Non-invasive Evaluation of Non-alcoholic Fatty Liver Disease Using Biochemical and Genetic Markers

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Abstract

Background and aims: Non-alcoholic fatty liver disease (NAFLD) is one of the most common chronic liver diseases worldwide. It encloses a wide disease spectrum from simple steatosis to non-alcoholic steatohepatitis (NASH). While simple steatosis is thought to be benign, NASH may progress to end-stage liver disease and hepatocellular carcinoma. Traditionally, the diagnosis of NAFLD and, particularly, NASH relies on liver biopsy. It is an invasive procedure with poor acceptance and risk of major complications such as hemorrhage. Non-invasive evaluations of NAFLD and NASH are urgently needed. In this study, we tested the performance of different biochemical and genetic markers in the diagnosis and monitoring of NAFLD and NASH.

Methods: This study included 147 patients with biopsy-proven NAFLD (hospital cohort), 51 of whom also had per protocol follow-up liver biopsies 3 years later. In addition, 922 subjects from a population screening project (community cohort) underwent proton-magnetic resonance spectroscopy to determine intrahepatic triglyceride content (IHTG). NAFLD was diagnosed when IHTG was over 5%. Subjects with IHTG less than 5% and 5 other subjects with normal liver histology served as controls. Furthermore, 154 NAFLD subjects from the community cohort were enrolled in a prospective single-blinded trial comparing a community-based lifestyle intervention programme (n=77) and standard care (n=77). Cytokeratin-18

(CK-18), adipocyte fatty acid binding protein (AFABP) and fibroblast growth factor 21 (FGF21) were measured by enzyme-linked immunosorbent assays. Patatin-like phospholipase domain containing 3 (*PNPLA3*) rs738409 gene polymorphism was determined by TaqMan® SNP Genotyping Assay.

Results: CK-18 (including apoptosis marker CK-18 M30 and 2 total cell death markers CK-18 M65 and CK-18 M65ED) and FGF21 had high accuracy in diagnosing NAFLD (area under the receiver-operating characteristics curves [AUROC] 0.84-0.94) and moderate accuracy in diagnosing NASH (AUROC 0.66-0.71). AFABP only had moderate accuracy in diagnosing NAFLD (AUROC 0.63) and NASH (AUROC 0.63). Combined application of CK-18 M30 and FGF21 using a 2-step approach further improved the negative predictive value and positive predictive value to around 80%. Changes of M30 and M65ED had high AUROC of over 0.8 in predicting disease progression in the 51 patients who underwent paired liver biopsies. Changes in AFABP and FGF21 did not correlate with disease progression.

The *PNPLA3* rs738409 GG genotype was associated with 2-fold increase in the risk of NAFLD independent of dietary pattern in the community. The GG genotype was also associated with more severe histological damage in hospital NAFLD patients. The community-based lifestyle intervention programme was sustainable and effective. Subjects with allele G were more sensitive to the programme. Patients with

GG genotype had an additional 6% absolute reduction in IHTG compared with those with CC genotype from lifestyle intervention. This reduction was accompanied with greater reduction in body weight, body mass index and total cholesterol.

Conclusion: Biomarkers CK-18 M30/M65/M65ED and FGF21 have high accuracy in diagnosing NAFLD and moderate accuracy in diagnosing NASH. A two-step approach combining CK-18 M30 and FGF21 further improves the accuracy in diagnosing NASH. Changes in CK-18 M30 and M65ED have high accuracy in predicting disease progression and may be used for serial monitoring. The GG genotype in *PNPLA3* rs738409 is associated with increased risk of NAFLD independent of dietary pattern. Those patients with GG genotype were more sensitive to lifestyle intervention and thus should be encouraged to participate in such programmes.

摘要

研究背景及實驗目的: 非酒精性脂肪性肝病（non-alcoholic fatty liver disease, NAFLD）是世界範圍內最常見的慢性肝病之一。NAFLD 包括從單純性脂肪肝到非酒精性脂肪性肝炎（non-alcoholic steatohepatitis, NASH）在內的一系列疾病譜。單純性脂肪肝通常不易進展，然而，NASH 卻會進展爲包括肝細胞肝癌在內的終末期肝病。現今 NAFLD，特別是 NASH 的確診需要借助肝活檢組織病理學實現。這是一個有創的檢查，接受程度低且存在出血等嚴重併發症的可能。NAFLD 及 NASH 的無創評估手段亟需建立。在這一研究中，我們評估了不同生物及遺傳標誌物在 NAFLD 及 NASH 診斷監控中的表現。

實驗方法: 本研究納入了 147 名活檢確診 NAFLD 患者（醫院人群），其中 51 名患者另接受了預先確認安排的相隔三年的配對肝活檢。同時，922 名來自一人群 NAFLD 篩查計劃的個體（社區人群）接受了正電子磁共振波譜測量肝內甘油三脂含量（intrahepatic triglyceride, IHTG）。當 IHTG 超過 5%時診斷為 NAFLD。IHTG 少於 5%的個體及另外 5 名肝活檢結果正常的個體作為實驗的對照組。另外，154 名社區人群中的 NAFLD 患者參與了一項隨機單盲臨床研究，以比較社區生活方式幹預療程組（77 名）和常規對照組（77 名）對於 NAFLD 治療的區別。血細胞角蛋白-18（CK-18），脂肪細胞脂肪酸結合蛋白（AFABP）和成纖維細胞生長因子（FGF21）由酶聯免疫吸附法測定。Patatin-like phospholipase domain containing 3 (PNPLA3) rs738409 基因多態性由 TaqMan® SNP 基因分型檢測決定。

結果: CK-18（包括凋亡相關標誌物 CK-18 M30 和全細胞死亡標誌物 CK-18 M65，CK-18 M65ED）及 FGF21 在診斷 NAFLD 上具有高度準確率（接受者操作特徵[receiver-operating characteristics curve, ROC]曲綫下面積 0.84-0.94）；在診斷 NASH 上具有中度準確率（ROC 曲綫下面積 0.66-0.71）。AFABP 在診斷 NAFLD 和 NASH 上均只有中度準確率（ROC 曲綫下面積均為 0.63）。採取一兩步法聯合應用 CK-18 M30 和 FGF21 進一步將陰性預測率及陽性預測率增加至約 80%。在 51 名接受配對肝活檢的患者中，M30 和 M65 的變化在預測疾病進展上具有高準確率，ROC 曲綫下面積大於 0.8。AFABP 和 FGF21 的變化和疾病進展沒有相關。

在社區人群中，*PNPLA3* rs738409 GG 基因型與兩倍的 NAFLD 風險相關，且這種相關性獨立於飲食結構不同而存在。GG 基因型也與醫院人群中 NAFLD 患者的組織學損害嚴重性相關。社區生活方式幹預療程可持續且有效治療 NAFLD。攜帶有等位基因 G 的個體對此療程更加敏感。從生活方式幹預療程中，GG 基因型的患者較之 CC 基因型的患者可得到絕對值多達 6%的額外 IHTG 降低。這一降低同時伴隨著更大的體重，體重指數及血總膽固醇的降低。

結論: 生物標誌物 CK-18 M30/M65/M65ED 和 FGF21 在診斷 NAFLD 上具有高準確率；在診斷 NASH 上具有中度準確率。兩步法聯合應用 CK-18 M30 和 FGF21 進一步增加診斷 NASH 的準確率。CK-18 M30 和 M65ED 的變化在預測疾病進展上具有高準確率，可被用於多次檢測監控疾病。*PNPLA3* rs738409 GG 基因型

獨立於飲食結構之外與 NAFLD 的高風險相關。GG 基因型的 NAFLD 患者對生活方式干預治療更加敏感，因此須鼓勵參加類似療程。

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Publications

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Abbreviations

^{1}H-MRS	Proton-magnetic resonance spectroscopy
AASLD	American Association for the Study of Liver Diseases
AFABP	Adipocyte fatty acid binding protein
ALT	Alanine aminotransferase
ApoA1	Apolipoprotein A1
APOC3	Apolipoprotein C3
ARFI	Acoustic radiation force impulse imaging
AST	Aspartate aminotransferase
ATP	Adenosine triphosphate
AUROC	Area under Receiver Operating Characteristic curve
BMI	Body-mass index
CI	Confidence interval
CK-18	Cytokeratin 18
CT	Computed tomography
DAG	Diacylglycerol
DGE-MRI	Dual gradient echo magnetic resonance imaging
ELF	Enhanced Liver Fibrosis
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
FDFT1	Farnesyl diphosphate farnesyl transferase 1

FGF21	Fibroblast growth factor 21
FLI	Fatty Liver Index
GC	Group-specific component
GCKR	Glucokinase regulatory protein
GGT	Gamma-glutamyltransferase
GWAS	Genome-wide association study
HBV	Hepatitis B
HCV	Hepatitis C
HDL	High-density lipoprotein
HOMA-IR	Homeostasis model assessment of insulin resistance
HR	Hazard ratio
IHTG	Intrahepatic triglyceride
IL-6	Interleukin 6
IQR	Interquartile range
LCP1	Lymphocyte cytosolic protein-1
LDL	Low-density lipoprotein
LSM	Liver stiffness measurement
LYPLAL1	Lysophospholipase-like 1
M30	CK-18 M30
M65	CK-18 M65
M65ED	CK-18 M65ED
MAG	Monoacylglycerol

MRE	Magnetic resonance elastography
MRI	Magnetic resonance imaging
MS	Metabolic syndrome
N.A.	Not available
NAFLD	Non-alcoholic fatty liver disease
NAS	NAFLD activity score
NASH	Non-alcoholic steatohepatitis
NASH CRN	Nonalcoholic Steatohepatitis Clinical Research Network
NCAN	Neurocan
NHANES III	Third National Health and Nutrition Examination Survey
NPV	Negative predictive value
OR	Odds ratio
PCOS	polycystic ovary syndrome
PIVENS	Pioglitazone or Vitamin E for NASH Study
PNPLA3	Patatin-like phospholipase domain containing 3
PPV	Positive predictive value
ROC	Receiver-operating characteristics
ROS	Reactive oxygen species
SNP	Single-nucleotide polymorphism
SREBP-1c	sterol regulatory element binding protein 1c
SWE	Shear wave elastography

T2DM	Type 2 diabetes mellitus
TAG	Triacylglycerol
TE	Transient elastography
TG	Triglycerides
TLR-4	Toll-like-receptor 4
TNF-α	Tumor necrosis factor α
US	Ultrasonography
HbA1c	Glycated hemoglobin
LR	Likelihood ratio
Non-NASH	Non-NASH NAFLD patients
GI	Glycaemic index
WHR	Waist-to-hip ratio
WC	Waist circumference

Chapter 1: General introduction

Non-alcoholic fatty liver disease (NAFLD) is one of the most common chronic liver diseases all over the world [1]. It includes a wide disease spectrum from simple steatosis to non-alcoholic steatohepatitis (NASH), which is the active form of NAFLD. While simple steatosis usually exhibits a benign process, NASH may progress to end-stage liver disease such as cirrhosis, liver failure and hepatocellular carcinoma [2-3].

The gold standard for diagnosing of NAFLD and NASH is liver biopsy. However, it is an invasive test. The risk for major complications and sampling bias cannot be avoided. Moreover, it is not very suitable for repeated evaluation. Several non-invasive approaches have been introduced, including different imaging technologies and biomarkers. Non-invasive imaging methods are mainly used to determine the grade of steatosis. Physical measurements correlated well with fibrosis. Clinical tests, biomarkers and together with several established prediction scores, provided potential options in distinguishing NASH and fibrosis; however, most of them still need to be further validated. With the advances in genome analysis, genetic determinants of NAFLD are also widely studied in the recent years using genome-wide association study (GWAS). Several genetic determinants of NAFLD, including genetic variants in patatin-like phospholipase domain-containing protein 3 (*PNPLA3*), apolipoprotein C3 (*APOC3*), farnesyl diphosphate farnesyl transferase 1

(*FDFT1*) were identified. The development of non-invasive tests of NAFLD serves 2 main purposes. First, this allows accurate diagnosis of NAFLD and evaluation of disease severity, and thus reduces the burden of liver biopsy. Second, the primary goal of managing chronic liver diseases is not improvement in liver histology but a reduction in the risk of hepatic complications. Therefore, it is also important to evaluate non-invasive tests as predictors of clinical outcomes.

The management of patients with NAFLD should not be limited to treating liver disease. Metabolic disorders, which are frequently associated with NAFLD, should be treated at the same time. Multiple modalities including lifestyle intervention, medical treatment as well as bariatric surgery are available for the management of NAFLD [3].

In this Chapter, I will review the epidemiology, natural history and pathogenesis of NAFLD. This is followed by an overview of the non-invasive tests and genetic determinants of NAFLD. The final section describes the management of NAFLD.

1.1 Definition, epidemiology, risk factors, natural history and pathogenesis of non-alcoholic fatty liver disease

1.1.1 Definition

Non-alcoholic fatty liver disease (NAFLD) is one of the most common chronic liver

diseases all over the world [1-2]. It is defined as the presence of hepatic steatosis with the absence of secondary causes for hepatic fat accumulation which are summarized in Table 1.1 [3]. It includes a wide disease spectrum from simple steatosis to non-alcoholic steatohepatitis (NASH). Patients with simple steatosis have only hepatic steatosis with no evidence of hepatic injury. NASH, which is the active form of NAFLD, is defined as hepatic steatosis with lobular inflammation and hepatocyte injury in the form of ballooning. NAFLD can affect both adults and children. Here we mainly focus on NAFLD in adults.

1.1.2 Epidemiology

The prevalence of NAFLD also differs widely depending on the study population and different diagnostic methods. Liver biopsy, which is the gold standard, is an invasive approach and cannot be adopted in population-based studies. Thus, prevalence of histological confirmed NAFLD can only be assessed from potential liver donors. Marcos et al. reported 11% (14/126) of living liver donors had >30% hepatic steatosis in the United States, which accounted for 20% of the excluded candidates [4]; while Lee et al. from Korea reported that 51% (303/589) of living liver donors had NAFLD defined as ≥5% steatosis in biopsy, including 10% (61/589) had >30% steatosis [5].

Several non-invasive approaches can be used to estimate the prevalence of NAFLD in general population. Ultrasound has satisfactory sensitivity and specificity in

detecting moderate to severe hepatic steatosis; however, it is not reliable when the amount of steatosis is less than 30% [6]. The prevalence of NAFLD defined by ultrasound is ranged between 17% and 46% [7-13]. A population based study in India found 194 NAFLD in 1,168 subjects (17%) [7]; while another study from Brooke Army Medical Center, United States, reported 151 NAFLD in 328 subjects recruited from the clinic (46%) [8]. Proton-magnetic resonance spectroscopy (^{1}H-MRS) quantifies hepatic steatosis which correlated well with the degree of steatosis by histology, and show superior accuracy than ultrasound [14]. In Dallas Heart Study, the prevalence of NAFLD was found to be 31% in 2,287 subjects [15-16]. A community-based study in Hong Kong revealed a NAFLD prevalence of 27% in 922 Chinese subjects [17]. Elevated aminotransferases is also an indication of suspected NAFLD. However, it can be normal in NAFLD patients and correlates poorly with histological findings [18]. The worldwide prevalence of NAFLD estimated by aminotransferases alone ranged from 3% to 21% [3]. The estimated prevalence of NAFLD is summarized in Table 1.2.

On the other hand, the definitive diagnosis of NASH relies on liver biopsy. Two studies mentioned above have reported the prevalence of histological confirmed NASH. Lee et al. from Korea reported 2.2% (13/589) of potential liver donors had NASH [5]. In the study from Brook Army Medical Center, a subgroup of 134 ultrasound diagnosed NAFLD patients received liver biopsies, 40 of them were diagnosed as NASH. However, since all subjects in this study were recruited from

the clinic, the prevalence of NASH might be overestimated.

Taken together, the estimated worldwide prevalence of NAFLD is ranged from 6.3 to 33%. The estimated worldwide prevalence of NASH is ranged from 3 to 5% [3].

The accurate incidence rate of NAFLD remains unknown. The reported incidence rate of NAFLD ranged widely from 29 cases per 100,000 person-years to 86 cases in 1000 person-years [19-21]. The large discrepancy clearly suggests that further studies are required to determine the accurate incidence of NAFLD across different ethic and geographic populations.

1.1.3 Risk factors for NAFLD

Male gender and Hispanic ethnicity are associated with higher prevalence of NAFLD [7-8 10-11 15-17]. In a population-based study in which NAFLD was defined by ^{1}H-MRS, Hispanics had a median intrahepatic triglyceride (IHTG) of 4.6%, significantly higher than whites (3.6%) and blacks (3.2%) ($p < 0.001$)[15]. The prevalence of NAFLD in Hispanics, whites and blacks was 45%, 33% and 31%, respectively. White males had significant higher prevalence of NAFLD compared with white females (42% vs. 24%). Moreover, several studies have shown the prevalence of NAFLD was higher in older patients [7 10 22-23]. However, older patients also have more NAFLD risk factors such as metabolic syndrome. It remains unclear whether the higher incidence in older patients is due to the duration of disease or age itself [2].

Metabolic syndrome (MS) is a well-recognized risk factor for NAFLD [3]. In a joint interim statement of the International Diabetes Federation Task Force on Epidemiology and Prevention; National Heart, Lung, and Blood Institute; American Heart Association; World Heart Federation; International Atherosclerosis Society; and International Association for the Study of Obesity, metabolic syndrome is defined as the existence of any three of the following: (1) Central obesity; (2) Hypertriglyceridemia; (3) Reduced high-density lipoprotein-cholesterol; (4) Elevated blood pressure; (5) Impaired fasting plasma glucose; or receiving treatment for the above metabolic abnormalities [24]. NAFLD is very common in obese individuals. In morbidly obese patients who received bariatric surgeries, the prevalence of NAFLD can be as high as 98% [25-30]. Notably, the prevalence of NASH in obese patients ranges from 10 to 56% with a median of 33% [2]. 67% of these patients have portal fibrosis [2]; 5% of them may even have unsuspected cirrhosis [3]. Type 2 diabetes mellitus (T2DM) also has a close relationship with NAFLD. Using both ultrasound and ^{1}H-MRS to evaluate 939 patients from Edinburgh Type 2 Diabetes Study, 42.6% of them were diagnosed as NAFLD [31]. Leite and colleagues found the prevalence of NAFLD defined using ultrasound was 69% in 180 T2DM patients in Brazil [32]; association of NAFLD with central obesity and hypertriglyceridemia was also observed. In another study from India, Prashanth et al. evaluated 204 T2DM patients and found 127 (62%) had hepatic fatty infiltration on ultrasound. Furthermore, 90 of these 127 patients received liver biopsy, of whom 87% had histologically confirmed

NAFLD, 63% had NASH and 37% had fibrosis [33]. Hypertriglyceridemia and low serum HDL levels are very common in NAFLD patients. Approximately, 50% of the subjects with dyslipidemia have NAFLD [34].

Sleep apnea and polycystic ovary syndrome (PCOS) are also suggested to be risk factors for NAFLD and NASH [3]. Several independent studies reported that sleep apnea was associated with elevated aminotransferases and histological severity in NAFLD patients [35-37]. A meta-analysis which pooled 2,183 subjects from 18 cross-sectional studies revealed that sleep apnea at least doubles the risk of NAFLD, NASH and fibrosis [38]. NAFLD is found in a great portion of patients with PCOS, and vice versa [39-45]. In a study which included 41 patients with PCOS and 31 age- and body-mass index (BMI)-matched control subjects, PCOS patients had significantly higher prevalence of NAFLD (41% vs. 19%) and insulin resistance (63% vs. 35.5%) [43]. In another study, Brzozowska et al. screened 14 consecutive female NAFLD patients, 10 (71%) of them had PCOS [42].

1.1.4 Natural history

1.1.4.1 Survival and mortality

While simple steatosis usually exhibits a benign process, its advanced form, NASH, may progress to end-stage liver disease such as cirrhosis, liver failure and hepatocellular carcinoma [2-3 46-47]. NAFLD patients have increased overall mortality compared to healthy controls. The leading cause of NAFLD patients are

cardiovascular disease, malignancy and liver-related death [2-3]. In a study based on the Third National Health and Nutrition Examination Survey (NHANES III) and its Linked Mortality File in the United States, Ong et al. showed the overall mortality was significantly higher in 817 NAFLD patients compared to 10,468 control subjects after adjusting for age, gender, race, education, income, BMI, hypertension disease, and diabetes (hazard ratio [HR]: 1.038; $p < 0.001$) with a median follow-up of 8.7 years. Liver-related mortality was even higher (HR: 9.32; $p < 0.001$) [48]. In another study which followed up 420 NAFLD patients with a mean duration of 7.6 years, the overall survival was lower than the expected survival for the general population (HR for mortality: 1.34; $p = 0.03$). Liver disease was the third leading cause of death, as compared with the thirteenth leading cause of death in the general population [49]. Furthermore, histology based studies suggest that the increased mortality are attributed to NASH patients alone other than all NAFLD patients. In a study from Sweden, 71 NASH and 58 non-NASH patients were followed up for 13.7 years. The overall survival of NAFLD was significantly lower than control population (78% vs. 84%, $p = 0.006$). However, non-NASH patients had similar survival compared to the corresponding reference population. In contrast, NASH patients had significant lower survival compared to its reference population (70% vs. 80%, $p = 0.01$) [50]. Also from Sweden, another study reported similar results that increased risk of death was only observed in patients with NASH after a 28-years follow-up [51]. Moreover, Matteoni et al. and Rafiq et al. both revealed the increased liver-related mortality in NASH patients compared to non-NASH patients using a same cohort [52-53]. All of the above

data suggest that patients with NASH are at risk of increased mortality compared to general population.

1.1.4.2 Disease progression

Patterns and risk factors of disease progression in patients with different stages of NAFLD can be revealed by paired liver biopsy studies. In one study, 12 patients with non-NASH NAFLD received paired liver biopsy after 11 years apart from the initial evaluation due to abnormal results of liver blood tests. None of them developed NASH [47]. However, a very different result was reported by showing that after 5 years, inflammation and ballooning were developed in all patients initially diagnosed as simple steatosis [54]. In a large scale prospective study, Wong et al. followed-up 52 biopsy-proven NAFLD patients and performed a second liver biopsy 3 years apart from first assessment [46]. A semi-quantitative score, NAFLD activity score (NAS) was used to describe the changes in disease status. At baseline, 13 patients had simple steatosis defined as NAS < 3. At month 36, 5 (39%) developed borderline NASH (defined as NAS = 3-4); 3 (23%) developed NASH (defined as NAS ≥ 5). 22 patients had borderline NASH at baseline and 5 (23%) of whom developed NASH at month 36. Interestingly, 2 (15%) simple steatosis patients regressed to normal liver; 4 (18%) borderline NASH patients regressed to simple steatosis; 1 (6%) and 6 (35) NASH patients regression to simple steatosis or borderline NASH, respectively. Reduction in body mass index and waist circumference were identified as independent predicting factors for non-progressive disease. Dysregulation of adipokines

(adiponectin, tumour necrosis factor a, interleukin 6 and leptin) were not associated with progression of NAFLD, although it is generally considered to be associated with inflammation.

1.1.4.3 Fibrosis progression

NAFLD, especially NASH, can develop fibrosis. In Western countries, burnt-out NASH is considered to be the leading etiology of cryptogenic cirrhosis [2 55]. Advanced fibrosis and its complications contribute to the increased liver-related mortality in NAFLD patients. The prevalence of fibrosis in NAFLD patients ranges from 38% to 72%; the prevalence of advanced fibrosis ranges from 9% to 27% (Table 1.3) [28 56-64]. Different risk factors have been identified in cross-sectional studies including older age, obesity, metabolic syndrome, abnormal aminotransferase, and histological necroinflammation and ballooning degeneration (Table 1.3). Risk factors of fibrosis progression were also identified by paired liver biopsy studies. In the study by Wong et al. [46], 26 of 52 (50%) patients had fibrosis at baseline. After 3 years, 14 (27%) patients had fibrosis progression, including 5 (10%) patients had fibrosis progression by at least 2 stages. 25 (48%) had stable disease and 13 (25%) patients had regression of fibrosis. Increase in waist circumference and high baseline low-density lipoprotein-cholesterol were independently associated with fibrosis progression. Adams and colleagues performed serial liver biopsy in 103 patients in a mean follow-up of 3.2 years [65]. 38 (37%) patients had fibrosis progression, including 14 (14%) patients had fibrosis progression by at least 2 stages. 35 (34%) had stable

disease and 30 (29%) had regression of fibrosis. History of diabetes, earlier fibrosis stage in the initial evaluation and higher BMI were independent risk factors associated with fibrosis progression. Notably, in the study by Adams, 50 patients were treated by ursodiol or clofibrate, although the treatment did not lead to significant change of fibrosis stage compared with placebo [65]. Other paired liver studies do not have enough statistical power to identify independent risk factors due to limited case numbers or follow-up duration [50 62 66-72]. Argo et al. summarized ten studies comprising 221 patients and performed pooled-analysis [50 62 65-72]. Their data showed that age and inflammation on initial biopsy are independent predictors of progression to advanced fibrosis [55]. The discrepancy indicates that the risk factors for fibrosis progression in NAFLD patients were still not well recognized. Large-scale prospective study is needed.

1.1.4.4 NAFLD and hepatocellular carcinoma

NAFLD patients are at increased risk for hepatocellular carcinoma (HCC) [73-76]. HCC could be a complication of advanced fibrosis and cirrhosis. Up to 27% of patients with NASH-related cirrhosis develop HCC [77]. Studies comparing HCC incidence in NAFLD and hepatitis C virus (HCV) patients showed discrepant results. While some studies showed lower risk for HCC in NAFLD patients compared with HCV patients [78-80], other studies showed comparable risk in both groups [76 81]. Moreover, indirect evidence suggested NAFLD itself could promote HCC development independent of cirrhosis [82]. Obesity and diabetes are closely associated with NAFLD. In animal

models, both genetic and dietary obesity could promote HCC tumorigenesis and growth [83]. A meta-analysis which pooled 13 cohort studies suggested diabetes can promote HCC before the development of cirrhosis [84]. In two studies in which patients with cirrhosis had been excluded, diabetes was still an independent risk factor for HCC [85-86]. In a study which compared metabolic syndrome (MS) related HCC patients with other chronic liver disease related HCC patients, the background liver had significantly less advance fibrosis in MS related HCC patients [87]. Moreover, some of the MS related malignant tumors were transformed from benign tumors.

1.1.5 Pathogenesis

Hepatic steatosis arises from the abnormal accumulation of triglycerides (TG) in the liver as a result of an imbalance between TG acquisition and removal [77]. The factors which initiate NAFLD pathogenesis and promote simple steatosis to NASH are not fully understood. The classic "Two-hit" hypothesis for the pathogenesis of NAFLD is introduced and modified by Day [88-89]. The "first hit" is steatosis, which is closely associated with obesity and insulin resistance. It increases the sensitivity of liver to a combination of both environmental and genetic "second hits", which leads to NASH and fibrosis.

1.1.5.1 The pathogenesis of hepatic steatosis

The fatty acids used for hepatic TG formation are derived from diet, de novo synthesis and influx from adipose tissue [77]. Accumulation of TG in the liver can arise

from disorders in all three ways [90-91]. Obese individuals have increased supply of fatty acids compared with lean individuals which may contribute to the NAFLD pathogenesis. Insulin resistance also plays an important role in the development of hepatic steatosis. Insulin promotes lipogenesis in the liver even in the presence of insulin resistance [92]. Hyperinsulinemia, as a consequence of insulin resistance, causes hepatic steatosis in different animal models [93]. Furthermore, Semple et al. [94] demonstrated that patients with mutations in *AKT2*, which plays a key role in insulin signaling pathway, result in elevated liver fat content.

Genetic disorders also contribute to the pathogenesis of hepatic steatosis. Inherited disease such as glycogen storage disease type 1a and citrin deficiency can independently lead to severe hepatic steatosis [95-96]. A missense mutation in patatin-like phospholipase domain–containing 3 gene (*PNPLA3*) rs738409 was found to be associated with NAFLD in a genome wide association study and was robustly validated in independent cohorts [97-98]. The underlying mechanism of *PNPLA3* rs738409 in pathogenesis of steatosis will be further discussed in 1.3.1.

Dietary pattern is also associated with hepatic steatosis [77]. For example, the increased consumption of fructose is parallel with increased prevalence of NAFLD. Unlike glucose, fructose cannot be used to synthesize glycogen; instead, it is converted to glyceraldehyde-3-phosphate, providing substrate for de novo lipogenesis. A high fructose diet is used to induce NAFLD in animal models [93]. In a study which

included 49 biopsy-proven NAFLD patients and 24 age/gender/BMI-matched controls, consumption of fructose in NAFLD patients was over 2-folds compared with control subjects. Hepatic lipogenesis was also increased in NAFLD patients indicated by higher level of hepatic mRNA level of fatty acid synthase [99]. Impaired recovery of hepatic adenosine triphosphate (ATP) induced by fructose may also contribute to the development and progression of NAFLD [100].

Emerging evidence has suggested the link between gut microbiota and pathogenesis of hepatic steatosis [101]. Transplantation of normal microbiota to germ-free mice led to over 2-folds increase in hepatic steatosis and development insulin resistance with reduced food intake [102]. Gut microbiota composition is also associated with the presence of obesity in human, which is a major risk factor for NAFLD [101].

1.1.5.2 NAFLD progression

The progression of NAFLD involves the development of inflammation, hepatocyte damage and fibrosis. Dysregulated secretion of cytokines and adipokines is closely associated with the disease progression of NAFLD. Increased pro-inflammatory cytokine such as tumor necrosis factor α (TNF-α) and interleukin 6 (IL-6), and decreased anti-inflammatory cytokine such as adiponectin, is observed in both NASH animal models and human NASH patients compared with those with simple steatosis [77 103]. The dysregulation of cytokines may be induced by insulin resistance and hepatic lipotoxicity of free fatty acids [103]. Excessive endoplasmic reticulum (ER)

stress also contributes to NAFLD progression through activating inflammatory pathways such as nuclear factor κB, c-Jun N-terminal kinase and oxidative stress pathways [104].

Apoptosis is a predominant feature of NASH [105]. Feldstein et al. clearly demonstrated that hepatic steatosis increases Fas-mediated hepatocyte apoptosis [106]. It is not only associated with the development of NASH but also fibrosis [107]. Mitochondrial dysfunction is also activated by Fas-mediated signaling pathway [105]. Excessive reactive oxygen species (ROS) generated by mitochondrial dysfunction and apoptosis may further exacerbate inflammation and tissue injury.

The genetic variants in *PNPLA3* rs738409 is also associated with disease severity of NAFLD [98]. Its role in promoting NAFLD progression will be further discussed in 1.3.1.

Gut microbiota is recently suggested to be an extrahepatic factor which can promote NAFLD progression. It may promote NASH through promoting obesity by improving energy yield; regulating gut permeability; inducing low-grade inflammation through increasing endotoxin production and activation of Toll-like-receptor 4 (TLR-4) signaling; causing immune imbalance; modulating choline and bile acid metabolism; and increasing endogenous ethanol [101].

Table 1.1. Common causes of secondary hepatic steatosis in adults.

Nutrition related	Significant alcohol consumption
	Parenteral nutrition
	Starvation
Viral hepatitis	Hepatitis C
Genetic and metabolic	Wilson's disease
	Lipodystrophy
	Abetalipoproteinemia
	Hypothyroidism
	Hypopituitarism
	Hypogonadism
Obstetric	Acute fatty liver of pregnancy
	HELLP syndrome
Medications	Corticosteroids
	Tamoxifen
	Amiodarone
	Methotrexate
	Anti-retroviral drugs

Table 1.2. Population prevalence of NAFLD.

Author	Year	Country	Diagnostic method	NAFLD	Total number	Prevalence
Marcos [4]	2000	Untied States	Biopsy (>30% steatosis)	14	126	11%
Lee [5]	2007	Korea	Biopsy (>5% steatosis)	303	589	51%
Browning [15]	2004	United States	^{1}H-MRS (IHTG >5.5%)	708	2,287	31%
Wong [17]	2012	China	^{1}H-MRS (IHTG >5%)	252	922	27%
Kojima [12]	2003	Japan	Ultrasound	N.A.	N.A.	30%
Bedogni [9]	2005	Italy	Ultrasound	135	598	23%
Amarapurkar [7]	2007	India	Ultrasound	194	1,168	17%
Caballeria [11]	2010	Spain	Ultrasound	198	766	26%
Williams [8]	2011	Untied States	Ultrasound	151	328	46%
Hu [10]	2012	China	Ultrasound	2,730	7,152	38%
Clark [108]	2003	United States	Aminotransferase	N.A.	15,676	7.9%

Ruhl [109]	2003	United States	Aminotransferase	N.A.	5,724	2.8%
Patt [110]	2003	United States	Aminotransferase	N.A.	1,309	21%
Suzuki [20]	2005	Japan	Aminotransferase	143	1,537	9.3%
Ioannou [111]	2006	United States	Aminotransferase	N.A.	6,823	7.3%

[1]H-MRS: Proton-magnetic resonance spectroscopy; IHTG: Intrahepatic triglyceride; N.A.: Not available.

Table 1.3. Prevalence and risk factors of fibrosis in NAFLD patients.

Author	Year	Country	Total number	Fibrosis	Advanced fibrosis	Risk factor identified
Angulo [57]	1999	United States	144	107 (74%)	39 (27%)	Age ≥50*
						BMI ≥31.1 (Male)/32.5 (Female)*
						Diabetes mellitus*
						AST/ALT ratio > 1*
Garcı´a-Monzo´n [56]	2000	Spain	46	28 (61%)	4 (9%)	Age ≥50
Ratziu [62]	2000	France	93	59 (63%)	28 (30%)	Age ≥50*
						BMI ≥28*
						Necroinflammation present*
Marchesini [61]	2003	Italy	163	111 (68%)	34 (21%)	Metabolic syndrome*
Gramlich [59]	2004	United States	132	50 (38%)	28 (21%)	Hepatocyte ballooning
						Mallory bodies present

Ong [28]	2005	United States	197	N.A.	17 (9%)	Waist-hip ratio*
						Abnormal AST*
						Focal necrosis*
Kleiner [60]	2005	United States	576	225 (39%)	121 (21%)	N.A.
Wong [64]	2009	China	173	113 (65%)	19 (11%)	Age*
						Fasting glucose*
						HOMA-IR*
Wong [63]	2010	China/France	246	176 (72%)	56 (23%)	N.A.
Brunt [58]	2011	United States	934	677 (72%)	215 (23%)	N.A.

BMI, body-mass index; AST, aspartate aminotransferase; ALT, alanine aminotransferase; HOMA-IR: Homeostasis model assessment of insulin resistance; N.A.: Not available. * Risk factors for advanced fibrosis, others for fibrosis.

1.2 Evaluation of non-alcoholic fatty liver disease

The gold standard for diagnosing of NAFLD and NASH is liver biopsy. Several histological evaluation systems can be used for NAFLD staging. However, it is an invasive test. The risk for major complications and sampling bias cannot be avoided. Moreover, it is not very suitable for repeated evaluation. Several non-invasive approaches have been introduced, including different imaging technologies and biomarkers.

1.2.1 Liver biopsy

Traditionally, liver biopsy is the gold standard for the diagnosis and assessment of NAFLD [112]. It is used to determine the degree of hepatic steatosis and exclude other fatty liver disease such as autoimmune hepatitis. It is also used to assess the presence of necroinflammation and hepatocyte injury, which are the key feature of NASH [113]. Fibrosis can also be evaluated and staged [113].

A high quality liver biopsy sample is essential for histological evaluation [112-113]. Adequacy of a liver biopsy sample can be assessed grossly by its length and diameter, as well as the number of portal tracts that can be visualized under microscope [113]. In a consensus meeting of the American Association for the Study of Liver Diseases (AASLD) [112] on the endpoints and clinical trial design for NASH, a needle core biopsy with a 16 or lower gauge needle is recommended. A tissue core at least 2 cm long and containing at least 10 portal tracts is considered to be of good quality.

Several histological evaluation systems are available. The pathologists' global

assessment, which was original described and modified by Matteoni and colleagues, is widely accepted [53 112]. It is well associated with disease severity and long term mortality [52-53]. In this assessment, fatty liver is defined by the presence of > 5% steatosis under light microscopic examination of a hematoxylin and eosin stained liver section. A definite NASH is defined by the presence of 1. > 5% steatosis; 2. hepatocellular ballooning of any grade; and 3. lobular inflammation of any grade. A borderline NASH is defined as disease more than simple steatosis but do not meet the criteria for definite NASH.

A semiquantitative scoring system, NAFLD activity score (NAS) is recommended to quantify disease activity [112]. NAS is the sum of the scoring of steatosis (0-3), lobular inflammation (0-3), and hepatocellular ballooning (0-2). The detailed scoring method is shown in Table 1.4. It is developed by Nonalcoholic Steatohepatitis Clinical Research Network (NASH CRN) in 2005 [60], suggesting that NAS of ≥ 5 correlated with a diagnosis of NASH, and NAS of < 3 correlated with a diagnosis of simple steatosis. However, NASH CRN reevaluated this system in 2011 and found the diagnosis does not correlate well with the cutoffs of NAS [58]. As a result, its role is only limited in clinical studies [114]. The high sensitivity of NAS to disease change made it a reliable tool in repeated biopsy studies [112].

Fibrosis staging described by NASH CRN is widely accepted to assess fibrosis in NAFLD patients (Table 1.4) [60 112]. Stage 3 (bridging fibrosis) and stage 4 (cirrhosis) fibrosis are generally considered as advanced fibrosis.

Liver biopsy is limited by its invasive nature. Although it is generally safe, major

complications, such as significant bleeding, biliary peritonitis and pneumothorax, can still occur. There is an estimated morbidity of 0.06% - 0.35% and mortality of 0.01% - 0.1% [113]. Some patients may refuse to have the procedure because of perceived pain and potential complications. Bleeding tendency and ascites, which are common situations in advanced liver disease, are relative contraindications for liver biopsy. It required in hospital treatment and observation. The cost is also high. In addition, it is also not very suitable for repeated evaluation due to increasing risk of complication.

Sampling bias is another limitation of liver biopsy. A biopsy sample considered adequate for histological assessment is only 1/50,000 to 1/65,000 of the whole liver mass [113]. Merriman et al. reviewed 51 patients who received liver biopsy from both the right and left lobes of liver. Histological results of the two biopsy samples were compared [115]. Agreement for steatosis was excellent, however it was only moderate for fibrosis and fair for lobular inflammation and hepatocyte ballooning. Intra-observer and inter-observer variability also existed.

1.2.2 Non-invasive assessment of NAFLD

Because of the limitations of liver biopsy, non-invasive assessment of NAFLD is urgently needed. An ideal non-invasive assessment should be accurate, easy to perform, reproducible and affordable. Its role, however, should not be confined to detecting steatosis. It is more important to distinguish NASH and fibrosis in NAFLD patients. For this purpose, several non-invasive tests are developed. Their advantages and limitations are discussed in this section.

1.2.2.1 Radiological imaging tests for hepatic steatosis

Ultrasonography (US) is the most common test for evaluating hepatic steatosis in daily clinical practice. It is generally available, easy to perform, radiation free with relatively low cost. For patients with hepatic fatty infiltration > 30% of the hepatocytes, US has over 90% sensitivity and specificity in detecting fatty liver [6]. Typical ultrasonographic features of fatty liver include diffuse increase in fine echoes in liver parenchyma and impaired visualization of intrahepatic vessels and diaphragm. However, changes in US are not associated with the presence of lobular inflammation, hepatocyte ballooning or Mallory-Denk body, which are features of NASH. In other words, it cannot distinguish NASH patients from patients with simple steatosis [6]. Besides, it is insensitive to fibrosis and early cirrhosis [6 116]. Since the severity of disease is associated with necroinflammation and fibrosis rather than the degree of steatosis, the role of US in predicting clinical outcome of NAFLD is limited.

Computed tomography (CT) had similar accuracy in detecting hepatic steatosis with US [6 14 116]. It cannot distinguish NASH either; fibrosis can only be detected in a cirrhotic stage [6]. Furthermore, the radiation exposure to patients limited its wide use for the evaluation of NAFLD.

New modalities of magnetic resonance imaging, including dual gradient echo magnetic resonance imaging (DGE-MRI) and proton magnetic resonance spectroscopy (^{1}H-MRS) largely improve the sensitivity in detecting hepatic steatosis [14]. Both methods have around 80% sensitivity and specificity for the detection of hepatic steatosis as low as 5% [14]. These tests have the ability to quantify hepatic steatosis with good accuracy and also are radiation free. Thus, they are preferred in

recent population based studies for NAFLD screening [15-17]. However, like US and CT, these tests cannot distinguish NASH and mild-moderate fibrosis [6]. They are expensive, not widely available, which limited their utility in clinical practice.[116]

1.2.2.2 Physical measurements for NAFLD related fibrosis

Transient elastography (TE) by Fibroscan (Echosens, Paris, France) is a novel and rapid non-invasive measurement of liver stiffness [117-118]. TE is equipped with a probe consisting of an ultrasonic transducer mounted on the axis of a vibrator. Once the probe is put at an intercostal space overlying the liver, it transmits a vibration of mild amplitude and low frequency to generate an elastic shear wave which propagates through liver parenchyma. In the meantime, the probe generates ultrasound wave to measure the velocity of the sheer wave. The denser the liver tissue, the faster the shear wave travels. Based on this principle, liver stiffness may be estimated [119]. The measurement is quantitative and highly reproducible [120]. Since liver stiffness significantly correlated with fibrosis stage, TE can be used as a non-invasive tool to assess fibrosis and cirrhosis in different liver diseases including NAFLD [63 120-123]. In NAFLD, the Area under Receiver Operating Characteristic curve (AUROC) ranges from 0.78-0.99 for moderate (≥F2) fibrosis and 0.87-1.0 for advanced (F3-4) fibrosis [116].

The main challenge for TE in NAFLD patients is the high prevalence of obesity in this population. Take Hong Kong Chinese as an example, 86% of biopsy-proven NAFLD patients have central obesity [64]. The transmission of shear wave and ultrasound into the liver parenchyma is affected by the thickness of subcutaneous and peri-hepatic fat. As a result, the success rate of liver stiffness measurement (LSM)

decreases in obese subjects. The failure rate of TE is reported to be ranging from 3% to 16% in NAFLD patients [116]. In the study by Wong et al., the successful rate was over 97% in patients with BMI lower than 30 kg/m^2, and dropped dramatically to 75% in patients with BMI of 30 kg/m^2 or higher [63]. To solve this problem, the manufacturer of Fibroscan has recently developed an XL probe specifically for obese subjects. The XL probe uses lower frequency ultrasound and more sensitive ultrasonic transducer to assess deeper liver parenchyma 35-75 mm from the skin surface [124]. It achieves higher success rate of measurement in obese subjects. Although the XL probe generates lower LSM compared with the traditional M probe [125-126], a study which included 193 biopsy-proven NAFLD patients revealed that LSM by XL probe was more likely to be performed successfully in NAFLD patients compared with M probe. AUROCs of XL probe for moderate (≥F2) fibrosis, advanced (≥F3) fibrosis and cirrhosis were 0.80, 0.85, and 0.91, respectively [126].

A real-time tissue elastography or shear wave elastography (SWE) is recently introduced to the non-invasive evaluation of fibrosis in NAFLD [127-128]. This system displays the color-coded elastography image over the B-mode image of selected regions of interest in real time. This enables real-time SWE to obtain measurements based on both anatomical and tissue stiffness information. In a pilot study which enrolled 121 biopsy-proven NAFLD patients, AUROC of real-time SWE for moderate fibrosis was 0.92, which was significantly higher than TE (0.84, $p = 0.002$) [127]. However, these results are still needed to be validated in larger populations; the cutoff values for different fibrosis stage also need to be confirmed.

Acoustic radiation force impulse imaging (ARFI, Virtual Touch Tissue

Quantification, Siemens ACUSON S2000) is a different method, which explores the elastic properties of a region of interest. Liver tissue is mechanically excited using short-duration acoustic pulses. The displacement tissue generated produces a propagating shear wave whose velocity is calculated and which is proportional to tissue elasticity. Friedrich-Rust and colleagues compared the performance of ARFI with TE in NAFLD patients. No significant difference was found in diagnostic accuracies of TE and ARFI imaging for fibrosis staging [129]. In another study, Yoneda et al. also reported similar results [130].

The advantage of ARFI is that it does not have the limitations in obese patients as TE. It is combined with regular ultrasound examination. Non-invasive assessment of fibrosis and surveillance of HCC can be done in the same time. The main limitations of AFRI are that the lack of standardized protocol for measurement; and the lack of precise definitions of ARFI failure [116].

Unlike TE and ARFI, magnetic resonance elastography (MRE) uses magnetic resonance imaging to evaluate the propagation of shear waves in the liver parenchyma. An active driver which is located outside the magnet room generates continuous low frequency vibrations, transmitted via a flexible tube to a transducer placed directly against the anterior right chest wall over the liver to generate shear waves. MRE is not affected by obesity. MRE has the appeal of becoming a one-stop service by combining it with regular magnetic resonance imaging (MRI) for structural examination and proton-magnetic resonance spectroscopy for hepatic steatosis assessment. Interestingly, a study showed that by applying a cut-off value of 2.74kPa, MRE could distinguish histological confirmed NASH form simple steatosis

with a sensitivity of 94% and specificity of 73% [131]. However, the long examination time, high cost and limited availability of facilities may be prohibitive. Besides, more experience is needed for using this technique in NAFLD patients.

1.2.2.3 Clinical tests and biomarkers

The progression from NAFLD and the development of fibrosis is sometimes accompanied by the changes in certain clinical tests such as elevated aminotransferase. However, the association is weak. In order to improve the accuracy for predicting NAFLD, NASH or fibrosis, several biomarkers and clinical prediction scores combining different clinical parameters have been developed. The utility of these clinical tests, biomarkers and their combines in non-invasive evaluation of NAFLD is discussed in this section.

Clinical tests

Aminotransferase, including alanine aminotransferase (ALT), aspartate aminotransferase (AST) and gamma-glutamyltransferase (GGT) are commonly measured in chronic liver disease patients. They also tend to be elevated in NAFLD patients compared with healthy subjects. However, the association is weak. Take ALT for example, it is commonly used to reflect hepatic inflammation and injury. In population studies, high ALT level is associated with long term increased risk of liver-related mortality and cardiovascular mortality [132-133]. Elevated serum ALT sometimes serves as a diagnostic method in population-based studies to estimate NAFLD prevalence [2]. However, serum ALT level can be normal in over half of NAFLD patients [134]. Its association with histological findings is also poor [18]. Even patients with persistently normal ALT may have lobular inflammation and hepatocyte

ballooning. The association between ALT level and fibrosis is also poor. In patients with fibrosis progression towards cirrhosis, serum ALT may even decrease [135]. However, although the correlation of ALT alone and severity of disease is weak, it could be further improved by be combined with other clinical parameters in different clinical prediction score.

Metabolic syndrome and its components are also associated with increased risk of NAFLD. In a large-scale community-based study, Wong showed that each component of the metabolic syndrome increased the risk of NAFLD in a dose-dependent manner [17]. The prevalence of NAFLD in subjects without any component of metabolic syndrome was only 5%. It increased with the number of components and reached to 80% in subjects with all five components. Metabolic syndrome is also associated with the diagnosis of NASH and the presence of fibrosis in NAFLD patients [64]. However, since simple steatosis patients may also have metabolic syndrome or its components, the accuracy of using metabolic syndrome to distinguish between patients with and without NASH or fibrosis is likely to be modest.

Other clinical tests, such as mean platelet volume [136], are also suggested as potential non-invasive tests for NAFLD. However, they still need to be validated.

Biomarkers

Large-scale screening methods, such as proteomics and liquid chromatography-tandem mass spectrometry, were used to identify potential biomarkers for NAFLD and NASH [229]. Several biomarkers have been introduced to

overcome the limited accuracy in predicting NASH of clinical tests (Table 1.5). These biomarkers detect different features of NASH development, including cell apoptosis, cell necrosis, dysregulated adipokines, excessive oxidative stress and systemic inflammation.

Hepatocyte apoptosis is a prominent feature of NASH [105]. Increase in hepatocyte cell apoptosis is typically present in both animal models of NASH and in human NASH patients. Effector caspases (mainly caspase-3) is activated in apoptotic process. Activated caspase-3 will cleave different intracellular substrate including cytokeratin 18 (CK-18), which is the major intracellular filament protein specific to hepatocytes. As a result, the amount of cleaved CK-18 fragment is increased both in the liver and in blood. The serum or plasma CK-18 level can be captured by specific antibody and measured using an immunoassay such as enzyme-linked immunosorbent assay (ELISA). The test is commercially available (M30-Apoptosense ELISA kit, PEVIVA, Bromma, Sweden), reliable with intra- and inter-test variable less than 10%. It is also rapid and easy-performing, which can be done in one working day. In a multicenter validation study which enrolled 44 simple steatosis, 26 borderline NASH and 69 NASH patients, Feldstein et al. showed that plasma CK-18 fragments M30 levels were significantly increased in patients with NASH [137]. CK-18 M30 had high accuracy with an AUROC of 0.83 in diagnosing NASH. By applying different cutoff values from 216 to 287 (U/l), the sensitivity of CK-18 M30 ranges between 65% and 77%, and the specificity ranges between 65% and 92%. The results were validated in different ethnic populations [46 138-142]. In a longitudinal paired liver biopsy study, the change of CK-18 M30 was also found to be correlated with disease progression [46]. Patients with increased NAS 3 years after initial evaluation had greater increase of

CK-18 M30 compared with those have static or decreased NAS.

The plasma level of another biomarker of apoptosis, soluble Fas, is also increased in NASH patients. In a study by Tamimi et al., the AUROC for the diagnosis of NASH was 0.86 for plasma soluble Fas in 95 biopsy-proven NAFLD patients [140]. The AUROC can be further increased to 0.93 when combined with M30. In a validation cohort of 82 obese patients received bariatric surgeries, the AUROC for combined application of soluble Fas and M30 was 0.79.

Recent studies suggest that other cell death markers may also be useful in the prediction of NASH. In addition to apoptosis, necrosis has also been proposed to be responsible for the disease progression in NAFLD patients [143]. Unlike CK-18 M30 (M30), the CK-18 M65 (M65) and CK-18 M65ED (M65ED) ELISA (M65 ELISA kit and M65 EpiDeath ELISA kit, PEVIVA, Bromma, Sweden) measures soluble CK-18 released from dying cells and can be used to assess overall cell death due to apoptosis and necrosis [144]. Both assays are based on two antibodies, M6 and M5, which are directed against two different epitopes of CK-18 and recognize total CK-18. The difference between M65 and M65ED assays is that M65 assay uses the M5 antibody for detection and M6 for capture; while M65ED assay uses these antibodies inversely. A study in USA suggested that the overall diagnostic accuracy of M65 for NASH was higher than that of M30 (AUROC: 0.81 for M65 vs. 0.71 for M30) [141]; in another study in Turkey, the performance was similar (AUROC: 0.81 for M65 vs. 0.78 for M30) [142]. Recently, Joka and colleagues suggested that M65/M65ED may have superior performance to M30 in detecting mild fibrosis and steatosis [139]. However, that study was limited by the inclusion of different liver

diseases and the small number of NAFLD patients [145]. As such, the performance of M65 and M65ED in detecting NASH as compared to M30 is still unclear. The clinical significance and test performance of M65/M65ED warrant further validation.

Adipokines are cytokines secreted by adipocytes. They have important roles in regulating metabolism and insulin resistance, contributing to chronic inflammation associated with the metabolic syndrome [146]. Most of the recognized adipokines are pro-inflammatory, while some of them are anti-inflammatory.

Adiponectin is one of the few adipokines that has anti-inflammatory effects. In a meta-analysis pooling 27 studies including totally 2243 subjects (698 control subjects and 1545 patients with NAFLD), blood adiponectin level was significantly lower in NASH patients compared with these non-NASH NAFLD patients or control subjects [147]. The blood level of adiponectin in control subjects and non-NASH NAFLD patients had no significant difference. Its level is also decreased in obese patients with diabetes and metabolic syndrome [148]. In a study including 80 NASH and 29 simple steatosis patients, Hui and colleagues showed adiponectin combined with HOMA-IR had an AUROC of 0.79 in differentiating NASH [149].

On the other hand, pro-inflammatory cytokines such as tumor necrosis factor-alpha and interleukin-6 are increased in NASH patients and yielding modest diagnostic accuracy [64]. Adipocyte fatty acid binding protein (AFABP) is involved in the interaction between adipocytes and macrophages, which leads to inflammation and insulin resistance [150]. In the study by Milner, serum AFABP was significantly higher in 69 NASH patients than in 31 simple steatosis patients and controls. It also

correlated with ballooning, lobular inflammation and fibrosis stage [150]. However, the authors did not perform standard c statistics to evaluate the diagnostic accuracy of AFABP.

NASH is characterized by heightened oxidative stress in the liver, which is the basis for the evaluation of anti-oxidant therapy such as vitamin E [151]. Fibroblast growth factor 21 (FGF21) is a hormone which regulates lipid oxidation in the liver and stimulates glucose uptake in the adipose tissue [152]. It is also termed as a "mitokine" due to its regulation by mitochondrial dysfunction and strong effect on increasing lipid oxidation and browning of white adipose tissue [153]. Li et al. found that in human liver, FGF21 mRNA expression level increased with steatosis grade; its serum level was significantly higher in 159 Chinese NAFLD patients compared with 553 healthy controls [154-155]. Similar results were reported by Yilmaz et al. [156]. However, the correlation was not found in children [157]. In a Japanese study of 57 patients, serum thioredoxin, a stress-inducible thiol-containing protein, was significantly increased in NASH patients [158]. The test had an AUROC of 0.79 in diagnosing NASH. Other biomarkers of oxidative stress including copper-to-zinc superoxide dismutase, glutathione peroxidase and vitamin E level have also been evaluated with conflicting results.

C-reactive protein is a non-specific inflammatory marker that is increased in various conditions such as bacterial infection and coronary artery disease. While it (particularly high specificity C-reactive protein) has been shown to increase in NASH patients (several but not all studies), the diagnostic accuracy is modest and its use is limited by the non-specific nature.

1.2.2.4 Prediction scores

In order to improve the predictive value of single tests, efforts are made to combine these tests and generate an algorithm for a clinical prediction score. Most of these procedures are based on multivariable logistic regression. Independent risk factors for the end point of interest will be used to generate the algorithm following the formula which is estimated by regression model. The main clinical prediction scores are summarized in Table 1.6 [159-171].

Among the prediction scores designed for NASH diagnosis, the NAFIC score has the highest overall accuracy [171]. It was generated from 177 biopsy-proven NAFLD patients and was further validated in 442 biopsy-proven NAFLD patients in Japan. It is a weighted sum of serum ferritin, fasting insulin and type IV collagen 7S level by gender specific cutoffs. The AUROC for predicting NASH was 0.85 in the training cohort and 0.78 in the validation cohort. However, further validations in other independent cohorts are still needed before the NAFIC score, together with other scores designed for NASH prediction, can be used in clinical practice.

The Fatty Liver Index (FLI) is a simple score comprising BMI, waist circumference, triglycerides, GGT [161]. It is designed for the prediction of fatty liver, with an AUROC of 0.84. FLI varies between 0 and 100. A FLI < 30 rules out fatty liver with a negative likelihood ratio of 0.2; while a FLI ≥60 predicts fatty liver with a positive likelihood ratio of 4.3. All parameters are routinely checked in clinical practice. Recently, the accuracy of FLI was validated in a large cohort with 2,652 subjects with an AUROC of 0.80 [172]. However, both study used ultrasonography as the

reference standard. In another study which determined fatty liver by ^{1}H-MRS in 220 diabetes patients, the AUROC of FLI was only 0.65 [173]. Therefore, its clinical usefulness is limited.

NashTest is a commercially available test comprising 13 parameters: age, gender, height, weight, and serum levels of triglycerides, cholesterol, alpha2-macroglobulin, apolipoprotein A1, haptoglobin, GGT, ALT, AST and total bilirubin. In a validation study led by the manufacturer, NashTest had an AUROC of 0.79 in diagnosing NASH [168].

The NAFLD fibrosis score was developed and validated in 733 NAFLD patients from USA, Europe and Australia, which is the largest cohort for fibrosis prediction so far. It is comprised of 6 simple clinical parameters: age, hyperglycemia, BMI, platelet, albumin, and AST/ALT ratio [160]. By applying different cutoffs (low: -1.455; high: 0.676), NAFLD fibrosis score has a negative predictive value (NPV) of 88% or a positive predictive value (PPV) of 82% in excluding or diagnosing advanced fibrosis. The negative predictive value of the low cutoff remains high at 91% when it is applied to the Chinese population; however, few Chinese patients have scores above the high cutoff value [174]. This may be because Asians are generally less obese than Caucasians. Further validation in different ethnic groups using ethnic-specific definitions for anthropometry is still needed.

Enhanced Liver Fibrosis panel (ELF) was validated in 196 European NAFLD patients for fibrosis predicting [164]. The ELF panel had AUROC of 0.90 for predicting severe (≥F3) fibrosis, 0.82 for moderate (≥F2) fibrosis and 0.76 for any fibrosis. By

adding simple clinical parameters, including age, BMI, diabetes or impaired fasting glucose, AST/ALT ratio, platelets, and albumin to the panel, the AUROC could be further improved to 0.98, 0.93, and 0.84 for distinguishing severe fibrosis, moderate fibrosis, and any fibrosis, respectively. Authors suggested at least 82% of liver biopsies for evaluating fibrosis in NAFLD patients could be spared by applying ELF.

FibroTest is a commercially available test panel comprising 5 parameters: α2-macroglobulin, apolipoprotein A1 (ApoA1), haptoglobin, total bilirubin and GGT. It was originally designed to estimate liver fibrosis in patients with chronic hepatitis C. When FibroTest was applied to 170 NAFLD patients, the AUROC for detecting F2-4 disease and F3-4 disease were 0.75 and 0.81, respectively [169]. Although these are biomarkers of liver fibrosis, its performance may be affected by other parameters. For example, intravascular hemolysis results in low haptoglobin level and raised bilirubin. GGT is also sensitive to recent alcohol consumption.

In three separate validation studies performing head-to-head comparison in NAFLD subjects from the United States (n=541), United Kingdom (n=145) and France/Hong Kong (n=246), FIB-4 index appeared to have the highest AUROC among all tested clinical prediction scores including AST/ALT ratio, APRI, BARD, FIB-4 and NAFLD fibrosis scores [63 166 175]. FIB-4 index is a formula comprised of 4 clinical parameters including age, platelet, AST and ALT. In all studies, the AUROCs of FIB-4 index for F3-4 fibrosis were all ≥0.80.

Among all these prediction scores, AST/ALT ratio is the simplest one first designed to predict fibrosis and cirrhosis in patients with chronic hepatitis C [176]. However, the

performance of AST/ALT score differed significantly in different validation studies in NAFLD patients. The accuracy in detecting advanced fibrosis was 83%, 74% and 66% in the head-to-head comparison studies mentioned above [63 166 175].

In summary, non-invasive imaging methods are mainly used to determine the grade of steatosis; accuracy in evaluating inflammation and fibrosis is poor. Physical measurements correlated well with fibrosis; however, they are limited by its availability. Clinical tests and biomarkers, together with those prediction scores, provided potential options in distinguishing NASH and fibrosis, however, most of them still need to be validated in large independent cohort. Furthermore, the effort in searching non-invasive tests is merely searching for surrogates for liver biopsy; however, the most important issue is the ability to predict whether a patient will develop hepatic complications in the future. As such, further studies are required to evaluate the performance of these non-invasive tests in predicting clinical events.

Table 1.4. NAFLD activity score and fibrosis staging system by Nonalcoholic Steatohepatitis Clinical Research Network.

Item	Definition	Score
Steatosis grade	<5%	0
	5%-33%	1
	>33%-66%	2
	>66%	3
Lobular inflammation	No foci	0
	<2 foci per 200X field	1
	2-4 foci per 201X field	2
	>4 foci per 202X field	3
Ballooning	None	0
	Few balloon cells	1
	Many cells/prominent ballooning	2
Fibrosis	None	0
	Perisinusoidal or periportal	1
	Mild, zone 3, perisinusoidal	1A
	Moderate, zone 3, perisinusoidal	1B
	Portal/periportal	1C
	Perisinusoidal and portal/periportal	2
	Bridging fibrosis	3
	Cirrhosis	4

Table 1.5. Biomarkers of NASH.

Mechanism	Biomarkers
Apoptosis	Cytokeratin-18 fragments
	Soluble Fas
Necrosis	Intact cytokeratin-18
Adipokines	Adiponectin
	Tumor necrosis factor-alpha
	Interleukin-6
	Adipocyte fatty acid-binding protein
Oxidative stress	Fibroblast growth factor 21
	Thioredoxin
	Copper-to-zinc superoxide dismutase
	Glutathione peroxidase
	Vitamin E
Systemic inflammation	C-reactive protein

Table 1.6. Clinical prediction scores for NAFLD, NASH diagnosis and fibrosis staging.

Purpose	Marker	Parameters	Endpoint	AUROC
NAFLD	Fatty liver index (FLI)	BMI, waist circumference, triglycerides, GGT	Fatty liver	0.84
NASH	Nash Tes	Age, gender, BMI, triglycerides, cholesterol, α-2-macroglobulin, GGT, AST, ALT, haptoglobin, apolipoprotein A1, total bilirubin	NAS ≥5	0.79
	Palekar index	Age ≥50 yrs, female gender, AST ≥45 U/L, AST/ALT ratio ≥0.8, BMI ≥30 Kg/m2 , hyaluronate ≥55 ug/l	NASH	0.76
	Shimada index	Serum adiponectin, HOMA-IR, serum type IV collagen 7S level	NAS ≥5	N.A.
	NAFIC score	Ferritin ≥200 ng/ml (female) or ≥300 ng/ml	NAS ≥5	0.85

		(male), fasting insulin ≥10.0 l U/ml, and type IV collagen 7S ≥5.0 ng/ml		
	NASH Clinical score for morbid obesity	Hypertension, diabetes, AST ≥27 IU/L, ALT ≥27 IU/L, obstructive sleep apnea and nonblack race	NAS ≥5	0.8
Fibrosis	FibroTest	α2-macroglobulin, GGT, apolipoprotein A1, haptoglobin, total bilirubin, age, gender	F ≥2	0.81
			F ≥3	0.88
	NAFLD fibrosis score	Age, fasting glucose , BMI, platelet count, albumin, AST/ALT ratio	F ≥3	0.82
	ELF	Age, hyaluronate, MMP-3, TIMP-1	F ≥1	0.76
			F ≥2	0.82
			F ≥3	0.9
	FibroMeter for NAFLD	Age, weight, platelet count, ferritin, glucose, AST,	F ≥2	0.94

	ALT		
BARD score	BMI ≥28, AST/ALT ≥0.82, diabetes	F ≥2	0.81
FIB-4	Age, AST, ALT, platelet count	F ≥2	0.74
		F ≥3	0.86
		F4	0.86
Hepascore	Age, gender, total bilirubin, GGT,	F ≥2	0.73
	α2-macroglobulin, hyaluronic acid	F ≥3	0.81
		F4	0.91
APRI	AST, platelet count	F ≥2	0.73
		F ≥3	0.79
		F4	0.75
AST/ALT ratio	AST, ALT	F ≥3	0.83

1.3 Genetic determinants of NAFLD

Although obesity and metabolic disorder are the major risk factor for NAFLD, it is not the only mechanism. As mentioned before, ethnicity also affects the prevalence of NAFLD [7-8 10-11 15-17]. The high prevalence in Hispanics and Indian Asians of NAFLD cannot be fully explained by ethnic differences in BMI or insulin resistance. Furthermore, NAFLD tends to cluster in families. Struben et al reported 8 index NASH or cryptogenic cirrhosis patients and 10 relatives with similar diseases in 8 kindreds [177]. Patterns of afflicted patients included mother-daughter, sister-sister, sister-brother, father-daughter, and male-female cousins, and these disorders were not associated with obesity or diabetes. Moreover, fatty liver was found to be more common in siblings and parents of children with NAFLD compared with overweight children without NAFLD [178]. The heritability of NAFLD was estimated to be 39% after adjusting for age, sex, race and BMI. All these data suggest that genetic determinants may play an important role in NAFLD development.

With the advances in genome analysis, genetic determinants of NAFLD are widely studied in the recent years. Genome-wide association study (GWAS) is a high-throughput genotyping technology, which scan genome-wide known single-nucleotide polymorphism (SNP) genetic markers in a case-control setting [179]. By comparing the different allele frequency between NAFLD patients and healthy controls, a number of potential genetic determinants have been introduced based on

GWAS (Table 1.7) [77 97 180-183].

1.3.1 Patatin-like phospholipase domain containing 3 (*PNPLA3*) rs738409

The nonsynonymous rs738409 I148M (C/G) variant located in human patatin-like phospholipase domain containing 3 gene (*PNPLA3*) is the first identified genetic variant associated with higher prevalence of NAFLD in over 2000 participants in the Dallas Heart Study [97]. NAFLD was diagnosed by ^{1}H-MRS. *PNPLA3* rs738409 was significantly associated with hepatic fat content after adjustment for BMI, diabetes status, alcohol use and ethnicity. Hepatic fat content was more than twofold higher in GG homozygotes than in CC homozygotes. Furthermore, Hispanics had larger proportion of risk allele carriers (49%) than African Americans (17%) and European Americans (23%), which could partly explain the higher risk of NAFLD in Hispanics [15].

The findings were subsequently confirmed by other GWAS [183-184]. Speliotes et al. reported *PNPLA3* rs738409 was associated with hepatic steatosis evaluated by CT in 7,176 subjects from United States[183]. Meanwhile, Kawaguchi and colleagues showed similar results in 529 histologically diagnosed NAFLD patients and 932 population controls from Japan [184]. It is further validated in many independent cohort studies from different countries and ethnicities [185-194]

As the association between *PNPLA3* rs738409 and hepatic steatosis is robustly

validated and widely accepted, whether it is associated with the histological severity seems to be a little controversial. The Japanese GWAS study which rediscover *PNPLA3* rs738409 had a subgroup of histological confirmed NAFLD patients, which allowed us to explore the association of *PNPLA3* rs738409 and NASH diagnosis as well as fibrosis [184]. In 529 histologically diagnosed NAFLD patients, *PNPLA3* rs738409 exhibited the strong association with the histological classifications proposed by Matteoni [53]. The distribution of *PNPLA3* rs738409 genotype was significantly different between patients with NASH and other NAFLD patients. Moreover, *PNPLA3* rs738409 also showed strong association with hyaluronic acid, HbA_{1c} and iron deposition in the liver in 3 independent clinical trials, which may have prognostic effect on NAFLD patients.

However, in another GWAS conducted in 236 biopsy-proven NAFLD patients, *PNPLA3* rs738409 did not exhibit any association with histological severity [182]. The authors strictly selected non-Hispanic white female NAFLD patients as study subjects. Farnesyl diphosphate farnesyl transferase 1(*FDFT1*) rs2645424 was found to be associated with NAS, which indicted overall disease severity; 4 other SNPs were associated with lobular inflammation or fibrosis. *PNPLA3* rs738409 was not associated with any of them. Authors attributed it to their relative small sample size and highly selected study subjects.

Other than GWAS, more cross-sectional studies which focused on single SNP of

PNPLA3 rs738409 have been reported. A number of them included histological confirmed NAFLD patients. Sookoian et al. first demonstrated the significant association of *PNPLA3* rs738409 with NASH diagnosis. 12 in 40 (30%) simple steatosis patients were GG homozygotes, while 33 of 63 (52%) NASH patients were GG homozygotes [191]. The association of *PNPLA3* rs738409 with fibrosis was not reported. In another larger study including 574 NAFLD patients received liver biopsy in Italy and United Kingdom [193], *PNPLA3* rs738409 was strongly associated with NASH (Odds ratio [OR]: 1.5, 95% confidence interval [CI]: 1.12-2.04) and moderate fibrosis (OR: 1.5, 95% CI: 1.09-2.12). The GG homozygotes also had significantly higher blood ALT, low-density lipoprotein (LDL), fasting insulin level, HOMA-IR and lower blood high-density lipoprotein (HDL) level compared with CC homozygotes. The study with the largest validation cohort was reported by Rotman et al., who evaluated 894 adult patients from NASH Clinical Research Network (NASH CRN) [190]. *PNPLA3* rs738409 was found to be strongly associated with steatosis, portal inflammation, lobular inflammation, Mallory-Denk bodies, NAS and fibrosis. GG homozygotes had a mean NAS of 4.5, which was significantly higher than CC homozygotes (4.1). GG homozygotes had a mean fibrosis stage of 4.5, which was also significantly higher than CC homozygotes (4.0). Each G allele had an adjusted OR of 1.5 for predicting advanced fibrosis, translating to 2.3 times of risk in GG homozygotes compared with CC homozygotes. Hotta et al. studied 253 patients with NAFLD, 189 of which had NASH [185]. Their data showed *PNPLA3* rs738409 was significantly associated with NASH diagnosis in NAFLD patients

even adjusting for age, gender and BMI. It was also associated with histological fibrosis stage in a regression model.

One study consisting of 678 NAFLD patients also from NASH CRN demonstrated *PNPLA3* rs738409 was associated with lobular inflammation and fibrosis. However, no association was found between *PNPLA3* rs738409 and grade of steatosis, ballooning or the diagnosis of NASH [192]. Interestingly, in the study jointly conducted in Italy and United Kingdom, the associated between *PNPLA3* rs738409 and NASH diagnosis could not be replicated in the Italian cohort alone [193].

A meta-analysis pooling 16 studies was performed for better understanding the association between *PNPLA3* rs738409 and histological severity of NAFLD [98]. It included both studies about adult and pediatric NAFLD. Table 1.8 listed the adult NAFLD studies enrolled in the meta-analysis. Histological data from over 2,000 patients were available for evaluating. The results showed that *PNPLA3* rs738409 had a strong association with a more aggressive disease. GG homozygotes had 3.5-fold greater risk of NASH; 3.3-fold greater risk of fibrosis; and 28% increase in serum ALT levels.

After the validation of the robust association between *PNPLA3* rs738409 and NAFLD, several studies have been conducted to elucidate the underlying mechanism. Two studies from Hoekstra and Huang [195-196] both demonstrated *PNPLA3* expression

in the liver of fasting mice is low, and the expression can increase significantly up to 90-fold with carbohydrate intake. The expression level of *PNPLA3* is correlated with genes associated with the lipogenesis [195]. Huang[196] further found the increased expression required the involvement of transcription factor sterol regulatory element binding protein 1c (*SREBP-1c*), which also promoted fatty acid synthesis.

Using purified and characterized recombinant human *PNPLA3* protein, Huang et al also found *PNPLA3* exhibited a strong hydrolytic activity against triacylglycerol (TAG), diacylglycerol (DAG), and monoacylglycerol (MAG). However, mutant *PNPLA3* protein with substitution of methionine for isoleucine at position 148, which was caused by the G allele in rs730409, significantly attenuated the hydrolytic activity [197]. These findings suggest the loss of function of *PNPLA3* contributes to the development of NAFLD in rs738409 GG homozygotes and CG heterozygotes.

Furthermore, Li and colleagues [198] generated liver transgenic mice overexpressing either wild-type *PNPLA3* or mutant *PNPLA3* to study their different function in lipid metabolism. Liver-specific expression of mutant *PNPLA3* caused increased formation and impaired hydrolysis of liver TAG, as well as depletion of TAG long-chain polyunsaturated fatty acids, compared with wild-type *PNPLA3*.Their findings indicate that *PNPLA3* plays an important role in remodeling TAG which is disrupted by the isoleucine to methionine changing in rs738409 GG homozygotes and CG heterozygotes. This disruption further leads to hepatic steatosis

development.

1.3.2 Other genetic determinants

Other than *PNPLA3* rs738409, several SNPs on different genes are also suggested to be genetic determinants for NAFLD and NASH. However, the associations are still in need of further validation by independent studies. Two SNPs (rs2854116 T-C and rs2854117 C-T) in apolipoprotein C3 (*APOC3*) were also found to be associated with NAFLD by Petersen et al. [181]. 76 among 95 Asian Indian men without known liver disease were found to be carriers of above variant *APOC3* alleles, 29 (38%) of them had NAFLD. None of the 19 (0%) subjects with wild-type homozygotes had NASH ($p < 0.001$). The association was also seen in a validation cohort including 163 non-Asian Indian men. NAFLD was found in 11 of 124 (9%) carriers of variant alleles and none of 39 (0%) wild-type homozygotes ($p = 0.02$). The carriers of variants also had increased blood triglyceride level and decreased triglyceride clearance. Fasting plasma *APOC3* level were elevated in variants carriers, which was found to increase the sensibility of high fat diet induced NAFLD in an animal model [199].

However, subsequent studies in different ethnic groups have failed to confirm the association of *APOC3* variants with NAFLD. Kozlitina et al. genotyped the two variants in 228 African Americans, 843 European Americans and 426 Hispanics (total 1,497 subjects) from the Dallas Heart Study [200]. No significant difference in

hepatic fat content was observed between carriers and others. The authors further genotyped 4,399 lean individuals from the Atherosclerosis Risk in Communities Study; no association was observed either. The lack of association was also reported in 253 French diabetes patients[201], 585 obese Italians [202], and 417 Finnish subjects as well [203]. Moreover, in 437 biopsy-proven NAFLD patients from Italy and 321 patients from United Kingdom, the two variants were not associated with histological severity [204].

The discordance results may be attributed to different ethnic groups and anthropometry profiles. Large studies across multiple ethnic populations, or larger studies in the Asian Indian population is needed for further validation of the association between *APOC3* variants and NAFLD.

In the GWAS reported by Speliotes et al. [183], Neurocan (*NCAN*) rs2228603 (C-T) and Glycogen binding subunit of protein phosphatase 1 (*PPP1R3B*) rs4240624 (A-G) were associated with NAFLD defined by CT in 7,176 individuals from 4 separated studies. *NCAN* rs2228603, Glucokinase regulatory protein (*GCKR*) rs780094 (C-T) and Lysophospholipase-like 1 (*LYPLAL1*) rs12137855 (C-T) were associated with histological NAFLD in 592 biopsy-proven NAFLD from the NASH CRN and 1,405 healthy controls from the Myocardial Genetics Consortium. In subsequent studies including 4,808 non-Hispanic subjects from United States, *PPP1R3B* rs4240624 and *NCAN* rs2228603 were found to be associated with NAFLD defined by ultrasound;

while *GCKR* rs780094 was associated with NAFLD with high serum ALT level [205]. The association of *GCKR* rs780094 and NAFLD was also found in 903 Chinese subjects [206]. In another GWAS study recently reported by Adams et al. [180], group-specific component (*GC*) rs222054 (G-C) and lymphocyte cytosolic protein-1 (*LCP1*) rs7324845 (G-A) were found to be associated with ultrasound defined NAFLD in 928 Australian adolescents. Further studies are needed to further validate their association with NAFLD and histological based studies are needed to determine their association with NASH or fibrosis.

Chalasani et al. reported the only GWAS study based on histological proven NAFLD for identifying SNPs which were associated with histological severity [182]. 236 non-Hispanic white women with NAFLD were enrolled. After adjusting for certain confounders such age, BMI, et al., the SNP rs2645424 on farnesyl diphosphate farnesyl transferase 1 (*FDFT1*) was the only SNP which was found to be associated with NAS. This study was limited to relatively small sample size and highly selected study subjects. The findings should be validated in larger and more diverse cohorts.

In summary, *PNPLA3* rs738409 is the only genetic determinant which is associated with both NAFLD and NASH. The association is validated in several independent studies including a large meta-analysis. Its functional role in promoting NAFLD is also partially revealed. Other potential genetic determinants are still lack of well validation. More studies are needed to identify the genetic variants which are

associated with NASH and fibrosis. In the future, whether these genetic determinants could predict disease progression should be studied. Since all GWAS and validation studied are performed in the recent several years, follow-up studies can be expected in 5-10 years.

Table 1.7. Genetic determinants for NAFLD and NASH.

Gene	Protein	Allele variant	Effective allele	OR for NAFLD	Association with NASH
PNPLA3	Patatin-like phospholipase domain-containing protein 3	rs738409	G	3.26	Yes
PPP1R3B	Glycogen binding subunit of protein phosphatase 1	rs4240624	A	0.93	N.A.
NCAN	Neurocan	rs2228603	T	1.65	N.A.
GCKR	Glucokinase regulatory protein	rs780094	T	1.45	N.A.
LYPLAL1	Lysophospholipase-like 1	rs12137855	C	1.37	N.A.
APOC3	Apolipoprotein C3	rs2854116	C	N.A.	N.A.
		rs2854117	T	N.A.	N.A.
GC	Group-specific component	rs222054	C	2.54	N.A.
LCP1	Lymphocyte cytosolic protein-1	rs7324845	A	3.29	N.A.

FDFT1	Farnesyl diphosphate farnesyl transferase 1	rs2645424	A	N.A.	Yes

N.A.; Not available.

Table 1.8. Studies on the association between the *PNPLA3* rs738409 and adult fatty liver disease listed in a meta-analysis.

Author	Year	Country	Population/Hospital based	Endpoint	Total Number	Histology (n)
Romeo [97]	2008	United States	Population	Steatosis by MRS	2,240	No
Sookoian [191]	2009	Argentina	Hospital	Histology	266	103
Kantartzis [186]	2009	Germany	Hospital	Steatosis by MRS	330	No
Kotronen [187]	2009	Finland	Hospital	Steatosis by MRS	291	No
Rotman [190]	2010	United States	Mixed	Histology	894	894
Speliotes [192]	2010	United States	Mixed	Histology	2,128	678
Petit [207]	2010	France	Hospital	Steatosis by MRS	218	No
Hotta [185]	2010	Japan	Hospital	Histology	831	253
Romeo [189]	2010	Italy	Hospital	Steatosis	678	No
Valenti [208]	2011	Italy/United Kingdom	Mixed	Histology	753	574
Wagenknecht [194]	2011	United States	Population	Steatosis by CT	1,214	No

1.4 Management of non-alcoholic fatty liver disease

As metabolic disorders are frequently associated with NAFLD, the management of patients with NAFLD should not be limited to treating liver disease. The accompanied disorders such as obesity, dislipidemia and diabetes should be treated equally. Multiple modalities including lifestyle intervention, medical treatment as well as bariatric surgery are available for the management of NAFLD as well as the co-morbidities. The treatment combination should be decided based on thorough evaluation in a most cost-effective way. Since most patients with simple steatosis follow a benign clinical course, treatments aimed at improving liver disease should only be conducted in NASH patients.

1.4.1 Lifestyle intervention

Lifestyle intervention or lifestyle modification aims to reduce body weight through consuming a reduced calorie diet and increasing physical activity [209]. Consumption of vegetables, fruits and whole grains is encouraged. Main dietary options include very low calorie diets, meal replacements, portion-controlled servings of conventional foods, low-carbohydrate diets and low gylcemic index diets. Increasing physical activity can be achieved with programmed or lifestyle activity. Programmed activity is exercise planned and completed in a discrete period of time at a relatively high-intensity level, such as swimming or jogging for half an hour. Lifestyle activity involves increasing energy expenditure throughout the daily life without concern for

the intensity of the activity, such as climbing stairs instead of taking an elevator. 10% reduction of initial body weight in obese individuals is recommended by World Health Organization and the National Institutes of Health [209].

Lifestyle intervention may reduce aminotransferases and improve hepatic steatosis [3]. In a multicenter clinical trial enrolled 96 overweight or obese diabetic subjects, lifestyle intervention group (n =46) achieved more body weight loss compared with diabetes support and education group (N =50) in a 12-months interval (8.5% vs. 0.05%, $p < 0.01$). Greater decline in hepatic steatosis measured by ^{1}H-MRS (50.8% vs. 22.8%, $p = 0.04$) was also observed in lifestyle intervention group [210]. Similar results are reported by many other independent studies [3 211-214]. An average reduction in hepatic steatosis of about 40% (ranging from 20-81%) was achieved with a body weight loss between 5-10% in these studies [3].

A few studies demonstrate that lifestyle intervention improves histological severity of NAFLD [215-216]. In a randomized controlled study with 31 biopsy-proven NASH patients, 21 were assigned to lifestyle intervention group and 10 were assigned to education group. After 48 weeks, 72% patients in lifestyle intervention group had improvements in NAS, compared with only 30% in the education group ($p = 0.03$). These improvements were in parallel with higher body weight loss (9.3% vs. 0.2%). Moreover, in patients who had body weight loss more than 7%, significant improvements in steatosis, lobular inflammation, ballooning and NAS were observed

compared with the others [216].

1.4.2 Medication

A number of pharmacological agents have been tested for the treatment of NASH. Among them, pioglitazone, vitamin E and omega-3 fatty acids hold some promise [3].

Pioglitazone is an insulin sensitizing agent. In a large multicenter randomized control trial, Pioglitazone or Vitamin E for NASH Study (PIVENS), 80 non-diabetic biopsy-proven NASH patients were assigned in pioglitazone (30 mg/day) group while 83 patients were assigned in placebo group [217]. 47% patients in pioglitazone group had NASH resolution after 96 weeks compared with 21% in placebo group (p = 0.001). No significant different in changes of fibrosis were observed between these two groups. However, a recent meta-analysis pooled 4 randomized control trials showed pioglitazone (n = 137) may improve fibrosis compared with placebo (n = 134) (OR 1.68; 95% CI, 1.02-2.77) [218]. The main side effect of pioglitazone is weight gain and possibly increasing long term risk of cardiovascular disease [3].

Vitamin E is an anti-oxidative agent. In the PIVENS trial, vitamin E was given at a dose of 800 IU daily in 84 subjects for 96 weeks. Primary endpoint (an improvement in NAS to less than 3 or at least by 2 points; with at least 1 point improvement in ballooning, and 1-point improvement in either the lobular inflammation or steatosis score; and no increase in the fibrosis score) and key secondary endpoints

(improvements in overall NAS and individual component scores) were all achieved in a significant great portion in vitamin E group compared with placebo group. No beneficial on fibrosis improvement was observed either [217]. The joint guideline by American Gastroenterological Association, American Association for the Study of Liver Diseases, and American College of Gastroenterology recommended vitamin E administered at daily dose of 800 IU/day as a first-line pharmacotherapy for non-diabetic biopsy-proven NASH patients [3]. The main side effects are potential increased risk in over-all mortality and prostate cancer [3].

Omega-3 fatty acids are polyunsaturated fatty acids. A meta-analysis pooled 9 studies including 4 randomized control trials revealed omega-3 acids may decrease hepatic steatosis, however, the optimal dose is currently not known [219]. Although it is premature to recommend omega-3 fatty acids for the specific treatment of NAFLD or NASH but they may be considered as the first line agents to treat hypertriglyceridemia in patients with NAFLD [3].

It should be noted that most subjects who participate in the above mentioned clinical trials are without diabetes. Administration of vitamin E in diabetic NASH patients is not recommended currently.

1.4.3 Bariatric surgery

Bariatric surgery such as gastric banding, bilio-intestinal bypass and gastric bypass is

considered in morbidly obese subjects. A 60-80% excess weight loss (defined as the excess weight over an optimal body weight, eg. a body weight for BMI =25) in the first year can be expected in patients who have received gastric bypass, while 50%–60% excess weight loss with long term stabilization can be expected [220]. The prevalence of NASH can be as high as 98% in patients received bariatric surgery [25]. Thus, in these patients, bariatric surgery may be a potential treatment of NASH. In a study of 99 patients with borderline or definite NASH undergoing bariatric surgery, a significant improvement in liver histology was observed 1 year after surgery and was still exist after 5 years after surgery [221]. There is no study comparing the influence of different types of bariatric surgery on the treatment of NASH yet.

Chapter 2: Study design

2.1 Aims of the study

2.1.1 Biomarkers for non-invasive evaluation of NAFLD and NASH

As mentioned in Chapter 1, the gold standard for diagnosing of NAFLD and NASH is liver biopsy. However, it is an invasive test with potential risks such as significant bleeding, biliary peritonitis and pneumothorax [113]. Some patients may refuse to have the procedure because of perceived pain and potential complications. Bleeding tendency and ascites, which are common situations in advanced liver disease, are relative contraindications for liver biopsy. It requires in-hospital treatment and observation and is therefore costly. It is also not very suitable for repeated evaluation due to increasing risk of complications. Besides, a typical biopsy sample is only 1/50,000 to 1/65,000 of the whole liver mass [113]. Discordance can exist between right or left lobe in the same patient [115]. Thus, sampling bias cannot be avoided in liver biopsy. Reliable non-invasive tests for NAFLD and NASH are urgently needed.

Currently, steatosis and fibrosis can be determined by non-invasive imaging or physical methods such as proton-magnetic resonance spectroscopy (^{1}H-MRS) and transient elastography (TE). However, the correlation between these tests and NASH diagnosis is weak. A number of biomarkers have been developed in this purpose such as cytokeratin-18 (CK-18), adipocyte fatty acid binding protein (AFABP) and

fibroblast growth factor 21 (FGF21). Among them, CK-18 is a group of cell death markers for both NAFLD and NASH diagnosis which has been validated in several independent studies [137-140 142]. However, it has not been evaluated in a Chinese cohort. The diagnostic thresholds in Chinese population are also unknown. Meanwhile, AFABP and FGF21 are found to be elevated in NAFLD patients compared with healthy controls [150 154]. Their close relationship with insulin resistance or oxidative stress makes them promising biomarkers for NASH. However, it is still not clear whether they could distinguish NASH from non-NASH NAFLD patients.

The aim of the first part of this study was to evaluate the performance of blood CK-18, AFABP and FGF21 as biomarkers for the diagnosis of NAFLD and NASH. Whether combination of these biomarkers could improve the diagnostic accuracy than individual biomarkers was also tested in this part.

2.1.2 Genetic marker *PNPLA3* rs738409, dietary pattern, lifestyle intervention and NAFLD

The genetic determinants play an important role in NAFLD development. With the advances in genome analysis, especially genome-wide association study (GWAS), genetic determinants of NAFLD are widely studied in recent years. The nonsynonymous rs738409 I148M (C/G) variant located in human patatin-like phospholipase domain containing 3 gene (*PNPLA3*) is the first identified genetic variant associated with higher prevalence of NAFLD in over 2,000 participants in the

Dallas Heart Study [97]. The association between *PNPLA3* rs738409 polymorphism and the development of NAFLD, as well as the disease severity, is validated in several independent cohorts [98]. However, many validation studies included study subjects from hospital clinics, who might have heavy metabolic burden and have been treated with various methods. The impact of PNPLA3 at the population level is uncertain.

Meanwhile, the dietary pattern, including the quantity and composition of food, is also associated with the development of NAFLD. The changes of dietary pattern is also thought to be the major reason of the increased global prevalence of NAFLD over the past decades [77]. *PNPLA3* rs738409 polymorphism affects lipid metabolism, however, it is unclear if the gene variant may indirectly affect the dietary pattern and thereby contribute to the development of NAFLD. The interaction between *PNPLA3* genotypes and dietary pattern has also not been adequately evaluated [222-223].

Another unsolved question is whether *PNPLA3* gene polymorphism may affect the response to lifestyle or pharmacological intervention. In a small study of 18 subjects (8 GG homozygotes and 10 CC homozygotes), GG homozygosity was associated with greater reduction in IHTG than subjects with the CC genotype (45% vs 18% reduction) after hypocaloric low-carbohydrate diet for 6 days [224]. The intriguing results need confirmation in bigger datasets. Moreover, the impact of *PNPLA3* gene polymorphism on response of sustainable lifestyle intervention for patients with

NAFLD in community is also largely unknown.

The aim of the second part of this study was to examine the association between *PNPLA3* gene polymorphism and NAFLD. We also aimed to test the effect of *PNPLA3* gene polymorphism on the dietary pattern of the subjects, and the possible interaction between the two in NAFLD development. The impact of *PNPLA3* gene polymorphism on the response of lifestyle intervention will also be evaluated.

2.2 Study populations

In order to evaluate the performance of blood biomarkers in diagnosing NAFLD and NASH, and to test the association of *PNPLA3* gene polymorphism, dietary pattern, lifestyle intervention and NAFLD, we included two well-characterized cohorts. The first cohort included 152 consecutive patients who underwent liver biopsy at Prince of Wales Hospital due to suspected NAFLD. The second cohort included 922 subjects from community who participated in a population screening for NAFLD [17].

2.2.1 Hospital cohort

152 consecutive patients without HBV (defined by positive blood hepatitis B surface antigen) or HCV (defined by positive blood antibody against hepatitis C virus) infection who underwent liver biopsy at Prince of Wales Hospital due to suspected NAFLD were prospectively included. Abnormal liver imaging findings and elevated blood aminotransferase were the main seasons for liver biopsy. Patients with excessive alcoholic consumption (>30 g/day for men and >20g/d for women) and secondary fatty liver (Table 1.1) were excluded. Five patients had normal liver histology. They were assigned to the control group. 147 histology confirmed NAFLD patients were assigned to the hospital NAFLD group (Figure 2.1).

Among patients in the hospital NAFLD group, 51 patients received prospectively planned paired liver biopsies to study the progression of NAFLD [46]. The second liver

biopsies were performed 36 months after the baseline evaluation. The second biopsy results from this subgroup served as a validation cohort to validate the diagnostic accuracy of serum biomarkers, as well as evaluated the utility of the biomarkers in predicting disease progression (Figure 2.1).

2.2.2 Community cohort

Potential study subjects from the community were randomly selected from the government census database. Subjects with conditions which might cause secondary fatty liver (Table 1.1) were excluded. Subjects with decompensated liver disease (defined as albumin < 35 g/l, bilirubin > 50 mmol/l, international normalised ratio > 1.3, platelet count < 150×10^9/l, or the presence of ascites or varices) were also excluded.

Invitation letters were sent to 3,000 subjects. Totally, 1,069 subjects responded to the invitation. These subjects underwent anthropometric measurements, blood tests for liver biochemistry and metabolic parameters, and ^{1}H-MRS to quantify intrahepatic triglyceride (IHTG) content. Among them, 91 had HBV infection; 3 had HCV infection. Fifty two subjects had contraindications to magnetic resonance imaging; 1 failed ^{1}H-MRS. Finally, 922 subjects were included in the community cohort (Figure 2.1).

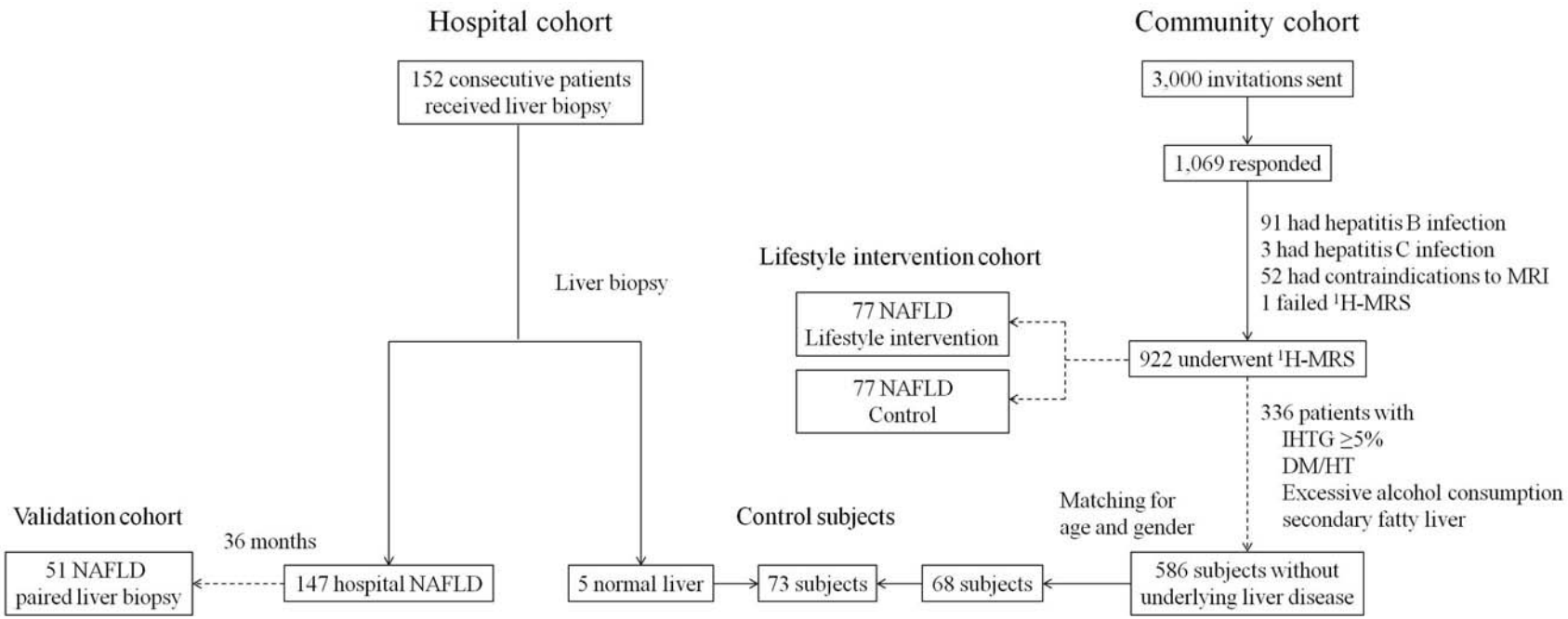
A subgroup of the community cohort served as controls for evaluating the

performance of biomarkers together with the hospital NAFLD cohort. We first excluded patients who with IHTG content above 5% measured by ^{1}H-MRS and who with type 2 diabetes mellitus or hypertension. Patients who met the excluding criteria applied in the hospital NAFLD group were also excluded. Five hundred and eighty-six subjects without underlying liver disease remained. After matching by age (±2 years) and gender, 68 control subjects were selected. Together with the 5 subjects with histological normal liver, 73 control subjects were finally enrolled (Figure 2.1).

154 subjects found to have both NAFLD and elevated blood alanine aminotransferase (ALT) levels from community cohort were invited to enter the lifestyle intervention trial. NAFLD was defined as IHTG of 5% or above by ^{1}H-MRS; elevated blood ALT was defined by plasma ALT above 30 IU/l in men and 19 IU/l in women. Enrolled patients were randomized in 1:1 ratio to participate in the lifestyle modification programme or receive usual care. Randomization was performed through the use of a computer-generated list of random numbers. Finally, 77 patients were assigned into intervention group and other 77 patients were assigned into control group.

The study protocol was approved by the Clinical Research Ethics Committee of The Chinese University of Hong Kong. All subjects gave informed written consents. The lifestyle intervention programme was registered at ClinicalTrials.gov (NCT00868933).

Figure 2.1 Study population.



MRI: Magnetic resonance imaging; ^{1}H-MRS: Proton-magnetic resonance spectroscopy; IHTG: Intrahepatic triglyceride; DM: Diabetes mellitus; HT: Hypertension disease; MS: Metabolic syndrome.

2.3 Methods

2.3.1 Clinical assessment

The medical history, including co-morbid illness and drug/herb intake, was recorded with a standard questionnaire. The subjects had anthropometric measurements including body weight, body height, waist and hip circumferences. Body mass index (BMI) was calculated as body weight (kg) divided by height (m) squared. Waist circumference was measured at a level midway between the lower rib margin and iliac crest with the tape all around the body in the horizontal position. Dietary intake was recorded using a locally validated food-frequency questionnaire that captures food intake over 7 days [225]. Daily nutrient intake was calculated using the Food Processor Nutrition Analysis and Fitness software version 7.9 (Esna Research, Salem, USA). All nutrient intakes were adjusted for dietary energy intake by the residual method for subsequent nutrient analysis [226].

2.3.2 Laboratory tests

Blood samples were taken after at least 8 hours of fasting. Tests for liver biochemistry, glucose and lipids were performed routinely. Metabolic syndrome was defined according to the ethnic-specific criteria by the International Diabetes Federation, which was modified from the National Cholesterol Education Program, Adult Treatment Panel III Guidelines, as any 3 of the followings: (1) waist circumference ≥90 cm in men and ≥80 cm in women; (2) triglycerides >1.7 mmol/L; (3) high-density lipoprotein-cholesterol <1.03 mmol/L in men and <1.29 mmol/L in women; (4) blood pressure ≥130/85 mmHg; and (5) fasting plasma glucose ≥5.6 mmol/L; or receiving treatment for the above metabolic abnormalities [24].

2.3.3 Liver histology

Percutaneous liver biopsy was performed using a 16-gauge Temno needle [63]. Liver biopsy specimens were fixed in formalin and embedded in paraffin. Histological slides were read by two experienced pathologists who were blinded to the clinical data. When there was discrepant interpretation between the two pathologists, they reviewed the slides together and came to a consensus. Liver histology was reported by both semiquantitative scoring according to the NASH Clinical Research Network system [60] and the pathologists' global assessment, which was modified from the original description by Matteoni and colleagues [53 112]. NAFLD activity score (NAS) was the sum of steatosis, lobular inflammation and hepatocellular ballooning scores (Table 1.4). Fibrosis was staged from 0 to 4, with stage 0=no fibrosis, 1=perisinusoidal or periportal fibrosis, 2=perisinusoidal and portal/periportal fibrosis, 3=bridging fibrosis and 4=cirrhosis. Stage 3 and 4 fibrosis was considered as advanced fibrosis. NASH was diagnosed for specimens with fatty liver, lobular inflammation and hepatocytes ballooning. NAFLD patients not fulfilling the criteria of NASH were labeled as non-NASH.

2.3.4 Proton-magnetic resonance spectroscopy (^{1}H-MRS)

^{1}H-MRS was performed to measure IHTG content. A whole-body 3.0 T scanner with a single voxel point-resolved spectroscopy sequence and an echo time of 40 ms and repetition time of 5000 ms was used. A survey scan was first performed in the abdominal region to help in positioning a volume measuring 20 (AP) 315 (RL) 340 (FH) mm within the liver. The scanner's built-in body coil was used for both signal transmission and reception. A no-water-suppressed spectrum was acquired using 32

signal averages and the data were exported for offline spectral analysis. Water (4.65 ppm) and lipid (1.3 ppm) peak amplitudes were measured to determine vertebral marrow fat content, which was defined as the relative fat signal amplitude in terms of a percentage of the total signal amplitude (water and fat) and calculated according to the following equation: fat content = $(I_{fat} / (I_{fat}+I_{water}))\times 100$, where I_{fat} and I_{water} are the peak amplitudes of fat and water, respectively. Correction for relaxation loss was not applied because of the relatively long repetition time and short echo time. An IHTG content of 5% was used to distinguish between patients with and without fatty liver [17].

2.3.5 Transient elastography (TE)

Liver stiffness measurement (LSM) was performed by transient elastography (Fibroscan, Echosens, France) according to the instructions and training by the manufacturer. Liver stiffness measurements were considered reliable only if 10 successful acquisitions were obtained, the success rate was above 60%, and the interquartile range-to-median ratio of the 10 acquisitions was smaller than 0.3. The median of 10 measurements represented the liver elastic modulus, and the interquartile range represented the variability of measurements. The operators had performed at least 50 examinations before the study and were blinded to the clinical data. A cutoff value of 7.9 kPa was used to estimate the proportion of patients with possible advanced fibrosis according to a local validation study using liver histology as the reference standard [63].

2.3.6 Serum biomarkers

During each clinic visit, part of the patients' serum samples was stored at -80°C, and

biomarker testing was performed in one batch afterwards. Serum level of CK-18 M30, M65 and M65ED was measured by the M30 Apoptosense enzyme-linked immunosorbent assay (ELISA) kit (PEVIVA, Bromma, Sweden), M65 ELISA kit (PEVIVA, Bromma, Sweden) and M65 EpiDeath ELISA kit (PEVIVA, Bromma, Sweden), respectively. Both assays are based on two antibodies, M6 and M5, which are directed against two different epitopes of CK-18 and recognize total CK-18. The difference between M65 and M65ED assays is that M65 assay uses the M5 antibody for detection and M6 for capture; while M65ED assay uses these antibodies inversely. AFABP and FGF21 was quantified by Human Adipocyte FABP ELISA kit and Human FGF21 ELISA kit (BioVendor Laboratory Medicine, Czech Republic), respectively. For all tests, both inter- and intra-assay coefficient of variations were less than 10% according to the manufacturers' instruction.

2.3.7 Genetic analysis

Genomic DNA was extracted from 100 μl buffy coat using QIAamp Blood DNA Mini Kit and QIAcube System (Qiagen, Germany). Extracted DNA was quantified using Nanodrop 1000 (Thermo Fisher Scientific, USA). For each patient, 20 ng of genomic DNA was used for *PNPLA3* rs738409 allelic discrimination using TaqMan® SNP Genotyping Assays (Life Technologies, USA) on the Applied Biosystems 7900HT Fast Real-Time PCR System (Life Technologies, USA).

2.3.8 Lifestyle modification programme

2.3.8.1 Trial design

This was a parallel group, superiority, single-blind randomized controlled trial comparing a community-based lifestyle modification programme with usual care in

NAFLD patients.

2.3.8.2 Intervention group

Patients randomized to the intervention group participated in a dietitian-led lifestyle modification programme for 12 months. The programme was held at 2 urban centres that are open to the public for the management of obesity and related disorders. The programme is based on a strategy of increasing energy expenditure and reducing caloric intake using lifestyle behavioral change to achieve long-lasting impact. The patients attended dietary consultation sessions weekly in the first 4 months, and monthly in the following 8 months. At the first session (about 1 hour), the dietitian carried out a complete behavioral assessment, covering important areas such as the patient's current eating and lifestyle patterns, specific eating-related behaviors, knowledge of risks associated with current eating patterns, and concerns and feelings about specific lifestyle changes. The dietitian also discussed the expected duration and specific dietary and lifestyle advices to achieve a desirable weight status with the patients.

In the follow-up sessions (about 20 minutes), the dietitian reviewed the patient's dietary practice and provided recommendations. Each patient was given an individualized menu plan. The dietary component and portion sizes of the menu plan were based on the recommendations of the American Dietetic Association [227]. A varied balanced diet with an emphasis on fruit and vegetables, and low-fat, low-glycaemic index (GI) and low-calorific products in appropriate portions was encouraged. Each patient was provided with two booklets, one for food portion size exchange and tips for eating out, and another listing the low-GI food options and

meal plans (GI <55). Moreover, techniques for coping at-risk situations such as parties and festival celebrations were taught. Recipes were also provided to the patients to encourage healthy cooking. Adherence to dietary intervention was assessed by calculating the percentage attendance to the intervention sessions and evaluating the dietary intakes and meal patterns using a weekly food record.

Besides, patients were encouraged to see an exercise instructor at least once during the lifestyle modification programme. During the first exercise consultation (about 30 minutes), the exercise instructor reviewed the patient's medical history and exercise habits, and designed a suitable exercise regime for the patient. In general, patients were first instructed to do moderate intensity aerobic exercise for 30 minutes 3 to 5 days a week and encouraged to increase daily physical activities. During subsequent appointments, the exercise instructor evaluated the patient's exercise progress on aerobic exercise and stretching during follow-ups. When patients were able to develop a routine exercise habit, they were instructed to perform resistance training to increase their muscle endurance and strength for better aerobic performance and liver fat reduction [228].

2.3.8.3 Control group

Patients in the control group received routine care at the medical clinic of the Prince of Wales Hospital, Hong Kong. At baseline, a clinician explained the laboratory test results and the natural history of NAFLD to the patients. The patients were encouraged to reduce carbohydrate and fat intake, and to exercise for at least 90 minutes per week.

2.3.8.4 Follow-up assessments

The patients attended the clinic at months 3, 6, 9 and 12 for metabolic assessment, and received further advice from a clinician at months 6 and 12. During each visit, new symptoms and drug intake were monitored by history and territory-wide computer prescription record. Anthropometric measurements, liver biochemistry, fasting glucose and lipids were assessed. Physical activities were recorded as the total duration of active exercise (minutes) per week. At baseline and month 12, ^{1}H-MRS and liver stiffness measurement were performed to assess hepatic steatosis and fibrosis, respectively.

2.3.8.5 Study outcomes

The primary outcome was remission of NAFLD at month 12 as evidenced by IHTG of less than 5% by ^{1}H-MRS. Secondary outcomes were reduction in IHTG and changes in liver stiffness by transient elastography, anthropometric measurements, liver biochemistry, fasting glucose and lipids.

2.3.9 Statistical analysis

With a sample size of 147 NAFLD patients, the inclusion of 73 control subjects would have the power to evaluate the performance of biomarkers in detecting NAFLD with standard errors of the area under the receiver operating characteristics curve (AUROC) between 0.03 and 0.04. According to our previous experience, 20-50% of NAFLD patients underwent liver biopsy had NASH [18]. A sample size of 147 NAFLD patients would have the power to evaluate the performance of biomarkers in detecting NASH with standard errors of the AUROC between 0.04 and 0.07.

Continuous variables were expressed as mean ± standard deviation or median (interquartile range [IQR]) as appropriate. Categorical clinical data between groups were compared by chi-squared test; Hardy-Weinberg equilibrium of alleles was also assessed by chi-squared test. Quantitative variables were analyzed using t test and one-way analysis of variance with post-hoc analysis for normal distributional data, or Mann-Whitney U test and Kruskal-Wallis test for highly skewed data. Spearman's or Pearson's correlation coefficient was used to estimate the association of factors of interest. Multiple linear regression was used to determine the independent factors associated with continuous variables. Binary logistic regression was used to determine the independent factors associated with NAFLD and NASH, as well as other binary categorical data. It is also used to calculate the predicted probability of combined utility of various biomarkers on predicting NAFLD/NASH. Receiver-operating characteristics (ROC) curve analysis was conducted to assess the performance of biomarkers and *PNPLA3* gene polymorphism in the diagnosis of NAFLD/NASH. Delong's test revealed no significant difference among various biomarkers. For each biomarker, 3 optimal cutoff values were selected based on high sensitivity >90%, high specificity >90%, and the best combined sensitivity and specificity according to the Youden's index.

All statistical tests were performed using the Statistical Package for Social Sciences version 16.0 (SPSS, Chicago, IL, USA) and Analyse-it Method Evaluation Edition version 2.26 (Analyse-it, Leeds, UK). A two-tailed p value of <0.05 was considered statistically significant. In multiple regression models, a p value of 0.05-0.10 was considered marginally significant.

Chapter 3: Assessment of non-alcoholic fatty liver disease using serum biomarkers

3.1 Serum apoptosis and total cell death markers

3.1.1 Background

A number of biomarkers have been developed for the non-invasive evaluation of NAFLD and NASH (Table 1.5). Among them, CK-18 is a group of cell death markers for both NAFLD and NASH diagnosis including apoptotic marker CK-18 fragment, CK18Asp396 neo-epitope (M30) and total cell death markers CK-18 M65, CK-18 M65ED.

Apoptosis is a prominent feature of NASH [105]. M30 is a fragment of CK-18, the major intracellular filament protein specific to hepatocytes, which is cleaved by activated caspases during cell apoptosis. M30 fragments are released into circulation and can be captured by specific antibody and measured. Thus, detecting serum M30 level reflects the degree of hepatocellular apoptosis. The utility of CK-18 M30 in diagnosing NAFLD and NASH has been validated in several independent cohorts (See Chapter 1, 1.2.2.3) [137-138 140]. However, it has not been evaluated in Chinese. The diagnostic thresholds in Chinese population are also unknown.

Meanwhile, CK-18 M65 and M65ED detect both caspase-cleaved and uncleaved CK-18. The serum M65/M65ED level reflects the degree of total hepatocyte death (mainly apoptosis and necrosis). Necrosis has also been proposed to be responsible

for the disease progression in NAFLD patients [144], and M65/M65ED is recently suggested to be useful in NAFLD non-invasive evaluation, or even superior to M30 in differentiating moderate steatosis and fibrosis from mild disease [139]. However, that study was limited by the inclusion of different liver diseases and the small number of NAFLD patients. Moreover, M65 and M65ED have not been validated in the Chinese population either.

The aim of this part of the study was to evaluate the performance of blood CK-18 M30, M65 and M65ED as biomarkers for the diagnosis of NAFLD and NASH. For this purpose, a hospital NAFLD cohort (147 biopsy-proven NAFLD patients); validation cohort (51 biopsy-proven NAFLD patients with prospectively scheduled paired liver biopsies 36 months apart); and 73 control subjects (5 subjects with normal liver histology and 68 subjects without underlying liver disease from community cohort) were enrolled in this part of the study. Details of the study subjects were described in Chapter 2, 2.2 and Figure 2.1.

3.1.2 Results

3.1.2.1 Patient characteristics

The clinical and pathological characteristics were shown in Table 3.1. The NAFLD patients and control subjects were well matched in age (47.4 ± 10.3 years vs. 47.7 ± 9.7 years, $p = 0.836$) and gender (male: 53.4% vs. 55.8%, p = 0.741). NAFLD patients were more obese than control subjects (BMI: 27.4 ± 3.9 kg/m^2 vs. 22.5 ± 2.7 kg/m^2, $p < 0.001$) and had a higher prevalence of metabolic syndrome (74.8% vs. 11.0%, $p < 0.001$). ALT, fasting glucose, glycated hemoglobin and triglyceride levels were also significantly higher in NAFLD patients. Sixty-nine (47%) NAFLD patients

had NASH by global pathological assessment. One control subject had diabetes and was from the 5 subjects with normal liver histology.

NASH patients had higher body mass index compared with non-NASH NAFLD patients (28.2 ± 4.0 kg/m^2 vs. 26.7 ± 3.7 kg/m^2, p = 0.019). They were also more likely to have metabolic syndrome (82.6% vs. 67.9%, p = 0.041). Other clinical characteristics were similar between these 2 groups.

3.1.2.2 Prediction of NAFLD

In control subjects, the median serum levels of M30, M65 and M65ED were 103 (IQR, 80-138) U/L, 309 (249-411) U/L and 47 (30-92) U/L, respectively. In NAFLD patients, the median serum levels of M30, M65 and M65ED were 354 (221-529) U/L, 770 (539-1010) U/L and 443 (202-801) U/L, respectively. The serum levels of all 3 biomarkers were significantly higher in NAFLD patients compared with control subjects, with all p value < 0.001 (Figure 3.1 A).

In the whole population, all 3 biomarkers were highly correlated with each other by spearman correlation test. They were all highly correlated with NAFLD diagnosis too (Table 3.2). M30, M65 and M65ED had high overall accuracy in diagnosing NAFLD with AUROC of 0.92 (95% CI: 0.87-0.96), 0.92 (0.89-0.96) and 0.94 (0.92-0.97), respectively (Table 3.3, Figure 3.2 A). Delong's test revealed no significant difference among the 3 biomarkers (M30 vs. M65, p = 0.670; M30 vs. M65ED, p = 0.205; M65 vs. M65ED, p = 0.084). The optimal cutoff values with the highest Youden index for M30, M65 and M65ED were 180 U/L, 523 U/L and 105 U/L respectively. By applying these optimal cutoffs, both high sensitivity and specificity

could be achieved in diagnosing NAFLD using M30 (sensitivity and specificity: 84.4% and 90.4%), M65 (76.9% and 95.9%) and M65ED (93.2% and 79.5%). High PPV and NPV could also be achieved at the same time (Table 3.3).

3.1.2.3 Prediction of NASH

In NAFLD patients, all 3 biomarkers increased in a stepwise fashion in different steatosis, lobular inflammation, ballooning and fibrosis levels (Figure. 3.3). While comparing between minimal and moderate diseases, all M30, M65 and M65ED were able to differentiate grade 1 from grade 0 lobular inflammation or ballooning; all of them were also able to differentiate stage 2-3 from stage 0-1 fibrosis. However, only M65 and M65ED could differentiate patients with grade 2 from grade 1 steatosis (p = 0.008 and 0.001, respectively), while M30 could not (p = 0.190). By Spearman correlation test, M30, M65 and M65ED were highly correlated with each other. They were also moderately correlated with NASH diagnosis or NAS (Table 3.2). All 3 biomarkers were independently associated with higher blood ALT level and higher lobular inflammation grade by multiple linear regression (Table 3.4). Moreover, M65 and M65ED were also independently associated with more severe steatosis and fibrosis.

In patients with non-NASH, the median serum levels of M30, M65 and M65ED were 277 (186-472) U/L, 637 (457-886) U/L and 271 (187-579) U/L, respectively. In patients with NASH, the median serum levels of M30, M65 and M65ED were 397 (264-657) U/L, 877 (671-1469) U/L and 572 (328-1070) U/L, respectively. All 3 biomarkers were significantly elevated in NASH patients compared with non-NASH patients ($p = 0.001$ for M30 and $p < 0.001$ for M65 and M65ED, Figure 3.1 B).

The AUROC of M30, M65 and M65ED in differentiating NASH were 0.66 (0.57-0.75), 0.71 (0.62-0.79) and 0.70 (0.62-0.79), respectively (Table 3.3, Figure 3.2 B). There was no significant difference by Delong's test (M30 vs. M65, $p = 0.056$; M30 vs. M65ED, $p = 0.169$; M65 vs. M65ED, $p = 0.806$). The optimal cutoff values with the highest Youden index for M30, M65 and M65ED were 338 U/L, 790 U/L and 309 U/L respectively. By applying these optimal cutoffs, moderate sensitivity and specificity could be achieved in predicting NASH using M30 (sensitivity and specificity: 66.7% and 60.3%), M65 (62.3% and 70.5%) and M65ED (79.7% and 57.7%).

In the 51 NAFLD patients with prospective paired liver biopsies as a validation cohort, significantly lower serum ALT level and higher diastolic blood pressure were observed at month 36 compared with baseline, while other clinical characteristics remained similar (Table 3.1). Eighteen (35%) patients had NASH at month 36. The AUROC of M30, M65 and M65ED in differentiating NASH at month 36 were 0.63 (0.47-0.79), 0.62 (0.46-0.77) and 0.64 (0.48-0.80). Delong's test revealed no significant difference (M30 vs. M65, $p = 0.748$; M30 vs. M65ED, $p = 0.772$; M65 vs. M65ED, $p = 0.261$).

3.1.3 Summary

Apoptosis and necrosis are both important modes of cell death in liver disease. Apoptotic biomarker CK-18 M30 was widely validated in independent cohorts [137-138] [140]. Recently, Joka and colleagues suggested that total cell death markers M65 and M65ED might be superior to M30 [139]. However, this study was limited by small

number of NAFLD patients. Here, we confirmed that M30, M65 and M65ED had similar overall accuracy in predicting NAFLD and NASH. The results were validated in the validation cohort. In our study, M65 and M65ED were superior in detecting mild steatosis and fibrosis, which was consisted with Joka's study. The overall accuracies in diagnosing NAFLD were all over 90% for these 3 biomarkers; thus, all of them can be used to diagnose or exclude NAFLD alone. The accuracy of all 3 biomarkers in predicting NASH is just moderate. However, almost half of the biopsy-proven NAFLD patients in our cohort had NASH. When the biomarkers are applied to primary care setting, in which only 3-5% are with NASH [2], the NPV in excluding NASH will be much higher.

On the other hand, the AUROCs of these biomarkers appear to be lower than those in other studies. For example, the AUROC of M30 in diagnosing NASH was over 0.80 in the original multicenter study and a follow-up report [137 140]. However, the better diagnostic performance may be explained by the inclusion of patients without NAFLD in the "non-NASH" group. For example, 18 of 54 patients in the "non-NASH" group of the study by Tamimi et al. had steatosis of less than 5%. In our cohort, if the entire study population including both NAFLD patients and controls is analyzed, the AUROC for M30 in diagnosing NASH is increased to 0.83.

Table 3.1. Clinical characteristics of all patients' population.

	Control	NAFLD	Non-NASH	NASH	Validation cohort	
					Baseline	Month 36
All	73	147	78	69	51	
Male gender	39 (53.4)	82(55.8)	46(59.0)	36(52.2)	34(66.7)	
Age (years) ¶¶	47.4(10.3)	47.7(9.7)	47.8(9.0)	47.7(10.5)	44.2(8.9)	47.2(9.0)
Body Weight (kg)**	63.0(8.4)	74.5(14.7)	72.9(14.4)	76.2(14.9)	75.8(12.8)	75.6(12.7)
BMI (kg/m^2)** §	22.5(2.7)	27.4(3.9)	26.7(3.7)	28.2(4.0)	27.5(3.7)	27.4(3.8)
Waist (cm)**	81(8)	94(11)	93(11)	95(11)	93(9)	91(10)
Systolic blood pressure (mmHg)**	126(15)	135(16)	135(17)	135(16)	133(16)	135(18)
Diastolic blood pressure (mmHg)¶	81(9)	81(11)	79(11)	82(10)	78(9)	81(12)
Diabetes**	1(1.4)	70(47.6)	34(43.6)	36(52.2)	26(51.0)	
Hypertension**	0(0)	63(42.9)	30(38.5)	33(47.8)	26(51.0)	
Metabolic syndrome** §	8(11.0)	110(74.8)	53(67.9)	57(82.6)	35(68.6)	

ALT (IU/L)** ¶¶	28(26)	73(45)	66(40)	80(49)	78(54)	58(30)
Fasting glucose (mmol/L)**	4.9(0.4)	6.5(2.4)	6.3(2.1)	6.8(2.7)	6.7(2.9)	6.5(2.1)
HbA_{1c} (%)**	5.3(0.4)	6.2(1.3)	6.0(1.3)	6.4(1.3)	6.5(1.5)	6.3(1.2)
LDL-cholesterol (mol/L)	3.0(0.9)	3.1(0.9)	3.1(1.0)	3.0(0.7)	3.0(1.1)	3.1(0.8)
Total cholesterol (mol/L)	5.2(1.2)	5.2(1.0)	5.3(1.2)	5.1(0.7)	5.3(1.3)	5.2(1.2)
Triglyceride (mmol/L)**	1.3(1.2)	2.2(1.2)	2.1(1.3)	2.3(1.1)	2.2(1.3)	2.6(5.6)
Liver TG content (%)	1.7(1.2)					
Biopsy length§§¶¶		1.9(0.6)	1.7(0.5)	2.1(0.5)	1.5(0.4)	1.8(0.4)
Steatosis grade 1/2/3§§¶¶		53/52/42	38/28/12	15/24/30	0/29/15/7	4/14/20/13
Lobular inflammation 0/1/2§§¶¶		46/95/6	46/30/2	0/65/4	15/34/2	32/16/3
Ballooning 0/1/2§§¶¶		56/82/9	56/21/1	0/61/8	35/15/1	12/38/1
Fibrosis 0/1/2/3/4§§		59/51/ 16/10/11	46/21/ 9/0/2	13/30/ 7/10/9	26/16/7/1/1	28/14/2/4/3
NASH					14(27.5)	18(35.3)

* Significant at $p<0.05$, ** Significant at $p<0.01$, between control and NAFLD patients;

§ Significant at $p<0.05$, §§ Significant at $p<0.01$, between Non-NASH and NASH patients;

¶ Significant at $p<0.05$, ¶¶ Significant at $p<0.01$, between baseline and month 36 in patients received paired liver biopsies.

Numbers in parentheses are percentage for categorical data or standard deviation for numerical data.

ALT, alanine aminotransferase; BMI, body mass index; HbA_{1c}, glycated hemoglobin; LDL, low density lipoprotein; NASH, non-alcoholic steatohepatitis; TG, triglycerides

Table 3.2. Correlations within M30, M65, M65ED and NAFLD/NASH diagnosis.

	M30		M65		M65ED	
	rho	*p* value*	rho	*p* value*	rho	*p* value*
All patients (n=220)						
M30			0.86	<0.001	0.80	<0.001
M65	0.86	<0.001			0.94	<0.001
M65ED	0.80	<0.001	0.94	<0.001		
NAFLD	0.68	<0.001	0.69	<0.001	0.73	<0.001
NAFLD (n=147)						
M30			0.86	<0.001	0.80	<0.001
M65	0.86	<0.001			0.94	<0.001
M65ED	0.80	<0.001	0.94	<0.001		
NASH	0.27	0.001	0.36	<0.001	0.35	<0.001
NAS	0.41	<0.001	0.50	<0.001	0.54	<0.001

**p* value corresponds to Ho: rho = 0

Table 3.3. Accuracy of CK-18 M30, M65 and M65ED in predicting NAFLD and NASH.

	Biomarker	AUROC	Cutoff (U/L)	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	LR+	LR-
NAFLD[a]	M30	0.92(0.87-0.96)	110	95.2	57.5	81.9	85.6	2.2	0.08
			180	84.4	90.4	94.7	74.2	8.8	0.17
			310	57.1	94.5	95.4	52.2	10.4	0.45
	M65	0.92(0.89-0.96)	360	94.6	64.4	84.3	85.6	2.7	0.08
			523	76.9	95.9	97.4	67.3	18.8	0.24
			523	76.9	95.9	97.4	67.3	18.8	0.24
	M65ED	0.94(0.92-0.97)	80	95.2	74.0	88.1	88.4	3.7	0.06
			105	93.2	79.5	90.2	85.3	4.5	0.09
			237	61.4	95.9	96.8	55.2	15.0	0.40
NASH[b]	M30	0.66(0.57-0.75)	203	89.9	32.1	53.9	78.2	1.3	0.33
			338	66.7	60.3	59.8	67.2	1.7	0.55

		670	24.6	89.7	67.9	57.4	2.4	0.84
M65	0.71(0.62-0.79)	501	91.3	34.6	55.3	81.8	1.4	0.25
		790	62.3	70.5	65.1	67.9	2.1	0.53
		1183	31.9	89.7	73.3	59.8	3.1	0.76
M65ED	0.70(0.62-0.79)	143	91.3	17.9	49.6	69.9	1.1	0.48
		309	79.7	57.7	62.5	76.3	1.9	0.35
		1000	27.5	91.0	73.0	58.7	3.1	0.80

a. Represent the performance for discriminating NAFLD from control cases;

b. Represent the performance for discriminating NASH from Non-NASH

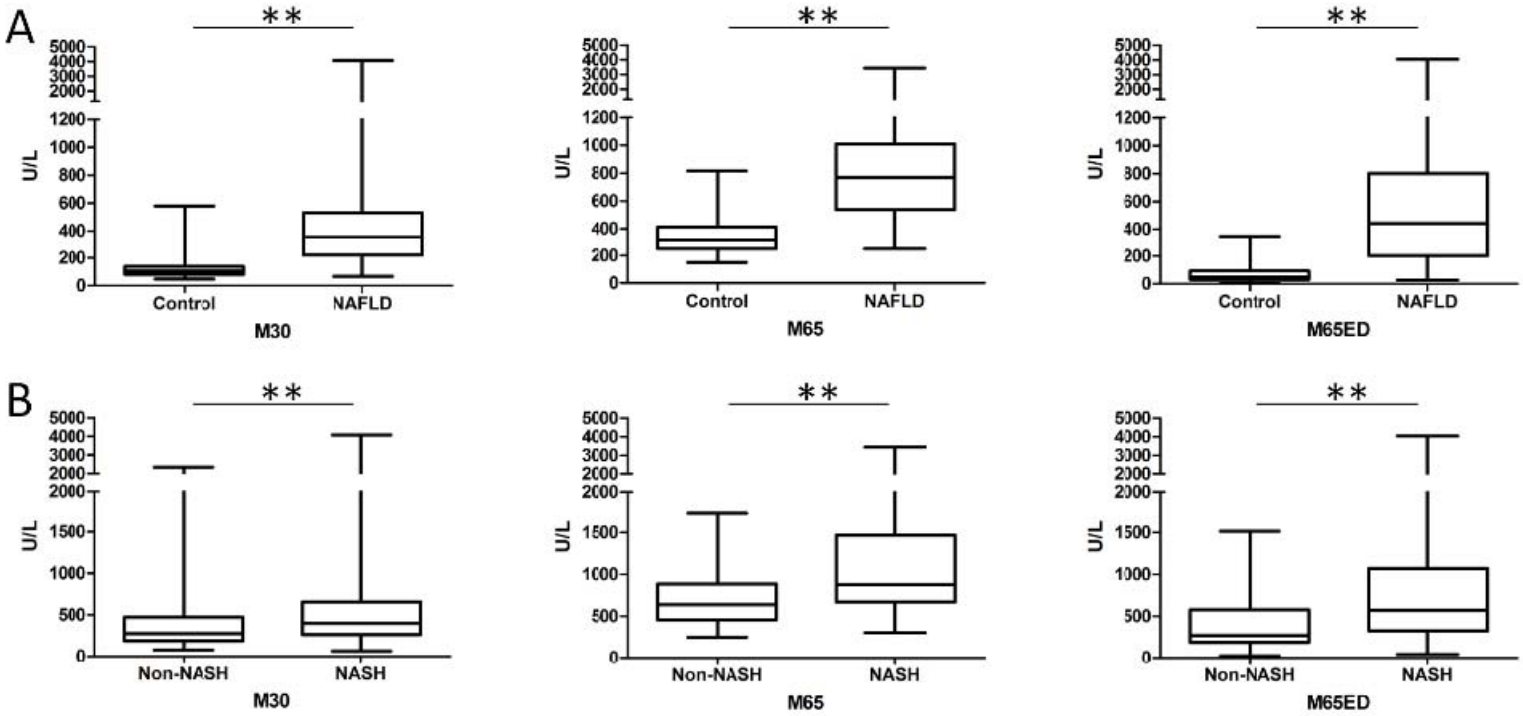
Cutoffs with high sensitivity, highest overall accuracy and high specificity were presented.

AUROC, area under receiver-operating characteristics curve; LR, likelihood ratio; NPV, negative predictive value; PPV, positive predictive value

Table 3.4. Multivariable analysis for independent factors associated with M30, M65 and M65ED in NAFLD patients.

		Beta	*p* value
M30	ALT	0.516	<0.001
	Lobular inflammation	0.260	<0.001
M65	ALT	0.581	<0.001
	Steatosis	0.196	0.002
	Lobular inflammation	0.172	0.007
	Fibrosis	0.217	0.001
M65ED	ALT	0.443	<0.001
	Glucose	0.245	0.034
	Steatosis	0.237	0.001
	Lobular inflammation	0.142	0.041
	Fibrosis	0.263	<0.001

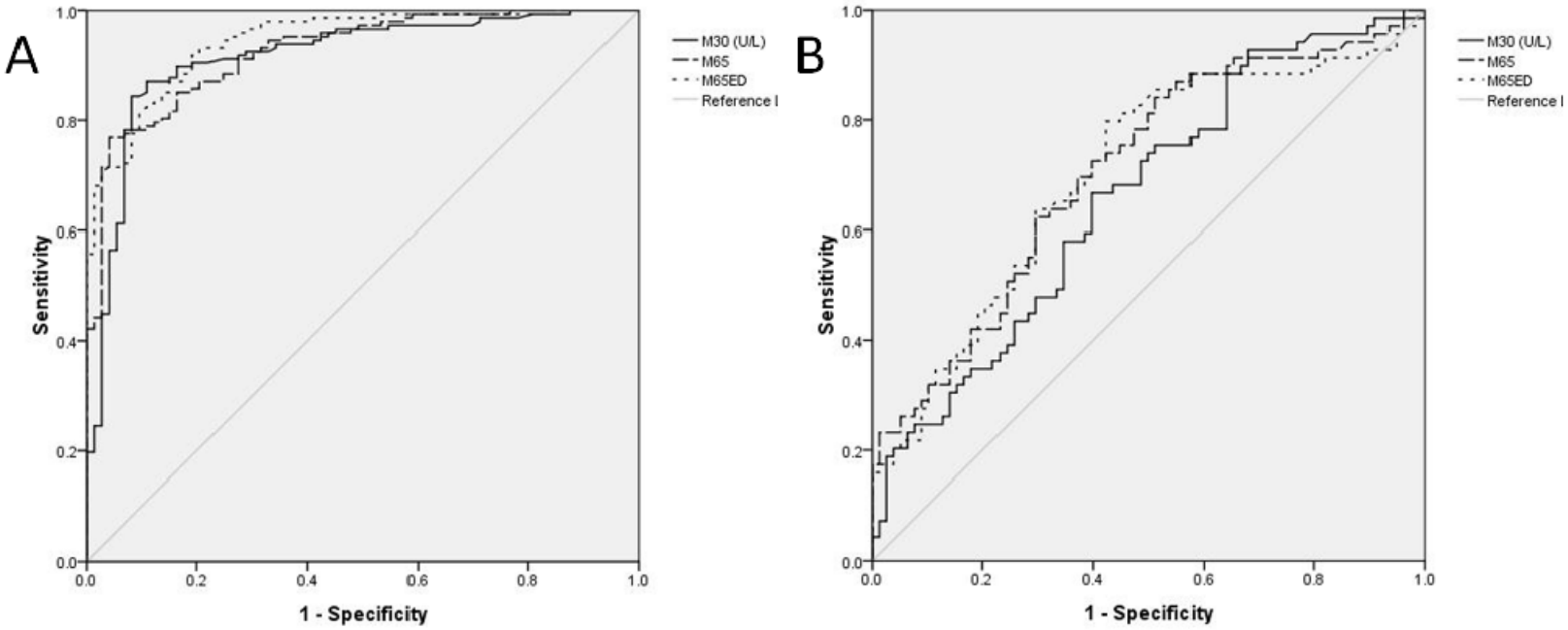
Figure 3.1. Serum level of CK-18 M30, M65 and M65ED in the whole population and NAFLD patients.



A. Comparison between control and NAFLD patients. B. Comparison between patients with non-NASH NAFLD and NASH.

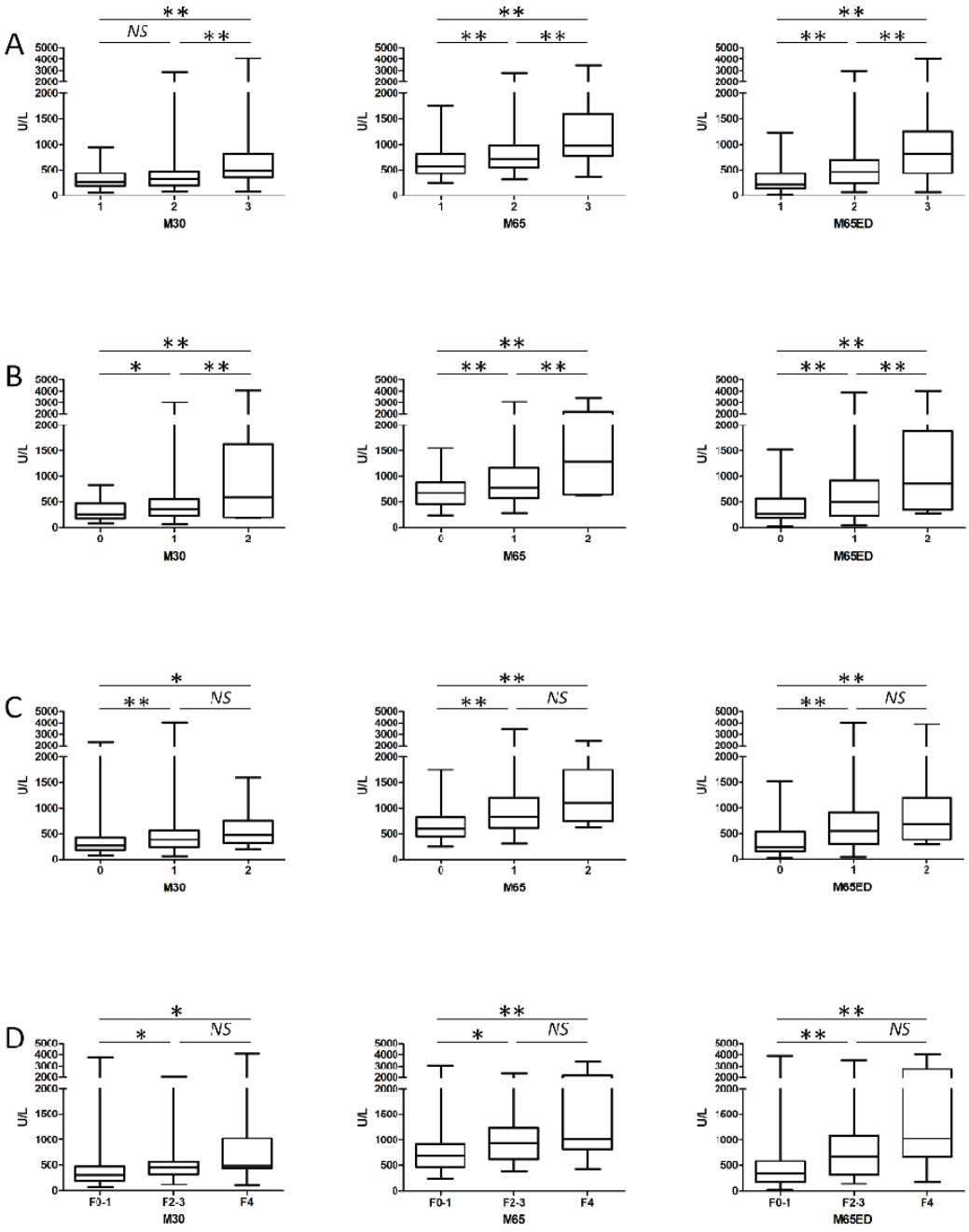
** Significant at $p < 0.01$.

Figure 3.2. ROC curves of CK-18 M30, M65 and M65ED in predicting NAFLD and NASH.



ROC curves in (A) distinguishing NAFLD patients from control subjects; and (B) distinguishing NASH from non-NASH NAFLD patients

Figure 3.3. Serum level of CK-18 M30, M65 and M65ED with different histological features in NAFLD patients.



Comparison among different (A) steatosis, (B) lobular inflammation, (C) ballooning and (D) fibrosis grades.

* Significant at $p < 0.05$; ** Significant at $p < 0.01$; *NS* Not statistically significant.

3.2 Evaluation of potential biomarkers AFABP and FGF21 for NAFLD and NASH

3.2.1 Background

Other than CK-18, several potential biomarkers for NAFLD and NASH were also developed. Adipocyte fatty acid binding protein (AFABP) is a pro-inflammatory cytokine. It is involved in the interaction between adipocytes and macrophages, which leads to inflammation and insulin resistance. Milner and colleagues found that serum AFABP level was significantly higher in 69 NASH patients compared with 31 patients with simple steatosis and 129 controls. It also correlated with individual histological features of NASH such as ballooning, lobular inflammation and fibrosis stage [150]. However, standard c statistics was not performed in this study. The diagnostic accuracy of AFABP in predicting NAFLD and NASH is still unknown.

Heightened oxidative stress in the liver is also a characteristic of NASH. Fibroblast growth factor 21 (FGF21) is a hormone which regulates lipid oxidation in the liver and stimulates glucose uptake in the adipose tissue [152]. It is also termed as a "mitokine" due to its regulation by mitochondrial dysfunction and strong effect on increasing lipid oxidation and browning of white adipose tissue [153]. Li et al. found that in human liver, FGF21 mRNA expression level increased with steatosis grade; its serum level was significantly higher in Chinese NAFLD patients defined by ultrasound [154-155]. Yilmaz et al. also reported elevated serum FGF21 levels in NAFLD

patients; however, FGF21 could not distinguish NASH in their cohort which included 82 NAFLD patients and 77 healthy controls [156].

Both AFABP and FGF21 hold great promise as non-invasive tests for NAFLD and NASH. However, they have not been adequately evaluated in independent cohorts. Their overall accuracies and optimal cutoff values in predicting NAFLD/NASH are largely unknown. The aim of this part of the study was to evaluate the performance of blood AFABP and FGF21 as biomarkers for the diagnosis of NAFLD and NASH. The same hospital NAFLD cohort; validation cohort; and control subjects as enrolled in Chapter 3, 3.1 were enrolled in this part of the study.

3.2.2 Results

3.2.2.1 Patient characteristics

Clinical and pathological characteristics were shown in Table 3.1. Details were described in 3.1.2.1 and 3.1.2.3.

3.2.2.2 Prediction for NAFLD using AFABP and FGF21

The median serum levels of AFABP in control subjects and NAFLD patients were 15.4 (12.5-19.0) ng/ml and 18.9 (13.9-25.3) ng/ml, respectively (p = 0.002) (Figure 3.4 A).Serum AFABP level significantly correlated with the NAFLD diagnosis (p = 0.002) with a correlation coefficient of 0.211. AFABP had a moderate accuracy in predicting NAFLD with AUROC of 0.63 (0.55-0.71) (Table 3.5, Figure 3.5 A). The

optimal cutoff value with the highest Youden index for AFABP was 18.9 ng/ml. By applying this optimal cutoff, AFABP had a sensitivity of 50.3% and a specificity of 74.0% in predicting NAFLD. PPV and NPV was 79.9% and 42.5%, respectively.

The median serum levels of FGF21 in control subjects and NAFLD patients were 106 (71-160) pg/ml and 297 (168-478) ng/ml, respectively ($p < 0.001$) (Figure 3.4 A).Serum FGF21 level significantly correlated with the NAFLD diagnosis ($p < 0.001$) with a moderate correlation coefficient of 0.552. FGF21 had a high accuracy in diagnosing NAFLD with AUROC of 0.84 (0.79-0.89) (Table 3.5, Figure 3.5 A). It was significantly higher than AFABP by Delong's test ($p < 0.001$). The optimal cutoff value with the highest Youden index for FGF21 was 191 pg/ml. By applying this optimal cutoff, FGF21 had both high sensitivity of 72.8% and specificity of 84.9% in predicting NAFLD. PPV and NPV was 90.7% and 60.8%, respectively.

3.2.2.3 Prediction for NASH using AFABP and FGF21

Although AFABP significantly correlated with lobular inflammation and ballooning; and FGF21 significantly correlated with steatosis and lobular inflammation by Spearman's correlation (Table 3.6); they could not differentiate each stage in most cases (Figure 3.6). Both AFABP and FGF21 were independently associated with higher lobular inflammation grade by multiple linear regression (Table 3.7). AFABP was also associated with higher ballooning grade, while FGF21 was also associated with higher blood ALT level.

The median serum levels of AFABP in non-NASH and NASH patients were 17.2 (12.3-23.8) ng/ml and 19.4 (16.1-27.5) ng/ml, respectively (p = 0.006) (Figure 3.4 B). AFABP was independently correlated with NASH diagnosis (p = 0.005) with a correlation coefficient of 0.230. The AUROC of AFABP in differentiating NASH was 0.63 (0.54-0.72) (Table 3.5, Figure 3.5 B). The optimal cutoff value with the highest Youden index for AFABP in predicting NASH was 13.7 ng/ml. By applying this optimal cutoff, AFABP had a sensitivity of 89.9% and a specificity of 34.6% in predicting NAFLD. PPV and NPV was 54.9% and 79.5%, respectively.

The median serum levels of FGF21 in non-NASH and NASH patients were 244 (119-393) pg/ml and 366 (232-579) pg/ml, respectively (p = 0.006) (Figure 3.4 B). FGF21 was independently correlated with NASH diagnosis (p = 0.001) with a correlation coefficient of 0.282. The AUROC of FGF21 in differentiating NASH was 0.66 (0.58-0.75) (Table 3.5, Figure 3.5 B). Delong's test revealed no significant difference between the AUROCs of FGF21 and AFABP (p = 0.616). The optimal cutoff value with the highest Youden index for FGF21 in predicting NASH was 332 pg/ml. By applying this optimal cutoff, FGF21 had a sensitivity of 58.0% and a specificity of 71.8% in predicting NAFLD. PPV and NPV was 64.5% and 65.9%, respectively.

In the validation cohort including 51 patients with paired liver biopsy, the AUROC of

AFABP and FGF21 in differentiating NASH at month 36 were 0.57 (0.38-0.76) and 0.63 (0.46-0.80), respectively.

3.2.3 Summary

While AFABP and FGF21 hold great promise as non-invasive tests for NAFLD and NASH, they were not validated in a well-characterized histological cohort. Here, we clearly demonstrated both serum AFABP and FGF21 levels were significantly increased in NAFLD patients compared with control subjects, or in NASH patients compared with non-NASH patients. Both biomarkers had similar moderate overall accuracy in predicting NASH. However, AFABP had a significantly lower accuracy in diagnosing NAFLD compared with FGF21. Moreover, the optimal cutoff value of AFABP in NASH predicting was even lower than which in NAFLD predicting. Both of these could be explained by the relatively high serum level of AFABP in control subjects, which would limit the application of AFABP in primary care for disease exclusion. On the other hand, FGF21 had a high accuracy of 84% in diagnosing NAFLD and a moderate accuracy of 66% in predicting NASH.

Table 3.5. Accuracy of AFABP and FGF21 in predicting NAFLD and NASH.

	Biomarker	AUROC	Cutoff	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	LR+	LR-
NAFLD[a]	AFABP	0.63(0.55-0.71)	9.5	90.5	11.0	67.2	36.5	1.0	0.86
	(ng/ml)		18.9	50.3	74.0	79.6	42.5	1.9	0.67
			25.8	22.4	90.4	82.5	36.6	2.3	0.86
	FGF21	0.84(0.79-0.89)	88	94.6	39.7	76.0	78.5	1.6	0.14
	(pg/ml)		191	72.8	84.9	90.7	60.8	4.8	0.32
			308	49.0	94.5	94.7	47.9	8.9	0.54
NASH[b]	AFABP	0.63(0.54-0.72)	12.4	91.3	25.6	52.1	76.9	1.2	0.34
	(ng/ml)		13.7	89.9	34.6	54.9	79.5	1.4	0.29
			29.0	24.6	91.0	70.7	57.7	2.7	0.83
	FGF21	0.66(0.58-0.75)	128	91.3	26.9	52.5	77.8	1.2	0.32
	(pg/ml)		332	58.0	71.8	64.5	65.9	2.1	0.58

	675	20.3	91.0	66.6	56.3	2.3	0.88

c. Represent the performance for discriminating NAFLD from control cases;

d. Represent the performance for discriminating NASH from Non-NASH

Cutoffs with high sensitivity, highest overall accuracy and high specificity were presented.

AUROC, area under receiver-operating characteristics curve; LR, likelihood ratio; NPV, negative predictive value; PPV, positive predictive value

Table 3.6. Correlations within AFABP, FGF21 and NASH histological features.

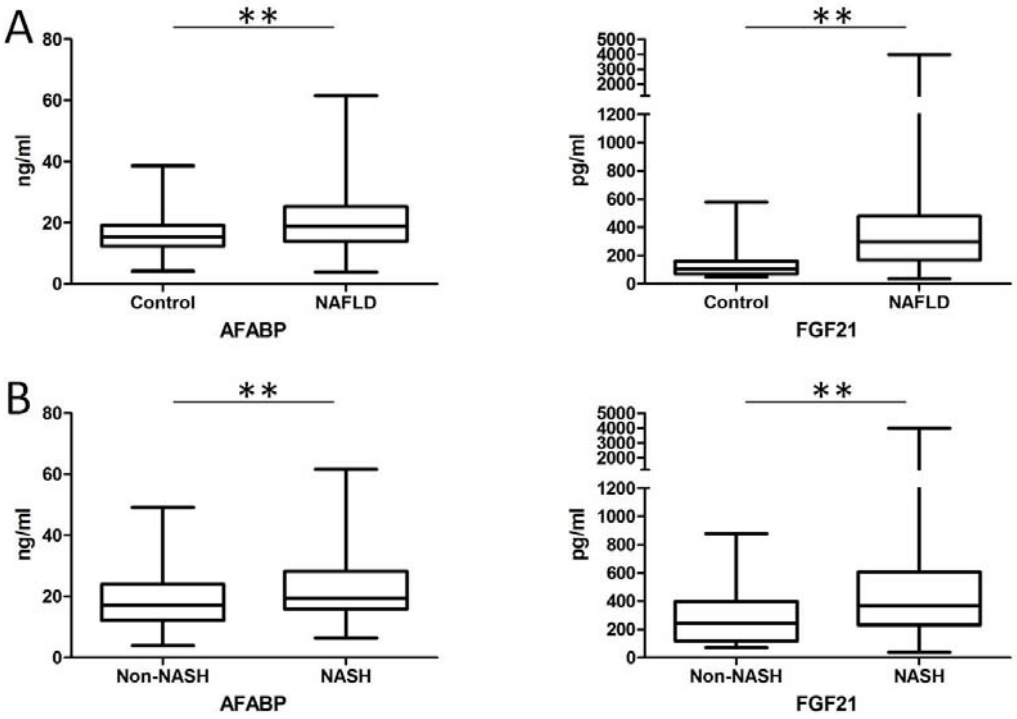
	Steatosis		Lobular inflammation		Ballooning		Fibrosis	
	rho	*p* value*	rho	*p* value*	rho	*p* value*	rho	*p* value*
AFABP	0.055	0.509	0.304	<0.001	0.229	0.005	0.108	0.195
FGF21	0.223	0.007	0.278	0.001	0.141	0.088	0.023	0.784

**p* value corresponds to Ho: rho = 0

Table 3.7. Multivariable analysis for independent factors associated with AFABP and FGF21 in NAFLD patients.

		Beta	*p* value
AFABP	Gender	0.366	<0.001
	Lobular inflammation	0.175	0.024
	Ballooning	0.184	0.016
FGF21	ALT	0.205	0.013
	Lobular inflammation	0.195	0.019

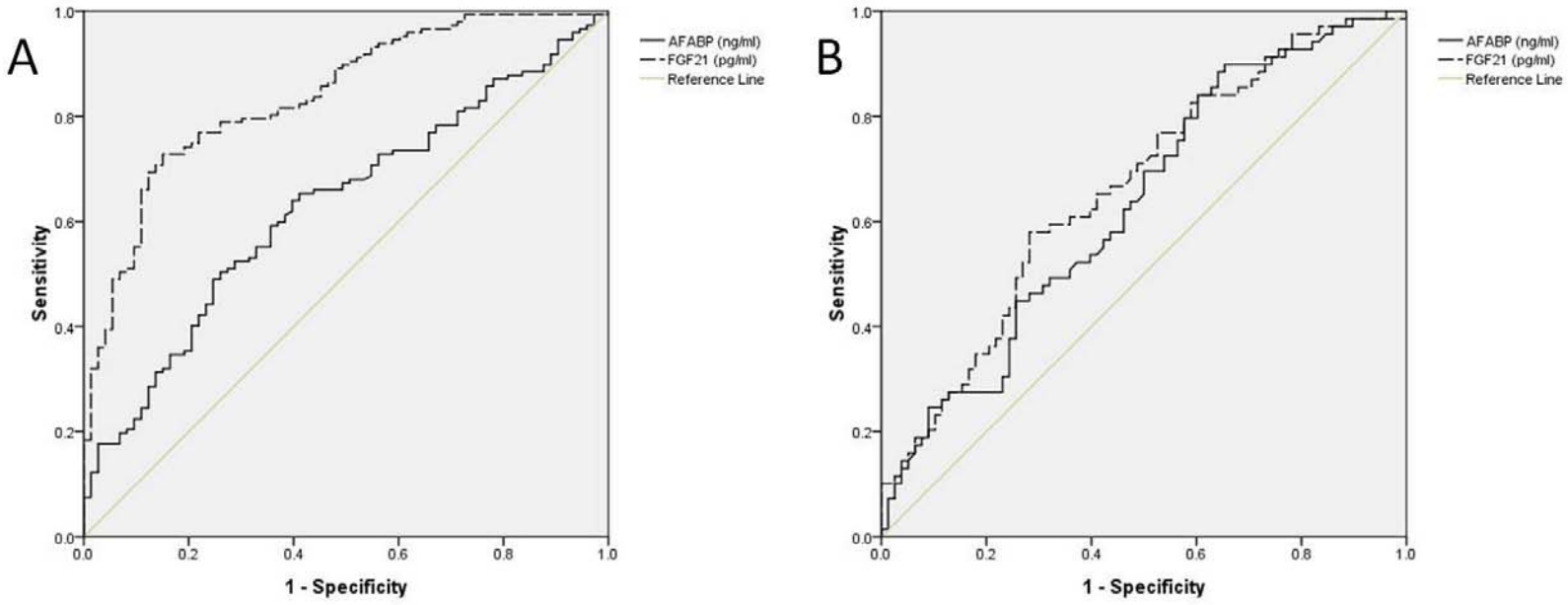
Figure 3.4. Serum level of AFABP and FGF21 in the whole population and NAFLD patients.



A. Comparison between control and NAFLD patients. B. Comparison between patients with non-NASH NAFLD and NASH.

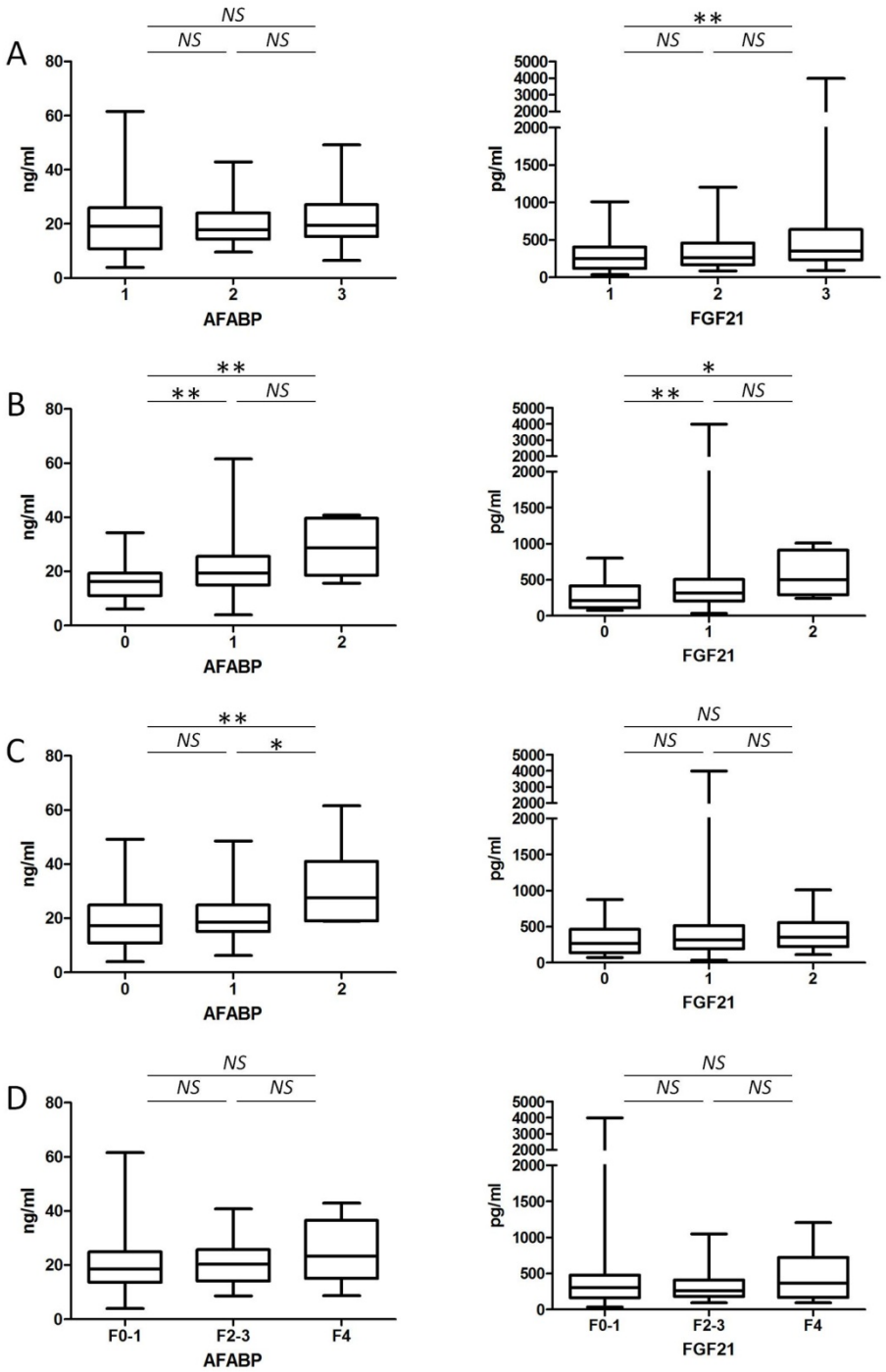
** Significant at $p < 0.01$.

Figure 3.5. ROC curves of AFABP and FGF21 in predicting NAFLD and NASH.



ROC curves in (A) distinguishing NAFLD patients from control subjects; and (B) distinguishing NASH from non-NASH NAFLD patients

Figure 3.6. Serum level of AFABP and FGF21 with different histological features in NAFLD patients.



Comparison among different (A) steatosis, (B) lobular inflammation, (C) ballooning and (D) fibrosis grades.

* Significant at p <0.05; ** Significant at p <0.01; *NS* Not statistically significant.

3.3 Assessment of disease progression using various biomarkers

3.3.1 Background

One advantage of biomarkers over liver biopsy is the non-invasive nature, which allows repeated measurements. If the change of a biomarker correlates well with change of disease status, it would be more suitable for serial monitoring than liver biopsy. However, previous studies on these biomarkers are cross-sectional designed. Whether these biomarkers can be used for serial monitoring could not be addressed.

In this part of the study, we aimed to evaluate whether the changes of CK-18, AFABP and FGF21 could reflect the NAFLD disease progression. We also aimed to explore the correlation between the changes of these biomarkers and changes of individual histological features of NASH. The prospective paired liver biopsy cohort including 51 NAFLD patients was included in this part of the study.

3.3.2 Results

3.3.2.1 Patient characteristics

Clinical and pathological characteristics were shown in Table 3.1. At month 36, significantly lower serum ALT level and higher diastolic blood pressure were observed compared with baseline. Twenty-five patients had increased NAS in 36 months. Ten patients progressed from non-NASH to NASH, and 14 patients had fibrosis progression for at least 1 stage.

3.3.2.2 Assessment of disease progression

The changes in M30, M65 and M65ED were all associated with NAS change and disease status change; however, only changes of M65 and M65ED were associated with changes of fibrosis stage (Table 3.8, Figure 3.7). Table 3.9 summarized the predictive performance and cutoff values with highest overall accuracy for changes of biomarkers. Delong's test revealed no significant difference among 3 biomarkers. At a single cutoff of 35 U/L, change in M30 had both sensitivity and specificity above 80% in predicting disease progression from non-NASH to NASH. When M65ED increased for no more than 62 U/L, the chance to have disease progression was only 10%. At a cutoff of 236 U/l, change in M65ED had sensitivity and specificity of 71.4% and 81.1% in predicting fibrosis progression, with NPV of 88.2% and PPV of 58.8%. On the other hand, the baseline levels of biomarkers could not predict disease progression.

There was no significant difference in changes of AFABP and FGF21 in patients with or without disease progression (Figure 3.8). By Spearman's correlation, neither change in AFABP nor FGF21 was associated with changes in NAS or disease status. Change in FGF21 was not associated with change in fibrosis; change in AFABP even exhibited a negative correlation with fibrosis change (Table 3.8). The predictive performance for changes of AFABP and FGF21 were not significantly higher than 0.5 to either end point (Table 3.9).

3.3.3 Summary

The changes in CK-18 M30, M65 and M65ED correlated well with changes in NAS and could be used to predict progression to NASH. Changes in M65 and M65ED were also associated with progression of liver fibrosis. Notably, the AUROCs for

predicting disease progression were both higher than 0.8 for M30 and M65ED. These results could be promising as non-invasive tests are more acceptable for long term and repeated monitoring of disease progression. Importantly, while the changes in CK-18 correlated with histological changes, baseline CK-18 level alone failed to predict disease progression. This indicates that NASH is a dynamic process. Change in disease activity is possible with lifestyle modifications. Therefore, serially performing these biomarkers will be helpful to monitor disease progression.

On the other hand, the changes in AFABP and FGF21 did not correlate with changes in NAS or disease status. Change in AFABP even negatively correlated with fibrosis progression. As serum AFABP levels were not significantly different among patients with different fibrosis stage in a larger cross sectional cohort (Figure 3.6), this negative correlation was controversial and might be meaningless. The utility of AFABP and FGF21 in disease progression prediction is limited.

Table 3.8. Correlations between change of biomarkers and disease progression in 51 patients with paired liver biopsy.

Change	NAS		Disease status		Fibrosis	
	rho	*p* value*	rho	*p* value*	rho	*p* value*
M30	0.51	<0.001	0.47	0.002	0.27	0.059
M65	0.49	<0.001	0.38	0.022	0.29	0.038
M65ED	0.50	<0.001	0.46	0.005	0.34	0.015
AFABP	0.03	0.832	0.07	0.650	-0.305	0.030
FGF21	0.221	0.119	0.242	0.087	0.208	0.143

**p* value corresponds to Ho: rho = 0

NAS, NAFLD activity score

Table 3.9. Prediction of disease progression using changes of biomarkers.

		Biomarker	AUROC	*p* value*	Cutoff (U/L)	Sensitivity	Specificity
Changes	NAS[a]	M30	0.75 (0.61-0.89)	0.002	3	68.0	76.9
		M65	0.72 (0.58-0.86)	0.006	139	68.0	73.1
		M65ED	0.72 (0.58-0.86)	0.007	56	68.0	69.2
		AFABP	0.53(0.37-0.69)	0.706			
		FGF21	0.62(0.46-0.78)	0.152			
	NASH[b]	M30	0.82 (0.65-0.99)	0.003	35	80.0	81.5
		M65	0.74 (0.56-0.93)	0.024	182	80.0	70.4
		M65ED	0.80 (0.62-0.98)	0.006	62	90.0	59.3
		AFABP	0.60(0.40-0.80)	0.356			
		FGF21	0.62(0.38-0.86)	0.274			

Fibrosis[c]	M30	0.68 (0.52-0.84)	0.050	123	42.9	89.2
	M65	0.72 (0.57-0.87)	0.016	1	78.6	45.9
	M65ED	0.77 (0.64-0.91)	0.003	236	71.4	81.1
	AFABP	0.33(0.16-0.50)	0.063			
	FGF21	0.61(0.43-0.78)	0.237			

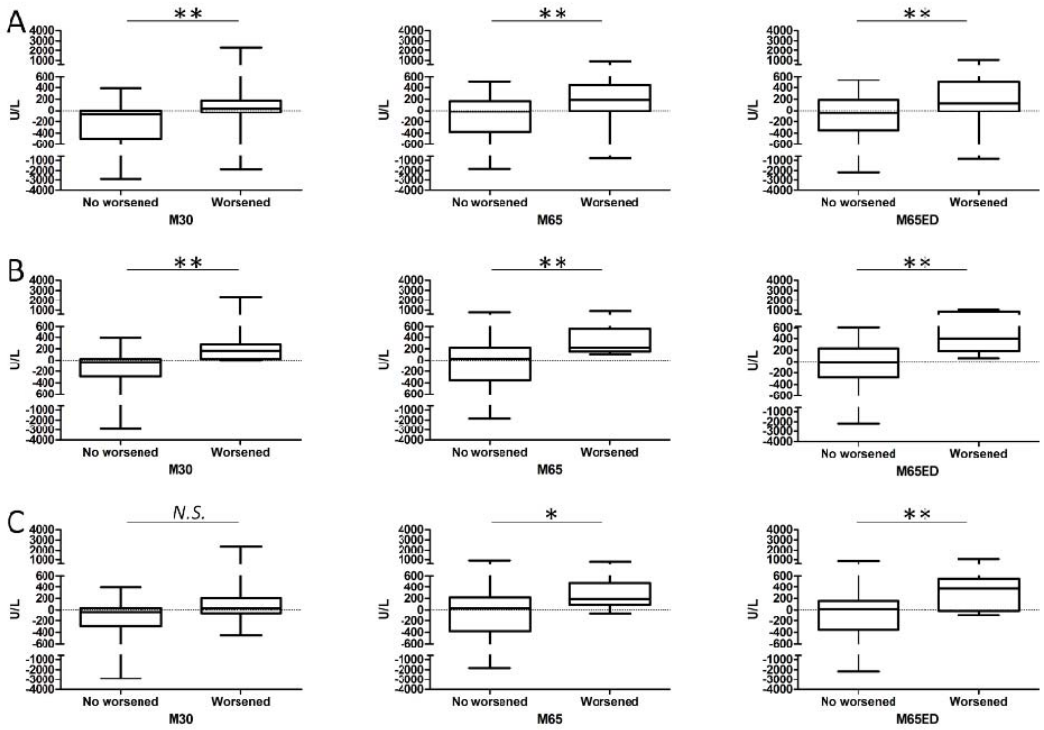
a. For prediction of increased NAFLD activity score

b. For prediction of disease progression, 14 patients who were diagnosed as NASH at baseline were excluded

c. For prediction of increased fibrosis stage

* Compared to AUROC =0.50

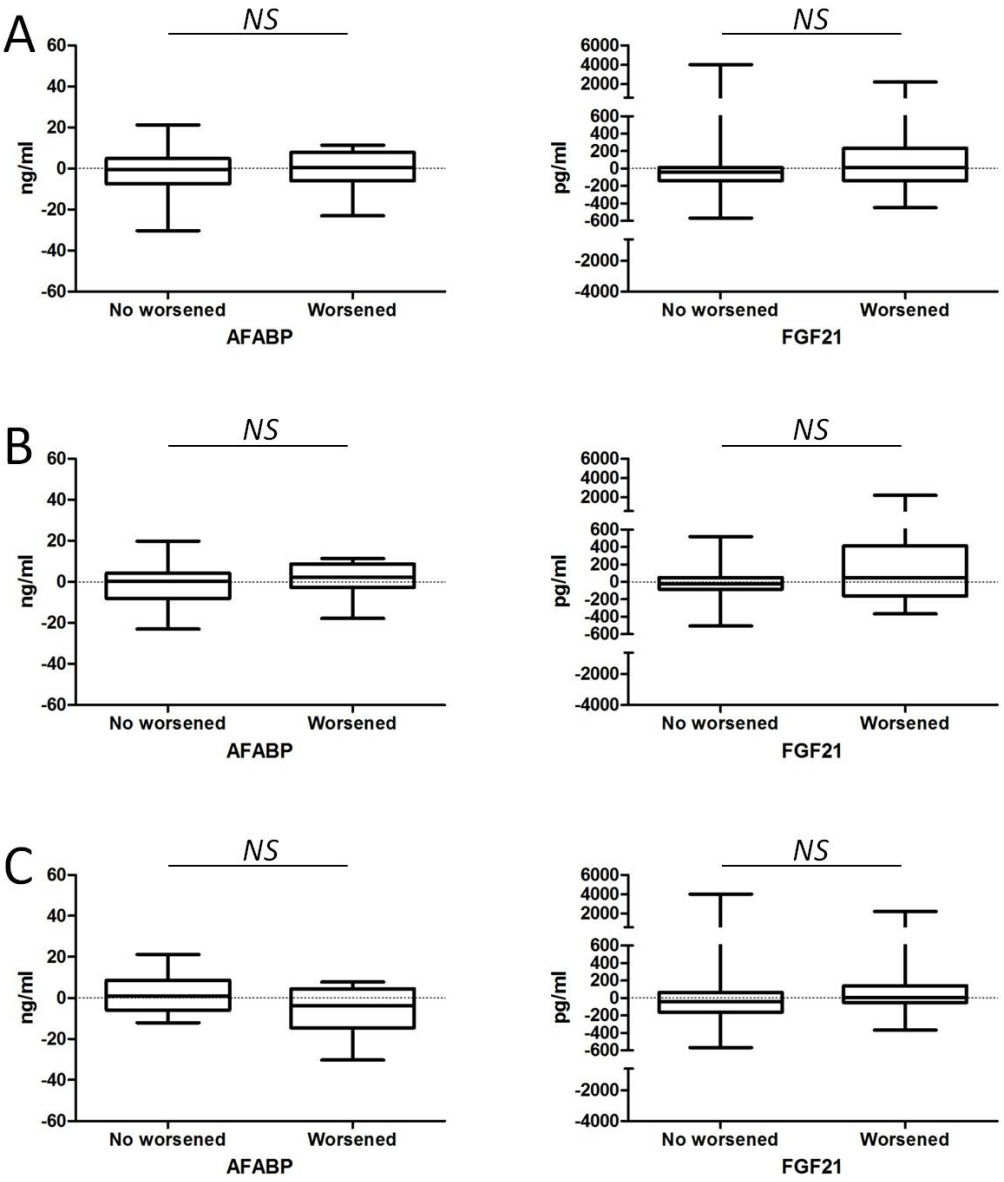
Figure 3.7. Changes of serum level of CK-18 M30, M65 and M65ED and disease progression in 51 patients received paired live biopsies.



Changes of patients with or without (A) NAFLD activity score worsened, (B) disease progression from non-NASH to NASH (14 patients who were diagnosed as NASH at baseline were excluded), and (C) fibrosis progression.

* Significant at p <0.05; ** Significant at p <0.01; *NS* Not statistically significant.

Figure 3.8. Changes of serum level of AFABP and FGF21 and disease progression in 51 patients received paired live biopsies.



Changes of patients with or without (A) NAFLD activity score worsened, (B) disease progression from non-NASH to NASH (14 patients who were diagnosed as NASH at baseline were excluded), and (C) fibrosis progression.

NS Not statistically significant.

3.4 Combined application and clinical use of biomarkers

3.4.1 Background

In previous sections, we cleared showed CK-18 M30, M65 and M56ED had similar AUROC in diagnosing NAFLD and NASH. Compared with M65 and M65ED, M30 was more widely validated as a biomarker for NASH. Thus, M30 will be used in the following analysis together with FGF21. AFABP will not be considered in the following analysis due to its relatively high serum level in control subjects.

Although CK-18 M30 and FGF21 have high overall accuracies over 0.9 in diagnosing NAFLD, their accuracies in predicting NASH are moderate. Since NAFLD can be easily diagnosed using non-invasive imaging techniques such as ultrasound and ^{1}H-MRS, predicting NASH is a more important role for biomarkers. However, neither of them is good enough to diagnose or exclude NASH alone. Previous studies suggested that the development of biomarker panels may improve the performance of individual biomarkers [140 229]. Since the CK-18 M30 and FGF21 reflect different aspects of the pathogenesis of NASH, it may be possible to combine them to achieve better diagnostic accuracy.

The aim of this part of study is to test whether combination of CK-18 M30 and FGF21 could improve the diagnostic performance. We also aimed to find an easy way to combine these biomarkers in clinical use. The same hospital NAFLD cohort

and validation cohort in Chapter 3, 3.1 were enrolled in this part of the study.

3.4.2 Results

3.4.2.1 Patient characteristics

Clinical and pathological characteristics of 147 biopsy-proven NAFLD patients and 51 patients received paired liver biopsies were shown in Table 3.1. Details were described in 3.1.2.1 and 3.1.2.3.

3.4.2.2 Combined application and clinical use of CK-18 M30 and FGF21

We used binary logistic regression to calculate the predicted probability of combined utility of CK-18 M30 and FGF21 on NASH diagnosis. AUROC of this predicted probability value was 0.69 (0.60-0.78). Although it was slightly higher than CK-18 M30 (0.66 [0.57-0.75]) or FGF21 (0.66 [0.58-0.75]), Delong's test revealed no significant improvement of accuracy in the combined probability (p = 0.385 and 0.243, compared with CK-18 M30 and FGF21, respectively). On the other hand, by applying the optimal cutoffs of CK-18 M30 (338 U/L) and FGF21 (332 pg/ml), among 47 patients had both biomarkers lower than the cutoffs, 37 did not have NASH, yielding a NPV of 79% and a sensitivity of 86%. 39 patients had both biomarkers above the cutoffs, 27 of them were with NASH, yielding a PPV of 69% and a specificity of 85%.

We further evaluated the performance of a 2-step approach in detecting NASH.

When CK-18 was used alone, 32 (22%) patients had level below the low cutoff value of 203 U/L (Figure 3.9 A). Twenty-five of these 32 patients did not have NASH, yielding a NPV of 78% and a sensitivity of 90%. In contrast, 17 of 25 patients with CK-18 above the high cutoff value of 670 U/L had NASH, yielding a PPV of 68% and a specificity of 90%. Ninety (61%) patients had CK-18 between the two cutoff values.

If FGF21 was added to the model, the overall accuracy could be further improved (Figure 3.9 A). Among patients with CK-18 level below 203 U/L, 24 had FGF21 below 332 pg/ml. Twenty of these 24 patients did not have NASH, yielding a negative predictive value of 83% and a sensitivity of 94%. Among 25 patients with CK-18 above 670 U/L, 16 had FGF21 above 332 pg/ml. Twelve of these 16 patients had NASH, yielding a positive predictive value of 75% and a specificity of 95%.

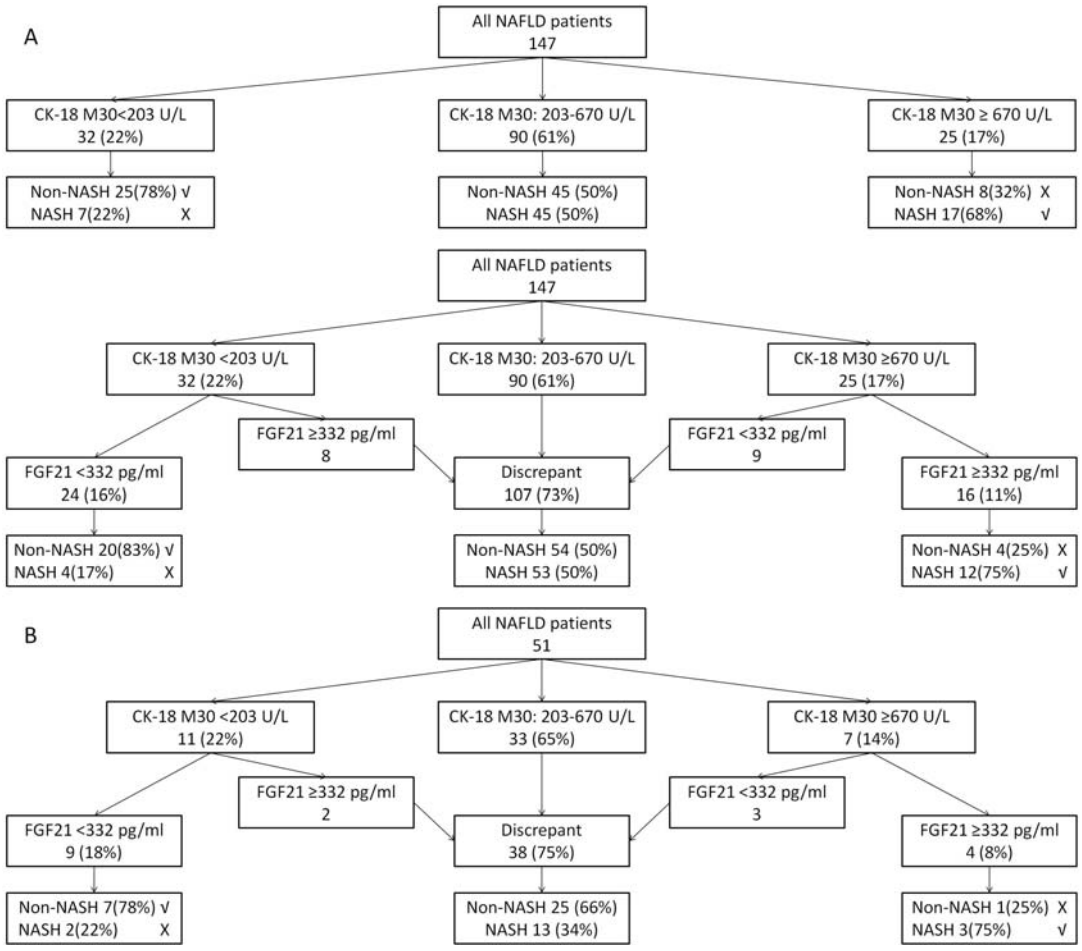
The 2-step approach was further validated in 51 patients with paired liver biopsies. When the 2-step approach was applied in this cohort, 7 of 9 patients with both biomarkers below the selected cutoffs did not have NASH, yielding a negative predictive value of 78% and sensitivity of 89%. Meanwhile, 3 of 4 patients with both biomarkers above the cutoffs had NASH, yielding a positive predictive value of 75% and specificity of 97% (Figure 3.9 B).

3.4.3 Summary

Combined application of CK-18 M30 and FGF21 could further improve the diagnostic accuracy in NASH than individual biomarkers. The 2-step approach provides an easy and accurate method in diagnosing and excluding NASH.

The predicted probability generated by logistic regression did not significantly improve overall accuracy; however, the equation itself would be too complex for clinical use. Simply by applying the optimal cutoff values did not achieve adequate predictive values either. By applying both optimal cutoffs of CK-18 M30 and FGF21, a NPV of 79% or a PPV of 69% can be achieved when both biomarker are lower or higher than the cutoff. However, all patients need both biomarkers tested; the overall cost would be high. After testing several strategies of combination, we found the 2-step approach would be cost-effective (Figure 3.9). In this model, patients with CK-18 M30 level in the gray zone would have inaccurate diagnosis even when another biomarker is added and should be considered for liver biopsy. In contrast, patients with CK-18 < 203 U/L or > 670 U/L can have the diagnosis of NASH further refined by the addition of FGF21 test. By doing so, a PPV and NPV of around 80% can be achieved. The adoption of this approach could potentially spare nearly 30% of NAFLD patients from liver biopsy. Only less than 40% of patients required both biomarkers evaluated. Also it can be well validated, both in the diagnostic accuracy and proportions of benefited population. Because of the high prevalence of NAFLD, this approach would have important clinical implications.

Figure 3.9. Diagnostic performance of two-step approach in NASH using CK-18 M30 and FGF21.



Prediction of NASH diagnosis by (A) CK-18 M30 only or a two-step approach combining CK-18 M30 and FGF21 in the main cohort, and (B) validation of the two-step approach in the validation cohort.

3.5 Summary of Chapter 3

In summary, CK-18 M30/M65/M65ED and FGF21 have high overall accuracy and can be used alone in diagnosing NAFLD. All of them have only moderate and similar accuracy in predicting NASH. The relatively high serum level of AFABP in control subjects limits its clinical use in NAFLD and NASH predicting.

Changes in CK-18 M30, M65 and M65ED correlate well with changes in NAS and can be used to predict progression to NASH. Changes in M65 and M65ED are also associated with progression of liver fibrosis. Notably, changes of M30 and M65ED have high accuracy of over 0.8 in predicting disease progression, indicating both biomarkers can be used for serial monitoring of disease progression. However, changes in AFABP and FGF21 do not correlate with changes in NAS or disease status.

Combine application of CK-18 M30 and FGF21 could further improve the diagnostic accuracy in NASH. A 2-step approach using CK-18 M30 and FGF21 can spare nearly 30% of NAFLD patients from liver biopsy. This approach would have important clinical implications based on the high prevalence of NAFLD.

Chapter 4: *PNPLA3* rs738409 gene polymorphism and non-alcoholic fatty liver disease

4.1 *PNPLA3* rs738409 gene polymorphism and non-alcoholic fatty liver disease in community and hospital patients

4.1.1 Background

Genetic determinants play an important role in NAFLD development. With advances in genome analysis, especially genome-wide association study (GWAS), genetic determinants of NAFLD are widely studied in recent years. The first and most important genetic factor associated with NAFLD is the nonsynonymous rs738409 I148M (C/G) variant located in human patatin-like phospholipase domain containing 3 gene (*PNPLA3*). It was first identified in a GWAS which included in over 2000 participants in the Dallas Heart Study [97]. Subsequent studies not only confirmed the association, but also showed that patients with the gene variant have more severe hepatic necroinflammation and fibrosis across different ethnic groups [98].

The expression of the PNPLA3 (also known as adiponutrin) protein is stimulated by high fat intake [195]. The protein hydrolyzes emulsified triglyceride in hepatocytes, and the I148M substitution abolishes the enzymatic activity [230-231]. This results in impaired secretion of very low density lipoproteins and hepatic insulin resistance

[232-233]. Moreover, patients with the I148M variant have reduced serum level of adiponectin [234], which is an adipokine that enhances insulin sensitivity and protects against obesity and NAFLD [235].

Although knowledge on *PNPLA3* rs738409 polymorphism has increased in the last few years, a number of important questions remain unanswered. First, many studies included NAFLD patients from hospital clinics. Some of these patients have heavy metabolic burden and have been treated with various methods. The impact of *PNPLA3* rs738409 polymorphism at the population level is uncertain. Second, previous studies were cross-sectional designed. The impact of *PNPLA3* rs738409 polymorphism on NAFLD progression is unknown.

The aim of this part of the study was to evaluate the association between *PNPLA3* rs738409 polymorphism and NAFLD in the general population. The association between *PNPLA3* rs738409 and NAFLD disease severity will also be studied in the histological cohort. We also aimed to investigate the impact of *PNPLA3* rs738409 on disease progression of NAFLD. For this purpose, the community cohort (922 subjects from community received ^{1}H-MRS evaluation), hospital NAFLD cohort (147 biopsy-proven NAFLD patients); and the prospective cohort (51 biopsy-proven NAFLD patients with prospectively scheduled paired liver biopsies 36 months apart) were enrolled in this part of the study. Details of the study subjects were described in Chapter 2, 2.2 and Figure 2.1.

4.1.2 Results

4.1.2.1 Patient characteristics

920 of 922 subjects in the community cohort and all 147 patients in the hospital cohort had sufficient blood samples for genomic DNA extraction. *PNPLA3* rs738409 allelic discrimination was successfully performed in all DNA samples. In all 1,067 study subjects, there were 400 (37.5%) CC homozygotes, 498 (46.7%) CG heterozygotes and 169 (15.8%) GG homozygotes. The alleles of *PNPLA3* rs738409 polymorphism were in Hardy-Weinberg equilibrium ($p = 0.782$).

In the community cohort, 366 (39.8%) subjects had the CC genotype, 429 (46.6%) had the CG genotype and 125 (13.6%) had the GG genotype. In the hospital cohort, 34 (23.1%) patients had the CC genotype, 69 (46.9%) had the CG genotype and 44 (29.9%) had the GG genotype. Allele G was more common in the hospital cohort than the community cohort ($p < 0.001$). In both cohorts, subjects with CC, CG or GG genotype had similar age, gender distribution, anthropometric measurements and history of metabolic syndrome. In the community cohort, allele G carriers had significantly higher blood ALT levels ($p = 0.010$); in the hospital cohorts, allele G carriers had significantly higher blood HDL levels ($p = 0.026$) (Table 4.1).

4.1.2.2 *PNPLA3* gene polymorphism and hepatic steatosis

In the community cohort, the median IHTG in subjects with GG genotype was 4.1% (1.5-9.8%), significantly higher than those with CC (1.7% [0.8-4.5%], $p < 0.001$) or CG genotype (2.0% [0.9-6.2%], $p < 0.001$). There was no significant difference in

IHTG between subjects with CC or CG genotype (p = 0.058). After excluding 30 subjects with significant alcohol consumption, 251 subjects were diagnosed as NAFLD and 639 subjects were healthy controls. The prevalence of GG genotype in control and community NAFLD was 10.6% and 20.7%, respectively (p < 0.001; Figure 4.1). Since subjects with CC and CG genotypes had similar degree of hepatic steatosis, these two groups were combined in the subsequent analysis.

In the hospital cohort, 17 of 44 (38.6%) patients with GG genotype had grade 3 steatosis, compared to 25 of 103 (24.3%) patients with CC or CG genotype (p = 0.037; Figure 4.2). The prevalence of GG genotype in hospital NAFLD patients was 29.9%, and was significantly higher than that in community NAFLD patients (20.7%; p = 0.038) and controls (10.6%; p < 0.001) (Figure 4.1).

Combining these two cohorts, there were 389 NAFLD and 639 control subjects in total. The overall accuracy of *PNPLA3* rs738409 polymorphism in predicting NAFLD diagnosis was 0.61 (95% CI: 0.57-0.64; p < 0.001, comparing with reference area = 0.5) estimated by ROC curve (Figure 4.3 A).

4.1.2.3 *PNPLA3* gene polymorphism and liver injury

In the community cohort, 759 patients had valid liver stiffness measured by transient elastography. Subjects with GG genotype had a median liver stiffness of 4.3 (3.5-5.5) kPa, which was similar to those with CC or CG genotype (4.2 [3.6-5.1] kPa, p =

0.672). Five of 101 (5.0%) subjects with the GG genotype and 30 of 658 (4.6%) subjects with the CC or CG genotypes had liver stiffness above 7.9 kPa (p = 0.800).

In the hospital cohort, GG genotype was not associated with the severity of lobular inflammation (p = 0.180) or ballooning (p = 0.520). However, it was significantly associated with the severity of fibrosis (p = 0.003) (Figure 4.2). Liver fibrosis was found in 75.0% of patients with GG genotype and 53.4% of those with CC or CG genotypes (p = 0.014). The prevalence of advanced fibrosis in patients with GG genotype and CC or GG genotype was 25.0% and 9.7%, respectively (p = 0.015).

In the hospital cohort, the AUROC of *PNPLA3* rs738409 polymorphism in differentiating NASH was 0.54 (0.45-0.64; p = 0.355) (Figure 4.3 B). The AUROC of *PNPLA3* rs738409 polymorphism in differentiating fibrosis and advanced fibrosis were 0.58 (0.48-0.67; p = 0.119) and 0.64 (0.51-0.77; p = 0.045), respectively (Figure 4.4).

4.1.2.4 *PNPLA3* gene polymorphism and disease progression

In the prospective cohort of 51 patients received paired liver biopsies (see patients' characteristics in Chapter 3, 3.3.2), 14 patients were with CC genotype, 24 were with CG genotype and 13 were with GG genotype. By Spearman's correlation, GG genotype was not significantly correlated with changes of NAS (correlation coefficient: -0.24; p = 0.093), disease status (correlation coefficient: -0.25; p = 0.076)

or fibrosis stage (correlation coefficient: -0.13; p = 0.357). *PNPLA3* rs738409 polymorphism could not predict worsening of NAS, disease progression from non-NASH to NASH or fibrosis progression by at least one stage in month 36. The AUROCs were 0.43 (0.27-0.59; p = 0.381), 0.39 (0.20-0.58; p = 0.305) and 0.47 (0.29-0.66; p = 0.768), respectively.

4.1.3 Summary

In this large population study, the *PNPLA3* rs738409 GG genotype conferred a 2-fold increase in the risk of NAFLD in the community. The magnitude of effect was similar to that observed in hospital NAFLD patients. At the population level, studies on the association between *PNPLA3* polymorphism and NAFLD were mainly conducted among children and adolescents, including 1 study from Taiwan using ultrasonography to detect fatty liver [194 222 236-237]. Our study adds to the current literature in using state-of-the-art non-invasive tests for hepatic steatosis and fibrosis in adults. We confirmed that subjects with the GG genotype had 2.4-fold increase in IHTG as compared with those with the CC genotype.

Although *PNPLA3* gene polymorphism did not affect liver stiffness in community subjects due to low prevalence of advanced fibrosis, the at risk genotype GG was associated with advanced fibrosis and cirrhosis in hospital NAFLD patients with liver biopsy. Furthermore, *PNPLA3* gene polymorphism had an overall accuracy of 0.64 in predicting advanced fibrosis. The hospital NAFLD cohort served as a

complement to the community cohort in this case.

PNPLA3 gene polymorphism was not associated with disease progression in the prospective cohort. However, these patients received lifestyle advice in a university centre, which might alter the natural history of the disease. These patients were also at a more advanced stage compared with the subjects in community cohort. Prospective studies based on the community cohort may better elucidate whether *PNPLA3* could affect disease progression.

Table 4.1. Clinical characteristics for subjects with different *PNPLA3* genotypes.

	Community cohort				Hospital cohort			
	All	CC	CG	GG	All	CC	CG	GG
Total number	920	366	429	125	147	34	69	44
Male gender, n (%)	389 (42.3)	148 (40.4)	185 (43.1)	56 (44.8)	65 (44.2)	10 (29.4)	34 (49.3)	21 (47.7)
Age (years)	48.1 (10.6)	48.3 (10.5)	47.6 (10.8)	49.3 (9.7)	47.7 (9.7)	45.3 (10.3)	47.8 (9.6)	49.5 (9.2)
BMI (kg/m^2)	22.8 (3.5)	22.9 (3.3)	22.7 (3.6)	23 (3.8)	27.4 (3.9)	29.2 (5.1)	27.0 (3.4)	26.7 (3.1)
Diabetes, n (%)	38 (4.1)	14 (3.8)	16 (3.8)	8 (6.4)	70 (47.6)	17 (50.0)	35 (50.7)	18 (40.9)
Hypertension, n (%)	142 (15.5)	44 (12.0)	75 (17.5)	23 (18.4)	63 (42.9)	13 (38.2)	30 (43.5)	20 (45.5)
Metabolic syndrome, n (%)	186 (20.2)	74 (20.2)	81 (18.9)	31 (24.8)	110 (74.8)	26 (76.5)	54 (78.3)	30 (68.2)
ALT (IU/L)*	26 (16)	25 (15)	26 (16)	30 (17)	73 (45)	68 (39)	69 (40)	83 (54)
Fasting glucose (mmol/L)	5.1 (0.9)	5.2 (0.9)	5.1 (0.9)	5.2 (1.0)	6.5 (2.4)	6.4 (2.5)	6.5 (1.7)	6.7 (3.3)
HbA_{1c} (%)	5.4 (0.6)	5.5 (0.6)	5.4 (0.7)	5.5 (0.7)	5.2 (1.0)	5.1 (1.0)	5.3 (1.1)	5.2 (0.9)
Total cholesterol (mmol/L)	5.2 (1.0)	5.2 (1.0)	5.1 (1.0)	5.3 (1.1)	2.2 (1.2)	2.4 (1.3)	2.2 (1.1)	2.0 (1.3)

HDL-C (mmol/L) [§]	1.5 (0.4)	1.5 (0.4)	1.5 (0.4)	1.5 (0.4)	1.2 (0.3)	1.1 (0.3)	1.2 (0.3)	1.3 (0.4)
LDL-C (mmol/L)	3.0 (0.9)	3.0 (0.9)	3.0 (0.9)	3.1 (0.9)	3.1 (0.9)	3.1 (0.9)	3.1 (0.9)	3.1 (0.9)
Triglyceride (mmol/L)	1.4 (1.2)	1.3 (1.0)	1.4 (1.4)	1.4 (1.1)	6.2 (1.3)	6.0 (1.0)	6.3 (1.3)	6.2 (1.5)

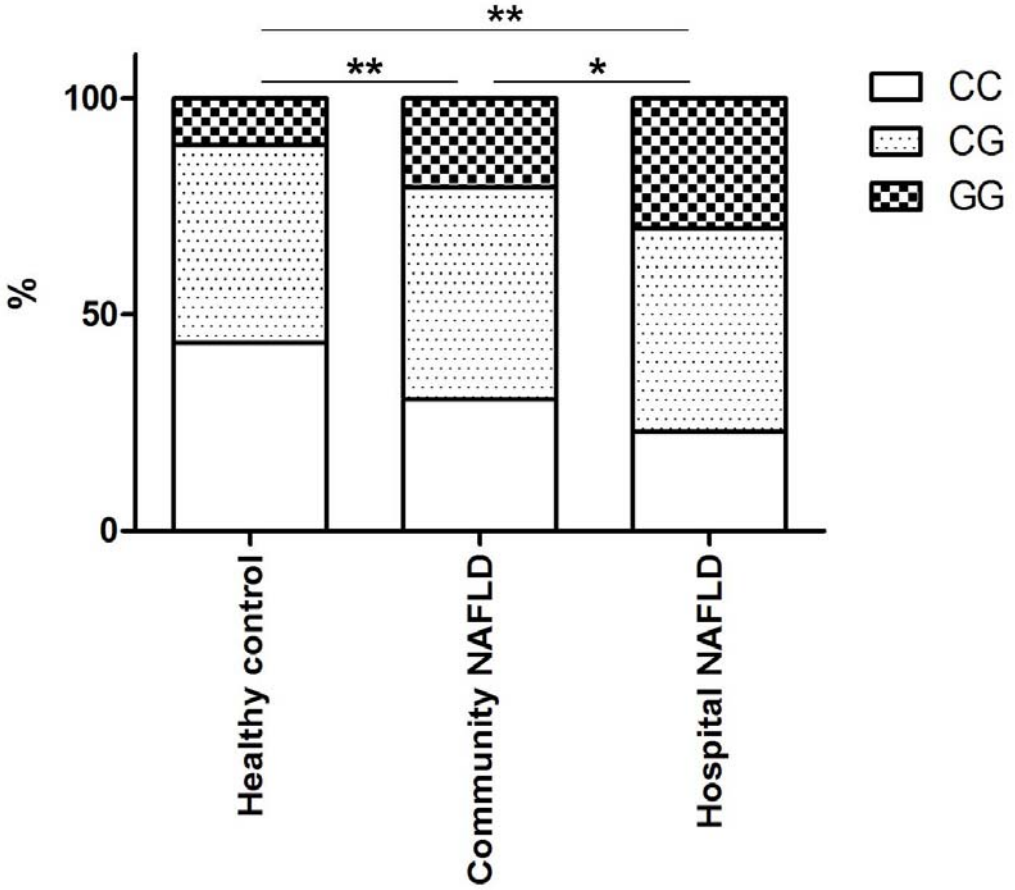
* Significant at $p < 0.05$, among subjects with different genotypes in community cohort;

[§] Significant at $p < 0.05$, among subjects with different genotypes in hospital cohort;

Numbers in parentheses are percentage for categorical data or standard deviation for numerical data.

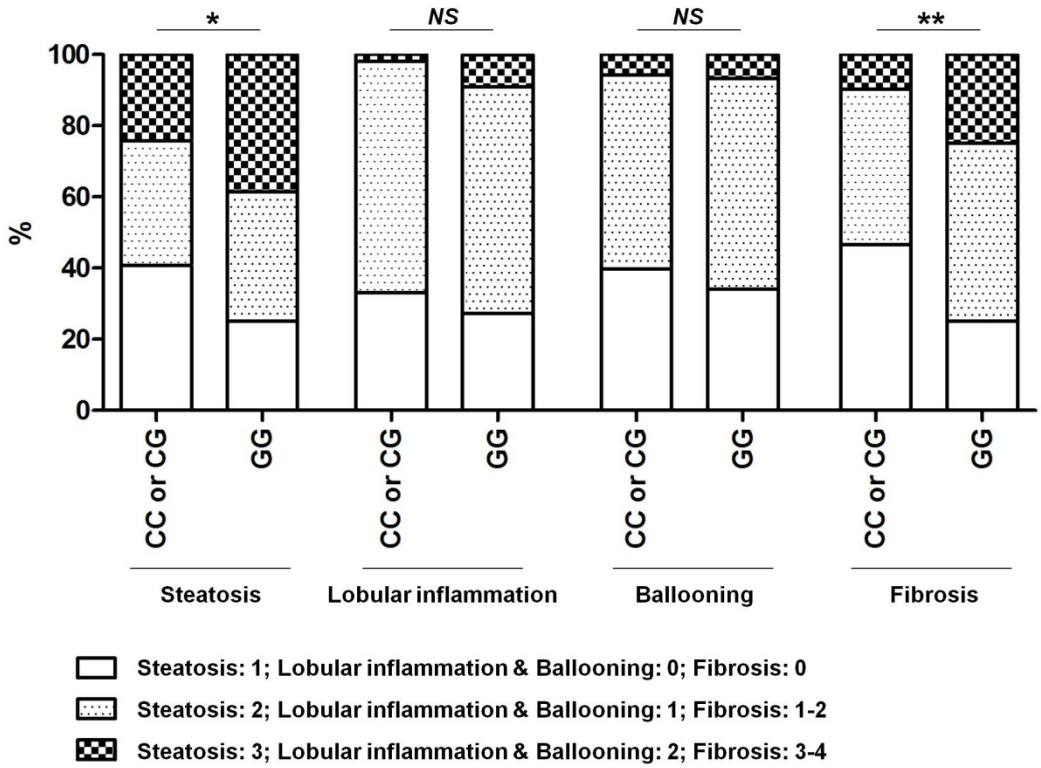
ALT, alanine aminotransferase; BMI, body mass index; HbA_{1c}, glycosylated hemoglobin, HDL-C, high density lipoprotein-cholesterol; LDL-C, low density lipoprotein-cholesterol

Figure 4.1. *PNPLA3* polymorphism in healthy control, community NAFLD and hospital NAFLD patients.



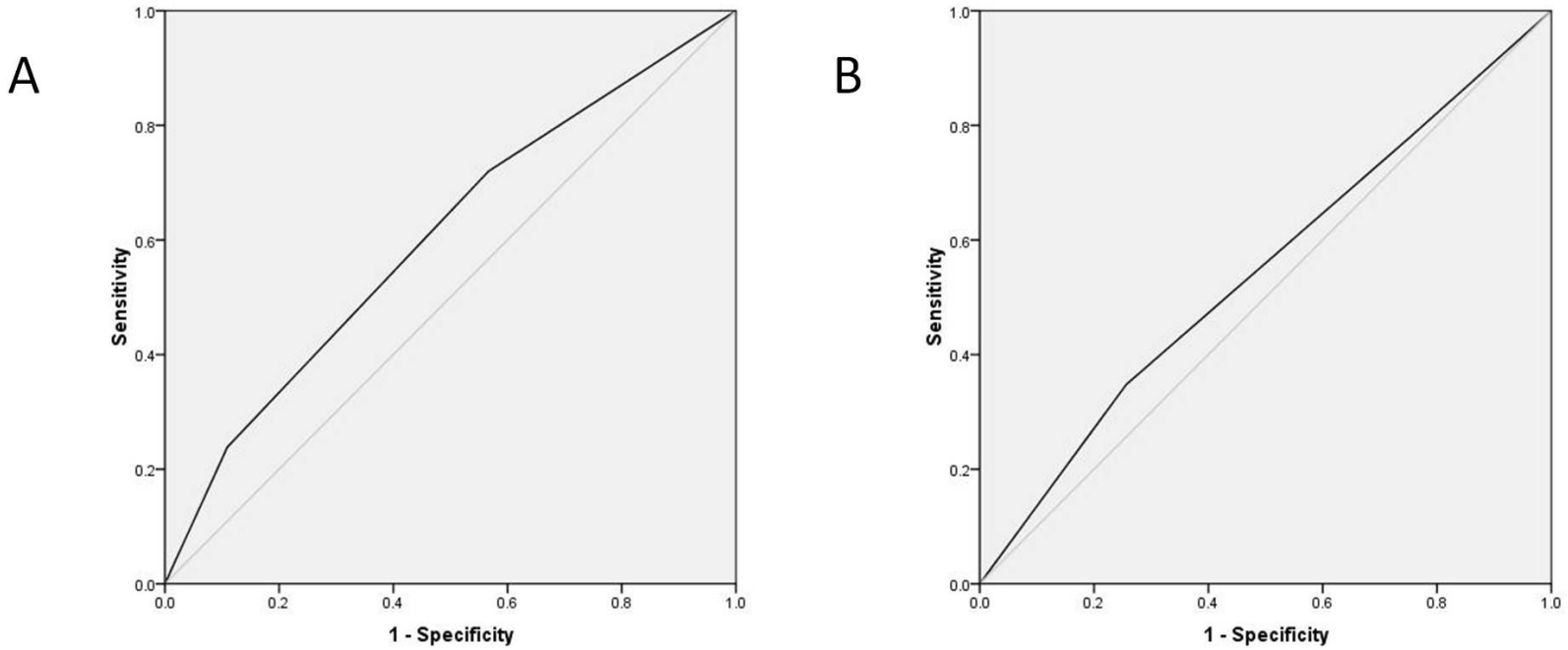
* Significant at $p < 0.05$, ** Significant at $p < 0.01$.

Figure 4.2. *PNPLA3* polymorphism and histological severity of disease in 147 hospital NAFLD patients.



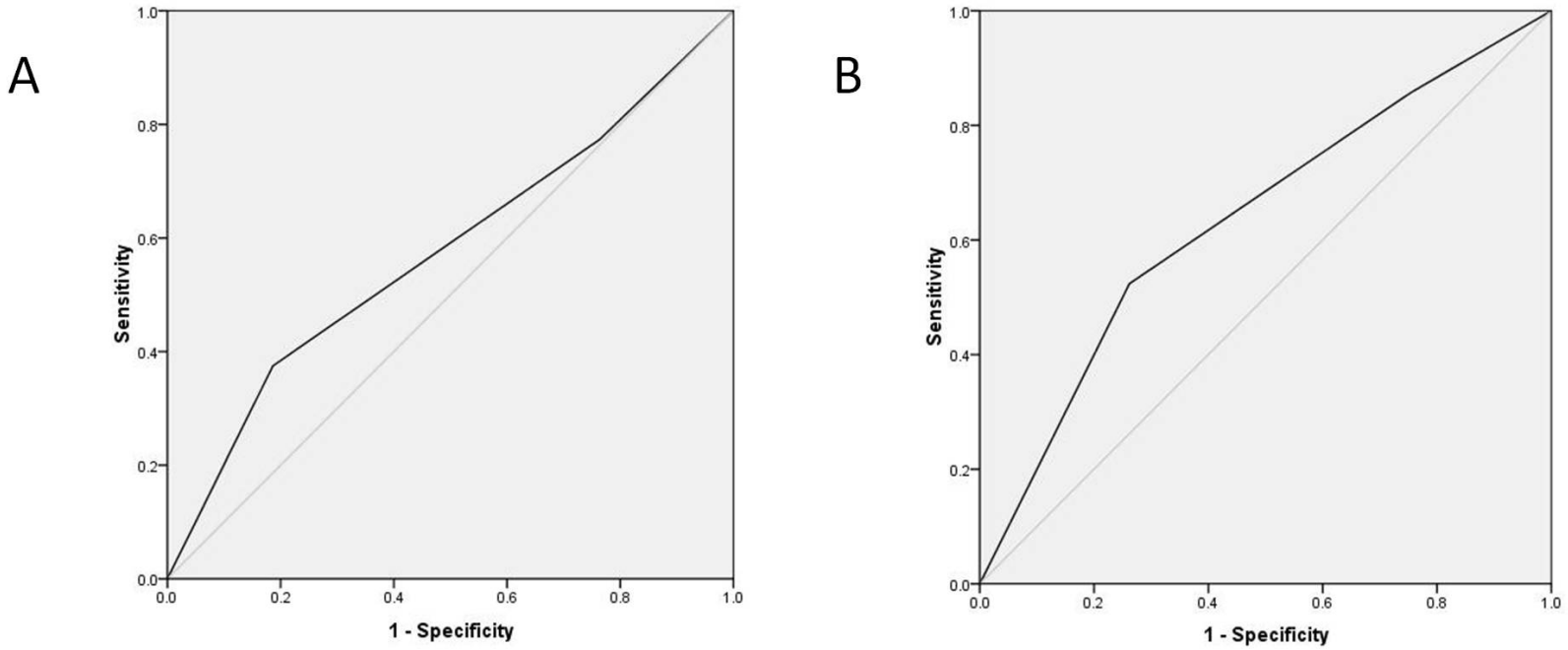
* Significant at $p < 0.05$; ** Significant at $p < 0.01$; *NS* Not statistically significant.

Figure 4.3. ROC curves of *PNPLA3* polymorphism in predicting NAFLD and NASH.



ROC curves in (A) distinguishing NAFLD patients in the community cohort + hospital NAFLD cohort; and (B) distinguishing NASH in the hospital NAFLD cohort.

Figure 4.4. ROC curves of *PNPLA3* polymorphism in predicting fibrosis in the hospital NAFLD cohort.



ROC curves in (A) distinguishing the presence of fibrosis; and (B) distinguishing the presence of advanced fibrosis in the hospital NAFLD cohort.

4.2 *PNPLA3* gene polymorphism, dietary pattern and non-alcoholic fatty liver disease

4.2.1 Background

Both *PNPLA3* gene polymorphism and western dietary pattern are associated with hepatic steatosis [77]. The expression of PNPLA3 protein can be stimulated by intake of high fat diet [195]; and *PNPLA3* gene polymorphism affects lipid metabolism [232-233]. However, it is not clear whether the gene variants can directly affect the dietary habit, thus contribute to the development of NAFLD. Meanwhile, the interaction between dietary habits and *PNPLA3* genotypes has also not been adequately evaluated.

In this part of the study, we aimed to evaluate the association of *PNPLA3* gene polymorphism, dietary pattern and NAFLD in general population. The community cohort (922 subjects from community received ^{1}H-MRS evaluation) were enrolled in this part of the study. Details of the study subjects were described in Chapter 2, 2.2 and Figure 2.1.

4.2.2 Results

4.2.2.1 Patient characteristics

The clinical and pathological were shown in Table 4.1. Details were described in 4.1.2.1.

4.2.2.2 *PNPLA3* and dietary pattern

Seven hundred and ninety-six subjects with sufficient clinical and dietary data were analyzed. There was no significant difference in the macronutrient intake among subjects with different *PNPLA3* genotypes in the whole community cohort (Table 4.2) or community NAFLD patients (Table 4.3). After adjusting for clinical parameters associated with NAFLD, *PNPLA3* genotype was found to be a predictor for NAFLD diagnosis independent of metabolic syndrome and macronutrient intake (Table 4.4). Subjects with the GG genotype had almost doubled risk (relative risk: 1.79; 95% CI: 1.10-2.90) of NAFLD compared with those with CC or CG genotype ($p = 0.019$). Among the macronutrients, less dietary fiber intake was associated with NAFLD.

An additive effect was observed between *PNPLA3* genotype and metabolic syndrome (Figure 4.5A). The prevalence of NAFLD was only 16.6% in subjects with CC or CG genotypes and no metabolic syndrome, and increased to 71.0% in those with both GG genotype and metabolic syndrome ($p < 0.001$). On the other hand, there was no interaction between *PNPLA3* genotype and dietary fiber intake (Figure 4.5B).

4.2.3 Summary

In this part of the study, we clearly demonstrated that *PNPLA3* rs738409 polymorphism was not associated with changes in dietary pattern. However, the GG genotype and low dietary fiber intake were independently associated with NAFLD.

Subjects with GG genotype did not develop fatty liver because of higher energy, carbohydrate or fat consumption. Rather, *PNPLA3* and dietary fat intake independently affect the development of NAFLD. In a small study of 153 Hispanic children, dietary carbohydrate and total sugar were associated with hepatic steatosis only in those with GG genotype but not in the CC or CG groups [222]. In another study of 127 children and adolescents of different ethnic background, dietary omega-6/omega-3 polyunsaturated fatty acids ratio was associated with fatty liver only in subjects with the GG genotype [223]. In this population based study, however, no interaction between *PNPLA3* gene polymorphism and diet was noted.

Table 4.2. Daily median macronutrient intake in community subjects.

	PNPLA3 genotype		
	CC (n=311)	CG (n=380)	GG (n=105)
Calories (kcal)	1964(1671;2424)	2017(1630;2391)	2116(1713;2576)
Protein (g)	81.7(65.5;101.3)	83.1(65.7;109.7)	84.9(71.0;111.6)
Carbohydrate (g)	244(198;302)	242(204;308)	259(205;315)
Fiber (g)	13.4(10.0;18.1)	14.1(10.5;18.3)	14.5(11.7;19.7)
Total fat (g)	71.5(57.5;89.2)	70.3(54.9;88.0)	75.8(57.8;91.7)
Saturated fat (g)	16.2(12.4;20.9)	15.6(12.2;20.2)	17.2(12.1;21.9)
Monounsaturated fat (g)	25.0(19.6;32.1)	25.2(18.7;31.0)	26.4(19.3;34.7)
Polyunsaturated fat (g)	15.9(11.3;21.4)	14.9(11.0;21.3)	15.7(12.1;21.7)
Cholesterol (mg)	273(198;367)	267(196;358)	289(209;360)
Alcohol (g)	0 (0;0)	0 (0;0)	0(0;0)

Numbers in parentheses are IQRs.

Table 4.3. Daily median macronutrient intake in community NAFLD patients.

	PNPLA3 genotype		
	CC (n=63)	CG (n=115)	GG (n=42)
Calories (kcal)	2077(1710;2502)	1970(1559;2440)	2163(1837;2668)
Protein (g)	83.4(70.9;107.2)	83.1(63.5;110.6)	93.9(73.8;123.5)
Carbohydrate (g)	250(198;337)	236(195;315)	245(205;330)
Fiber (g)	13.5(9.3;20.2)	14.8(9.6;17.3)	14.6(11.4;20.4)
Total fat (g)	74.6(58.3;89.9)	73.4(53.9;88.6)	78.8(62.3;100.8)
Saturated fat (g)	17.3(13.0;22.0)	16.2(11.8;20.2)	17.8(12.1;25.7)
Monounsaturated fat (g)	25.8(19.6;33.2)	26.8(19.3;32.3)	32.5(20.2;36.4)
Polyunsaturated fat (g)	16.9(11.0;21.4)	15.3(10.3;21.6)	17.4(13.5;23.7)
Cholesterol (mg)	299(200;401)	269(183;361)	293(210;407)
Alcohol (g)	0(0;0)	0(0;0)	0(0;0)

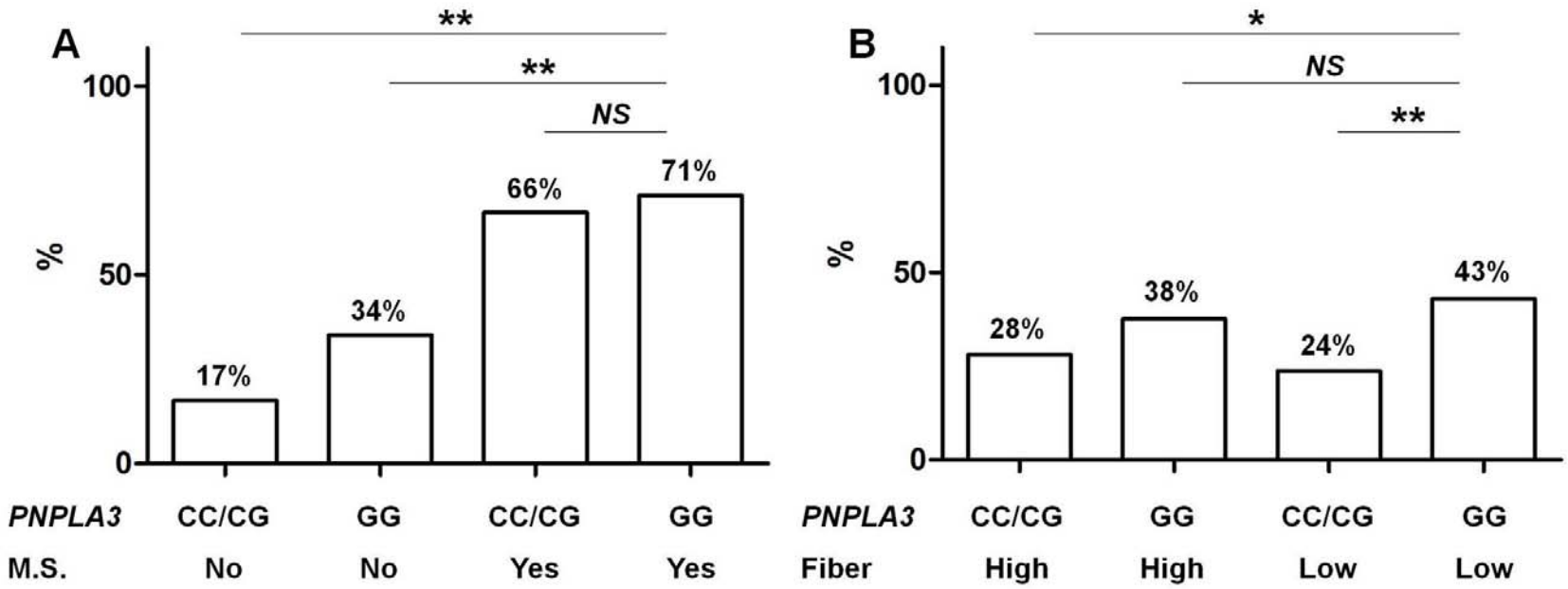
Numbers in parentheses are IQRs.

Table 4.4. Multivariable analysis for NAFLD diagnosis in community subjects.

Dependent	Independent risk factor	RR	95% CI	*p* value
NAFLD	*PNPLA3* (GG to CC or CG)	1.79	1.10-2.90	0.019
	Gender (male to female)	1.90	1.33-2.72	<0.001
	Age	1.02	1.00-1.04	0.016
	Metabolic syndrome (yes to no)	9.43	6.17-14.40	<0.001
	Adjusted dietary fiber	0.96	0.93-1.00	0.028

Parameters entered: *PNPLA3*; gender; age; metabolic syndrome; energy adjusted intake of protein, carbohydrate, fiber, total fat, saturated fat, monounsaturated fat, polyunsaturated fat and cholesterol intake.

Figure 4.5. NAFLD prevalence in patients with different *PNPLA3* genotype and A. with or without metabolic syndrome (M.S.); B. with dietary fiber intake ≥ median (High) or < median (Low).



* Significant at $p < 0.05$; ** Significant at $p < 0.01$; *NS* Not statistically significant.

4.3 *PNPLA3* gene polymorphism and lifestyle intervention in patients with non-alcoholic fatty liver disease

4.3.1 Background

Lifestyle intervention is one of the major management options for NAFLD patients [3]. It may reduce aminotransferases and improve hepatic steatosis, as well as the histological severity of NAFLD. Since *PNPLA3* gene polymorphism has a close relationship with lipid metabolism, it is possible that the gene variants may affect the response to lifestyle intervention. In a pilot study, Sevastianova et al. treated 18 non-diabetic NAFLD patients (8 GG homozygotes and 10 CC homozygotes) with hypocaloric low-carbohydrate diet for 6 days [224]. GG homozygotes exhibited significantly greater reduction (45%) in IHTG than CC homozygotes (18%). The intriguing results need confirmation in bigger datasets. Moreover, the diet used in this pilot study was tightly controlled. It is unlikely to adopt such dietary treatment in general community to achieve a sustainable lifestyle. The impact of *PNPLA3* gene polymorphism on the response of long-term lifestyle intervention is also unknown.In this part of the study, we aimed to test whether *PNPLA3* gene polymorphism may affect the response of lifestyle intervention in NAFLD patients. The lifestyle intervention cohort (154 NAFLD patients derived from the community cohort who received lifestyle intervention or routine care for 12 months) were enrolled in this part of the study. Details of the study subjects were described in Chapter 2, 2.2 and Figure 2.1.

4.3.2 Results

4.3.2.1 Patient characteristics

One hundred and fifty-four subjects with both NAFLD and abnormal ALT level from community cohort joined the trial. 77 patients were randomized to the lifestyle modification programme and 77 received usual care. Three patients in the intervention group and 6 in the control group were lost to follow-up, but all randomized patients were included in the intention-to-treat analysis. Missing values were treated using the last-observation-carried-forward method and were considered failure for that outcome. At baseline, the two groups were well-matched in demographic characteristics, clinical and laboratory data, IHTG and liver stiffness measurements (Table 4.5). Each group had 22 *PNPLA3* rs738409 CC homozygotes, 38 CG heterozygotes and 17 GG homozygotes. In the intervention group, hypertension was less common among GG carriers. In the control group, GG carriers had lower body weight and smaller waist circumference. Other clinical-pathological features were all similar.

64 (83%) patients in the intervention group attended more than 80% of the dietary consultation sessions. 65 (84%) patients in the intervention group attended exercise consultation sessions. The patients in the intervention group or control group exercised for 56 ± 49 minutes or 45 ± 52 minutes per week at baseline, respectively (p = 0.19); and 62 ± 56 minutes or 58 ± 60 minutes at month 12, respectively (p =

0.63). During the study period, none of the patients were started on insulin sensitizers, weight loss agents, lipid lowering drugs, vitamin E and omega-3 supplements.

4.3.2.2 Primary outcome

The primary outcome of remission of NAFLD at month 12 occurred in 49 of 77 (64%) patients in the intervention group and 15 of 77 (20%) patients in the control group ($p = 0.001$) (Table 4.6). In the intervention group, 11 (50%) of patients with CC genotype, 26 (68%) patients with CG genotype and 12 (71%) of patients with GG genotype had remission of NAFLD ($p = 0.165$). In the control group, 4 (18%) of patients with CC genotype, 6 (16%) patients with CG genotype and 5 (29%) of patients with GG genotype had remission of NAFLD ($p = 0.426$). By multivariable logistic regression, *PNPLA3* gene polymorphism did not emerge as an independent predictor for NAFLD remission in either group (Table 4.7).

4.3.2. 3 Secondary outcomes

At month 12, IHTG was significantly lower in the intervention group (5.5 ± 5.9%) than the control group (10.1 ± 6.7%; $p < 0.001$) (Table 4.6). In the intervention group, patients with CC, CG, and GG genotype had IHTG of 7.1 ± 8.0%, 5.2 ± 5.2% and 4.3 ± 4.2%, respectively ($p = 0.306$). In the control group, patients with CC, CG, and GG genotype had IHTG of 9.9 ± 6.6%, 10.6 ± 6.8% and 9.0 ± 6.8%, respectively ($p = 0.697$).

The mean reduction in IHTG from baseline to month 12 was 6.7 ± 6.1% in the intervention group and 2.1 ± 6.4% in the control group ($p < 0.001$). In the intervention group, patients with CC, CG, and GG genotype had reduction in IHTG of 3.7 ± 5.2%, 6.5 ± 3.6% and 11.3 ± 8.8%, respectively. The reduction in IHTG correlated significantly with the presence of allele G in the intervention group (Pearson correlation, 0.44; $p < 0.001$) (Figure 4.6 A). In the control group, patients with CC, CG, and GG genotype had reduction in IHTG of 2.0 ± 4.6%, 0.8 ± 5.7% and 5.2 ± 7.2%, respectively (Pearson correlation, 0.16; $p = 0.163$). By multivariable linear regression, *PNPLA3* gene polymorphism (Beta = 2.97 [95%CI: 1.19-4.76]; $p = 0.001$) and reduction in BMI (Beta = 2.05 [1.12-2.99]; $p < 0.001$) were independently associated with reduction in IHTG (Table 4.7). Reduction in BMI was also independently associated with reduction in IHTG in the control group (Beta = 1.56 [95%CI: 0-3.12]; $p = 0.050$).

In addition, patients in the intervention group had greater reduction in body weight ($p <0.001$), BMI ($p = 0.014$) and waist circumference ($p < 0.001$) (Table 4.6). 59 (77%) patients in the intervention group and 23 (30%) patients in the control group had weight loss of 3% or more ($p < 0.001$). 30 (39%) patients in the intervention group and no patient in the control group had weight loss of 10% or more ($p < 0.001$). Furthermore, the intervention group had greater reduction in ALT ($p = 0.011$) and liver stiffness ($p = 0.016$) than the control group (Table 4.6). 41 (53%) patients

in the intervention group and 19 (25%) patients in the control group achieved ALT normalization at month 12 ($p < 0.001$); 30 (39%) patients in the intervention group and 5 (7%) patients in the control group achieved both NAFLD remission and ALT normalization at month 12 ($p < 0.001$).

In the intervention group, the presence of allele G was significantly correlated with greater reduction in body weight ($p = 0.022$), BMI ($p = 0.042$), waist circumference ($p = 0.021$), waist-to-hip ratio ($p = 0.012$) and total cholesterol ($p = 0.022$) (Table 4.6 and Figure 4.6 B-F upper panels). Body weight and BMI were significantly reduced in patients with all genotypes at month 12; waist circumference and total cholesterol were significantly reduced in patients with CG and GG genotype; waist-to-hip ratio was significantly reduced only in patients with GG genotype (Figure 4.6 B-F lower panels). In the control group, the *PNPLA3* gene polymorphism did not correlate with any changes in the secondary outcomes (Table 4.6).

PNPLA3 gene polymorphism was independently associated with IHTG reduction in the intervention group ($p = 0.001$) (Table 4.7). The presence of each allele G brought 2.97% (95%CI: 1.19-4.76%) more absolute reduction in IHTG. Reduction in BMI was associated with IHTG reduction in both the intervention and control groups.

4.3.3 Summary

The community-based lifestyle modification programme resulted in 44% more remission of NAFLD compared with control group. Remarkably, 64% of patients in the intervention group achieved NAFLD remission. The baseline features were comparable among study subjects with different genotypes in *PNPLA3* rs738409. Although *PNPLA3* gene polymorphism did not correlated with the remission of NAFLD, it significantly correlated with several secondary outcomes including the reduction of IHTG, body weight, BMI, waist circumference, waist-hip-ratio, and total cholesterol. In the intervention group each allele G brought almost 3% more absolute reduction in IHTG, translating to nearly 6% more reduction in IHTG in GG homozygotes compared with CC homozygotes.

Here, we convincingly showed that subjects with allele G in *PNPLA3* rs738409 gene are more sensitive to lifestyle intervention. Because the GG genotype is associated with more severe disease, these patients may receive additional benefit from lifestyle intervention programme.

Table 4.5. Baseline clinical and pathological characteristics for subjects joined lifestyle intervention trial.

	Intervention group (n=77)				Control group (n=77)			
PNPLA3	All	CC(n=22)	CG(n=38)	GG(n=17)	All	CC(n=22)	CG(n=38)	GG(n=17)
Demographics and baseline measurements								
Age (years)	51(9)	50(10)	53(9)	49(7)	51(9)	51(9)	51(8)	50(9)
Male gender	41(52)	10(46)	19(50)	10(59)	31(41)	12(55)	12(32)	8(47)
Body weight (kg)[§]	70.6(11.9)	72.3(12.0)	68.4(10.3)	73.4(14.6)	68.4(9.8)	72.3(8.7)	67.9(9.7)	64.2(9.8)
BMI (kg/m^2)	25.5(3.9)	26.1(2.9)	25(3.5)	25.8(5.5)	25.3(3.2)	26.3(2.8)	25.3(3.4)	23.9(2.9)
Male[§]	24.9(3.4)	26.4(3.7)	24.3(3.1)	24.8(3.5)	25.1(2.2)	25.9(2.3)	24.7(2.2)	24.4(2.0)
Female	26.1(4.2)	26.0(2.1)	25.7(3.7)	27.2(7.7)	25.4(3.7)	26.8(3.4)	25.6(3.8)	23.4(3.5)
Waist circumference (cm)[§]	89(9)	91(10)	89(8)	87(9)	88(8)	92(7)	87(7)	85(10)
Male	89(9)	91(13)	90(8)	86(7)	90(5)	93(5)	89(4)	87(3)

Female	89(8)	91(7)	88(8)	89(11)	87(9)	90(8)	86(8)	84(13)
Waist-to-hip ratio	0.91(0.05)	0.91(0.06)	0.92(0.05)	0.89(0.03)	0.90(0.06)	0.91(0.05)	0.90(0.06)	0.90(0.07)
Male	0.92(0.06)	0.90(0.07)	0.93(0.05)	0.90(0.03)	0.92(0.05)	0.94(0.04)	0.91(0.04)	0.91(0.06)
Female	0.91(0.05)	0.92(0.06)	0.90(0.05)	0.89(0.02)	0.89(0.07)	0.88(0.06)	0.89(0.06)	0.90(0.09)
SBP (mmHg)	136(22)	133(15)	141(23)	131(25)	136(21)	137(20)	136(23)	132(18)
DBP (mmHg)	87(13)	85(10)	90(14)	82(12)	86(13)	89(11)	86(13)	82(15)
ALT (IU/l)	43(28)	43(25)	43(33)	45(18)	40(23)	41(26)	37(19)	44(26)
AST (IU/l)	26(12)	27(10)	26(14)	27(10)	25(12)	23(10)	25(12)	28(12)
AST/ALT ratio	0.71(0.31)	0.70(0.25)	0.74(0.38)	0.64(0.19)	0.70(0.21)	0.64(0.19)	0.75(0.23)	0.68(0.15)
Alkaline phosphatase (IU/l)	66(25)	58(26)	72(23)	63(26)	66(19)	65(17)	67(21)	65(17)
Fasting glucose (mmol/l)	5.4(1.1)	5.5(0.7)	5.5(1.4)	5.2(0.5)	5.6(1.5)	5.5(1.8)	5.8(1.7)	5.2(0.7)
HbA_{1c} (%)	5.7(0.8)	5.7(0.4)	5.7(1.0)	5.7(0.3)	5.8(1.0)	5.9(1.0)	5.8(1.0)	5.8(1.1)
Total cholesterol (mmol/l)	5.2(1.0)	5.3(1.0)	5.2(1.0)	5.1(0.9)	5.5(1.1)	5.6(0.8)	5.4(1.1)	5.3(1.2)

HDL-cholesterol (mmol/l)	1.3(0.3)	1.3(0.4)	1.4(0.3)	1.3(0.1)	1.3(0.3)	1.3(0.3)	1.3(0.3)	1.3(0.3)
LDL-cholesterol (mmol/l)	3.2(1.1)	3.2(0.9)	3.2(1.3)	3.2(0.8)	3.3(0.9)	3.4(0.8)	3.2(0.8)	3.4(1.1)
Triglycerides (mmol/l)	1.8(1.4)	2.0(1.3)	1.9(1.6)	1.5(0.6)	2.2(2.4)	2.1(1.5)	2.3(3.2)	1.9(0.9)
IHTG (%)	12.3(6.6)	10.8(5.6)	11.7(6.5)	15.6(7.2)	12.2(6.8)	11.9(6.3)	11.4(5.0)	14.2(10.4)
Liver stiffness (kPa)[a]	5.1(1.8)	4.8(1.4)	5.3(2.1)	4.8(1.4)	5.0(1.7)	5.0(1.5)	5.1(2.0)	5.0(1.0)
Medical history								
Type 2 diabetes	4(5)	1(5)	2(5)	1(6)	8(10)	1(5)	4(11)	3(18)
Hypertension**	23(30)	4(18)	18(47)	1(6)	21(27)	4(18)	11(29)	6(35)
Current smoking	6(8)	3(14)	2(5)	1(6)	11(14)	5(23)	3(8)	2(12)
Median (range) alcohol consumption (g per week)	0(0-50)	0(0-50)	0(0-60)	0(0-0)	0(0-60)	0(0-0)	0(0-40)	0(0-0)

Drug history								
Metformin	2(3)	2(9)	0	0	5(7)	0	3(8)	2(12)
Sulphonylurea	1(1)	1(5)	0	0	4(5)	0	2(5)	2(12)
Thiazolidinedione	0	0	0	0	0	0	0	0
Insulin	0	0	0	0	0	0	0	0
Statin	3(4)	1(5)	2(5)	0	6(8)	0	5(13)	1(6)

Values are mean (SD) or numbers (percentages) unless otherwise specified.

a. Included 65 patients in the intervention group and 73 patients in the control group with reliable liver stiffness measurement.

** Significant at $p < 0.01$, among patients with different *PNPLA3* genotypes in intervention group.

§ Significant at $p < 0.05$, among patients with different *PNPLA3* genotypes in control group.

SBP, systolic blood pressure; DBP, Diastolic blood pressure; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; HDL, high density lipoprotein; LDL, low density lipoprotein; IHTG, intrahepatic triglyceride content.

Table 4.6. Study outcomes and metabolic changes at month 12.

	Intervention group (n=77)				Control group (n=77)			
PNPLA3	All	CC (n=22)	CG (n=38)	GG (n=17)	All	CC (n=22)	CG (n=38)	GG (n=17)
Body weight (kg)[§]	65.0 (11.0)	67.9 (13.0)	63.1 (8.5)	65.7 (12.6)	67.8 (9.9)	72.0 (9.3)	67.3 (9.5)	63.3 (10.1)
Change in body weight (kg)*††	-5.6 (4.4)	-4.4 (4.1)	-5.4 (4.3)	-7.7 (4.6)	-0.6 (2.5)	-0.3 (2.3)	-0.7 (2.6)	-0.9 (2.5)
BMI (kg/m^2)[§]	24.0 (5.7)	24.5 (3.0)	23.1 (3.2)	23.1 (4.8)	25.4 (4.5)	26.1 (2.8)	25.0 (3.2)	23.6 (3.1)
Change in BMI (kg/m^2)*†	-1.5 (4.5)	-1.6 (1.5)	-1.9 (1.5)	-2.7 (1.8)	0.2 (3.8)	-0.2 (0.8)	-0.3 (1.0)	-0.3 (0.9)
WC (cm)*[§§]†	86 (9)	90 (10)	86 (8)	83 (9)	89 (8)	92 (6)	90 (7)	84 (9)
Change in WC (cm)* ††	-3.0 (6.0)	-0.6 (6.0)	-3.2 (6.3)	-5.5 (6.2)	1.0 (5.0)	0.3 (4.7)	2.7 (5.9)	-1.5 (4.3)
WHR*[§]	0.90 (0.06)	0.92 (0.06)	0.90 (0.05)	0.86 (0.05)	0.91 (0.06)	0.90 (0.05)	0.92 (0.07)	0.87 (0.06)
Change in WHR*	0 (0.06)	0.01 (0.06)	-0.01 (0.06)	-0.04 (0.04)	0 (0.06)	0 (0.04)	0.02 (0.06)	-0.03 (0.04)
ALT (IU/l)††	26 (13)	27 (13)	24 (13)	31 (15)	33 (17)	37 (24)	30 (11)	33 (16)

Change in ALT (IU/l)†	-17 (30)	-17 (22)	-18 (36)	-14 (20)	-7 (19)	-4 (16)	-7 (17)	-11 (25)
AST (IU/l)	22 (8)	21 (5)	21 (7)	25 (11)	22 (8)	22 (10)	22 (5)	24 (9)
Change in AST (IU/l)	-4 (12)	-7 (9)	-4 (15)	-2 (7)	-3 (11)	-2 (6)	-3 (13)	-4 (9)
Fasting glucose (mmol/l)	5.4 (1.3)	5.6 (0.9)	5.4 (1.6)	5.0 (0.4)	5.7 (1.3)	5.7 (1.0)	5.7 (1.3)	5.5 (1.7)
Change in fasting glucose (mmol/l)	0 (0.5)	0.1 (0.6)	0 (0.5)	-0.2 (0.4)	0.1 (1.3)	0.2 (1.8)	0 (1.0)	0.3 (1.4)
Total cholesterol (mmol/l)**	4.9 (0.9)	5.4 (0.9)	4.8 (0.8)	4.6 (0.8)	5.2 (0.9)	5.3 (0.7)	5.3 (1.1)	5.0 (1.0)
Change in total cholesterol (mmol/l)*	-0.3 (0.8)	0 (0.8)	-0.4 (0.8)	-0.5 (0.6)	-0.3 (0.7)	-0.3 (0.5)	-0.2 (0.7)	-0.4 (0.9)
HDL-c (mmol/l)	1.4 (0.3)	1.4 (0.3)	1.4 (0.3)	1.4 (0.3)	1.3 (0.3)	1.3 (0.3)	1.4 (0.3)	1.3 (0.3)
Change in HDL-c (mmol/l)	0.1 (0.2)	0.1 (0.2)	0.1 (0.2)	0.2 (0.3)	0 (0.2)	0 (0.2)	0.1 (0.2)	0 (0.2)
LDL-c (mmol/l)*†	2.9 (0.8)	3.3 (0.8)	2.7 (0.8)	2.6 (0.7)	3.1 (0.8)	3.1 (0.7)	3.2 (0.9)	3.0 (0.8)
Change in LDL-c (mmol/l)	-0.3 (1.0)	0 (0.7)	-0.4 (1.2)	-0.5 (0.4)	-0.2 (0.6)	-0.2 (0.6)	0 (0.5)	-0.4 (0.9)

Triglycerides (mmol/l)	1.5 (1.4)	1.9 (2.3)	1.3 (1.0)	1.1 (0.6)	1.7 (1.0)	1.9 (1.5)	1.5 (0.8)	1.4 (0.5)
Change in triglycerides (mmol/l)	-0.3 (1.5)	0 (2.6)	-0.5 (0.9)	-0.4 (0.7)	-0.5 (2.1)	-0.1 (0.9)	-0.7 (2.9)	-0.5 (0.6)
IHTG (%)††	5.5 (5.9)	7.1 (8.0)	5.2 (5.2)	4.3 (4.2)	10.1 (6.7)	9.9 (6.6)	10.6 (6.8)	9.0 (6.8)
Change in IHTG (%)**††	-6.7 (6.1)	-3.7 (5.2)	-6.5 (3.6)	-11.3 (8.8)	-2.1 (6.4)	-2.0 (6.4)	-0.8 (5.7)	-5.2 (7.2)
Liver stiffness (kPa)[a]†	4.6 (1.4)	4.8 (1.2)	4.4 (1.6)	4.7 (1.4)	5.2 (1.9)	5.5 (1.8)	5.2 (2.2)	4.7 (1.3)
Change in liver stiffness (kPa)†	-0.5 (1.4)	0 (1.1)	-0.9 (1.3)	0 (1.5)	0.2 (1.7)	0.5 (1.7)	0.2 (1.9)	-0.2 (1.2)
Resolution of NAFLD[b]††	49 (64)	11 (50)	26 (68)	12 (71)	15 (20)	4 (18)	6 (16)	5 (29)
ALT normalization[c]††	41 (53)	13 (59)	20 (53)	8 (47)	19 (25)	6 (27)	7 (18)	6 (35)
Resolution of NAFLD and ALT normalization††	30(39)	8 (36)	15 (40)	7 (41)	5(7)	2 (9)	2 (5)	1 (6)

Values are mean (SD) or numbers (percentages).

One way ANOVA for measurements at month 12; binary pearson correlation test for changes at month 12; and linear-by-linear association for categorical variables.

a. Included 65 patients in the intervention group and 73 patients in the control group with reliable liver stiffness measurement.

b. Resolution of NAFLD was defined as IHTG less than 5.0% at month 12.

c. ALT normalization was defined as ALT below 30 IU/l in men and 19 IU/l in women at month 12.

* Significant at $p < 0.05$, ** Significant at $p < 0.01$, among patients with different *PNPLA3* genotypes in intervention group.

§ Significant at $p < 0.05$, §§ Significant at $p < 0.01$, among patients with different *PNPLA3* genotypes in control group.

† Significant at $p < 0.05$, †† Significant at $p < 0.01$, between patients in intervention group and control group.

WC, waist circumstance; WHR, Waist-to-hip ratio ; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; HDL-c, high density lipoprotein-cholesterol; LDL-c, low density lipoprotein-cholesterol; IHTG, intrahepatic triglyceride content.

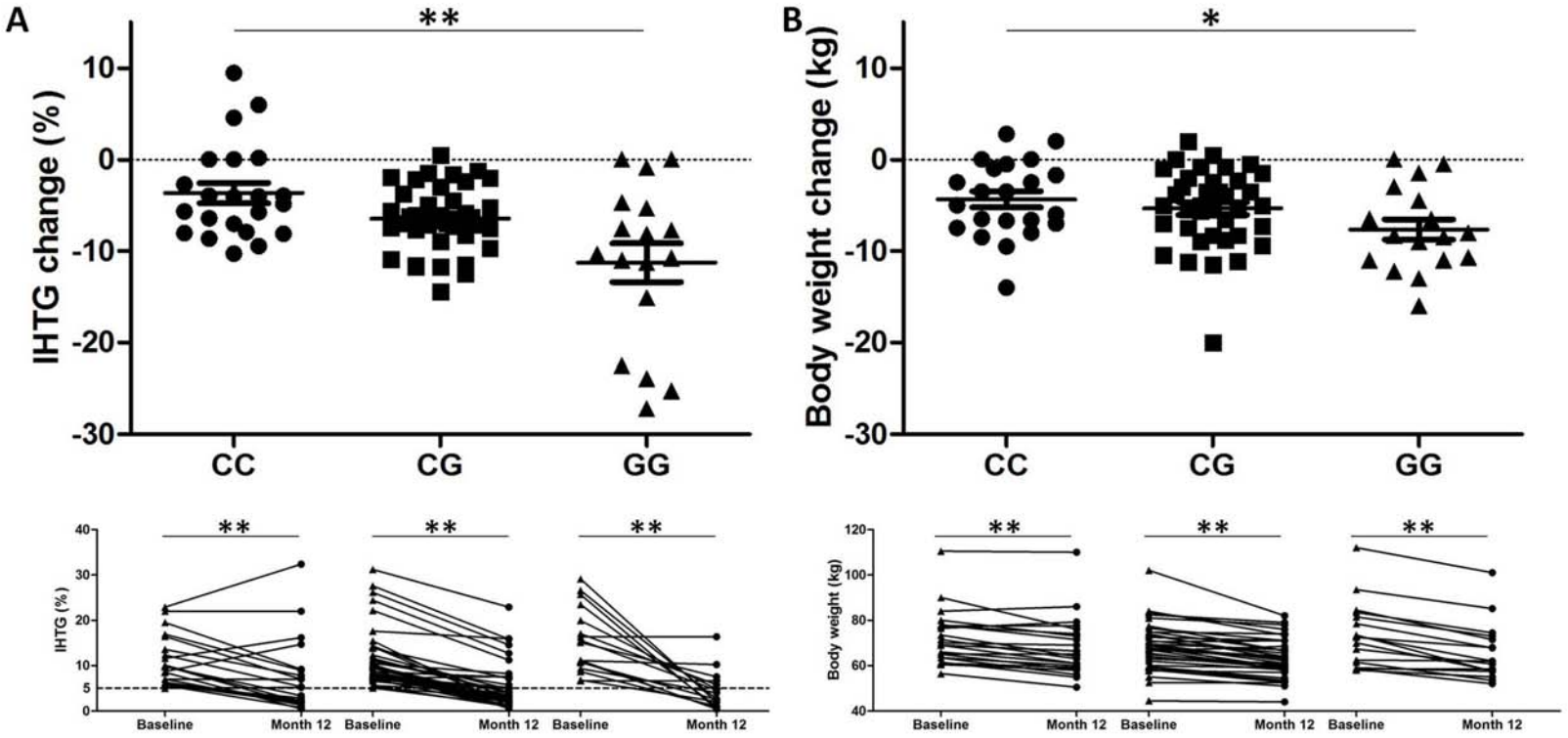
Table 4.7. Independent predictors for NAFLD remission and changes in IHTG in the intervention group and control group.

	Intervention group				Control group			
Outcomes	Factor(s)	RR	95% CI	*p* value	Factor(s)	RR	95% CI	*p* value
NAFLD remission[a]	BMI reduction	3.20	1.82-5.63	<0.001	ALT reduction	0.96	0.92-1.00	0.042
IHTG reduction[b]	*PNPLA3*	2.97	1.19-4.76	0.001	BMI reduction	1.56	0-3.12	0.050
	BMI reduction	2.05	1.12-2.99	<0.001				

a. By binary logistic regression; b. By multivariable linear regression

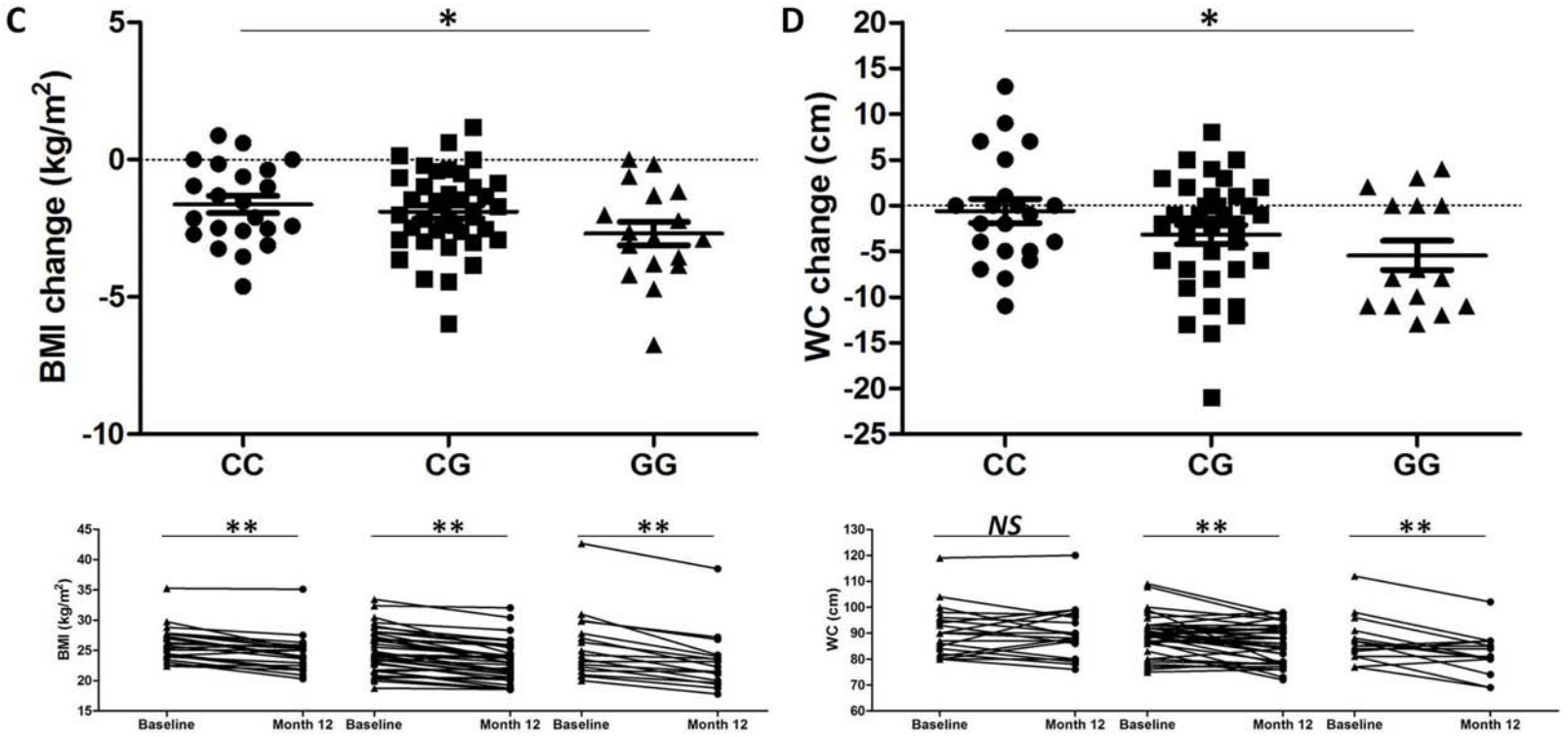
Variables entered on regression models: *PNPLA3* genotype (CC=1, CG=2, GG=3), gender, age, changes in body weight, BMI, waist circumference, ALT, AST, fasting glucose, total cholesterol and triglycerides.

Figure 4.6 (A-B). Secondary outcomes in different genotypes of *PNPLA3* rs738409 polymorphism in the intervention group.



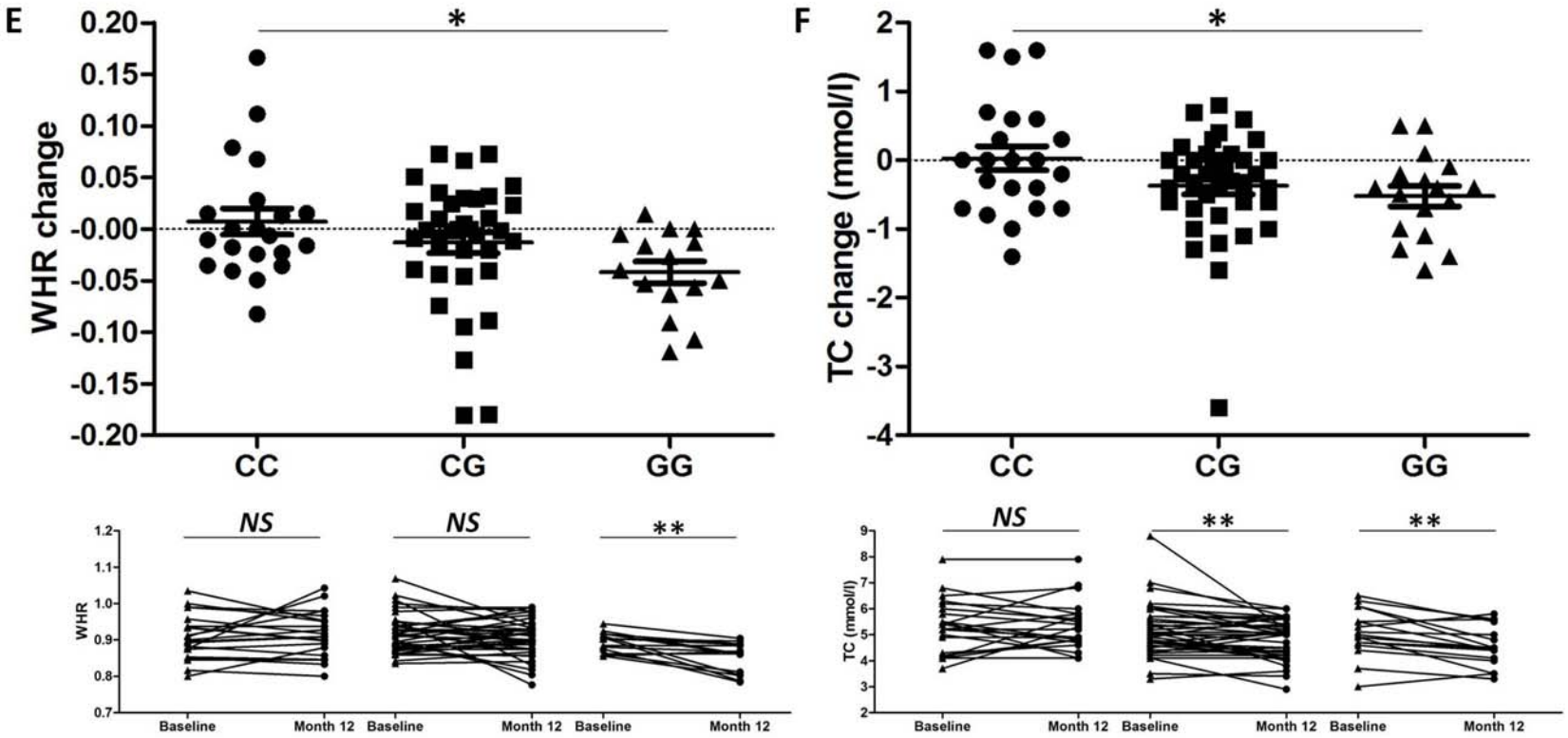
Upper: Changes of IHTG (A), body weight (B), BMI (C), waist circumference (WC) (D), waist-to-hip ratio (WHR) (E), and total cholesterol (TC) (F) in different genotypes; Lower: Individual changes of each patient for upper rows.

Figure 4.6 (C-D). Secondary outcomes in different genotypes of *PNPLA3* rs738409 polymorphism in the intervention group (Continue).



Upper: Changes of IHTG (A), body weight (B), BMI (C), waist circumference (WC) (D), waist-to-hip ratio (WHR) (E), and total cholesterol (TC) (F) in different genotypes; Lower: Individual changes of each patient for upper rows.

Figure 4.6 (E-F). Secondary outcomes in different genotypes of *PNPLA3* rs738409 polymorphism in the intervention group (Continue).



Upper: Changes of IHTG (A), body weight (B), BMI (C), waist circumference (WC) (D), waist-to-hip ratio (WHR) (E), and total cholesterol (TC) (F) in different genotypes; Lower: Individual changes of each patient for upper rows.

4.4 Summary of Chapter 4

In summary, *PNPLA3* rs738409 GG genotype increases the risk of NAFLD in the community independent of the change of dietary pattern. It is also associated with more severe histological damage in NAFLD patients. Our data does not suggest a predictive role of *PNPLA3* gene polymorphism on the disease progression in the prospective cohort. However, these patients were at a more advanced stage compared with the subjects in community cohort, and received advice on lifestyle. Prospective studies based on the community cohort should be conducted to determine whether *PNPLA3* gene polymorphism could affect disease progression.

The sustainable, community-based lifestyle intervention is effective in reducing and normalizing liver fat in NAFLD patients. Subjects with allele G are more sensitive to lifestyle intervention. Patients with GG genotype, who usually have more severe disease, may gain additional 6% absolute reduction in IHTG compared with those with CC genotype from lifestyle intervention. This reduction is accompanied with more reduction in body weight, BMI and total cholesterol.

Chapter 5: Conclusions

5.1 Discussion

This study clearly shows that biomarkers CK-18 M30/M65/M65ED and FGF21, and genetic marker *PNPLA3* rs738409 gene polymorphism have their important and different roles in the non-invasive evaluation of NAFLD.

Apoptotic biomarkers CK-18 M30, total cell death biomarker CK-18 M65/M65ED, and "mitokine" FGF21 all have high accuracies in diagnosing NAFLD (AUROC: 0.84-0.94) and moderate accuracy in diagnosing NASH (AUROC: 0.66-0.71) alone. Combined application of CK-18 M30 and FGF21 using a 2-step approach further improves the NPV and PPV to around 80%. It can spare nearly 30% of NAFLD patients from liver biopsy while only 40% of NAFLD patients need both biomarkers tested. On the other hand, the role of AFABP in non-invasive evaluation of NAFLD is limited by the relatively poor diagnostic performance.

Both apoptosis and necrosis are important cell death models in liver diseases. In NAFLD, while apoptosis was thought to be a characteristic feature of NASH [105 238], Joka et al. suggested recently that necrosis might also contribute to the liver damage in NAFLD and NASH by showing biomarkers detecting total cell death including apoptosis and necrosis might be superior to detecting apoptosis alone [139]. However,

this study included patients with chronic viral hepatitis, and NAFLD patients were underrepresented. The role of different cell death markers was also discussed in other liver diseases such as HBV infection and acute liver failure [239-240]. A study in USA suggested that the overall diagnostic accuracy of CK-18 M65 for NASH was higher than that of M30 [141], but that was not confirmed by another study in Turkey [142]. Here, we showed that the overall accuracies of M30/M65/M65ED in diagnosing NAFLD and NASH were similar. Furthermore, we demonstrated the correlation between the biomarkers and individual histological features of NAFLD, in particular the evaluation of mild disease, which was not reported before. M65 and M65ED had higher discriminating power in detecting mild steatosis and fibrosis. On the other hand, the AUROC of CK-18 M30 in diagnosing NASH was 0.66, which appears to be lower than the AUROC of over 0.80 in the original multicenter study and a follow-up report [137] [140]. However, the better diagnostic performance may be explained by the inclusion of patients without NAFLD in the "non-NASH" group. For example, 18 of 54 patients in the "non-NASH" group of the study by Tamimi et al. had steatosis of less than 5%. In our cohort, if the entire study population including both NAFLD patients and controls is analyzed, the AUROC for CK-18 in diagnosing NASH is increased to 0.83.

Fibroblast growth factor 21 (FGF21) is a hormone which regulates lipid oxidation in the liver and stimulates glucose uptake in the adipose tissue [152]. It is also termed as a "mitokine" due to its regulation by mitochondrial dysfunction and strong effect on

increasing lipid oxidation and browning of white adipose tissue [153]. Li et al. found that in human liver, FGF21 mRNA expression level increased with steatosis grade; its serum level was significantly higher in 159 Chinese NAFLD patients compared with 553 healthy controls [154-155]. Similar results were reported by Yilmaz et al. [156]. However, the association of FGF21 and NASH was not determined. Our study confirmed that FGF21 had high accuracy of 84% in diagnosing NAFLD; and extended the original findings by demonstrating that FGF21 was associated with lobular inflammation in patients with NAFLD. The AUROC of FGF21 in diagnosing NASH was 0.66. Since studies on biopsy-proven NAFLD usually include patients with risk factors of advanced disease such as metabolic syndrome, the proportion of patients with NASH is relatively high. When CK-18 M30/M65/M65ED and FGF21 are applied to primary care setting, the NPV in excluding NASH will be even higher.

In contrast, the overall diagnostic performance of AFABP was inferior to CK-18 and FGF21. The relative high level of AFABP in control subjects largely limited the role of AFABP in the non-invasive evaluation of NAFLD.

The prospective paired liver biopsy cohort allowed us to study the utility of these biomarkers in predicting disease progression. Changes in CK-18 M30, M65 and M65ED correlated well with disease progression. Notably, changes of M30 and M65ED had high accuracy of over 80% in predicting disease progression, indicating both biomarkers may be used for serial monitoring of disease progression. Changes

of M65 and M65ED also correlated with fibrosis progression. Li and colleagues recently reported that baseline FGF21 serum level was an independent risk factor for development of NAFLD defined by ultrasound in healthy Chinese subjects [155]. However, in this study, neither baseline nor changes in FGF21 correlated with histological disease progression in NAFLD patients.

Genetic marker *PNPLA3* rs738409 GG genotype is associated with 2-folds increased risk of NAFLD in the community. This association is independent of the change of dietary pattern. Subjects with GG genotype develop NAFLD not because they tend to consume more than or different to others. The GG genotype is also associated with more severe histological damage in NAFLD patients.

In a meta-analysis of 16 studies including 2,651 patients with NAFLD, GG homozygosity is associated with not only increased hepatic fat content but also around 3-fold increase in the risk of steatohepatitis, high necroinflammatory grade and fibrosis stage [98]. At the population level, studies on the association between *PNPLA3* polymorphism and NAFLD were mainly conducted among children and adolescents, including 1 study from Taiwan using ultrasonography to detect fatty liver [194 222 236-237]. Our study adds to the current literature in using state-of-the-art non-invasive tests for hepatic steatosis and fibrosis. We confirmed that subjects with the GG genotype had 2.4-fold increase in IHTG as compared with those with the CC genotype.

This study also aimed at evaluating the interaction between *PNPLA3* and dietary pattern. We convincingly showed that subjects with the GG genotype did not develop fatty liver because of higher energy, carbohydrate or fat consumption. Rather, *PNPLA3* and dietary fat intake independently affect the development of NAFLD. In a small study of 153 Hispanic children, dietary carbohydrate and total sugar were associated with hepatic steatosis only in those with GG genotype but not in the CC or CG groups [222]. In another study of 127 children and adolescents of different ethnic background, dietary omega-6/omega-3 polyunsaturated fatty acids ratio was associated with fatty liver only in subjects with the GG genotype [223]. In our study, however, no interaction between *PNPLA3* gene polymorphism and diet was noted.

In a small study of 18 subjects, GG homozygosity was associated with greater reduction in IHTG than subjects with the CC genotype (45% vs 18% reduction) after hypocaloric low-carbohydrate diet for 6 days [224]. We further confirmed this intriguing finding in a larger prospective clinical trial which enrolled 77 patients received an effective, community based, sustainable lifestyle intervention programme. Subjects with allele G are more sensitive to the lifestyle itervention. Patients with GG genotype may gain additional 6% absolute reduction in IHTG compared with those with CC genotype from lifestyle intervention. This reduction is accompanied with more reduction in body weight, BMI and total cholesterol.

Because GG genotype is associated with more advanced disease, these patients will receive additional benefit and should be highly encouraged to take lifestyle intervention.

5.2 Limitations of this study and plans for future works

Our study has certain limitations. First, in the hospital NAFLD cohort, liver biopsy was used as the reference standard, which might lead to sampling bias. However, it is currently the gold standard, and biopsy specimens were reviewed by two experienced pathologists who were blind to the clinical data. Second, liver biopsy was not performed in community subjects. However, biopsy on healthy people is unethical. Instead, liver disease was evaluated in community subjects stringently by history, laboratory tests and ^{1}H-MRS. Third, the prospective paired liver biopsies cohort was relatively small. These patients also received lifestyle advice, which might change the natural disease history. However, it is unethical to not providing such advice. Fourth, the number of subjects with advanced fibrosis in the community cohort was small. We were thus unable to evaluate the impact of *PNPLA3* polymorphism on liver injury at the population level. However, we adopted the hospital NAFLD cohort as a completion of the community cohort and proved the association of *PNPLA3* polymorphism and liver fibrosis. Fifth, dietary habit and hepatic steatosis may change over time. Here, we minimized the bias by using a food-frequency questionnaire that takes into account of the average food intake over

a typical week. Finally, in the lifestyle intervention study, blinding of patients was impossible because of the nature of intervention. Unmeasured changes other than intervention might cause bias in this study.

For further assessment of the clinical utility of both biomarkers and genetic markers in the non-invasive evaluation of NAFLD, prospective studies should be designed to test whether these markers could predict the disease outcome. Development of HCC, end stage liver disease, as well as other complications such as cardiovascular diseases should be studied as the outcomes. Studies based on both community and hospital should be conducted. Meanwhile, more biomarkers and genetic markers should be explored for the non-invasive evaluation of NAFLD and NASH.

5.3 Conclusions

Biomarkers CK-18 M30/M65/M65ED and FGF21 have high accuracy in diagnosing NAFLD and moderate accuracy in diagnosing NASH. A two-step approach combining CK-18 M30 and FGF21 further improves the accuracy in diagnosing NASH. Changes in CK-18 M30 and M65ED have high accuracy in predicting disease progression and may be used for serial monitoring. The GG genotype in *PNPLA3* rs738409 is associated with increased risk of NAFLD independent of dietary pattern. Those patients with GG genotype were more sensitive to lifestyle intervention and thus should be encouraged to participate in such programmes.

Statement of contributions

This is a comprehensive study which cannot be done without the numerous efforts of many other investigators. My contributions to this study include study concept and design; biomarkers measurement and *PNPLA3* gene single nucleotide polymorphism discrimination; analysis and interpretation of data; statistical analysis and drafting of the manuscript.

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