
B2	46.4 %	35.7 %	1	–	–
c) Fat					
All patients					
A1	18.4 %	11.8 %	3.00	1.03 – 8.88	0.024
A2	41.5 %	36.2 %	2.22	0.89 – 5.61	0.060
B1	6.8 %	13.2 %	1	–	–
B2	33.3 %	38.8 %	1.66	0.66 – 4.23	0.238
Patients with central obesity					
A1	19.3 %	10.4 %	4.03	1.12 – 14.99	0.015
A2	44.5 %	35.4 %	2.73	0.94 – 8.05	0.038
B1	6.7 %	14.6 %	1	–	–
B2	29.4 %	39.6 %	1.61	0.55 – 4.83	0.339
Patients without central obesity					
A1	14.3 %	14.3 %	1.50	0.14 – 17.50	1.000
A2	28.6 %	37.5 %	1.14	0.15 – 10.30	1.000
B1	7.1 %	10.7 %	1	–	–
B2	50.0 %	37.5 %	2.00	0.29 – 16.87	0.688

A1 = low level of physical activity and highest quartile of percentage calorie intake from corresponding macronutrient

A2 = low level of physical activity and lower quartiles of percentage calorie intake from corresponding macronutrient

B1 = moderate or high level of physical activity and highest quartile of percentage calorie intake from corresponding macronutrient

B2 = moderate or high level of physical activity and lower quartiles of percentage calorie intake from corresponding macronutrient

Table 5.6 Level of physical activity and percentage calorie intake from high sugar, high cholesterol and high SFA food in patients with and without central obesity and the prevalence of NAFLD

	NAFLD	No NAFLD	OR	95 % CI	p
a) High sugar food					
All patients					
A1	15.0 %	11.2 %	2.05	0.92 – 4.58	0.055
A2	44.9 %	36.8 %	1.86	1.07 – 3.25	0.019
B1	10.9 %	7.2 %	2.30	0.90 – 5.91	0.053
B2	29.3 %	44.7 %	1	–	–
Patients with central obesity					
A1	16.8 %	9.4 %	3.23	1.19 – 8.89	0.010
A2	47.1 %	36.5 %	2.32	1.19 – 4.55	0.008
B1	10.1 %	7.3 %	2.49	0.79 – 7.99	0.080
B2	26.1 %	46.9 %	1	–	–
Patients without central obesity					
A1	7.1 %	14.3 %	0.48	0.06 – 3.12	0.469
A2	35.7 %	37.5 %	0.91	0.29 – 2.87	0.862
B1	14.3 %	7.1 %	1.92	0.32 – 11.59	0.443
B2	42.9 %	41.1 %	1	–	–
b) High cholesterol food					
All patients					
A1	21.1 %	9.9 %	2.83	1.30 – 6.24	0.004
A2	38.8 %	38.2 %	1.35	0.77 – 2.36	0.269
B1	8.8 %	8.8 %	1.11	0.45 – 2.74	0.799
B2	31.3 %	31.3 %	1	–	–
Patients with central obesity					
A1	21.8 %	9.4 %	3.61	1.37 – 9.72	0.004
A2	42.0 %	36.5 %	1.79	0.90 – 3.54	0.072
B1	9.2 %	12.5 %	1.15	0.40 – 3.24	0.777

	NAFLD	No NAFLD	OR	95 % CI	p
B2	26.9 %	41.7 %	1	–	–
Patients without central obesity					
A1	17.9 %	10.7 %	1.37	0.29 – 6.48	0.732
A2	25.0 %	41.1 %	0.50	0.15 – 1.65	0.203
B1	7.1 %	7.1 %	0.82	0.09 – 6.39	1.000
B2	50.0 %	41.1 %	1	–	–
c) High SFA food					
All patients					
A1	19.7 %	12.5 %	2.24	1.06 – 4.74	0.021
A2	40.1 %	35.5 %	1.60	0.91 – 2.82	0.080
B1	9.5 %	8.6 %	1.58	0.63 – 3.99	0.287
B2	30.6 %	43.4 %	1	–	–
Patients with central obesity					
A1	21.0 %	12.5 %	2.67	1.08 – 6.67	0.019
A2	42.9 %	33.3 %	2.04	1.03 – 4.08	0.028
B1	9.2 %	11.5 %	1.28	0.45 – 3.69	0.611
B2	26.9 %	42.7 %	1	–	–
Patients without central obesity					
A1	14.3 %	12.5 %	1.10	0.22 – 5.37	1.000
A2	28.6 %	39.3 %	0.70	0.21 – 2.25	0.504
B1	10.7 %	3.6 %	2.88	0.33 – 29.05	0.344
B2	46.4 %	44.6 %	1	–	–

A1 = low level of physical activity and highest quartile of percentage calorie intake from corresponding food type

A2 = low level of physical activity and lower quartiles of percentage calorie intake from corresponding food type

B1 = moderate or high level of physical activity and highest quartile of percentage calorie intake from corresponding food type

B2 = moderate or high level of physical activity and lower quartiles of percentage calorie intake from corresponding food type

5.4 Discussion

Obesity is the result of energy intake in excess of expenditure over time. Given sufficient time, even a relatively small imbalance between energy intake and expenditure can lead to obesity. Moreover, such imbalance and the resultant weight change may occur at some time but not others (Roberts et al., 1998). Consequently, detecting and linking differences in dietary intake and physical activity to the development of obesity and its associated conditions such as NAFLD is difficult even under the best circumstances. Hence, it came as no surprise that we did not detect any significant differences in the calorie intake, the intake of macronutrients, and the percentage of calorie intake from each macronutrient and the percentage of calorie intake from high sugar food, high cholesterol food and high SFA food in our cross-sectional study of diabetic patients with and without NAFLD.

In a retrospective analysis of data from a large population-based cross-sectional study that used physical activity monitors, Gerber and colleagues showed that average physical activity and moderate/vigorous physical activity was significantly lower in patients with NAFLD or diabetes mellitus compared to those without either conditions. Average physical activity and moderate/vigorous physical activity were lowest among patients with coexisting NAFLD and diabetes mellitus (Gerber et al., 2012). This is consistent with our findings, that diabetic patients with NAFLD were more likely to have low level of physical activity compared to diabetic patients without NAFLD. Whether low level of physical activity is the cause or effect of NAFLD is unclear. Reduced energy expenditure due to low level of physical activity can theoretically contribute to energy excess, weight gain, obesity and NAFLD. On the other hand, obese patients have lower cardio-respiratory fitness and lower level of physical activity. These associations can potentially form a self-perpetuating vicious cycle that promotes NAFLD.

We also found that low level of physical activity and high percentage calorie intake from fat, high cholesterol food and high SFA food was associated with NAFLD in centrally obese but not in lean diabetic patients. Obese patients have increased insulin resistance. As elucidated in Chapter 2, insulin resistance results in impaired insulin-mediated glucose uptake in adipose tissue and skeletal muscle. The resultant increase in blood glucose concentration leads to increased glucose uptake in the liver, a process which is insulin-independent. This leads to increase de novo lipogenesis (DNL). In patients with NAFLD, free fatty acids from DNL are a significant source of accumulated fat in the liver (Donnelly et al., 2005). Insulin resistance also results in impaired insulin-mediated suppression of triglyceride hydrolysis in adipose tissue leading to increased non-esterified fatty acids (Donnelly et al., 2005; Finelli et al., 2012). Low level of physical activity aggravates insulin resistance (Mayer-Davis et al., 1998). This, along with increased dietary fat makes obese diabetic patients more susceptible to NAFLD compared to their non-obese counterparts. Dietary cholesterol has been shown to exacerbate hepatic steatosis and inflammation in animal model (Subramanian et al., 2011). In a study comparing non-obese and obese NAFLD patients, dietary cholesterol was superabundant in non-obese NAFLD patients suggesting an important role in development of NAFLD. Accumulation of SFA in the liver also plays an important role in the pathogenesis of NAFLD and has been shown to exacerbate liver inflammation and injury in various studies at the cellular and molecular level (Gentile et al., 2011).

Findings from this study are novel and complement that from other previous studies. However, as the study population consisted of diabetic patients only, the findings may not be generalized to non-diabetic patients. Further studies should be performed to see if similar findings are observed in other populations. Despite our effort, there were several other limitations in our study. Dietary intake and physical activity were self-reported and could be

subjected to bias. However, strict and continuous measurement of dietary intake and physical activity over time would not be practical in cross-sectional studies involving fairly large number of patients like this and has its own inherent way of causing bias too. We did use previously validated questionnaires to capture information on dietary intake and physical activity. Dietary composition for many Malaysian food items was incomplete so we were not able to look at the sugar, cholesterol and SFA content of each individual food items. Instead, we categorized food items into high sugar food, high cholesterol food and high SFA food for analysis. Similarly, we were also not able to look at the role of dietary n-3 and n-6 PUFAs and trans fatty acids in our patients. Lastly, diagnosis of fatty liver was based on ultrasound and not histopathological examination of liver biopsy specimen. While the latter is more accurate to diagnose fatty liver, it is invasive and not feasible in our study. Ultrasonography is by far the most common method to diagnose fatty liver in clinical practice and in epidemiological studies with good sensitivity and specificity in moderate and severe fatty liver.

5.5 Conclusion

Based on findings from this study, we conclude that low level of physical activity and high percentage calorie intake from fat, high cholesterol food and high SFA food is associated with NAFLD in centrally obese but not in lean diabetic patients. We are not implying that there should be different lifestyle recommendations for centrally obese and lean diabetic patients in regards to NAFLD, but are simply putting forward direct evidence from a cross-sectional study that low level of physical activity and poor dietary habits have different impact on NAFLD in diabetic patients with and without central obesity.

Note: A poster on the findings from this study was presented at The 3rd Asian-Pacific Topic Conference in Tokyo, Japan in 2012. A poster on the findings from this study was also presented at the Malaysian Society of Gastroenterology and Hepatology Annual Scientific Meeting in 2013. The full article has been accepted for publication in the Asian Pacific Journal of Clinical Nutrition (Chan et al., 2014).

Chapter 6

NAFLD and ischemic heart disease in diabetics

6.1 Introduction

As discussed in Chapter 2, non-alcoholic fatty liver disease (NAFLD) is considered the liver manifestation of metabolic syndrome, which is a constellation of closely related cardiovascular risk factors. Hence, it is not surprising that studies have shown that NAFLD is associated with increased risk of cardiovascular disease. However, there is substantial heterogeneity in the design of published studies on this matter. Some studies utilized serum alanine aminotransferase (ALT) or gamma glutamyl transpeptidase (GGT) levels as a surrogate marker of NAFLD (Lee et al., 2006; Ruttmann et al., 2005; Schindhelm et al., 2007; Wannamethee et al., 1995; Yun et al., 2009). These studies reported NAFLD to be associated with cardiovascular disease independent of traditional risk factors. However, it is known that these enzymes lack sensitivity and specificity for diagnosis of NAFLD. On the other hand, some studies were on patients with liver biopsy. These studies reported increased mortality in patients with NAFLD compared to the general population with cardiovascular disease as a leading cause of death (Ekstedt et al., 2006; Soderberg et al., 2010). However, patients with NAFLD who are subjected to a liver biopsy may arguably have more severe liver disease and findings from studies on these patients may not be generalized to all patients with NAFLD. Ultrasonography is by far the most widely used modality for diagnosis of NAFLD as it is widely available and relatively inexpensive. Studies utilizing ultrasonography similarly reported association of NAFLD with cardiovascular disease (Adams et al., 2005; Hamaguchi et al., 2007; Targher et al., 2006; Targher et al, 2012; Wong et al., 2011).

While most of the studies mentioned above were population-based (Adams et al., 2005; Hamaguchi, et al., 2007; Lee et al., 2006; Ruttmann et al., 2005; Schindhelm et al., 2007; Wannamethee et al., 1995; Yun et al., 2009), some were hospital-based (Ekstedt et al., 2006; Soderberg et al., 2010) or included exclusive populations e.g. patients with diabetes mellitus (Targher et al., 2006; Targher et al., 2012), patients undergoing coronary angiography (Wong et al., 2011). Targher and colleagues have made tremendous contributions in the study of the association of NAFLD with cardiovascular diseases in patients with diabetes mellitus (Targher et al., 2006; Targher et al., 2007; Targher et al., 2012). However, there were no published studies from other centers on this matter. NAFLD and cardiovascular diseases are both common among patients with diabetes mellitus and we found it difficult to appreciate that NAFLD is associated with increased risk for cardiovascular diseases independent of the other traditional risk factors. Hence, we embarked on this study to determine if ultrasonography-diagnosed NAFLD is associated with ischemic heart disease (IHD) in our hospital clinic patients with diabetes mellitus.

6.2 Patients and methods

All patients who were included in the study described in Chapter 4 were assessed for IHD at the same setting by their attending endocrinologists. The medical record for each of the patients was carefully reviewed for documented IHD. Documented IHD was defined as previous admission for acute coronary syndrome (i.e. ST segment elevation myocardial infarction, non-ST segment elevation myocardial infarction or unstable angina), previous coronary intervention (i.e. coronary angioplasty with/without stent placement or coronary artery by-pass grafting), previous coronary angiography showing coronary artery disease, or under follow-up and medical treatment for IHD. Patients without documented IHD were interviewed by their attending endocrinologists for any symptoms suggestive of IHD and

sent for an electrocardiography. History of chest pain or discomfort that was precipitated by exertion and relieved by rest was considered suggestive of IHD. Pathological Q wave, ST segment depression and/or deep T wave inversion were considered suggestive of IHD. Patients with symptoms and/or electrocardiographic changes suggestive of IHD were referred to a cardiologist for further evaluation. The endocrinologists who assessed patients for IHD were blinded to ultrasonography findings, and the operator who performed ultrasonography was blinded to the endocrinologists' assessment for IHD.

Statistical analysis

Data were analyzed using SPSS 15.0. Continuous variables were expressed as mean ± standard deviation or median (inter-quartile range), and analyzed using student's t-test or Mann-Whitney U test where appropriate. Categorical variables were expressed as percentage and analyzed using chi-square test or Fisher's exact test where appropriate. All variables which were significant on univariate analysis were entered into multivariate logistic regression analysis to identify independent factors associated with IHD. Significance was assumed at p-value < 0.05.

6.3 Results

Patient characteristics

Data for 399 patients were analyzed. Mean age of the study population was 62.8 ± 10.5 years and consisted of 43.1 % male. Mean duration of diabetes mellitus was 16.2 ± 9.7 years and mean serum glycated hemoglobin (HbA1c) level was 8.1 ± 1.8 %. The prevalence of NAFLD was 49.6 %. Ninety-two patients had documented IHD while six patients were newly diagnosed to have IHD (**Figure 6.1**). Eight patients who had history and/or ECG changes suggestive of IHD but missed their cardiology appointment were assumed to have IHD for the analysis. The results of analyses were similar whether these patients were completely excluded or assumed to have or not to have IHD.

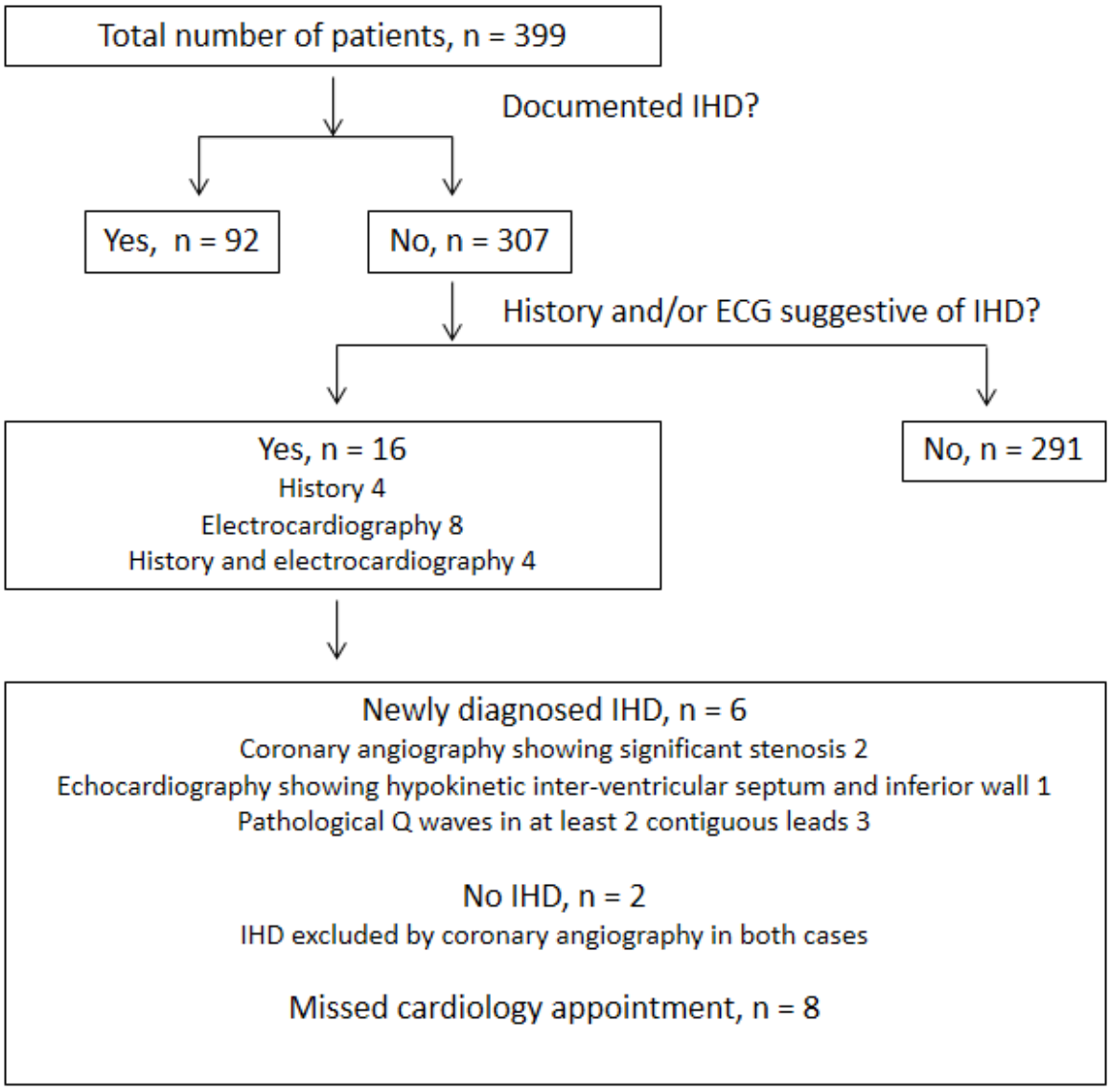

Figure 6.1 Flow chart illustrating cases of IHD in the study population

Characteristics of patients with and without IHD

Characteristics of patients with and without IHD are as shown in **Table 6.1**. The prevalence of IHD was highest among the Indians (34.1 %) followed by the Malays (29.2 %) and the Chinese (20.1 %). Patients with IHD were older, less active physically and more likely to have metabolic syndrome. They were diagnosed with diabetes mellitus for a longer duration and seemed to have poorer control as reflected by a higher HbA1c level. They also had greater body mass index (BMI), waist circumference (WC) and systolic blood pressure (SBP) compared to those without IHD. Patients with IHD were less likely to be on metformin but more likely to be on anti-platelet, beta-blocker and angiotensin converting enzyme inhibitor (ACE-i) or angiotensin receptor blocker (ARB).

Patients with ultrasound-diagnosed NAFLD were not more likely to have IHD. Elevated serum alanine aminotransferase (ALT) level was not associated with IHD regardless of whether the laboratory or a more stringent cut-off was used. Elevated serum gamma glutamyl transpeptidase (GGT) level was also not associated with IHD. No association was found between ultrasonography-diagnosed NAFLD and IHD, and between elevated serum ALT and GGT levels and IHD, even when analyzed according to the different ethnic groups (**Table 6.2**).

Table 6.1 Characteristics of patients with and without IHD

	IHD		p
	Yes n = 106	No n = 293	
Age, years	66.4 ± 8.9	61.5 ± 10.7	0.000
Male	50.0 %	40.6 %	0.095
Race Malay Chinese Indian Others	 24.5 % 33.0 % 42.5 % 0.0 %	 21.5 % 47.4% 29.7 % 1.4 %	0.025
Education – secondary and above	79.2 %	84.0 %	0.271
Income/household person/month, RM	1000 (625 – 1813)	1176 (667 – 2000)	0.117
Smoking	2.8 %	4.1 %	0.768
Calorie intake†, kcal per day	1296 (932 – 1609)	1247 (1002 – 1618)	0.590
Physical activity Low Moderate High	 61.3 % 35.8 % 2.8 %	 51.2 % 34.1 % 14.7 %	0.004
NAFLD	46.2 %	50.9 %	0.414
Metabolic syndrome	100.0 %	93.5 %	0.007
Duration of diabetes mellitus, years	19.0 ± 9.6	15.1 ± 9.5	0.001
Fasting blood sugar, mmol/L	8.2 ± 3.7	8.0 ± 3.1	0.671
HbA1c, %	8.4 ± 2.1	7.9 ± 1.6	0.024
Hypertension	99.1%	88.4 %	0.000
Systolic blood pressure ≥130 mmHg	75.5 %	61.1 %	0.008
Diastolic blood pressure ≥80 mmHg	46.2 %	41.3 %	0.379
Dyslipidemia‡	100.0 %	96.2 %	0.042
Body mass index, kg per m^2	29.3 ± 8.3	27.6 ± 6.4	0.031
Waist circumference, cm	95.8 ± 11.4	91.4 ± 12.9	0.002

	IHD		p
	Yes n = 106	No n = 293	
Medications§			
Metformin	72.6 %	81.9 %	0.043
ACE-i or ARB	85.8 %	67.6 %	0.000
Beta-blocker	42.5 %	21.5 %	0.000
Aspirin	59.4 %	47.8 %	0.040
Elevated serum alanine aminotransferase (lab cut-off)	1.0 %	4.9 %	0.080
Elevated serum alanine aminotransferase (stringent cut-off)	69.5 %	72.3 %	0.593
Elevated serum gamma glutamyl transpeptidase	24.8 %	19.4 %	0.251

†No significant difference in intake of carbohydrate, protein and fat and percentage calorie intake from these macronutrients between patients with and without IHD

‡No significant difference in total cholesterol, low- and high-density lipoprotein, and triglyceride between patients with and without IHD

§No significant difference in use of other medications – sulphonylurea, insulin, calcium channel blocker, hydrochlorothiazide, statin and fibrate between patients with and without IHD

IHD =ischemic heart disease, ACE-i = angiotensin converting enzyme inhibitor, ARB = angiotensin receptor blocker

Table 6.2 No association was found between ultrasonography-diagnosed NAFLD and IHD, and between elevated serum ALT and GGT levels and IHD when analyzed according to the different ethnic groups

	Malay, n = 88			Chinese, n = 168			Indian, n = 45		
	IHD	No IHD	p	IHD	No IHD	p	IHD	No IHD	p
NAFLD	46.2 %	66.7 %	0.072	37.1 %	43.2 %	0.519	53.3 %	50.6 %	0.764
Elevated serum ALT (laboratory cut-off)	3.8 %	4.8 %	1.000	0 %	6.0 %	0.207	0 %	3.5 %	0.550
Elevated serum ALT (stringent cut-off)	69.2 %	72.6 %	0.751	77.1 %	76.7 %	0.955	63.6 %	66.3 %	0.764
Elevated serum GGT	38.5 %	30.6 %	0.477	25.7 %	19.8 %	0.450	15.9 %	11.6 %	0.493

IHD = ischemic heart disease, ALT = alanine aminotransferase, AST = aspartate aminotransferase, GGT = gamma glutamyl transpeptidase

Univariate and multivariate analyses of factors associated with IHD

The results of univariate and multivariate analyses of factors associated with IHD are shown in **Table 6.3**. The number of subjects of other races, without metabolic syndrome and without dyslipidemia were small, and there were no patients with IHD in these groups of patients. Hence, these variables were not included in univariate and multivariate analyses. BMI and WC were not entered together in multivariate analysis as there parameters are closely associated with each other. On multivariate analysis, independent factors associated with IHD were older age, lower levels of physical activity, greater WC and higher HbA1c levels.

Table 6.3 Univariate and multivariate analyses on factors associated with IHD

	Univariate analysis		Multivariate analysis	
	OR (95 % CI)	p	OR (95 % CI)	p
Age	1.05 (1.03 – 1.08)	0.000	1.05 (1.01 – 1.08)	0.004
Race				
Malay	1.64 (0.91 – 2.95)	0.098	1.42 (0.72 – 2.79)	0.309
Chinese	1	–	1	–
Indian	2.05 (1.23– 3.44)	0.006	1.70 (0.96 – 3.00)	0.071
Physical activity				
Low	6.15 (1.84 – 20.56)	0.001	4.13 (1.18 – 14.42)	0.026
Moderate	5.37 (1.57 – 18.38)	0.003	3.66 (1.01 – 13.23)	0.048
High	1	–	1	–
Duration of diabetes mellitus	1.04 (1.02 – 1.06)	0.001	1.02 (0.99 – 1.04)	0.277
Diagnosis of non-alcoholic fatty liver disease	1.19 (0.76 – 1.86)	0.450	–	–
Body mass index	1.03 (1.00 – 1.07)	0.038	–	–
Waist circumference	1.03 (1.01 – 1.05)	0.002	1.03 (1.00 – 1.05)	0.021
Systolic blood pressure ≥130 mmHg	2.00 (1.21 – 3.31)	0.008	1.50 (0.86 – 2.60)	0.150
HbA1c	1.16 (1.02 – 1.31)	0.026	1.17 (1.02 – 1.35)	0.028
Serum alanine aminotransferase level	0.98 (0.97 – 1.00)	0.054	0.99 (0.97 – 1.00)	0.112

IHD = ischemic heart disease

6.4 Discussion

In this cross-sectional study of diabetic patients in our hospital clinic, ultrasonography-diagnosed NAFLD was not found to be associated with IHD. In a cross-sectional study of diabetic patients, Targher et al found ultrasonography-diagnosed NAFLD to be associated with prevalent cardiovascular disease independent of classical risk factors, glycemic control, medications and metabolic syndrome features (Targher et al., 2006). We believe the explanation for this contrasting finding lies in important differences in the study populations. Although both study populations consisted of patients of similar age, patients in our study population had diabetes mellitus for longer durations (16.2 years vs. 11.1 years) with poorer control as reflected by higher serum HbA1c levels (8.1 % vs. 7.2 %) and possibly more advanced NAFLD. Patients with more advanced NAFLD may have increased co-morbidities that could have limited their survival and/or attendance to clinic. This could have in turn resulted in their lower representation in our study population. Moreover, hepatic steatosis decreases as NAFLD progresses (Adams et al., 2005). Ultrasonography is good to detect moderate to severe but not milder hepatic steatosis (Saadeh et al., 2002). Therefore, NAFLD patients with more advanced disease/fibrosis may be missed on ultrasonography. Both of these factors would explain the lower prevalence of ultrasonography-diagnosed NAFLD in our study population as compared with that of Targher et al (49.6 % vs. 69.5 %) and the decreasing prevalence of NAFLD with advancing age in our study population (see Chapter 4).

Histology-based studies have suggested that cardiovascular disease is mainly associated with more severe forms of NAFLD (Ekstedt et al., 2006; Soderberg et al., 2010). NASH is associated with a more severe inflammatory and insulin-resistant state that promotes atherosclerosis (Bhatia et al., 2012). On the other hand, advanced fibrosis is the

result of long-standing non-alcoholic steatohepatitis (NASH) and may indirectly reflect exposure to risk factors for cardiovascular disease. The current gold standard for assessing severity of NAFLD is by histopathological examination of a liver biopsy specimen. However, performing a liver biopsy in NAFLD patients for risk stratification of cardiovascular disease is not justified. A recently published study that characterized severity of NAFLD patients using non-invasive methods i.e. the NAFLD fibrosis score, the aspartate aminotransferase to platelet ratio index and FIB-4 score clearly demonstrated that those with higher probability of advanced fibrosis based on these scores had a significantly higher mortality that was almost entirely from cardiovascular causes. NAFLD as a whole was not associated with higher mortality (Kim et al., 2013). Controlled attenuation parameter has been shown to correlate well with degree of hepatic steatosis (Sasso et al., 2010) while measurement of liver stiffness using transient elastography has been shown to have high negative predictive value for advanced fibrosis (Wong et al., 2010). On the other hand, measurement of cytokeratin-18 fragment levels in the blood has been shown to predict histological NASH (Feldstein et al., 2009). Use of these non-invasive methods to better characterize NAFLD patients may allow more accurate risk stratification for cardiovascular disease and deserves further study. These are further discussed in Chapter 8.

To the best of our knowledge, there were no published study on ultrasonography-diagnosed NAFLD and IHD among patients with diabetes mellitus from the Asian-Pacific region at the time that this thesis was written. There were only two studies looking at ultrasonography-diagnosed NAFLD and cardiovascular disease from the Asian-Pacific region. In a cohort study of healthy subjects, Hamaguchi et al found NAFLD to be an independent factor associated with incident cardiovascular disease (Hamaguchi et al., 2007). In a study on patients undergoing coronary angiography, Wong et al reported that NAFLD was associated with coronary artery disease independent of other metabolic factors (Wong et

al., 2011). None of the patients in the former study and only 32 % of patients in the latter study had diabetes mellitus. We were also able to compare data from the three major ethnic groups in Malaysia, namely the Malays, Indians and Chinese. The prevalence of IHD was found to be highest among Indians followed by Malays and Chinese, similar to previously reported in other studies (Danaraj et al., 1959; Lee et al., 2001). In our study, the absence of association between ultrasonography-diagnosed NAFLD and IHD was consistent across the different ethnic groups. We were also able to look at dietary intake and level of physical activity between diabetic patients with and without IHD. We found lower level of physical activity to be an independent factor associated with IHD among diabetic patients but could not determine causality due to cross-sectional nature of our study.

Despite our best effort, this study has several limitations. We could not subject all patients without documented IHD to coronary angiography for objective assessment of IHD due to ethical reason. Nevertheless, we did interview and perform electrocardiography for each of these patients and refer those suspected to have IHD to a cardiologist for further evaluation so that the diagnosis of IHD would be reflective of that in real clinical practice. The IHD status could not be determined for 8 patients who did not attend their cardiology appointment. Nevertheless, subsequent analyses were found to be unaffected whether these patients were completely excluded or assumed to have or not to have IHD. NAFLD may precede by years the clinical diagnosis of ischemic heart disease. A follow-up study of our cohort of patients would elucidate whether ultrasonography-diagnosed NAFLD is associated with increased coronary event in diabetic patients with and without IHD at baseline. Secondly, diagnosis of fatty liver was based on ultrasonography and not histopathological examination of liver biopsy specimen. While the latter is more accurate to diagnose fatty liver, it is invasive and not feasible in our study. Retrospectively, it would have been better if we have used a non-invasive method e.g. transient elastography, NAFLD fibrosis score

etc. to detect patients with more advanced NAFLD and to see if this correlated well with cardiovascular disease. Lastly, dietary intake and physical activity were self-reported and could be subjected to bias. Nevertheless, we did use previously validated methods to capture the information.

6.5 Conclusion

Ultrasonography-diagnosed NAFLD was not found to be associated with IHD among long-standing poorly-controlled diabetics in a hospital clinic setting. Independent factors associated with IHD identified in this study were older age, lower levels of physical activity, greater WC and higher HbA1c levels. Better characterization of patients using non-invasive methods may allow more accurate risk stratification for cardiovascular disease and deserves further studies.

Note: A poster on the findings from this study was presented at the Malaysian Society of Gastroenterology and Hepatology Annual Scientific Meeting in 2013. A poster on the findings from this study was also presented at the World Congress of Gastroenterology 2013 in Shanghai, China, and the abstract was published in a supplementary issue of the Journal of Gastroenterology and Hepatology (Chan et al., 2013). The full article has been published in Clinics and Research in Hepatology and Gastroenterology (Chan et al., 2014).

Chapter 7

Progression of liver disease in NAFLD

7.1 Introduction

As elucidated in Chapter 3, non-alcoholic fatty liver disease (NAFLD) has rapidly increased over the years and is estimated to affect up to 30 % of the general population in the Asian-Pacific region (Chan et al., 2013). In Malaysia, the prevalence of NAFLD in the general population has been estimated to be 22.7 % based on a study on individuals attending a health-check in a suburban medical facility (Goh et al., 2012). The prevalence of NAFLD among diabetics is higher and has been estimated to be 49.6 % based on a separate study on a hospital clinic population (Chan et al., 2013) (as presented in Chapter 4).

NAFLD is not entirely benign. It encompasses a spectrum of liver conditions ranging from simple steatosis to its more severe form known as non-alcoholic steatohepatitis (NASH) which can lead to fibrosis and cirrhosis. In fact, NASH has been recognized as an important cause of cryptogenic cirrhosis (Maheshwari et al., 2006) and is associated with increased risk of hepatocellular carcinoma, even in patients without cirrhosis (Page et al., 2009). In a study on etiology of cirrhosis and association with hepatocellular carcinoma in our center, cryptogenic cause which is believed to be due to NASH, contributed to 15.4 % of cases of cirrhosis and was an independent predictor of hepatocellular carcinoma (Qua et al., 2011).

While there have been quite a number of studies on the natural history of NAFLD, few utilized paired liver biopsy for evaluation of disease status. Apart from being invasive and is associated with a small risk of complications, liver biopsy requires technical expertise, from obtaining a good specimen to processing and accurately interpreting the result. Nevertheless, histopathological examination of liver biopsy specimen is the current best

standard for evaluation of NAFLD. The use of a standardized scoring system such as that by the NASH Clinical Research Network (NASH CRN) enables objective quantification of all the important components of NAFLD i.e. steatosis, lobular inflammation, hepatocyte ballooning and fibrosis.

A descriptive study on a cohort of biopsy-proven NAFLD patients was conducted at our center between June 2003 and May 2005(Malik et al., 2007). We performed a follow-up study on this cohort of patients to elucidate the natural history of NAFLD and to determine factors associated with disease progression.

7.2 Patients and methods

Seventy-five NAFLD patients from the previous study were considered for inclusion into the current study. The patients were contacted by phone and if unsuccessful, by post, using information available in the hospital registry and in their medical records. When these measures have failed, a check was made at the National Registration Department for patient's status and address. Another letter was sent if the address was different from that earlier available. Patients who agreed for a repeat liver biopsy were included into the current study. For patients who have died, the cause of death was ascertained at the National Registration Department. The study was approved by the University of Malaya Medical Centre's Medical Ethics Committee and all patients who participated provided informed consent.

Demographic and anthropometric data and relevant clinical and laboratory data at baseline were retrieved from the database of the earlier study. The follow-up study was conducted between October 2009 and June 2010. Corresponding data were obtained using a standard protocol during the follow-up study. Alcohol intake was estimated using the quantity-frequency method (Goddard, 2007). Significant alcohol intake was defined as more than 21 units per week for men and more than 14 units per week for women (Chalasani et al.,

2012). Weight and height were measured using standardized equipment. Body mass index (BMI) was calculated by dividing weight in kilogram by the square of height in meters. Patients with BMI ≥ 25.0 kg per m^2 were considered obese (Anuurad et al., 2003). Waist circumference (WC) was measured at the mid-point between the lowest margin of the least palpable rib and the top of the iliac crest in the standing position. Central obesity was defined as WC > 90 cm for men and > 80 cm for women (Alberti et al., 2005). Blood pressure was measured in the sitting position using standardized equipment. A patient was considered hypertensive if there was a self-reported history of hypertension, if the patient was on anti-hypertensive medication(s), if the systolic blood pressure was ≥ 130 mmHg, or if the diastolic blood pressure was ≥ 85 mmHg.

All patients had venous blood drawn after an overnight fast for blood sugar, glycated hemoglobin (HbA1c), lipid profile, liver profile and tests for viral hepatitis B and C infection. Biochemical measurements were performed using standard laboratory procedures. A patient was considered to have diabetes mellitus if there was a self-reported history of diabetes mellitus or if the patient was on anti-diabetic medication(s). Patients who were not known to have diabetes mellitus were subjected to an oral glucose tolerance test. Patients were diagnosed to have diabetes mellitus if the fasting blood sugar was ≥ 7.0 mmol/L or if the 2-hour post-glucose challenge blood sugar was ≥ 11.1 mmol/L. Impaired fasting glucose was defined as fasting blood sugar ≥ 6.1 mmol/L but < 7.0 mmol/L while impaired glucose tolerance was defined as 2-hour post-glucose challenge ≥ 7.8 mmol/L but < 11.1 mmol/L. A patient was considered to have dyslipidemia if there was a self-reported history of dyslipidemia, if the patient was on lipid-lowering medication(s), if the serum total cholesterol (TC) was ≥ 5.2 mmol/L, if the serum triglyceride (TG) was ≥ 1.7 mmol/L, if the serum high-density lipoprotein (HDL) was < 1.0 mmol/L for men or < 1.3 mmol/L for women, or if the serum low-density lipoprotein (LDL) was ≥ 3.4 mmol/L. A patient was considered to have

metabolic syndrome if three or more of the following were present: impaired fasting glucose/impaired glucose tolerance/diabetes mellitus, central obesity, hypertension, hypertriglyceridemia and low serum HDL (according to the aforementioned cut-offs) (Alberti et al., 2009). Our laboratory's upper limit of normal for liver enzymes were as follows: alkaline phosphatase (ALP) 136 IU/L, aspartate aminotransferase (AST) 37 IU/L, alanine aminotransferase (ALT) 65 IU/L and gamma-glutamyl transpeptidase (GGT) 55 IU/L. Serum ALP, AST, ALT and GGT above these levels were considered as elevated. The Elecsys HBsAg II assay and the Elecsys Anti-HCV II assay (Roche, Mannheim, Germany) were used to test for viral hepatitis B and C infection, respectively.

Liver biopsy and histopathological assessment

Liver biopsies were performed using 18 G Terumo liver biopsy needle. Liver biopsy specimens were processed using standard laboratory procedures. Liver biopsy slides were stained with hematoxylin and eosin stain and masson trichrome stain. Paired liver biopsy slides were examined by an experienced histopathologist (PLC) who was blinded to clinical data and order of liver biopsy slides. Histopathological findings were reported according to the NASH CRN scoring system (Kleiner et al., 2005). The NAFLD activity score (NAS) is the sum of scores for hepatic steatosis (0 – 3), lobular inflammation (0 – 3) and hepatocyte ballooning (0 – 2). NAS 0 – 2 is not diagnostic of NASH, 3 – 4 is borderline NASH and 5 – 8 is definite NASH. Fibrosis was staged 0 – 4 (0 = no fibrosis, 1 = mild fibrosis, 2 = moderate fibrosis, 3 = severe fibrosis, 4 = cirrhosis). Typical histological findings are shown in **Figures 7.1a and 7.1b**.

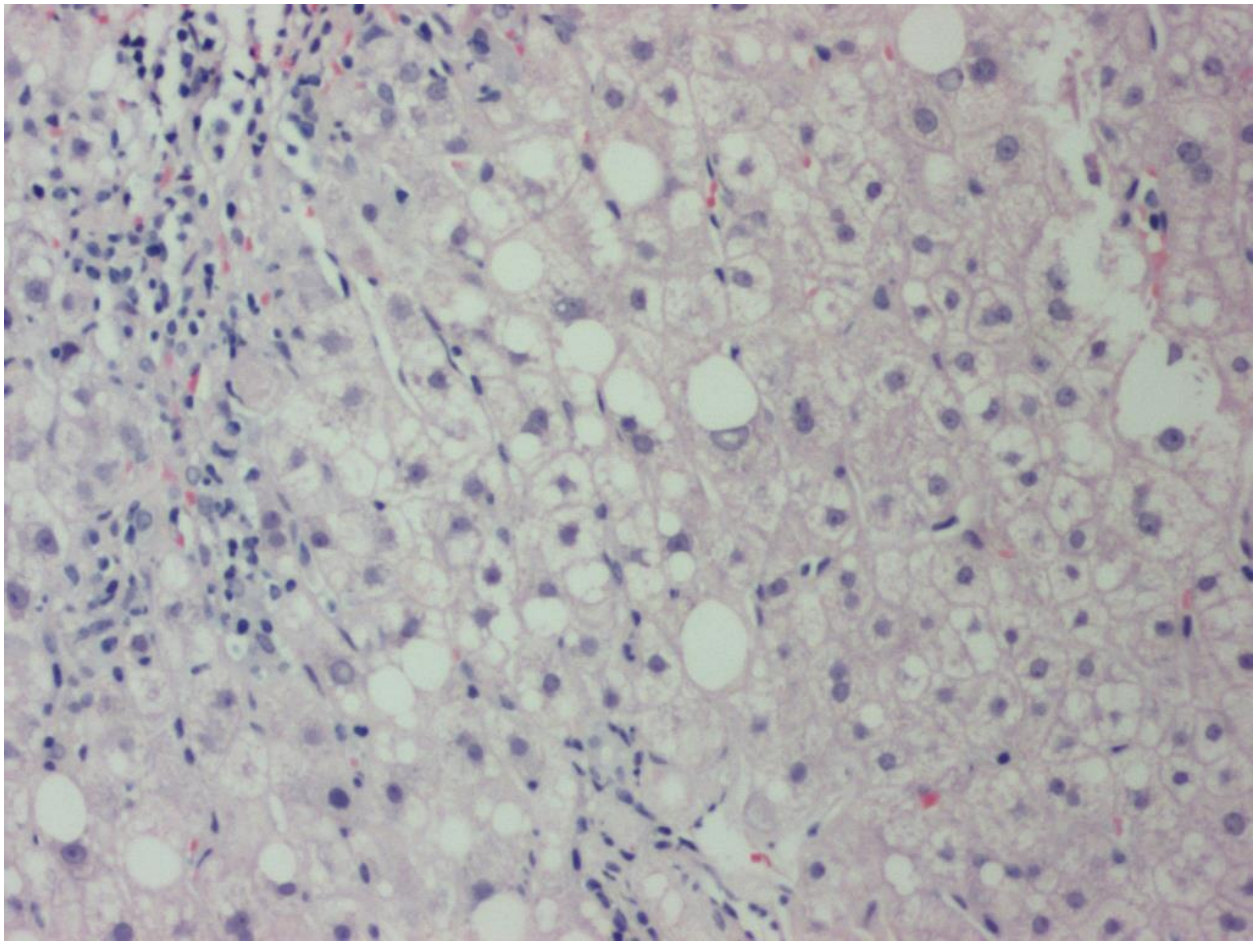

Figure 7.1a Hematoxylin and eosin staining shows a constellation of steatosis, inflammation and ballooning in a case of NAFLD

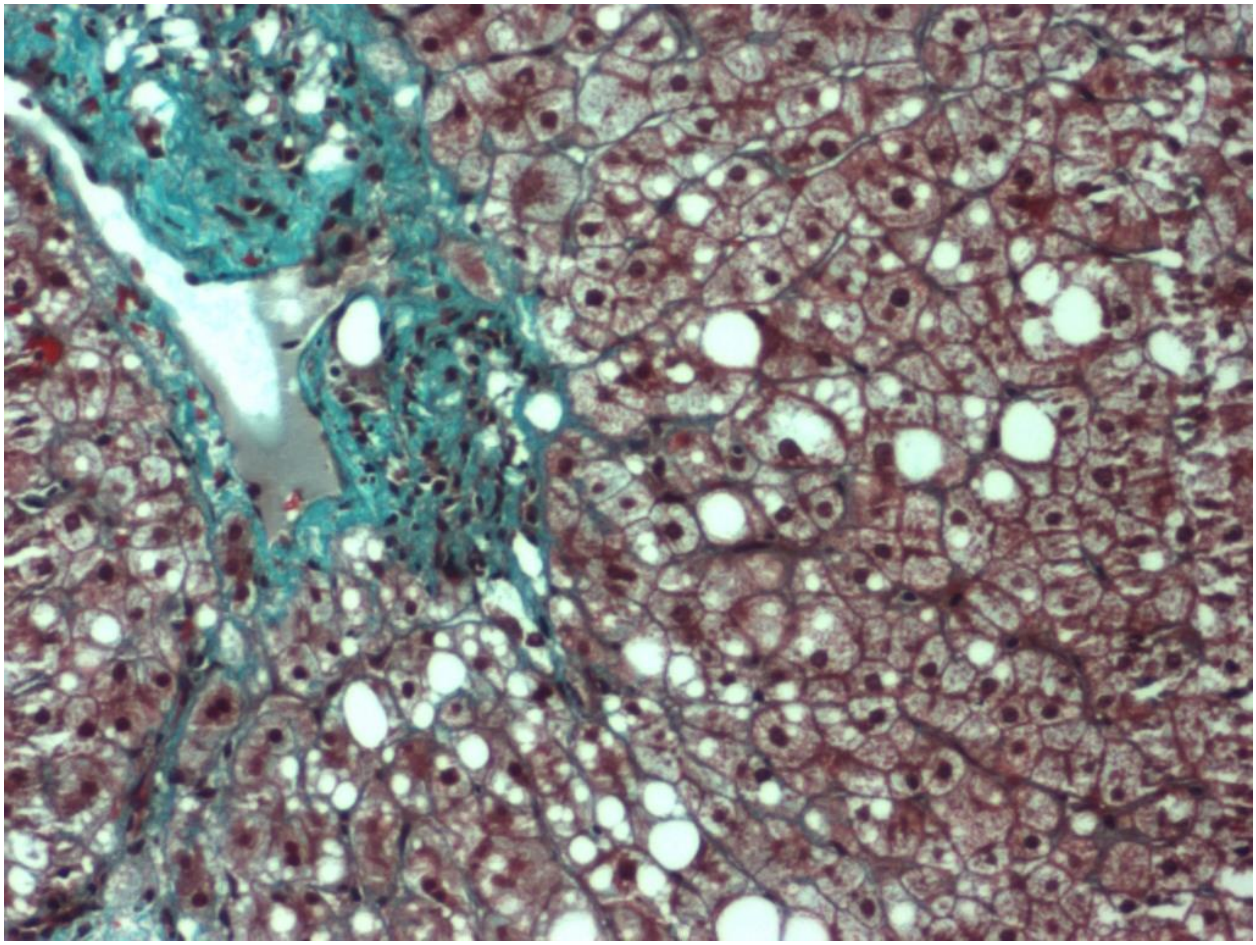

Figure 7.1b Masson trichrome highlighting the fibrosis

Statistical analysis

Data were analyzed using SPSS 15.0. Continuous variables were expressed as mean ± standard deviation or median (inter-quartile range), and analyzed using unpaired student's t-test or Mann-Whitney U test, as appropriate. Changes in continuous variables were analyzed using paired student's t-test or Wilcoxon rank-sum test, as appropriate. Categorical variables were expressed as percentage and analyzed using chi-square test or Fisher exact test, as appropriate. Univariate and multivariate analyses were performed to identify factors associated with worsened NAS and fibrosis. Significance was assumed when $p < 0.05$.

7.3 Results

Patient characteristics

Thirty-nine patients agreed for repeat liver biopsy (**Figure 7.2**). However, slides for the initial liver biopsy were missing for 4 patients. Hence, paired liver biopsies were available for 35 patients and data for these patients were analyzed. Mean age of the patients at baseline was 47.5 ± 10.9 years old and consisted of 40.0 % male. None of the patients had significant alcohol intake. At baseline, 62.9 % of patients had metabolic syndrome. Obesity and central obesity was present in 74.3 % and 85.7 %, respectively. Dyslipidemia, diabetes mellitus and hypertension was present in 97.1 %, 54.3 % and 31.4 %, respectively. At follow-up, 94.3 % of patients had metabolic syndrome. One patient developed obesity but the prevalence of central obesity remained unchanged. The prevalence of diabetes mellitus and hypertension increased to 80.0 % and 68.6 %, respectively, while the prevalence of dyslipidemia remained unchanged. Mean interval between the paired liver biopsies was 6.4 ± 0.8 years. Mean number of portal tracts for the initial and repeat liver biopsy was 8.6 ± 4.4 and 6.6 ± 4.6, respectively.

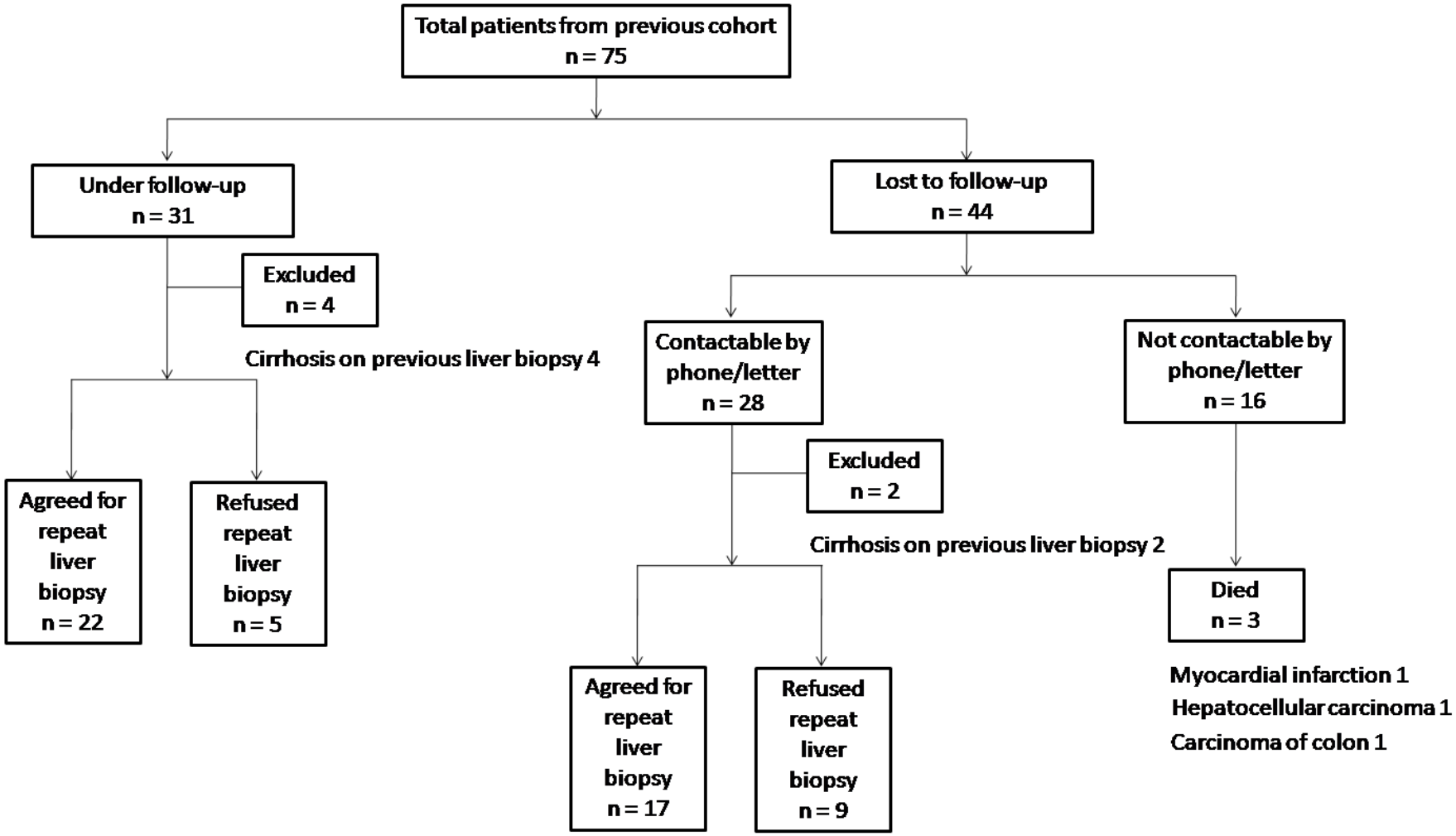

Figure 7.2 Flow chart detailing patients from the original cohort who were included/excluded from the current study

Changes in NAS and associated factors

At baseline, 1 patient had simple steatosis, 11 patients had probable NASH while 23 patients had definite NASH. At follow-up, 12 patients had probable NASH while 23 patients had definite NASH. NAS worsened in 13 patients, remained unchanged in 9 patients and improved in 13 patients. Distribution of NAS at baseline and at follow-up is shown in **Table 7.1.** Characteristics of patients with worsened, and unchanged or improved NAS are shown in **Table 7.2**. Although patients with worsened NAS had significantly lower BMI and WC at baseline and at follow-up, there was no significant change in BMI and WC between baseline and follow-up in both groups of patients. Metformin use was significantly higher among patients with unchanged or improved NAS. Patients with worsened NAS had significantly lower HbA1c at baseline but showed a significant increase in HbA1c so that there was no longer a significant difference in both groups during follow-up. Patients with unchanged or improved NAS had significant decrease in TC, TG, ALP, ALT, AST and GGT so that these variables were significantly lower at follow-up compared to patients with worsened NAS.

Elevated serum ALT, AST and GGT levels was seen in 53.8 %, 69.2 % and 92.3 % of patients with worsened NAS at follow-up compared to only 13.6 %, 13.6 % and 31.8 % of patients with unchanged or improved NAS. On univariate analysis, elevated serum ALT, AST and GGT levels at follow-up were associated with worsened NAS. On multivariate analysis, only elevated serum AST and GGT levels were associated with worsened NAS (**Table 7.3**).

Table 7.1 Distribution of NAS at baseline and at follow-up

		NAS at follow-up			
		0 – 2	3 – 4	5 – 8	Total
NAS at baseline	0 – 2	0	1	0	1
	3 – 4	0	3	8	11
	5 – 8	0	8	15	23
	Total	0	12	23	35

NAS = non-alcoholic fatty liver disease activity score

Table 7.2 Characteristics of patients with worsened NAS and patients with unchanged or improved NAS

	Patients with worsened NAS n = 13			Patients with unchanged or improved NAS n = 22		
	Baseline	Follow-up	p	Baseline	Follow-up	p
Age, years	44.1 ± 12.0	–	–	50.5 ± 12.0	–	–
Male	46.2	–	–	36.4	–	–
Ethnicity						
Malay	69.2	–	–	45.5	–	–
Chinese	23.1	–	–	27.3	–	–
Indian	7.7	–	–	27.3	–	–
Anthropometry						
BMI, kg per m^2 †, ‡	25.1 ± 3.6	25.7 ± 5.1	NS	29.2 ± 4.6	29.0 ± 3.9	NS
WC, cm †, ‡	87.4 ± 8.5	88.5 ± 11.9	NS	95.5 ± 10.4	96.5 ± 9.3	NS
Glycemic profile						
FBS, mmol/L	5.3 (5.0 – 6.0)	6.0 (5.1 – 7.8)	NS	5.9 (5.3 – 7.3)	6.6 (5.4 – 7.6)	NS
HbA1c, % †	5.47 (5.26 – 6.07)	7.12 (6.30 – 7.83)	0.018	6.31 (5.94 – 7.46)	7.10 (6.08 – 8.35)	NS
Lipid profile						
TC, mmol/L ‡	5.9 (5.7 – 6.7)	5.0 (4.7 – 6.0)	NS	5.7 (5.1 – 6.3)	4.4 (3.8 – 4.8)	< 0.001
HDL, mmol/L	1.1 (1.0 – 1.4)	1.2 (1.0 – 1.6)	NS	1.3 (1.1 – 1.6)	1.2 (1.1 – 1.5)	NS
LDL, mmol/L	3.9 (3.6 – 4.2)	3.0 (1.9 – 3.7)	NS	3.3 (2.9 – 4.3)	2.4 (1.8 – 2.7)	< 0.001
TG, mmol/L ‡	1.9 (1.2 – 2.9)	2.0 (1.4 – 2.7)	NS	1.8 (1.2 – 2.4)	1.2 (1.0 – 1.7)	0.049
Liver profile						
ALP, IU/L ‡	83 (79 – 117)	86 (76 – 113)	NS	84 (66 – 95)	56 (39 – 90)	0.014
ALT, IU/L §	92 (79 – 115)	67 (41 – 208)	NS	109 (71 – 132)	40 (31 – 52)	< 0.001
AST, IU/L §	44 (38 – 59)	45 (24 – 129)	NS	50 (35 – 71)	26 (18 – 31)	< 0.001
GGT, IU/L §	88 (72 – 275)	98 (61 – 320)	NS	78 (50 – 158)	35 (28 – 95)	0.009
Interval between paired liver biopsies, years	–	6.3 ± 0.6	–	–	6.5 ± 0.9	–

	Patients with worsened NAS n = 13			Patients with unchanged or improved NAS n = 22		
	Baseline	Follow-up	p	Baseline	Follow-up	p
Number of portal tracts	8.6 ± 4.8	7.3 ± 4.8	NS	8.6 ± 4.3	6.2 ± 4.5	NS

† $p < 0.05$ comparing patients with worsened NAS and patients with unchanged or improved NAS at baseline

‡ $p < 0.05$ comparing patients with worsened NAS and patients with unchanged or improved NAS at follow-up

§ $p < 0.01$ comparing patients with worsened NAS and patients with unchanged or improved NAS at follow-up

NAS = non-alcoholic fatty liver disease activity score, BMI = body mass index, WC = waist circumference, FBS = fasting blood sugar, TC = total cholesterol, TG = triglyceride, HDL = high-density lipoprotein, LDL = low-density lipoprotein, ALP = alkaline phosphatase, ALT = alanine aminotransferase, AST = aspartate aminotransferase, GGT = gamma glutamyl transpeptidase

Table 7.3 Univariate and multivariate analysis of factors associated with worsened NAS

	OR (95 % CI)	p	OR (95 % CI)	p
Age	1.05 (0.98 – 1.12)	0.155	1.03 (0.93 – 1.13)	0.609
Male gender	1.50 (0.37 – 6.05)	0.569	1.45 (0.16 – 12.85)	0.738
Elevated serum ALT level at follow-up	7.39 (1.44 – 37.88)	0.016	0.83 (0.08 – 8.80)	0.876
Elevated serum AST level at follow-up	14.25(2.62 – 77.54)	0.002	10.74 (1.00 – 115.86)	0.050
Elevated serum GGT at follow-up	25.71 (2.77 – 238.79)	0.004	16.10 (1.30 – 198.90)	0.030

NAS = non-alcoholic fatty liver disease activity score, ALT = alanine aminotransferase, AST = aspartate aminotransferase, GGT = gamma glutamyl transpeptidase

Changes in fibrosis and associated factors

Distribution of fibrosis stage at baseline and at follow-up is as shown in **Table 7.4**. Fibrosis stage worsened in 18 patients and remained unchanged in 17 patients. None of the patients had improvement in fibrosis stage. Nearly two thirds of patients without significant fibrosis (F0/F1) at baseline developed significant fibrosis ($F \geq 2$) at follow-up. Two patients developed cirrhosis at follow-up. Characteristics of patients with worsened fibrosis and patients with unchanged fibrosis are shown in **Table 7.5**. Patients with worsened fibrosis had lower FBS and HbA1c compared to patients with unchanged fibrosis but the difference was only significant for FBS at baseline and HbA1c at follow-up. Both groups of patients had increase in FBS and HbA1c at follow-up but the increase was only significant for HbA1c for patients with worsened fibrosis. Both groups of patients had significant improvement in TC, LDL and ALT. However, there was no significant difference in these parameters between the groups at baseline and at follow-up. Overall, no plausible factors were found to be associated with worsened fibrosis.

Table 7.4 Distribution of fibrosis stage at baseline and at follow-up

		Follow-up					
		F0	F1	F2	F3	F4	Total
Baseline	F0	0	0	1	0	0	1
	F1	0	6	7	1	1	15
	F2	0	0	7	7	0	14
	F3	0	0	0	3	1	4
	F4	0	0	0	0	1	1
	Total	0	6	15	11	3	35

Table 7.5 Characteristics of patients with worsened fibrosis and patients with unchanged fibrosis

	Patients with worsened fibrosis n = 13			Patients with unchanged fibrosis n = 22		
	Baseline	Follow-up	p	Baseline	Follow-up	p
Age, years	48.3 ± 11.8	–	–	46.7 ± 10.2	–	–
Male	38.9	–	–	41.2	–	–
Ethnicity						
Malay	44.4	–	–	64.7	–	–
Chinese	27.8	–	–	23.5	–	–
Indian	27.8	–	–	11.8	–	–
Anthropometry						
BMI, kg per m^2	27.1 ± 3.1	27.5 ± 4.2	NS	28.3 ± 5.9	28.0 ± 5.1	NS
WC, cm	90.4 ± 7.3	92.2 ± 10.5	NS	94.7 ± 12.7	94.9 ± 11.4	NS
Glycemic profile						
FBS, mmol/L †	5.5 (5.0 – 5.9)	5.8 (4.8 – 7.1)	NS	6.4 (5.2 – 8.6)	7.3 (5.7 – 8.2)	NS
HbA1c, % §	5.98 (5.60 – 6.34)	6.30 (6.00 – 7.11)	0.028	6.24 (5.46 – 8.74)	7.40 (7.10 – 9.18)	NS
Lipid profile						
TC, mmol/L	5.8 (5.1 – 6.3)	4.7 (3.8 – 5.2)	0.004	6.0 (5.3 – 7.0)	4.6 (3.9 – 4.9)	0.004
HDL, mmol/L	1.3 (1.0 – 1.6)	1.2 (1.1 – 1.7)	NS	1.2 (1.0 – 1.5)	1.2 (1.0 – 1.4)	NS
LDL, mmol/L	3.6 (3.0 – 4.1)	2.4 (1.9 – 2.8)	0.003	3.8 (3.3 – 4.7)	2.6 (1.8 – 3.1)	0.004
TG, mmol/L	2.0 (1.2 – 2.7)	1.6 (0.9 – 2.6)	NS	1.7 (1.1 – 2.3)	1.6 (1.1 – 1.9)	NS
Liver profile						
ALP, IU/L	86 (79 – 102)	83 (56 – 90)	NS	83 (70 – 95)	76 (38 – 103)	NS
ALT, IU/L	94 (78 – 144)	45 (37 – 81)	0.028	104 (74 – 118)	49 (33 – 69)	0.018
AST, IU/L	50 (38 – 70)	30 (23 – 40)	NS	45 (34 – 70)	27 (19 – 45)	NS
GGT, IU/L	85 (66 – 199)	69 (32 – 191)	NS	76 (51 – 142)	57 (31 – 93)	NS
Interval between paired liver biopsies, years	–	6.5 ± 0.8	–	–	6.3 ± 0.9	–
Number of portal tracts ‡	6.1 ± 2.9	7.2 ± 5.5	NS	11.3 ± 4.3	6.0 ± 3.4	0.001

† $p < 0.05$ comparing patients with worsened fibrosis and patients with unchanged fibrosis at baseline

‡ $p < 0.01$ comparing patients with worsened fibrosis and patients with unchanged fibrosis at baseline

§ $p < 0.05$ comparing patients with worsened fibrosis and patients with unchanged fibrosis at follow-up

NAS = non-alcoholic fatty liver disease activity score, BMI = body mass index, WC = waist circumference, FBS = fasting blood sugar, TC = total cholesterol, TG = triglyceride, HDL = high-density lipoprotein, LDL = low-density lipoprotein, ALP = alkaline phosphatase, ALT = alanine aminotransferase, AST = aspartate aminotransferase, GGT = gamma glutamyl transpeptidase

Baseline NAS and changes in NAS and overall histology

The patient with simple steatosis at baseline developed probable NASH but had no change in fibrosis stage at follow-up. Of the 11 patients with probable NASH at baseline, 8 patients developed definite NASH while 3 patients continued to have probable NASH at follow-up. NAS worsened in 8 patients (72.7 %) and remained unchanged in 3 patients (27.3 %). Fibrosis stage worsened in 6 patients (54.5 %), occurring in 5 patients (62.5 %) with worsened NAS and 1 patient (33.3 %) with unchanged NAS. Of the 23 patients with definite NASH at baseline, 8 patients had probable NASH while 15 patients continued to have definite NASH at follow-up. NAS worsened in 4 patients (17.4 %), remained unchanged in 6 patients (26.1 %) and improved in 13 patients (56.5 %). Fibrosis stage worsened in 11 patients (47.8%), occurring in 2 patients with worsened NAS (50.0. %), 2 patients with unchanged NAS (33.3 %) and 7 patients with improved NAS (53.8 %). Patients with borderline NASH at baseline were significantly more likely to have worsened NAS at follow-up compared to patients with definite NASH (OR = 12.67, 95 % CI = 2.29 – 70.02, p = 0.004). However, both groups of patients had similar likelihood of having worsened fibrosis stage at follow-up.

Three of the six patients who were found to have cirrhosis on histological examination of liver biopsy specimen in the previous study developed decompensated cirrhosis. All three patients had esophageal varices and ascites. Two of these patients had hepatic encephalopathy. Three other patients from the initial cohort have died due to carcinoma of colon, myocardial infarction and hepatocellular carcinoma, respectively.

7.4 Discussion

The largest prospective longitudinal study of NAFLD patients with paired liver biopsy in the Asian-Pacific to date is that by Wong and colleagues (Wong et al., 2010).

Of the 52 patients in the study, 13 patients had simple steatosis, 22 patients had borderline NASH and 17 patients had NASH at baseline. After 3 years, fibrosis stage had worsened in 14 patients (27 %), remained unchanged in 25 patients (48 %) and improved in 13 patients (25 %). In comparison, fibrosis stage had worsened in 18 patients (51.4 %) and remained unchanged in 17 patients (48.6 %) in our study. None of the patients in our study had improvement in fibrosis stage. This difference may be due to the substantial proportion of patients with simple steatosis and the shorter duration of follow-up in the study by Wong and colleagues. Moreover, none of the patients in our study were on specific interventions with nearly half of the patients lost to follow-up subsequent to the initial liver biopsy. Following the diagnosis of NASH, our patients were only given general advice on diet and encouraged to exercise and lose weight which was the standard of care at that time. In addition, nearly half of the patients were lost to follow-up subsequent to the initial liver biopsy. Longitudinal data for such patients would be increasingly rare with more and more intervention trials targeting these patients.

The study by Hui and colleagues (Hui et al., 2005), which is the first and only other longitudinal study on NAFLD patients with paired liver biopsy in the Asian-Pacific till date, similarly found that none of their patients had improvement in fibrosis stage while 53 % had worsened fibrosis stage over a median follow-up of 6.1 years. Of the 17 patients in the study, only 3 patients had steatosis alone while the remainder had necroinflammation at baseline. A systematic review summarized longitudinal studies of NAFLD patients with paired liver biopsy but most of these studies consisted of Caucasian patients and small number of subjects (Argo et al., 2009).

Findings from our study of a cohort of mostly NASH patients show that a substantial proportion of NASH patients undergo significant progression and suggest that fibrosis is irreversible without specific interventions. Hence, it is important that all patients diagnosed with NASH be considered for specific interventions to prevent disease

progression. While many studies have shown lifestyle interventions i.e. dietary restrictions, exercise and weight loss to be effective in NASH (Thoma et al., 2012), these measures are difficult to implement in practice. Pioglitazone and vitamin E have been shown useful in NASH but specific concerns have limited their use (Sanyal et al., 2010). Until a safe and effective drug becomes available, NASH patients may be considered for interventional trials where these are available.

We found that greater baseline BMI and WC did not predict worsening of NAS. Although patients with worsened NAS had significantly lower BMI and WC at baseline, there was a trend towards increasing BMI and WC among these patients. On the other hand, patients with unchanged or improved NAS had a trend towards decreasing BMI. In their study, Wong and colleagues showed that reduction in BMI and WC was independently associated with non-progressive disease activity and fibrosis (Wong et al., 2010). Therefore, NAFLD patients with greater BMI and WC should not be discouraged to undergo lifestyle interventions. Patients with worsened NAS had significant increase in serum HbA1c level at follow-up, reflecting poorer control of diabetes mellitus with increasing severity of liver disease. An excellent review on the intricate relationship between diabetes mellitus and liver disease can be found elsewhere (Moscatiello et al., 2007). We also found that patients with unchanged or improved NAS have significant decrease and normalization of serum ALP, ALT, AST and GGT levels at follow-up. Patients with elevated serum AST and GGT at follow-up were more likely to have worsened NAS. Therefore, NAFLD patients with persistently elevated serum AST and GGT levels during follow-up should be suspected of having worsened NAS.

Three of the six patients who were incidentally found to have cirrhosis on histology in the previous study have progressed to decompensated cirrhosis while 3 other patients have died due to carcinoma of colon, myocardial infarction and hepatocellular carcinoma, respectively. Patients with NAFLD have been shown to have lower survival

with higher rate of liver-related morbidity and mortality compared to the general population (Adams et al., 2005). Malignancy and cardiovascular diseases were shown to be the leading causes of death followed by liver-related complications in NAFLD patients. Although progressive disease is mainly associated with NASH, patients with simple steatosis should also be followed as they too may develop NASH and progressive disease as clearly reported by Wong and colleagues (Wong et al., 2010).

This study had several limitations. First, we were not able to perform repeat liver biopsy for all patients from the original cohort as some patients were not contactable while others were not willing due to the invasive nature of the procedure. However, patients who had the repeat liver biopsy and who were included in our analysis were well characterized. Baseline and follow-up data were prospectively captured during the previous and current study, and were therefore complete and robust. Second, the analysis of factors associated with worsened NAS and fibrosis may be limited by the sample size. However, this is considered a fairly large number of subjects for a longitudinal study of NAFLD patients with paired liver biopsy. Moreover, we did identify some interesting associations in our analysis. Third, histopathological examination of liver biopsy specimen may be limited by sampling variability as the liver biopsy specimen only represents approximately 1 in 50000 of the total liver volume (Ratziu et al., 2005). However, until more accurate methods become available, histopathological examination of liver biopsy specimen remains the best standard for evaluation of NAFLD status.

7.5 Conclusion

NAFLD patients with persistently elevated serum AST and GGT levels during follow-up should be suspected of having worsened NAS. NASH patients can undergo significant disease progression over a relatively short period of time and fibrosis is irreversible without specific interventions. Hence, it is important that all patients diagnosed with NASH be considered for specific interventions to prevent disease progression.

Note: A poster on the findings from this study was presented at the Asia-Pacific Digestive Week 2012 in Bangkok, Thailand, and the abstract was published in a supplementary issue of the Journal of Gastroenterology and Hepatology (Chan et al., 2012). The findings from this study was also presented at the Malaysian Society of Gastroenterology and Hepatology Annual Scientific Meeting in 2013 and won the Best Paper Award. The full article has been published in the Journal of Digestive Diseases (Chan et al., 2014).

Chapter 8

Non-Invasive Assessment of NAFLD

8.1 Introduction

The prevalence of non-alcoholic fatty liver disease (NAFLD) has increased rapidly over the years parallel to the increase in metabolic syndrome and it is recognized as one of the most common causes of chronic liver disease worldwide (Chan et al., 2013). NAFLD encompasses a spectrum of liver conditions, ranging from benign steatosis to non-alcoholic steatohepatitis (NASH) to fibrosis and cirrhosis (Ekstedt et al., 2006). NASH has been recognized as an important cause of cryptogenic cirrhosis (Maheshwari et al., 2006) and is associated with an increased risk of hepatocellular carcinoma, even in patients without cirrhosis (Page et al., 2009). In a study on etiology of cirrhosis and association with hepatocellular carcinoma in our centre, cryptogenic cause which is believed to be due to NASH, contributed to 15.4 % of cases of cirrhosis and was an independent predictor of hepatocellular carcinoma (Qua et al., 2011).

Ultrasonography is by far the most common method used to diagnose fatty liver in clinical practice and in epidemiological studies. However, ultrasonography is accurate only when fatty liver is moderate to severe (Saadeh et al., 2002). Moreover, ultrasonography is not able to distinguish NASH from simple steatosis and to assess the severity of fibrosis. Both factors carry important prognostic implications in NAFLD patients. Histopathological examination of a liver biopsy specimen is the current best standard for assessment of NAFLD. It confirms the diagnosis and helps exclude other causes of liver disease in some cases. It also distinguishes NASH from simple steatosis and allows assessment of the severity of fibrosis. However, liver biopsy is invasive and associated with a small risk of complications. It may also be limited by sampling

variability (Ratziu et al., 2005) and intra- and inter-observer variability (Younossi et al., 1998).

In this chapter, findings from studies on several non-invasive methods for assessment of NAFLD are presented and discussed, namely the use of controlled attenuation parameter (CAP) for the detection and quantification of hepatic steatosis, plasma cytokeratin-18 fragment level for the diagnosis of NASH, and NAFLD fibrosis score and liver stiffness measurement for the estimation of hepatic fibrosis.

8.2 CAP for the detection and quantification of hepatic steatosis

Recently, a novel technology called transient elastography has been used to estimate liver stiffness, which has been shown to correlate well with histopathological fibrosis stage. This has allowed non-invasive and accurate estimation of fibrosis stage in NAFLD patients (Yoneda et al., 2007). The decrease in amplitude of ultrasound as it is propagated through the liver tissue can be estimated using the same radio-frequency data that is used for estimation of liver stiffness using Fibroscan (Echosens, Paris, France), an ultrasound-based vibration-controlled transient elastography device. This is called controlled attenuation parameter (CAP) and it has been suggested to correlate well with hepatic steatosis (Sasso et al., 2010). Several publications have explored CAP for estimation of hepatic steatosis in patients with chronic liver disease (Chon et al., 2013; de Ledinghen et al., 2012; Kumar et al., 2013; Masaki et al., 2013; Myers et al., 2012; Sasso et al., 2012). All of these studies were on patients with chronic liver disease of various etiologies with limited number of NAFLD patients, except one study which consisted of a homogeneous cohort of chronic hepatitis C patients (Sasso et al., 2012). We conducted a prospective study to evaluate the diagnostic performance of CAP in estimation of hepatic steatosis specifically in NAFLD patients.

8.2.1 Methods

Consecutive adult patients (aged ≥ 18 years) with NAFLD who were scheduled for a liver biopsy were prospectively recruited between November 2012 and October 2013 for this study. The diagnosis of NAFLD was based on ultrasonography finding of fatty liver and exclusion of significant alcohol intake, use of medications that can cause fatty liver, viral hepatitis B and C infection, and other causes of chronic liver disease where indicated (Chalasani et al., 2012). An additional 60 subjects who did not have signs of fatty liver on ultrasonography were recruited as controls. Percutaneous liver biopsy was not performed for controls due to ethical considerations but all other relevant data were obtained. This study was approved by the University of Malaya Medical Centre's Ethics Committee and all patients who participated provided informed consent.

Demographic, anthropometric, relevant clinical and laboratory data were obtained using a standard protocol on the day of the liver biopsy procedure. Weight and height were measured using standard equipment. Body mass index (BMI) was calculated by dividing weight in kilogram by the square of height in meters. Subjects with BMI ≥ 25.0 kg per m^2 were considered obese (Anuurad et al., 2003). Waist circumference (WC) was measured at the mid-point between the lowest margin of the least palpable rib and the top of the iliac crest in the standing position. Central obesity was defined as WC > 90 cm for men and > 80 cm for women (Alberti et al., 2005). Blood pressure was measured in the sitting position using standardized equipment. A subject was considered hypertensive if there was a self-reported history of hypertension, if the subject was on anti-hypertensive medication(s), if the systolic blood pressure was ≥ 130 mmHg, or if the diastolic blood pressure was ≥ 85 mmHg.

All subjects had venous blood drawn after an overnight fast for complete blood count, blood glucose, glycated hemoglobin (HbA1c), lipid profile, liver profile and tests for viral hepatitis B and C infection. Biochemical measurements were performed using

standard laboratory procedures. A subject was considered to have diabetes mellitus if there was a self-reported history of diabetes mellitus, if the subject was on anti-diabetic medication(s), or if fasting blood sugar (FBS) was ≥ 7.0 mmol/L. A subject was considered to have dyslipidemia if there was a self-reported history of dyslipidemia, if the subject was on lipid-lowering medication(s), if the serum total cholesterol (TC) was ≥ 5.2 mmol/L, if the serum triglyceride (TG) was ≥ 1.7 mmol/L, if the serum high-density lipoprotein (HDL) was < 1.0 mmol/L for men or < 1.3 mmol/L for women, or if the serum low-density lipoprotein (LDL) was ≥ 3.4 mmol/L. The Elecsys HBsAg II assay and the Elecsys Anti-HCV II assay (Roche, Mannheim, Germany) were used to test for viral hepatitis B and C infection, respectively.

Liver biopsy and histological assessment

Ultrasonography-guided percutaneous liver biopsy was performed by either one of two experienced operators (WKC, SM) using 18 G Temno ® II semi-automatic biopsy needle (Cardinal Health, Dublin, Ohio, USA) (**Figure 8.1**). Liver biopsy slides were stained with hematoxylin and eosin stain and masson trichrome stain. Liver biopsy slides were examined by an experienced histopathologist (NRNM) who was blinded to clinical data. Histopathological findings were reported according to the Non-Alcoholic Steatohepatitis Clinical Research Network Scoring System (Kleiner et al., 2005). The NAFLD activity score (NAS) is the sum of scores for hepatic steatosis (0 – 3), lobular inflammation (0 – 3) and hepatocyte ballooning (0 – 2). NAS 0 – 2 is not diagnostic of NASH, 3 – 4 is borderline NASH and 5 – 8 is definite NASH. Hepatic steatosis were graded as follows: S0 = steatosis < 5 %, S1 = steatosis 5 % – 33 %, S2 = steatosis 33 % – 66 %, and S3 = steatosis > 66 % (**Figure 8.2a – d**). Steatosis was considered significant at a grade of ≥ S1. Fibrosis was staged 0 – 4 (0 = no fibrosis, 1 = mild fibrosis, 2 = moderate fibrosis, 3 = severe fibrosis, 4 = cirrhosis).

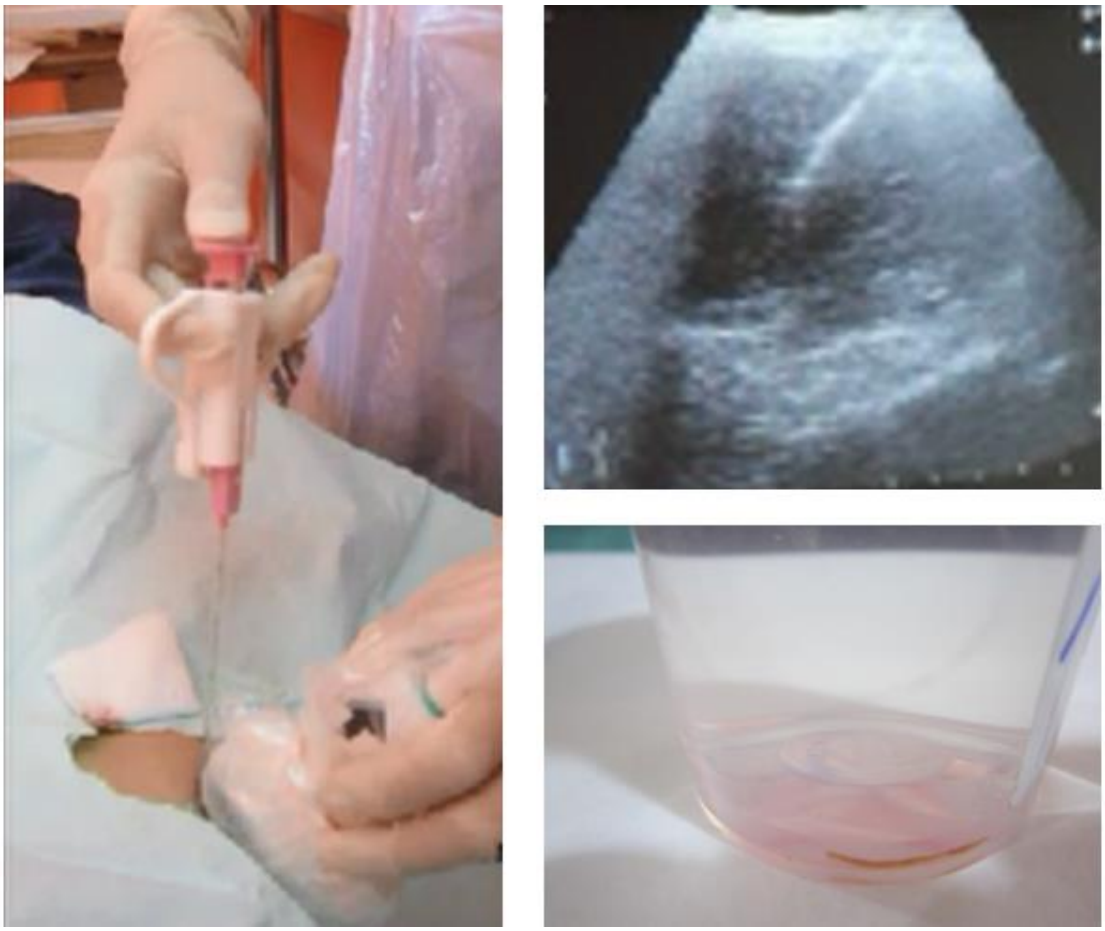

Figure 8.1 (clockwise from left): Ultrasound-guided percutaneous liver biopsy; ultrasound image showing the echogenic liver biopsy needle within the liver parenchyma; a liver biopsy specimen placed in a container containing formalin.

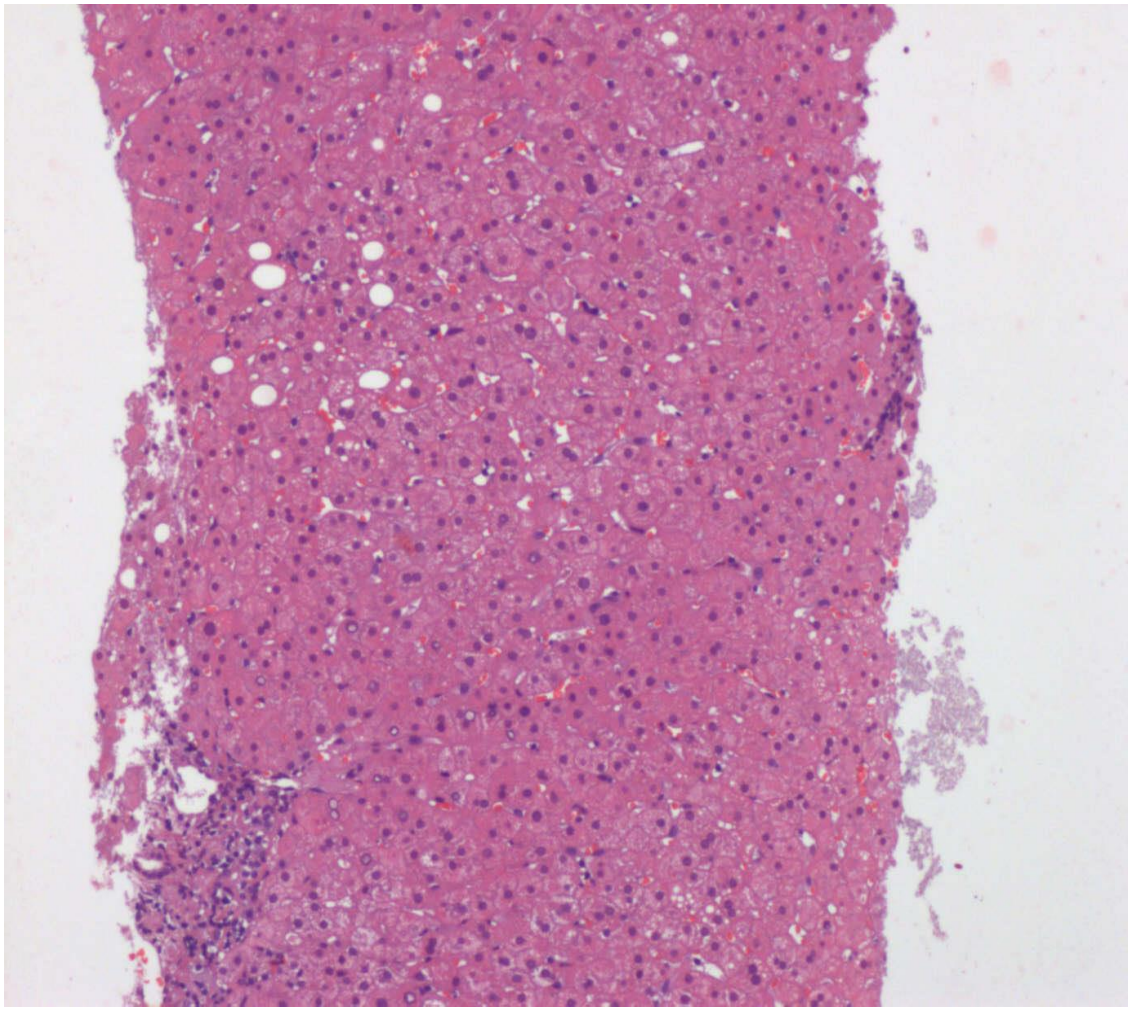

Figure 8.2a Steatosis grade S0.

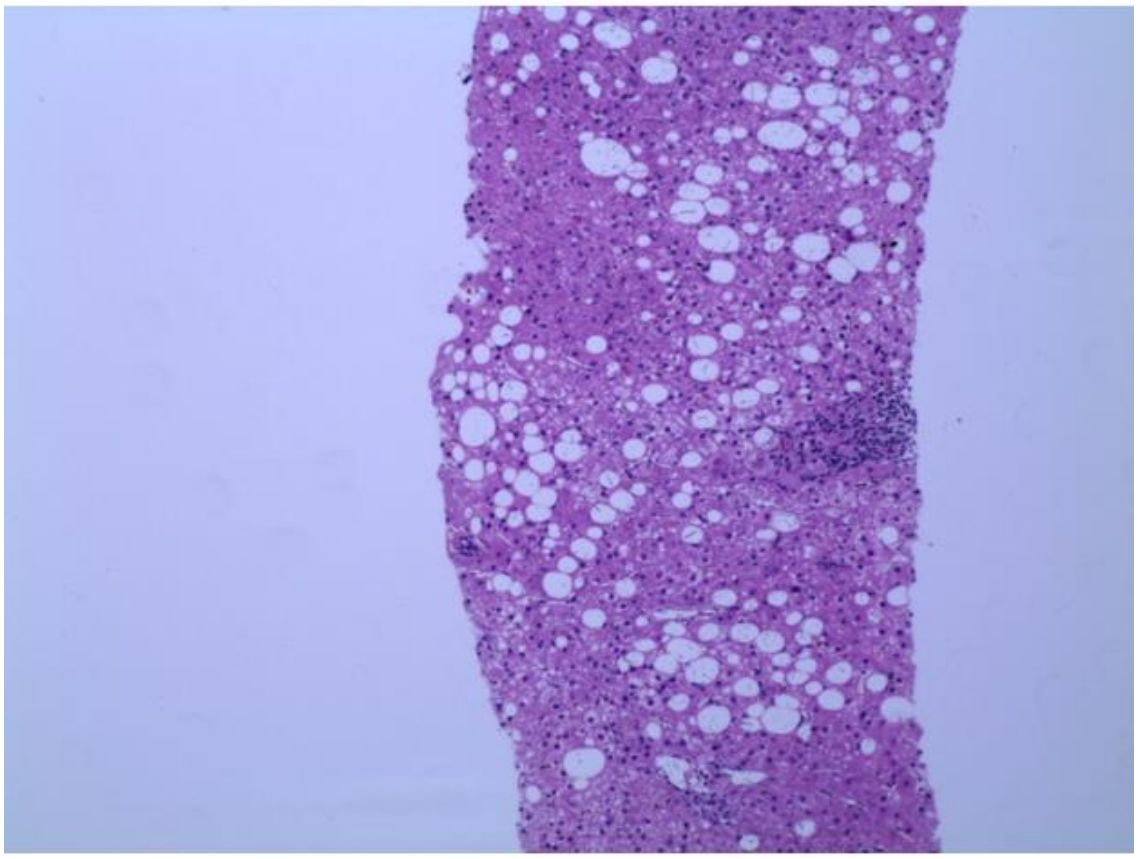

Figure 8.2b Steatosis grade S1.

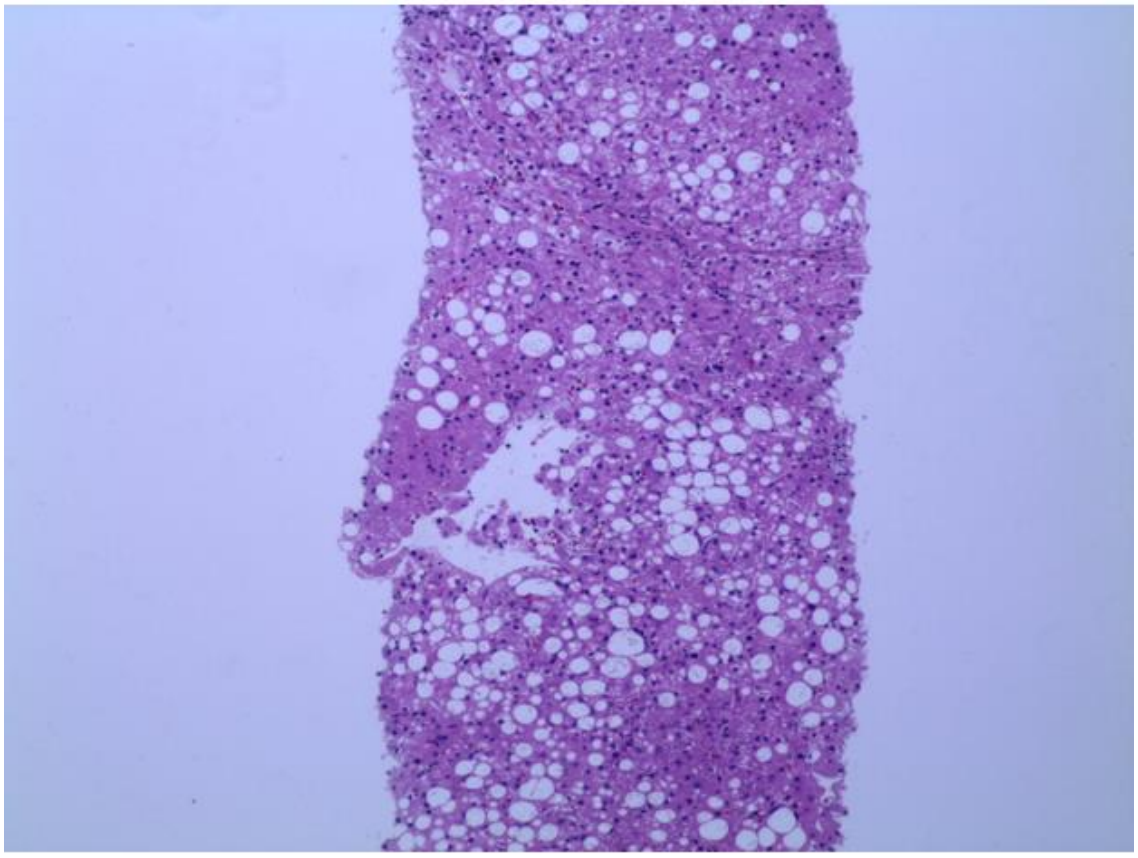

Figure 8.2c Steatosis grade S2

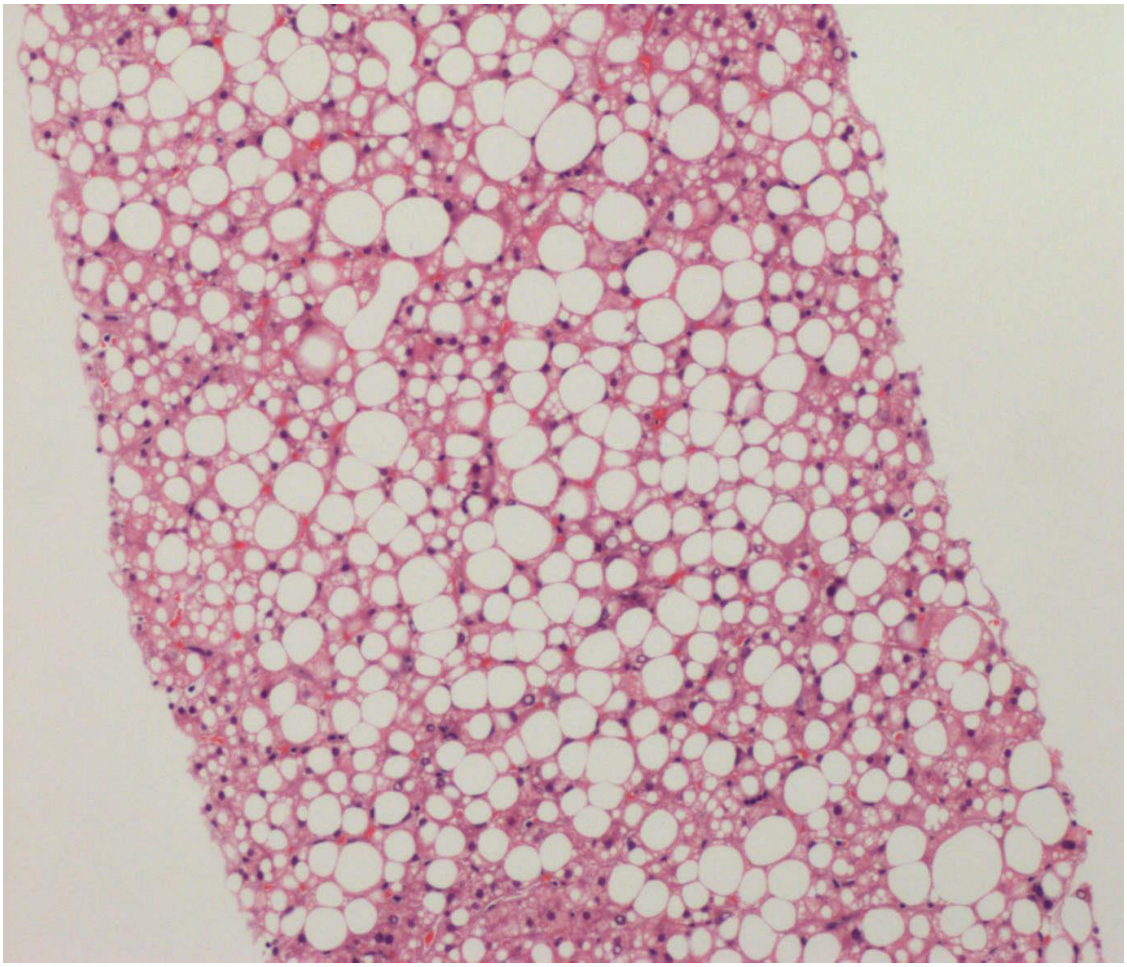

Figure 8.2d Steatosis grade S3

Transient elastography

Transient elastography was performed by either one of two experienced operators (WKC, SM) using Fibroscan 502 Touch with M probe (EchoSens, Paris, France) on the same day of the liver biopsy procedure (**Figure 8.3**). Ten measurements were obtained for each patient. Adequate pressure of the probe on the skin surface, good layering on TM mode and a straight imaginary line on A mode were ensured for each measurement. An examination was considered successful when valid measurements were ≥ 80 % and IQR/median for liver stiffness estimation was ≤ 30 %. Subjects with unsuccessful examination were excluded from analysis.

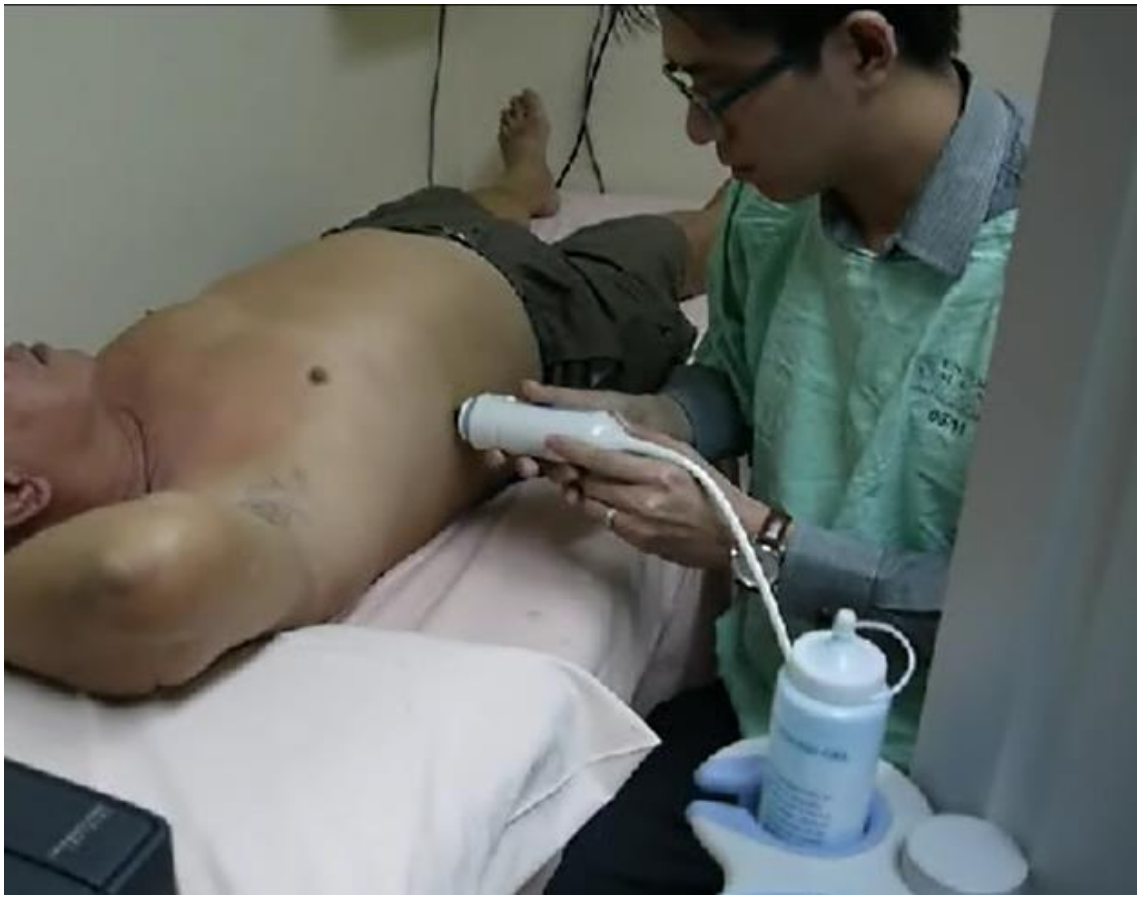

Figure 8.3 Transient elastography using Fibroscan 502 Touch with M probe

Statistical analysis

Data were analyzed using SPSS 15.0. Continuous variables were expressed as mean ± standard deviation or median (interquartile range) as appropriate. Categorical variables were expressed as percentages. Simple (i.e. univariate) and multiple (i.e. multivariate) linear regression analyses were performed to identify factors associated with CAP. Boxplots were used to show the distribution of CAP according to grades of steatosis, lobular inflammation and ballooning, and stages of fibrosis. CAP values for different groups were compared using Mann-Whitney U test and Kruskal-Wallis test. Univariate and multivariate logistic regression analyses were performed to identify factors associated with significant hepatic steatosis. Significance was assumed when $p < 0.05$.

The performance of CAP for diagnosis of hepatic steatosis was determined using areas under receiver operating characteristics curves (AUROC). AUROCs were interpreted as follows: 0.90 – 1.00 = excellent, 0.80 – 0.90 = good, 0.70 – 0.80 = fair, < 0.70 = poor. Optimal cut-off values for CAP for diagnosis of hepatic steatosis were the values that provided the greatest sum of sensitivity and specificity. The sensitivity, specificity, positive predictive value and negative predictive value using the optimal cut-off values were determined. For the purposes of analysis, all controls were regarded as having steatosis grade S0.

8.2.2 Results

Patient and control characteristics

A total of 105 NAFLD patients had liver biopsy during the study period. Four patients were excluded as transient elastography was unsuccessful. Data for 101 patients were analyzed. The mean age was 50.3 ± 11.3 years old and consisted of 51.5 % male. Ninety seven (87.1 %) patients were obese. Eight patients had 1 invalid transient elastography measurement before 10 valid measurements were successfully obtained i.e. success rate 91 %. All other patients had 10 consecutive valid measurements. The frequency of various steatosis grades were as follows: S0 = 63 (60 controls and 3 patients with liver biopsy), S1 = 33, S2 = 51, S3 = 14. Patient and control characteristics are shown in **Table 8.1**.

Table 8.1 Characteristics of non-NAFLD controls and NAFLD patients

	Non-NAFLD controls n = 60	NAFLD patients n = 101
Age, years	24.1 ± 0.9	50.3 ± 11.3
Male, %	36.7	51.5
Body mass index, kg per m^2	20.8 ± 3.4	29.4 ± 3.9
Obesity, %	6.7	87.1
Waist circumference, cm	71.7 ± 9.2	97.7 ± 9.5
Central obesity, %	8.3	96.0
Diabetes mellitus, %	0	52.5
Hypertension, %	15.0	88.1
Dyslipidemia, %	31.7	95.0
Fasting blood glucose, mmol/L	4.6 ± 0.3	6.3 ± 2.3
HbA1c, %	–	6.7 ± 1.6
Triglyceride, mmol/L	0.83 ± 0.35	1.76 ± 0.76
Total cholesterol, mmol/L	4.74 ± 0.67	5.00 ± 1.17
High-density lipoprotein, mmol/L	1.56 ± 0.34	1.12 ± 0.23
Low-density lipoprotein, mmol/L	2.80 ± 0.64	3.12 ± 1.03
Alkaline phosphatase, IU/L	62 ± 15	80 ± 23
Alanine aminotransferase, IU/L	21 (17 – 24)	71 (44 – 115)
Aspartate aminotransferase, IU/L	18 (15 – 21)	41 (29 – 65)
Gamma glutamyl transpeptidase, IU/L	19 (16 – 25)	75 (47 – 125)
Transient elastography		
Success rate, %	98.1 ± 3.7	99.2 ± 2.6
Median E, kPa	4.8 (4.0 – 6.1)	7.8 (5.9 – 11.7)
IQR/median for E, %	12.5 (9.0 – 16.0)	1.8 (0.9 – 12.0)
Median CAP, dB/m	184 (149 – 217)	318 (287 – 345)
IQR/median for CAP, %	20 (15 – 34)	16 (8 – 26)
Liver biopsy length, mm	–	14.7 ± 3.9
Number of portal tracts	–	8.3 ± 2.7
Steatosis score, %		
S0	–	3.0
S1	–	32.7
S2	–	50.5
S3	–	13.9
NAFLD activity score, %		
0 – 2	–	5.9
3 – 4	–	48.5
5 – 8	–	45.5
Fibrosis score, %		
F0	–	30.7
F1	–	44.6
F2	–	5.9
F3	–	15.8
F4	–	3.0

E = estimated liver stiffness, IQR = interquartile range

Factors associated with CAP on univariate and multivariate analyses

Factors associated with CAP on univariate (i.e. simple) and multivariate (i.e. multiple) linear regression analyses are shown in **Table 8.2**. On univariate analysis, age, BMI, WC, ALT, AST, FBS, TG, TC and HDL were associated with CAP but gender and LDL were not. Steatosis grade and the NAFLD activity score were associated with CAP. The association between NAFLD activity score and CAP was contributed by the steatosis component of the score as the NAFLD activity score was no longer associated with CAP when the steatosis component was removed from the score. Lobular inflammation and ballooning grades, and fibrosis stage were not associated with CAP. Age was not included in the multivariate analysis as non-NAFLD controls were significantly younger than NAFLD patients due to the selection process of the former. BMI and WC were not analyzed together in multivariate analysis as these parameters were closely associated. BMI was entered into multivariate analysis instead of WC as the former had a stronger association with CAP on univariate analysis. On multivariate analysis, only BMI, TG and steatosis grade remained significantly associated with CAP.

Table 8.2 Univariate (i.e. simple) and multivariate (i.e. multiple) linear regression analyses of factors associated with CAP

	Univariate		Multivariate	
	OR (95 % CI)	p	OR (95 % CI)	p
Age	3.31 (2.72 – 3.90)	< 0.001	–	–
Gender	-18.48 (-42.67 – 5.70)	0.133	–	–
Body mass index	9.94 (8.45 – 11.44)	< 0.001	4.34 (2.46 – 6.22)	< 0.001
Waist circumference	3.71 (3.20 – 4.22)	< 0.001	–	–
Fasting blood glucose	13.64 (7.96 – 19.32)	< 0.001	2.51 (-1.56 – 6.57)	0.225
Triglyceride	57.59 (44.82 – 70.36)	< 0.001	13.59 (0.85 – 26.33)	0.037
Total cholesterol	18.09 (6.45 – 29.84)	0.003	4.15 (-4.07 – 12.37)	0.320
High-density lipoprotein cholesterol	-116.78 (-146.65 – -86.91)	< 0.001	-17.40 (-46.29 – 11.49)	0.236
Low-density lipoprotein cholesterol	20.58 (7.48 – 33.68)	0.092	–	–
Alkaline phosphatase	1.05 (0.53 – 1.58)	< 0.001	0.08 (-0.29 – 0.45)	0.660
Alanine aminotransferase	0.79 (0.58 – 1.01)	< 0.001	-0.17 (-0.47 – 0.13)	0.264
Aspartate aminotransferase	1.24 (0.87 – 1.61)	< 0.001	0.47 (0.00 – 0.95)	0.050
Gamma glutamyl transpeptidase	0.38 (0.23 – 0.53)	< 0.001	-0.07 (-0.19 – 0.05)	0.241
Steatosis grade	56.36 (48.43 – 64.29)	< 0.001	29.16 (17.96 – 40.37)	< 0.001
Lobular inflammation grade	7.96 (-7.00 – 22.92)	0.294	–	–
Ballooning grade	0.54 (-14.10 – 15.17)	0.942	–	–
NAS	8.05 (1.17 – 14.94)	0.022	–	–
NAS without steatosis component	2.65 (-5.72 – 11.00)	0.532	–	–
Fibrosis stage	1.03 (-7.33 – 9.40)	0.807	–	–

CAP = controlled attenuation parameter, NAS = non-alcoholic fatty liver disease activity score

CAP for the detection and quantification of hepatic steatosis

CAP according to steatosis grade is shown in **Figure 8.4a**. The median CAP and its interquartile range for steatosis grades S0, S1, S2 and S3 was 184 (152 – 218), 305 dB/m (276 dB/m – 340 dB/m), 320 dB/m (305 dB/m – 346 dB/m) and 324 dB/m (291 dB/m – 351 dB/m), respectively. Although there was an overall significant increase in CAP across the steatosis grades ($p < 0.001$), the difference in CAP was only significant between steatosis grades S0 and S1 ($p < 0.001$). The AUROC, optimal cut-off for CAP, sensitivity, specificity, positive predictive value and negative predictive value for estimation of steatosis grades ≥ S1, S2 and S3 are shown in **Table 8.3**. The AUROC for estimation of steatosis grades ≥ S1, S2 and S3 was similar when only patients with liver biopsy specimen ≥ 15 mm and ≥ 6 portal tracts were analyzed (0.98, 0.65 and 0.59, respectively).

CAP according to steatosis grades in non-obese and obese subjects are illustrated in **Figure 8.4b** and **Figure 8.4c**, respectively. The diagnostic performance of CAP for estimation of steatosis grades in obese and non-obese subjects is shown in **Tables 8.4a and 8.4b**, respectively. The diagnostic performance of CAP was poor in obese subjects compared to non-obese patients with the exception of detection of significant hepatic steatosis. Among non-obese patients, the AUROC for estimation of steatosis grades ≥ S1 and S2 were 0.99 and 0.99, respectively. Among obese patients, the AUROC for estimation of steatosis grades ≥ S1, S2 and S3 were 0.92, 0.64 and 0.58, respectively. There was no significant trend in CAP according to grades of lobular inflammation and ballooning, and stages of fibrosis (**Figure 8.5a – 8.5c**).

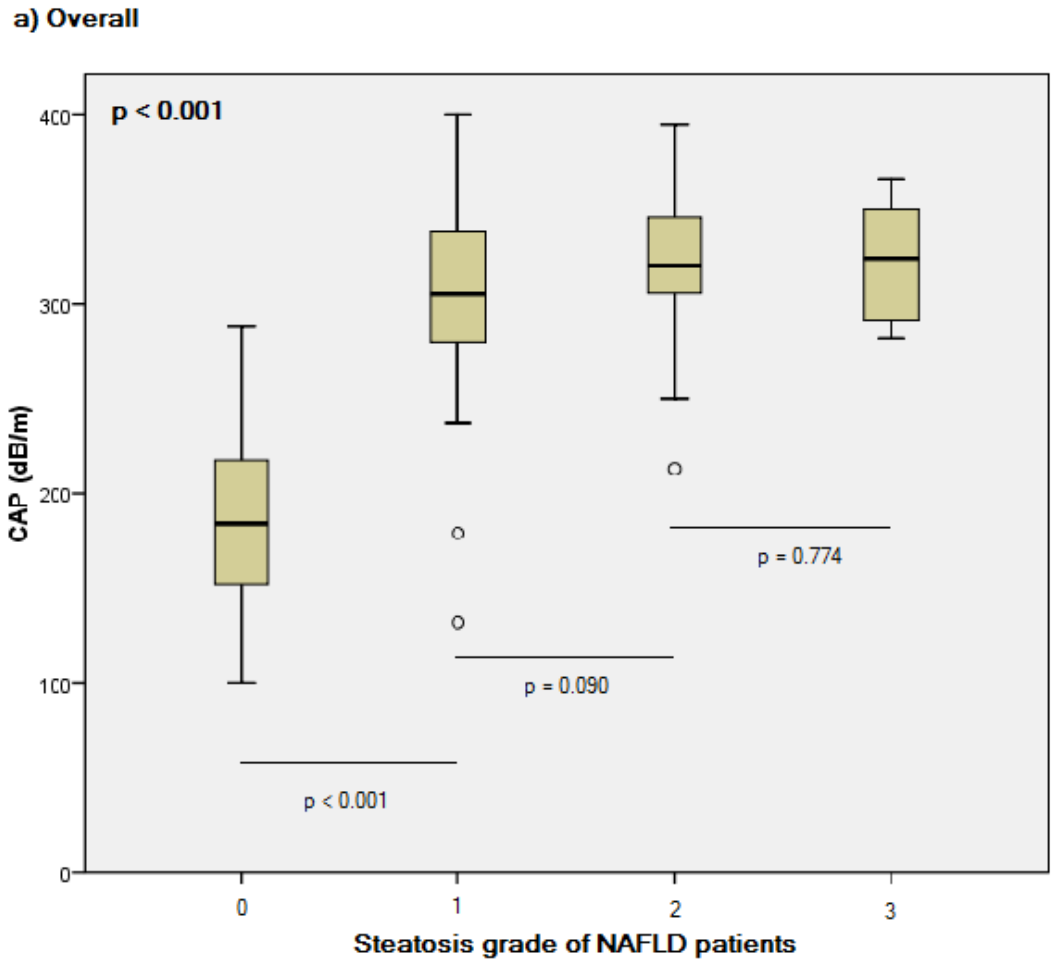

Figure 8.4a Boxplot showing CAP according to steatosis grades in the overall study population

Number of subjects for each of the steatosis grades: S0 = 63, S1 = 33, S2 = 51, S3 = 14.

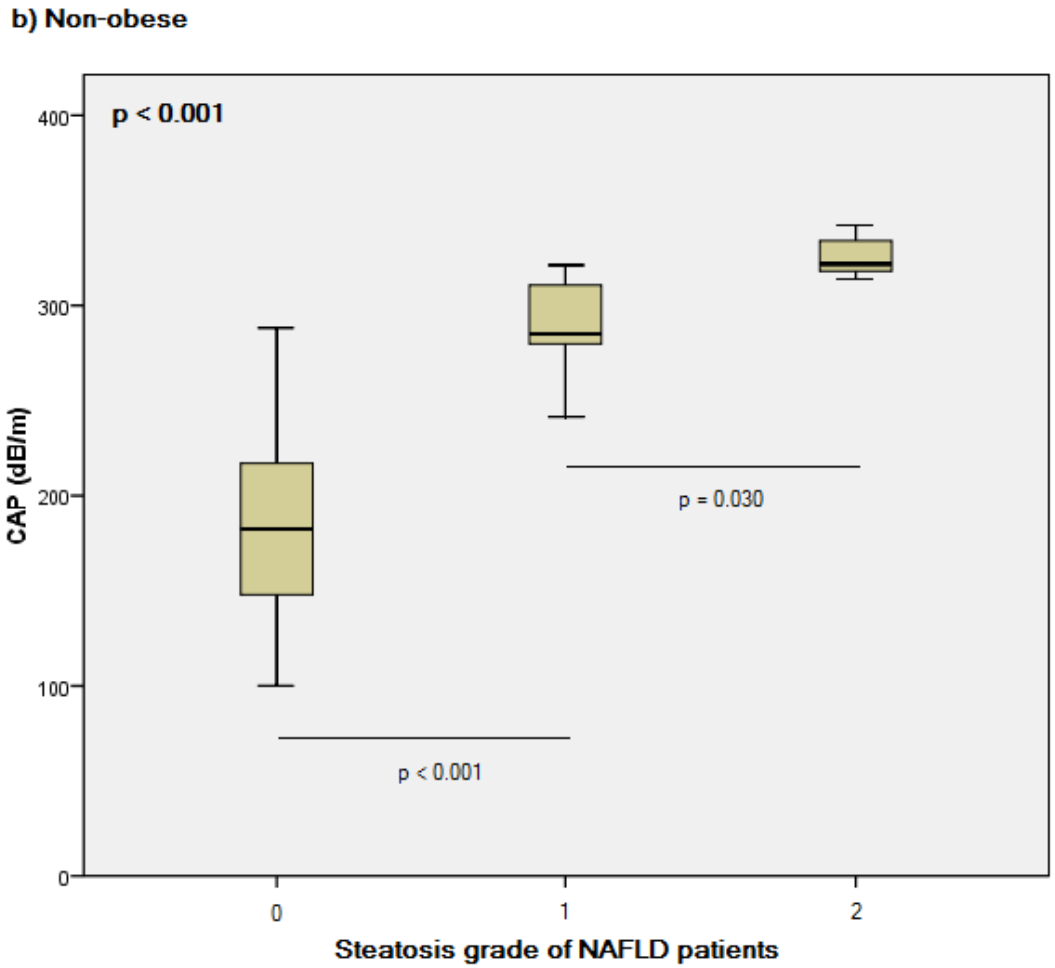

Figure 8.4b Boxplot showing CAP according to steatosis grades among non-obese subjects

Number of subjects for each of the steatosis grades: S0 = 58, S1 = 5, S2 = 6.

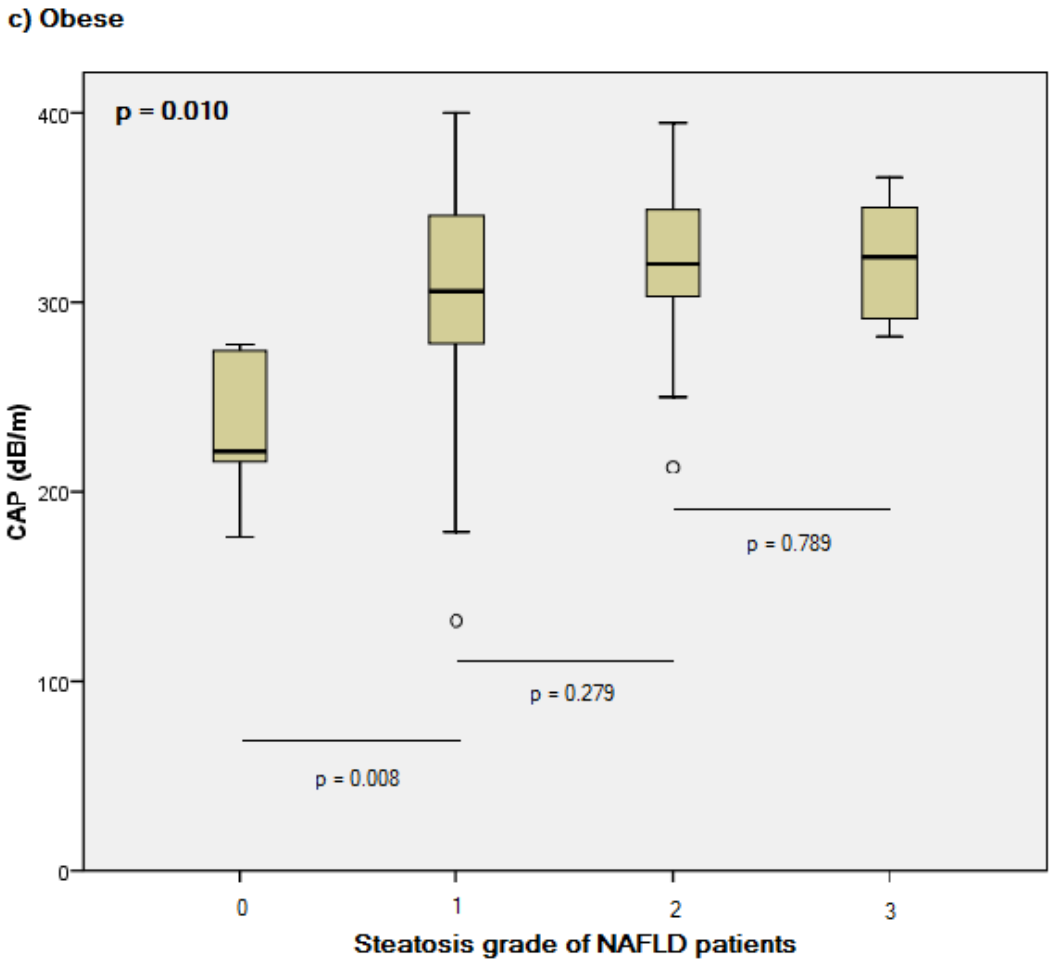

Figure 8.4c Boxplot showing CAP according to steatosis grades among obese subjects

Number of subjects for each of the steatosis grades: S0 = 5, S1 = 28, S2 = 45, S3 = 14.

Table 8.3 The AUROC, the optimal cut-offs for CAP, and the sensitivity, specificity, positive predictive value and negative predictive value for estimation of steatosis grades 1, 2 and 3

	S1	S2	S2*	S3	S3*
AUROC	0.97	0.86	0.86	0.75	0.75
Optimal cut-off, dB/m	263	263	281	281	283
Sensitivity	91.8	96.9	89.2	100.0	92.9
Specificity	93.7	67.7	74.0	53.1	54.4
Positive predictive value	95.7	67.0	69.9	16.9	16.2
Negative predictive value	88.1	97.0	91.0	100.0	98.8

Optimal cut-off is the value for CAP that provided the greatest sum of sensitivity and specificity for estimation of steatosis equal to or greater than the respective grades.

*Alternative optimal cut-off is the next CAP value above the optimal cut-off that provided the greatest sum of sensitivity and specificity for estimation of steatosis equal to or greater than the respective grades.

AUROC = area under the receiver operating characteristics curve, CAP = controlled attenuation parameter, S = steatosis grade

Table 8.4a The AUROC, the optimal cut-offs for CAP, and the sensitivity, specificity, positive predictive value and negative predictive value for estimation of hepatic steatosis grades 1, 2 and 3 in non-obese subjects (n = 69)

	S1	S2	S3
N	11	6	0
AUROC	0.99	0.99	–
Optimal cut-off, dB/m	239	313	–
Sensitivity, %	100	100	–
Specificity, %	91.4	98.4	–
Positive predictive value, %	68.8	85.7	–
Negative predictive value, %	91.4	98.4	–

Optimal cut-off is the value for CAP that provided the greatest sum of sensitivity and specificity for estimation of steatosis equal to or greater than the respective grades.

Estimation of AUROC could not be performed for diagnosis of hepatic steatosis grade 3 in non-obese subjects as there were no non-obese subjects with hepatic steatosis grade 3.

AUROC = area under the receiver operating characteristics curve, CAP = controlled attenuation parameter, S = steatosis grade, N = number of subjects with corresponding steatosis grade and above

Table 8.4b The AUROC, the optimal cut-offs for CAP, and the sensitivity, specificity, positive predictive value and negative predictive value for estimation of hepatic steatosis grades 1, 2 and 3 in obese subjects (n = 92)

	S1	S2	S3
N	87	59	14
AUROC	0.92	0.64	0.58
Optimal cut-off, dB/m	280	309	278
Sensitivity, %	83.9	69.5	100.0
Specificity, %	100.0	63.6	23.1
Positive predictive value, %	100.0	77.4	18.9
Negative predictive value, %	26.3	53.8	100.0

Optimal cut-off is the value for CAP that provided the greatest sum of sensitivity and specificity for estimation of steatosis equal to or greater than the respective grades.

AUROC = area under the receiver operating characteristics curve, CAP = controlled attenuation parameter, S = steatosis grade, N = number of subjects with corresponding steatosis grade and above

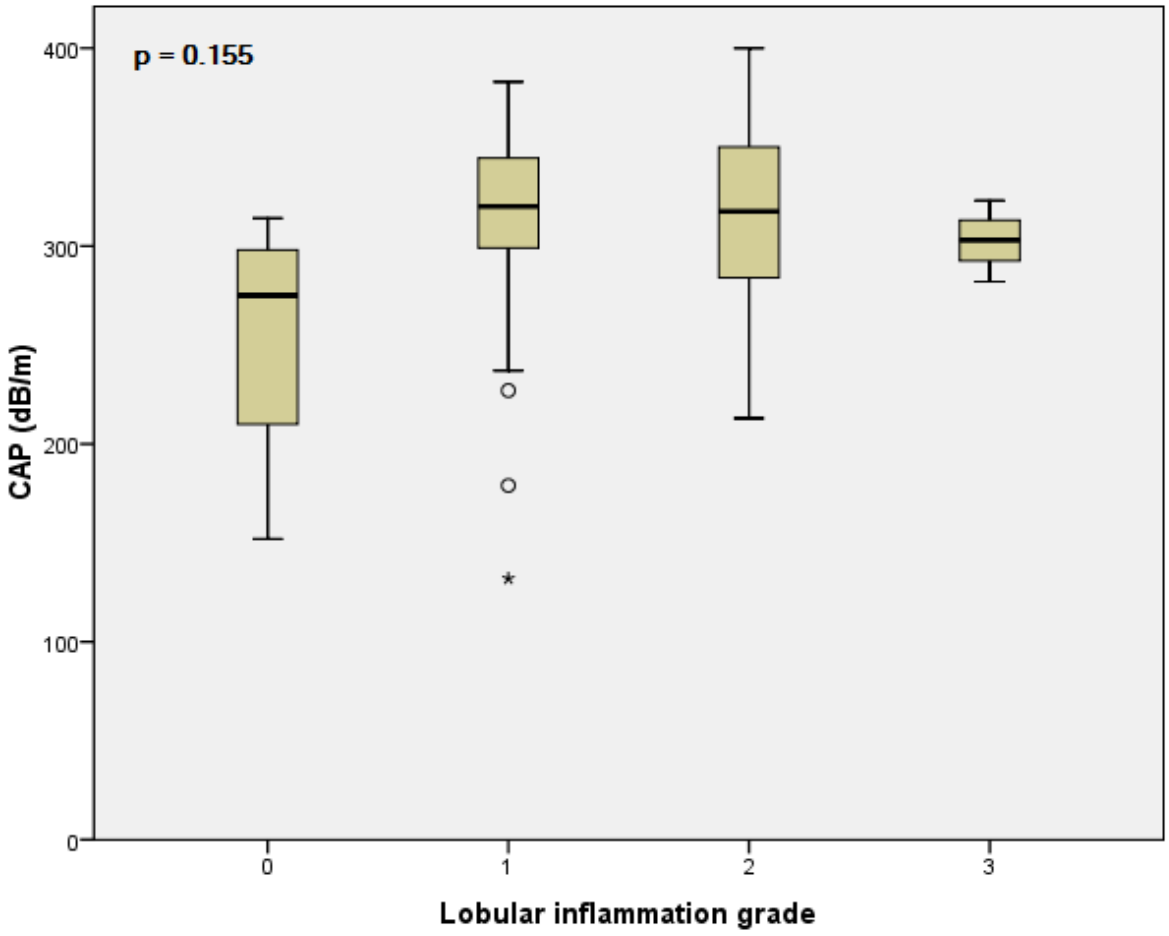

Figure 8.5a Boxplots showing CAP according to lobular inflammation grades

Number of subjects for each of the lobular inflammation grades: grade 0 = 4, grade 1 = 52, grade 2 = 42, grade 3 = 3.

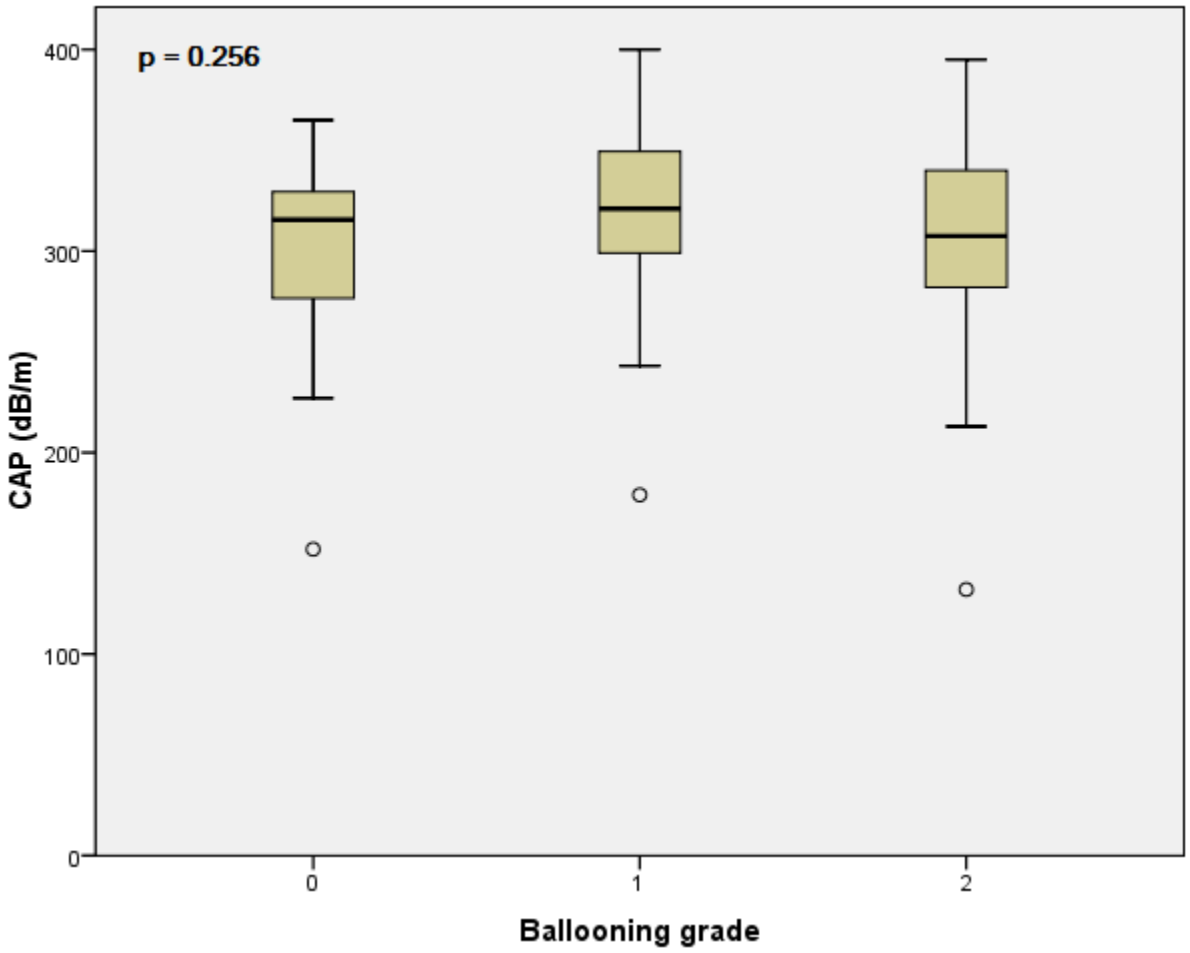

Figure 8.5b Boxplots showing CAP according to ballooning grades

Number of subjects for each of the ballooning grades: grade 0 = 16, grade 1 = 59, grade 2 = 26.

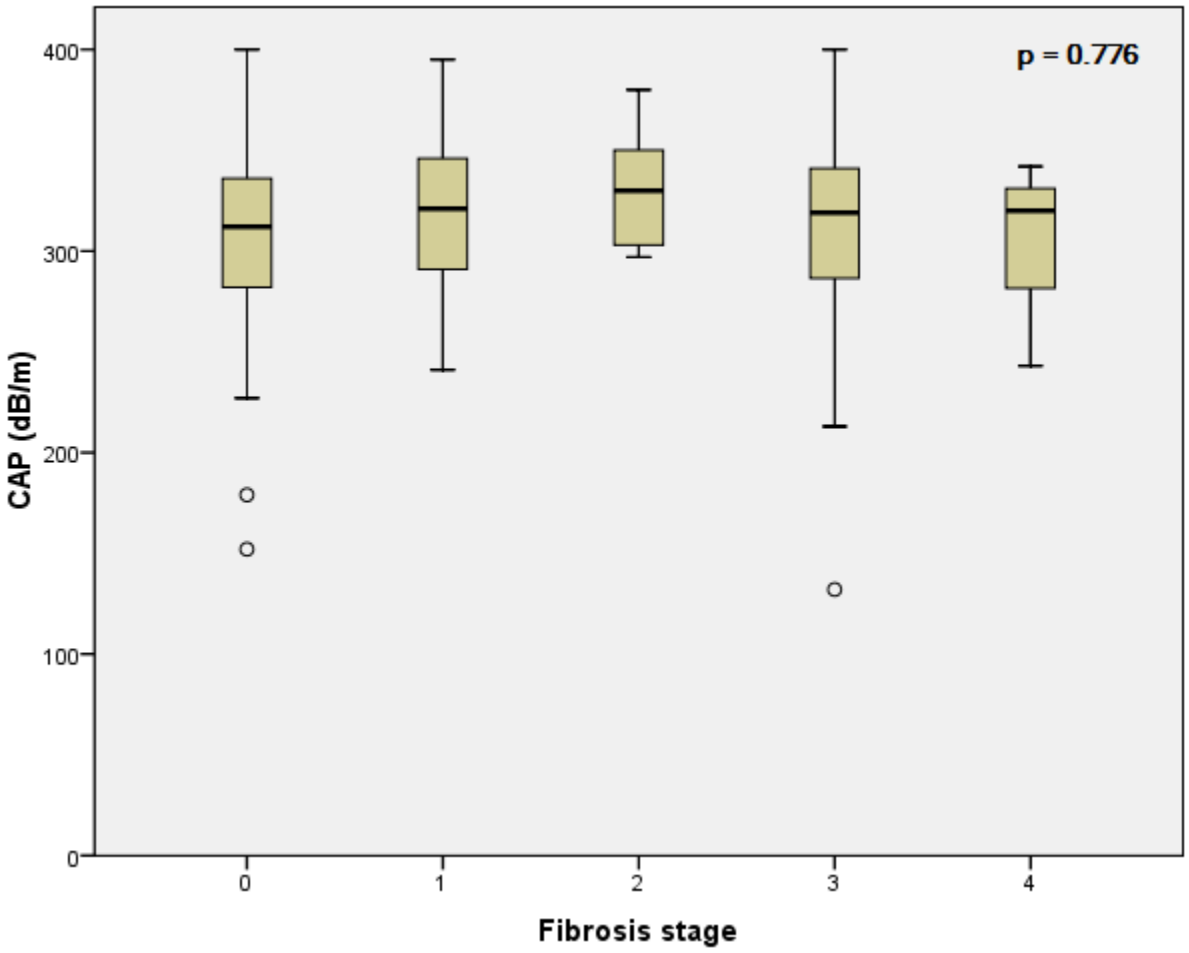

Figure 8.5c Boxplots showing CAP according to fibrosis stages

Number of subjects for each of the fibrosis stages: F0 = 31, F1 = 45, F2 = 6, F3 = 16, F4 = 3.

Factors associated with significant hepatic steatosis on univariate and multivariate analyses

Factors that were associated with significant hepatic steatosis on univariate analysis were age, BMI, WC, FBS, TG, HDL, LDL, ALP, ALT, AST, GGT and CAP. However, on multivariate analysis, only CAP remained an independent determinant of significant hepatic steatosis (**Table 8.5**).

Table 8.5 Univariate and multivariate logistic regression analyses of factors associated with significant hepatic steatosis

	Univariate analysis		Multivariate analysis	
	OR (95 % CI)	p	OR (95 % CI)	p
Age	1.31 (1.19 – 1.44)	< 0.001	–	
Male gender	1.89 (0.99 – 3.61)	0.055	–	
Body mass index	1.86 (1.54 – 2.25)	< 0.001	1.20 (0.85 – 1.69)	0.293
Waist circumference	1.30 (1.19 – 1.41)	< 0.001	–	
Fasting blood sugar	6.04 (2.87 – 12.71)	< 0.001	1.40 (0.54 – 3.64)	0.975
Triglyceride	88.97 (22.57 – 350.79)	< 0.001	46.85 (0.26 –8483)	0.147
Total cholesterol	1.33 (0.96 – 1.84)	0.092	–	
High-density lipoprotein cholesterol	0.00 (0.00 – 0.027)	< 0.001	0.01 (0.00 – 12.02)	0.194
Low-density lipoprotein cholesterol	1.50 (1.03 – 2.19)	0.034	0.20 (0.03 – 1.31)	0.093
Alkaline phosphatase	1.05 (1.03 – 1.07)	< 0.001	1.04 (0.96 – 1.13)	0.365
Alanine aminotransferase	1.09 (1.06 – 1.12)	< 0.001	1.03 (0.96 – 1.11)	0.408
Aspartate aminotransferase	1.12 (1.08 – 1.17)	< 0.001	0.94 (0.84 – 1.05)	0.942
Gamma glutamyl transpeptidase	1.08 (1.05 – 1.11)	< 0.001	1.03 (1.00 – 1.06)	0.094
Controlled attenuation parameter	1.05 (1.04 – 1.07)	< 0.001	1.05 (1.02 – 1.10)	0.008

8.2.3 Discussion

In this study on NAFLD patients, we found that CAP was excellent for the diagnosis of hepatic steatosis ≥ S1 (AUROC of 0.97) but less accurate for the diagnosis of hepatic steatosis ≥ S2 and S3 with an AUROC of 0.86 and 0.75, respectively. CAP was reported to be excellent for the diagnosis of steatosis in the first published study on this technique by Sasso et al. The AUROC for the diagnosis of hepatic steatosis grades ≥ S1, S2 and S3 was reported to be 0.91, 0.95 and 0.89, respectively (Sasso et al., 2010). However, such excellent results have not been reproduced in most subsequent studies. Overall, the performance of CAP was good for the diagnosis of hepatic steatosis grade ≥ S1 with AUROC of 0.79 – 0.89, but was only fair for steatosis grade ≥ S2 and S3 with AUROC of 0.72 – 0.79 and 0.70 – 0.76, respectively (Chon et al., 2013; de Ledinghen et al., 2012; Kumar et al., 2013; Masaki et al., 2013; Myers et al., 2012).

To date, all published studies on CAP included patients with chronic liver disease of various etiologies except one which included only patients with chronic hepatitis C (Sasso et al., 2012). In patients with chronic hepatitis C, the performance of CAP was good with an AUROC of 0.80, 0.86 and 0.88 for estimating hepatic steatosis equal to or greater than S1, S2 and S3, respectively (Sasso et al., 2012). In contrast, the performance of CAP was lower in our cohort of NAFLD patients due to substantial overlap in CAP among grades S1, S2 and S3 of hepatic steatosis. In addition, we found that the performance of CAP appeared to be compromised by an increased BMI. We demonstrated that CAP was independently associated with BMI and that the ability of CAP to estimate hepatic steatosis dropped remarkably in obese subjects when compared to non-obese subjects. We suspect that the increased subcutaneous tissue thickness in subjects with greater BMI may have affected CAP measurement. Further studies are needed to confirm this observation and to see if adjustments

can be made to improve the performance of CAP in NAFLD patients with an increased BMI particularly when non-invasive estimation of hepatic steatosis is arguably more important in these patients.

Liver stiffness measurement with Fibroscan has been shown to provide an excellent estimate of fibrosis stage in NAFLD patients (Yoneda et al., 2007). The procedure is non-invasive, operator-independent, and provides immediate result. CAP has been incorporated in the newer models of Fibroscan and is derived from the same radio-frequency data that is used for liver stiffness measurement so that the result will also be available immediately at the end of the examination. However, in clinical practice, there is really little added benefit of knowing the CAP when performing liver stiffness measurement for patients already diagnosed with NAFLD, particularly when CAP is not reliable in distinguishing the different grades of hepatic steatosis. CAP also does not appear useful to follow changes in hepatic steatosis over time in NAFLD patients due to the substantial overlap in CAP between the different grades of steatosis. However, in cases where hepatic steatosis has reduced to become insignificant, the change can be reliably detected by CAP. Otherwise, numerical change in CAP is difficult to interpret and may be due to reasons other than a change in degree of hepatic steatosis e.g. a different site of measurement, a different operator. A study showed that while CAP was operator-independent with an absolute difference of 20 dB which was not significant between operators, this difference was sufficient to result in poor concordance for the classification of hepatic steatosis (Recio et al., 2013).

Ultrasound examination of the liver is reasonably good for the detection of moderate to severe hepatic steatosis but is operator- and machine-dependent, and less reliable for detecting mild steatosis (Saadeh et al., 2002). While CAP is able to detect the presence of mild steatosis, it does not provide images with anatomical details. In clinical practice, patients

with elevated serum aminotransferase level would have an ultrasound examination which will be able to demonstrate fatty liver in most cases of NAFLD and exclude other pathology at the same time. However, incorporating a method to measure ultrasound attenuation similar to CAP during ultrasound examination will theoretically remove operator- and machine-dependence in diagnosis of fatty liver and improve detection of mild steatosis. The usefulness of CAP as a screening tool to detect significant hepatic steatosis in the population is unclear and deserves further study. CAP may also be useful as a non-invasive method to assess for the presence of significant hepatic steatosis in potential liver donor.

To date, this is the largest study on CAP on a homogenous cohort of NAFLD patients. The study was carried out prospectively according to a planned protocol so that the data collected was robust. Liver biopsy and Fibroscan were performed on the same day to minimize differences in findings due to changes over time. The procedures were carried out by experienced operators to ensure good quality of specimens and measurements. However, as in any study using liver histology as the reference, the study may be limited by sampling and observer variability. Nevertheless, we performed further analysis including only patients with "better" liver biopsy specimens and found no difference in our study findings. Our controls in this study did not have a liver biopsy for ethical reasons, and the cases with grade S0 steatosis in this study may have been over-estimated. However, the fact that CAP was able to delineate clearly between S0 and S1 in this study implies that the control cases truly had no steatosis.

8.2.4 Conclusion

CAP is excellent for the detection of significant hepatic steatosis but is less useful for distinguishing the different grades of significant hepatic steatosis in NAFLD patients. CAP appears to be affected by an increased BMI and further studies to address this limitation is necessary to improve the diagnostic performance of CAP. CAP is non-invasive, operator-independent and provides immediate results. However, its usefulness in NAFLD patients may be limited in clinical practice, unless its diagnostic performance is improved.

Note: Findings from this study was presented at the Asian Pacific Association for the Study of the Liver 2014 Meeting in Brisbane, Australia, and was listed as a Poster of Distinction. The abstract was published in a supplementary issue of Hepatology International (Chan et al., 2014). The findings from this study was also presented at the Malaysian Society of Gastroenterology and Hepatology Annual Scientific Meeting in 2014. The full article has been published in the Journal of Gastroenterology and Hepatology (Chan et al., 2014).

8.3 Plasma cytokeratin-18 fragment level for the diagnosis of NASH

Cytokeratin 18 (CK-18) is the major intermediate filament protein in liver cells and it is cleaved by caspases that are activated during apoptosis of liver cells, a process which plays an important role in non-alcoholic steatohepatitis (NASH) (Feldstein et al., 2003). CK-18 fragment, namely CK18Asp396 (M30), has been studied for the diagnosis of NASH with varying results (Cusi et al., 2014; Diab et al., 2008; Feldstein et al., 2009; Shen et al., 2012; Shen et al., 2012; Tamimi et al., 2011; Wieckowska et al., 2006; Yilmaz et al., 2007). In this study, we aim to evaluate plasma M30 as well as regular serum liver enzymes, namely alanine aminotransferase (ALT), aspartate aminotransferase (AST) and gamma glutamyl transpeptidase (GGT) for the diagnosis of NASH.

8.3.1 Methods

Consecutive adult patients (aged ≥ 18 years) with non-alcoholic fatty liver disease (NAFLD) who were scheduled for a liver biopsy were prospectively recruited between November 2012 and October 2013 for this study. The diagnosis of NAFLD was based on ultrasonography finding of fatty liver and exclusion of significant alcohol intake, use of medications that can cause fatty liver, viral hepatitis B and C infection, and other causes of chronic liver disease where indicated (Chalasani et al., 2012). This study was approved by the University of Malaya Medical Centre's Ethics Committee and all patients who participated provided informed consent.

Demographic, anthropometric, relevant clinical and laboratory data were obtained using a standard protocol on the day of the liver biopsy procedure. Weight and height were measured using standard equipment. Body mass index (BMI) was calculated by dividing weight in kilogram by the square of height in meters. Waist circumference (WC) was

measured at the mid-point between the lowest margin of the least palpable rib and the top of the iliac crest in the standing position. Blood pressure was measured in the sitting position using standardized equipment. A patient was considered hypertensive if there was a self-reported history of hypertension, if the patient was on anti-hypertensive medication(s), if the systolic blood pressure was ≥ 130 mmHg, or if the diastolic blood pressure was ≥ 85 mmHg.

All patients had venous blood drawn after an overnight fast on the day of the liver biopsy procedure for complete blood count, blood sugar, glycated hemoglobin (HbA1c), lipid profile, liver profile, tests for viral hepatitis B and C infection, and for measurement of plasma M30 level. Biochemical measurements were performed using standard laboratory procedures. A patient was considered to have diabetes mellitus if there was a self-reported history of diabetes mellitus, if the patient was on anti-diabetic medication(s), or if fasting blood sugar was ≥ 7.0 mmol/L. A patient was considered to have dyslipidemia if there was a self-reported history of dyslipidemia, if the patient was on lipid-lowering medication(s), if the serum total cholesterol (TC) was ≥ 5.2 mmol/L, if the serum triglyceride (TG) was ≥ 1.7 mmol/L, if the serum high-density lipoprotein (HDL) was < 1.0 mmol/L for men or < 1.3 mmol/L for women, or if the serum low-density lipoprotein (LDL) was ≥ 3.4 mmol/L. Our laboratory's upper limit of normal for liver enzymes were as follow: alkaline phosphatase (ALP) 136 IU/L, AST 37 IU/L, ALT 65 IU/L and GGT 55 IU/L. The Elecsys HBsAg II assay and the Elecsys Anti-HCV II assay (Roche, Mannheim, Germany) were used to test for viral hepatitis B and C infection, respectively.

Controls were recruited from persons attending the Endoscopy Unit, University of Malaya Medical Centre for investigation of dyspepsia or screening colonoscopy. All controls had no history of chronic liver disease and had an ultrasound examination to exclude fatty liver. The presence of hypertension, diabetes mellitus and dyslipidemia was based on self-

report. BMI and WC was determined as described above. Venous blood was drawn after an overnight fast for liver profile and for measurement of plasma M30 level. Percutaneous liver biopsy was not performed for controls due to ethical considerations.

Measurement of plasma M30 level

The blood sample for measurement of plasma M30 level was collected in a plain tube on the same day of the liver biopsy procedure. The blood sample was processed to plasma and stored at – 80 ˚C until further analysis. The plasma was subsequently used for quantitative measurement of M30 using the M30-Apoptosense ELISA kit (PEVIVA, Bromma, Sweden). The test was performed for all samples in a single session by a single investigator (PS).

Liver biopsy and histological assessment

Ultrasonography-guided percutaneous liver biopsy was performed by either one of two experienced operators (WKC, SM) using 18 G Temno ® II semi-automatic biopsy needle (Cardinal Health, Dublin, Ohio, USA). Liver biopsy specimens were processed using standard laboratory procedures. Liver biopsy slides were stained with hematoxylin and eosin stain and masson trichrome stain. Liver biopsy slides were examined by an experienced histopathologist (NRNM) who was blinded to clinical data. Histopathological findings were reported according to the Non-Alcoholic Steatohepatitis Clinical Research Network Scoring System (Kleiner et al., 2005). The NAFLD activity score (NAS) is the sum of scores for hepatic steatosis (0 – 3), lobular inflammation (0 – 3) and hepatocyte ballooning (0 – 2). Specifically, lobular inflammation was graded as follows: grade 0 = none, grade 1 = less than 2 foci, grade 2 = 2 – 4 foci, grade 3 = more than 4 foci (**Figure 8.6a – d**), while ballooning was graded as follows: grade 0 = none, grade 1 = few or mild, grade 2 = many or prominent

(**Figure 8.7a – c**). NAS 0 – 2 is not diagnostic of NASH, 3 – 4 is borderline NASH and 5 – 8 is definite NASH. Patients with NAS < 5 was considered as non-NASH while patients with NAS ≥ 5 were considered to have NASH (**Figure 8.8a – b**). Fibrosis was staged 0 – 4 (0 = no fibrosis, 1 = mild fibrosis, 2 = moderate fibrosis, 3 = severe fibrosis, 4 = cirrhosis).

Statistical analysis

Data were analyzed using a standard statistical software program (SPSS 15.0). Continuous variables were expressed as mean ± standard deviation or median (interquartile range) and analyzed using student's t-test, Mann-Whitney U test or Kruskal-Wallis test, as appropriate. Categorical variables were expressed as percentages and analyzed using chi-square test. Significance was assumed when $p < 0.05$. Boxplots were used to compare the distribution of plasma M30 and serum ALT, AST and GGT levels between healthy controls and NAFLD patients, and between NASH and non-NASH patients. The performance of plasma M30 and serum ALT, AST and GGT levels for prediction of NAFLD and NASH was determined using area under receiver-operating characteristics curve (AUROC). AUROC was interpreted as follows: 0.90 – 1.00 = excellent, 0.80 – 0.90 = good, 0.70 – 0.80 = fair, < 0.70 = poor. The sensitivity, specificity, positive predictive value and negative predictive value using cut-off values for high sensitivity, highest overall accuracy and high specificity were determined. Boxplots were also used to compare the distribution of plasma M30 and serum ALT, AST and GGT levels across the different grades of steatosis, lobular inflammation and ballooning, and across the different stages of fibrosis.

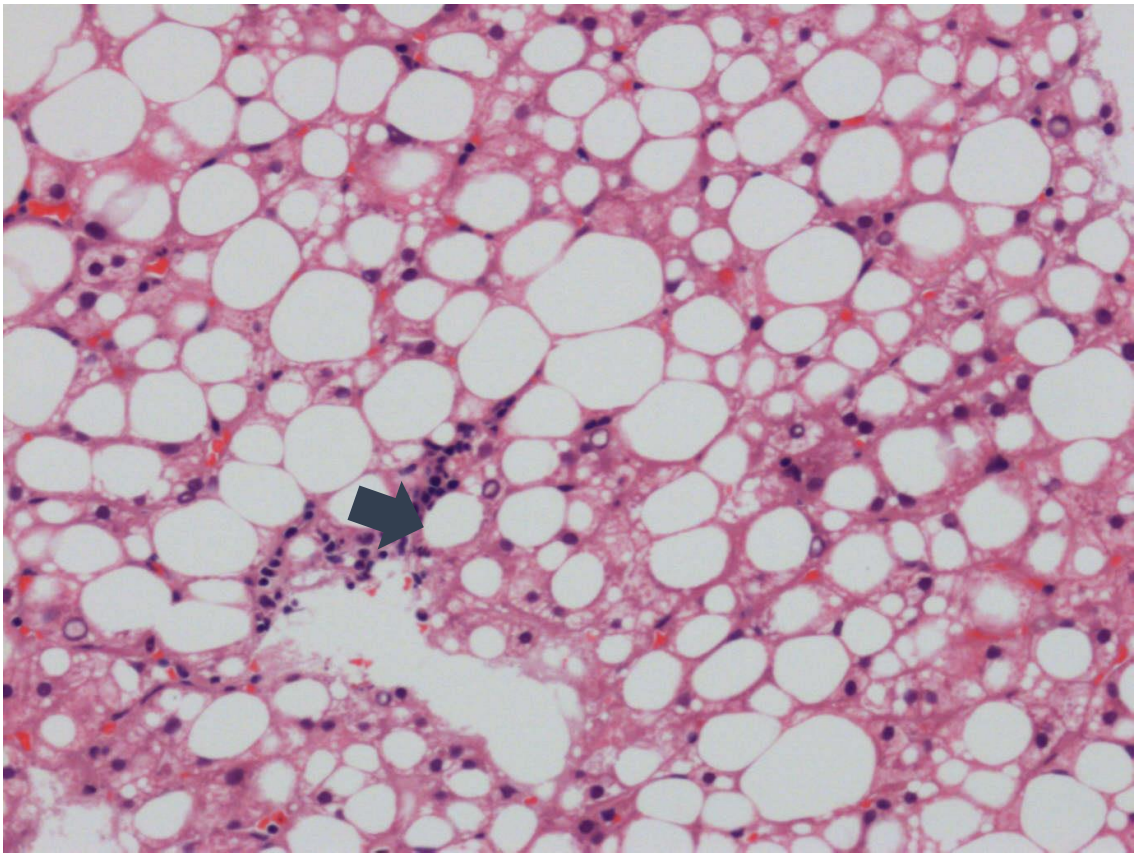

Figure 8.6a Lobular inflammation (arrow)

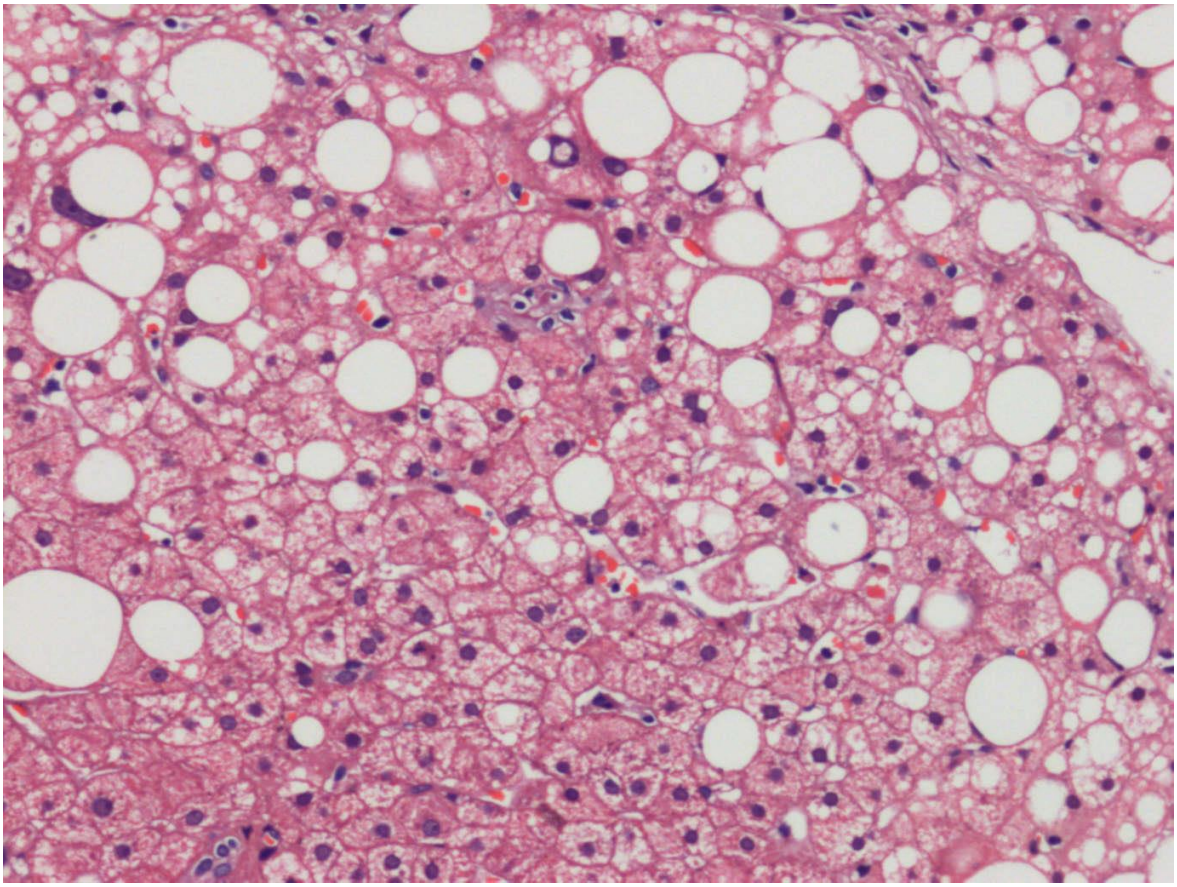

Figure 8.6b Lobular inflammation grade 1

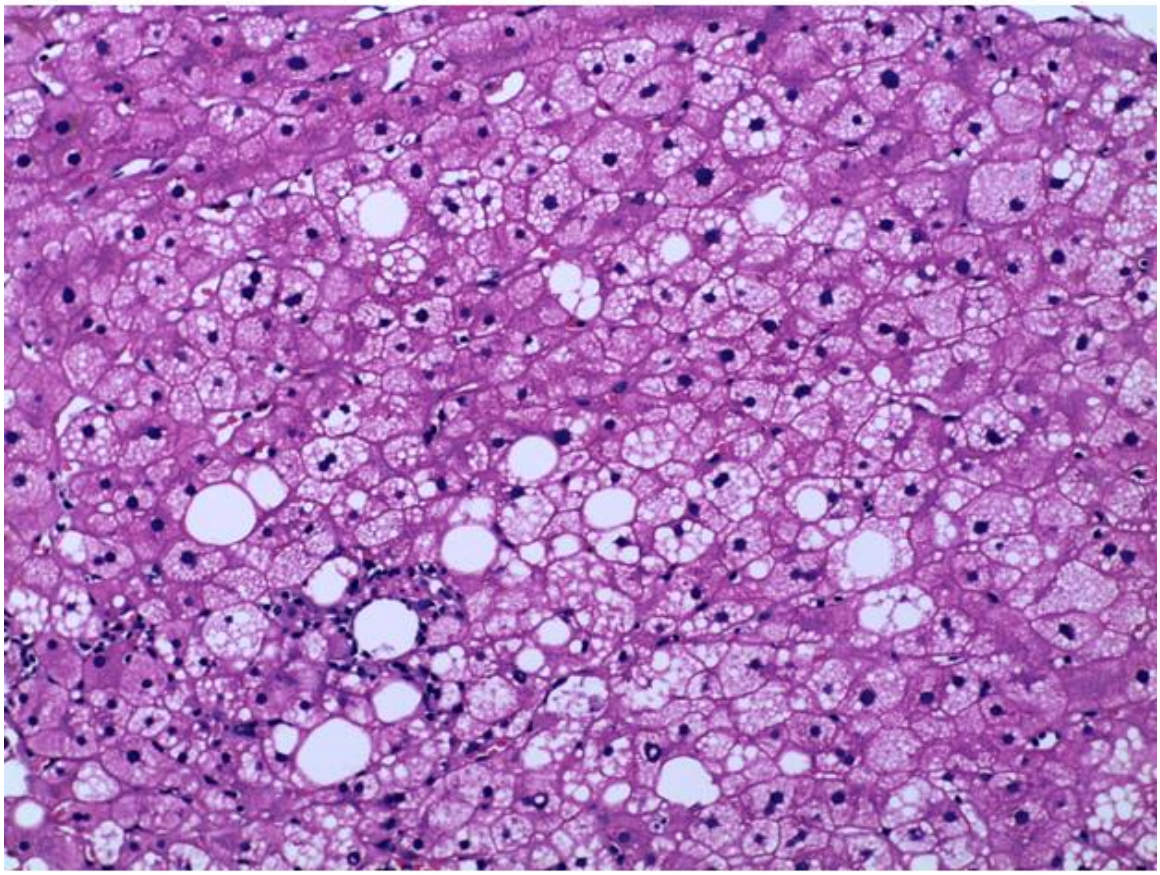

Figure 8.6c Lobular inflammation grade 2

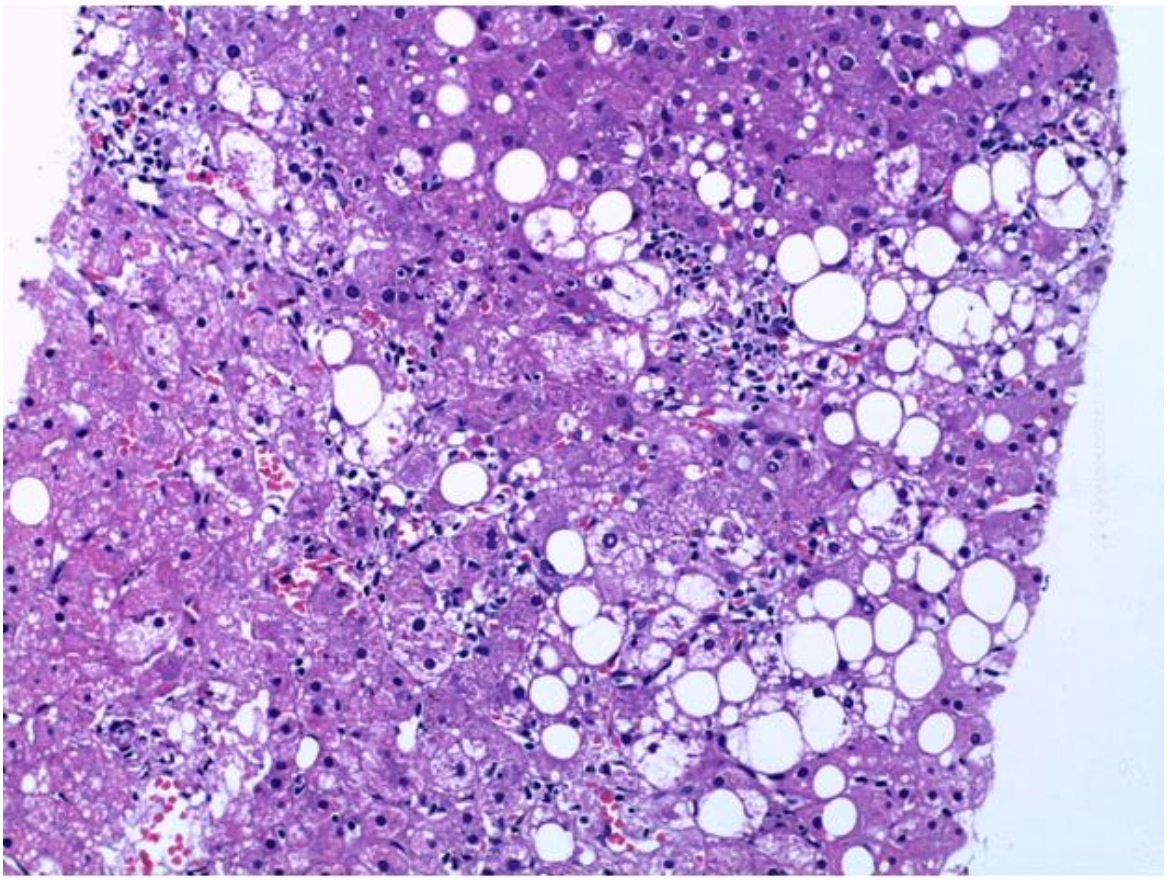

Figure 8.6d Lobular inflammation grade 3

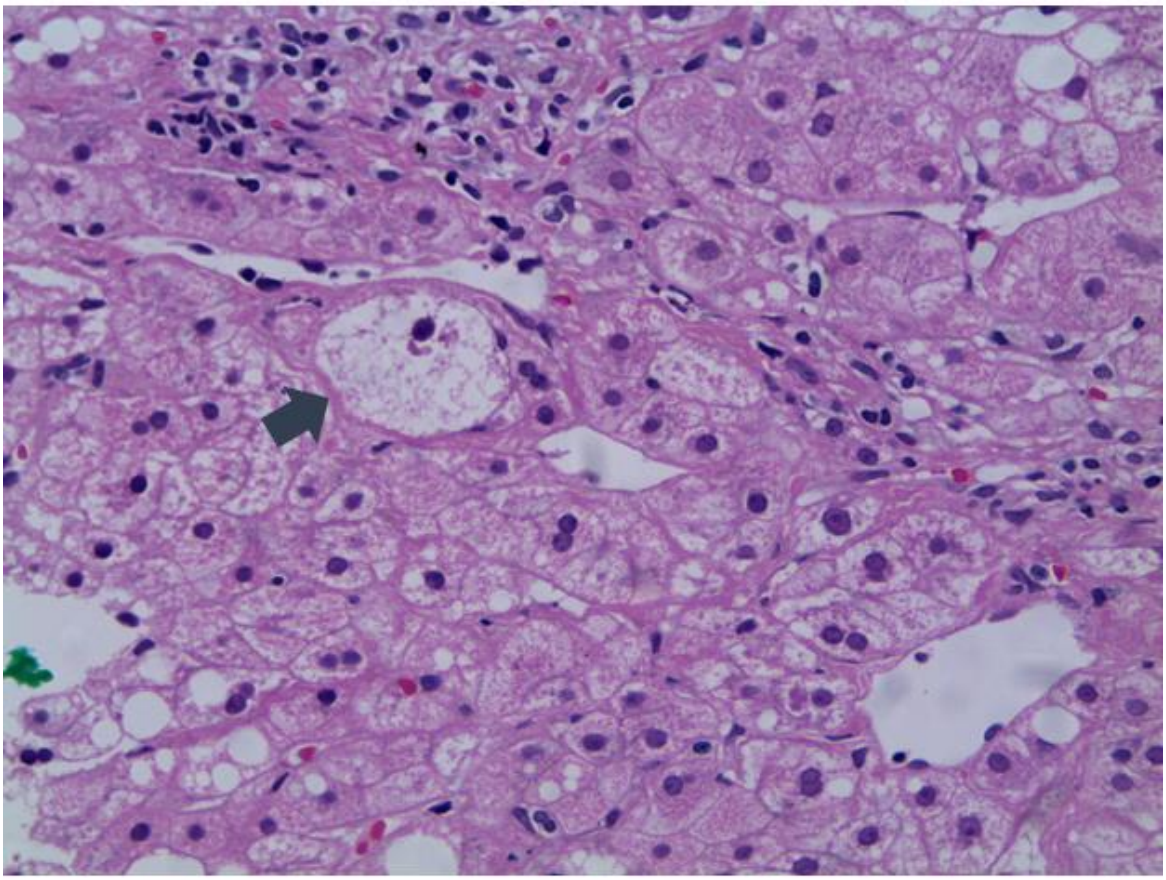

Figure 8.7a Hepatocyte ballooning (arrow)

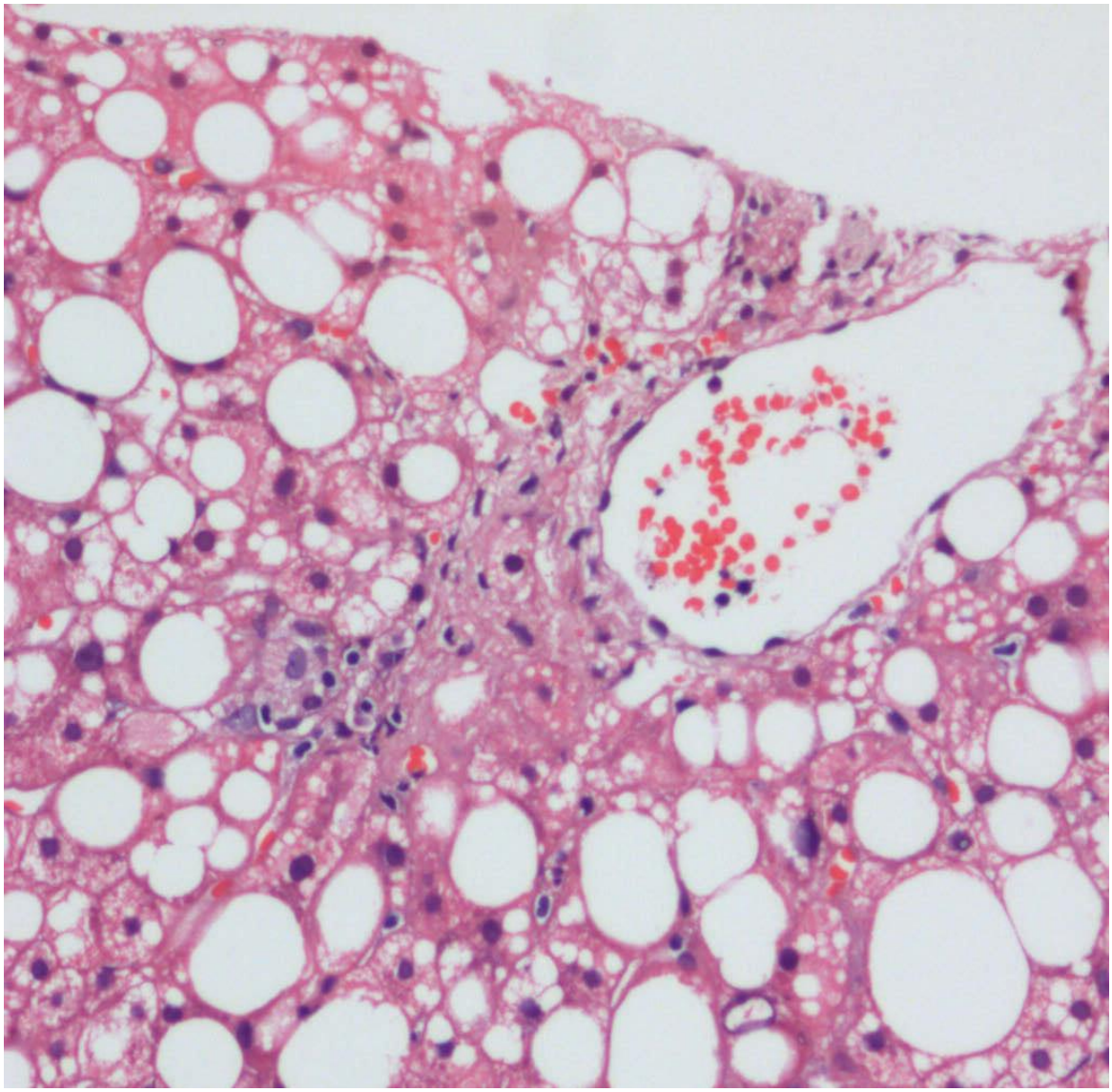

Figure 8.7b Hepatocyte ballooning grade 1

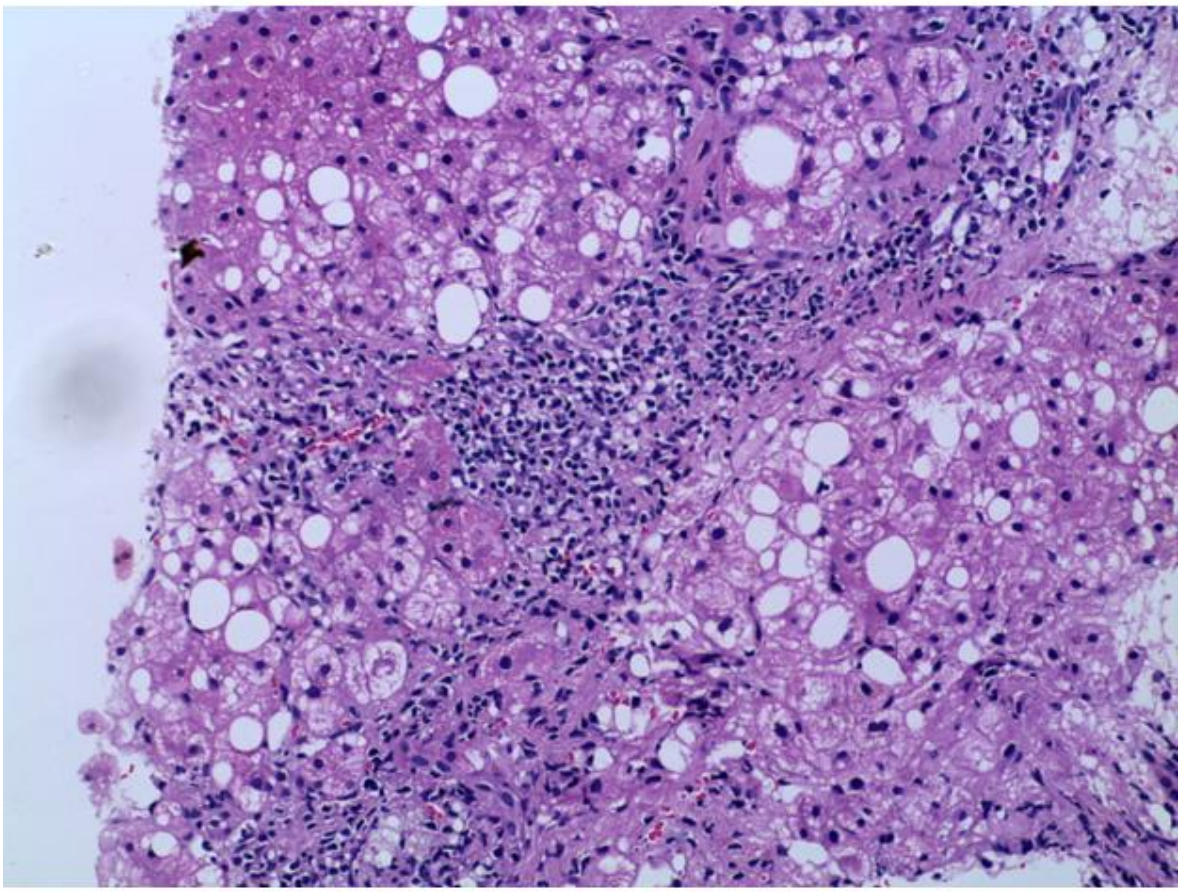

Figure 8.7c Hepatocyte ballooning grade 2

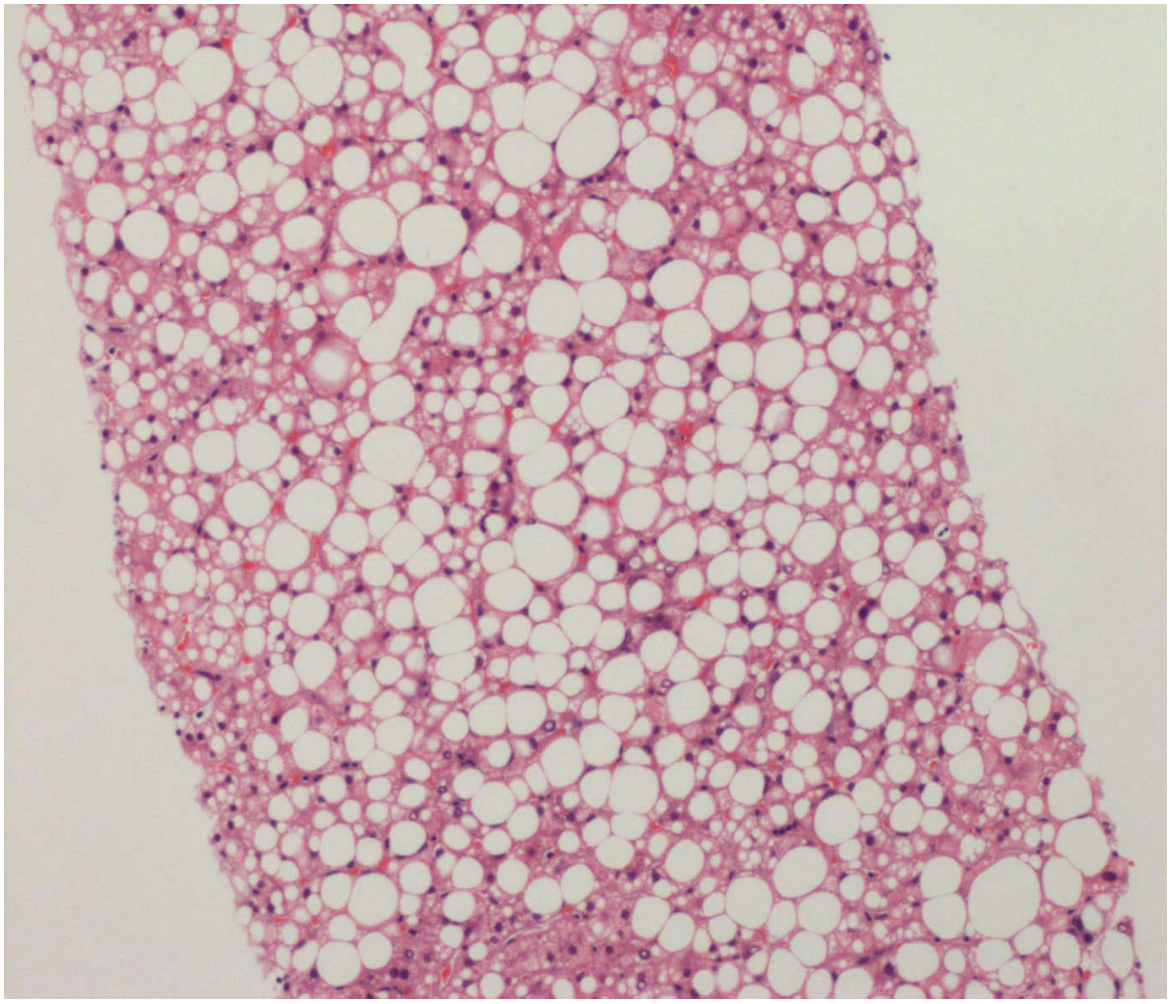

Figure 8.8a Non-NASH

Patients with NAS < 5 was considered as non-NASH while patients with NAS ≥ 5 were considered to have NASH.

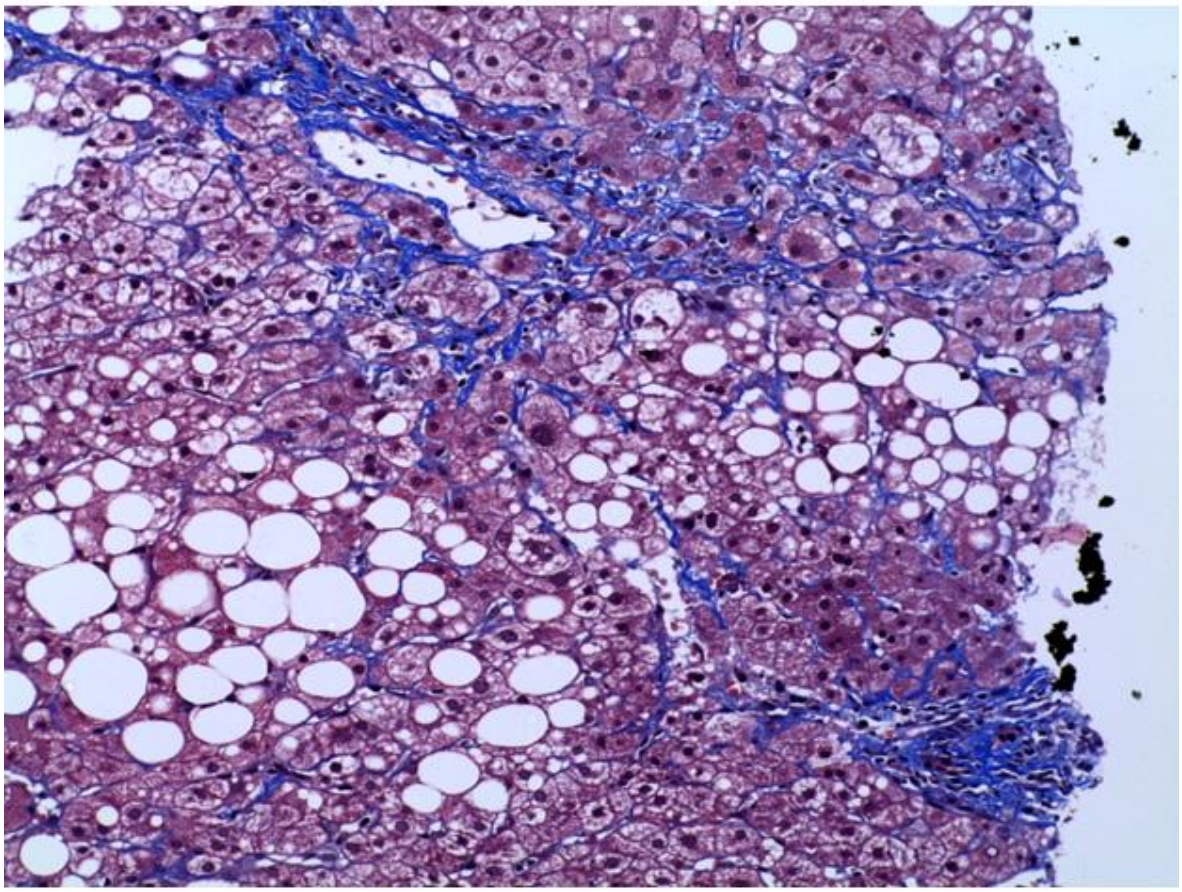

Figure 8.8b NASH

Patients with NAS < 5 was considered as non-NASH while patients with NAS ≥ 5 were considered to have NASH.

8.3.2 Results

Patient characteristics

Ninety-three NAFLD subjects and 20 controls were recruited during the period of study. The characteristics of subjects are shown in **Table 8.6**. The controls and NAFLD subjects were well-matched in age and gender. NAFLD subjects had greater BMI and WC and had higher prevalence of diabetes mellitus, hypertension and dyslipidemia compared to controls. There was a lower proportion of males among NASH subjects compared to non-NASH subjects. NASH and non-NASH subjects were similar in age, BMI, WC and prevalence of diabetes mellitus, hypertension and dyslipidemia. The quality of liver biopsy specimen as reflected by its length and the number of portal tracts, were also similar between NASH and non-NASH subjects. NASH subjects showed greater steatosis, lobular inflammation, ballooning and fibrosis.

Table 8.6 Characteristics of controls and NAFLD patients

	Controls n = 20	NAFLD patients n = 93	Non-NASH patients n = 54	NASH patients n = 39
Age, years	50.6 ± 16.8	51.0 ± 11.1	50.2 ± 11.3	52.2 ± 10.8
†Male, %	30.0	51.6	63.0	35.9
*BMI, kg per m^2	22.5 ± 2.8	29.4 ± 3.8	29.1 ± 3.7	29.8 ± 4.0
*WC, cm	81.8 ± 7.6	97.7 ± 9.7	96.8 ± 9.7	98.8 ± 9.7
*Diabetes mellitus, %	0	59.1	51.9	69.2
*Hypertension, %	20.0	88.2	85.2	92.3
*Dyslipidemia, %	40.0	96.8	94.4	100
Liver biopsy length, mm	–	15.0 ± 3.9	14.5 ± 4.2	15.7 ± 3.5
Number of portal tracts	–	8 (7 – 10)	8 (6 – 10)	9 (7 – 11)
†Steatosis				
S0	–	3.2	5.6	0
S1	–	34.4	42.6	23.1
S2	–	47.3	48.1	46.2
S3	–	15.1	3.7	30.8
‡Lobular inflammation				
0	–	4.3	7.4	0
1	–	53.8	81.5	15.4
2	–	38.7	11.1	76.9
3	–	3.2	0	7.7
‡Ballooning				
0	–	14.0	24.1	0
1	–	60.2	70.4	46.2
2	–	25.8	5.6	53.8
‡Fibrosis				
F0	–	30.1	44.4	10.3
F1	–	43.0	42.6	43.6
F2	–	6.5	1.9	12.8

	Controls n = 20	NAFLD patients n = 93	Non-NASH patients n = 54	NASH patients n = 39
F3	–	18.3	7.4	33.3
F4	–	2.2	3.7	0

*Significant at $p < 0.001$ between healthy controls and NAFLD patients

†Significant at $p < 0.05$

‡Significant at $p < 0.001$, between non-NASH and NASH patients

Patients with NAS < 5 was considered as non-NASH while patients with NAS ≥ 5 were considered to have NASH.

BMI = body mass index, WC = waist circumference, NAS = non-alcoholic fatty liver disease activity score

Plasma M30 and serum ALT, AST and GGT levels in controls and NAFLD patients

Plasma M30 levels were significantly higher in patients with NAFLD (median 349 U/L, IQR 257 U/L – 612 U/L) than in controls (median 162 U/L, IQR 103 U/L – 215 U/L, $p < 0.001$) (**Figure 8.9a**). Although plasma M30 levels were higher in patients with NASH (median 435 U/L, IQR 279 U/L – 758 U/L) compared to non-NASH patients (median 332 U/L, IQR 249 U/L – 534 U/L), the difference was not significant statistically ($p = 0.145$) (**Figure 8.10a**).

Serum ALT levels were significantly higher in patients with NAFLD (median 70 IU/L, IQR 44 IU/L – 109 IU/L) than in controls (median 26 IU/L, IQR 22 IU/L – 32 IU/L, $p < 0.001$) (**Figure 8.9b**). More importantly, serum ALT levels were significantly higher in NASH patients (median 86 IU/L, IQR 55 IU/L – 121 IU/L) compared to non-NASH patients (median 61 IU/L, IQR 44 IU/L – 93 IU/L, $p < 0.05$) (**Figure 8.10b**).

Serum AST levels were significantly higher in patients with NAFLD (median 41 IU/L, IQR 28 IU/L – 64 IU/L) than in controls (median 20 IU/L, IQR 18 IU/L – 27 IU/L, $p < 0.001$) (**Figure 8.9c**). Serum AST levels were also significantly higher in NASH patients (median 58 IU/L, IQR 38 IU/L – 78 IU/L) compared to non-NASH patients (median 34 IU/L, IQR 25 IU/L – 46 IU/L, $p < 0.001$) (**Figure 8.10c**).

Serum GGT levels were significantly higher in patients with NAFLD (median 75 IU/L, IQR 47 IU/L – 125 IU/L) than in controls (median 33 IU/L, IQR 22 IU/L – 45 IU/L, $p < 0.001$) (**Figure 8.9d**). Serum GGT levels were also significantly higher in NASH patients (median 97 IU/L, IQR 53 IU/L – 151 IU/L) compared to non-NASH patients (median 56 IU/L, IQR 40 IU/L – 101 IU/L, $p < 0.05$) (**Figure 8.10d**).

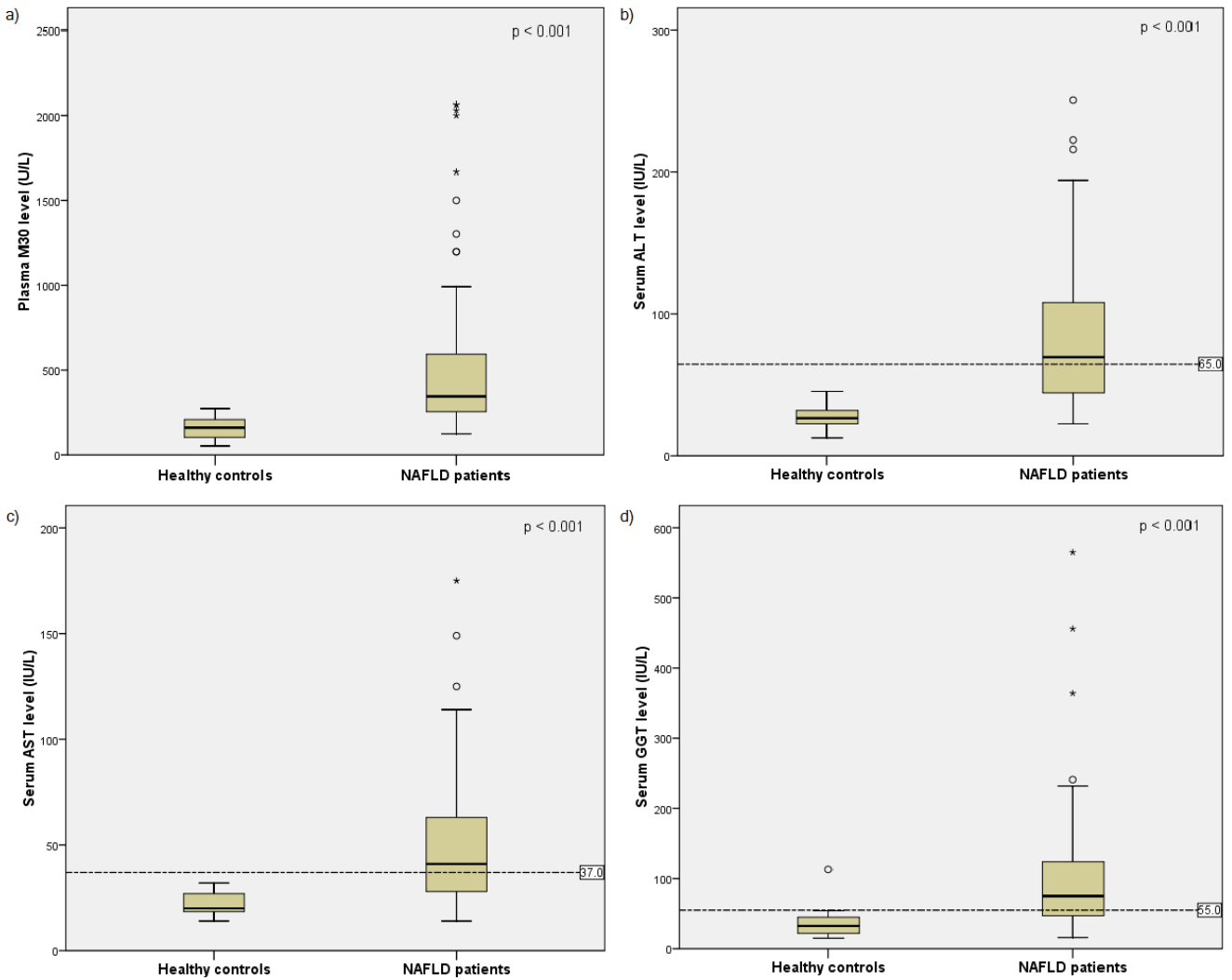

Figure 8.9 Plasma M30 and serum ALT, AST and GGT levels in healthy controls and NAFLD patients

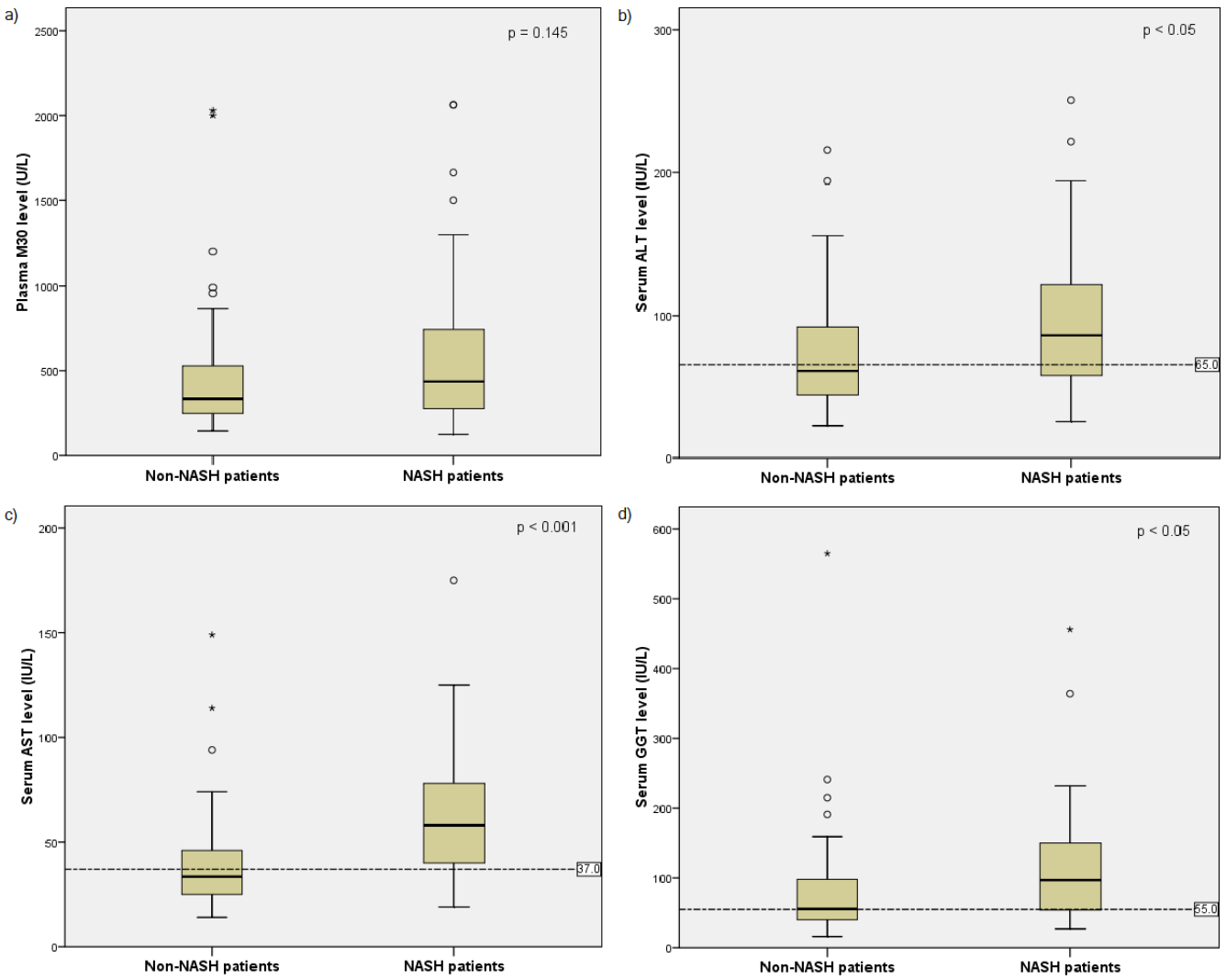

Figure 8.10 Plasma M30 and serum ALT, AST and GGT levels in non-NASH and NASH patients

Prediction of NAFLD and NASH

The receiver operating characteristic curves of plasma M30 and serum ALT, AST and GGT for prediction of NAFLD and NASH are shown in **Figures 8.11a and 8.11b**, respectively. Plasma M30 and serum ALT levels were excellent for prediction of NAFLD with AUROC of 0.91 and 0.95, respectively. Serum AST and GGT levels were good for prediction of NAFLD with AUROC of 0.87 and 0.85, respectively. Serum AST level was fair for prediction of NASH among NAFLD patients with AUROC of 0.75. Plasma M30 and serum ALT and GGT levels were poor for prediction of NASH among NAFLD patients with AUROC of 0.59, 0.64 and 0.68, respectively. The sensitivity, specificity, positive predictive value and negative predictive value when using the different cut-offs of plasma M30 and serum ALT, AST and GGT levels for prediction of NAFLD and NASH are shown in **Tables 8.7 and 8.8**, respectively.

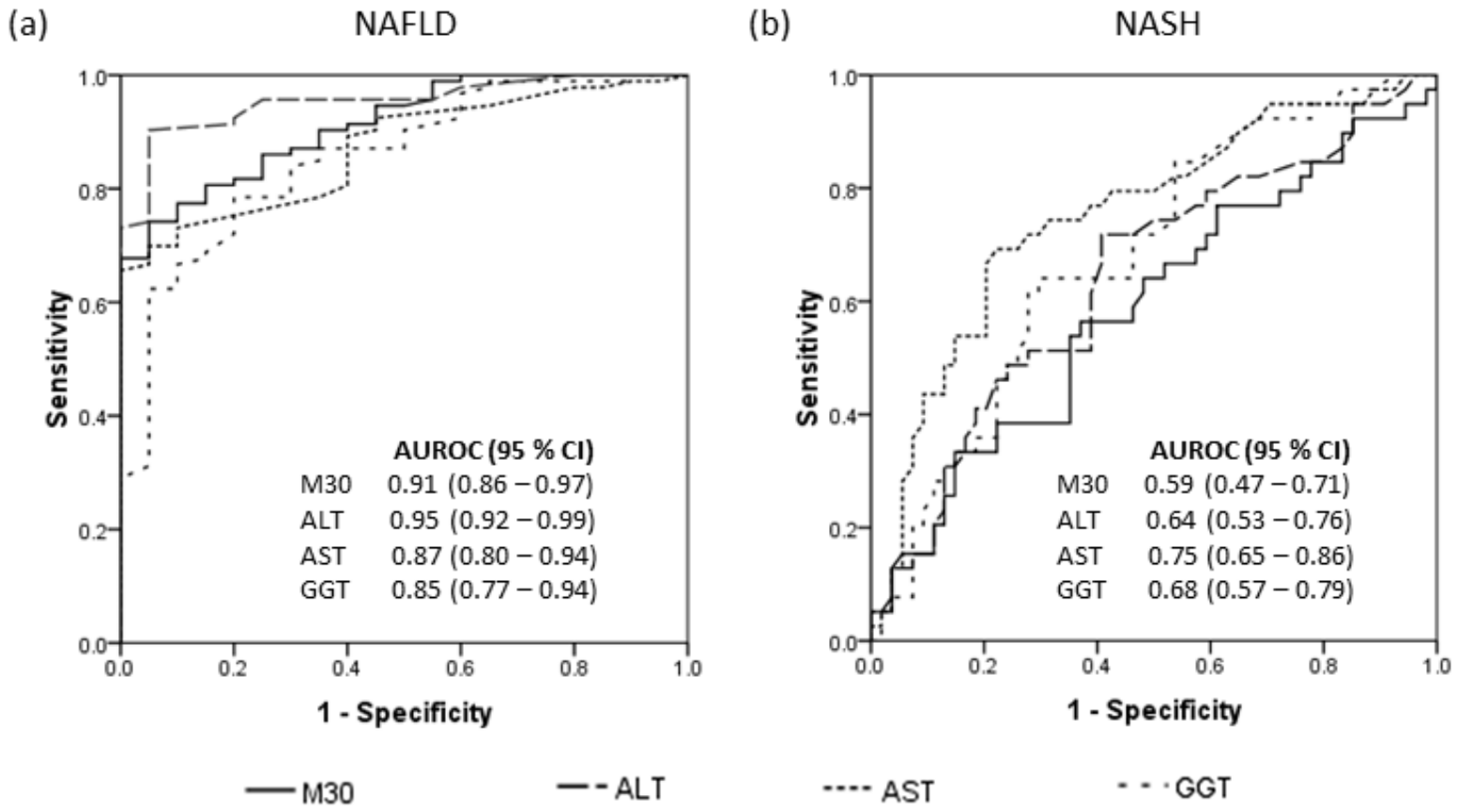

Figure 8.11 The receiver operating characteristic curves of plasma M30 and serum ALT, AST and GGT for prediction of (a) NAFLD, and (b) NASH

AUROC: 0.90 – 1.00 = excellent, 0.80 – 0.90 = good, 0.70 – 0.80 = fair, < 0.70 = poor

Table 8.7 The sensitivity, specificity, positive predictive value and negative predictive value when using the different cut-offs of plasma M30 and serum ALT, AST and GGT for prediction of NAFLD

	*Cut-off, U/L or IU/L	Sensitivity, %	Specificity, %	PPV, %	NPV, %
Plasma M30	162	95.7	45.0	89.0	69.2
	263	74.2	95.0	98.6	44.2
	278	67.7	100	100	40.0
Serum ALT	27	95.7	55.0	90.8	73.3
	35	90.3	95.0	98.8	67.9
	47	73.1	100	100	44.4
Serum AST	21	92.5	55.0	90.5	61.1
	33†	65.6	100	100	38.5
Serum GGT	32	90.3	50.0	89.4	52.6
	46	78.5	80.0	94.8	44.4
	55	62.4	95.0	98.3	35.2

*Cut-off with high sensitivity, highest overall accuracy and high specificity were presented

†Cut-off for highest overall accuracy and high specificity were the same

ALT = alanine aminotransferase, AST aspartate aminotransferase, GGT = gamma glutamyl transpeptidase, PPV = positive predictive value, NPV = negative predictive value

Table 8.8 The sensitivity, specificity, positive predictive value and negative predictive value when using the different cut-offs of plasma M30 and serum ALT, AST and GGT for prediction of NASH

	*Cut-off, U/L or IU/L	Sensitivity, %	Specificity, %	PPV, %	NPV, %
Plasma M30	293	71.8	40.7	46.7	66.7
	432	56.4	63.0	52.4	66.7
	474	43.6	64.8	47.2	61.4
Serum ALT	53	79.5	40.7	49.2	73.3
	67	71.8	59.3	56.0	74.4
	100	41.0	79.6	59.3	65.2
Serum AST	30	84.6	40.7	50.8	78.6
	48	69.2	77.8	69.2	77.8
	65	43.6	90.7	77.3	69.0
Serum GGT	49	84.6	42.6	51.6	79.3
	84	64.1	70.4	61.0	73.1
	109	46.2	77.8	60.0	66.7

*Cut-off with high sensitivity, highest overall accuracy and high specificity were presented

ALT = alanine aminotransferase, AST aspartate aminotransferase, GGT = gamma glutamyl transpeptidase, PPV = positive predictive value, NPV = negative predictive value

Plasma M30 and serum ALT, AST and GGT according to steatosis, ballooning, lobular inflammation and fibrosis

Plasma M30 and serum ALT and AST levels did not show any significant trend when analyzed according to steatosis grades. Although serum GGT level showed a significant trend when analyzed according to steatosis grades, the difference in serum GGT level was only significant between patients with grade 2 and 3 steatosis (**Figure 8.12**).

Serum ALT and AST levels showed significant increasing trend with increasing grades of lobular inflammation. However, this was not seen with plasma M30 and serum GGT levels. Serum ALT and AST levels were significantly higher in patients with grade 2 compared to grade 1 lobular inflammation. However, serum ALT and AST levels were not significantly different between patients with grade 2 and grade 3 lobular inflammation, and between patients with grade 1 and patients without lobular inflammation (**Figure 8.13**).

Serum ALT and AST levels showed significant increasing trend with increasing grades of ballooning. However, this was not seen with plasma M30 and serum GGT levels. Serum ALT levels were significantly higher in patients with grade 1 compared to patients without ballooning. However, serum ALT levels were not significantly different between patients with grade 1 and grade 2 ballooning. Serum AST levels were significantly higher in patients with grade 2 compared to grade 1 ballooning and in patients with grade 1 compared to patients without ballooning (**Figure 8.14**).

Plasma M30 level did not show any significant trend when analyzed according to fibrosis stages. There was significant difference in serum ALT, AST and GGT levels across fibrosis stages. However, only the difference in serum ALT and AST for stage 1 and stage 2 fibrosis was significant (**Figure 8.15**).

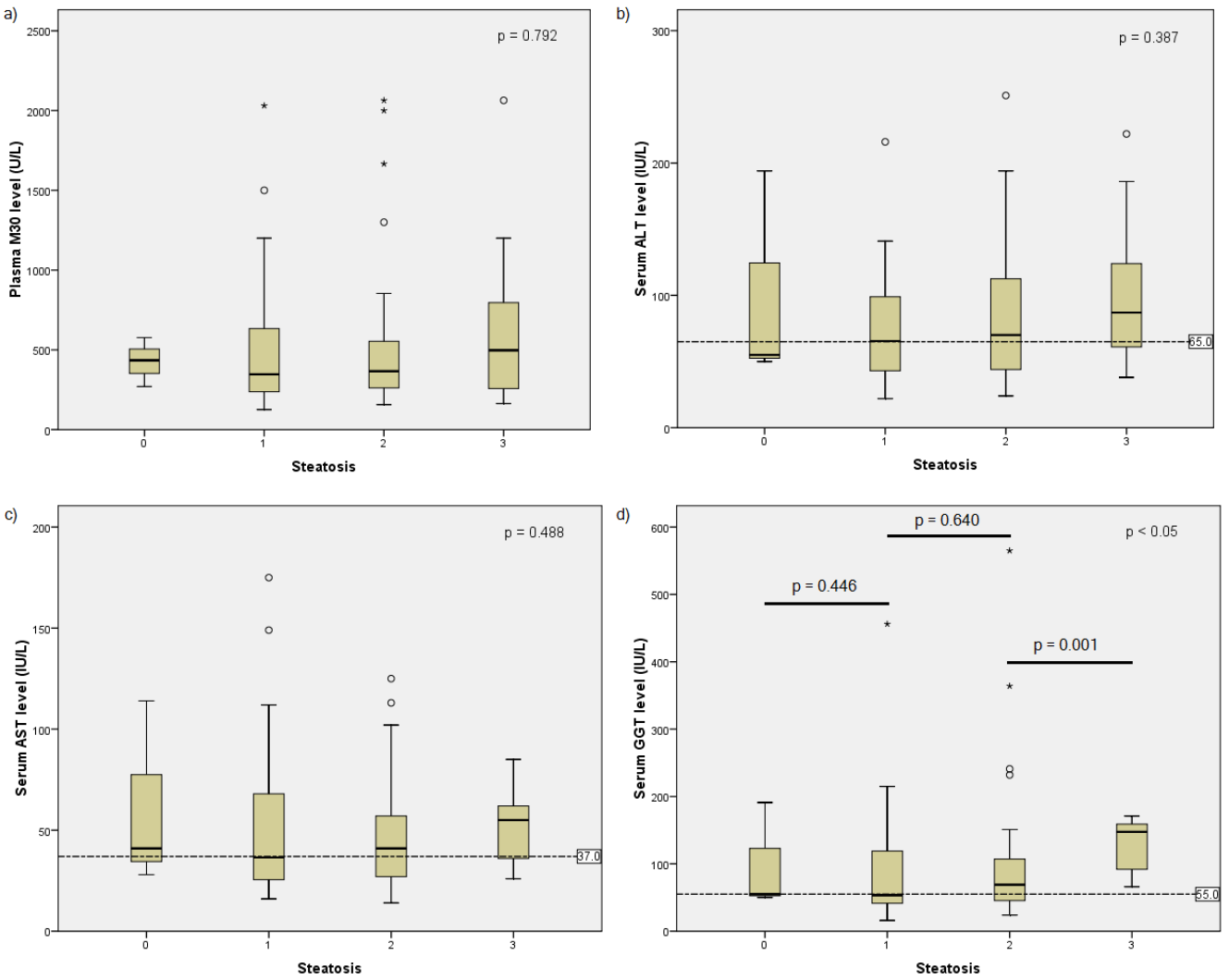

Figure 8.12 Plasma M30 and serum ALT, AST and GGT levels according to steatosis grades

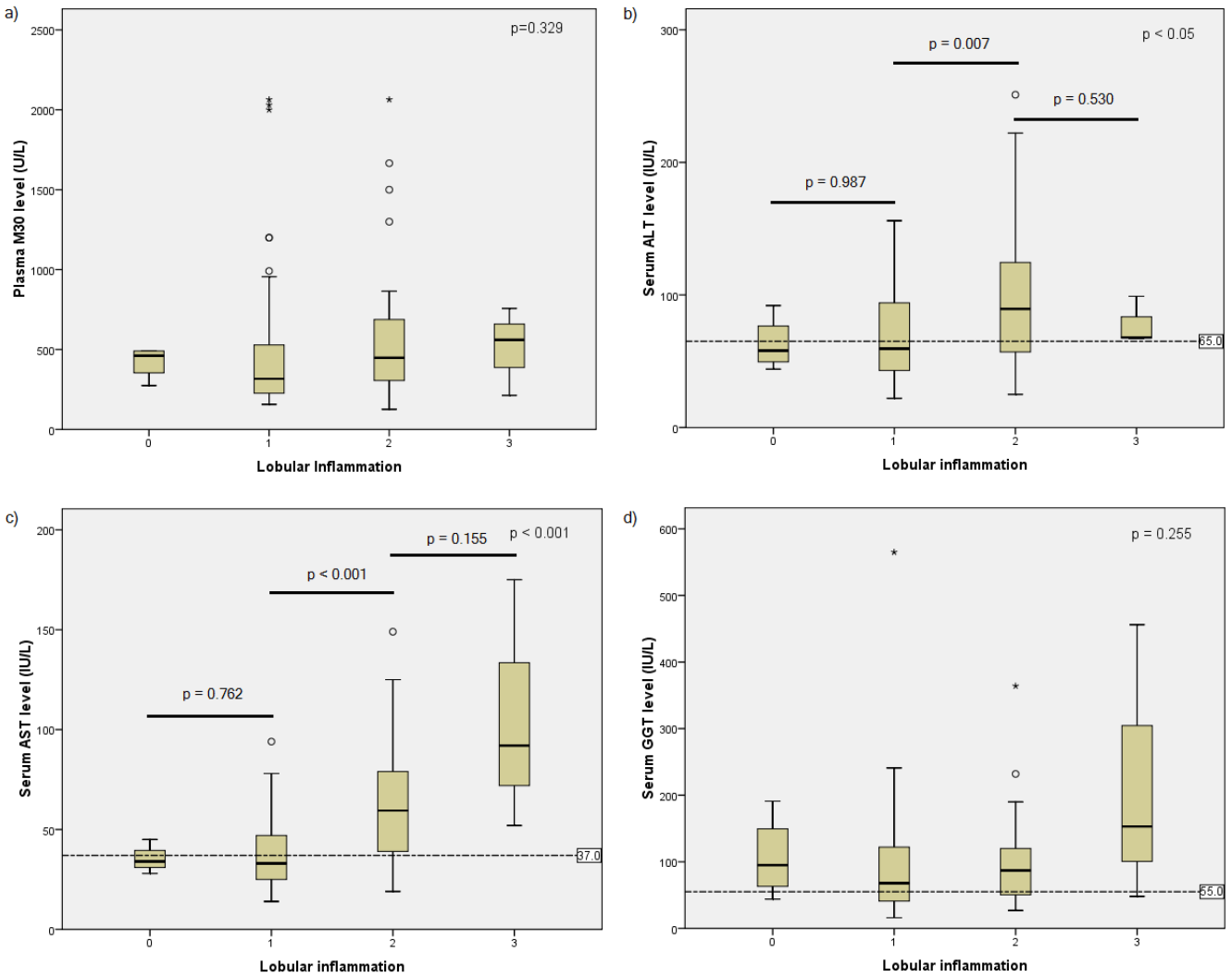

Figure 8.13 Plasma M30 and serum ALT, AST and GGT levels according to lobular inflammation grades

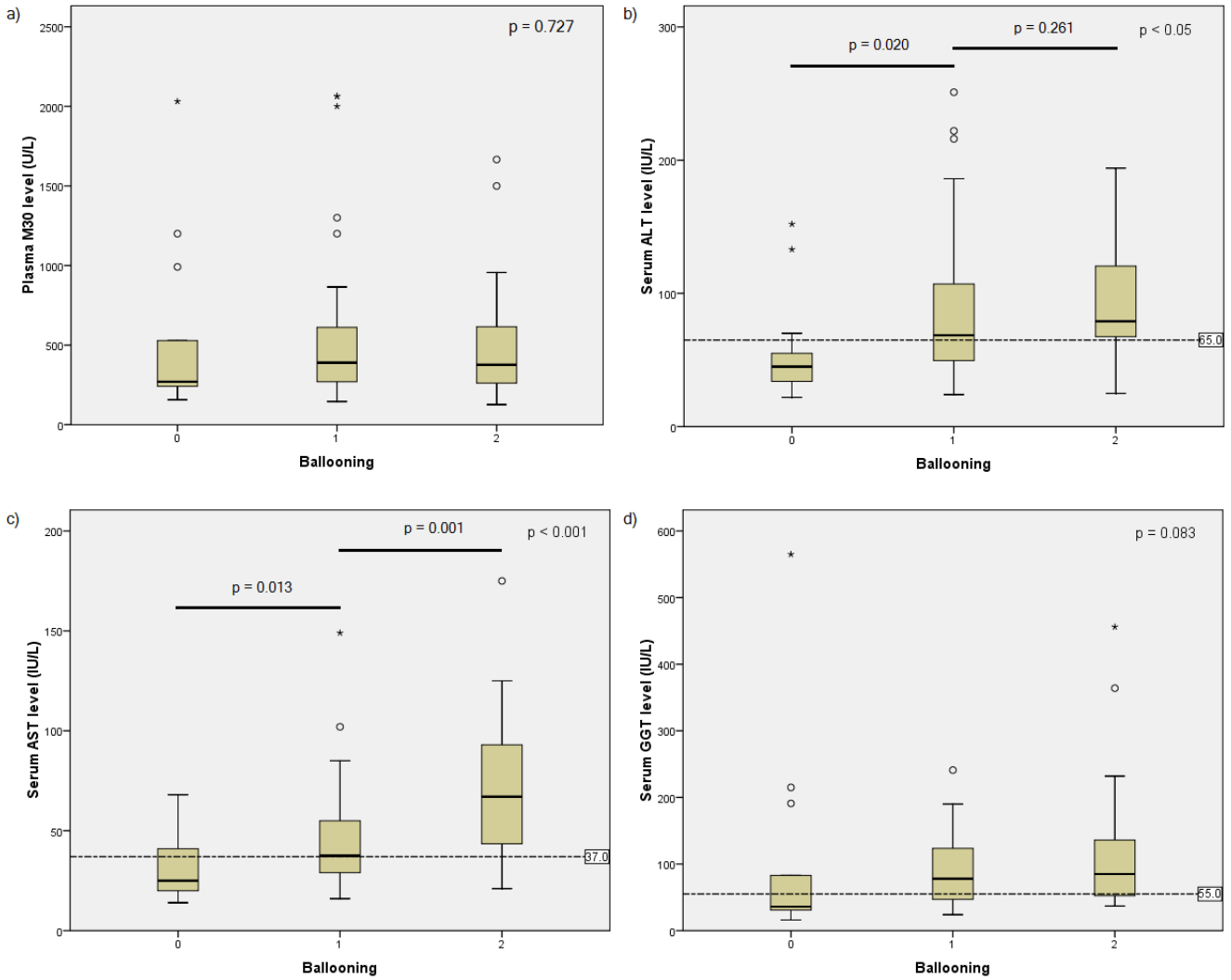

Figure 8.14 Plasma M30 and serum ALT, AST and GGT levels according to ballooning grades

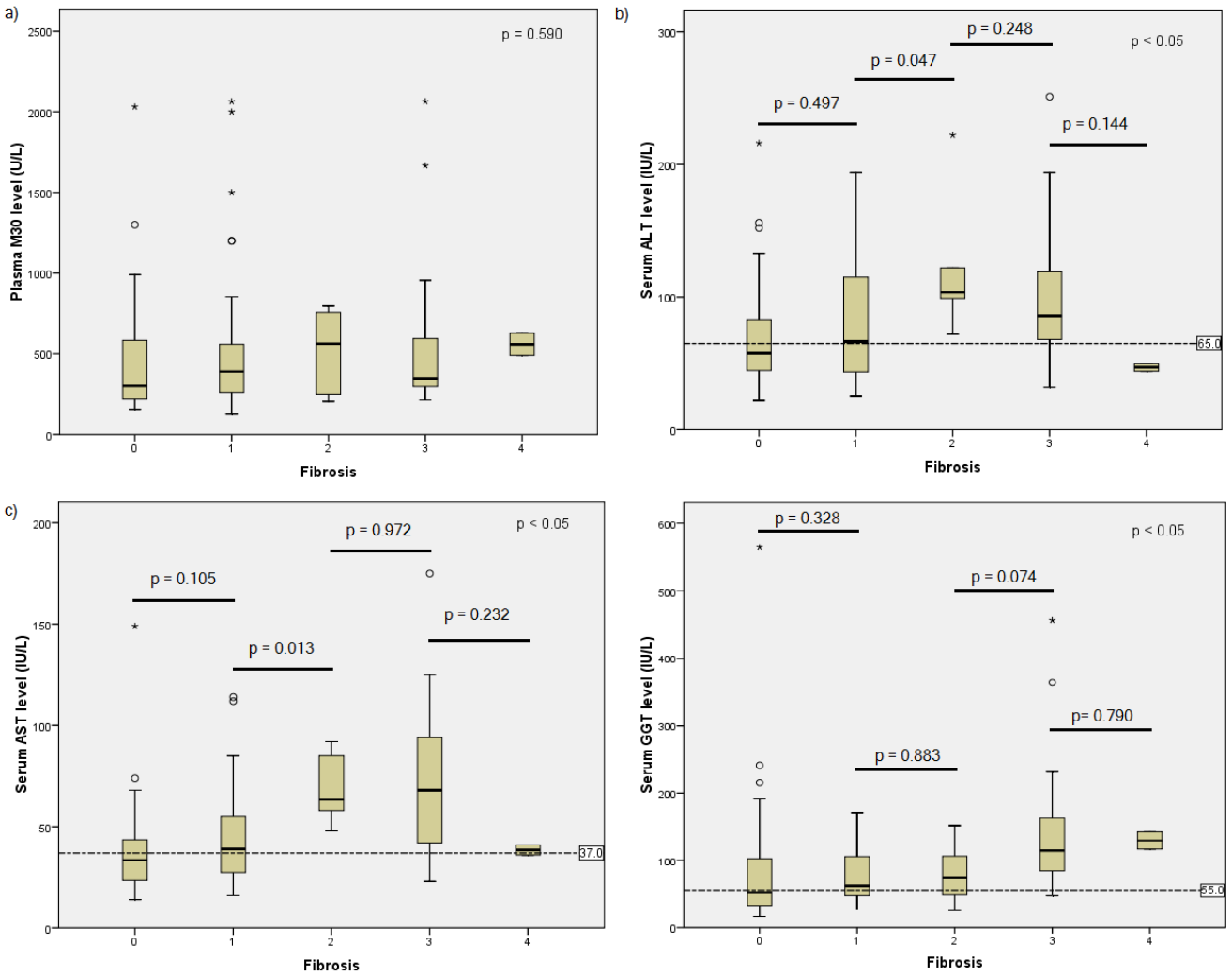

Figure 8.15 Plasma M30 and serum ALT, AST and GGT levels according to fibrosis stages

Prediction of ballooning and lobular inflammation

In view of the above findings, analysis was carried out to determine the accuracy of plasma M30 and serum ALT, AST and GGT levels for prediction of presence of ballooning and presence of more severe lobular inflammation. Lobular inflammation grade 0 and grade 1 were considered less severe while grade 2 and grade 3 were considered more severe. The receiver operating characteristic curves of plasma M30 and serum ALT, AST and GGT for prediction of more severe lobular inflammation and presence of ballooning are shown in **Figures 8.16a and 8.16b**, respectively. Serum ALT and AST levels were fair for prediction of presence of ballooning with AUROC of 0.72 and 0.77, respectively. Serum AST level was fair for prediction of presence of more severe lobular inflammation with AUROC of 0.78. Plasma M30 and serum GGT levels were poor for prediction of presence of ballooning and presence of more severe lobular inflammation. The sensitivity, specificity, positive predictive value and negative predictive value when using the different cut-offs of plasma M30 and serum ALT, AST and GGT levels for prediction of more severe lobular inflammation and presence of ballooning are shown in **Tables 8.9 and 8.10**, respectively.

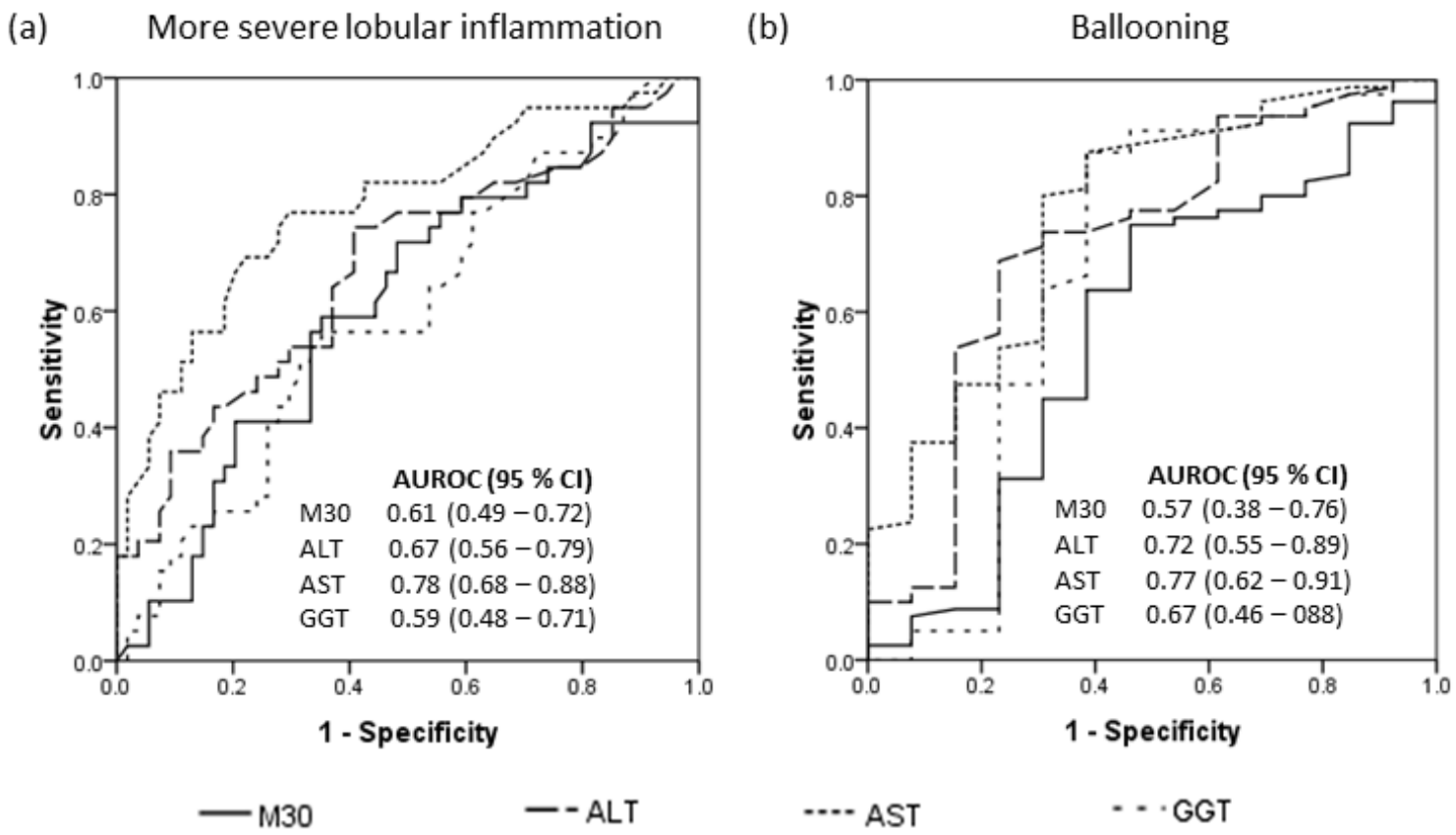

Figure 8.16 The receiver operating characteristic curves of plasma M30 and serum ALT, AST and GGT for prediction of (a) more severe lobular inflammation*, and (b) ballooning

*Lobular inflammation grade 0 and 1 were considered less severe while grade 2 and 3 were considered more severe

AUROC: 0.90 – 1.00 = excellent, 0.80 – 0.90 = good, 0.70 – 0.80 = fair, < 0.70 = poor

Table 8.9 The sensitivity, specificity, positive predictive value and negative predictive value when using the different cut-offs of plasma M30 and serum ALT, AST and GGT for prediction of presence of ballooning

	*Cut-off, U/L or IU/L	Sensitivity, %	Specificity, %	PPV, %	NPV, %
Plasma M30	268	76.3	46.2	89.7	24.0
	317	63.8	61.5	91.1	21.6
	474	40.0	69.2	88.9	15.8
Serum ALT	45	77.5	46.2	89.9	25.0
	57	68.8	76.9	94.8	28.6
	89	40.0	84.6	94.1	18.6
Serum AST	25	90.0	46.2	91.1	42.9
	29	80.0	69.2	94.1	36.0
	51	40.0	84.6	94.1	18.6
Serum GGT	37	91.2	53.8	92.4	50.0
	42	87.5	61.5	93.3	44.4
	95	38.8	76.9	91.2	16.9

*Cut-off with high sensitivity, highest overall accuracy and high specificity were presented

ALT = alanine aminotransferase, AST aspartate aminotransferase, GGT = gamma glutamyl transpeptidase, PPV = positive predictive value, NPV = negative predictive value

Table 8.10 The sensitivity, specificity, positive predictive value and negative predictive value when using the different cut-offs of plasma M30 and serum ALT, AST and GGT for prediction of presence of more severe lobular inflammation*

	Cut-off, U/L or IU/L †	Sensitivity, %	Specificity, %	PPV, %	NPV, %
Plasma M30	277	79.5	40.7	49.2	73.3
	432	59.0	64.8	54.8	68.6
	560	38.5	79.6	57.7	64.2
Serum ALT	53	79.5	40.7	49.2	73.3
	66	74.4	59.3	56.9	76.2
	109	38.5	85.2	65.2	65.7
Serum AST	30	84.6	40.7	50.8	78.6
	42	76.9	70.4	65.2	80.9
	69	38.5	94.4	83.3	68.0
Serum GGT	53	69.2	40.7	45.8	64.7
	84	56.4	64.8	53.7	67.3
	111	38.5	74.1	51.7	62.5

*Lobular inflammation grade 0 and 1 were considered less severe while grade 2 and 3 were considered more severe

†Cut-off with high sensitivity, highest overall accuracy and high specificity were presented

ALT = alanine aminotransferase, AST aspartate aminotransferase, GGT = gamma glutamyl transpeptidase, PPV = positive predictive value, NPV = negative predictive value

8.3.3 Discussion

The potential use of plasma M30 level as a non-invasive test to determine histological disease severity in NAFLD patients was first reported by Wieckowska et al. In their study of 44 consecutive patients with suspected NAFLD at the time of liver biopsy, plasma CK-18 levels were markedly increased in patients with NASH compared to patients with simple steatosis or normal liver biopsies. Plasma M30 level was excellent to distinguish patients with NASH from patients with simple steatosis or normal liver biopsies with an AUROC of 0.93. Two patients with borderline NASH were not included in the analysis (Wieckowska et al., 2006). In a subsequent multi-centre validation study consisting of 139 patients, Feldstein et al reported that plasma M30 level was good to distinguish NAFLD patients with NASH from those without NASH or with borderline NASH with an AUROC of 0.83. However, it is important to note that this population consisted of a relatively small percentage of patients with borderline NASH (19 %) (Feldstein et al., 2009). Subsequently, Shen et al reported an AUROC of 0.66 for plasma M30 level to distinguish NAFLD patients with NASH from patients without NASH. Interestingly, the study population consisted of a larger percentage of patients with borderline NASH (49.7 %) (Shen et al., 2012). In our study population which consisted of a similar percentage of patients with borderline NASH (52.7 %), we too found that plasma M30 was less useful for distinguishing NAFLD patients with NASH from those without NASH with an AUROC of 0.59. In a recently published study consisting of 318 patients, Cusi et al similarly reported that plasma M30 level was less useful for NASH diagnosis with an AUROC of 0.65(Cusi et al., 2014).

We found that serum AST level was fair in distinguishing NAFLD patients with NASH from those without NASH or with borderline NASH with an AUROC of 0.75. Serum AST level was fair in predicting the presence of ballooning and the presence of more severe

lobular inflammation with an AUROC of 0.77 and 0.78, respectively. Serum ALT and GGT levels were less useful. Other tests for diagnosis of NASH such as measurement of total cell death markers M65 and M65ED, adipocyte fatty acid binding protein (AFABP) and fibroblast growth factors 21 (FGF21) have been studied but were not better with AUROC of 0.71, 0.70, 0.59 and 0.62, respectively. It appears that an accurate non-invasive test for NASH remains elusive. However, we should not forget that NASH is a continuous spectrum and markers may be variably expressed in each individual so that finding a test that confirms the presence or absence of NASH using a pre-determined cut-off may be difficult if not impossible. It may be more realistic to aim for a test that would reflect changes in severity of NASH when followed over time. For example, Suzuki et al reported that the combination of baseline and rate of change of serum ALT and AST levels had an AUROC of 0.72 and 0.73, respectively, in predicting improvement, and an AUROC of 0.75 and 0.77, respectively, in predicting worsening of histological inflammation in NASH patients. The AUROC improved to 0.88 and 0.89, respectively, when baseline histology was taken into consideration (Suzuki et al., 2006). In a separate study of 36 patients without NASH at baseline among which 10 patients developed NASH at 36 months, Shen and colleagues showed that changes in M30 was good in predicting development of NASH with an AUROC of 0.82. Using 35 U/L as the cut-off for increment in M30, development of NASH could be predicted with sensitivity and specificity of 80.0 % and 81.5 %, respectively (Shen et al., 2012). The use of changes in plasma M30 and serum ALT and AST levels to predict changes in histology, particularly inflammation and ballooning, should be compared and deserves further studies in larger group of patients.

Our study was carried out prospectively according to a planned protocol so that the data collected was robust. Collection of blood sample was done on the same day as the liver

biopsy procedure to minimize difference in findings due to changes over time. Despite our best effort, the study had several limitations. Firstly, as in any study using histopathological examination of liver biopsy specimen as reference, our study may be limited by sampling variability and observer variability. Secondly, we were not able to perform liver biopsy in controls due to ethical reason and the absence of NAFLD was based on ultrasonography which may lack sensitivity in detection of mild hepatic steatosis. Nevertheless, this would only reduce the difference seen between controls and NAFLD patients, which remained highly significant.

8.3.4 Conclusion

Neither plasma M30 nor serum ALT, AST or GGT levels were good enough for diagnosis of NASH among NAFLD patients. While other more accurate yet simple and non-invasive tests are needed for diagnosis of NASH, the use of changes in plasma M30 and serum ALT and AST levels to predict changes in histology, particularly inflammation and ballooning, should be compared and deserves further studies in larger group of patients.

Note: A poster on the findings from this study was presented at the Asia-Pacific Digestive Week 2014 in Bali, Indonesia, and the abstract was published in a supplementary issue of the Journal of Gastroenterology and Hepatology (Chan et al., 2014). The findings from this study was also presented at the Malaysian Society of Gastroenterology and Hepatology Annual Scientific Meeting in 2014. The full article has been published in PLoS One (Chan et al., 2014).

8.4 NAFLD fibrosis score and liver stiffness measurement for the estimation of hepatic fibrosis

Liver fibrosis and cirrhosis is the common end result of chronic liver disease. The degree of liver fibrosis gives an idea about the severity of the chronic liver disease. This provides information on prognosis and helps identify patients for intervention. Histopathological examination of a liver biopsy specimen is the best standard for assessment of liver fibrosis. However, liver biopsy is invasive and associated with a small risk of complications. Technical expertise is also required, from obtaining a good specimen to processing and accurately interpreting the result. It is not practical to subject all non-alcoholic fatty liver disease (NAFLD) patients to a liver biopsy to assess liver fibrosis, particularly when the disease is so common. It is also not practical to subject NAFLD patients to repeated liver biopsies to monitor disease status in clinical practice.

Several non-invasive tests are available for assessment of liver fibrosis in NAFLD patients. The NAFLD fibrosis score is calculated from readily available parameters and can be used to predict the absence or presence of advanced fibrosis in NAFLD patients (Angulo et al., 2007). In a meta-analysis of 13 studies consisting of 3064 patients, the NAFLD fibrosis score had an area under the receiver operating characteristic curve (AUROC) of 0.85 for predicting advanced fibrosis. However, using the NAFLD fibrosis score alone, 20 % – 58 % of patients will fall in the indeterminate group (Musso et al., 2011). These patients will require further evaluation, for example by histopathological examination of a liver biopsy specimen.

Transient elastography has also been used to measure liver stiffness, which has been shown to correlate well with hepatic fibrosis. In a meta-analysis of 5 studies, transient elastography had an AUROC of 0.94 for predicting advanced fibrosis (Musso et al., 2011).

Combining the NAFLD fibrosis score and liver stiffness measurement for the prediction of advanced fibrosis in NAFLD patients seemed feasible and various algorithms have been proposed recently based on previously reported performance of the individual tests (Machado et al., 2013; Musso et al., 2011). We aimed to prospectively evaluate the combination of NAFLD fibrosis score and liver stiffness measurement in predicting advanced fibrosis in NAFLD patients.

8.4.1 Methods

Consecutive adult patients (aged ≥ 18 years) with NAFLD who were scheduled for a liver biopsy were prospectively recruited between November 2012 and October 2013 for the training cohort and between November 2013 and April 2014 for the validation cohort of this study. The diagnosis of NAFLD was based on ultrasonography finding of fatty liver and exclusion of significant alcohol intake, use of medications that can cause fatty liver, viral hepatitis B and C infection, and other causes of chronic liver disease where indicated. This study was approved by the University of Malaya Medical Centre's Ethics Committee and all patients who participated provided informed consent.

Demographic, anthropometric, relevant clinical and laboratory data were obtained using a standard protocol on the day of the liver biopsy procedure. Weight and height were measured using standard equipment. Body mass index (BMI) was calculated by dividing weight in kilogram by the square of height in meters. Patients with BMI ≥ 25.0 kg per m^2 were considered obese (Anuurad et al., 2003). Waist circumference (WC) was measured at the mid-point between the lowest margin of the least palpable rib and the top of the iliac crest in the standing position. Central obesity was defined as WC > 90 cm for men and > 80 cm for women (Alberti et al., 2005). Blood pressure was measured in the sitting position using

standardized equipment. A patient was considered hypertensive if there was a self-reported history of hypertension, if the patient was on anti-hypertensive medication(s), if the systolic blood pressure was ≥ 130 mmHg, or if the diastolic blood pressure was ≥ 85 mmHg.

All patients had venous blood drawn after an overnight fast for complete blood count, blood sugar, glycated hemoglobin (HbA1c), lipid profile, liver profile and tests for viral hepatitis B and C infection. Biochemical measurements were performed using standard laboratory procedures. A patient was considered to have diabetes mellitus if there was a self-reported history of diabetes mellitus, if the patient was on anti-diabetic medication(s), or if fasting blood sugar was ≥ 7.0 mmol/L. A patient was considered to have dyslipidemia if there was a self-reported history of dyslipidemia, if the patient was on lipid-lowering medication(s), if the serum total cholesterol (TC) was ≥ 5.2 mmol/L, if the serum triglyceride (TG) was ≥ 1.7 mmol/L, if the serum high-density lipoprotein (HDL) was < 1.0 mmol/L for men or < 1.3 mmol/L for women, or if the serum low-density lipoprotein (LDL) was ≥ 3.4 mmol/L. Our laboratory's upper limit of normal for liver enzymes were as follow: alkaline phosphatase (ALP) 136 IU/L, aspartate aminotransferase (AST) 37 IU/L, alanine aminotransferase (ALT) 65 IU/L and gamma-glutamyl transpeptidase (GGT) 55 IU/L. Serum ALP, AST, ALT and GGT above these levels were considered as elevated. The Elecsys HBsAg II assay and the Elecsys Anti-HCV II assay (Roche, Mannheim, Germany) were used to test for viral hepatitis B and C infection, respectively.

Liver biopsy and histological assessment

Ultrasonography-guided percutaneous liver biopsy was performed by either one of two experienced operators (WKC, SM) using an 18 G Terumo ® II semi-automatic biopsy needle (Cardinal Health, Dublin, Ohio, USA). Liver biopsy specimens were processed using standard laboratory procedures. Liver biopsy slides were stained with hematoxylin and eosin stain and masson trichrome stain. Liver biopsy slides were examined by an experienced histopathologist (NRNM) who was blinded to clinical data. Histopathological findings were reported according to the Non-Alcoholic Steatohepatitis Clinical Research Network Scoring System (Kleiner et al., 2005). The NAFLD activity score (NAS) is the sum of scores for hepatic steatosis (0 – 3), lobular inflammation (0 – 3) and hepatocyte ballooning (0 – 2). NAS 0 – 2 is not diagnostic of NASH, 3 – 4 is probable NASH and 5 – 8 is definite NASH. Fibrosis was staged 0 – 4 (0 = no fibrosis, 1 = mild fibrosis, 2 = moderate fibrosis, 3 = severe fibrosis, 4 = cirrhosis) (**Figure 8.17a – d**). Advanced fibrosis was defined as fibrosis stage ≥ F3.

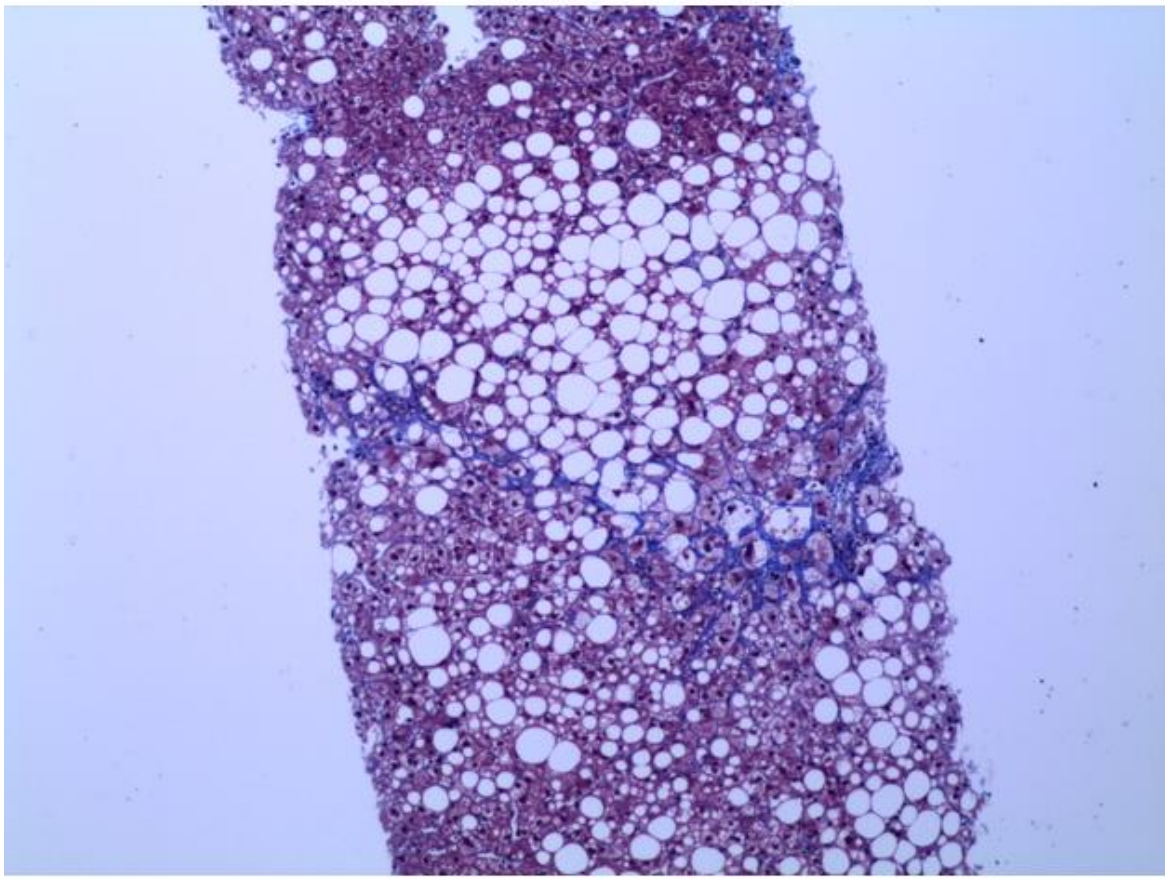

Figure 8.17a Fibrosis stage F1

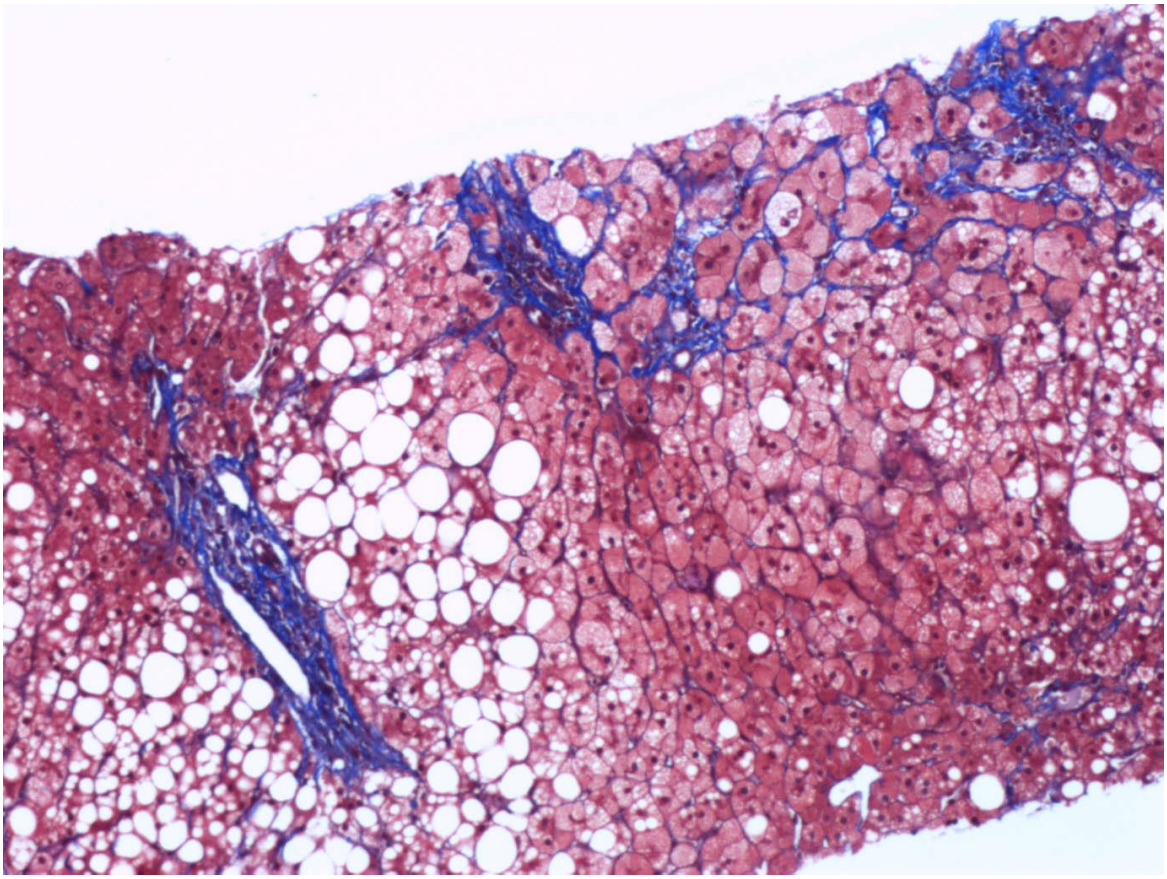

Figure 8.17b Fibrosis stage F2

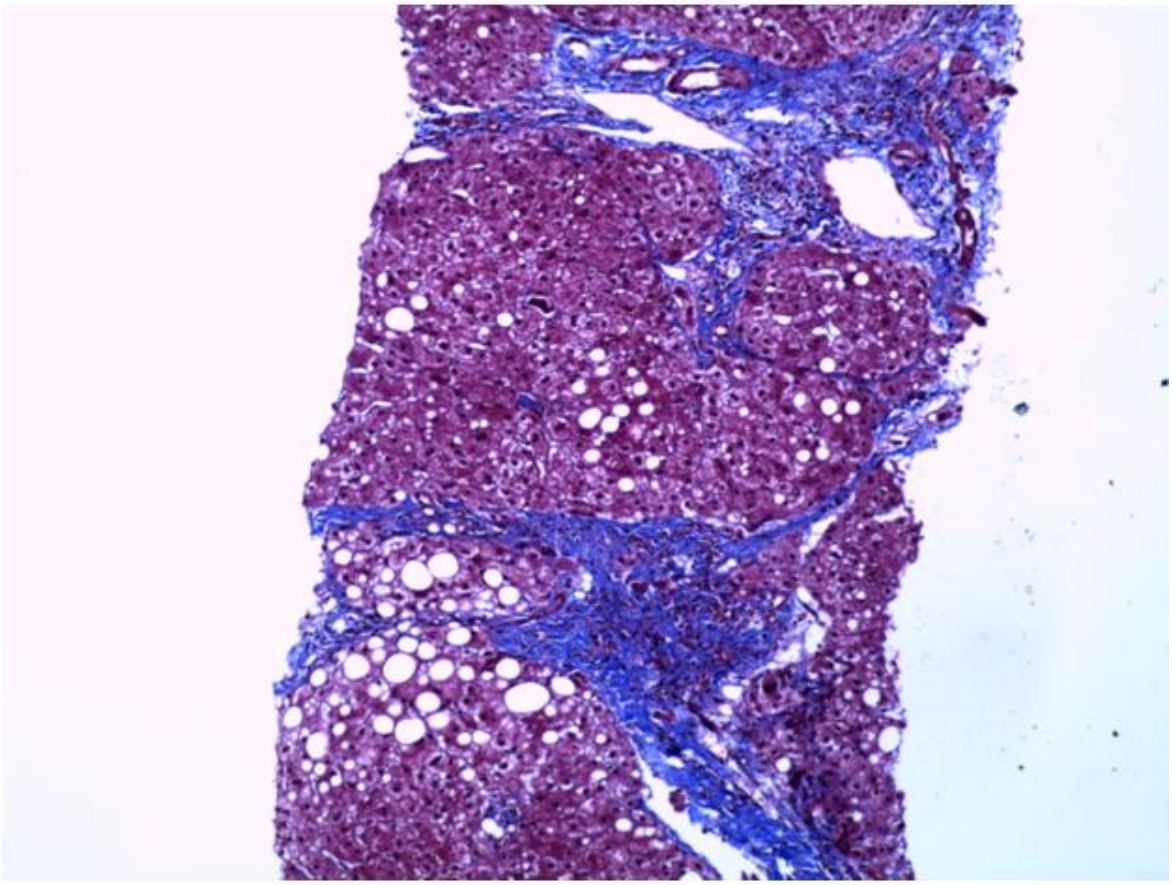

Figure 8.17c Fibrosis stage F3

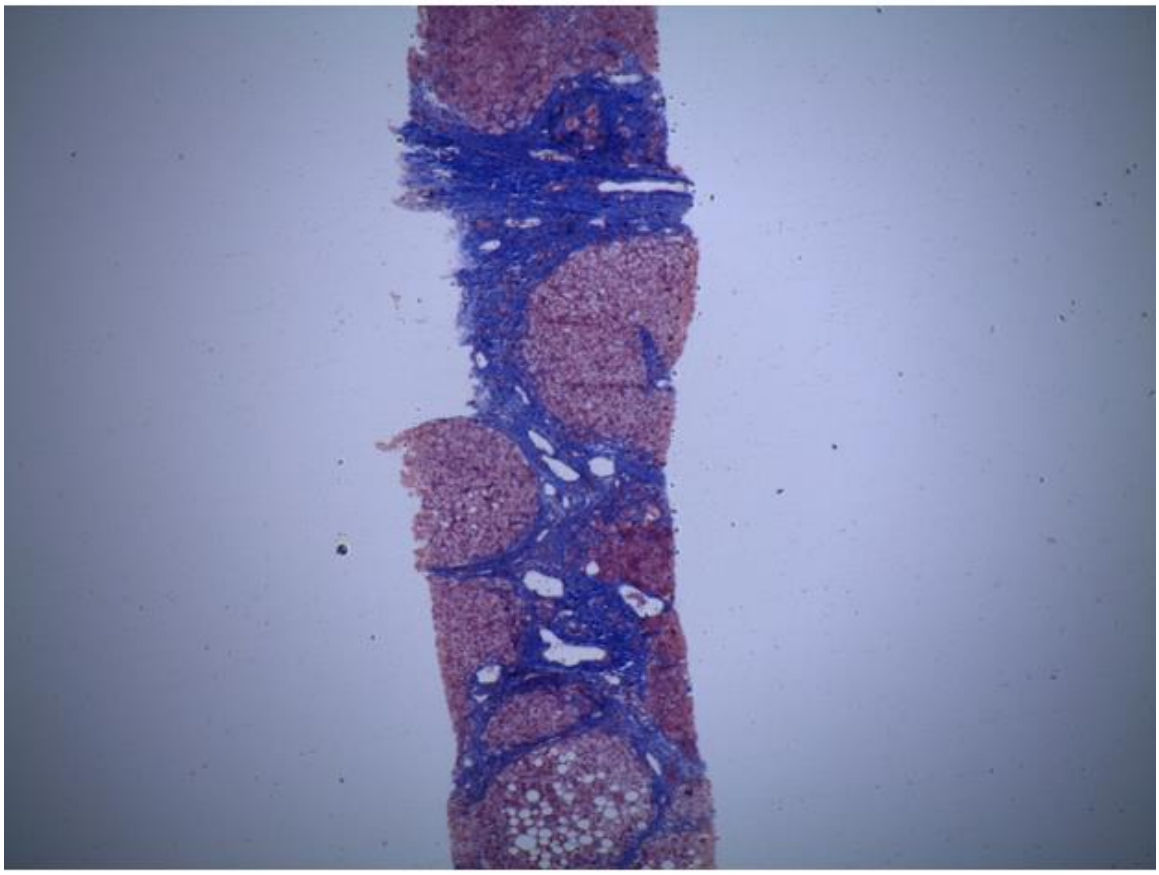

Figure 8.17d Fibrosis stage F4

NAFLD fibrosis score

The NAFLD fibrosis score was calculated using the formula: – 1.675 + 0.037 x age (years) + 0.094 x BMI (kg/m2) + 1.13 x impaired fasting glucose/diabetes (yes = 1, no = 0) + 0.99 x AST/ALT ratio – 0.013 x platelet (109/L) – 0.66 x albumin (g/dL). A score of < – 1.455 was considered as predictive of absence of advanced fibrosis (F0 – F2) while a score of > 0.675 was considered predictive of presence of advanced fibrosis (F3 – F4). A score between – 1.455 and 0.675 was considered indeterminate (Angulo et al., 2007). The use of NAFLD fibrosis score for prediction of advanced fibrosis was evaluated.

Transient elastography

Transient elastography was performed by either one of two experienced operators (WKC, SM) using Fibroscan 502 Touch with M probe (EchoSens, Paris, France) on the same day of the liver biopsy procedure. Ten measurements were obtained for each patient. Adequate pressure of the probe on the skin surface, good layering on TM mode and a straight imaginary line on A mode were ensured for each measurement. An examination was considered successful when valid measurements were ≥ 80 % and IQR/median for liver stiffness measurement was ≤ 30 %. Patients with unsuccessful examination were excluded from analysis. Previously reported optimal cut-offs for estimation of the different stages of liver fibrosis were used (Yoneda et al., 2007). The use of liver stiffness measurement for prediction of different stages of fibrosis was evaluated. The optimal cut-off for advanced fibrosis was 8 kPa. A higher cut-off to predict the presence of advanced fibrosis was determined using data from the training cohort and tested in the validation cohort. This model reduced the false positive rate of liver stiffness measurement for predicting the presence of advanced fibrosis but resulted in a grey zone.

Combining NAFLD fibrosis score and liver stiffness measurement for prediction of advanced fibrosis

The combination of NAFLD fibrosis score and liver stiffness measurement for prediction on advanced fibrosis was evaluated. An algorithm combining the NAFLD fibrosis score and liver stiffness measurement was developed based on findings from the training cohort and subsequently tested in the validation cohort. The percentages of misclassifications and number of patients requiring liver biopsy were evaluated when using the NAFLD fibrosis score alone, liver stiffness measurement alone, both tests for all patients and the algorithm.

Statistical analysis

Data were analyzed using SPSS 15.0. Continuous variables were expressed as mean ± standard deviation or median (interquartile range), and analyzed using student's t-test or Mann-Whitney test, as appropriate. Categorical variables were expressed as percentages, and analyzed using chi-square test or Fisher's exact test, as appropriate. Boxplots were used to show the distribution of liver stiffness measurements for each fibrosis stage. Liver stiffness measurements between and across fibrosis stages were compared using Mann-Whitney U test and Kruskal-Wallis test, respectively. Significance was assumed when $p < 0.05$. The sensitivity, specificity, positive predictive value and negative predictive value of the NAFLD fibrosis score, liver stiffness measurement and the 2 combination models for predicting liver fibrosis were determined.

8.4.2 Results

Patient characteristics

A total of 105 NAFLD patients had liver biopsy during the study period for the training cohort. Four patients were excluded as transient elastography was unsuccessful. Data for 101 patients were analyzed. A total of 48 NAFLD patients underwent a liver biopsy during the study period for the validation cohort. Two patients were excluded as transient elastography was unsuccessful. Data for 46 patients were analyzed. Patient characteristics are shown in **Table 8.11**. Demographic, anthropometric, clinical and laboratory data were comparable between the two groups.

Table 8.11 Patient characteristics

	Overall n = 147	Training cohort n = 101	Validation cohort n = 46	p
Age, years	50.5 ± 11.7	50.3 ± 11.3	50.9 ± 12.5	0.775
Male, %	54.4	51.5	60.9	0.289
Body mass index, kg per m^2	29.3 ± 4.5	29.6 ± 3.9	28.8 ± 5.7	0.313
Obesity, %	83.7	87.1	76.1	0.093
Waist circumference, cm	98.2 ± 10.1	97.7 ± 9.5	99.3 ± 11.2	0.370
Central obesity, %	95.2	96.0	93.5	0.678
Diabetes mellitus, %	52.4	52.5	52.2	0.973
Hypertension, %	89.1	89.9	91.3	1.000
Dyslipidemia, %	94.6	95.0	93.5	0.706
Fasting blood glucose, mmol/L	6.3 ± 2.2	6.3 ± 2.3	6.5 ± 1.9	0.557
HbA1c, %	6.5 ± 1.5	6.7 ± 1.6	6.3 ± 1.3	0.169
Triglyceride, mmol/L	1.73 ± 0.74	1.76 ± 0.76	1.66 ± 0.68	0.432
Total cholesterol, mmol/L	4.98 ± 1.15	5.00 ± 1.17	4.92 ± 1.12	0.663
High-density lipoprotein, mmol/L	1.15 ± 0.26	1.12 ± 0.23	1.21 ± 0.30	0.066
Low-density lipoprotein, mmol/L	3.07 ± 1.03	3.12 ± 1.03	2.96 ± 1.03	0.363
Alkaline phosphatase, IU/L	84 ± 27	80 ± 23	91 ± 33	0.026
Alanine aminotransferase, IU/L	71 (48 – 111)	71 (44 – 115)	68 (49 – 107)	0.874
Aspartate aminotransferase, IU/L	41 (29 – 66)	42 (29 – 66)	40 (29 – 70)	0.820
Gamma glutamyl transpeptidase, IU/L	82 (47 – 128)	75 (47 – 125)	94 (56 – 135)	0.152
Liver biopsy length, mm	14.9 ± 3.7	14.7 ± 3.9	15.3 ± 3.2	0.369
Number of portal tracts	8.4 ± 2.9	8.3 ± 2.7	8.4 ± 3.2	0.857
Steatosis score, %				
S0, less than 5 %	2.0	3.0	0	0.045
S1, 5 – 33 %	31.3	32.7	28.3	
S2, 34 – 66 %	46.9	50.5	39.1	
S3, more than 66 %	19.7	13.9	32.6	

	Overall n = 147	Training cohort n = 101	Validation cohort n = 46	p
NAFLD activity score, %				
0 – 2, not diagnostic of NASH	35.4	6.0	10.9	0.362
3 – 4, probable NASH	33.3	48.5	36.9	
5 – 8, definite NASH	31.3	45.5	52.2	
Fibrosis score, %				
0, no fibrosis	29.3	30.7	26.1	0.198
1, mild fibrosis	41.5	44.6	34.8	
2, moderate fibrosis	8.2	5.9	13.0	
3, severe fibrosis	19.0	15.8	26.1	
4, cirrhosis	2.0	3.0	0	
Transient elastography				
Success rate, %	98.3 ± 7.1	99.2 ± 2.6	96.3 ± 11.9	0.020
Median E, kPa	7.8 (5.9 – 11.8)	7.8 (5.9 – 11.4)	8.0 (5.9 – 11.9)	0.545
IQR/median for E, %	13 (9 – 17)	12 (8 – 16)	15 (10 – 20)	0.015
Median CAP, dB/m	321 (294 – 346)	315 (285 – 343)	332 (309 – 352)	0.046
IQR/median for CAP, %	7 (5 – 10)	7 (5 – 11)	7 (6 – 10)	0.634

*p value comparing the training and validation cohort

S = steatosis grade, NAFLD = non-alcoholic fatty liver disease, NASH = non-alcoholic steatohepatitis, E = estimated liver stiffness, IQR = interquartile range, CAP = controlled attenuation parameter

NAFLD fibrosis score for prediction of advanced fibrosis

In the training cohort, the NAFLD fibrosis score predicted absence of advanced fibrosis in 66 patients and presence of advanced fibrosis in 4 patients. The NAFLD fibrosis score was indeterminate for 31 patients. Of the 66 patients predicted as not having advanced fibrosis, 63 patients were correctly identified while 3 patients were not. Of the 4 patients predicted to have advanced fibrosis, 2 patients were correctly identified while 2 patients were not. The sensitivity, specificity, positive predictive value and negative predictive value for advanced fibrosis was 40.0 %, 96.9 %, 50.0 % and 95.5 %, respectively.

In the validation cohort, the NAFLD fibrosis score predicted absence of advanced fibrosis in 32 patients and presence of advanced fibrosis in 1 patient. The NAFLD fibrosis score was indeterminate for 13 patients. Of the 32 patients predicted as not having advanced fibrosis, 28 patients were correctly identified while 4 patients were not. The patient predicted to have advanced fibrosis did have advanced fibrosis. The sensitivity, specificity, positive predictive value and negative predictive value for advanced fibrosis was 20.0 %, 100.0 %, 100.0 % and 87.5 %, respectively.

Liver stiffness measurement for estimation of fibrosis stage

Liver stiffness measurements according to histological fibrosis stage are shown in **Figure 8.18**. The median liver stiffness measurements (with 95 % confidence intervals) for F0, F1, F2, F3 and F4 were 5.40 kPa (4.40 kPa – 7.20 kPa), 6.95 kPa (5.90 kPa – 9.58 kPa), 9.90 kPa (8.63 kPa – 12.35 kPa), 13.90 kPa (10.70 kPa – 17.70 kPa) and 26.30 kPa (20.20 kPa – 35.30 kPa), respectively. There were significant increases in liver stiffness measurements with increasing histological fibrosis stage ($p < 0.001$). The sensitivity, specificity, positive predictive value and negative predictive value of liver stiffness measurement for estimating the various fibrosis stages in the training cohort is as shown in **Table 8.12**.

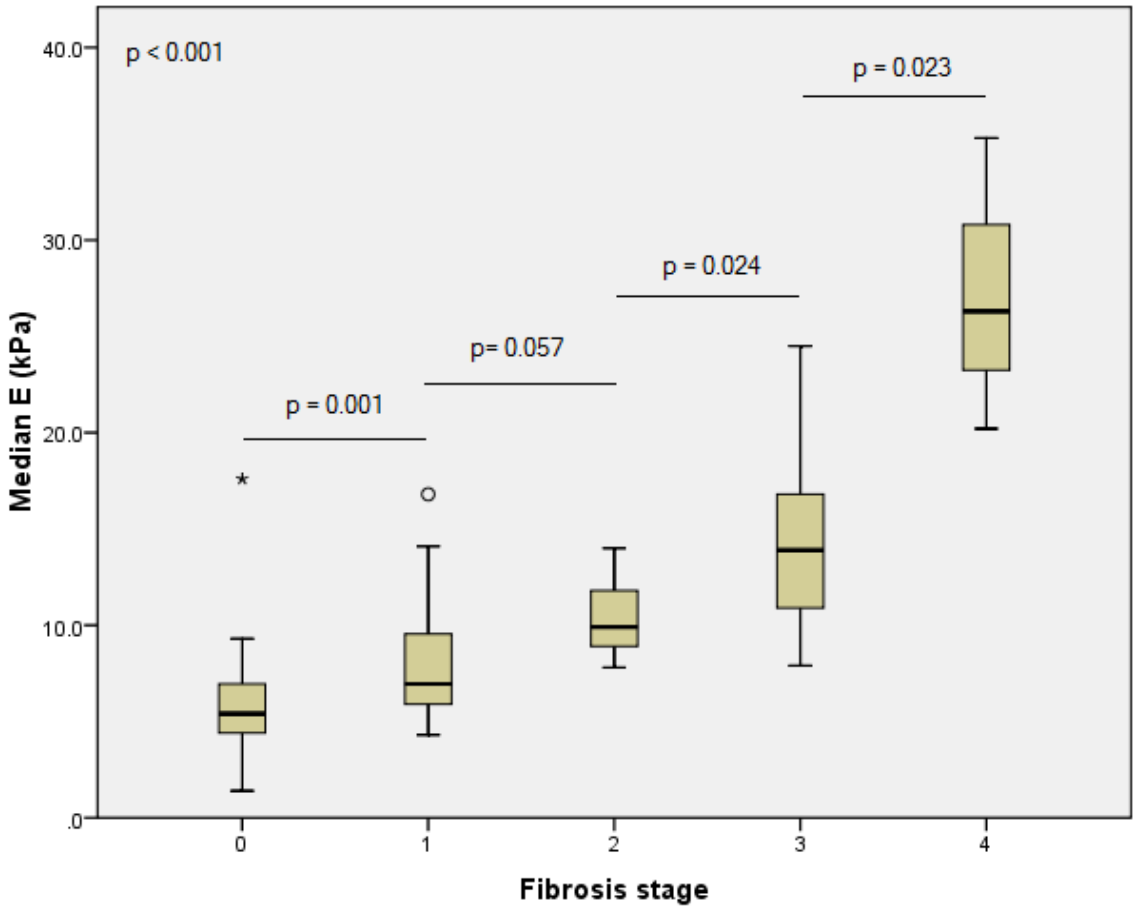

Figure 8.18 Liver stiffness measurements according to histological fibrosis stage

Table 8.12 The sensitivity, specificity, positive predictive value and negative predictive value of liver stiffness measurement for estimation of fibrosis stage equal to or greater than F1, F2, F3 and F4 in the training cohort using the cut-offs reported by Yoneda et al (Yoneda et al., 2007)

	F1	F2	F3	F4
Optimal cut-off, kPa	5.60	6.65	8.00	17.0
Sensitivity, %	89.4	100.0	95.0	100.0
Specificity, %	81.4	70.0	80.2	96.2
Positive predictive value, %	81.9	44.1	45.2	37.5
Negative predictive value, %	89.1	100.0	98.9	100.0

Liver stiffness measurement for prediction of advanced fibrosis

In the training cohort, 55 patients were predicted not to have advanced fibrosis while 46 patients were predicted to have advanced fibrosis. Of the 55 patients predicted not to have advanced fibrosis, 54 patients were correctly identified whilst 1 patient was not. Of the 46 patients predicted to have advanced fibrosis, 18 patients were correctly identified whilst 28 patients were not. A higher cut-off with greater specificity for prediction of advanced fibrosis was determined using the training cohort. The cut-off determined was 17 kPa. Using this higher cut-off, 9 patients were predicted to have advanced fibrosis while 37 patients were in the grey zone of 8 – 17 kPa. Of the 9 patients predicted to have advanced fibrosis using this higher cut-off, 8 patients were correctly identified while 1 patient was not. Using liver stiffness measurement < 8 kPa to predict absence of advanced fibrosis and ≥ 17 kPa to predict presence of advanced fibrosis, the sensitivity, specificity, positive predictive value and negative predictive value was 88.9 %, 98.2 %, 88.9 % and 98.2 %, respectively.

In the validation cohort, 23 patients were predicted not to have advanced fibrosis whilst 23 patients were predicted to have advance fibrosis. Of the 23 patients predicted not to have advanced fibrosis, 22 patients were correctly identified and 1 patient was not. Of the 23 patients predicted to have advanced fibrosis, 11 patients were correctly identified whilst 12 patients were not. Using the 17 kPa cut-off, 4 patients were predicted to have advanced fibrosis and 19 patients were in the grey zone of 8 – 17 kPa. All 4 patients who were predicted to have advanced fibrosis had advanced fibrosis on histology. Using liver stiffness measurement < 8 kPa to predict absence of advanced fibrosis and ≥ 17 kPa to predict presence of advanced fibrosis, the sensitivity, specificity, positive predictive value and negative predictive value was 80.0 %, 100 %, 100 % and 98.2 %, respectively.

Combining the NAFLD fibrosis score and liver stiffness measurement for prediction of advanced fibrosis

In the training cohort, liver stiffness measurement was unhelpful in patients already predicted not to have advanced fibrosis using the NAFLD fibrosis score (**Figure 8.19**). If NAFLD fibrosis score alone was used, only 3 of the 66 patients (4.5 %) identified not to have advanced fibrosis would be misclassified. Although the combination of NAFLD fibrosis score and liver stiffness measurement could accurately identify patients without advanced fibrosis when they agree with each other, there was disagreement in 22 of the 66 patients (33.3 %). In other words, a substantial proportion of patients would need to undergo a liver biopsy to confirm their fibrosis stage when liver stiffness measurement was used in addition to the NAFLD fibrosis score to identify the small percentage of patients that would otherwise be misclassified as not having advanced fibrosis based on the NAFLD fibrosis score alone.

There were only 4 patients predicted to have advanced fibrosis using the NAFLD fibrosis score, making it difficult to draw any conclusions regarding the role of liver stiffness measurement in this group of patients. While further studies with larger number of such patients are needed, it seemed reasonable that patients in whom the methods agree would not require a liver biopsy to confirm the presence of advanced fibrosis while patients in whom the methods disagree should have a liver biopsy to confirm the presence or absence of advanced fibrosis.

As for patients in the indeterminate group based on the NAFLD fibrosis score, using the 8 and 17 kPa cut-offs for liver stiffness measurement increased the accuracy to predict the absence and presence of advanced fibrosis. All 7 patients predicted to have advanced fibrosis using this higher cut-off had advanced fibrosis. On the other hand, only 1 of the 8

patients identified as not having advanced fibrosis, actually had advanced fibrosis. The 16 patients in the grey zone of 8 – 17 kPa should be considered for a liver biopsy.

Based on these findings, we developed a 2-step algorithm for non-invasive prediction of advanced fibrosis in NAFLD patients (**Figure 8.20**). The sensitivity, specificity, positive predictive value and negative predictive value using this strategy to predict advanced fibrosis in the training cohort was 69.2 %, 98.6 %, 90.0 % and 94.6 %, respectively. Seventeen patients (16.8 %) would be considered for a liver biopsy. This strategy halved the number of patients requiring a liver biopsy compared to using the NAFLD fibrosis score alone while maintaining the accuracy for prediction of advanced fibrosis.

The distribution of patients according to their NAFLD fibrosis score and liver stiffness measurement, and the status of advanced fibrosis in the validation cohort is as shown in **Figure 8.21**. In the validation cohort, the sensitivity, specificity, positive predictive value and negative predictive value was 42.9%, 100 %, 100 % and 88.6 %, respectively. Eight patients (17.4 %) would be considered for a liver biopsy.

The percentages of misclassifications and patients requiring a liver biopsy using the NAFLD fibrosis score alone, liver stiffness measurement alone, both tests for all patients and the 2-step algorithm in the training and validation cohorts are shown in **Tables 8.13 and 8.14**, respectively. In the training cohort, the combination of NAFLD fibrosis score and liver stiffness measurement for all patients provided no advantage over using either of the tests alone. This was confirmed in the validation cohort.

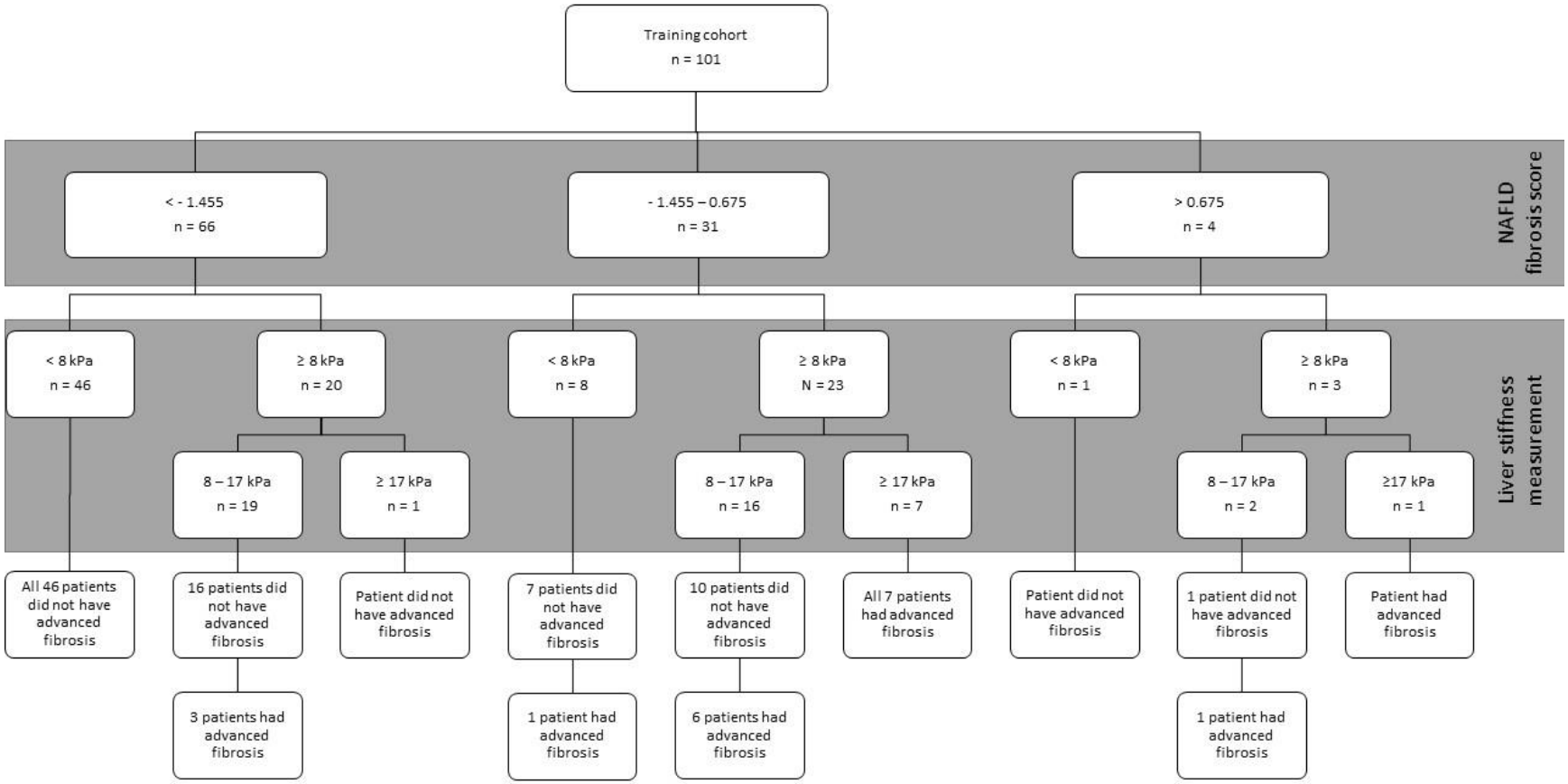

Figure 8.19 Distribution of patients according to their NAFLD fibrosis score and liver stiffness measurement, and the status of advanced fibrosis in the training cohort

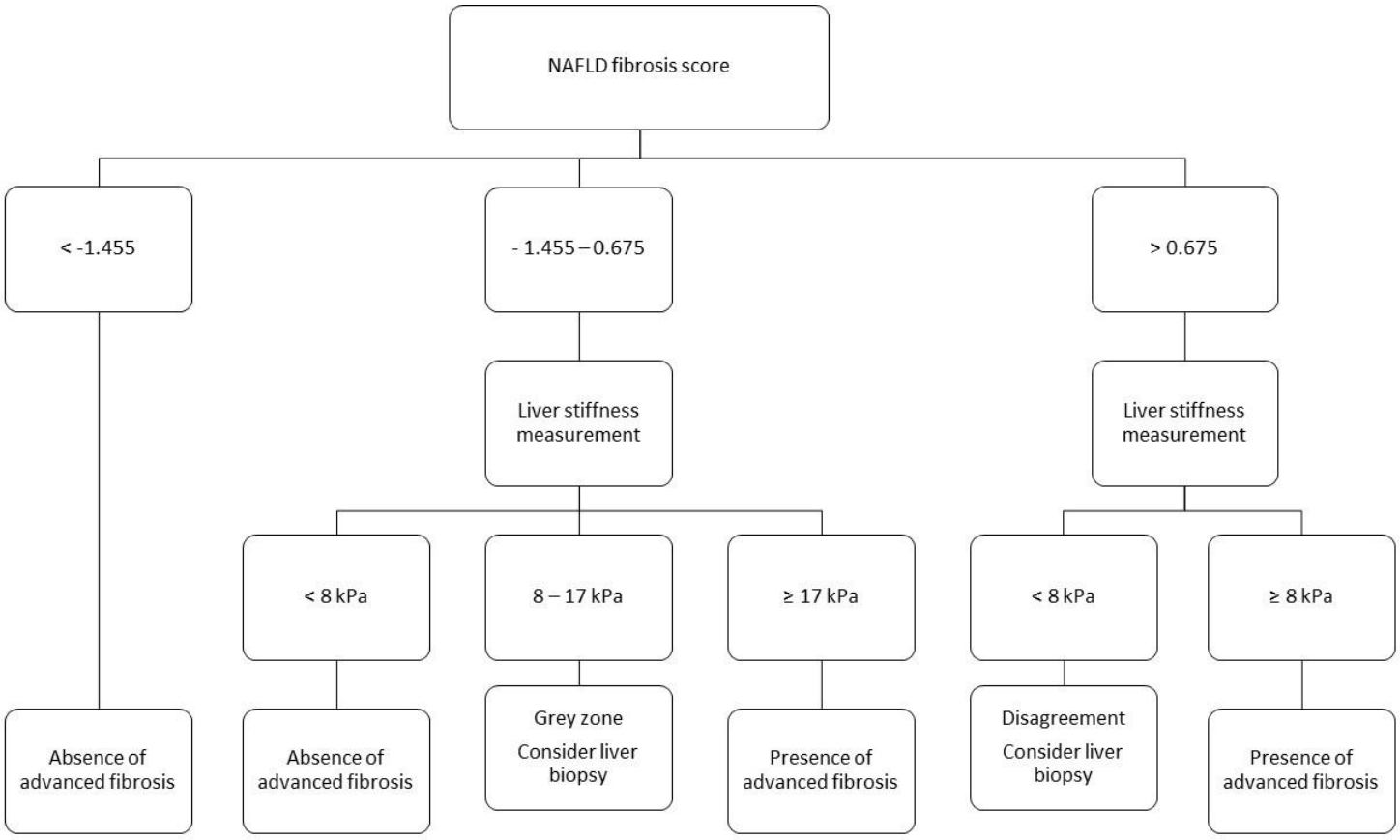

Figure 8.20 Proposed algorithm for the prediction of advanced fibrosis using a combination of NAFLD fibrosis score and liver stiffness measurement

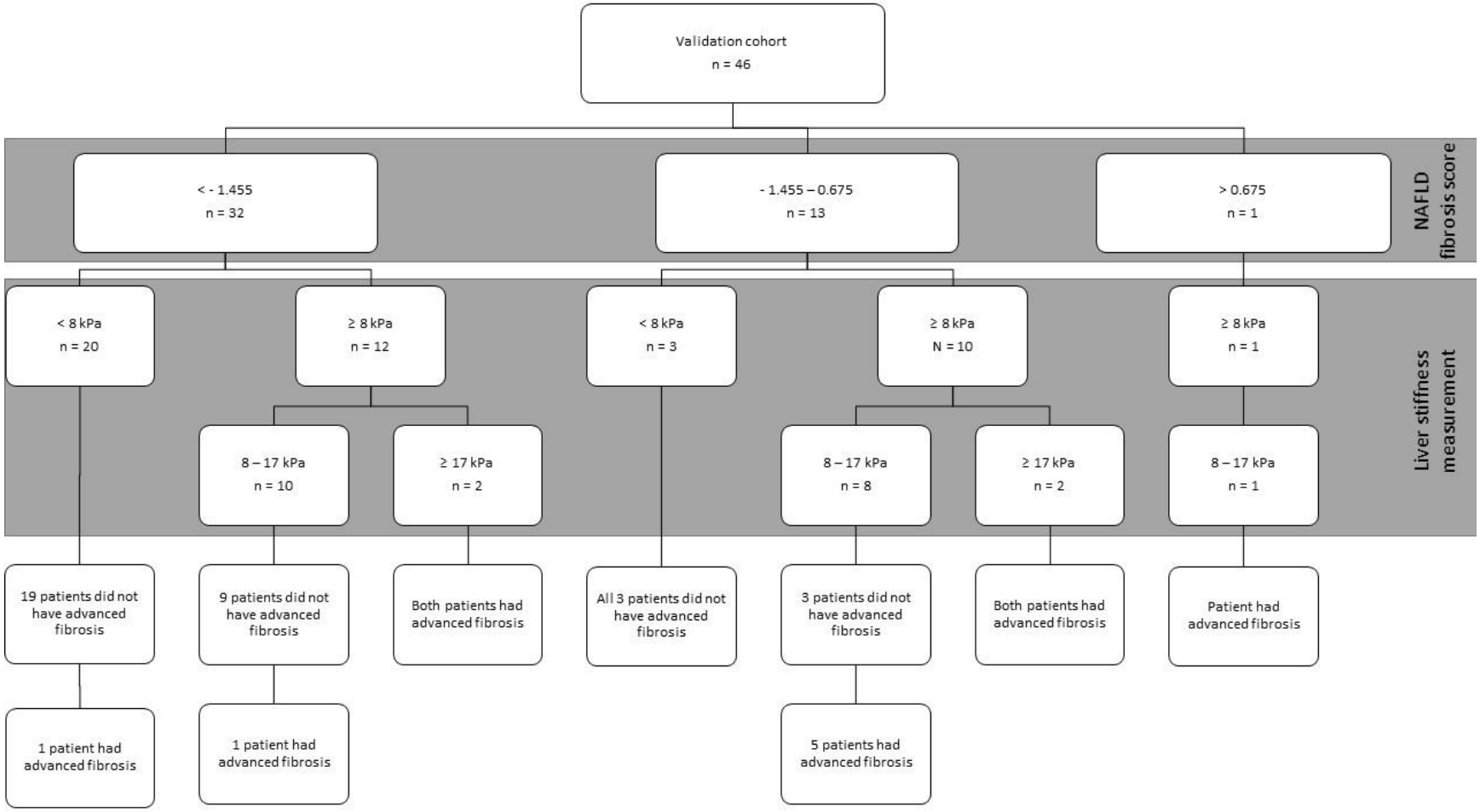

Figure 8.21 Distribution of patients according to their NAFLD fibrosis score and liver stiffness measurement, and the status of advanced fibrosis in the validation cohort

Table 8.13 The percentages of misclassifications and patients requiring a liver biopsy using the NAFLD fibrosis score alone, transient elastography alone, and the 2 models combining the NAFLD fibrosis score and liver stiffness measurement in the training cohort

	Misclassifications, %	Patients requiring a liver biopsy, %
NAFLD fibrosis score alone	7.1	30.7
Liver stiffness measurement alone	30.7	0
Liver stiffness measurement alone (with grey zone)	2.0	36.6
Both tests for all patients	2.0	36.6
2-step approach	6.0	16.8

Table 8.14 The percentages of misclassifications and patients requiring a liver biopsy using the NAFLD fibrosis score alone, transient elastography alone, and the 2 models combining the NAFLD fibrosis score and liver stiffness measurement in the validation cohort

	Misclassifications, %	Patients requiring a liver biopsy, %
NAFLD fibrosis score alone	8.7	28.3
Liver stiffness measurement alone	28.3	0
Liver stiffness measurement alone (with grey zone)	2.2	41.3
Both tests for all patients	2.2	43.5
2-step approach	8.7	17.4

8.4.3 Discussion

While the survival of patients with simple steatosis approach that of the general population, patients with NASH have a higher mortality which is mainly attributed to progression of the liver disease. In a meta-analysis, patients with NASH had a liver-related mortality of 11 % – 17.5 % compared to 1.7 % – 2.7 % in patients with simple steatosis. Furthermore, NASH patients with advanced fibrosis had an even higher liver-related mortality compared to those without advanced fibrosis (Musso et al., 2011). Hence, assessing the severity of liver fibrosis in NAFLD patients is important to guide prognosis and to plan management. Due to the limitations of liver biopsy, non-invasive tests for estimation of liver fibrosis are gaining popularity. Evidence to support the use of non-invasive tests to predict patient outcomes are also beginning to emerge. In a retrospective study of 320 patients of which nearly 50 % had NASH with advanced fibrosis, Angulo and colleagues showed that the NAFLD fibrosis score predicted adverse liver-related outcomes with an AUROC of 0.86 (Angulo et al., 2013).

The strength of the NAFLD fibrosis score lies in its convenience of use and accuracy. Besides using readily available parameters, a freely-available and user-friendly on-line calculator makes it even more appealing (Mofrad et al., 2003). However, using the NAFLD fibrosis score alone, a substantial proportion of patients will fall in the indeterminate group. In the training cohort of our study population, the NAFLD fibrosis score resulted in misclassifications in only 7.1 % of patients but 30.7 % of patients were in the indeterminate group and would have required further evaluation with a liver biopsy. On the other hand, liver stiffness measurement was associated with a high false positive rate for advanced fibrosis resulting in misclassifications in 30.7 % of patients. A higher cut-off for predicting the presence of advanced fibrosis was determined and tested in our study to overcome the

high false positive rate. While misclassifications declined to 2.0 %, 38.6 % of patients were in the grey zone and would have required further evaluation with a liver biopsy.

Our study clearly demonstrated that using both the NAFLD fibrosis score and liver stiffness measurement for all patients provided no advantage over using either of the tests alone. However, a 2-step algorithm using the NAFLD fibrosis score followed by liver stiffness measurement for patients with indeterminate and high NAFLD fibrosis score could reduce the number of patients requiring a liver biopsy whilst maintaining the accuracy of predicting advanced fibrosis. Combinations of non-invasive tests to predict fibrosis have been studied for other chronic liver diseases, such as the combination of liver stiffness measurement with Fibrometer for chronic hepatitis C (Boursier et al., 2011), and with Enhanced Liver Fibrosis for chronic hepatitis B (Wong et al., 2014). To the best of our knowledge, this is the first study to prospectively evaluate the combination of liver stiffness measurement and NAFLD fibrosis score for predicting advanced fibrosis in NAFLD patients.

The study was carried out prospectively according to a planned protocol so that the data collected was robust. Liver biopsy and transient elastography were performed on the same day to minimize differences in findings due to changes over time. The procedures were carried out by experienced operators to ensure a good quality of specimens and measurements. However, as in any study using liver histology as the gold standard reference, the study may be limited by sampling and observer variability. Further studies should be carried out to determine the feasibility and cost-effectiveness of using this 2-step approach to identify NAFLD patients with advanced fibrosis to guide management and whether it could be used to predict patient outcomes.

8.4.4 Conclusion

The use of liver stiffness measurement for patients with indeterminate and high NAFLD fibrosis scores allows accurate prediction of advanced fibrosis and reduced the number of patients requiring a liver biopsy. The combination of NAFLD fibrosis score and liver stiffness measurement for all patients provided no advantage over using either of the tests alone.

Note: A poster on the findings from this study was presented at the Asia-Pacific Digestive Week 2014 in Bali, Indonesia, and the abstract was published in a supplementary issue of the Journal of Gastroenterology and Hepatology (Chan et al., 2014). The findings from this study was also presented at the Malaysian Society of Gastroenterology and Hepatology Annual Scientific Meeting in 2014. The full article has been accepted for publication in Hepatology International (Chan et al., 2014).

Chapter 9

NAFLD in young adults

9.1 Introduction

As elucidated in Chapter 3 and reiterated in other earlier chapters of this thesis, non-alcoholic fatty liver disease (NAFLD) has been rapidly increasing in the Asian-Pacific and has been estimated to affect up to 30 % of the general population (Chan et al., 2013). In Malaysia, the prevalence of NAFLD in the general population has been estimated to be 22.7 % based on a study on individuals attending a health-check in a suburban medical facility (Goh et al., 2012). The prevalence of NAFLD among diabetics has been estimated to be 49.6 % based on a separate study on a hospital clinic population (Chan et al., 2013) (see Chapter 4). Both studies found an inordinately high prevalence of NAFLD among the Malays and Indians compared to the Chinese. There has been no published study on the prevalence of NAFLD among young adults in Malaysia. Whether the prevalence of NAFLD is different among young adults of different ethnic origin is unknown. Moreover, published studies that looked specifically at the prevalence of NAFLD and associated factors among young adults were limited in the existing literature. Hence, we embarked on this study to determine the prevalence of NAFLD among young adults and to identify associated factors. We also aimed to see if the prevalence of NAFLD were different among young adults of different ethnic origin.

9.2 Patients and Methods

This was a cross-sectional study on students pursuing their tertiary education at the Faculty of Medicine, University of Malaya who responded to an advertisement put up to

invite students to participate in the study. The study was approved by the University of Malaya Medical Centre's Medical Ethics Committee and informed consent was obtained from all included subjects.

Demographic and anthropometric data and relevant clinical and laboratory data were obtained using a standard protocol. Alcohol intake was estimated using the quantity-frequency method (Goddard, 2007). Alcohol intake was estimated based on subject's self-reported frequency and quantity of intake of each of the 3 main types of alcoholic beverages i.e. beer, wine and spirit. Frequency of intake was divided into 7 categories i.e. almost every day, 5 or 6 days a week, 3 or 4 days a week, once or twice a week, once or twice a month, once every couple of months and once or twice a year. Each of these categories provided a multiplying factor for calculation of alcohol intake per week. Information on average intake during each drinking session was captured using common serving measurements and this was translated into units of alcohol based on the volume consumed and the alcohol by volume for each of the types of alcoholic beverages. Units of alcohol consumed in a week in the form of beer, wine and spirit was calculated separately and summed up to give an estimate of alcohol intake per week for each patient. Significant alcohol intake was defined as more than 21 units per week for men and more than 14 units per week for women (Chalasani et al., 2012).

Frequency and duration of physical activities of moderate and vigorous intensity were determined for each student. According to recommendations by the American College of Sports Medicine and the American Heart Association, the following are the minimal amount of physical activity required to achieve substantial health benefits over and above the routine light-intensity physical activities of daily living: (1) 30 minutes of moderate-intensity physical activity 5 days per week, (2) 20 minutes of vigorous-intensity physical activity 3

days per week, or (3) a combination of moderate- and vigorous-intensity physical activity of more than 450 MET-minutes per week (Haskell et al., 2007). The term "physically active" was used to refer to students who reported any of these levels of physical activity in the study.

Weight and height were measured using standardized equipment. BMI was calculated by dividing weight in kilogram by the square of height in meters. Subjects were categorized as underweight (BMI < 18.5 kg per m^2), normal (18.5 kg per m^2 ≤ BMI < 23.0 kg per m^2), overweight (23.0 kg per m^2 ≤ BMI < 25.0 kg per m^2) or obese (BMI ≥ 25.0 kg per m^2) (Anuurad et al., 2003). Waist circumference (WC) was measured at the mid-point between the lowest margin of the least palpable rib and the top of the iliac crest in the standing position. Central obesity was defined as WC > 90 cm for men and > 80 cm for women (Alberti et al., 2005). Blood pressure was measured in the sitting position using standardized equipment.

All subjects had venous blood drawn after an overnight fast for blood glucose, glycated hemoglobin (HbA1c), lipid profile, liver profile, and viral hepatitis B and C serology. Biochemical measurements were performed using standard laboratory procedures. Impaired fasting glucose (IFG) was defined as fasting blood glucose (FBS) ≥ 5.5 mmol/L. A patient was considered to have dyslipidemia if the serum total cholesterol (TC) was ≥ 5.2 mmol/L, if the serum triglyceride (TG) was ≥ 1.7 mmol/L, if the serum high-density lipoprotein (HDL) was < 1.0 mmol/L for men or < 1.3 mmol/L for women, or if the serum low-density lipoprotein (LDL) was ≥ 3.4 mmol/L. A patient was considered to have metabolic syndrome if three or more of the following were present: (1) central obesity, (2) systolic blood pressure ≥ 130 mmHg and/or diastolic blood pressure ≥ 85 mmHg, (3) IFG, (4) hypertriglyceridemia, or (5) low serum HDL (according to the aforementioned cut-offs) (Alberti et al., 2009). Our laboratory's upper limit of normal for liver enzymes were as

follow: alkaline phosphatase (ALP) 136 IU/L, aspartate aminotransferase (AST) 37 IU/L, alanine aminotransferase (ALT) 65 IU/L and gamma-glutamyl transpeptidase (GGT) 55 IU/L. Serum ALP, AST, ALT and GGT above these levels were considered as elevated. In addition, a more stringent cut-off of 30 IU/L for men and 19 IU/L for women was used for serum ALT level during data analysis. The Elecsys HBsAg II assay and the Elecsys Anti-HCV II assay (Roche, Mannheim, Germany) were used to test for viral hepatitis B and C infection, respectively.

Diagnosis of NAFLD was by trans-abdominal ultrasonography and following exclusion of significant alcohol intake, use of medications known to cause fatty liver and other causes of chronic liver disease. The following criteria were used for ultrasonographic diagnosis of fatty liver: increased echogenicity, posterior attenuation and loss of intra-hepatic architectural details (Joy et al., 2003). Investigators involved in other parts of the study were blinded to the ultrasonography findings, vice versa.

Statistical analysis

With an estimated prevalence of 12.5 % based on a previous study that included adolescents (Alavian et al., 2009), a sample size of 169 patients was needed to estimate the prevalence with 95 % confidence and 5 % precision. Data were analyzed using SPSS 15.0. Continuous variables were expressed as mean ± standard deviation or median (inter-quartile range), and analyzed using student's t-test or Mann-Whitney U test where appropriate. Categorical variables were expressed as percentage and analyzed using chi-square test or Fisher's exact test where appropriate. Independent factors associated with NAFLD were identified using multiple logistic regression analysis. Significance was assumed at $p < 0.05$.

9.3 Results

Subject characteristics

Four hundred and seventy two subjects were included in the analysis (**Figure 9.1**). Mean age of the study population was 23.2 ± 2.4 years old comprising of 40.5 % men. The racial distribution was as follow: Chinese 53.6 %, Malay 30.3%, Indian 15.5 % and others 0.6 %. Central obesity was seen in 18.2 % of the study population. Thirteen subjects (2.8 %) had the metabolic syndrome.

Prevalence of NAFLD and associated factors

The prevalence of NAFLD was 8.1 % (38/472). Characteristics of subjects with and without NAFLD are shown in **Table 9.1**. Subjects with NAFLD were older, had greater BMI and WC, and recorded higher SBP and DBP. They had higher FBS, serum TG and LDL levels and lower serum HDL level. Serum ALP, ALT, AST and GGT levels were higher in subjects with NAFLD. All subjects who had NAFLD had insulin resistance. Family history of diabetes mellitus, dyslipidemia or hypertension was not found to be associated with NAFLD. Prevalence of NAFLD was not significantly different between subjects who were "physically active" and those who were not.

The prevalence of NAFLD was significantly higher among males compared to females (17.9 % vs. 3.3 %, $p < 0.001$). The prevalence of NAFLD was highest among the Indians followed by the Malays and the Chinese. This paralleled the prevalence of obesity among the different ethnic groups (**Table 9.2**). Highest prevalence of NAFLD was seen among Indian and Malay males at 33.3 % and 25.5 %, respectively. The prevalence of NAFLD among Chinese males was 6.8 %.

Univariate and multivariate analyses of factors associated with NAFLD

Univariate and multivariate analyses of factors associated with NAFLD are shown in **Table 9.3**. Obesity (instead of central obesity) and elevated serum ALT level using the standard laboratory cut-off (instead of the more stringent cut-off) were entered into the multivariate analysis as these had stronger association with NAFLD. Independent factors associated with NAFLD were: age, male gender, obesity and elevated serum ALT level.

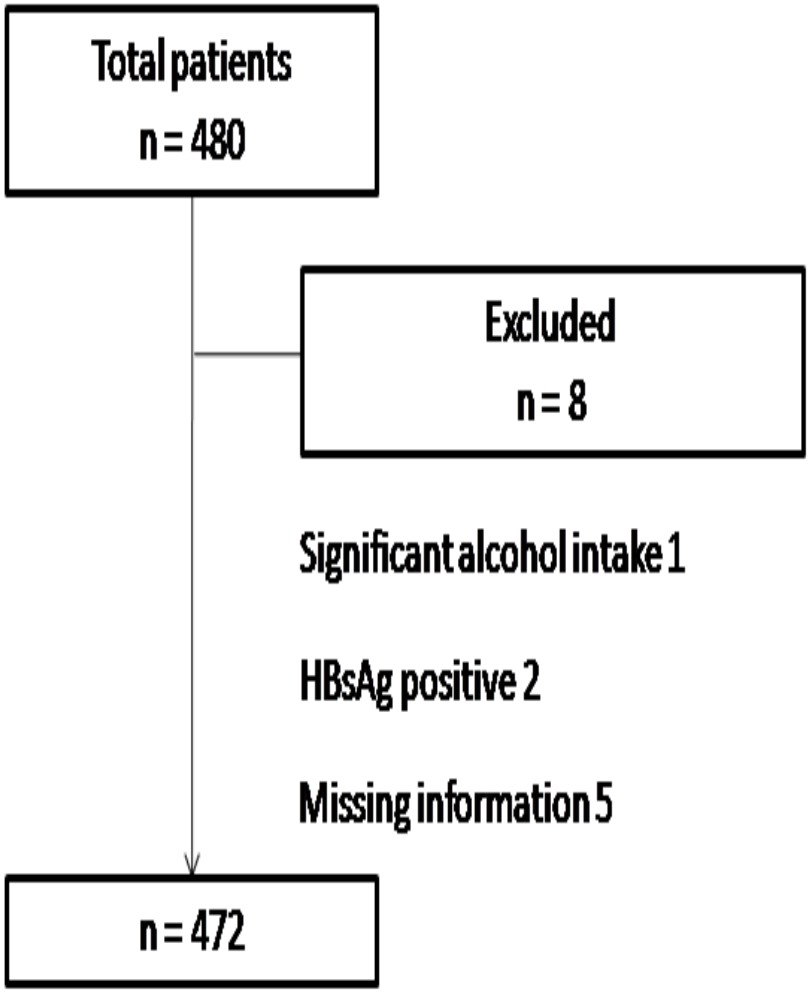

Figure 9.1 Flow chart illustrating the details of subjects included/excluded in the analysis

Table 9.1 Characteristics of subjects with and without NAFLD

	NAFLD		P
	Yes	No	
Age, years	25.2 ± 4.5	23.0 ± 2.1	< 0.001
Male	76.3 %	37.3 %	< 0.001
Race			
Malay	44.7 %	29.0 %	0.001
Chinese	23.7 %	56.2 %	
Indian	28.9 %	14.3 %	
Others	2.6 %	0.5 %	
Family history of diabetes mellitus	57.9 %	69.5 %	0.139
Family history of hypertension	52.6 %	56.8 %	0.618
Family history of ischemic heart disease	15.8 %	8.3 %	0.122
"Physically active"	34.2 %	41.7 %	0.368
Smoking	5.3 %	1.4 %	0.076
Body mass index, kg per m^2	28.7 ± 4.5	20.9 ± 3.2	< 0.001
Obese	81.6 %	8.8 %	< 0.001
Waist circumference, cm	96.4 ± 10.1	74.4 ± 9.5	< 0.001
Centrally obese	81.6 %	12.7 %	< 0.001
Metabolic syndrome	18.9 %	1.2 %	< 0.001
Systolic blood pressure (SBP), mmHg	124 ± 12	115 ± 11	< 0.001
SBP ≥ 130 mmHg	37.8 %	11.6%	< 0.001
Diastolic blood pressure (DBP), mmHg	79 ± 9	75 ± 9	0.003
DBP ≥ 85 mmHg	21.6 %	12.3 %	0.105
Fasting blood sugar, mmol/L	4.9 ± 0.4	4.6 ± 0.4	< 0.001
Impaired fasting glucose	5.4 %	1.4 %	0.126
HOMA IR*	3.6 (2.2 – 4.9)	1.5 (1.0 – 2.1)	< 0.001
Insulin resistant	100.0 %	57.5 %	< 0.001
Total cholesterol (TC), mmol/L	4.7 (4.2 – 5.3)	4.6 (4.1 – 5.1)	0.322

	NAFLD		P
	Yes	No	
TC ≥ 5.2 mmol/L	29.7 %	22.1 %	0.291
High-density lipoprotein (HDL), mmol/L	1.2 (1.1 – 1.4)	1.5 (1.3 – 1.7)	< 0.001
HDL < 1.0 mmol/L for men and < 1.3 mmol/L for women	24.3 %	9.0 %	0.003
Low-density lipoprotein (LDL), mmol/L	2.9 (2.6 – 3.4)	2.7 (2.3 – 3.2)	0.018
LDL ≥ 3.4 mmol/L	21.6 %	16.8 %	0.495
Triglyceride (TG), mmol/L	1.0 (0.7 – 1.6)	0.7 (0.5 – 0.9)	< 0.001
TG ≥ 1.7 mmol/L	21.6 %	2.6 %	< 0.001
Alkaline phosphatase (ALP), IU/L	82 (65 – 95)	69 (58 – 81)	0.001
ALP ≥ 136 IU/L	2.7 %	0.7 %	0.281
Alanine aminotransferase (ALT), IU/L	49 (40 – 72)	27 (21 – 33)	< 0.001
ALT ≥ 65 IU/L	29.7 %	2.1 %	< 0.001
ALT ≥ 30 IU/L for men and ≥ 19 IU/L for women	97.3 %	66.7 %	< 0.001
Aspartate aminotransferase (AST), IU/L	24 (21 – 32)	17 (14 – 21)	< 0.001
AST ≥ 37 IU/L	8.1 %	3.2 %	0.143
Gamma glutamyl transpeptidase (GGT), IU/L	35 (25 – 42)	21 (17 – 26)	< 0.001
GGT ≥ 55 IU/L	5.4 %	1.6 %	0.154

*n = 228 for HOMA IR and insulin resistance

Table 9.2 Prevalence of NAFLD and obesity according to race and gender

	NAFLD	Central obesity
Malay		
Overall	11.9 % (17/143)	22.4 % (32/143)
Male	25.5 % (12/47)	42.6 % (20/47)
Female	5.2 % (5/96)	12.5 % (12/96)
Chinese		
Overall	3.6 % (9/253)	8.3 % (21/253)
Male	6.8 % (8/118)	11.0 % (13/118)
Female	0.7 % (1/135)	5.9 % (8/135)
Indian		
Overall	15.1 % (11/73)	20.5 % (15/73)
Male	33.3 % (8/24)	33.3 % (8/24)
Female	6.1 % (3/49)	14.3 % (7/49)

Table 9.3 Univariate and multivariate analyses of factors associated with NAFLD

	Univariate analysis			Multivariate analysis		
	OR	95 % CI	p	OR	95 % CI	p
Age	1.30	1.16 – 1.46	< 0.001	1.18	1.00 – 1.38	0.046
Male	5.41	2.50 – 11.72	< 0.001	4.92	1.44 – 16.79	0.011
Race						
Malay	3.66	1.59 – 8.44	0.002	2.82	0.85 – 9.41	0.091
Chinese	1	–	–	1	–	–
Indian	4.81	1.91 – 12.12	0.001	4.14	0.98 – 17.45	0.053
Obese	29.38	12.31 – 70.12	< 0.001	19.24	6.94 – 53.32	< 0.001
SBP ≥ 130 mmHg	5.01	2.45 – 10.23	< 0.001	1.15	0.35 – 3.81	0.816
TG ≥ 1.7 mmol/L	11.85	4.55 – 30.88	< 0.001	5.11	0.94 – 27.73	0.058
HDL < 1.0 mmol/L for men and < 1.3 mmol/L for women	3.14	1.39 – 7.12	0.006	2.00	0.49 – 8.14	0.332
ALT ≥ 65 mmol/L	19.19	7.33 – 50.28	< 0.001	9.96	2.42 – 40.95	0.001

SBP = systolic blood pressure, TG = triglyceride, HDL = high-density lipoprotein, ALT = alanine aminotransferase

9.4 Discussion

As discussed in Chapter 3, the prevalence of NAFLD among young children has been reported to be between 2.1 % and 4.5 % in studies from the Asian-Pacific region. In studies that included adolescents, the prevalence was higher and ranged between 7.1 % and 16.9 %. Hence, the estimated prevalence of 8.1 % for young Malaysian adults is still consistent with the fact that prevalence of NAFLD increases with increasing age. While the overall prevalence is relatively low, it is alarming to note the very high prevalence of NAFLD among young Indian and Malay males, which were estimated to be 33.3 % and 25.5 %, respectively. The inordinately higher prevalence of NAFLD among the Indians and Malays has been observed in two separate studies on multi-racial Malaysian populations (Goh et al., 2012; Chan et al., 2013). The current study confirms that differences in prevalence of NAFLD among the different ethnic groups in Malaysia can be observed as early as young adulthood. This supports that genetic differences probably have a role in the difference in prevalence of NAFLD among the different ethnic groups. As presented in Chapter 4, dietary differences among the different ethnic groups may also play an important role (Chan et al., 2013).

This study also clearly showed that the prevalence of NAFLD was significantly higher in males compared to females across the different ethnic groups. In younger populations, NAFLD has been consistently shown to be more prevalent among men than women, but such trend was no longer observed in older populations suggesting the potential influence of sex hormones in the development of the disease. This study also clearly showed that NAFLD is associated with traditional risk factors for cardiovascular diseases. The difference in prevalence of NAFLD in the different ethnic groups and the clustering of cardiovascular risk factors in patients with NAFLD would explain the higher prevalence of

ischemic heart disease in Indians and Malays compared to Chinese that has been reported in previous studies (Danaraj et al., 1959; J. Lee et al., 2001). Although none of the subjects with NAFLD had diabetes mellitus based on fasting glucose, the diagnosis of diabetes mellitus in subjects with NAFLD can only be reliably excluded with an oral glucose tolerance test. A study from Hong Kong demonstrated that nearly half of NAFLD patients with diabetes mellitus or impaired glucose tolerance had normal fasting glucose (Wong et al., 2006).

All young adults with NAFLD in our study had insulin resistance. Insulin resistance is important in the pathogenesis of NAFLD (Dowman et al., 2010). Over half of subjects without NAFLD also had insulin resistance. These subjects may be at increased risk of developing NAFLD. Although serum ALT level was significantly higher in subjects with NAFLD and elevated serum ALT level remained an independent factor associated with NAFLD on multivariate analysis, it is not accurate enough for diagnosis of NALFD. When our laboratory cut-off was used, the sensitivity and specificity for diagnosis of NALFD was 29.7 % and 97.9 %, respectively. When the more stringent cut-off was used, sensitivity increased to 97.3 % but at the expense of specificity which dropped to 33.3 %.

There are limited published studies that looked specifically at the prevalence of NAFLD and associated factors among young adults. Moreover, our study compared young adults of different ethnic origins. However, as our study population consisted of subjects who were pursuing their tertiary education and who volunteered, the study population may be arguably more health conscious. Hence, the true prevalence of NAFLD may have been underestimated. Nevertheless, this study provided useful insights into the epidemiology of NAFLD in a young adult population and the differences among the different ethnic groups. The diagnosis of NAFLD was by ultrasonography and following careful exclusion of other causes of chronic liver disease. Ultrasonography is by far the most common method to

diagnose fatty liver in clinical practice and in epidemiological studies with good sensitivity and specificity. Ultrasonography is better than serum aminotransferase level alone in diagnosis of NALFD but is not as accurate as histopathological examination of liver biopsy specimen. However, a liver biopsy is invasive and is not feasible in a study of this nature.

9.5 Conclusion

The overall prevalence of NAFLD among young Malaysian adults was found to be relatively low. However, an inordinately high prevalence of NALFD was observed among Indian and Malay males consistent with the higher prevalence of obesity in these groups. This study confirms that differences in prevalence of NAFLD among the different ethnic groups in Malaysia can be observed as early as young adulthood. Independent factors associated with NAFLD were: age, male gender, obesity and elevated serum ALT level.

Note: The findings from this study was presented at the Malaysian Society of Gastroenterology and Hepatology Annual Scientific Meeting in 2013. A poster on the findings from this study was also presented at the World Congress of Gastroenterology 2013 in Shanghai, China, and the abstract was published in a supplementary issue of the Journal of Gastroenterology and Hepatology (Chan et al., 2013). The full article has been published in Hepatology International (Chan et al., 2013).

Chapter 10

Summary and conclusions

This thesis describes the findings from epidemiological and clinical studies on non-alcoholic fatty liver disease (NAFLD) that I had the opportunity to carry out over the few years that I was working in the Gastroenterology and Hepatology Unit of the University of Malaya and the University of Malaya Medical Centre.

In Chapter 2, a short review on the historical aspects and the current concepts of NAFLD, including the definition, pathogenesis, association with the metabolic syndrome and diagnosis were presented. This was followed by an extensive review of the epidemiology of the disease in the Asian-Pacific in Chapter 3. The prevalence of NAFLD has increased rapidly over the years and is now comparable to that in Western countries.

The prevalence of NAFLD among patients with diabetes mellitus was studied and the findings were presented in Chapter 4. Half of the patients attending the diabetic clinic had NAFLD and it was independently associated with central obesity and elevated serum alanine aminotransferase (ALT) level. The prevalence of NAFLD was found to be higher among the Malays and Indians compared to the Chinese consistent with the higher prevalence of central obesity and the higher percentage of calorie intake from fat in the former groups of patients.

In Chapter 5, findings from further analysis on dietary intake and physical activity of diabetic patients with and without NAFLD were presented. Low level of physical activity and high percentage calorie intake from fat, high cholesterol food and high saturated fatty acid food was associated with NAFLD in centrally obese but not in lean

diabetic patients suggesting that low level of physical activity and poor dietary habits have different impact on NAFLD in diabetic patients with and without central obesity.

In Chapter 6, ultrasonography-diagnosed NAFLD was found not to be associated with ischemic heart disease (IHD) among diabetic patients. Possible explanations for this finding were discussed. Independent factors associated with IHD identified were older age, lower levels of physical activity, greater WC and higher HbA1c levels.

The progressive nature of non-alcoholic steatohepatitis (NASH), the more severe form of NAFLD, was clearly demonstrated in the cohort study presented in Chapter 7. Based on the findings from this study, NAFLD patients with persistently elevated serum liver enzymes should be suspected of having worsened NAFLD activity score and all patients diagnosed with NASH should be considered for specific interventions to prevent disease progression.

In Chapter 8, we evaluated the use of non-invasive methods for assessment of severity of liver disease in NAFLD. Controlled attenuation parameter is excellent for the detection of significant hepatic steatosis but is less useful for distinguishing the different grades of significant hepatic steatosis in NAFLD patients. Neither plasma M30 nor serum ALT, aspartate aminotransferase (AST) or gamma glutamyl transpeptidase (GGT) levels were good enough for diagnosis of NASH among NAFLD patients. A more accurate yet simple and non-invasive test for diagnosis and follow-up of NASH is needed. The NAFLD fibrosis score, combined with liver stiffness measurement when necessary, allows accurate prediction of advanced fibrosis and reduces the need for liver biopsy in NAFLD patients.

In Chapter 9, findings from the study on the prevalence of NAFLD among young adults were presented. The prevalence of NAFLD among young adults was relatively low but an inordinately high prevalence of NALFD was observed among Indian and Malay

males consistent with the higher prevalence of obesity in these groups. The study also confirmed that differences in prevalence of NAFLD among the different ethnic groups in Malaysia can be observed as early as young adulthood.

The studies that have been performed for the writing of this thesis have yielded useful information on NAFLD and represent the majority of studies of their kind performed in Malaysia to date. However, there is no doubt that further studies are necessary to solve many of the unanswered questions, particularly in the treatment of this increasingly common and potentially serious condition that will continue to inflict and affect many Malaysians in time to come.

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Appendix

Appendix 1 The Global Physical Activity Questionnaire (GPAQ)

Appendix 2 The semi-quantitative food frequency questionnaire (FFQ)

Appendix 3 Publications

SOAL SELIDIK KEKERAPAN PENGAMBILAN MAKANAN

Sekarang saya akan bertanya mengenai pengambilan makanan anda dalam tempoh satu tahun lepas. Cuba ingat kembali keadaan dan suasana pada ketika itu yang mungkin mempengaruhi tabiat makan puan (contohnya tempat tinggal, pekerjaan, waktu makan dan tempat makan yang biasa).

Perlu diingatkan, kami berminat untuk mengetahui pengambilan makanan **BIASA** puan, yang bererti makanan dan minuman yang puan ambil lima (5) kali atau lebih dalam jangka masa satu tahun. Tandakan **(✓) bagi makanan tersebut dan biarkan kosong jika tidak berkaitan.**

Saya juga ingin mengetahui **kekerapan** pengambilan makanan tersebut dan saiz hidangan yang selalu puan amalkan.

Bagi kekerapan pengambilan makanan, sila nyatakannya dengan mengisikan bilangan kekerapan (berapa kali ambil) dan bulatkan kod kekerapan yang sesuai **SAMA ADA IA DIAMBIL SETIAP**

H (HARI) jika puan mengambil makanan tersebut setiap hari secara puratanya
atau
M (MINGGU) jika puan tidak mengambil makanan tersebut setiap hari tetapi mengambil makanan tersebut setiap minggu
atau
B (BULAN) jika puan tidak mengambil makanan tersebut setiap minggu tetapi mengambil makanan tersebut setiap bulan
atau
T (TAHUN) jika puan tidak mengambil makanan tersebut setiap bulan tetapi mengambil makanan tersebut setiap tahun

CONTOH

BIL	TANDAKAN (✓) JIKA ANDA BIASA MENGAMBIL <u>MINUMAN</u> BERIKUT UNTUK SARAPAN PAGI	NYATAKAN KEKERAPAN PENGAMBILAN DENGAN MENGISIKAN BILANGAN KEKERAPAN PADA KOTAK DAN BULATKAN KOD KEKERAPAN (H = HARI ; M = MINGGU ; B = BULAN ; T = TAHUN) YANG SESUAI	BERAPAKAH JUMLAH SAIZ HIDANGAN YANG SELALU ANDA MAKAN *(ISIKAN BILANGAN)*	SAIZ HIDANGAN PIAWAI	KOSONGKAN *(UNTUK KEGUNAAN PENYELIDIK)*
1	Susu segar ☐	3 KALI PER **H** **M** **B** **T**	1	1 gelas	

Contoh ini menunjukkan bahawa susu segar **BIASA** diambil dan ia diambil **3 kali dalam satu minggu**. Sebanyak **1 gelas** diambil pada setiap pengambilan tersebut.

NOTA: Saya mahu mengetahui apa yang puan biasa makan dalam masa satu minggu. Puan mungkin, sebagai contohnya mengambil sama ada ayam goreng berlada atau kari ayam pada hari Rabu. Tolong jangan rekodkan kedua-dua jenis makanan tersebut dalam satu minggu. Adalah digalakkan puan melaporkan sesetengah jenis makanan dengan kod kekerapan bulan dari kod kekerapan minggu.

Sekarang kita mulakan dengan pengambilan minuman dan makanan semasa sarapan pagi. Sila baca arahan yang diberikan pada setiap awalan sebelum soalan. Terima kasih atas kerjasama yang diberikan.

SARAPAN PAGI

BIL	TANDAKAN (✓) JIKA ANDA BIASA MENGAMBIL <u>MINUMAN</u> BERIKUT UNTUK SARAPAN PAGI	NYATAKAN KEKERAPAN PENGAMBILAN DENGAN MENGISIKAN BILANGAN KEKERAPAN PADA KOTAK DAN BULATKAN KOD KEKERAPAN (H = HARI ; M = MINGGU ; B = BULAN ; T = TAHUN) YANG SESUAI	BERAPAKAH JUMLAH SAIZ HIDANGAN YANG SELALU ANDA MAKAN *(ISIKAN BILANGAN)*	SAIZ HIDANGAN PIAWAI	KOSONGKAN *(UNTUK KEGUNAAN PENYELIDIK)*
1	Susu segar ☐	☐ KALI PER **H** **M** **B** **T**	☐	1 gelas	
2	Susu berperisa (contoh: Coklat, Starwbery) ☐	☐ KALI PER **H** **M** **B** **T**	☐	1 gelas	
3	Susu rendah lemak ☐	☐ KALI PER **H** **M** **B** **T**	☐	1 gelas	
4	Susu soya ☐	☐ KALI PER **H** **M** **B** **T**	☐	1 gelas	
5	Susu tepung penuh krim ☐	☐ KALI PER **H** **M** **B** **T**	☐	1 sudu makan	
6	Susu tepung skim ☐	☐ KALI PER **H** **M** **B** **T**	☐	1 sudu makan	
7	Susu pekat manis ☐	☐ KALI PER **H** **M** **B** **T**	☐	1 sudu makan	
8	Serbuk teh ☐	☐ KALI PER **H** **M** **B** **T**	☐	1 sudu teh	
9	Serbuk kopi (contoh: Nescafe) ☐	☐ KALI PER **H** **M** **B** **T**	☐	1 sudu teh	
10	Serbuk coklat (contoh: Milo) ☐	☐ KALI PER **H** **M** **B** **T**	☐	1 sudu makan	
11	Gula ☐	☐ KALI PER **H** **M** **B** **T**	☐	1 sudu makan	

BIL	TANDAKAN (✓) JIKA ANDA BIASA MENGAMBIL <u>MAKANAN</u> BERIKUT UNTUK SARAPAN PAGI		NYATAKAN KEKERAPAN PENGAMBILAN DENGAN MENGISIKAN BILANGAN KEKERAPAN PADA KOTAK DAN BULATKAN KOD KEKERAPAN (H = HARI ; M = MINGGU ; B = BULAN ; T = TAHUN) YANG SESUAI	BERAPAKAH JUMLAH SAIZ HIDANGAN YANG SELALU ANDA MAKAN *(ISIKAN BILANGAN)*	SAIZ HIDANGAN PIAWAI	KOSONGKAN *(UNTUK KEGUNAAN PENYELIDIK)*
12	Roti putih ☐		☐ KALI PER **H** **M** **B** **T**	☐	1 keping	
13	Roti mil penuh ☐		☐ KALI PER **H** **M** **B** **T**	☐	1 keping	
14	Sapuan roti	Mentega ☐	☐ KALI PER **H** **M** **B** **T**	☐	1 sudu teh	
		Margerin ☐	☐ KALI PER **H** **M** **B** **T**	☐	1 sudu teh	
		Keju kepingan ☐	☐ KALI PER **H** **M** **B** **T**	☐	1 keping	
		Mentega kacang ☐	☐ KALI PER **H** **M** **B** **T**	☐	1 sudu makan	
		Kaya ☐	☐ KALI PER **H** **M** **B** **T**	☐	1 sudu makan	
		Jem buah-buahan ☐	☐ KALI PER **H** **M** **B** **T**	☐	1 sudu makan	
15	Roti canai ☐		☐ KALI PER **H** **M** **B** **T**	☐	1 keping	
16	Roti canai telur ☐		☐ KALI PER **H** **M** **B** **T**	☐	1 keping	
17	Kuah roti canai	Kuah dhal ☐	☐ KALI PER **H** **M** **B** **T**	☐	1 sudu makan	
		Kuah kari ☐	☐ KALI PER **H** **M** **B** **T**	☐	1 sudu makan	
		Kuah sambal ☐	☐ KALI PER **H** **M** **B** **T**	☐	1 sudu makan	
18	Nasi lemak (biasa) ☐		☐ KALI PER **H** **M** **B** **T**	☐	1 pinggan	
19	Nasi goreng (biasa) ☐		☐ KALI PER **H** **M** **B** **T**	☐	1 pinggan	

20	Mee/ Bihun/ Kuih-teow goreng (biasa)	☐	☐	KALI	PER	**H**	**M**	**B**	**T**	☐	1 pinggan	
21	Soto ayam	☐	☐	KALI	PER	**H**	**M**	**B**	**T**	☐	1 mangkuk	
22	Lontong dengan masak lodeh	☐	☐	KALI	PER	**H**	**M**	**B**	**T**	☐	1 mangkuk	
23	Emping jagung (bijirin sahaja) (contoh: Corn Flakes)	☐	☐	KALI	PER	**H**	**M**	**B**	**T**	☐	½ cawan	
24	Bijirin coklat (bijirin sahaja) (contoh: Coco Crunch)	☐	☐	KALI	PER	**H**	**M**	**B**	**T**	☐	½ cawan	
25	Bijirin oats (bijirin sahaja) (contoh: Quacker Oats)	☐	☐	KALI	PER	**H**	**M**	**B**	**T**	☐	½ cawan	
26	Bijirin segera (bijirin sahaja) (contoh: Nestum)	☐	☐	KALI	PER	**H**	**M**	**B**	**T**	☐	½ cawan	
27	Cappati	☐	☐	KALI	PER	**H**	**M**	**B**	**T**	☐	1 keping	
28	Dosai	☐	☐	KALI	PER	**H**	**M**	**B**	**T**	☐	1 keping	

MAKAN TENGAH HARI / MALAM

BIL	TANDAKAN (✓) JIKA ANDA BIASA MENGAMBIL JENIS NASI BERIKUT UNTUK MAKAN TENGAHARI/ MALAM (TANPA LAUK KECUALI DINYATAKAN – LAUK DIMASUKKAN PADA BAHAGIAN SOALAN SETERUSNYA)	NYATAKAN KEKERAPAN PENGAMBILAN DENGAN MENGISIKAN BILANGAN KEKERAPAN PADA KOTAK DAN BULATKAN KOD KEKERAPAN (H = HARI ; M = MINGGU ; B = BULAN ; T = TAHUN) YANG SESUAI	BERAPAKAH JUMLAH SAIZ HIDANGAN YANG SELALU ANDA MAKAN *(ISIKAN BILANGAN)*	SAIZ HIDANGAN PIAWAI	KOSONGKAN *(UNTUK KEGUNAAN PENYELIDIK)*
29	Nasi putih ☐	☐ KALI PER **H** **M** **B** **T**	☐	2 senduk	
30	Nasi dagang ☐	☐ KALI PER **H** **M** **B** **T**	☐	1 pinggan	
31	Nasi tomato ☐	☐ KALI PER **H** **M** **B** **T**	☐	1 pinggan	
32	Nasi minyak ☐	☐ KALI PER **H** **M** **B** **T**	☐	1 pinggan	
33	Nasi briyani ☐	☐ KALI PER **H** **M** **B** **T**	☐	1 pinggan	
34	Nasi kerabu ☐	☐ KALI PER **H** **M** **B** **T**	☐	1 pinggan	
35	Nasi ayam (dengan ayam, sup, timun, salad, sos dan kicap) ☐	☐ KALI PER **H** **M** **B** **T**	☐	1 pinggan	
BIL	**TANDAKAN (✓) JIKA ANDA BIASA MENGAMBIL MAKANAN ASAS BERIKUT**	**NYATAKAN KEKERAPAN PENGAMBILAN DENGAN MENGISIKAN BILANGAN KEKERAPAN PADA KOTAK DAN BULATKAN KOD KEKERAPAN (H = HARI ; M = MINGGU ; B = BULAN ; T = TAHUN) YANG SESUAI**	**BERAPAKAH JUMLAH SAIZ HIDANGAN YANG SELALU ANDA MAKAN *(ISIKAN BILANGAN)***	**SAIZ HIDANGAN PIAWAI**	**KOSONGKAN *(UNTUK KEGUNAAN PENYELIDIK)***
36	Ayam ☐	☐ KALI PER **H** **M** **B** **T**	☐	1 ketul	
37	Ikan ☐	☐ KALI PER **H** **M** **B** **T**	☐	1 ekor sederhana	
38	Daging lembu/ kambing ☐	☐ KALI PER **H** **M** **B** **T**	☐	1 kotak mancis	

39	Organ dalaman ☐		☐ KALI PER **H M B T**	☐	1 ketul	
40	Makanan laut ☐		☐ KALI PER **H M B T**	☐	1 ketul	
41	Sayur-sayuran ☐		☐ KALI PER **H M B T**	☐	1 sudu makan	
42	Buah-buahan ☐		☐ KALI PER **H M B T**	☐	1 biji	
BIL	**SEKARANG, FIKIRKAN MENGENAI <u>AYAM YANG DIMASAK, CARA MEMASAK DAN KUAHNYA</u>. TANDAKAN (✓) JIKA ANDA BIASA MENGAMBIL AYAM DAN CARA MASAK BERIKUT. (LAUK YANG DITAMBAH PADA JENIS MASAKAN LAIN JUGA PERLU DISERTAKAN)**		**NYATAKAN KEKERAPAN PENGAMBILAN DENGAN MENGISIKAN BILANGAN KEKERAPAN PADA KOTAK DAN BULATKAN KOD KEKERAPAN (H = HARI ; M = MINGGU ; B = BULAN ; T = TAHUN) YANG SESUAI**	**BERAPAKAH JUMLAH SAIZ HIDANGAN YANG SELALU ANDA MAKAN *(ISIKAN BILANGAN)***	**SAIZ HIDANGAN PIAWAI**	**KOSONGKAN *(UNTUK KEGUNAAN PENYELIDIK)***
43	Ayam goreng ☐		☐ KALI PER **H M B T**	☐	1 ketul	
44	Ayam goreng berlada ☐	Ayam ☐	☐ KALI PER **H M B T**	☐	1 ketul	
		Kuah sambal ☐	☐ KALI PER **H M B T**	☐	1 sudu makan	
45	Kari ayam ☐ **(pilih satu jenis cara masak kuah kari)*	Ayam ☐	☐ KALI PER **H M B T**	☐	1 ketul	
		*Kuah kari (bersantan) ☐	☐ KALI PER **H M B T**	☐	1 sudu makan	
		*Kuah kari (bersusu) ☐				
		*Kuah kari (tanpa santan/susu) ☐				
46	Ayam masak lemak ☐	Ayam ☐	☐ KALI PER **H M B T**	☐	1 ketul	
		Kuah masak lemak ☐	☐ KALI PER **H M B T**	☐	1 sudu makan	
47	Ayam masak kurma ☐	Ayam ☐	☐ KALI PER **H M B T**	☐	1 ketul	
		Kuah kurma ☐	☐ KALI PER **H M B T**	☐	1 sudu makan	

48	Ayam masak kicap ☐	Ayam ☐	☐ KALI PER **H** **M** **B** **T**	☐	1 ketul	
		Kuah masak kicap ☐	☐ KALI PER **H** **M** **B** **T**	☐	1 sudu makan	
49	Rendang ayam ☐	Ayam ☐	☐ KALI PER **H** **M** **B** **T**	☐	1 ketul	
		Kuah rendang ☐	☐ KALI PER **H** **M** **B** **T**	☐	1 sudu makan	
50	Sup ayam ☐	Ayam ☐	☐ KALI PER **H** **M** **B** **T**	☐	1 ketul	
		Kuah sup ☐	☐ KALI PER **H** **M** **B** **T**	☐	1 sudu makan	
51	Ayam bakar ☐		☐ KALI PER **H** **M** **B** **T**	☐	1 ketul	
52	Ayam kukus ☐		☐ KALI PER **H** **M** **B** **T**	☐	1 ketul	
53	Satay ayam ☐		☐ KALI PER **H** **M** **B** **T**	☐	5 cucuk	

BIL	**SEKARANG, FIKIRKAN MENGENAI <u>IKAN YANG DIMASAK, CARA MEMASAK DAN KUAHNYA</u>. TANDAKAN (✓) JIKA ANDA BIASA MENGAMBIL IKAN DAN CARA MASAK BERIKUT. (LAUK YANG DITAMBAH PADA JENIS MASAKAN LAIN JUGA PERLU DISERTAKAN)**		**NYATAKAN KEKERAPAN PENGAMBILAN DENGAN MENGISIKAN BILANGAN KEKERAPAN PADA KOTAK DAN BULATKAN KOD KEKERAPAN (H = HARI ; M = MINGGU ; B = BULAN ; T = TAHUN) YANG SESUAI**	**BERAPAKAH JUMLAH SAIZ HIDANGAN YANG SELALU ANDA MAKAN *(ISIKAN BILANGAN)***	**SAIZ HIDANGAN PIAWAI**	**KOSONGKAN *(UNTUK KEGUNAAN PENYELIDIK)***
54	Ikan goreng ☐		☐ KALI PER **H** **M** **B** **T**	☐	1 ekor sederhana	
55	Ikan goreng berlada ☐	Ikan ☐	☐ KALI PER **H** **M** **B** **T**	☐	1 ekor sederhana	
		Kuah sambal ☐	☐ KALI PER **H** **M** **B** **T**	☐	1 sudu makan	
56	Kari ikan **(pilih satu jenis cara masak kuah kari)* ☐	Ikan ☐	☐ KALI PER **H** **M** **B** **T**	☐	1 ekor sederhana	
		*Kuah kari (bersantan) ☐	☐ KALI PER **H** **M** **B** **T**	☐	1 sudu makan	
		*Kuah kari (bersusu) ☐				

		*Kuah kari (tanpa santan/susu) ☐				
57	Ikan masak lemak ☐	Ikan ☐	☐ KALI PER **H** **M** **B** **T**	☐	1 ekor sederhana	
		Kuah masak lemak ☐	☐ KALI PER **H** **M** **B** **T**	☐	1 sudu makan	
58	Ikan masak kicap ☐	Ikan ☐	☐ KALI PER **H** **M** **B** **T**	☐	1 ekor sederhana	
		Kuah masak kicap ☐	☐ KALI PER **H** **M** **B** **T**	☐	1 sudu makan	
59	Ikan masak masam manis ☐	Ikan ☐	☐ KALI PER **H** **M** **B** **T**	☐	1 ekor sederhana	
		Kuah masam manis ☐	☐ KALI PER **H** **M** **B** **T**	☐	1 sudu makan	
60	Ikan masak asam/ sup/ cuka ☐	Ikan ☐	☐ KALI PER **H** **M** **B** **T**	☐	1 ekor sederhana	
		Kuah masak asam/ sup/ cuka ☐	☐ KALI PER **H** **M** **B** **T**	☐	1 sudu makan	
61	Ikan bakar ☐		☐ KALI PER **H** **M** **B** **T**	☐	1 ekor sederhana	
62	Ikan kukus ☐		☐ KALI PER **H** **M** **B** **T**	☐	1 ekor sederhana	
BIL	**SEKARANG, FIKIRKAN MENGENAI <u>DAGING LEMBU YANG DIMASAK, CARA MEMASAK DAN KUAHNYA</u>. TANDAKAN (✓) JIKA ANDA BIASA MENGAMBIL DAGING DAN CARA MASAK BERIKUT. (LAUK YANG DITAMBAH PADA JENIS MASAKAN LAIN JUGA PERLU DISERTAKAN)**		**NYATAKAN KEKERAPAN PENGAMBILAN DENGAN MENGISIKAN BILANGAN KEKERAPAN PADA KOTAK DAN BULATKAN KOD KEKERAPAN (H = HARI ; M = MINGGU ; B = BULAN ; T = TAHUN) YANG SESUAI**	**BERAPAKAH JUMLAH SAIZ HIDANGAN YANG SELALU ANDA MAKAN *(ISIKAN BILANGAN)***	**SAIZ HIDANGAN PIAWAI**	**KOSONGKAN *(UNTUK KEGUNAAN PENYELIDIK)***
63	Daging goreng ☐		☐ KALI PER **H** **M** **B** **T**	☐	1 kotak mancis	
64	Daging goreng berlada ☐	Daging ☐	☐ KALI PER **H** **M** **B** **T**	☐	1 kotak mancis	
		Kuah sambal ☐	☐ KALI PER **H** **M** **B** **T**	☐	1 sudu makan	

65	Kari daging **(pilih satu jenis cara masak kuah kari)* ☐	Daging ☐	☐ KALI PER **H** **M** **B** **T**	☐	1 kotak mancis	
		*Kuah kari (bersantan) ☐	☐ KALI PER **H** **M** **B** **T**	☐	1 sudu makan	
		*Kuah kari (bersusu) ☐				
		*Kuah kari (tanpa santan/susu) ☐				
66	Daging masak lemak ☐	Ayam ☐	☐ KALI PER **H** **M** **B** **T**	☐	1 kotak mancis	
		Kuah masak lemak ☐	☐ KALI PER **H** **M** **B** **T**	☐	1 sudu makan	
67	Daging masak kicap ☐	Daging ☐	☐ KALI PER **H** **M** **B** **T**	☐	1 kotak mancis	
		Kuah masak kicap ☐	☐ KALI PER **H** **M** **B** **T**	☐	1 sudu makan	
68	Lain-lain masakan daging ☐		☐ KALI PER **H** **M** **B** **T**	☐	1 kotak mancis	
69	Satay daging ☐		☐ KALI PER **H** **M** **B** **T**	☐	5 cucuk	
BIL	**SEKARANG, FIKIRKAN MENGENAI <u>DAGING KAMBING YANG DIMASAK, CARA MEMASAK DAN KUAHNYA</u>. TANDAKAN (✓) JIKA ANDA BIASA MENGAMBIL DAGING KAMBING DAN CARA MASAK BERIKUT. (LAUK YANG DITAMBAH PADA JENIS MASAKAN LAIN JUGA PERLU DISERTAKAN)**		**NYATAKAN KEKERAPAN PENGAMBILAN DENGAN MENGISIKAN BILANGAN KEKERAPAN PADA KOTAK DAN BULATKAN KOD KEKERAPAN (H = HARI ; M = MINGGU ; B = BULAN ; T = TAHUN) YANG SESUAI**	**BERAPAKAH JUMLAH SAIZ HIDANGAN YANG SELALU ANDA MAKAN *(ISIKAN BILANGAN)***	**SAIZ HIDANGAN PIAWAI**	**KOSONGKAN *(UNTUK KEGUNAAN PENYELIDIK)***
70	Kari daging kambing **(pilih satu jenis cara masak kuah kari)* ☐	Daging kambing ☐	☐ KALI PER **H** **M** **B** **T**	☐	1 kotak mancis	
		*Kuah kari (bersantan) ☐	☐ KALI PER **H** **M** **B** **T**	☐	1 sudu makan	
		*Kuah kari (bersusu) ☐				
		*Kuah kari (tanpa santan/susu) ☐				
71	Lain-lain masakan daging kambing ☐		☐ KALI PER **H** **M** **B** **T**	☐	1 kotak mancis	

BIL	SEKARANG, FIKIRKAN MENGENAI <u>ORGAN DALAMAN YANG DIMASAK, CARA MEMASAK DAN KUAHNYA</u>. TANDAKAN (✓) JIKA ANDA BIASA MENGAMBIL AYAM DAN CARA MASAK BERIKUT. (LAUK YANG DITAMBAH PADA JENIS MASAKAN LAIN JUGA PERLU DISERTAKAN)		NYATAKAN KEKERAPAN PENGAMBILAN DENGAN MENGISIKAN BILANGAN KEKERAPAN PADA KOTAK DAN BULATKAN KOD KEKERAPAN (H = HARI ; M = MINGGU ; B = BULAN ; T = TAHUN) YANG SESUAI	BERAPAKAH JUMLAH SAIZ HIDANGAN YANG SELALU ANDA MAKAN *(ISIKAN BILANGAN)*	SAIZ HIDANGAN PIAWAI	KOSONGKAN *(UNTUK KEGUNAAN PENYELIDIK)*
72	Paru lembu goreng ☐		☐ KALI PER **H** **M** **B** **T**	☐	1 keping (saiz kotak mancis)	
73	Paru lembu goreng berlada ☐	Paru lembu ☐	☐ KALI PER **H** **M** **B** **T**	☐	1 keping (saiz kotak mancis)	
		Kuah sambal ☐	☐ KALI PER **H** **M** **B** **T**	☐	1 sudu makan	
74	Hati ayam goreng ☐		☐ KALI PER **H** **M** **B** **T**	☐	1 ketul	
75	Lain-lain organ yang dimasak ☐		☐ KALI PER **H** **M** **B** **T**	☐	1 ketul	
BIL	**TANDAKAN (✓) JIKA ANDA BIASA MENGAMBIL <u>MAKANAN LAUT DAN CARA MASAK</u> BERIKUT. (LAUK YANG DITAMBAH PADA JENIS MASAKAN LAIN JUGA PERLU DISERTAKAN)**		**NYATAKAN KEKERAPAN PENGAMBILAN DENGAN MENGISIKAN BILANGAN KEKERAPAN PADA KOTAK DAN BULATKAN KOD KEKERAPAN (H = HARI ; M = MINGGU ; B = BULAN ; T = TAHUN) YANG SESUAI**	**BERAPAKAH JUMLAH SAIZ HIDANGAN YANG SELALU ANDA MAKAN *(ISIKAN BILANGAN)***	**SAIZ HIDANGAN PIAWAI**	**KOSONGKAN *(UNTUK KEGUNAAN PENYELIDIK)***
76	Ikan bilis goreng ☐		☐ KALI PER **H** **M** **B** **T**	☐	1 sudu makan	
77	Ikan bilis sambal *(selain bersama nasi lemak dan kuah roti canai)* ☐		☐ KALI PER **H** **M** **B** **T**	☐	1 sudu makan	
78	Sardin dalam tin masak sambal ☐	Sardin ☐	☐ KALI PER **H** **M** **B** **T**	☐	1 potong	
		Kuah sambal ☐	☐ KALI PER **H** **M** **B** **T**	☐	1 sudu makan	
79	Sotong sambal ☐	Sotong ☐	☐ KALI PER **H** **M** **B** **T**	☐	1 ekor sederhana	
		Kuah sambal ☐	☐ KALI PER **H** **M** **B** **T**	☐	1 sudu makan	

80	Sotong masak lain-lain ☐		☐ KALI PER **H** **M** **B** **T**	☐	1 ekor sederhana	
81	Udang sambal ☐	Udang ☐	☐ KALI PER **H** **M** **B** **T**	☐	1 ekor sederhana /5 ekor kecil	
		Kuah sambal ☐	☐ KALI PER **H** **M** **B** **T**	☐	1 sudu makan	
82	Udang masak lain-lain ☐		☐ KALI PER **H** **M** **B** **T**	☐	1 ekor sederhana /5 ekor kecil	
83	Kerang sambal ☐	Kerang ☐	☐ KALI PER **H** **M** **B** **T**	☐	10 ekor	
		Kuah sambal ☐	☐ KALI PER **H** **M** **B** **T**	☐	1 sudu makan	
84	Kerang masak lain-lain ☐		☐ KALI PER **H** **M** **B** **T**	☐	10 ekor	
85	Kari ketam ☐ **(pilih satu jenis cara masak kuah kari)*	Ketam ☐	☐ KALI PER **H** **M** **B** **T**	☐	1 ekor	
		*Kuah kari (bersantan) ☐	☐ KALI PER **H** **M** **B** **T**	☐	1 sudu makan	
		*Kuah kari (bersusu) ☐				
		*Kuah kari (tanpa santan/susu) ☐				
86	Ketam masak lain-lain ☐		☐ KALI PER **H** **M** **B** **T**	☐	1 ekor	
BIL	**TANDAKAN (✓) JIKA ANDA BIASA <u>TELUR DAN CARA MASAK</u> BERIKUT. (LAUK YANG DITAMBAH PADA JENIS MASAKAN LAIN JUGA PERLU DISERTAKAN)**		**NYATAKAN KEKERAPAN PENGAMBILAN DENGAN MENGISIKAN BILANGAN KEKERAPAN PADA KOTAK DAN BULATKAN KOD KEKERAPAN (H = HARI ; M = MINGGU ; B = BULAN ; T = TAHUN) YANG SESUAI**	**BERAPAKAH JUMLAH SAIZ HIDANGAN YANG SELALU ANDA MAKAN *(ISIKAN BILANGAN)***	**SAIZ HIDANGAN PIAWAI**	**KOSONGKAN *(UNTUK KEGUNAAN PENYELIDIK)***
87	Telur ayam goreng (termasuk dadar) ☐		☐ KALI PER **H** **M** **B** **T**	☐	1 biji	
88	Telur ayam rebus *(selain bersama nasi lemak)* ☐		☐ KALI PER **H** **M** **B** **T**	☐	1 biji	
89	Telur asin ☐		☐ KALI PER **H** **M** **B** **T**	☐	½ biji	

BIL	TANDAKAN (✓) JIKA ANDA BIASA MENGAMBIL <u>SAYURAN BERDAUN HIJAU DAN BERWARNA</u> BERIKUT	NYATAKAN KEKERAPAN PENGAMBILAN DENGAN MENGISIKAN BILANGAN KEKERAPAN PADA KOTAK DAN BULATKAN KOD KEKERAPAN (H = HARI ; M = MINGGU ; B = BULAN ; T = TAHUN) YANG SESUAI	BERAPAKAH JUMLAH SAIZ HIDANGAN YANG SELALU ANDA MAKAN *(ISIKAN BILANGAN)*	SAIZ HIDANGAN PIAWAI	KOSONGKAN *(UNTUK KEGUNAAN PENYELIDIK)*
90	Sayur sawi ☐	☐ KALI PER **H** **M** **B** **T**	☐	1 sudu makan	
91	Sayur bayam ☐	☐ KALI PER **H** **M** **B** **T**	☐	1 sudu makan	
92	Sayur bayam merah ☐	☐ KALI PER **H** **M** **B** **T**	☐	1 sudu makan	
93	Sayur kangkung ☐	☐ KALI PER **H** **M** **B** **T**	☐	1 sudu makan	
94	Sayur kailan ☐	☐ KALI PER **H** **M** **B** **T**	☐	1 sudu makan	
95	Pucuk paku ☐	☐ KALI PER **H** **M** **B** **T**	☐	1 sudu makan	
96	Pucuk ubi kayu ☐	☐ KALI PER **H** **M** **B** **T**	☐	1 sudu makan	
97	Cekur manis ☐	☐ KALI PER **H** **M** **B** **T**	☐	1 sudu makan	
98	Cendawan ☐	☐ KALI PER **H** **M** **B** **T**	☐	1 sudu makan	
99	Taugeh ☐	☐ KALI PER **H** **M** **B** **T**	☐	1 sudu makan	

BIL	TANDAKAN (✓) JIKA ANDA BIASA MENGAMBIL <u>SAYURAN KRUSIFERUS</u> BERIKUT	NYATAKAN KEKERAPAN PENGAMBILAN DENGAN MENGISIKAN BILANGAN KEKERAPAN PADA KOTAK DAN BULATKAN KOD KEKERAPAN (H = HARI ; M = MINGGU ; B = BULAN ; T = TAHUN) YANG SESUAI	BERAPAKAH JUMLAH SAIZ HIDANGAN YANG SELALU ANDA MAKAN *(ISIKAN BILANGAN)*	SAIZ HIDANGAN PIAWAI	KOSONGKAN *(UNTUK KEGUNAAN PENYELIDIK)*
100	Kobis ☐	☐ KALI PER **H** **M** **B** **T**	☐	1 sudu makan	
101	Bunga kobis ☐	☐ KALI PER **H** **M** **B** **T**	☐	1 sudu makan	
102	Brokoli ☐	☐ KALI PER **H** **M** **B** **T**	☐	1 sudu makan	
103	Petola ☐	☐ KALI PER **H** **M** **B** **T**	☐	1 sudu makan	
104	Peria ☐	☐ KALI PER **H** **M** **B** **T**	☐	1 sudu makan	
105	Lobak merah ☐	☐ KALI PER **H** **M** **B** **T**	☐	1 sudu makan	
106	Tomato ☐	☐ KALI PER **H** **M** **B** **T**	☐	½ biji sederhana	
107	Terung ☐	☐ KALI PER **H** **M** **B** **T**	☐	1 potong	
108	Kucai ☐	☐ KALI PER **H** **M** **B** **T**	☐	1 sudu makan	
109	Labu merah ☐	☐ KALI PER **H** **M** **B** **T**	☐	1 sudu makan	
110	Kapsicum (Paprika) ☐	☐ KALI PER **H** **M** **B** **T**	☐	1 sudu makan	
111	Sengkuang ☐	☐ KALI PER **H** **M** **B** **T**	☐	1 keping	
112	Kentang ☐	☐ KALI PER **H** **M** **B** **T**	☐	½ biji sederhana	
113	Kacang panjang ☐	☐ KALI PER **H** **M** **B** **T**	☐	1 sudu makan	
114	Kacang buncis ☐	☐ KALI PER **H** **M** **B** **T**	☐	1 sudu makan	

115	Kacang botor ☐	☐ KALI PER **H M B T**	☐	1 batang	
116	Kacang pea (pis) ☐	☐ KALI PER **H M B T**	☐	1 sudu makan	
BIL	**TANDAKAN (✓) JIKA ANDA BIASA MENGAMBIL <u>ULAM</u> BERIKUT**	**NYATAKAN KEKERAPAN PENGAMBILAN DENGAN MENGISIKAN BILANGAN KEKERAPAN PADA KOTAK DAN BULATKAN KOD KEKERAPAN (H = HARI ; M = MINGGU ; B = BULAN ; T = TAHUN) YANG SESUAI**	**BERAPAKAH JUMLAH SAIZ HIDANGAN YANG SELALU ANDA MAKAN *(ISIKAN BILANGAN)***	**SAIZ HIDANGAN PIAWAI**	**KOSONGKAN *(UNTUK KEGUNAAN PENYELIDIK)***
117	Ulam raja ☐	☐ KALI PER **H M B T**	☐	1 tangkai	
118	Petai ☐	☐ KALI PER **H M B T**	☐	10 biji	
119	Timun ☐	☐ KALI PER **H M B T**	☐	3 potong/ hiris	
BIL	**SEKARANG, FIKIRKAN MENGENAI SEMUA <u>SAYUR YANG DIMASAK, CARA MEMASAK DAN KUAHNYA</u>. TANDAKAN (✓) JIKA ANDA BIASA MENGAMBIL SAYUR DAN CARA MASAK BERIKUT**	**NYATAKAN KEKERAPAN PENGAMBILAN DENGAN MENGISIKAN BILANGAN KEKERAPAN PADA KOTAK DAN BULATKAN KOD KEKERAPAN (H = HARI ; M = MINGGU ; B = BULAN ; T = TAHUN) YANG SESUAI**	**BERAPAKAH JUMLAH SAIZ HIDANGAN YANG SELALU ANDA MAKAN *(ISIKAN BILANGAN)***	**SAIZ HIDANGAN PIAWAI**	**KOSONGKAN *(UNTUK KEGUNAAN PENYELIDIK)***
120	Sayur goreng/ tumis air *(stir-fry)* ☐	☐ KALI PER **H M B T**	☐	1 sudu makan	
121	Sup/ Tom yam sayur ☐ — Sayur dalam sup ☐	☐ KALI PER **H M B T**	☐	1 sudu makan	
	Kuah sup/ tom yam ☐	☐ KALI PER **H M B T**	☐	1 sudu makan	
122	Sayur masak lemak ☐ — Sayur dalam masak lemak ☐	☐ KALI PER **H M B T**	☐	1 sudu makan	
	Kuah masak lemak ☐	☐ KALI PER **H M B T**	☐	1 sudu makan	
123	Sayur celur/ rebus ☐	☐ KALI PER **H M B T**	☐	1 sudu makan	

BIL	TANDAKAN (✓) JIKA ANDA BIASA MENGAMBIL <u>BUAH-BUAHAN</u> BERIKUT	NYATAKAN KEKERAPAN PENGAMBILAN DENGAN MENGISIKAN BILANGAN KEKERAPAN PADA KOTAK DAN BULATKAN KOD KEKERAPAN (H = HARI ; M = MINGGU ; B = BULAN ; T = TAHUN) YANG SESUAI	BERAPAKAH JUMLAH SAIZ HIDANGAN YANG SELALU ANDA MAKAN *(ISIKAN BILANGAN)*	SAIZ HIDANGAN PIAWAI	KOSONGKAN *(UNTUK KEGUNAAN PENYELIDIK)*
124	Belimbing ☐	☐ KALI PER **H** **M** **B** **T**	☐	1 biji	
125	Betik ☐	☐ KALI PER **H** **M** **B** **T**	☐	1 potong	
126	Kiwi ☐	☐ KALI PER **H** **M** **B** **T**	☐	1 biji	
127	Lai/ pear ☐	☐ KALI PER **H** **M** **B** **T**	☐	1 biji	
128	Pic ☐	☐ KALI PER **H** **M** **B** **T**	☐	1 biji	
129	Epal (hijau dan merah) ☐	☐ KALI PER **H** **M** **B** **T**	☐	1 biji	
130	Jambu batu ☐	☐ KALI PER **H** **M** **B** **T**	☐	½ biji	
131	Oren ☐	☐ KALI PER **H** **M** **B** **T**	☐	1 biji	
132	Limau manis/ mandrin ☐	☐ KALI PER **H** **M** **B** **T**	☐	1 biji	
133	Mangga ☐	☐ KALI PER **H** **M** **B** **T**	☐	1 biji	
134	Tembikai ☐	☐ KALI PER **H** **M** **B** **T**	☐	1 potong	
135	Anggur ☐	☐ KALI PER **H** **M** **B** **T**	☐	10 biji	
136	Laici ☐	☐ KALI PER **H** **M** **B** **T**	☐	3 biji	
137	Nenas ☐	☐ KALI PER **H** **M** **B** **T**	☐	1 potong	
138	Pisang ☐	☐ KALI PER **H** **M** **B** **T**	☐	1 biji	

139	Rambutan ☐	☐ KALI PER **H** **M** **B** **T**	☐	5 biji	
140	Langsat ☐	☐ KALI PER **H** **M** **B** **T**	☐	10 biji	
141	Mata kucing ☐	☐ KALI PER **H** **M** **B** **T**	☐	10 biji	
142	Nangka ☐	☐ KALI PER **H** **M** **B** **T**	☐	3 ulas	
BIL	**TANDAKAN (✓) JIKA ANDA BIASA MENGAMBIL <u>BUAH-BUAHAN KERING</u> BERIKUT**	**NYATAKAN KEKERAPAN PENGAMBILAN DENGAN MENGISIKAN BILANGAN KEKERAPAN PADA KOTAK DAN BULATKAN KOD KEKERAPAN (H = HARI ; M = MINGGU ; B = BULAN ; T = TAHUN) YANG SESUAI**	**BERAPAKAH JUMLAH SAIZ HIDANGAN YANG SELALU ANDA MAKAN *(ISIKAN BILANGAN)***	**SAIZ HIDANGAN PIAWAI**	**KOSONGKAN *(UNTUK KEGUNAAN PENYELIDIK)***
143	Kismis (raisin/ sultana) ☐	☐ KALI PER **H** **M** **B** **T**	☐	1 biji	
144	Tamar (buah kurma/ dates) ☐	☐ KALI PER **H** **M** **B** **T**	☐	1 potong	
BIL	**TANDAKAN (✓) JIKA ANDA BIASA MENGAMBIL <u>KEKACANG</u> BERIKUT SETELAH DIMASAK**	**NYATAKAN KEKERAPAN PENGAMBILAN DENGAN MENGISIKAN BILANGAN KEKERAPAN PADA KOTAK DAN BULATKAN KOD KEKERAPAN (H = HARI ; M = MINGGU ; B = BULAN ; T = TAHUN) YANG SESUAI**	**BERAPAKAH JUMLAH SAIZ HIDANGAN YANG SELALU ANDA MAKAN *(ISIKAN BILANGAN)***	**SAIZ HIDANGAN PIAWAI**	**KOSONGKAN *(UNTUK KEGUNAAN PENYELIDIK)***
145	Kacang dhal ☐	☐ KALI PER **H** **M** **B** **T**	☐	¼ cawan	
146	Kacang hijau ☐	☐ KALI PER **H** **M** **B** **T**	☐	¼ cawan	
147	Kacang kuda ☐	☐ KALI PER **H** **M** **B** **T**	☐	¼ cawan	

BIL	TANDAKAN (✓) JIKA ANDA BIASA MENGAMBIL <u>KACANG</u> BERIKUT	NYATAKAN KEKERAPAN PENGAMBILAN DENGAN MENGISIKAN BILANGAN KEKERAPAN PADA KOTAK DAN BULATKAN KOD KEKERAPAN (H = HARI ; M = MINGGU ; B = BULAN ; T = TAHUN) YANG SESUAI	BERAPAKAH JUMLAH SAIZ HIDANGAN YANG SELALU ANDA MAKAN *(ISIKAN BILANGAN)*	SAIZ HIDANGAN PIAWAI	KOSONGKAN *(UNTUK KEGUNAAN PENYELIDIK)*
148	Badam ☐	☐ KALI PER **H** **M** **B** **T**	☐	¼ cawan	
149	Kacang gajus ☐	☐ KALI PER **H** **M** **B** **T**	☐	¼ cawan	
150	Kacang tanah ☐	☐ KALI PER **H** **M** **B** **T**	☐	¼ cawan	
151	Kacang pistacio ☐	☐ KALI PER **H** **M** **B** **T**	☐	¼ cawan	
152	Walnut ☐	☐ KALI PER **H** **M** **B** **T**	☐	¼ cawan	
BIL	**TANDAKAN (✓) JIKA ANDA BIASA MENGAMBIL <u>PRODUK SOYA</u> BERIKUT**	**NYATAKAN KEKERAPAN PENGAMBILAN DENGAN MENGISIKAN BILANGAN KEKERAPAN PADA KOTAK DAN BULATKAN KOD KEKERAPAN (H = HARI ; M = MINGGU ; B = BULAN ; T = TAHUN) YANG SESUAI**	**BERAPAKAH JUMLAH SAIZ HIDANGAN YANG SELALU ANDA MAKAN *(ISIKAN BILANGAN)***	**SAIZ HIDANGAN PIAWAI**	**KOSONGKAN *(UNTUK KEGUNAAN PENYELIDIK)***
153	Tauhu ☐	☐ KALI PER **H** **M** **B** **T**	☐	1 keping	
154	Tempe ☐	☐ KALI PER **H** **M** **B** **T**	☐	½ cawan (kiub)	
155	Tau foo fah ☐	☐ KALI PER **H** **M** **B** **T**	☐	1 mangkuk	

BIL	TANDAKAN (✓) JIKA ANDA BIASA MENGAMBIL <u>MAKANAN SIAP</u> BERIKUT	NYATAKAN KEKERAPAN PENGAMBILAN DENGAN MENGISIKAN BILANGAN KEKERAPAN PADA KOTAK DAN BULATKAN KOD KEKERAPAN (H = HARI ; M = MINGGU ; B = BULAN ; T = TAHUN) YANG SESUAI	BERAPAKAH JUMLAH SAIZ HIDANGAN YANG SELALU ANDA MAKAN *(ISIKAN BILANGAN)*	SAIZ HIDANGAN PIAWAI	KOSONGKAN *(UNTUK KEGUNAAN PENYELIDIK)*
156	Mee/ Bihun/ Kuih-teow sup ☐	☐ KALI PER **H M B T**	☐	1 mangkuk	
157	Mee/ Bihun bandung ☐	☐ KALI PER **H M B T**	☐	1 mangkuk	
158	Mee kari ☐	☐ KALI PER **H M B T**	☐	1 mangkuk	
159	Mee hailam ☐	☐ KALI PER **H M B T**	☐	1 mangkuk	
160	Mee wantan ☐	☐ KALI PER **H M B T**	☐	1 mangkuk	
161	Laksa ☐	☐ KALI PER **H M B T**	☐	1 mangkuk	
162	Mee segera (contoh : Maggi Mee) ☐	☐ KALI PER **H M B T**	☐	1 mangkuk	
163	Yong tau fu ☐	☐ KALI PER **H M B T**	☐	1 mangkuk	
BIL	**TANDAKAN (✓) JIKA ANDA BIASA MENGAMBIL <u>PRODUK TERPROSES/ MAKANAN SEGERA</u> BERIKUT**	**NYATAKAN KEKERAPAN PENGAMBILAN DENGAN MENGISIKAN BILANGAN KEKERAPAN PADA KOTAK DAN BULATKAN KOD KEKERAPAN (H = HARI ; M = MINGGU ; B = BULAN ; T = TAHUN) YANG SESUAI**	**BERAPAKAH JUMLAH SAIZ HIDANGAN YANG SELALU ANDA MAKAN *(ISIKAN BILANGAN)***	**SAIZ HIDANGAN PIAWAI**	**KOSONGKAN *(UNTUK KEGUNAAN PENYELIDIK)***
164	Burger ayam ☐	☐ KALI PER **H M B T**	☐	1 biji	
165	Burger daging ☐	☐ KALI PER **H M B T**	☐	1 biji	
166	Sosej/ frankfurter ☐	☐ KALI PER **H M B T**	☐	1 ketul	

167	Nuggets	☐	☐	KALI	PER	**H**	**M**	**B**	**T**	☐	1 ketul	
168	French fries	☐	☐	KALI	PER	**H**	**M**	**B**	**T**	☐	1 cawan	
169	Mc Donald (burger sahaja)	☐	☐	KALI	PER	**H**	**M**	**B**	**T**	☐	1 biji	
170	Kentucky Fried Chicken (ayam sahaja)	☐	☐	KALI	PER	**H**	**M**	**B**	**T**	☐	2 ketul	

MINUMAN

BIL	**TANDAKAN (✓) JIKA ANDA BIASA MENGAMBIL <u>MINUMAN</u> BERIKUT**	**NYATAKAN KEKERAPAN PENGAMBILAN DENGAN MENGISIKAN BILANGAN KEKERAPAN PADA KOTAK DAN BULATKAN KOD KEKERAPAN (H = HARI ; M = MINGGU ; B = BULAN ; T = TAHUN) YANG SESUAI**	**BERAPAKAH JUMLAH SAIZ HIDANGAN YANG SELALU ANDA MAKAN *(ISIKAN BILANGAN)***	**SAIZ HIDANGAN PIAWAI**	**KOSONGKAN *(UNTUK KEGUNAAN PENYELIDIK)***
171	Sirap ☐	☐ KALI PER **H** **M** **B** **T**	☐	1 gelas	
172	Sirap bandung ☐	☐ KALI PER **H** **M** **B** **T**	☐	1 gelas	
173	Limau ais ☐	☐ KALI PER **H** **M** **B** **T**	☐	1 gelas	
174	Teh O limau ais ☐	☐ KALI PER **H** **M** **B** **T**	☐	1 gelas	
175	Minuman berkarbonat (contoh : Coke, Pepsi) ☐	☐ KALI PER **H** **M** **B** **T**	☐	1 tin	
176	Yogurt penuh krim (biasa) ☐	☐ KALI PER **H** **M** **B** **T**	☐	1 cawan	
177	Yogurt rendah lemak ☐	☐ KALI PER **H** **M** **B** **T**	☐	1 cawan	

MINUM PAGI / PETANG

BIL	TANDAKAN (✓) JIKA ANDA BIASA MENGAMBIL <u>KUIH BERGORENG</u> BERIKUT UNTUK MINUM PAGI/ PETANG	NYATAKAN KEKERAPAN PENGAMBILAN DENGAN MENGISIKAN BILANGAN KEKERAPAN PADA KOTAK DAN BULATKAN KOD KEKERAPAN (H = HARI ; M = MINGGU ; B = BULAN ; T = TAHUN) YANG SESUAI	BERAPAKAH JUMLAH SAIZ HIDANGAN YANG SELALU ANDA MAKAN *(ISIKAN BILANGAN)*	SAIZ HIDANGAN PIAWAI	KOSONGKAN *(UNTUK KEGUNAAN PENYELIDIK)*
178	Cekodok pisang ☐	☐ KALI PER **H** **M** **B** **T**	☐	5 biji kecil	
179	Pisang goreng ☐	☐ KALI PER **H** **M** **B** **T**	☐	1 biji	
180	Cucur udang/ tepung ☐	☐ KALI PER **H** **M** **B** **T**	☐	1 biji	
181	Karipap ☐	☐ KALI PER **H** **M** **B** **T**	☐	1 biji	
182	Popia goreng ☐	☐ KALI PER **H** **M** **B** **T**	☐	1 biji	
183	Kuih kacang hijau ☐	☐ KALI PER **H** **M** **B** **T**	☐	1 biji	
184	Donat ☐	☐ KALI PER **H** **M** **B** **T**	☐	1 biji	
185	Vadai kacang dhal ☐	☐ KALI PER **H** **M** **B** **T**	☐	1 biji	
BIL	**TANDAKAN (✓) JIKA ANDA BIASA MENGAMBIL <u>KUIH MANIS</u> BERIKUT UNTUK MINUM PAGI/ PETANG**	**NYATAKAN KEKERAPAN PENGAMBILAN DENGAN MENGISIKAN BILANGAN KEKERAPAN PADA KOTAK DAN BULATKAN KOD KEKERAPAN (H = HARI ; M = MINGGU ; B = BULAN ; T = TAHUN) YANG SESUAI**	**BERAPAKAH JUMLAH SAIZ HIDANGAN YANG SELALU ANDA MAKAN *(ISIKAN BILANGAN)***	**SAIZ HIDANGAN PIAWAI**	**KOSONGKAN *(UNTUK KEGUNAAN PENYELIDIK)***
186	Kuih apam ☐	☐ KALI PER **H** **M** **B** **T**	☐	1 biji besar	
187	Kuih seri muka ☐	☐ KALI PER **H** **M** **B** **T**	☐	1 potong	

188	Kuih apam balik ☐	☐ KALI PER **H** **M** **B** **T**	☐	1 potong tebal	
189	Lain-lain kuih manis ☐	☐ KALI PER **H** **M** **B** **T**	☐	1 biji/potong	
BIL	**TANDAKAN (✓) JIKA ANDA BIASA MENGAMBIL <u>KEK</u> BERIKUT UNTUK MINUM PAGI/ PETANG**	**NYATAKAN KEKERAPAN PENGAMBILAN DENGAN MENGISIKAN BILANGAN KEKERAPAN PADA KOTAK DAN BULATKAN KOD KEKERAPAN (H = HARI ; M = MINGGU ; B = BULAN ; T = TAHUN) YANG SESUAI**	**BERAPAKAH JUMLAH SAIZ HIDANGAN YANG SELALU ANDA MAKAN *(ISIKAN BILANGAN)***	**SAIZ HIDANGAN PIAWAI**	**KOSONGKAN *(UNTUK KEGUNAAN PENYELIDIK)***
190	Kek biasa *(Butter cake)* ☐	☐ KALI PER **H** **M** **B** **T**	☐	1 potong	
191	Kek coklat ☐	☐ KALI PER **H** **M** **B** **T**	☐	1 potong	
192	Lain-lain jenis kek ☐	☐ KALI PER **H** **M** **B** **T**	☐	1 potong	
BIL	**TANDAKAN (✓) JIKA ANDA BIASA MENGAMBIL <u>PRODUK PASTRY/ BAKERI</u> BERIKUT UNTUK MINUM PAGI/ PETANG**	**NYATAKAN KEKERAPAN PENGAMBILAN DENGAN MENGISIKAN BILANGAN KEKERAPAN PADA KOTAK DAN BULATKAN KOD KEKERAPAN (H = HARI ; M = MINGGU ; B = BULAN ; T = TAHUN) YANG SESUAI**	**BERAPAKAH JUMLAH SAIZ HIDANGAN YANG SELALU ANDA MAKAN *(ISIKAN BILANGAN)***	**SAIZ HIDANGAN PIAWAI**	**KOSONGKAN *(UNTUK KEGUNAAN PENYELIDIK)***
193	Muffin ☐	☐ KALI PER **H** **M** **B** **T**	☐	1 biji	
194	Croissant ☐	☐ KALI PER **H** **M** **B** **T**	☐	1 biji	
195	Bun	Cheese ☐	☐ KALI PER **H** **M** **B** **T**	☐	1 biji	
		Coklat ☐	☐ KALI PER **H** **M** **B** **T**	☐	1 biji	
		Kacang merah ☐	☐ KALI PER **H** **M** **B** **T**	☐	1 biji	

MAKANAN RINGAN / SNEK

BIL	TANDAKAN (✓) JIKA ANDA BIASA MENGAMBIL <u>MAKANAN RINGAN/ SNEK</u> BERIKUT	NYATAKAN KEKERAPAN PENGAMBILAN DENGAN MENGISIKAN BILANGAN KEKERAPAN PADA KOTAK DAN BULATKAN KOD KEKERAPAN (H = HARI ; M = MINGGU ; B = BULAN ; T = TAHUN) YANG SESUAI	BERAPAKAH JUMLAH SAIZ HIDANGAN YANG SELALU ANDA MAKAN *(ISIKAN BILANGAN)*	SAIZ HIDANGAN PIAWAI	KOSONGKAN *(UNTUK KEGUNAAN PENYELIDIK)*
196	Coklat bersusu/ berkismis/ berkacang ☐	☐ KALI PER **H** **M** **B** **T**	☐	1 bar kecil	
197	Ais krim pelbagai perisa ☐	☐ KALI PER **H** **M** **B** **T**	☐	1 scoop	
198	Biskut cream crackers/ wholemeal ☐	☐ KALI PER **H** **M** **B** **T**	☐	1 keping	
199	Biskut coklat/ berkrim manis ☐	☐ KALI PER **H** **M** **B** **T**	☐	1 keping	
200	Pelbagai jenis gula-gula ☐	☐ KALI PER **H** **M** **B** **T**	☐	5 biji	

SEKIAN, TERIMA KASIH DI ATAS KERJASAMA ANDA

2 The questionnaire

Physical Activity		
Next I am going to ask you about the time you spend doing different types of physical activity in a typical week. Please answer these questions even if you do not consider yourself to be a physically active person. Think first about the time you spend doing work. Think of work as the things that you have to do such as paid or unpaid work, study/training, household chores, harvesting food/crops, fishing or hunting for food, seeking employment. *[Insert other examples if needed].* In answering the following questions 'vigorous-intensity activities' are activities that require hard physical effort and cause large increases in breathing or heart rate, 'moderate-intensity activities' are activities that require moderate physical effort and cause small increases in breathing or heart rate.		
Question	**Response**	**Code**
Work		
Does your work involve vigorous-intensity activity that causes large increases in breathing or heart rate like *[carrying or lifting heavy loads, digging or construction work]* for at least 10 minutes continuously? *[INSERT EXAMPLES] (USE SHOWCARD)*	Yes 1 No 2 *If No, go to P 4*	P1
In a typical week, on how many days do you do vigorous-intensity activities as part of your work?	Number of days ⌊__⌋	P2
How much time do you spend doing vigorous-intensity activities at work on a typical day?	Hours : minutes ⌊__⌋⌊__⌋ : ⌊__⌋⌊__⌋ hrs mins	P3 (a-b)
Does your work involve moderate-intensity activity, that causes small increases in breathing or heart rate such as brisk walking *[or carrying light loads]* for at least 10 minutes continuously? *[INSERT EXAMPLES] (USE SHOWCARD)*	Yes 1 No 2 *If No, go to P 7*	P4
In a typical week, on how many days do you do moderate-intensity activities as part of your work?	Number of days ⌊__⌋	P5
How much time do you spend doing moderate-intensity activities at work on a typical day?	Hours : minutes ⌊__⌋⌊__⌋ : ⌊__⌋⌊__⌋ hrs mins	P6 (a-b)
Travel to and from places		
The next questions exclude the physical activities at work that you have already mentioned. Now I would like to ask you about the usual way you travel to and from places. For example to work, for shopping, to market, to place of worship. *[Insert other examples if needed]*		
Do you walk or use a bicycle *(pedal cycle)* for at least 10 minutes continuously to get to and from places?	Yes 1 No 2 *If No, go to P 10*	P7
In a typical week, on how many days do you walk or bicycle for at least 10 minutes continuously to get to and from places?	Number of days ⌊__⌋	P8
How much time do you spend walking or bicycling for travel on a typical day?	Hours : minutes ⌊__⌋⌊__⌋ : ⌊__⌋⌊__⌋ hrs mins	P9 (a-b)

Continued on next page

Physical Activity, Continued		
Question	**Response**	**Code**
Recreational activities		
The next questions exclude the work and transport activities that you have already mentioned. Now I would like to ask you about sports, fitness and recreational activities (leisure), *[Insert relevant terms]*.		
Do you do any vigorous-intensity sports, fitness or recreational *(leisure)* activities that cause large increases in breathing or heart rate like *[running or football]* for at least 10 minutes continuously? *[INSERT EXAMPLES] (USE SHOWCARD)*	Yes 1 No 2 *If No, go to P 13*	P10
In a typical week, on how many days do you do vigorous-intensity sports, fitness or recreational *(leisure)* activities?	Number of days ⌊__⌋	P11
How much time do you spend doing vigorous-intensity sports, fitness or recreational activities on a typical day?	Hours : minutes ⌊__⌋⌊__⌋ : ⌊__⌋⌊__⌋ hrs mins	P12 (a-b)
Do you do any moderate-intensity sports, fitness or recreational *(leisure)* activities that cause a small increase in breathing or heart rate such as brisk walking, *[cycling, swimming, volleyball]* for at least 10 minutes continuously? *[INSERT EXAMPLES] (USE SHOWCARD)*	Yes 1 No 2 *If No, go to P16*	P13
In a typical week, on how many days do you do moderate-intensity sports, fitness or recreational *(leisure)* activities?	Number of days ⌊__⌋	P14
How much time do you spend doing moderate-intensity sports, fitness or recreational *(leisure)* activities on a typical day?	Hours : minutes ⌊__⌋⌊__⌋ : ⌊__⌋⌊__⌋ hrs mins	P15 (a-b)
Sedentary behaviour		
The following question is about sitting or reclining at work, at home, getting to and from places, or with friends including time spent sitting at a desk, sitting with friends, traveling in car, bus, train, reading, playing cards or watching television, but do not include time spent sleeping. *[INSERT EXAMPLES] (USE SHOWCARD)*		
How much time do you usually spend sitting or reclining on a typical day?	Hours : minutes ⌊__⌋⌊__⌋ : ⌊__⌋⌊__⌋ hrs mins	P16 (a-b)

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REVIEW ARTICLE

Epidemiology of a fast emerging disease in the Asia-Pacific region: non-alcoholic fatty liver disease

Chan Wah-Kheong · Goh Khean-Lee

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Abstract Non-alcoholic fatty liver disease (NAFLD) is rapidly increasing in the Asia-Pacific and affects up to 30 % of the general population. In younger children, prevalence has been reported to be between 2.1 and 4.5 %. The prevalence of NAFLD increases with increasing age. NAFLD is more prevalent in men than women, but this trend fades in older age group. NAFLD is one of the most common causes of raised serum ALT levels and the latter is closely related to the presence of features of metabolic syndrome. NAFLD may contribute to metabolic syndrome in a similar way as visceral adiposity and can be an early predictor of metabolic disorders. NAFLD increases the risk of developing diabetes mellitus and is closely related to degree of glucose intolerance. A significant proportion of patients with NAFLD have impaired glucose tolerance or diabetes mellitus but with normal fasting blood glucose, highlighting the importance of oral glucose tolerance test in NAFLD patients with normal fasting blood glucose. Besides liver-related complications, NAFLD has been associated with cardiovascular complications, hyperuricemia, gout, chronic kidney disease, gallstone disease, colorectal adenomatous polyp, and polycystic ovarian syndrome. NAFLD seems to be related to host metabolic factors rather than viral factors and does not seem to affect severity of the liver disease in patients with chronic hepatitis B. On the other hand, hepatic steatosis may be related to both host metabolic and viral factors in patients with chronic hepatitis C and seems to adversely impact on the severity of liver disease and possibly response to antiviral therapy.

Keywords Asia-Pacific · Epidemiology · NAFLD

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Introduction

Non-alcoholic fatty liver disease (NAFLD) encompasses a spectrum of liver conditions that occur in individuals who do not consume alcohol or who consume alcohol but in amounts regarded as insufficient to cause liver damage. At one end of this spectrum is steatosis or accumulation of fat in the liver. This is followed by steatohepatitis, the more severe form of the disease which may in time result in fibrosis and eventually cirrhosis. Individuals with cirrhosis due to NAFLD are at risk of developing hepatocellular carcinoma. The purpose of this paper is to review the literature and provide a current overview of the epidemiology of NAFLD in the Asia-Pacific region.

Methods

The authors searched via PubMed using MeSH terms "non-alcoholic fatty liver disease" or "fatty liver" and "epidemiology" or "prevalence" in November 2011. The search yielded 495 articles. Out of these, 139 articles were from the Asia-Pacific region. The abstracts of the articles were examined and where doubt existed as to the relevance of an article to the review, the full paper was examined. In total, 63 articles were deemed relevant and were included in the review.

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HEPATOLOGY

Non-alcoholic fatty liver disease in diabetics – prevalence and predictive factors in a multiracial hospital clinic population in Malaysia

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Key words
diabetes mellitus, dietary intake, epidemiology, ethnicity, non-alcoholic fatty liver disease, physical activity.

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Abstract

Background and Aim: There is currently no published study comparing prevalence of non-alcoholic fatty liver disease (NAFLD) and associated factors among diabetics of different ethnicity in the Asia-Pacific region.

Methods: Cross-sectional study of consecutive patients in the Diabetic Clinic in University of Malaya Medical Centre. The Global Physical Activity Questionnaire and a semi-quantitative food-frequency questionnaire were used to assess physical activity and dietary intake, respectively. Diagnosis of NAFLD was ultrasound-based and following exclusion of significant alcohol intake.

Results: Data for 399 patients were analyzed (mean age 62.3 ± 10.5 years, 43.1% men). The racial distribution was Chinese 43.6%, Indian 33.1%, Malay 22.3%, and others 1.0%. The prevalence of NAFLD was 49.6%. On univariate analysis, factors associated with NAFLD were age < 65 years, race, obesity, central obesity, glycated hemoglobin ≥ 7.0%, and elevated serum alanine aminotransferase (ALT) and gamma-glutamyl transpeptidase levels. Patients with low physical activity were more likely to have NAFLD (odds ratio [OR] = 1.67, 95% confidence interval [CI] = 1.06–2.63, $P = 0.020$). The prevalence of NAFLD was highest among Malays (60.7%), followed by Indians (51.5%), and lowest among Chinese (42.0%) consistent with higher prevalence of central obesity and higher percentage calorie intake from fat in the former groups of patients. On multivariate analysis, independent factors associated with NAFLD were central obesity (OR = 2.20, 95% CI = 1.29–3.75, $P = 0.004$) and elevated serum ALT level (OR = 1.98, 95% CI = 1.21–3.25, $P = 0.007$).

Conclusions: NAFLD was seen in half of a cohort of diabetic patients and was independently associated with central obesity and elevated serum ALT level. Prevalence of NAFLD was different and paralleled the difference in prevalence of central obesity and in percentage calorie intake from fat among the different ethnic groups.

Introduction

Non-alcoholic fatty liver disease (NAFLD) is rapidly increasing in the Asia-Pacific region and is estimated to affect up to 30% of the general population.[1] In the only published study on prevalence of NAFLD in the general population from Malaysia, Goh *et al.* reported a prevalence of 22.7% among individuals attending a health check in a suburban medical facility.[2] The study also reported an inordinately high prevalence of NAFLD among the Malays and Indians compared with the Chinese.

NAFLD is closely associated with diabetes mellitus (DM) and obesity. The prevalence of NAFLD is higher in patients with DM and has been estimated to be between 55% and 70% in previous studies from other parts of the world.[3–5] The prevalence of NAFLD is even higher among the morbidly obese and has been reported to be over 90%.[6]

In Malaysia, the prevalence of DM and obesity has reached epidemic proportions over the years. The Third National Health and Morbidity Survey (NHMS III) estimated the prevalence of DM among adults aged 30 years old and above to have almost doubled from 8.3% in 1996 to 14.9% in 2006.[7] Yet to be published, the Fourth NHMS found that this figure has increased to 20% in 2011. The NHMS III also reported that 43.1% of adult Malaysians were overweight or obese in 2006, almost double that reported 10

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ORIGINAL ARTICLE

Non-alcoholic fatty liver disease in a young multiracial Asian population: a worrying ethnic predilection in Malay and Indian males

Wah-Kheong Chan · Norhaniza Bahar · Hamizah Razlan · Anushya Vijayananthan · Pavai Sithaneshwar · Khean-Lee Goh

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Abstract
Purpose Previous studies on multiracial Malaysian populations found inordinately high prevalence of NAFLD among Malays and Indians. Whether the prevalence of NAFLD is different among young adults of different ethnic origins is not known. We aimed to determine racial differences in NAFLD in a young multiracial Malaysian population and associated factors.
Methods This was a cross-sectional study on medical students from the University of Malaya. Diagnosis of NAFLD was by transabdominal ultrasonography and following exclusion of significant alcohol intake and other causes of chronic liver disease.
Results Data of 469 subjects were analyzed (mean age 23.2 ± 2.4 years, 40.3 % male). The racial distribution was: Chinese 53.9 %, Malay 30.5 % and Indian 15.6 %. The overall prevalence of NAFLD was 7.9 %. Subjects with NAFLD were older, had greater BMI and WC, higher SBP and DBP, higher FBS, serum TG and LDL levels, and lower serum HDL level. The prevalence of NAFLD was higher among males compared to females (17.9 % vs. 3.3 %, $p < 0.001$). The highest prevalence of NAFLD was seen among Indian and Malay males at 33.3 and 25.5 %, respectively, compared to Chinese males at 6.8 % ($p < 0.001$). No significant difference was seen among females of different races. Independent factors associated with NAFLD were male gender, obesity and hypertriglyceridemia.
Conclusions The difference in prevalence of NAFLD among the different ethnic groups can be observed as early as young adulthood. An inordinately high prevalence of NAFLD was observed among Malay and Indian males consistent with the higher prevalence of obesity in these groups.

Keywords Non-alcoholic fatty liver disease · Epidemiology · Young adults · Ethnicity · Asian

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Introduction

Non-alcoholic fatty liver disease (NAFLD) is rapidly increasing in the Asia-Pacific region and has been estimated to affect up to 30 % of the general population [1]. In a study on a suburban population in Malaysia, the prevalence of NAFLD was found to be 22.7 % [2]. The prevalence of NAFLD among diabetics has been estimated to be 49.6 % based on a separate study on a hospital clinic population [3]. Both studies found an inordinately high prevalence of NAFLD among the Malays and Indians compared to the Chinese. In a study on the etiology of liver cirrhosis in Malaysia, a cryptogenic cause, believed to be due to NAFLD, was identified as the cause of liver cirrhosis in a significantly larger proportion of Malay and Indian patients compared to Chinese patients [4]. Besides being one of the most common causes of chronic liver disease, NAFLD has been closely associated with risk

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HEPATOLOGY

Controlled attenuation parameter for the detection and quantification of hepatic steatosis in nonalcoholic fatty liver disease

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Key words
controlled attenuation parameter, hepatic steatosis, nonalcoholic fatty liver disease, noninvasive assessment.

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Abstract

Background and Aim: Controlled attenuation parameter (CAP) has been suggested as a noninvasive method for detection and quantification of hepatic steatosis. We aim to study the diagnostic performance of CAP in nonalcoholic fatty liver disease (NAFLD) patients.
Methods: Transient elastography was performed in consecutive NAFLD patients undergoing liver biopsy and non-NAFLD controls. The accuracy of CAP for the detection and quantification of hepatic steatosis was assessed based on histological findings according to the Nonalcoholic Steatohepatitis Clinical Research Network Scoring System.
Results: Data for 101 NAFLD patients (mean age 50.3 ± 11.3 years old, 51.5% male) and 60 non-NAFLD controls were analyzed. CAP was associated with steatosis grade (odds ratio [OR] = 29.16, $P < 0.001$), body mass index (BMI; OR = 4.34, $P < 0.001$) and serum triglyceride (OR = 13.59, $P = 0.037$) on multivariate analysis. The median CAP for steatosis grades S0, S1, S2, and S3 were 184 dB/m, 305 dB/m, 320 dB/m, and 324 dB/m, respectively. The areas under receiver operating characteristics curves (AUROC) for estimation of steatosis grades ≥ S1, S2, and S3 were 0.97, 0.86, and 0.75, respectively. The optimal CAP cutoffs for estimation of steatosis grades ≥ S1, S2, and S3 were 263 dB/m, 281 dB/m, and 283 dB/m, respectively. Among non-obese patients, the AUROC for estimation of steatosis grades ≥ S1 and S2 were 0.99 and 0.99, respectively. Among obese patients, the AUROC for estimation of steatosis grades ≥ S1, S2, and S3 were 0.92, 0.64, and 0.58, respectively.
Conclusions: CAP is excellent for the detection of significant hepatic steatosis. However, its accuracy is impaired by an increased BMI, and it is less accurate to distinguish between the different grades of hepatic steatosis.

Introduction

Nonalcoholic fatty liver disease (NAFLD) encompasses a spectrum of liver conditions, ranging from benign steatosis to nonalcoholic steatohepatitis (NASH) to fibrosis and cirrhosis.[1] NASH has been recognized as an important cause of cryptogenic cirrhosis[2] and is associated with an increased risk of hepatocellular carcinoma, even in patients without cirrhosis.[3] In a study on etiology of cirrhosis and association with hepatocellular carcinoma in our center, cryptogenic cause, which is believed to be due to NASH, contributed to 15.4% of cases of cirrhosis and was an independent predictor of hepatocellular carcinoma.[4] The prevalence of NAFLD has increased rapidly over the years parallel to the increase in metabolic syndrome, and it is recognized as one of the most common causes of chronic liver disease worldwide.[5]

Ultrasonography is by far the most common method used to diagnose fatty liver in clinical practice and in epidemiological studies. However, ultrasonography is accurate only when fatty liver is moderate to severe.[6] Moreover, ultrasonography is not able to distinguish NASH from simple steatosis and to assess the severity of fibrosis. Both factors carry important prognostic implications in NAFLD patients. Histopathological examination of a liver biopsy specimen is the current best standard for assessment of NAFLD. It confirms the diagnosis and helps exclude other causes of liver disease in some cases. It also distinguishes NASH from simple steatosis and allows assessment of the severity of fibrosis. However, liver biopsy is invasive and associated with a small risk of complications. It may also be limited by sampling variability[7] and intra- and interobserver variability.[8]

Recently, a novel technology called transient elastography has been used to estimate liver stiffness, which has shown to correlate well with histopathological fibrosis stage. This has allowed noninvasive and accurate estimation of fibrosis stage in NAFLD patients.[9] The decrease in amplitude of ultrasound as it is

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ORIGINAL ARTICLE

Ultrasonography-diagnosed non-alcoholic fatty liver disease is not associated with prevalent ischemic heart disease among diabetics in a multiracial Asian hospital clinic population☆

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Summary
Background: Non-alcoholic fatty liver disease (NAFLD) and cardiovascular diseases are both common among patients with diabetes mellitus.
Objective: The aim of this study is to determine if ultrasonography-diagnosed NAFLD is associated with prevalent ischemic heart disease (IHD) among patients with diabetes mellitus.
Methods: This is a cross-sectional study on consecutive patients seen at the Diabetic Clinic, University of Malaya Medical Centre. The medical record for each patient was reviewed for documented IHD. Patients without documented IHD but had symptoms and/or electrocardiographic changes suggestive of IHD were referred for cardiac evaluation.
Results: Data for 399 patients were analyzed. Mean age was 62.8 ± 10.5 years with 43.1% male. NAFLD and IHD were present in 49.6 and 26.6%, respectively. The prevalence of IHD among patients with and without NAFLD was 24.7 and 28.4%, respectively ($P = 0.414$). The prevalence

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Original article

Progression of liver disease in non-alcoholic fatty liver disease: A prospective clinicopathological follow-up study

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OBJECTIVE: To perform a follow-up study on non-alcoholic fatty liver disease (NAFLD) patients in our previous study using paired liver biopsy.

METHODS: Patients who were included in our previous study on NAFLD and agreed to receive a repeat liver biopsy were included in the study. Their clinical characteristics, laboratory examination results and histological analysis on the repeat liver biopsied specimens were prospectively collected and compared with those in the previous study.

RESULTS: Data from 35 patients (mean age 47.5 ± 10.9 years, male 40.0%) were analyzed. The mean interval between the liver biopsies was 6.4 ± 0.8 years. NAFLD activity score (NAS) worsened in 13, remained unchanged in 9 and ameliorated in 13. Fibrosis worsened in 18 and remained unchanged in 17. Two patients who were confirmed with cirrhosis at baseline developed decompensated cirrhosis. On multivariate analysis, elevated serum aspartate aminotransferase (AST) (odds ratio [OR] 10.74, 95% confidence interval [CI] 1.00–115.86, $P = 0.050$) and γ-glutamyl transpeptidase (γ-GT) (OR 16.10, 95% CI 1.30–198.90, $P = 0.030$) at follow-up were associated with worsened NAS. Patients with borderline NASH at baseline were more likely to have worsened NAS at follow-up than those with definite NASH (OR 12.67, 95% CI 2.29–70.02, $P = 0.004$). However, both groups had a similar likelihood of having worsened fibrosis at follow-up. No plausible factors were found to be associated with worsened fibrosis.

CONCLUSIONS: NAFLD patients with persistently elevated serum AST and γ-GT levels during follow-up should be suspected of having worsened NAS. NASH patients can have significant disease progression over a relatively short period of time and fibrosis might be irreversible without specific interventions.

KEY WORDS: disease progression, histology, NAFLD, NASH, non-alcoholic fatty liver disease, non-alcoholic steatohepatitis.

INTRODUCTION

The prevalence of non-alcoholic fatty liver disease (NAFLD) has rapidly increased over the decades and the disease is estimated to affect up to 30% of the general population in the Asia–Pacific region.[1] In Malaysia Goh *et al.*[2] reported a prevalence of NAFLD of 22.7% based on a group of suburban individuals who attended health check. We found that the prevalence of NAFLD (49.6%) among patients with

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Conflicts of interest: None.

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PLOS ONE

Limited Utility of Plasma M30 in Discriminating Non-Alcoholic Steatohepatitis from Steatosis – A Comparison with Routine Biochemical Markers

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Abstract

Introduction: The utility of Cytokeratin-18 fragment, namely CK18Asp396 (M30), for the diagnosis of non-alcoholic steatohepatitis (NASH) is currently uncertain. We aimed to provide further data in this area among multi-ethnic Asian subjects with NAFLD.

Materials and Methods: The accuracy of M30 for detecting NASH was compared with serum alanine aminotransferase (ALT), aspartate aminotransferase (AST) and gamma glutamyl transpeptidase (GGT) levels in consecutive adult subjects with biopsy-proven non-alcoholic fatty liver disease (NAFLD).

Results: Data for 93 NAFLD subjects (mean age 51.0±11.1 years old and 51.6% males) and 20 healthy controls (mean age 50.2±16.4 years old and 33.3% males) were analyzed. There were 39 NASH subjects (41.9%) and 54 non-NASH subjects (58.1%) among the NAFLD subjects. Plasma M30 (349 U/L vs. 162 U/L), and serum ALT (70 IU/L vs. 26 IU/L), AST (41 IU/L vs. 20 IU/L) and GGT (75 IU/L vs. 33 IU/L) were significantly higher in NAFLD subjects than in healthy controls. Serum ALT (86 IU/L vs. 61 IU/L), AST (58 IU/L vs. 34 IU/L) and GGT (97 IU/L vs. 56 IU/L) were significantly higher in NASH subjects compared to non-NASH subjects, but no significant difference was observed with plasma M30 (435 U/L vs. 331 U/L). The accuracy of plasma M30, and serum ALT, AST and GGT was good for predicting NAFLD (AUROC 0.91, 0.95, 0.87 and 0.85, respectively) but less so for NASH (AUROC 0.59, 0.64, 0.75 and 0.68, respectively). Serum ALT and AST, but not plasma M30 showed a significant trend with increasing grades of ballooning and lobular inflammation.

Conclusion: The utility of M30 in the detection of NASH in clinical practice appears limited, in comparison to routine biochemical markers.

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Data Availability: The authors confirm that all data underlying the findings are fully available without restriction. All relevant data are within the paper and its Supporting Information files.

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Competing Interests: The authors have declared that no competing interests exist.

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Introduction

The prevalence of non-alcoholic fatty liver disease (NAFLD) has increased rapidly over the years, parallel to the increase in metabolic syndrome, and it is recognized as one of the most common causes of chronic liver disease worldwide [1]. NAFLD encompasses a spectrum of liver conditions, ranging from simple steatosis to non-alcoholic steatohepatitis (NASH) to fibrosis and cirrhosis. While simple steatosis is generally considered benign, NASH may lead to fibrosis and eventually cirrhosis, with an increased risk of morbidity and mortality [2,3].

The diagnosis of NASH is made by histopathological examination of a liver biopsy specimen. However, liver biopsy is invasive and it is associated with a small risk of serious complications [4]. It is not practical to subject all subjects with NAFLD to a liver biopsy to diagnose NASH. Furthermore, repeated liver biopsies to monitor disease progression in clinical practice is not acceptable either. A simple and reliable non-invasive test is needed for the diagnosis and follow-up of NASH.

Cytokeratin 18 (CK-18) is the major intermediate filament protein in liver cells and it is cleaved by caspases that are activated during apoptosis of liver cells, a process which plays an important role in NASH [5]. CK-18 fragment, namely CK18Asp396 (M30), has been studied for the diagnosis of NASH with varying results [6–13]. Whilst some studies have suggested that specific cut-off levels of CK-18 can reliably detect NASH in a cohort of NAFLD subjects [6–10], others have not shown such promising results [11–13]. These contrasting data may have been due to studies with a

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ORIGINAL ARTICLE

A novel 2-step approach combining the NAFLD fibrosis score and liver stiffness measurement for predicting advanced fibrosis

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Abstract
Background The non-alcoholic fatty liver disease (NAFLD) fibrosis score (NFS) is indeterminate in a proportion of NAFLD patients. Combining the NFS with liver stiffness measurement (LSM) may improve prediction of advanced fibrosis. We aim to evaluate the NFS and LSM in predicting advanced fibrosis in NAFLD patients.
Methods The NFS was calculated and LSM obtained for consecutive adult NAFLD patients scheduled for liver biopsy. The accuracy of predicting advanced fibrosis using either modality and in combination were assessed. An algorithm combining the NFS and LSM was developed from a training cohort and subsequently tested in a validation cohort.
Results There were 101 and 46 patients in the training and validation cohort, respectively. In the training cohort, the percentages of misclassifications using the NFS alone, LSM alone, LSM alone (with grey zone), both tests for all patients and a 2-step approach using LSM only for patients with indeterminate and high NFS were 5.0, 28.7, 2.0, 2.0 and 4.0 %, respectively. The percentages of patients requiring liver biopsy were 30.7, 0, 36.6, 36.6 and 18.8 %, respectively. In the validation cohort, the percentages of misclassifications were 8.7, 28.3, 2.2, 2.2 and 8.7 %, respectively. The percentages of patients requiring liver biopsy were 28.3, 0, 41.3, 43.5 and 19.6 %, respectively.
Conclusions The novel 2-step approach further reduced the number of patients requiring a liver biopsy whilst maintaining the accuracy to predict advanced fibrosis. The combination of NFS and LSM for all patients provided no apparent advantage over using either of the tests alone.

Keywords Non-alcoholic fatty liver disease (NAFLD) · Liver fibrosis · Non-invasive test · NAFLD fibrosis score · Liver stiffness measurement · Fibroscan

Introduction

Non-alcoholic fatty liver disease (NAFLD) encompasses a spectrum of liver conditions, ranging from benign steatosis to non-alcoholic steatohepatitis (NASH) to fibrosis and cirrhosis [1]. The degree of liver fibrosis provides information on prognosis and helps identify patients for intervention. For example, NASH patients with advanced fibrosis have a much higher liver-related mortality compared to those without advanced fibrosis [2]. On the other hand, the US multi-society practice guideline on diagnosis and management of NAFLD recommends that patients with NASH-related cirrhosis be screened for gastroesophageal varices and hepatocellular carcinoma [3].

Histopathological examination of a liver biopsy specimen is the best standard for assessment of liver fibrosis. However, liver biopsy is invasive and associated with a small risk of complications [4]. Technical expertise is also required, from obtaining a good specimen to processing and accurately interpreting the result. It is not practical to subject all NAFLD patients to a liver biopsy to assess liver

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