MONASH University

Clinical and pathophysiological aspects of nonalcoholic fatty liver disease in bariatric surgery

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Abstract

Nonalcoholic fatty liver disease (NAFLD) is a substantial burden in obesity, particularly in bariatric surgical cohorts. NAFLD can progress to nonalcoholic steatohepatitis (NASH), cirrhosis and liver failure. This is especially the case when fuelled by metabolic comorbidities. Despite its prevalence, there is a limited understanding of NAFLD in the context of severe obesity.

Bariatric surgery presents an ideal platform for NAFLD research. This cohort is a high-risk population, with detailed clinical data and easy access to liver histology and specimens. Furthermore, this population provides the opportunity to observe changes with weight loss.

This thesis aimed to address key knowledge deficiencies in a range of areas encompassing the full spectrum of NAFLD in the setting of obesity. Four research themes investigated were: (1) the epidemiology of NAFLD in obesity, (2) diagnostic tests, (3) weight loss as a primary therapy and (4) lipids as a pathophysiological mechanism. These aspects were studied via a systematic review, a prospective follow-up study (Metabolic Syndrome Study, 2008-2012) and a prospective cohort study (NAFLD and Obesity Study, 2015-2017).

The first research theme investigated NAFLD epidemiology in obesity. Whilst overall NAFLD prevalence was high (74.1%), more significant disease was less common (12% NASH, 5.1% significant fibrosis). Metabolic disease and increasing obesity increased the odds of disease by threefold. Being both super obese and having metabolic disease had a multiplicative effect, with nearly 10 times the odds of NASH.

The second research theme examined diagnostic tests for NAFLD in obese cohorts. A systematic review of the literature showed that there were few studies assessing diagnostic accuracy of commonly used NAFLD investigations in obesity. Major limitations of current tests included feasibility issues, poor accuracy, or insufficient validation in obesity.

Subsequent studies demonstrated that obesity can alter feasibility and diagnostic accuracy of tests. These studies showed how modification or combination of existing tests can help to improve accuracy.

Magnetic resonance spectroscopy (MRS) has excellent diagnostic accuracy for steatosis with sensitivity 81.3%, specificity 87.5% and AUROC 0.852 ($p=0.001$). However, low feasibility

due to obesity (65.3% success) substantially decreased the accuracy when using an intention-to-diagnose analysis (AUROC 0.688, sensitivity 84.8%, specificity 47.2%). Algorithms combining imaging tests (such as transient elastography and controlled attenuation parameter, with Forn index and alanine aminotransferase) yielded reasonable overall accuracy.

Intraoperatively, a simple and structured tool based on liver appearance (visual liver score) assisted in stratifying patients for an intraoperative biopsy. Comparison of intraoperative core and wedge biopsy techniques showed significant variation in fibrosis stage between all biopsy sites and techniques (κ=0.223-0.496, $p<0.01$). Neither wedge nor core biopsy were shown to better assess NAFLD.

Collectively, the Metabolic Syndrome Studies demonstrated the importance of achieving 10% total body weight loss (TBWL) as an initial weight loss goal for significant improvement in NAFLD, metabolic syndrome and hypercholesterolaemia. Further weight loss resulted in greater odds of resolution in most NAFLD and metabolic parameters.

Lipidomic analysis of NAFLD in obesity identified specific lipids associated with disease progression. In particular, ceramides, dihydroceramides, and other sphingolipids were significantly increased, and strongly associated with liver steatosis and NASH. This study showed that the liver lipidome, but not adipose tissue lipidome, correlated significantly with plasma lipids. Therefore, these findings indicated the potential for use of the plasma lipidome as a biomarker of NAFLD.

Overall, this thesis highlighted the unique nature of NAFLD within the obese cohort. It leveraged the large, well-characterised and high-risk bariatric surgical population, with relatively safe access to liver biopsies for diagnosis and research. These studies significantly contribute to an improved understanding of the presentation, diagnosis and treatment of NAFLD in this context. Importantly, it has also established vital collaborations that can advance research efforts. Ultimately, these findings can improve clinical practice by providing specific tools and knowledge for management of NAFLD in the growing obese population.

General Declaration

This thesis contains no material which has been accepted for the award of any other degree or diploma at any university or equivalent institution and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

Signature:

Print Name: Geraldine Jia-Ping Ooi

Date:

Thesis including published works declaration

I hereby declare that this thesis contains no material which has been accepted for the award of any other degree or diploma at any university or equivalent institution and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

This thesis includes eight original papers published in peer reviewed journals and two unpublished publications. The core theme of the thesis is nonalcoholic fatty liver disease and related metabolic disorders in the setting of obesity and bariatric surgical patients, specifically investigating diagnosis, treatment with weight loss and pathophysiology. The ideas, development and writing up of all the papers in the thesis were the principal responsibility of myself, the student, working within The Department of Surgery, Central Clinical School under the supervision of Professor Wendy Brown, Mr Paul Burton, Professor Stuart Roberts and Dr William Kemp.

The inclusion of co-authors reflects the fact that the work came from active collaboration between researchers and acknowledges input into team-based research. In the case of chapters including published works my contribution to the work involved the following:

Thesis Chapter	Chapter Title	Status	Nature and % of student contribution	Co-author names, nature and % of contribution	Monash student?
6	Effect of metabolic health and adipose tissue inflammation on the severity of nonalcoholic fatty liver disease in bariatric surgical patients: A prospective study	Published (Obes Surg)	90%. Overall responsibility for all aspects of study - designing, planning, patient recruitment, conducting studies, recording and analysing data, writing and revising manuscript.	PR Burton (2% Designing, data collection, revising manuscript) J Bayliss (1% Data collection, tissue analysis, revising) A Raajendiran (0.5% Data collection, tissue analysis, revising) A Earnest (1% Data analysis, revising) C Laurie (0.5% Patient recruitment, data collection, reviewing manuscript) W Kemp (1% Design, data collection, revising) C McLean (1% Data collection, biopsy review, review) S Roberts (1% Designing, data collection, revising) M Watt (1% Designing, planning, data collection, revising) W Brown (1% Designing, planning, collection of patient data, revising)	None
7	Systematic review and meta-analysis: Non-invasive detection of nonalcoholic fatty liver disease related fibrosis in the obese	Published (Obes Rev)	88%. Overall responsibility for all aspects of study - designing, planning, conducting literature search and study screening, extracting and presenting data, writing and revising manuscript.	S Mgaieth (5% 2nd reviewer for study screening, revising) G Eslick (3% Meta-analysis, revising) P Burton (1% Design and concept, revising) W Kemp (1% Design and concept, revising) S Roberts (1% Design and concept, revising) W Brown (1% Design and concept, revising)	None
8	Modified thresholds for fibrosis risk scores in nonalcoholic fatty	Published (Obes Surg)	89%. Overall responsibility for all aspects of study - designing,	PR Burton (2% Design, recruitment, data collection, revising) L Doyle (0.5% Design, recruitment, data collection)	None

	liver disease are necessary in the obese		planning, patient recruitment, conducting studies, recording and analysing data, writing and revising manuscript.	J Wentworth (0.5% Design, revising) P Bhathal (3% Design, data collection, revising) K Sikaris (0.5% Design, concept, revising) M Cowley (0.5% Design, revising) S Roberts (1% Design, revising) W Kemp (1% Design, revising) P O'Brien (1% Design, recruitment, data collection, revising) W Brown (1% Design, planning, concept, recruitment, data collection, revising)	
9	Evaluating feasibility and accuracy of non-invasive tests for nonalcoholic fatty liver disease in obese patients	Published (Int J Obes)	90%. Overall responsibility for all aspects of study - designing, planning, patient recruitment, conducting studies, recording and analysing data, writing and revising manuscript.	A Earnest (2% Data analysis, presentation, revising) W Kemp (1% Design, data collection, revising) P Burton (1% Design, data collection, revising) C Laurie (1% Data collection, planning) A Majeed (1% Data collection, planning) N Johnson (1% Data collection, analysis, revising) C McLean (1% Data collection, reviewing) S Roberts (1% Design, data collection, revising) W Brown (1% Design, concept, data collection, revising)	None
10	Visual liver score to stratify nonalcoholic steatohepatitis risk and determine selective intraoperative liver biopsy in obesity	Published (Obes Surg)	90%. Overall responsibility for all aspects of study - designing, planning, patient recruitment, conducting studies, recording and analysing data, writing and revising manuscript.	P Burton (3% Concept, design, data collection, revising) A Earnest (1% Data analysis, revising) C Laurie (1% data collection, revising) W Kemp (0.5% design, revising) P Nottle (0.5% data collection, revising) C McLean (2% Data collection, reviewing) S Roberts (1% Design, data collection, revising) W Brown (1% Design, concept, data collection, revising)	None
11	Evaluation of the histologic variability of nonalcoholic fatty liver disease	Submitted (Hepatol Int)	85%. Overall responsibility for all aspects of study - designing, planning, patient recruitment, conducting studies, recording and analysing data, writing and revising manuscript.	A Clouston (3% Data collection, reviewing) C McLean (2% Data collection, reviewing) Y Johari (1% Analysis, revising) C Laurie (1% Data collection, revising) W Kemp (2% Concept, design, revising) S Roberts (2% Design, data collection, revising) W Brown (2% Design, concept, data collection, revising) P Burton (2% Concept, design, data collection, revising)	None
12	Effects of bariatric surgery on liver function tests in patients with nonalcoholic fatty liver disease	Published (Obes Surg)	88%. Development of specific research question. Overall responsibility for data cleaning, analysis, writing and revising manuscript.	PR Burton (1% Design, recruitment, data collection, revising) L Doyle (0.5% Design, recruitment, data collection) J Wentworth (0.5% Design, revising) P Bhathal (3% Design, data collection, revising) K Sikaris (0.5% Design, concept, revising) M Cowley (0.5% Design, revising) S Roberts (1% Design, revising) W Kemp (1% Design, revising) A Earnest (2% Planning, data analysis, revising) P O'Brien (1% Design, recruitment, data collection, revising) W Brown (1% Design, planning, concept, recruitment, data collection, revising)	None
13	Weight loss after laparoscopic adjustable gastric band and resolution of the metabolic syndrome and its components	Published (Int J Obes)	78%. Development of specific research question. Overall responsibility for data cleaning, analysis, writing and revising manuscript.	L Doyle (1% Design, recruitment, data collection) T Tie (2% Data analysis, revising) J Wentworth (1% Design, revising) C Laurie (1% Data collection, revising) A Earnest (5% Planning, data analysis, revising) K Sikaris (1% Design, concept, revising) M Cowley (0.5% Design, revising) C LeRoux (0.5% Design, revising) PR Burton (2% Design, recruitment, data collection, revising) P O'Brien (3% Design, recruitment, data collection, revising) W Brown (5% Design, planning, concept, recruitment, data collection, revising)	T Tie

14	Detailed description of change in serum cholesterol profile with incremental weight loss after restrictive bariatric surgery	Published (Obes Surg)	84%. Development of specific research question. Overall responsibility for data cleaning, analysis, writing and revising manuscript.	A Earnest (5% Data analysis, revising) L Doyle (1% Design, recruitment, data collection) C Laurie (1% Planning, data collection) J Wentworth (0.5% Design, revising) K Sikaris (2% Design, concept, revising) C LeRoux (0.5% Design, revising) PR Burton (1% Design, recruitment, data collection, revising) P O'Brien (2% Design, recruitment, data collection, revising) W Brown (3% Design, planning, concept, recruitment, data collection, revising)	None

I have renumbered sections of submitted or published papers in order to generate a consistent presentation within the thesis.

Student signature: **Date:**

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the student's and co-authors' contributions to this work. In instances where I am not the responsible author, I have consulted with the responsible author to agree on the respective contributions of the authors.

Main Supervisor signature: **Date:**

Publications arising from this thesis

1. **Effect of body mass index, metabolic health and adipose tissue inflammation on the severity of nonalcoholic fatty liver disease in bariatric surgical patients: a prospective study**
   G Ooi, P Burton, J Bayliss, A Raajendiran, A Earnest, C Laurie, W Kemp, C McLean, S Roberts, M Watt, W Brown
   Obesity Surgery. Accepted 2 Sept 2018. [Epub ahead of print.]

2. **Systematic review and meta-analysis: Non-invasive detection of nonalcoholic fatty liver disease in the obese**
   G Ooi, S Mgaieth, G Eslick, P Burton, W Kemp, S Roberts, W Brown.
   Obesity Reviews, 2018. 19(2):281-294. DOI: 10.1111/obr.12628

3. **Modified thresholds for fibrosis risk scores in nonalcoholic fatty liver disease are necessary in the obese**
   G Ooi, P Burton, L Doyle, J Wentworth, P Bhathal, K Sikaris, M Cowley, S Roberts, W Kemp, P O'Brien, W Brown.
   Obesity Surgery, 2017. 27(1):115-125. DOI 10.1007/s11695-016-2246-5.

4. **Evaluating feasibility and accuracy of non-invasive tests for nonalcoholic fatty liver disease in severe and morbid obesity**
   G Ooi, A Earnest, W Kemp, P Burton, C Laurie, A Majeed, N Johnson, C McLean, S Roberts, W Brown
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5. **Visual liver score to stratify nonalcoholic steatohepatitis risk and determine selective intraoperative liver biopsy in obesity**
   G Ooi, PR Burton, A Earnest, C Laurie, WW Kemp, PD Nottle, CA McLean, SK Roberts, WA Brown
   Obesity Surgery, 2018. 28(2):427-436. DOI: 10.1007/s11695-017-2859-3.

6. **Weight loss after laparoscopic adjustable gastric band and resolution of the metabolic syndrome and its components**
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   International Journal of Obesity, 2017. 41(6):902-908. DOI: 10.1038/ijo.2017.59.

7. **Detailed description of change in serum cholesterol profile with incremental weight loss after restrictive bariatric surgery**
   G Ooi, A Earnest, L Doyle, C Laurie, J Wentworth, K Sikaris, C Le Roux, P O'Brien, P Burton, W Brown.
   Obesity Surgery, 2018. 28(5):1351-1362. DOI: 10.1007/s11695-017-3015-9

8. **Effects of bariatric surgery on liver function tests in patients with nonalcoholic fatty liver disease**
   G Ooi, P Burton, L Doyle, J Wentworth, P Bhathal, K Sikaris, M Cowley, S Roberts, W Kemp, A Earnest, P O'Brien, W Brown
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List of Tables

Abbreviations

Abbreviation	Full
5-HT	5-hydroxytryptamine (serotonin)
AAR	AST to ALT ratio
AASLD	American Association for the Study of Liver Disease
ALP	alanine phosphatase
ALT	alanine aminotransferase
APRI	AST to platelet ratio index
ARB	angiotensin receptor blocker
ARFI	acoustic radiation force index
AST	aspartate aminotransferase
ATGL	adipose triglyceride lipase
ATPIII	adult treatment panel III
AUROC	area under the receiver operator characteristic curve
BA	bile acids
BDI	Beck Depression Inventory
BMI	body mass index
BP	blood pressure
BPD	biliopancreatic diversion
BSL	blood sugar level
CAP	controlled attenuation parameter
CC	correctly classified
CCK	cholecystokinin
CCL	chemokine (C-C motif) ligand
CD	cluster differentiation protein
CE	cholesterol ester
Cer	ceramide
ChREBP	carbohydrate response element binding protein
CI	confidence interval
CK-18	cytokeratin 18
CLD	chronic liver disease
COH	free cholesterol
CRN	Clinical Research Network
CT	computed tomography
CVD	cardiovascular disease
DBP	diastolic blood pressure
DCAT2	diacylglycerol acyltransferase 2
DG	diacylglycerol
DHC	dihexosylceramide, Hex2Cer
dhCer	dihydroceramide
DNA	deoxyribonucleic acid
DNL	*de novo* lipogenesis
DOR	diagnostic odds ratio
DS	duodenal switch
ECM	extracellular matrix
eGFR	estimated glomerular filtration rate
ELF	enhanced liver fibrosis score
ELISA	enzyme-linked immunosorbent assays
ER	endoplasmic reticulum
ETOH	ethanol
EWL	excess weight loss
FBE	full blood examination
FBG	fasting blood glucose
FC	free cholesterol
FDR	false discovery rate
FFA	free fatty acid
FIB-4	fibrosis-4 score
FLI	fatty liver index

Abbreviation	Full
FRC	functional residual capacity
FRE	Framingham risk equation
FWER	family-wise error rate
FXR	farsenoid X receptor
GABA	γ-aminobutyric acid
GEE	generalised estimating equation
GGT	gamma-glutamyl transferase
GI	glycaemic index
GIP	glucose-dependent insulinotropic polypeptide
GL	glycerolipid
GLP-1	glucagon-like peptide 1
GLUT4	glucose transporter 4
GORD	gastroesophageal reflux disease
GWAS	genome-wide association studies
H&E	hematoxylin eosin
HA	hyaluronic acid
HbA1c	glycosylated haemoglobin
HBV	hepatitis B virus
HCC	hepatocellular carcinoma
HCV	hepatitis C virus
HDL	high density lipoprotein
HIF-1a	hypoxia-inducible factor 1 alpha
HIS	hepatitis steatosis index
HIV	human immunodeficiency virus
HOMA2-IR	homeostatic model of assessment 2 insulin resistance
HR	hazard ratio
HREC	Human Research Ethics Committee
HRT	hormone replacement therapy
HSC	hepatic stellate cells
hsCRP	highly sensitive C-reactive protein
HSL	hormone sensitive lipase
HTN	hypertension
IDF	International Diabetes Federation
IFG	impaired fasting glucose
IGT	impaired glucose tolerance
IKK-β	IκB kinase β
IL	interleukin
IQR	interquartile range
ITT	intention to treat
JAK-1	Janus kinase 1
JIB	jejunoileal bypass
JNK	c-Jun amino-terminal kinase
LABS	Longitudinal Assessment of Bariatric Surgery
LAGB	laparoscopic adjustable gastric band
LAP	lipid accumulation product
LDL	low density lipoprotein
LFT	liver function tests
LPC	lysophosphatidylcholine
LPC(O)	lysoalkylphosphatidylcholine
LPE	lysophosphatidylethanolamine
LPE(P)	lysoalkenylphosphatidylethanolamine
LPI	lysophosphatidylinositol
LPL	lipoprotein lipase
LPS	lipopolysaccaride
LSM	liver stiffness measurement
M1	classically activated macrophage

Abbreviation	Full
M2	alternatively activated macrophage
MAO	metabolically abnormal obese
MAP	mitogen-activated protein
MCH	melanin-concentrating hormone
MEN2	multiple endocrine neoplasia 2
MetS	metabolic syndrome
MF	myofibroblast
MG	monoacylglycerol
MHC	monohexosylceramide, Hex1Cer
MHO	metabolically healthy obese
MIP-1a	macrophage inflammatory protein 1α
MMP	matrix metalloproteinase
MRE	magnetic resonance spectroscopy
MRI	magnetic resonance imaging
MRS	magnetic resonance spectroscopy
MUFA	mono-unsaturated fatty acid
NAFLD	nonalcoholic fatty liver disease
NAS	NAFLD activity score
NASH	nonalcoholic steatohepatitis
NCEP	National Cholesterol Education Program
NF-κB	nuclear factor kB
NFS	NAFLD fibrosis score
NHANES	National Health and Nutrition Examination Survey
NIH	National Institute of Health
NNT	number needed to treat
NPV	negative predictive value
ns	not significant
NVDPA	National Vascular Disease Prevention Alliance
OA	osteoarthritis
OBS	observational study
OCP	oral contraceptive pill
OGTT	oral glucose tolerance test
OHG	oral hypoglycaemic
OR	odds ratio
OSA	obstructive sleep apnoea
OXM	oxyntomodulin
PAI-1	plasminogen activator inhibitor-1
PC	phosphatidylcholine
PC(O)	alkylphosphatidylcholine
PC(P)	alkenylphosphatidylcholine
PCA	principal component analysis
PCOS	polycystic ovarian syndrome
PCR	polymerase chain reaction
PE	phosphatidylethanolamine
PE(O)	alkylphosphatidylethanolamine
PE(P)	alkenylphosphatidylethanolamine
PG	phosphatidylglycerol
PI	phosphatidylinositol
PI(3)K	phosphatidylinositol-3-OH kinase
PIIINP	procollagen II amino-terminal peptide
PL	phospholipid
PNPLA3	patatin-like phospholipase domain-containing protein 3
PP	pancreatic polypeptide
PPAR	peroxisome proliferator-activated receptor
PPV	positive predictive value
PRESS	point resolved spectroscopy

Abbreviation	Full
PRISMA	Preferred Reporting Items for Systematic Reviews and Meta-Analyses guidelines
PS	phosphatidylserine
PUFA	poly-unsaturated fatty acid
PYY	polypeptide YY
QOL	quality of life
QUADAS-2	Quality Assessment of Diagnostic Accuracy Studies 2
RBP4	retinol binding protein 4
RCT	randomised controlled trial
RNA	ribonucleic acid
ROS	reactive oxygen species
RR	relative risk
RYGB	Roux-en-Y gastric bypass
SAF	steatosis, activity, fibrosis score
SAGB	single anastomosis gastric bypass
SAT	subcutaneous adipose tissue
SBP	systolic blood pressure
SCD1	stearoyl-CoA desaturase-1
SD	standard deviation
sdLDL	small dense low density lipoprotein
SF-36	short form survey 36
SFA	saturated fatty acid
SG	sleeve gastrectomy
SL	sphingolipid
SM	sphingomyelin
SOS	Swedish Obesity Study
SPT	serine palmitoyl-CoA transferase
SREBP1c	sterol response element binding protein 1c
SROC	summary receiver operator characteristic
SSA	serum amyloid A
STAT	signal transducer and activators of transcription
STEAM	stimulated-echo acquisition mode
SVF	stromal vascular fraction
SVS	single-voxel spectroscopy
SWE	shearwave elastography
T2DM	type II diabetes mellitus
TBWL	total body weight loss
TC	total cholesterol
TC:HDL	total cholesterol to HDL ratio
TE	transient elastography
TG or TAG	triglycerides
THC	trihexosylceramide, Hex3Cer
TIMP1	tissue inhibitor of matrix metalloproteinase 1
TNF-a	tumour necrosis factor alpha
TLR-4	toll-like receptor 4
TSH	thyroid stimulating hormone
UCP-1	uncoupling protein-1
ULN	upper limit of normal
UPR	unfolded protein response
VAT	visceral adipose tissue
VLCD	very low calorie diet
VLDL	very low-density lipoprotein
VLS	visual liver score
WAT	white adipose tissue
WHO	World Health Organisation
WHR	waist-hip ratio

1 Synopsis

Nonalcoholic fatty liver disease and obesity: A snapshot

Nonalcoholic fatty liver disease (NAFLD) is now the most common cause of chronic liver disease worldwide (1, 2). The hallmark of NAFLD is the accumulation fat within the liver (steatosis >5%), in the absence of other liver disease (3). The more severe form of NAFLD, nonalcoholic steatohepatitis (NASH), manifests with inflammatory change with or without fibrosis (steatofibrosis). The incidence and severity of NAFLD has risen in parallel with overwhelming worldwide increase in obesity. NAFLD will soon rival alcoholic and viral hepatitis as the primary cause for liver failure and transplantation (1).

Obesity (defined as a body mass index (BMI) over 30kg/m^2) has become increasingly prevalent over the last few decades. Currently, 28.3% of the Australian adult population are obese, with another 35.1% being overweight (4). Obesity predisposes to significant metabolic comorbidities. Ultimately, it is associated with increased all cause morbidity and mortality (5).

Obesity and NAFLD are intricately linked. The pathophysiological consequences of obesity and associated metabolic disease directly and indirectly exacerbate liver steatosis and inflammation (6). Up to 71-98% of obese patients are reported to have NAFLD, compared to 10-51% in the general population (7). The full spectrum of NAFLD is pervasive in the bariatric surgical population, due to the increased severity of obesity and metabolic disease.

Framing the problem: Dilemmas in nonalcoholic fatty liver disease in the setting of obesity

Despite obesity and NAFLD being intimately associated, there are key knowledge deficiencies in a range of areas encompassing the characteristics, management and pathophysiology of NAFLD in the setting of morbid obesity. This is exemplified in the bariatric surgical cohort, where surgeons face issues related to fatty liver disease in everyday practice, from incidental liver function test derangements, to lack of surgical access and unexpected liver abnormalities found intraoperatively. In these cases, NAFLD is commonly suspected, however few data currently exist to guide subsequent management. Key areas of knowledge deficiency include:

1. ***NAFLD epidemiology in obesity***: Studies suggest that there is an increased prevalence of NAFLD in obesity compared to the general population (8). However, significant discrepancy in reported prevalence exists, particularly for the more severe forms of disease. The prevalence of NASH is commonly cited at 25-56% in obesity (9-11). However, more recently, studies of well-characterised bariatric surgical cohorts report prevalence as low as 7.3-11% (12, 13).

   The influence of other obesity-related factors on NAFLD likely account for this, but have not been well described. Obesity severity, metabolic disease, insulin sensitivity (14) and adipose tissue characteristics (15, 16) can significantly affect NAFLD risk, and may explain the differences in recorded prevalence in obese cohorts.
2. ***Diagnosing and grading NAFLD in bariatric surgical patients***: Liver biopsy is the current gold standard, and only reliable means of diagnosing and grading fibrosis. However, liver biopsy is an impractical initial diagnostic test for screening the large at-risk obese population. There are many non-invasive diagnostic tools available, mainly developed and tested within the viral hepatitis or liver clinic setting. Obesity-related differences in clinical, biochemical and physiological profiles can significantly impact these serum and imaging based investigations (17). Their application in the obese is not well validated.
3. ***Weight loss and bariatric surgery as a primary therapy for NAFLD and metabolic disease***: Treatment options for NAFLD are limited. Weight loss is universally advocated, as it has known benefits in reversing metabolic and systemic consequences of obesity (18). Bariatric surgery is a proven, long-term and safe means of attaining weight loss, with proven metabolic benefits. Over 22,000 Australians currently undergo bariatric surgery every year (19). Whilst significant weight loss is known to benefit NAFLD, the effects of modest or incremental weight loss on NAFLD and related disorders have not been well defined.
4. ***Pathophysiology of NAFLD in obesity***: The pathogenesis of NAFLD, and factors that drive progression to steatohepatitis are not fully understood. The process is likely multifactorial, with evidence suggesting the contribution of adipose tissue inflammation, immunity, gut microbiota, genetics and hormonal influences.

   Lipotoxicity has recently been explored as a potential driver of disease. It is defined as the accumulation of lipids leading to cellular dysfunction and death (20). Ongoing advances in lipidomic techniques now enable measurement of hundreds of lipid species, permitting in-depth exploration of the lipid profile of NAFLD.

A key contributing factor to the challenges in studying NAFLD is the current requirement for a liver biopsy to diagnose NAFLD. This is particularly risky in individuals with obesity, further deterring specific study in this population. This has contributed to the lack of knowledge and established guidelines for NAFLD, as compared to other obesity-related diseases, such as type II diabetes and cardiovascular disease.

Bariatric surgery provides a pivotal platform for investigation of NAFLD, due to the relative ease of obtaining a liver biopsy for histological diagnosis. It is also an opportunity to collect tissue and blood specimens for laboratory studies to further our understanding of pathophysiological changes of NAFLD. Ultimately, pairing the opportunity for liver histology and tissue collection, with detailed clinical data in a high-risk population can provide a powerful means of studying clinical and pathophysiological aspects of NAFLD.

2 Thesis overview

This thesis centres around the bariatric surgical population with the intention of addressing key clinical and pathophysiological questions on nonalcoholic fatty liver disease (NAFLD) in severe obesity. It is divided into several sections:

- *Literature Review:* This literature review provides detailed background and evidence on NAFLD, its interaction with obesity and metabolic disease, pathogenesis and relevance in the bariatric surgical context.
- *Summary, Rationale for Research Direction and Aims:* This section contextualises the key areas of knowledge deficiency identified within the literature review, specifies the aims of the thesis and frames them against the research opportunities provided by bariatric surgery. It further highlights the development of the research themes and links them to feasible methodologies.
- *Research Design:* This chapter provides an overview of the main methods used for the thesis studies. This includes a systematic literature review, a study based on metabolic syndrome in obesity and the effects of weight loss, and a prospective study focusing on NAFLD and obesity.
- *Thesis Studies:* These studies are presented as individual papers, either published, accepted or submitted for publication. These are grouped into four major research themes:
    1. Current scope of the problem: NAFLD, obesity and metabolic disease
    2. Challenges of diagnosing NAFLD in obesity
    3. Impact of bariatric surgery on NAFLD and related metabolic diseases
    4. Developing an understanding of pathophysiological drivers of NAFLD in obesity
- *Conclusions:* Finally, the conclusions are presented, with discussion of the vital future research directions for furthering our understanding of NAFLD in obesity and bariatric surgery.

3 Literature review

Overview of literature review

This literature review has been divided into four sections over nine chapters:

- ***Section 1***: Obesity, its related comorbidities and the burden of disease
- ***Section 2***: Weight loss strategies and the rise of bariatric surgery
- ***Section 3***: Nonalcoholic fatty liver disease, including its relationship to obesity and metabolic disease, pathophysiology, diagnosis and current treatment
- ***Section 4***: The intersection of bariatric surgery, NAFLD and metabolic disease

Section 1*: Obesity and related comorbidities*

Chapter 3.1 introduces the current worldwide problem of obesity. It discusses the consequences of obesity and provides an overview of common related metabolic conditions, such as hyperlipidaemia, insulin resistance, diabetes and metabolic syndrome. *Chapter 3.2* reviews the current evidence on adipose tissue dysfunction and its association with metabolic consequences of obesity, including insulin resistance and lipid metabolism.

Section 2*: Weight loss*

Weight loss is a powerful means of tackling obesity-related comorbidities. The benefits of weight loss and strategies for weight management in obesity are reviewed in *Chapter 3.3*.

Bariatric surgery is currently the most effective means of long-term weight loss. *Chapter 3.4* reviews the commonly performed bariatric surgical procedures and their mechanisms of inducing weight loss.

Section 3: *Nonalcoholic fatty liver disease in obesity*

Chapter 3.5 is an introduction to nonalcoholic fatty liver disease, the burden of disease and the consequences.

Chapter 3.6 is a summary of current diagnostic techniques for NAFLD. It reviews the evidence for the diagnosis of the three main components of NAFLD – steatosis,

inflammation and fibrosis – and summarises current deficiencies in their use in the setting of obesity.

The current evidence on treatment of nonalcoholic fatty liver disease is considered in *Chapter 3.7*, including lifestyle, pharmacological and non-operative weight loss options.

Chapter 3.8 reviews our current understanding of the complex pathophysiology behind NAFLD. Lipotoxicity is an interesting and new pathway theorised to contribute to pathogenesis. This chapter reviews basic lipid physiology and advancing lipidomic technology. It finally reviews current evidence on the contribution of lipotoxicity to NAFLD progression.

Section 4: *The intersection between bariatric surgery, NAFLD and metabolic disease*

Nonalcoholic fatty liver disease is a key obesity-related comorbidity affecting the majority of bariatric patients, and a central issue in bariatric surgical practice. *Chapter 3.9* summarises our current understanding on the benefits of bariatric surgery, particularly its effects on NAFLD and metabolic disease. It reviews international guidelines on NAFLD and bariatric surgery, and identifies pertinent aspects of NAFLD in clinical bariatric surgery.

Overall, this literature review summarises the importance of NAFLD in the bariatric surgical population, identifies deficiencies in our current knowledge that limit our management, and discusses the promising role of bariatric surgery in both clinical and research aspects of this disease.

3.1 Obesity

The rising rates of obesity represent a serious public health challenge in Australia and worldwide. It has such a large impact on health that the World Health Organisation (WHO) labelled obesity as an epidemic in 2000 (21). It currently outranks smoking as a leading risk factor for burden of disease (22), and is a key contributor to cardiovascular disease, cancer, metabolic disease, disability and all-cause mortality. Over the last few decades, as the prevalence of obesity increased, so to have these comorbidities. There is not only a high individual burden, but obesity places significant strains on health care systems, the workforce and the economy (5).

3.1.1 Definitions

Obesity is defined as a condition where there is "abnormal or excessive fat accumulation in adipose tissue, to an extent that health may be impaired" (21). Obese individuals not only differ in the absolute percentage of adiposity they carry, but also in the distribution and storage of adipose tissues. Central obesity is particularly associated with increased risk.

3.1.1.1 Measurement of obesity

Body mass index (BMI)

Body mass index is defined as weight (in kilograms) divided by height (in metres) squared (unit: kg/m^2). This is a useful measure by which the World Health Organisation has used to define weight category (**Table 3.1**) (21). Overweight is defined as a BMI $\geq$25 kg/m^2, and obesity is a BMI $\geq$30 kg/m^2.

Table 3.1: The World Health Organisation classification of weight by body mass index (21)

Classification	BMI	Risk of comorbidities
Underweight	<18.50 kg/m^2	Low (but risk of other clinical problems increased)
Normal range	18.50 – 24.99 kg/m^2	Average
Overweight	≥25.00 kg/m^2	
Pre-obese	25.00 – 29.99 kg/m^2	Increased
Obese class I	30.00 – 34.99 kg/m^2	Moderate
Obese class II	35.00 – 39.99 kg/m^2	Severe
Obese class III	≥40.00 kg/m^2	Very severe (morbid)

BMI – body mass index

Notably, BMI as a measure of body adiposity has its shortcomings. Firstly, these proposed weight categories are independent of age and gender. Secondly, specific cut-off points for BMI should be used for different populations to reflect health-related risk (23). Finally, BMI does not distinguish between adipose mass and muscle mass (21), nor does it distinguish patterns of body fat distribution, which may be an important predictor of disease (24).

3.1.1.2 Central obesity

Central adiposity is commonly measured by waist circumference and waist-hip ratio (**Table 3.2**). Both waist circumference (24, 25) and waist-hip ratio (WHR) (26) have been found to have similar or better predictive power for CVD risk than BMI (27).

Table 3.2: The World Health Organisation classification of waist circumference and waist-hip ratio, and risk of metabolic complications associated with obesity in Caucasians[a] (28)

Indicator	Cut-off points	Risk of metabolic complications
Waist circumference	>94 cm (male); >80 cm (female)	Increased
	>102 cm (male); >88 cm (female)	Substantially increased
Waist-hip ratio	≥0.90 (male); ≥0.85 (female)	Substantially increased

[a]*Identification of risk using waist circumference is population specific*

3.1.2 Epidemiology

3.1.2.1 Prevalence and trends

Worldwide, around 39% of all adult are now overweight and 13% are obese (4). There is a global trend over the last three decades for an increase in prevalence in all countries (29). In Australia, 63.4% of the adult population were overweight or obese, of which 28.3% were obese. This number has increased in the last two decades, from 56.3% in 1995 to 61.2% in 2007-2008 (30). Most alarmingly, the rates of more severe and morbid obesity (BMI≥40 kg/m^2) have also increased substantially over this time (30).

3.1.2.2 Associated factors

Large epidemiological studies have shown various demographic associations with overweight and obesity, including age, gender, socioeconomic status and ethnicity.

Obesity and overweight increases with advancing age, with a 74.7% rate of overweight or obese in those aged 65-74 years, compared to 38.4% of people aged 18-24 years (30). Men are more affected by overweight than women, with 42% of men having a body mass index (BMI) ≥25 kg/m^2 compared to 35% of women (30).

Socioeconomic status influences obesity rates. In 2004-05, there was a 22% prevalence of obesity in the most disadvantaged, compared to 13% in the least disadvantaged (30). Rates of overweight or obese in major cities are 52%, which rises to 56% in inner regional and 60% in outer regional locations (30). Additionally, Aboriginal and Torres Strait Islanders have double the prevalence of obesity compared to the general population (31).

3.1.3 **Aetiology of obesity**

3.1.3.1 Energy imbalance

Body weight is maintained at a remarkably stable level in normal individuals despite daily changes in intake and expenditure. When energy input is increased or decreased, compensatory mechanisms are activated. However, energy homeostasis is biased against weight loss, preferentially tipping towards weight gain for survival benefit. This ultimately results in increased hunger, decreased metabolic rate and decrease energy use (32).

Physiologically, obesity is simply an imbalance of energy homeostasis between energy intake and output (33). However, this simplicity of this model does not account for the complexities of how food intake or energy expenditure is regulated, how and where fat is stored, and the genes and hormones that control these processes. The factors that contribute to obesity can be divided into two broad fields (**Table 3.3**): (1) environmental agents that promote obesity and (2) host susceptibility (5).

Table 3.3: Environmental and host factors contributing to obesity (5).

Environmental factors	**Host susceptibility**
Intrauterine factors Neonatal environment Adiposity rebound Drug-induced weight gain Diet Physical inactivity Smoking Viruses Microbiome	Genetic predisposition Neurophysiologic factors Endocrine factors

Environmental factors

Diet

Overall increased energy intake has been clearly implicated in the current obesity epidemic. Highly palatable energy-rich food is now cheap and abundant. A comparison of the 1995 National Nutrition Survey with the 1983 National Dietary Survey of adults showed a 3-4% increase in energy intake per day (350kJ or one slice of bread extra per day) (34). Consumption of sweetened beverages and liberal use of high-fructose corn syrup have also been linked to obesity (35).

Physical inactivity

Daily physical activity has decreased due to changes in work and leisure activities, and modernization of transportation (5). The 2011-12 National Health Survey by the Australian Institute of Health and Welfare showed that 56% of adults do not exercise sufficiently to meet physical activity guidelines (**Table 3.4**) (36).

Table 3.4: Guidelines on physical activity and sedentary behaviour (36)

Australian 2014 Physical Activity and Sedentary Behaviour Guidelines for adults 18-64 years old
• Be active on most, preferably all, days every week • Accumulate 150-300 minutes of moderate intensity physical activity or 75-150 minutes of vigorous intensity activity, or equivalent, each week • Do muscle-strengthening activities on at least 2 days each week • Minimise the amount of time spent in prolonged sitting • Break up long periods of sitting as often as possible

Medications

Several drug and drug classes that are used for the treatment of chronic diseases have been linked to weight gain (**Table 3.5**) (37).

Table 3.5: Medications associated with weight gain (37)

Class	**Examples**
Antipsychotic medications	Clozapine, olanzapine, risperidone
Antidepressants	Amitriptyline, imipramine, paroxetine
Antidiabetic medications	Insulin, sulphonylureas, thiazolidinedione
Anticonvulsants	Valproate, gabapentin
Antihypertensives	Beta-blockers

Smoking

Evidence shows that smokers have a lower BMI than non-smokers, due to the thermogenic nature of smoking, the reduction of hunger and alteration of taste. The cessation of smoking is associated with weight gain (38).

Other factors

Several intrauterine, maternal and childhood factors have been implicated in future overweight, obesity and metabolic disease (39, 40). Parental obesity is also significantly associated with childhood obesity (OR 10.44) (40). Several viruses have been implicated as a possible aetiological cause or precipitant of obesity (41). Most recently, evidence suggests that gut microbiota plays a key role in energy homeostasis, by helping to extract and store calories in a manner reflective of its composition (42).

Host susceptibility

Genetic causes

It is estimated that 40-70% of individual susceptibility to obesity is due to genetic differences (43). In the last four years, genome wide association studies have identified 52 genetic loci unequivocally associated with five obesity-related traits, including BMI, waist circumference, waist-to-hip ratio, body fat percentage and extreme and early-onset obesity. Some have been described in **Table 3.6**.

Table 3.6: Some genes implicated in obesity susceptibility (44)

Gene	Full name	Description
FTO	Fat mass and obesity associated gene	First discovered in 2007 in studies comprising more than 10,000 participants. Consistently associated with BMI, obesity risk, body fat percentage, and waist circumference. Of all the obesity- related genetic loci, FTO has the largest effect on obesity-susceptibility, increasing obesity risk by 1.20 fold.
MC4R	Melanocortin 4 receptor	Identified in a meta-analysis of more 16,876 white European participants. Associated with extreme childhood obesity. Also associated with increased waist circumference and related traits in Indian Asians and other populations, including South Asians, East Asians, and African Americans.
SH2B1	SH2B adaptor protein 1	Encodes a protein implicated in leptin signaling, which is important in appetite control, body fat storage and energy expenditure. SH2B1 knockout mice are obese.

Despite the highly significant associations of genes to obesity, the predictive ability of gene loci is poor (45). This is likely due to the substantial contribution of lifestyle choices.

Neurophysiologic factors

There are several neuropeptides that play a role in the control of appetite, food intake and body weight. Changes or differential signalling have been theorised to affect obesity, and modulation of their activity has been studied as treatment for obesity (**Table 3.7**) (5).

Table 3.7: Peptides involved in neurophysiological control of food intake (5, 46)

Peptide	Effect on appetite	Origin and receptors	Function
Peripheral peptides			
Leptin	Decrease	*Origin*: Adipose tissue. Coded by the OB gene, chromosome 7 (7q31.3) *Receptor*: Crosses blood-brain barrier to act on LEPR receptors in arcuate nucleus.	Reduces food intake and body weight, and increases energy expenditure. Inhibits production of orexigenic peptides: neuropeptide Y (NPY), agouti-related peptide (AGRP). Enhances production of anorexigenic peptides: α-melanocytic stimulating hormone
Brain peptides			
Melanin-concentrating hormone (MCH)	Decrease	*Origin:* Lateral hypothalamus.	Decreases food intake.
Orexin (or hypocretin)	Increase	*Origin:* Perifornical, lateral and dorsal hypothalamus	Wide range of function. Influences appetite, sleep, locomotor activity and grooming.
Intestinal peptides			
Ghrelin	Increase	*Origin:* Gastric fundus, duodenum. *Receptor*: Growth hormone secretagogue receptor in hypothalamus and brain stem	Stimulates appetite and food intake (~30% more in experiments). Increases pre-meal and during fasting. Baseline ghrelin lower in obesity but rises after diet-induced weight loss.
Glucagon-like peptide (GLP-1)	Decrease	*Origin:* Intestinal L-cells, small intestine and colon. *Receptors*: GLP1 receptor (stomach, brainstem, hypothalamus)	Anorexigenic effect, with reduced gastric emptying and suppression of gastric acid secretion. Proportional to caloric intake. Delay in GLP-1 release in obesity.
Cholecystokinin (CCK)	Decrease	*Origin:* Intestinal L-cells, duodenum and jejunum *Receptors:* CCK_A and CCK_B.	Plasma levels increase within 15 minutes from meal initiation and decreases meal size and duration.
Pancreatic polypeptide (PP)	Decrease	*Origin:* Pancreas/colon *Receptor:* Y receptors, with Y4 being the most effective at appetite suppression.	Reduces appetite and food intake, increases energy expenditure. Low in fasting, rises proportional to caloric intake.
Polypeptide YY (PYY)	Decrease	*Origin:* Intestinal L-cells *Receptor:* Y receptors, with preference for Y2 receptor	Decreases caloric intake. Elevated within 1 hour from meal initiation. Low levels in obesity.

Leptin is one of the most important peripherally produced adipose tissue derived hormone, with plasma concentrations directly related to adipose tissue mass or severity of obesity. It increases satiety, decreases energy intake, and increases thermogenesis (47). It has direct central effect that regulates both orexigenic ("hunger": neuropeptide Y, agouti-related peptide) and anorexigenic ("satiation": α-melanocyte stimulating hormone, cocaine and amphetamine-related transcript, corticotropin-releasing hormone) peptides. Leptin resistance and alterations in leptin metabolism have been implicated in the development of obesity (48).

Several important gastrointestinal hormones play roles in the regulation of appetite. Ghrelin, popularly known as the "hunger hormone", is produced mainly in the fundus of the stomach, and increases appetite (5).

3.1.4 Comorbidities associated with obesity

Increased body mass index is related to increased risk of comorbidities (25). This risk continues to increase with increasing body adiposity (49). This section summarises common obesity-related comorbidities, with the exception of nonalcoholic fatty liver disease. This is discussed in greater detail in **Chapter 3.5 – Nonalcoholic fatty liver disease**.

3.1.4.1 Insulin resistance and diabetes

Insulin resistance is the condition describing the inability of insulin to effectively increase cellular glucose uptake and utilisation. When the exocrine pancreas is still able to compensate, hyperinsulinaemia overcomes insulin resistance and achieve similar peripheral insulin effects. As insulin resistance increases and pancreatic insulin production fails to compensate, glucose levels increase leading first to impaired glucose tolerance (IGT), then type II diabetes (T2DM) (50).

Glucose intolerance is a spectrum, ranging from normal glucose tolerance, to 'prediabetes' then diabetes. The diagnosis is made with a fasting plasma glucose, glycated haemoglobin A1c (HbA1c) and a two-hour plasma glucose after an oral glucose tolerance test (OGTT).

The American Diabetes Association defines the following states of 'pre-diabetes' (51):

1. Fasting blood glucose (FBG) of 5.6-6.9 mmol/L
    a. Otherwise known as ***impaired fasting glucose*** or IFG

2. Two hour glucose on OGTT of 7.8-11.0 mmol/L
    a. Otherwise known as ***impaired glucose tolerance*** or IGT
3. HbA1c 5.7-6.4%

Diabetes is defined by one of four possible criteria (51):

1. HbA1c ≥6.5%, or
2. Fasting blood glucose ≥7.0 mmol/L (fasting defined as no caloric intake for ≥8 hours), or
3. Two hour plasma glucose ≥11.1 mmol/L during OGTT
4. In patients with classic symptoms of hyperglycaemia or hyperglycaemic crisis, a random plasma glucose ≥11.1mmol/L.

The significance of diabetes is that it is associated with increased microvascular and macrovascular disease. Macrovascular disease includes myocardial infarction, stroke and peripheral vascular disease, with microvascular disease being retinopathy, neuropathy and nephropathy. Those with prediabetes are mostly at risk of macrovascular complications, whereas those who develop diabetes are at risk of both (51).

Obesity is a known risk factor for T2DM, and interferes with insulin sensitivity and insulin production (52). Diabetes is seven times as prevalent in obese populations compared to normal weight populations. Incident diabetes is also higher in overweight and obese cohorts, with an annual incidence that is 2-5 times higher than in normal weight individuals (53). The risk of T2DM increases exponentially with increasing severity obesity above 30kg/m^2, with a prevalence of 1.8-2.9% with BMI 20 kg/m^2, 4.8% at BMI 30 kg/m^2, and 10.3-11.4% in those with a BMI above 40 kg/m^2 (54).

3.1.4.2 Dyslipidaemia

Dyslipidaemia describes the abnormalities in lipoprotein metabolism that give rise to abnormalities in total cholesterol (TC), low-density lipoprotein (LDL) cholesterol, high density lipoprotein (HDL) cholesterol, triglyceride levels and non-HDL cholesterols (25). Recommended guidelines for cholesterol levels are shown in (**Table 3.8**).

Table 3.8: Guideline recommendations on optimal cholesterol levels for primary prevention of cardiovascular disease (25).

Cholesterol variable	Normal level (mmol/L)	Comment
Triglycerides (TG)	<1.7	National Cholesterol Education Program (NCEP) (55)
High density lipoprotein (HDL)	Male: >1.0 Female: >1.3	NCEP
Total cholesterol (TC)	<4.0-5.5	National Vascular Disease Prevention Alliance (NVDPA) (25)
Non-HDL	<2.5	NVDPA
Low density lipoprotein (LDL)	<1.8-2.6	NVDPA, NCEP
Total cholesterol to HDL ratio (TC:HDL)	<4.5 (ideal) 4.5-8.0 (medium) >8.0 (high)	NVDPA Combines the contributions of HDL and LDL into a single figure, weighing up 'bad' (LDL) transport to the tissues against 'good' (HDL) cholesterol return to the liver, so called 'reverse cholesterol transport'. There is a continuous graded association with CVD risk, and it is the single most useful predictive lipid index of CVD risk. (56)

In 2011-2012, 63.2% of all Australians have dyslipidaemia, equating to 13.8% taking cholesterol lowering medication and 49.4% with at least one abnormal cholesterol parameter. Obese individuals had five times the risk of high triglycerides (25.3% vs 5.3%), twice as likely to have subnormal HDL levels (36.2% vs 14.1%) and twice as likely to have elevated total cholesterol (35.5% vs 16.4%) than normal weight individuals (53).

3.1.4.3 Hypertension

Hypertension is defined as a blood pressure of >140/90 mmHg in general populations, or >130/80 mmHg in those with diabetes or albuminuria (25). Hypertension is a major risk factor for ischaemic heart disease, stroke, kidney disease and heart failure.

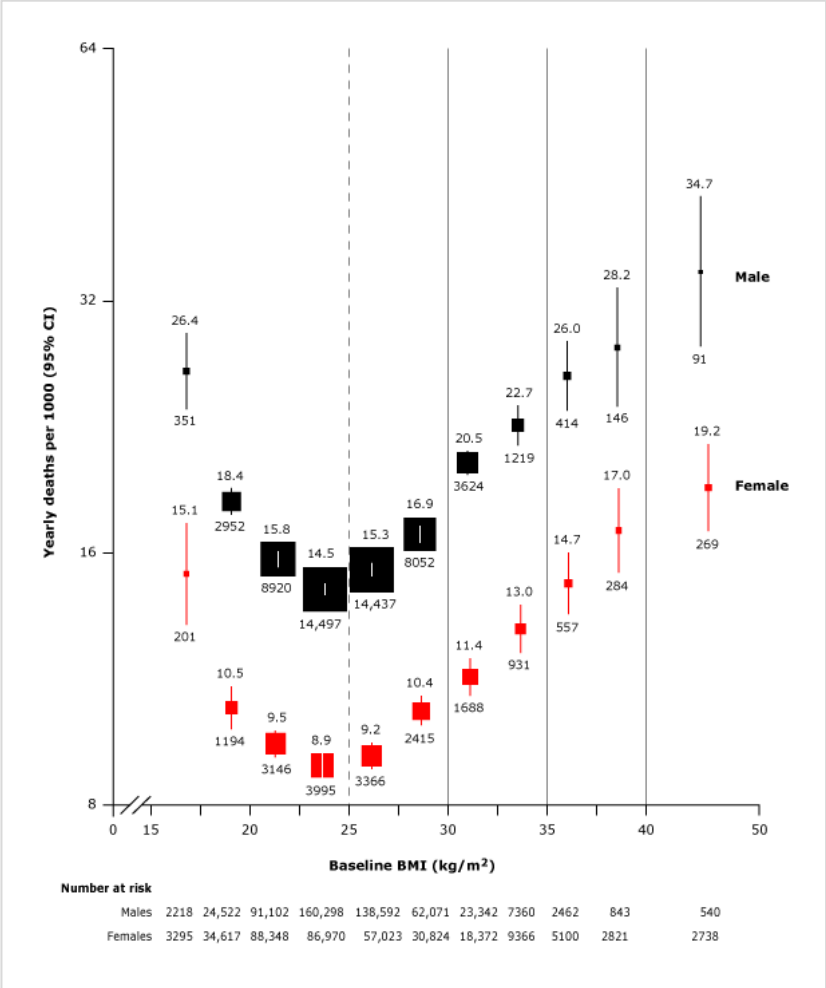
Obesity can directly lead to hypertension via alterations in renal function, particularly sodium resorption, impaired pressure natriuresis and alterations in sympathetic nervous system activation (57). In 2011-2012, 31.6% of adult Australians had hypertension, comprising 10.1% taking antihypertensive medication and 21.5% with high blood pressure. Of those with hypertension, obese and overweight individuals were heavily represented, comprising 76.3%

of hypertensive individuals (58). Obese individuals were 2.2 times as likely to have hypertension compared to normal weight individuals (41.2% vs 18.5%). Resistant hypertension is also five times as likely in those with obesity (59).

3.1.4.4 Mortality

Several large epidemiological studies (5, 60, 61) have shown a J-shaped relationship between BMI and all-cause and cardiovascular mortality (**Figure 3.1**). The lowest risk occurs between 21-30 kg/m^2 and rises incrementally as BMI increases. In adults over 50 years of age, the increase in mortality occurs at a higher BMI of 25kg/m^2 for women and 30kg/m^2 for men (62).

Figure 3.1: All-cause mortality vs body mass index (54)



From Whitlock G et al, Body-mass index and cause-specific mortality in 900,000 adults: collaborative analyses of 57 prospective studies. The Lancet. 2009;373:1083-1096

3.1.4.5 Cardiovascular disease

Obesity predisposes to a plethora of cardiac atherosclerotic risk factors, including insulin resistance, hypertension, dyslipidaemia, and obstructive sleep apnoea. Obesity is present in up to 86% of patients with heart failure, and is a risk factor, independent of comorbidities. Duration of obesity over 10 years impacts prevalence rates significantly, with 70% prevalence after 20 years of obesity, and 90% after 30 years (63).

Obesity also has direct physiological links with cardiovascular damage and heart failure. Its impact on haemodynamic function predisposes to cardiac remodelling and ventricular dysfunction. These abnormalities increase with obesity severity, and may be present even in the absence of comorbidities such as hypertension and coronary artery disease (63).

3.1.4.6 Pulmonary function

Pulmonary function is significantly affected by obesity, even in the absence of any specific respiratory disease. The primary abnormality is excess adipose tissue around and within the thorax and abdomen. This restricts functional residual capacity (FRC) and expiratory reserve volumes, increasing airway closure. Subsequently, oxygen saturations may be lower in obese subjects (64).

Obesity has strong epidemiological links with asthma, with a 2-3.8 times increased risk. The relationship between obesity and asthma is hypothesised to be partly because of obesity-related systemic inflammation, which individually sensitises airways (65).

3.1.4.7 Sleep disorders and sleep apnoea

Sleep apnoea is the recurrent occlusion of the upper airway during sleep that results in desaturation and arousal from sleep. It is independently associated with the development of diabetes mellitus, hypertension and cardiovascular disease. Obesity is one of the strongest risk factors for sleep apnoea. Whilst approximately 2-4% of the general population suffers from sleep apnoea, this increases to 30-60% in overweight and obese populations (66).

3.1.4.8 Cancer

Epidemiological studies have linked obesity with several types of cancers. The strongest links are with endometrial, postmenopausal breast, colon, kidney, oesophageal, pancreas, gallbladder, liver and haematological malignancies (67, 68). Obesity is predicted to account for 14% of all cancer deaths in men and 20% in women (68). In addition, the presence of obesity leads to poorer outcomes, worse prognosis and increased cancer-related death.

3.1.4.9 Other obesity related comorbidities

Gastro-oesophageal reflux disease (GORD)

A meta-analysis of studies assessing GORD and obesity showed that there was a significant association of BMI with GORD symptoms, erosive gastritis, oesophageal adenocarcinoma and gastric cardia adenocarcinoma. For every 5 kg/m^2 above 25 kg/m^2, there is an increase in odds of GORD symptoms (69).

Gallstones

In the morbidly obese, there is a relative risk of 5-6 for the development of gallstones (70), with an increased rate of cholecystectomy, and gallbladder cancer. The pathophysiological mechanisms behind this are likely the super-saturation of bile with cholesterol, fuelled by higher levels of insulin associated with obesity, prompting hepatic cholesterol synthesis.

Osteoarthritis

Obesity is a significant risk factor for osteoarthritis of the lower limbs. The prevalence of knee osteoarthritis (OA) increases with increasing weight. A meta-analysis by Jiang *et al* in 2012 showed a 1.22-1.54 increase in odds with every 5-unit increase in BMI (71).

Fertility

Obesity affects the fecundity of women by at least 18% (72) and is closely related to polycystic ovarian syndrome (PCOS), which affects 50% of obese women (73). Excess adipose tissue in men increases the conversion of testosterone into oestradiol, which can suppress the reproductive axis causing secondary hypogonadism (74).

Mental health and quality of life

Health related quality of life (QOL), assessed by the SF-36 questionnaire, shows considerably lower scores for individuals with obesity compared to community norms. These QOL scores decrease with increasing weight. There are multiple psychological disorders closely associated with obesity, including depression and anxiety (75).

3.1.5 Metabolic syndrome

3.1.5.1 Definition of the metabolic syndrome

The term 'metabolic syndrome' refers to a cluster of metabolic risk factors that are predictive of progression to both type II diabetes mellitus and coronary heart disease (76). There are differing definitions of the metabolic syndrome (MetS) (**Table 3.9**), however, all share a common focus on elevated glucose, triglyceride and blood pressure levels and/or low HDL cholesterol (77). Clustering most commonly occurs in the setting of obesity, insulin resistance (77) and a sedentary lifestyle (78). The most broadly utilised definition is the National Cholesterol Education Program (NCEP) Adult Treatment Panel III (ATPIII) (55), as there is no requirement for a formal glucose tolerance test, nor clamp test.

Metabolic syndrome in clinical practice

The metabolic syndrome serves as a simple measure of metabolic derangements associated with obesity. It is a useful and practical tool for physicians and patients, to easily identify those at greatest risk (79).

The metabolic syndrome does not predict absolute risk, but relative risk. Therefore, compared to those without the MetS, the relative risk of a major cardiovascular event is twofold, and the lifetime risk of developing type II diabetes mellitus is fivefold (80). The METS-GREECE study showed that presence of increasing numbers of risk factors is associated with incremental increases in atherosclerosis and cardiovascular disease endpoints (81). Treatment and resolution of the MetS and its components correlate with improved health outcomes including improvements in cardiovascular risk and overall mortality (82).

Table 3.9: Commonly used definitions of the metabolic syndrome

Clinical measure	NCEP Adult Treatment Panel III (ATPIII), 2001 (55)	WHO definition, 1998 (83)	IDF consensus, 2005 (84)
Insulin resistance	No requirement.	IGT, IFG, T2DM, or insulin resistance (assessed by clamp studies).	No requirement.
	Any three of the following five:	**Plus two of the following criteria:**	
Body weight	• Waist circumference >102 cm (40 in) in men and >88 cm (35 in) in women	• Waist-to-hip ratio of >0.90 in men and >0.85 in women	Central obesity (defined as waist circumference >94 cm for Europid men and >80 cm for Europid women, with ethnicity specific values for other groups)
			Plus any two of the following four factors:
Lipids	• Serum TG >150 mg/dL (1.7 mmol/L)	• Serum TG >150 mg/dL (1.7 mmol/L), and/or HDL cholesterol <35 mg/dL (0.9 mmol/L) in men and <39 mg/dL mmol/L in women.	• Raised TG level >150 mg/dL (1.7 mmol/L), or specific treatment for this lipid abnormality
	• HDL cholesterol <40 mg/dL (1.0 mmol/L) in men and <35 mg/dL (1.3 mmol/L) in women		• Reduced HDL cholesterol <40 mg/dL (0.9 mmol/L) in males and <50 mg/dL (1.1 mmol/L) in females, or specific treatment for this lipid abnormality.
Blood pressure	• BP >130/85 mmHg	• BP >140/90	• Raised BP: systolic BP>130 or diastolic BP >85 mmHg, or treatment of previously diagnosed hypertension
Glucose	• Serum glucose >100 mg/dL (5.6 mmol/L) changed in 2004 from 110 mg/dl (6.1 mmol/L)	(IGT, IFG or T2DM, as specified above)	• Raised fasting blood sugar level (BSL) >100 mg/dL (5.6 mmol/L), or previously diagnosed type II diabetes. If above 5.6 mmol/L or 100 mg/dL, oral glucose tolerance test (OGTT) strongly recommended but is not necessary to define presence of the syndrome.
Other		• Urinary albumin excretion rate >20 ug/min or albumin:creatinine ratio >30 mg/g	

NCEP – National Cholesterol Education Program; WHO – World Health Organization; IDF – International Diabetes Federation; IGT – impaired glucose tolerance; IFG – impaired fasting glucose; T2DM – type II diabetes mellitus; TG – triglycerides; HDL – high density lipoprotein; BP – blood pressure; BSL – blood sugar level; OGTT – oral glucose tolerance test

Metabolic syndrome and obesity

Morbid obesity and metabolic dysfunction are closely linked (85). Measures of obesity form at least one component of MetS in all frequently used definitions (**Table 3.9**). Obesity-related visceral fat accumulation and inflammation is one of the postulated pathogenic mechanisms behind the MetS (86). Visceral fat production of bioactive substances, such as leptin, resistin, tumour necrosis factor-α (TNF-α), interleukin-6 (IL-6) and angiotensin II have all been related to the development of vascular disease (87). Via these mechanisms, obesity is thought to promote a metabolically dysfunctional state and thereby induce the metabolic syndrome.

The prevalence of MetS increases with increasing weight. Whilst only 4.6% of normal weight men are affected, 22.4% and 59.6% of overweight and obese men respectively have the MetS (88). There was a similar pattern in women.

The effects of weight loss or dynamic weight change on the MetS has not been established.

3.1.5.2 Controversy regarding the metabolic syndrome

The metabolic syndrome as a distinct diagnostic entity is controversial. The major limitations have been extensively discussed and its value debated (89). Firstly, there is no consensus definition for MetS, with numerous agencies publishing a variety of criteria and threshold values (**Table 3.9**). These criteria also require some modification depending on ethnicity. As such, studies examining the prevalence and change in MetS have significant heterogeneity depending on the criteria that is used (90).

Secondly, there is ongoing debate about whether MetS represents an entity or a surrogate of combined risk factors. There is no current unifying pathogenic hypothesis behind the components that comprise MetS, although there is ongoing investigation into the role of insulin resistance and obesity-related adipose tissue inflammation (86).

Thirdly, MetS dichotomises each risk factor. Many argue that specific cut-offs may oversimplify risk and previously have not been evidence based. However, recent updates have defined thresholds that are evidence-based and widely accepted (76). Some argue that the use of individual metabolic factors, such as the presence of diabetes, hypertension or dyslipidaemia, adequately evaluate risk, and clustering these into a single entity, such as MetS, does not add value.

However, proponents have suggested a multiplicative model of MetS, showing independent risk of cardiovascular disease (CVD), myocardial infarction and stroke associated with presence of MetS compared to those without (91). Additionally, some studies have found unaccounted additional risk after treatment of component metabolic risk factors, suggesting that MetS as a sum is probably greater than its parts (92).

Practically, MetS is a useful measure of metabolic derangements associated with obesity that correlates with the risk of cardiovascular disease, nonalcoholic fatty liver disease and type 2 diabetes (93). It is easily applicable in the clinical setting for primary care physicians and

those not subspecialised in the field. It can be applied widely and in almost every clinical setting at no significant cost. Identification of MetS can lead to further risk assessment with tools such as the Framingham score, which can quantify absolute risk (94). In addition, there is considerable clinical utility in identifying and treating each of these risk factor components of MetS (92). Ultimately, resolution of MetS and its components correlates with better health outcomes (92).

3.2 Pathophysiology of obesity

The pathophysiology of obesity originates from the excessive fat produced as a result of energy imbalance, and subsequently stored predominantly in fat cells (adipocytes) as well as ectopic regions. Enlarged fat cells produce excess adipokines as well as inflammatory markers. These factors have significant autocrine, paracrine and endocrine effects, and can influence a variety of distant organ systems, including the liver. Additionally, expanded fat stores exert mechanical stress on adjacent structures and organs, altering normal physiological function and accelerating degeneration.

This chapter summarises the physiological changes that occur with obesity, and the mechanisms by which obesity gives rise to disease.

3.2.1 Adipose fat

3.2.1.1 Development of adipose tissue

Adipose fat develops in the 14th week of gestation, with proliferation slowly decreasing in late gestation. Up to the age of 10 years old, adiposity is achieved by filling existing predetermined cells. During adolescence, increasing adiposity occurs via cellular proliferation. This growth generally determines the cellular level of adiposity in adulthood, which is maintained throughout life (95).

3.2.1.2 Structure and composition

Adipose tissue comprises adipocytes and the stromal vascular fraction (SVF), which contains fibroblasts, endothelial cells, blood cells, macrophages, pericytes and preadipocytes (96). Although adipocytes make up more than 90% of the fat pad volume, they comprise only 20-40% of cellular content, with the stromal vascular fraction comprising the majority of cells.

Adipocytes

Adipocytes have a unique structure, in that more than 95% of its cell body is made of the lipid droplet and one organelle. This lipid droplet is made of triglycerides that are synthesised and stored by the adipocyte, and can subsequently be broken down and exported as required.

This lipid droplet can dramatically increase and decrease the size of the adipocyte, from 25µm to 200µm in diameter (96).

There are three types of adipocytes - white, brown and the most recently discovered, beige. White adipose tissue (WAT) is the most abundant adipose tissue, and the primary storage depot for lipids (96). They produce various factors (adipokines) that can have substantial hormonal influence around the body. Particularly important, is the production of leptin, which controls our food intake and energy expenditure (97).

Brown adipocytes are highly specialised (98). They are a thermogenic source, converting chemical energy into heat via the actions of uncoupling protein-1 (UCP-1) located within the mitochondria. Significant brown fat depots exist in the neonatal period, but almost completely disappear in adults unless challenged by chronic cold or states of catecholaminergic excess (e.g. phaeochromocytoma) (99). Small pockets of brown fat remain in adults around the supraclavicular and spinal regions. They are controlled via the sympathetic nervous system, circulating hormones (e.g. triiodothyronine), bile salts, cardiac hormones and irisin.

Beige adipocytes are found within white fat depots and can switch thermogenic levels depending on stimulation. Beige cells are usually in a resting state, with low basal UCP-1 expression and uncoupled respiration. However, stimulation by beta-adrenergic agonists via the sympathetic nervous system can induce UCP-1 in beige fat to brown fat levels (100). Rodent experimental models exposed to prolonged periods of cold were seen to induce the appearance of thermogenic UCP-1^{+} beige cell clusters within white adipose tissues (101).

Stromal vascular cells

The cells of the stromal vascular fractions (i.e. non-adipocytes) constitute the majority of cellular content of adipose tissue (albeit, a small volume). They are physiologically active and have a number of important functions for adipose tissue homeostasis (102).

The majority of these non-adipocyte cells are immune cells, such as monocytes and macrophages (103). They clear necrotic adipocytes, but importantly, mediate endocrine and paracrine signalling, as well as inflammation. There are two types of macrophages – M1, which is “classically activated” and M2, which is “alternatively activated” (104). M1 express

the surface marker CD11c, and are pro-inflammatory, producing cytokines such as TNF-α, IL-6 and IL-1β. M2 macrophages have CD206 and CD301 markers, and assist in tissue remodelling and wound healing, by secreting the anti-inflammatory cytokines, IL-10 and IL-1 receptor antagonist.

Other immune cells exist, including neutrophils, mast cells, B-lymphocytes, and various T-lymphocytes. A reservoir of pluripotent cells, including preadipocyte cells, exists within adipose tissue. Endothelial cells and pericytes create the vasculature that enables adipocyte growth. This is controlled by adipocyte-secreted vascular endothelial growth factor (VEGF), which mediates angiogenesis (105).

3.2.1.3 Function

Adipose tissue is a remarkably complex organ with substantial effects on physiology and pathophysiology (102). It produces a multitude of hormones and factors that influence other adipocytes and exert a systemic effect on distant systems. Adipose tissues additionally play an important mechanical function, protecting delicate organs such as the heart and kidneys, and cushioning body parts exposed to high levels of mechanical stress.

These three main functions of adipose tissue are discussed below: (1) adipocyte metabolism, (2) adipokine production and (3) mechanical function.

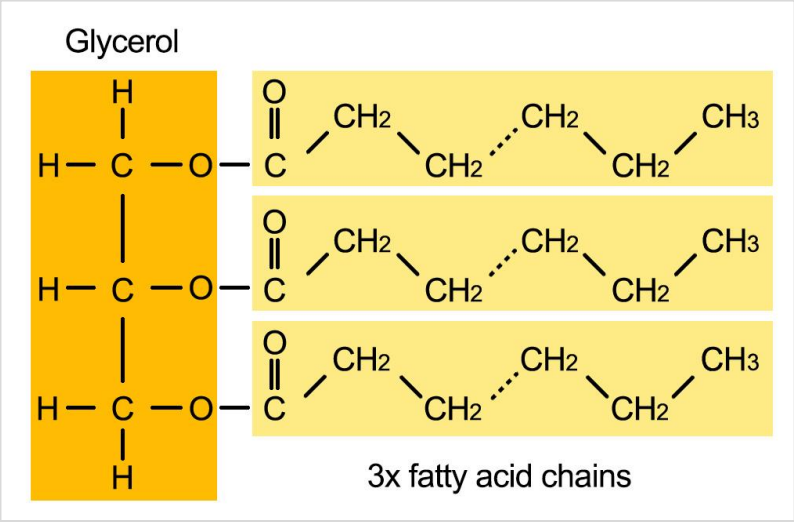
Adipocyte metabolism

Energy balance and nutritional homeostasis is a vitally important function of adipose tissue, and is coordinated by complex and critical local and systemic processes (102). Triglycerides (TG) are the main form of energy storage in adipocytes. The breakdown (lipolysis) and synthesis of triglycerides within adipose tissue is described below.

Triglyceride synthesis and storage

Triglycerides are formed and stored by adipocytes after being synthesised from free fatty acids (FFA) esterified to a glycerol backbone (**Figure 3.2**).

Figure 3.2: Generic structure of a triglyceride molecule, with glycerol backbone and three fatty acid chains attached.



Free fatty acids (FFA) are delivered from three pathways – (1) from circulating chylomicrons (from gastrointestinal tract) or very low-density lipoprotein (VLDL) (derived from liver), (2) direct uptake from circulating FFA or (3) de novo lipogenesis (DNL) by producing fatty acids from glucose and other precursors, under control of sterol-response-element-binding protein 1c (SREBP1c, found in liver) and carbohydrate-response-element-binding protein (ChREBP, found in white adipose tissue) (102).

Lipolysis

Lipolysis is the process where free fatty acids and glycerol are liberated from their triglyceride form. Triglycerides (TG) are hydrolysed into diacylglycerol (DG) by adipose TG lipase (ATGL), then into monoacylglycerol (MG) by hormone sensitive lipase (HSL). The final process is the breakdown of MG into FFA and glycerol by monoacylglycerol lipase. This pathway accounts for >90% of lipolytic activity. Insulin plays a substantial role in physiological control of this lipolytic process (102).

Free fatty acids bind to albumin in the circulation and are delivered to muscle (for oxidation), liver (for TG synthesis or oxidation) and adipocytes (for re-esterification).

Adipokine production

Adipocytes secrete various peptide hormones and bioactive molecules (**Table 3.10**). The two most notable and most studied adipocyte-derived hormones are leptin and adiponectin. They influence appetite, metabolism, innate immunity and reproduction (106). In addition, they exert a protective effect against lipotoxic damage to lean tissues (107).

Table 3.10: Factors produced by adipose tissue and dysfunction in obese states.

Adipokines	Function	Protective or detrimental	Change with obesity
Adipocyte secrete hormones			
Leptin	**Decreases hunger** and **increases energy expenditure** through suppression of orexigenic signals (neuropeptide Y and agouti-related peptide) and stimulation of appetite suppressors (α-melanocytic stimulating hormone). **Stimulates release** of thyrotropin releasing hormone, corticotropin releasing hormone and oxytocin (108).	Protective - ↓hunger - ↑energy expend	Increased
Adiponectin	Produced exclusively by adipose tissue. Structurally, but not functionally, related to tumour necrosis factor α (TNF-α). Acts predominantly on adipose, but also brain, liver and muscle (109). **Increases insulin sensitivity**, and therefore affects glucose metabolism and lipid metabolism. **Increases energy expenditure**. Strong **anti-inflammatory** action. In liver, induces fatty acid oxidation and decreases hepatic glucose output via **decreased gluconeogenesis** (110).	Protective - ↑insulin sensitivity - ↑energy expend - ↓inflam	Decreased
Lipocalin 2	Increases in obesity in humans and animal models. Expressed in adipocytes in response to inflammation. Significantly associated with **insulin resistance** and **increased inflammation** (111).	Detrimental - ↑insulin resistance - ↑inflam	Increased
Adipocyte secrete inflammatory markers			
Retinol binding protein 4 (RBP4)	Major vitamin A transporting protein in serum. Highly expressed in adipose tissue (mainly visceral) and serum in response to obesity. **Promotes insulin resistance** via inflammatory pathways and activation of cytokine receptor Stra6 (102).	Detrimental - ↑insulin resistance - ↑inflam	Increased
Serum amyloid A (SAA)	**Pro-inflammatory protein** highly expressed in adipocytes and liver. Upregulated in obesity and decreases with weight loss (112). Stimulates release of cytokines from endothelial cells and adipose tissue, increases lipolysis and decreases adiponectin production (113).	Detrimental - ↑inflam	Increased
Non-adipocyte factors			
Omentin	Produced by omental VAT. Decreases in response to obesity and insulin resistance. *In vitro* experiments show that it may **increase insulin sensitivity** (114).	Protective - ↑insulin sensitivity	Decreased
Visfatin	VAT specific adipokine. Levels increase with obesity, **insulin resistance** and **metabolic syndrome** (114).	Detrimental - ↑insulin resistance	Increased
Resistin	Small protein found in white adipose tissue and serum. Levels directly related to **insulin resistance** in animal models (102). Its role is less defined in humans.	Detrimental - ↑insulin resistance	Increased
TNF-α	Produced mainly by adipose tissue macrophages and other immune cells. Greater expression in subcutaneous than visceral fat. Mediates **obesity-related inflammation and insulin resistance** (114), via effects on gene expression and alteration of insulin signaling.	Detrimental - ↑insulin resistance - ↑inflam	Increased
IL-6	IL-6 receptor is homologous to the leptin receptor. Both IL-6 and the IL-6 receptor are expressed by adipocytes and adipose tissue matrix. One third of circulating Il-6 is from adipose tissue, and concentrations correlate with obesity. Visceral fat has 2-3 times concentration compared to subcutaneous fat. Induces hyperlipidaemia, hyperglycaemia and insulin resistance. Predicts the development of type 2 diabetes and cardiovascular disease (114).	Detrimental - ↑insulin resistance - ↑lipids	Increased

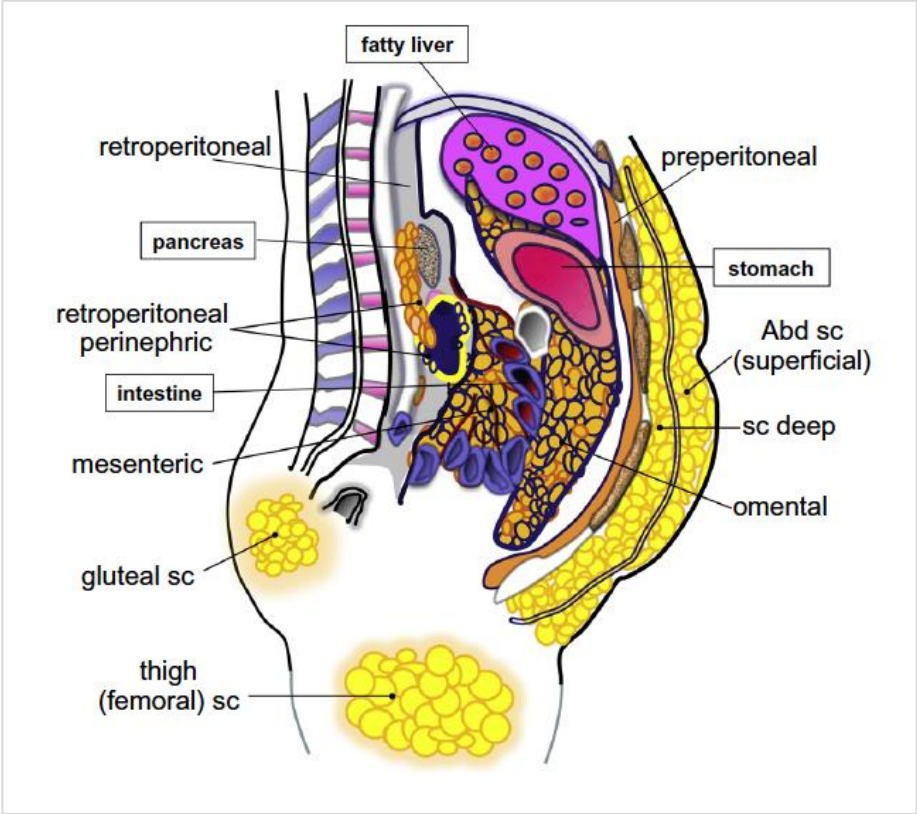
Adipocytes also influence systemic inflammation and insulin resistance through complement factors and acute phase proteins, including serum amyloid A (SSA) (113) and retinol binding

protein 4 (RBP4) (115). The non-adipocyte cells (or stromal vascular fraction) within adipose tissue produce additional factors, including omentin, visfatin, resistin, TNF-α, IL-6 and IL-8 (116).

3.2.1.4 Adipose location

Adipose tissues are present in discrete locations throughout the body. The two rough distinctions are the subcutaneous adipose tissue (SAT) and visceral adipose tissue (VAT). These can be further divided into subgroups within each, such as abdominal, gluteofemoral subcutaneous adipose tissues, and omental, mesenteric and epiploic visceral adipose tissues. Smaller subspecialised adipose depots exist, such as the epicardial and intermuscular depots, and specific brown adipose tissue depots exist around the cervical-supraclavicular, perirenal and paravertebral regions (**Figure 3.3**).

Figure 3.3: Major adipose fat depots in humans (117).



sc – subcutaneous. From Lee MJ et al. Adipose tissue heterogeneity: implications of depot differences in adipose tissue for obesity complications. Mol Aspects Med. 2013;34:1-11.

There are clear distinctions in characteristics and function of adipose tissue according to location. The differences between general subcutaneous (SAT) and visceral adipose tissue

(VAT) have been most commonly studied (**Table 3.11**). These differences can be described according to: (1) cellular content, (2) adipocyte metabolism and (3) adipokine production.

Table 3.11: Differences between subcutaneous and visceral adipose tissue

Characteristic	Subcutaneous adipose tissue (SAT)	Visceral adipose tissue (VAT)
Cellular differences		
Cellular size	Generally larger cell size	Smaller cell size
Cellular composition	More pre-adipocytes (118)	More non-adipocytes (stromal vascular fraction) (119)
Cellular growth	Upper body (abdominal): Hypertrophy Lower body (gluteal, femoral): Hyperplasia	
Metabolic differences		
Lipolysis (affected by innervation, blood flow, enzyme expression) (120)	Higher basal lipolysis rate. Upper body lipolysis (~70% FFA) is greater than lower body lipolysis (~30% FFA). More responsive to anti-lipolytic effects of α-2 adrenergic agonists.	Lower basal lipolysis rate. Elevated response to adrenergic agonists. Less sensitive to insulin (anti-lipolytic).
Triglyceride storage (121)	Women: Higher lipoprotein lipase (LPL) Men (normal or moderately obese): Higher LPL activity	Women: Lower LPL activity Men (normal or moderately obese): Lower LPL activity
Free fatty acid (FFA) handling (122)	Upper body vs lower body SAT: Greater FFA uptake after meals.	Preferential FFA uptake in VAT compared to SAT
Glycerol synthesis (for triglyceride synthesis) (120)		Potentially greater GLUT4 protein and blood flow, facilitating glucose uptake and conversion to glycerol backbone.
Adipokine differences		
Inflammatory markers (123)	Greater IP-10.	Greater expression of inflammatory cytokines (IL-6, IL-8, CCL2, CCL5, MIP-1α, PAI-1), acute phase reactants and complement factors
Hormones (116)	Greater leptin	Omentin is produced exclusively by non-adipocytes in omental VAT, decreasing with obesity and insulin resistance.

SAT – subcutaneous adipose tissue; VAT – visceral adipose tissue; FFA – free fatty acid; LPL – lipoprotein lipase; GLUT4 – glucose transporter 4; IP-10 – interferon-gamma inducible protein 10; IL-6 – interleukin 6; IL-8 – interleukin 8; CCL2 – chemokine (C-C motif) ligand 2; CCL5 – chemokine (C-C motif) ligand 5; MIP-1α – macrophage inflammatory protein 1α; PAI-1 – plasminogen activator inhibitor-1.

3.2.2 **Adipose dysfunction in obesity**

Storage of excess energy in the form of adipose tissue is necessary for survival during nutritionally poor periods, such as in times of starvation. However, prolonged periods of food abundance can lead to excessive storage of fat, and subsequently, obesity (124).

Over the last few decades, increasing evidence has revealed the importance and role of adipose tissue in the pathophysiology of obesity. As increasing amounts of fat are stored, adipose tissue becomes increasingly dysfunctional, affecting its many physiological roles.

Although there are still significant gaps in knowledge around adipose biology in obesity, the main mechanisms are summarised below.

3.2.2.1 Adipose tissue expansion in obesity

Adipose tissue has a unique ability to change its size and remodel. In the setting of obesity, expansion of the adipose tissue depots can occur by hypertrophy (enlargement of cells) or hyperplasia (recruitment of preadipocytes). Hyperplasia occurs in childhood and early adulthood after months of overnutrition (125). By early adulthood, adipocyte numbers are generally fixed, regardless of weight loss or gain (126). Therefore, the main mechanism whereby fat depots increase in adults is via hypertrophy.

This, however, does not mean that adipocytes do not die. Whilst the overall number of adipocytes remain the same, there is an ongoing turnover of cells, at a rate of ~8% per year (127). Animal studies suggest that there is an overall increase in adipocyte death rate in obesity, with balanced increases in proliferation rates (128). Macrophages play a key role in the regulation of this process, with possible contributions from M1 and M2 subtypes (see **Section 3.2.2.2 - Immunomodulation**) (128).

Hypoxia

Expansion of the adipose tissue depot can result in hypoxia. Normally, adipose tissue promotes neovascularisation in order to achieve adequate perfusion during growth (129). This is achieved through various adipocyte-derived factors, including oxygen-sensitive transcription factor hypoxia-inducible factor 1α (HIF-1α). Similar to cancer, adipose tissue can outgrow its blood supply and hypoxia can develop during rapid expansion when formation of new blood vessels does not match rate of growth (**Figure 3.4**). This can result in inflammation and adipocyte death (102).

The overexpression of HIF-1α in obese adipocytes is, itself, associated with metabolic dysfunction. The proposed mechanisms for this include reduction in adiponectin (130), and promotion of fibrosis and inflammation (131).

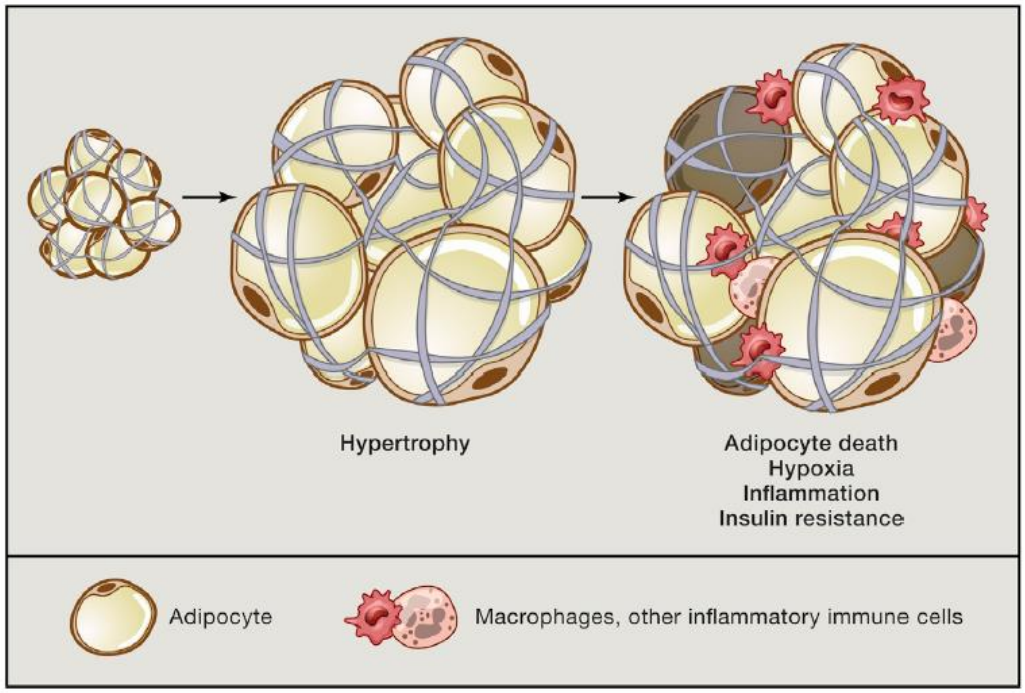
Fibrosis

Fibrosis has recently been hypothesised to contribute to general health of adipose tissue. Extracellular matrix maintains the structure of fat, holding the cells in a mesh. Adipocytes

produce this collagen-based matrix, and also possess the enzymes required to break them down for remodelling. This process is highly regulated by changes in nutrient availability (132).

Pathology exists when adipocytes increase their size until the matrix limits expansion (**Figure 3.4**). This produces further fibrotic changes, resulting in increased hypoxia, activation of stress-related pathways, and inflammation (133). Experiments in *ob/ob* mice show that disruption of adipose fibrosis, via disruption of collagen VI, results in reduced inflammation, better glycaemic control and an improved lipid profile (134).

Figure 3.4: Adipocytes increase their size during overnutrition until further expansion becomes limited by the matrix, which undergoes fibrotic changes. This triggers hypoxia, inflammation and cell death (102).



From Rosen ED, Spiegelman BM. What we talk about when we talk about fat. Cell. 2014;156(1): 20-44.

3.2.2.2 Immunomodulation

The role of cytokines in obesity was first described in 1993 by Hotamisligil *et al*, who showed that TNF-α and other cytokines were produced in adipose tissue, and increased with overnutrition (135). These factors were shown to directly affected insulin sensitivity locally and systemically in the liver and muscle. Further studies showed that these inflammatory markers were produced from adipose tissue macrophages rather than the adipocytes themselves (103).

Obesity is accompanied by significant changes in composition and function of adipose tissue macrophages. In histological sections, macrophages appear as “crown-like structures” clustering around dead adipocytes (136). Two adipose tissue macrophage phenotypes exist – M1 “classically-activated” and M2 “alternatively activated” (137). Obesity is characterised by excess accumulation of M1 macrophages, a proinflammatory phenotype that produces TNF-α, IL-6 and IL-1β. M1 macrophages decrease insulin sensitivity through paracrine and endocrine mechanisms. On the other hand, M2 macrophages are anti-inflammatory, immunosuppressive and assist with tissue remodelling and healing. They produce anti-inflammatory cytokines IL-10 and TGF-β. New evidence suggests M2 macrophages are involved in promoting white adipose tissue browning (138). Obesity affects adipose tissue macrophages by increasing overall macrophage volume, and shifting the balance of M1/M2 in favour of a classically activated M1 proinflammatory state (104).

Almost all types of inflammatory cells have been implicated in influencing adipose tissue inflammation and exacerbating insulin resistance (139). The exact sequence of events that brings immune cells into adipose tissue is still unclear. Increased oxidative stress, endoplasmic reticulum (ER) stress, and toll-like receptor activation could potentially upregulate chemokines and recruit immune cells to the fat pad. Adipocytes, macrophages or other antigen presenting cells are also known to activate resident T-cells and facilitate inflammation (140). Specific antigens are yet undefined.

3.2.2.3 Hormonal and inflammatory marker disturbances

Alterations in adipose-derived hormones and inflammatory markers exist in states of obesity (**Table 3.10**).

The expression of adiponectin decreases with increases in obesity (141). Reduction in adiponectin has been associated with the development of insulin resistance, dyslipidaemia and atherosclerosis in both rodent models and humans. Weight loss significantly elevates plasma adiponectin, with resultant increased insulin sensitivity (109).

Leptin increases with increasing adiposity. Evidence shows that it has both beneficial and detrimental effects (142). It exerts some control over weight gain by signalling the central and peripheral nervous system, to reduce body weight via decreased food intake and increased energy expenditure (102). Leptin can enhance insulin sensitivity in muscle and

liver, limiting ectopic accumulation of triglycerides (143). However, it also has a role as a proinflammatory mediator, and promotes angiogenesis at other sites (142). Its influence on growth and cellular proliferation also links elevated leptin levels with cancer development and progression (144).

Adipose tissue inflammatory markers, such as TNF-α, IL-6, retinol binding protein 4 (RBP4), serum amyloid A (SSA), visfatin and resistin, have detrimental effects on glucose metabolism pathways and inflammation. Increased levels result in increased insulin resistance, hyperglycaemia and lipid mobilisation (114).

3.2.2.4 Ectopic fat deposition

The 'spill-over hypothesis' suggests that adipose tissue, once saturated, will 'spill over' and deposit excess lipids and energy in other tissues including liver, skeletal muscle and pancreas (145). Excessive ectopic fat stores subsequently cause insulin resistance via defects in insulin signalling and reduced insulin-stimulated glucose transport (146). This interplay is seen in experimental mice models of lipodystrophy, where fat is unable to be stored in adipose tissue. In these models, positive energy balance causes fat deposition in muscle and liver, resulting in profound insulin resistance. Implantation of normal fat from healthy mice resolves muscle and liver fat deposition, and normalises insulin signalling and glucose levels (146).

Ectopic fat deposition can cause direct disturbances in liver immunometabolism, inflammation, mitochondrial and endoplasmic reticulum (ER) function, ultimately leading to liver damage (See **Section 3.8.2 - Pathophysiological mechanisms for steatohepatitis**).

3.2.3 Impact of obesity on insulin resistance and type II diabetes

Excess adipose tissue in states of obesity can heavily influences insulin sensitivity. Several mechanisms are listed below, and includes release of free fatty acids and glycerol, adipokines including leptin and adiponectin, and proinflammatory cytokines.

- *Free fatty acids (FFA)*: Increased release of FFAs are seen in obesity. Experiments that infuse FFA into human subjects have shown that insulin resistance increases within hours, due to extracellular inhibition of peripheral glucose transport and phosphorylation (147). Intracellular FFA accumulation inhibits pathways of glucose metabolism (148). Chronic exposure to high levels of FFAs can result in impaired insulin secretion and

decreased β-cell insulin synthesis. Furthermore, increased FFA delivery alters the intracellular lipid profile, creating metabolites such as diacylglycerol and ceramides, which can, in turn, impair insulin sensitivity (149).

- *Retinol-binding protein-4 (RBP4):* RBP4 is produced by adipose tissue and augmented in obesity. It increases insulin resistance via reduced phosphatidylinositol-3-OH kinase (PI(3)K) signalling in muscle and increased gluconeogenesis in the liver (115).
- *Adiponectin*: Adiponectin is an insulin sensitiser, via AMP-activated protein kinase (AMPK) and peroxisome proliferator activated receptor α (PPAR-α). In obesity, adiponectin is decreased, thereby reducing its protective effect (150).
- *Cytokines*: Adipose tissue macrophages release increasing amounts of TNF-α, IL-6 and CCL-2 in response to obesity. These act on the c-Jun amino-terminal kinase (JNK) and IκB kinase-β (IKK-β)/nuclear factor-κB (NF-κB) pathways, which increase insulin resistance (151).
- *Hyperglycaemia:* Extreme elevation in blood glucose can cause disease progression via glucotoxic effects on β-cells.

3.2.4 Impact of obesity on lipid metabolism and dyslipidaemia

3.2.4.1 Normal lipid metabolism

After a meal, triglycerides (TG) are hydrolysed into free fatty acids (FFA) and monoacylglycerol (MG), and cholesterol esters are de-esterified into free cholesterol. These are emulsified by bile acid into micelles for absorption. Once inside the enterocytes, triglycerides are reassembled and packaged with cholesterol into chylomicrons for circulation.

Lipoprotein lipase (LPL) in the capillary vasculature of adipose and muscle tissue converts 90% of triglycerides within chylomicrons into glycerol and FFA. These are taken up by muscle cells and adipocytes for energy use or storage. The remaining chylomicron remnant is cleared by the liver (152).

The liver provides lipids to the peripheries for energy synthesis when required. This is achieved via the synthesis of very low-density lipoproteins (VLDL), which contain triglycerides and cholesterol. Peripheral lipoprotein lipase (LPL) breakdown TG within

VLDL for uptake by peripheral tissues. The level of LPL activity, stimulated by insulin, determines the amount of TG and FFA liberated (152).

After LPL processing of VLDLs, intermediate-density lipoproteins (IDL) are created. These are cholesterol rich VLDLs, and are either cleared by the liver, or metabolised into low density lipoproteins (LDL) by hepatic lipase.

Low density lipoproteins (LDL) have the highest cholesterol content of all lipoproteins. Approximately 40-60% of LDLs are cleared by the liver via apo-B and hepatic LDL receptors. The remainder are taken up by either (1) hepatic LDL receptors or (2) non-hepatic LDL receptors. Hepatic LDL receptor concentration is down-regulated by increased dietary saturated fats, and upregulated with decreased dietary fats. Non-hepatic LDL receptors reside mostly on macrophages, which take up excess circulating LDLs and form foam cells (152). These can subsequently contribute to atherosclerotic disease.

High density lipoproteins (HDL) are synthesised by liver and enterocytes, and initially contain no cholesterol. They collect and transport cholesterol from peripheral tissues and lipoproteins, and deliver it where it is required, in a process named reverse cholesterol transport. As such, its overall effect is anti-atherogenic (153).

Role of insulin in lipoprotein metabolism

Insulin plays an important role in lipoprotein metabolism. Firstly, it increases triglyceride uptake into peripheral tissues. Insulin stimulates the activity of lipoprotein lipase (LPL) in muscle and adipose tissue to facilitate triglyceride hydrolysis for release and uptake of FFA and glycerol. Insulin also increases the scavenger receptor CD36 expression, which transports FFA into muscle and adipose tissue (153).

Secondly, the postprandial insulin rise importantly regulates fuel storage via inhibition of hormone-sensitive lipase (HSL). Hormone-sensitive lipase hydrolyses intracellular lipids within adipose tissue, mobilising FFA for energy use. Its activity is upregulated in fasting states, to meet the energy requirements of peripheral tissues. In the postprandial state, HSL is inhibited by insulin (153).

3.2.4.2 Changes in lipid metabolism with obesity and atherogenic effects

The hallmark change in obesity is an increase in triglyceride levels. Triglyceride levels are increased due to increased FFA delivery to the liver from both endogenous and exogenous sources. This leads to increased triglyceride accumulation in liver, which subsequently synthesises large amounts of VLDL for export. Furthermore, in the setting of obesity, TG clearance from the circulation is impaired, due to a reduction in LPL expression in adipose tissue (154), reduction in LPL activity in skeletal muscle and competition for lipolysis between VLDL and chylomicrons (155).

Hypertriglyceridaemia result in large LDLs with high triglyceride content (156). As triglycerides are removed from these LDLs via hepatic lipase, small dense LDL (sdLDL) are formed. Small dense LDL are significantly atherogenic due to their long circulating time (five days), and inability to be cleared by hepatic LDL receptors. Furthermore, their small size enables easy migration through the endothelium, where they have increased affinity to arterial proteoglycans. This increases phagocytosis by macrophages, and production of foam cells, leading to atherosclerosis (153).

Triglyceride rich remnant chylomicrons and VLDL have a direct negative effect on vessel walls, via impaired endothelial function mediated by elevated FFA and increased action of LPL (153). Activation of leucocytes has also been theorised, and results in increased cytokine generation and oxidative stress. These changes have been associated with development of coronary, cerebral and peripheral atherosclerosis (157).

HDL metabolism is also strongly affected by obesity. High levels of triglyceride-rich lipoproteins increase peripheral TG transfer to HDL (158). Subsequent lipolysis of TG-rich HDL in the liver results in small HDL, which lowers HDL levels, and impairs reverse cholesterol transport (159).

Additional changes of obesity that have been studied include postprandial hyperlipidaemia associated with visceral obesity (160), decreased catabolism of chylomicron remnant (161), and reduced LDL receptor expression (162).

3.3 Weight loss

The management of obesity is complex. This is partly due to the combination of causes and contributing factors to obesity. Therefore, a multifaceted and step-wise approach is often required for successful weight loss.

3.3.1 Benefits of weight loss

Weight loss has innumerable benefits in obesity. There is substantial evidence to suggest that long-term weight loss can reverse many of the consequences of obesity. This section summarises the evidence for benefits of non-surgical weight loss in common obesity-related comorbidities and conditions. The evidence around improvement in nonalcoholic fatty liver disease is discussed in greater detail in **Chapter 3.7 – Treatment of nonalcoholic fatty liver disease**, and benefits of surgical weight loss are presented in **Chapter 3.9 – Bariatric surgery, NAFLD and metabolic disease**.

3.3.1.1 Insulin resistance and diabetes

Weight loss has proven beneficial effects on insulin resistance, with studies showing that weight loss improves HbA1c and glycaemic control in diabetes, leading to remission in some, and decreased rates of incident diabetes (163). Improvements in adipose, liver and muscle insulin sensitivity, as well as increased β-cell function is seen with weight loss. Greater effects are observed with increased weight loss (18).

Two landmark randomised controlled trials studied the efficacy of lifestyle modification and weight reduction on diabetes. The Diabetes Prevention Program Research Group compared the medium term outcomes of lifestyle modification, metformin or placebo (164). Lifestyle interventions were significantly more effective than metformin for prevention of diabetes, with NNT of 6.9 vs 13.9 persons. Tuomilehto *et al* showed significant decrease in weight with lifestyle modification over two years, with significantly reduced risk of diabetes by 58% ($p<0.001$) (165).

Longer-term metabolic effects of intensive lifestyle intervention versus standard care in overweight and obese type II diabetic patients are reported in the Look AHEAD (Action for Health in Diabetes) study. This showed increased odds of HbA1c reduction with 5-10% weight loss (OR 3.52 (CI 2.81-4.40)).

3.3.1.2 Dyslipidaemia

Weight loss improves almost all cholesterol parameters. The Look AHEAD study showed reductions in both triglyceride levels and HDL cholesterol after 1 year of lifestyle or standardised treatment, which was strongly correlated with the magnitude of weight loss. The average weight loss at this stage was 4.7±3.0kg. LDL measurements did not change significantly (166). This has been shown in multiple other studies (167, 168), although the mechanisms behind this are unknown.

3.3.1.3 Cardiovascular risk and disease

Physiologically, weight loss decreases the haemodynamic load in obesity. Substantial weight loss decreases the total and circulating blood volume, oxygen consumption, arterio-venous oxygen difference, cardiac output, ventricular work and stroke volume (63). Significant physiological changes in ventricular function have been demonstrated after weight loss, with decreased left ventricular (LV) size and wall thickness. It is hypothesised that changes in the renin-angiotensin-aldosterone system, sympathetic nervous system, improvements in insulin resistance and changes in leptin levels can also assist in cardiac remodelling.

Documented reductions in systolic and diastolic blood pressure, triglyceride levels, increases in HDL levels and resolution of T2DM contribute to improved cardiovascular risk and health (166). Dependence on medication also decreases, with greater reductions in use of diabetic, hypertensive and lipid lowering drugs (169). Ultimately, substantial weight loss in obesity leads to reductions in overall cardiovascular adverse events (OR 0.54 (0.41-0.70)), myocardial infarction (OR 0.46 (0.30-0.69)) and stroke (OR 0.49 (0.32-0.75)) (170).

3.3.1.4 Cancer

Weight loss is associated with decreased cancer incidence and mortality. A systematic review in 2012 by Birks *et al* (171) summarised 34 studies examining weight loss and cancer incidence. Nearly half of the studies showed a decrease in cancer incidence or mortality with weight loss, with the remainder showing a null finding. Almost all studies examining intentional weight loss showed a significant decrease in incidence, with hazard ratios (HR) between 0.22-0.76. The greatest benefit was seen in women, and in obesity-related cancers such as breast and endometrial cancer.

3.3.1.5 Mortality

Overall improvements in metabolic and cardiovascular risk factors with intentional weight loss can result in improvement in mortality risk in obese individuals (166). A meta-analysis in 2009 showed a relative risk (RR) of 0.84 associated with intentional weight loss (172).

3.3.1.6 Quality of life (QOL)

Weight loss measures, by medical or surgical methods, significantly improve quality of life. Modest weight loss of as little as 5% total body weight loss can result in significant improvements in health related quality of life, particularly the physical component, vitality and bodily pain (173).

3.3.1.7 Others

Other associations with weight loss include improvements in osteoarthritis, and subsequently improved mobility (174), improvements in obstructive sleep apnoea (OSA) (66), decreased rates of infertility (175), improvements in gastrointestinal diseases such as gastro-oesophageal reflux disease (GORD) (176), and improved pulmonary function and OSA (177).

3.3.2 **Management of obesity**

The WHO Guidelines ‘Obesity: Preventing and Managing the Global Epidemic’ describes the actions required to tackle this health epidemic (21). Strategies should be multifactorial, based on (1) societal, cultural, political, and structural environment in the population at large, (2) programs to focus on individuals and groups at high risk of obesity and (3) individual management of subjects with existing obesity.

Management of existing obesity can be divided into lifestyle modification, pharmaceutical treatment and surgical treatment. A step-up approach is generally used, due to increasing risk with increasing invasiveness. However, major differences arise around the extent and sustainability of weight loss with each of these treatment strategies.

3.3.2.1 Prevention

Long term successful management of the obesity epidemic on a societal level involves obesity prevention. This involves not only prevention of normal weight individuals from becoming overweight and obese, but also preventing weight gain universally.

Prevention strategies can be addressed in three levels: (1) public health prevention targeting everyone, (2) selective prevention, targeting high risk individuals and (3) targeted prevention, targeting those with existing weight problems and at high risk of comorbidities (21). Many strategies have been implemented by health and government bodies to improve lifestyle choice, treat and prevent obesity. These include education programs for children and families, advertising simple healthy behaviour strategies, limiting food advertising, decreasing availability of sugar-sweetened drinks or implementing a tax on unhealthy foods. The evidence for the efficacy of such strategies is thus far unknown (178).

3.3.2.2 Lifestyle modification

Dietary

Education of overweight and obese patients about food and eating habits is an essential part of weight management. Strategies and eating plans aiming to reduce energy intake by 2000-2500kJ/day can result in larger weight loss in the long-term compared to severe energy restriction. Diets that provide less than 4200 kJ per day can achieve a weight loss of up to 15% over 10-20 weeks (179). Better results are observed with active follow-up and combination with physical therapy, active support networks and behavioural therapy.

Very low calorie diets (VLCD) results in successful rapid short-term weight loss (180). This lifestyle and weight loss are rarely maintained after the VLCD is ceased, and weight regain varies from -7% to 122% at 1 year, and 26% to 121% at 5 years (181).

Exercise

Physical activity prevents weight gain and achieves weight loss in a dose dependent manner. Exercise together with diet is more effective than either measure alone. It limits loss of lean tissues with weight loss, improves metabolic rate, reduces weight regain and beneficially affects body fat distribution (21). A position statement by the American College of Sports Medicine (182) reports that 150-200 minutes of physical activity per week prevents weight

gain greater than 3% in most adults, and 225-420 minutes per week results in 5-7.5kg weight loss.

Limitations of lifestyle modification

Lifestyle modification is effective at changing behaviour and weight loss in the short-term. Individuals lose up to 5-10% total body weight loss, with greater efficacy in intensive lifestyle programs. However, lifelong behaviour change is required to sustain results, with long-term compliance being a significant issue. Unfortunately, due to this, regardless of lifestyle modification strategy used, many patients return to pre-treatment baseline within 5 years (21).

3.3.2.3 Pharmacology

Pharmacological therapies for weight loss are used as an adjunct when lifestyle modifications have not been effective. They can promote long-term weight maintenance, ameliorate comorbidities, and improve physical function. Most weight loss medications target appetite suppression, with the exception of orlistat.

The Endocrine Society Clinical Practice guidelines recommend close monitoring of efficacy and safety for the first three months, then three-monthly thereafter. Efficacy of medications generally only occurs during use, and compliance remains a major issue due to adverse effects of medications (183). Medications, action, efficacy and side effects are listed in **Table 3.12**.

3.3.2.4 Endoscopic methods

There have been numerous endoscopic strategies developed for weight loss. These are roughly divided into (1) space-occupying devices, (2) malabsorptive endoscopic procedures, (3) gastric restrictive methods and (4) regulation of gastric emptying (185).

The intragastric balloon is currently the most common endoscopic device used for obesity. There is significant evidence supporting short-term efficacy and safety. The procedure involves insertion of a silicone balloon into the stomach and inflating it with 400-700ml of saline/methylene blue. It is often used as a pre-treatment to bariatric surgery, to reduce weight and decreased perioperative complications. These devices are often used for 3-6 months, with necessary removal thereafter. Weight loss achieved varies between 8-25kg

during therapy. Short-term weight regain has been reported, with reductions in excess weight loss from 14-50.9% at 6 months post-removal, to 14.2-27.2% at 12 months post-removal (186). Few studies have looked at longer-term weight loss outcomes. The most common complication is persistent nausea and vomiting. The balloon can also cause gastric outlet obstruction, gastric erosion and ulceration.

Table 3.12: Common weight loss medications (184)

Medication	Action	Weight loss	Approval	Side effects and contraindications
Short term use				
Phentermine (AdipexP™)	Norepinephrine-releasing agent, causing appetite suppression	3.6kg in 2-24 weeks	Short term use, since 1960s	Headache, hypertension, palpitations, dry mouth, constipation, insomnia, anxiety, altered libido. Contraindicated in anxiety disorders, heart disorders, severe hypertension, glaucoma, hyperthyroidism, pregnancy, concurrent MAOi use.
Diethylpropion (Tenuate™)	Norepinephrine-releasing agent, causing appetite suppression	3.0kg in 6-52 weeks	Short term use, since 1960s	
Long term use				
Orlistat	Pancreatic and gastric lipase inhibitor, causing excretion of ~30% of ingested triglycerides in stool	2.9-3.4kg in 1 year	Long term use, since 1999	Steatorrhoea, flatulence, bloating, oily spotting, decreased absorption of fat-soluble vitamins
Lorcaserin (Belviq™)	Highly selective serotonin 5-HT2c receptor agonist, causing appetite suppression	3.6kg in 1 year	Long term use, since 2012	Headache, fatigue, dry mouth, nausea, dizziness, constipation
Phentermine/ topiramate (Qsymia™)	GABA receptor modulation plus norepinephrine releasing agent, causing appetite suppression	6.6-8.6kg in 1 year	Long term use, since 2012	Insomnia, dry mouth, constipation, paraesthesia, dizziness.
Naltrexone/ bupropion	Reuptake inhibitor of dopamine and norepinephrine, and opioid antagonist	4.8kg in 1 year	Long term use, since 2014	Nausea, vomiting, constipation, headache, dizziness
Liraglutide	GLP-1 agonist, causing appetite suppression	5.8kg in 1 year	Long term use, since 2014	Pancreatitis, nausea, vomiting Contraindicated in medullary thyroid cancer history, MEN2 history

MAOi – monoamine oxidase inhibitor; GABA - γ-aminobutyric acid; GLP-1 – glucagon-like protein-1; 5-HT – 5-hydroxytryptamine (serotonin); MEN2 – multiple endocrine neoplasia 2. Adapted from Yanovski SZ, Yanovski JA, Long term drug treatment for obesity. A systematic and clinical review. JAMA. 311(1):74-86.

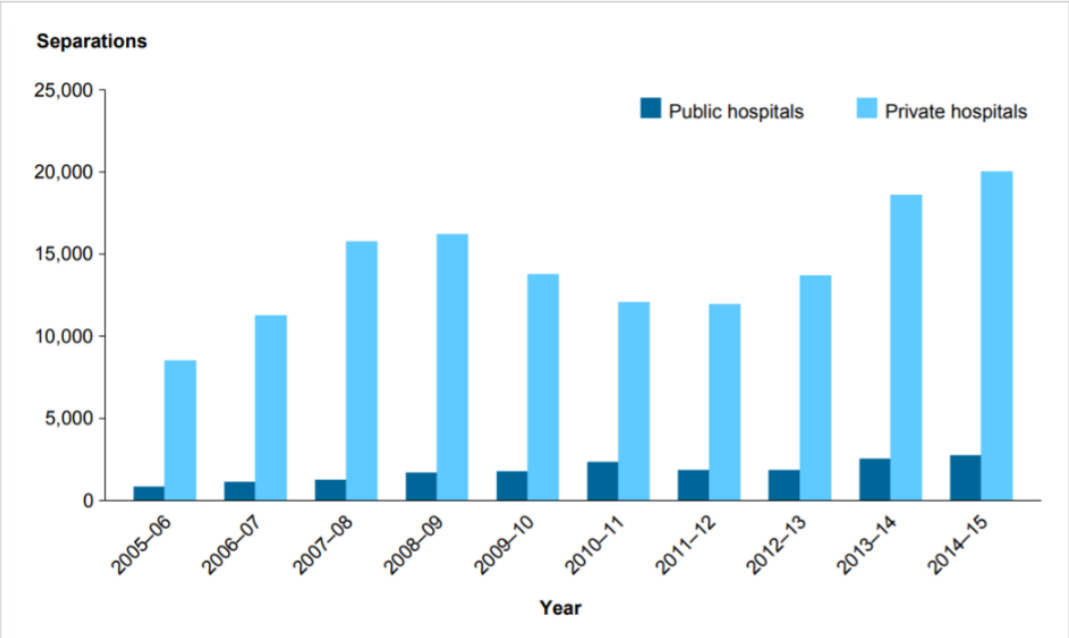
3.4 Overview of bariatric surgery

The first meaningful attempts at surgical management of obesity began in the 1950s (187). Although various techniques and procedures have been developed since, the fundamental goals of bariatric operations remain the same – to maximise weight loss, whilst maintaining and achieving nutritional health.

The advent of laparoscopic surgery in the 1990s improved the safety profile and acceptability of bariatric surgery. Since then, its popularity has increased, in part due to the improved documentation of effectiveness in weight loss and greater recognition of health benefits (188). Bariatric surgery is now one of the fastest growing operative procedures worldwide. In Australia, between 1998 and 2015, the number of weight loss operations increased from 535 to 22,700 (**Figure 3.5**) (189).

Surgical interventions currently provide the only means of achieving substantial and sustained weight loss in morbidly obese individuals, with weight loss in the vicinity of 50% excess weight loss (190, 191). With this weight loss, patients have experienced substantial reductions in metabolic and cardiovascular risk factors, and improvements in quality of life and overall survival.

Figure 3.5: Number of hospital separation codes for weight loss surgery, by public and private hospitals, 2005-06 to 2014-15 (189)



From the Australian Institute of Health and Welfare. Weight loss surgery in Australia 2014-15: Australian hospital statistics. Category no. HSE 186. Canberra, 2016.

3.4.1 Indications for bariatric surgery

The most universally followed guidelines for bariatric surgery are the National Institute of Health (NIH) criteria, developed in 1992 at a consensus conference in the United States (192). These criteria state that bariatric surgery can be offered to those with BMI over 40 kg/m^2, or to those with BMI 35-40 kg/m^2 with comorbidities such as diabetes or hypertension. Recently, there has been increasing evidence and support for the use of bariatric surgery in cohorts of class I obesity (BMI 30-35 kg/m^2), with evidence showing health and economic benefit, particularly in diabetic patients (193).

3.4.2 Bariatric surgical procedures

Various anatomical modifications of the gastrointestinal tract have been developed for the surgical treatment of obesity. Each technique has its own unique efficacy, risk and durability profile. Indications for each procedure depend on multiple factors, including patient demographics, past history, risks and comorbidities (194). Currently, there are four widely accepted procedures. These include the laparoscopic adjustable gastric band (LAGB), sleeve gastrectomy (SG), gastric bypass (Roux-en-Y gastric bypass (RYGB) or single anastomosis gastric bypass (SAGB)) and the biliopancreatic diversion (BPD).

3.4.2.1 Laparoscopic adjustable gastric band

The laparoscopic adjustable gastric band (LAGB) is the least invasive and least risky of the bariatric surgical procedures. A band is placed around the upper stomach, 2cm from the gastro-oesophageal junction, to create a small gastric pouch (**Figure 3.6a**). This band is inflatable via a port, which is placed on the abdominal wall beneath the subcutaneous tissue. The restriction offered by the band can thus be adjusted to an optimal level.

The main mechanism of weight loss by the gastric band is postulated to be due to peripheral satiety mechanisms and vagal stimulation (195), thereby leading to overall lower caloric intake. This is opposed to other procedures, which significantly alter the gastrointestinal anatomy and produce a range of hormonal changes (196). The gastric band may therefore better mimic non-operative weight loss measures, and could be a better model for studying the effects of weight loss on metabolic disease.

Of all the bariatric operations, the laparoscopic adjustable gastric band has the lowest operating time, length of stay, perioperative complication and mortality rate (197). Longer

term complications are more frequent, and complications specific to the gastric band commonly include proximal gastric enlargements, erosion and access port problems. These require reoperations, which can be performed safely and effectively with relatively low morbidity (188).

3.4.2.2 Sleeve gastrectomy

Laparoscopic sleeve gastrectomy is currently one of the most popular bariatric procedures worldwide, owing to the simplicity and its medium-term efficacy. Historically, the sleeve gastrectomy (SG) was the first stage of a two-stage bariatric procedure called the duodenal switch (DS), which was divided into two operations due to operative time and risk in high BMI cohorts. It became a stand-alone procedure after clinicians noted significant weight loss post-operatively, without completing the DS procedure (198).

The sleeve gastrectomy involves the creating of a gastric tube along the lesser curve of the stomach, typically reducing the stomach capacity from 1.0-1.5L to 100ml (**Figure 3.6b**). In addition to its restrictive nature, the sleeve gastrectomy alters the hormonal milieu of the gut due to the removal of the gastric fundus. The resultant decrease in ghrelin production decreases hunger, increases satiety and improves insulin sensitivity (199). Furthermore, the gastric sleeve demonstrates accelerated gastric and duodenal emptying, which is postulated to act as a functional duodenal bypass (200).

Despite its simplicity, the sleeve gastrectomy can result in significant and life-threatening complications. The post-operative complication rate varies from 0-23.8% (201). The most significant early complication is the staple-line leak, which usually occurs at the proximal staple line. Difficulty healing this defect is reflected by the multitude of endoscopic and surgical management techniques reported in the literature, which have varying success rates. Common long term complications include gastroesophageal reflux, weight regain and sleeve stenosis (202).

3.4.2.3 Gastric bypass

The gastric bypass has often been considered the gold standard bariatric procedure, particularly in North America. Long-term weight loss outcomes rival both the LAGB and sleeve gastrectomy at 61.6% excess weight loss, with an acceptable long term safety and

adverse effects profile (197). It has profound metabolic benefits, particularly in the remission and improvement of type II diabetes (203).

There are two common variations of the gastric bypass currently in use – the Roux-en-Y gastric bypass (RYGB) and the single anastomosis gastric bypass (SAGB) (**Figure 3.6c-d**). Both use the same principle of gastric restriction, by creating a small gastric pouch, and upper small bowel bypass.

The RYGB involves partitioning the stomach to create a small 20-30ml pouch at the proximal stomach. The small bowel is transected 30-50cm below the ligament of Treitz and a Roux-en-Y fashioned to restore continuity. Variation exist in the gastric pouch size, method of anastomosis, length of alimentary limb and closure of mesenteric defects (204).

The SAGB differs from the RYGB in the shape of the gastric pouch, as well as the position and number of anastomoses. Since it was first performed in 1997, it has grown in popularity due to reports of shorter operation times, low complication rates and comparable outcomes. The gastric pouch created is longer and narrower than in the RYGB. A single anastomosis is created with small bowel, most commonly 200cm distal to the ligament of Treitz (205).

Given the technical complexity of the gastric bypass, the associated complication rate is approximately 21%, with a reoperation rate of 8-38% (188, 206). The risks of early complications are relatively low (gastrointestinal bleeding 0.8-4.4%, anastomotic leak 2-4.4%). Medium to long term complications include marginal ulcers, fistula, anastomotic stricture, obstruction (from internal hernia, abdominal wall hernias, adhesions, kinking), and nutritional deficiencies (207).

3.4.2.4 Biliopancreatic diversion

The biliopancreatic diversion (BPD) has some similarities to the gastric bypass. It, however, mediates weight loss primarily through malabsorption. The modern day BPD was developed by Nicola Scopinaro (208). It consists of a horizontal partial gastrectomy leaving a 500ml pouch of stomach, a non-restrictive gastrojejunostomy with a 250cm Roux alimentary limb and anastomosis of the long biliopancreatic limb to the Roux limb 50cm proximal to the ileocaecal valve (**Figure 3.6e**). This creates an extremely short common channel in which pancreatic enzymes and bile can facilitate absorption of dietary fats. As the bowel absorbs

almost no fats and little starch, steatorrhoea and flatulence will occur if eating habits are not changed.

The biliopancreatic diversion with duodenal switch (BPD/DS) differs from the Scopinaro BPD in that the stomach is sleeved and the pylorus preserved and anastomosed to the Roux limb, and it utilises a 100cm common channel. Some studies show lower incidence of stomal ulceration and diarrhoea than the BPD alone (209).

Weight loss is excellent, but significant malabsorption may result in a variety of nutritional problems and deficiencies, that are difficult to manage. Common biochemical abnormalities include deficiencies in Vitamin D3 (45.8% of patients), zinc (38.2%), Vitamin B12 and folate. Of these, deficiencies in B complex vitamins may produce severe neurological symptoms including encephalopathy, acute visual loss and peripheral neuropathy (208).

Malabsorptive procedures have gained limited popularity outside a few centres. This is due to their high operative risk, reduced gastrointestinal quality of life and risk of ongoing complications, such as severe nutritional deficiencies (208).

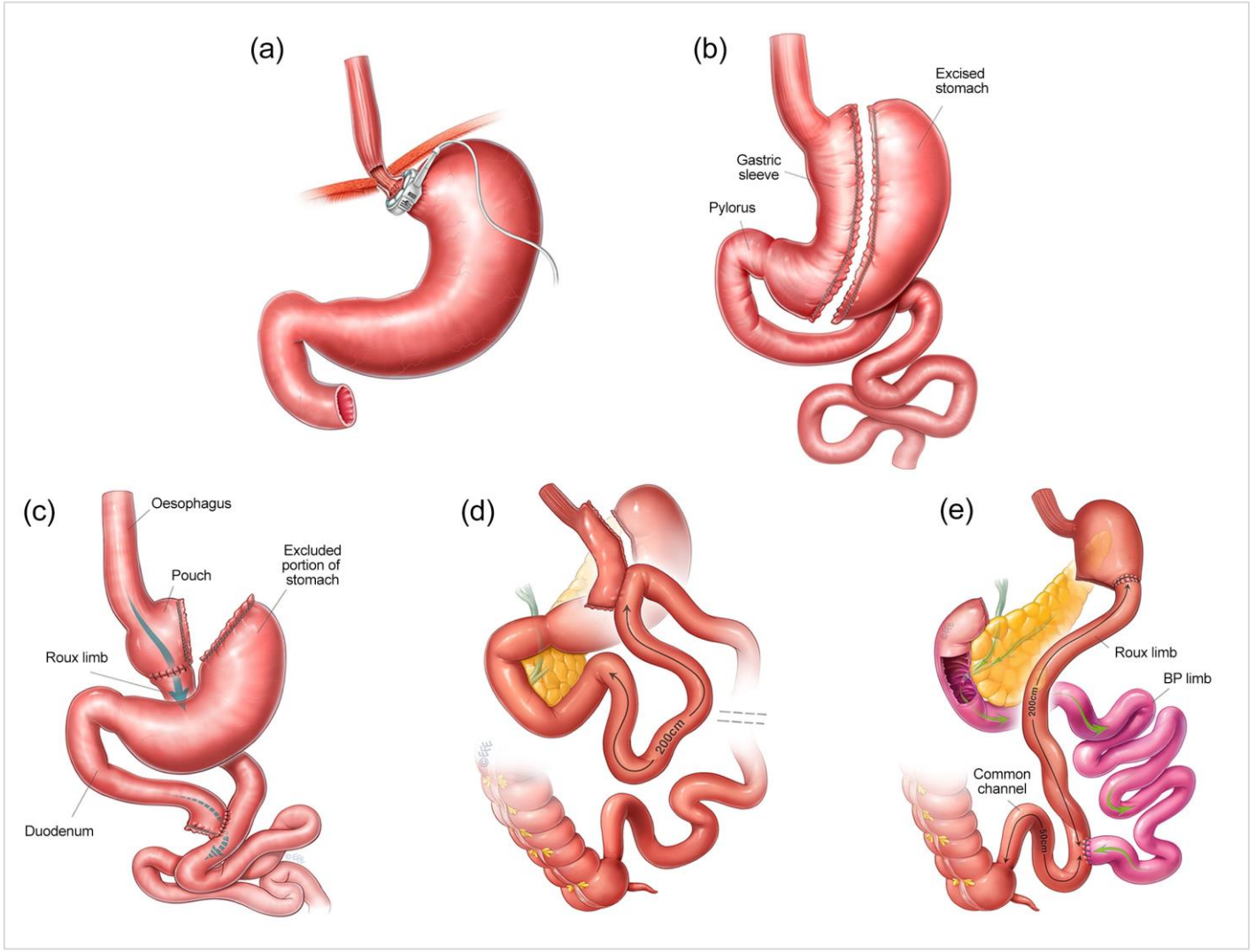
3.4.3 Mechanisms of weight loss

Weight loss via bariatric surgery was traditionally classified as restrictive, malabsorptive or a combination of both. However, it is clear that caloric restriction alone cannot be the sole reason for the reduction in weight, as metabolic compensation and increased hunger would compensate. We now know that the underlying mechanisms are likely more complex, involving neural feedback pathways and hormonal changes. The mechanisms of weight loss via bariatric surgery are discussed below.

3.4.3.1 Restriction

The reduction of food intake via restriction in gastric size is an initial mechanism for weight loss in many bariatric procedures. The sudden caloric restriction to 800-1200 kcal per day results in significant weight loss, similarly seen in subjects undergoing very low calorie diets (180).

Figure 3.6: (a) Laparoscopic adjustable gastric band. (b) Sleeve gastrectomy. (c) Roux-en-Y gastric bypass. (d) Single anastomosis gastric bypass. (e) Biliopancreatic diversion. Illustration commissioned from L.Efe for Centre for Obesity Research and Education.



However, after this short-term period, the course of bariatric surgical subjects diverges significantly compared to those undergoing lifestyle modifications with similar caloric restriction. Faster and more substantial weight loss is achieved after gastric bypass, sustained over longer periods of time. Furthermore, the magnitude of weight loss is substantially larger (25-35% vs 5-10% TBWL with lifestyle) (190).

Studies into the physiology of the laparoscopic adjustable gastric band (LAGB) have revealed that its mechanism of weight loss is not consistent with restriction alone, as gastric transit and emptying is not significantly altered (210). A study by Burton *et al* proposed that peripheral satiety mechanisms are triggered by repeated peristaltic contractions of the oesophagus as a food bolus is passed through the band (211).

There is current controversy about whether the size of the gastric pouch or sleeve restricts food intake and, therefore, affects weight loss. Some studies have found correlation between a larger sized pouch and poor weight loss, whilst others have shown no relationship between the two (196). More recent kinetic and scintigraphy studies indicate that nutrients are facilitated through the gastric pouch rapidly, rather than restricted within it (212, 213).

3.4.3.2 Malabsorption

Malabsorption was initially thought to be an important mechanism of many bariatric procedures, including the gastric bypass and BPD. These procedures diverted nutrients away from the duodenum, and variable amounts of small bowel, thus decreasing absorptive area and gastrointestinal secretions (214).

More recently, studies have found that this is not the main mechanism of weight loss. Only 21-31% of weight loss can be attributed to fat malabsorption (215). Experimental models show other factors are likely more important for inducing weight loss, including alteration and exaggeration of gut hormone release, increased energy expenditure despite caloric restriction, and diversion of bile play (216).

3.4.3.3 Vagal signalling

Afferent vagal fibres within oesophageal, gastric and small bowel respond to mechanical stretch, and detect the volume of food ingested (217). These fibres could play a role in satiation in both gastric bypass and laparoscopic adjustable gastric band (LAGB) surgery.

In gastric bypass and BPD, disruption of vagal fibres distal to the gastric pouch after gastric bypass has been theorised to affect the gut-brain axis, and alter hormonal and neural signalling (218). Furthermore, early transit from the gastric pouch into the midgut likely activates mechanoreceptors within the alimentary limb. These increased pressures within the alimentary limb correlate with early termination of meals, resulting in lower caloric intake (219).

3.4.3.4 Altered gastric emptying

Various factors are thought to contribute to alterations in gastric emptying after bariatric surgery. Some studies have shown rapid gastric emptying after RYGB (196). The gastric sleeve also appears to affect gastric and intestinal transit. Nuclear medicine studies show rapid gastric emptying, as well as decreased small bowel transit time after sleeve gastrectomy (200). These effects are thought to be responsible for the exaggerated release of gastrointestinal hormones and vagal signalling, which may induce meal cessation and decreased caloric intake.

3.4.3.5 Gastrointestinal-derived hormones

Appetite regulation is controlled heavily by the gut-brain axis, which can directly convey information about energy intake to satiety centres. These hormones either suppress appetite (anorexigenic) or stimulate appetite (orexigenic). Significant alterations in secretion occur after bariatric surgery (**Table 3.13**) (216, 220). For example, the early transit of food into the small bowel following bypass surgery is hypothesized to stimulate an exaggerated release of anorexigenic peptides, that promote meal termination and increase energy expenditure.

The mechanisms by which this occurs are not entirely known, however, diversion of nutrients or bile towards the hindgut, altered gastric emptying, and altered vagal signalling is thought to be a significant mechanism.

3.4.3.6 Change in bile acids

After gastric bypass, bile travels down the biliopancreatic limb, undiluted by food. Bile acids (BA) bind to cell-membrane G protein coupled receptor (TGR5), which augment GLP-1 and PYY release, thereby mediating post-prandial satiety. Increased activation of farsenoid X receptor (FXR) facilitates the effects of bile acids on energy homeostasis, leading to

increased metabolic rate and decreased adiposity (221). FXR activation promotes carbohydrate and lipid metabolism, and improves insulin sensitivity. It is also known to modulate hepatic growth and regeneration after injury. Increased serum BA concentrations can increase energy expenditure via promotion of intracellular thyroid hormone activation (222).

Table 3.13: Gastrointestinal-derived hormones: Summary of their action and changes with bariatric surgery (220)

Hormone	Location	Secretion	Action	Change with bariatric surgery
Ghrelin (199)	Gastric fundus, duodenum. Less so in small bowel.	Increases pre-meal and during fasting. Rapidly suppressed by food intake. Baseline levels lower in obese subjects, rises after diet-induced weight loss	Increases hunger and decreases with intake. Action modulated by intact vagus (218).	Conflicting evidence. Decreases with SG (223). Conflicting evidence on levels after RYGB. Increase after LAGB.
Peptide YY (PYY)	Distal small bowel	Released post-prandial. Secreted with other hormones (GLP-1, OXM).	Increases post-prandial satiety. Inhibits GI motility. Inhibits gastric, intestinal and pancreatic secretions.	Long term increased secretion of PYY with RYGB and JIB, even after weight loss. No change with LAGB.
Glucose-like peptide-1 (GLP-1)	Distal small bowel	Released post-prandial. Secreted with other hormones (PYY, OXM).	Increases post-prandial satiety. Slows gastric emptying. Incretin– Improves glucose metabolism and expands pancreatic β-cell population.	Long term increased secretion of GLP-1 with RYGB and JIB, accompanied by better glucose and insulin control
Oxyntomodulin (OXM) (199)	Distal small bowel	Released post-prandial, especially in response to glucose. Secreted with other hormones (PYY, GLP-1).	Increases satiety. Associated with dumping syndrome.	Markedly increased after RYGB and JIB.
Glucose-dependent insulinotropic polypeptide (GIP)	Duodenum and jejunum	Secreted in response to food.	Incretin– Improves glucose metabolism, insulin secretion and expands pancreatic β-cell population. Enhances bone formation, increases energy storage in adipose tissues (224).	Conflicting evidence on GIP levels after RYGB and jejunoileal bypass, showing increased or decreased levels.
Cholecystokinin (CCK) (199)	Duodenum	Secreted in response to food.	Increases satiety, inhibits gastric emptying and mobility.	No change after RYGB. Increased peak levels after JIB.
Pancreatic polypeptide (PP) (199)	Pancreas	Secreted in response to food.	Decreases gastric emptying. Increases energy expenditure.	No significant change.

LAGB – laparoscopic adjustable gastric band; RYGB – Roux-en-Y gastric bypass; SG – sleeve gastrectomy; JIB – jejunoileal bypass

3.5 Nonalcoholic fatty liver disease

In 1980, Ludwig and colleagues gave the term ‘nonalcoholic steatohepatitis’ to a clinicopathologic syndrome that occurred in obese diabetic females who denied alcohol use, but in whom the hepatic histology was consistent with alcoholic hepatitis. The more general term, nonalcoholic fatty liver disease (NAFLD), encompasses the full spectrum of metabolic fatty liver disorders. Our current disease concept and terminology of NAFLD have been developed on the groundwork of this landmark paper (225).

The hallmark of NAFLD is the presence of hepatic steatosis in the absence of any secondary cause for hepatic fat accumulation. Today, NAFLD has become one of the most common causes of chronic liver disease, with an estimated one in every three people in the Western world with some degree of NAFLD. Its prevalence has increased substantially in response to the growing obesity epidemic, with rates of NAFLD doubling over the last 20 years. This compares to other causes of chronic liver disease, which have remained stable or even decreased over that time (1). The burden of this disease is significant, due to its potential to progress to cirrhosis, liver failure and hepatocellular carcinoma (2).

3.5.1 **Definition of NAFLD**

According to the American Association for the Study of Liver Diseases (AASLD), the definition of nonalcoholic fatty liver disease requires two components:

a) Evidence of hepatic steatosis (by imaging or biopsy)
b) No other cause for hepatic fat accumulation, such as significant alcohol consumption, use of steatogenic medication or hereditary disorders. (3)

For clinical trials, >21 drinks per week for men and >14 drinks per week for women is the definition used, and considered a reasonable limit for clinical practice. Common secondary causes for hepatic steatosis are listed in **Table 3.14**, and need to be excluded prior to diagnosis of NAFLD.

Table 3.14: Common causes of secondary hepatic steatosis

Type of steatosis	Secondary causes of hepatic steatosis
Macrovesicular steatosis	Excessive alcohol consumption Viral hepatitis Nutritional factors: starvation, parenteral nutrition, Medications: e.g. amiodarone, methotrexate, tamoxifen, corticosteroids Hereditary: Wilson's disease, lipodystrophy, abetalipoproteinaemia.
Microvesicular steatosis	Medications: e.g. valproate, anti-retroviral medications Acute fatty liver of pregnancy Reye's syndrome HELLP syndrome Inborn errors of metabolism: e.g. LCAT deficiency, cholesterol ester storage disease, Wolman disease

HELLP – hypertension, elevated liver function and low platelet syndrome; LCAT – lecithin cholesterol acyltransferase

3.5.2 **Epidemiology**

Challenges in studying NAFLD prevalence

The true incidence and prevalence of NAFLD and NASH is largely unknown. This is due to the current lack of an accurate non-invasive diagnostic test, especially for the diagnosis of steatohepatitis (226). Liver biopsy is considered the gold standard (227), but is obviously not applicable in population-based studies for practical and ethical reasons. Subsequently, studies reporting NAFLD prevalence are based on a variety of imperfect diagnostic techniques, including aminotransferase levels and ultrasound (228, 229).

As steatohepatitis can only be diagnosed histologically, accurate reports of NASH prevalence are restricted to settings where liver biopsies are readily available. This is limited to specific populations with clear indication for liver biopsy, such as individuals with abnormal liver function tests or significant metabolic disease (8). Subsequently, over-estimation of liver disease may occur in these populations.

3.5.2.1 NAFLD prevalence

The reported prevalence of NAFLD has risen rapidly in parallel with the dramatic rise in obesity and diabetes, and is now the most common causes of liver disease in Western countries (1). Two large population based studies, the Dallas Heart Study in the United States (230) and the Dionysos study in Northern Italy (231) have cited rates of NAFLD in the

general population between 23-31%. These studies used non-invasive assessment tools, such as ultrasound and aminotransferase levels. A meta-analysis by Younossi *et al* (232), using only studies with histologically confirmed NAFLD concur with these results, showing the global prevalence of NAFLD to be 25%. Of these patients, 59.1% had NASH.

Demographic variables contribute to the prevalence of NAFLD. Risk increases with age, with a reported prevalence of 22.4% in 30-39 year age bracket, up to 34.0% prevalence in those who were 70-79 year (232). More males who were overweight or obese were affected than females (56.6% vs 44.3%) (233). Ethnic group and geographic location also played a role. The highest incidence of NAFLD was seen in South America, Central America and the Middle East, with lowest prevalence in Africa and in those of African descent (232). Genetic and environmental factors have been implied as causative through observation of familial clustering (234) and identification of genetic contributors such as patatin-like phospholipase domain-containing protein 3 (PNPLA3) (235).

Of concern is the high prevalence of NAFLD in the paediatric population (236). There is a 3-11% prevalence in the general paediatric population, rising to 40-70% in obese children.

3.5.2.2 Prevalence in obesity

Obesity has strong epidemiological links with NAFLD. Studies consistently show that obesity is a risk factor for NAFLD (237). In obese study cohorts, the prevalence of NAFLD is 71-98% for any degree of NAFLD, with 7.3-56% showing signs of NASH and 1-2% having cirrhosis (7, 12). Significant heterogeneity exists due to variation in study design, patient selection and drawbacks of non-invasive tests.

Notably, obesity is also additive to other factors that predispose to liver steatosis, such as alcohol intake where obesity conferred a twofold increase (CI 1.5-3.0 fold) in risk (237).

3.5.2.3 Prevalence with metabolic disease

In addition to obesity, type II diabetes, dyslipidaemia and the metabolic syndrome are commonly associated with NAFLD (232, 238).

Insulin resistance and nonalcoholic liver disease share a pathophysiological link (see C**hapter 3.8 – Pathophysiology of nonalcoholic fatty liver disease**), with evidence

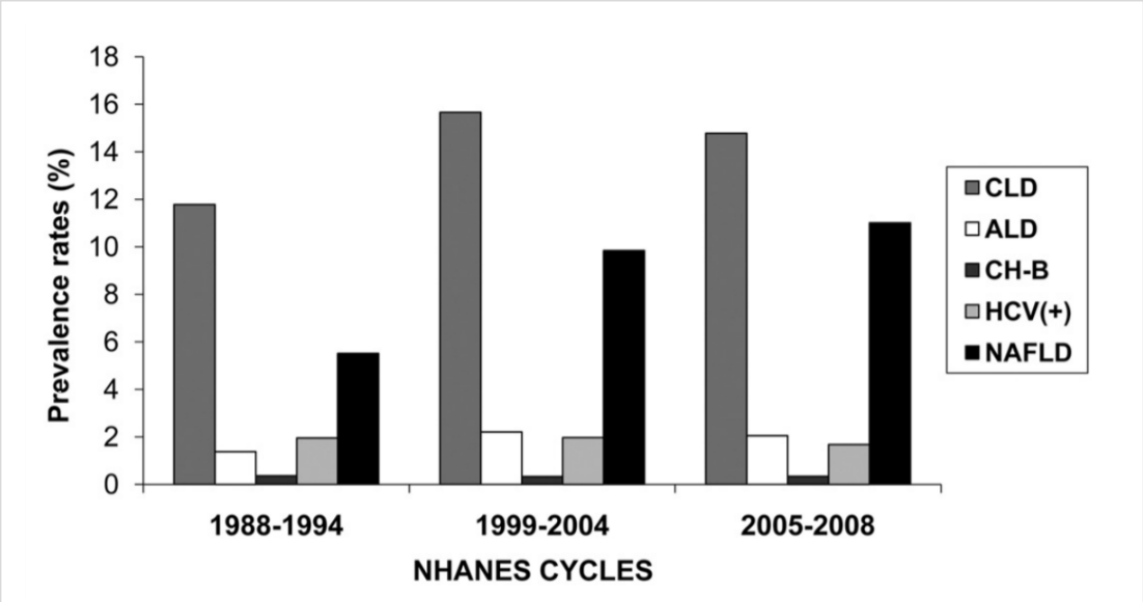
suggesting that each may fuel the other (6). The epidemiology is consistent with this, showing that T2DM prevalence among those with NAFLD and NASH is 22.5% and 43.6% respectively (232). Correspondingly, in a population of patients with T2DM, the rates of NAFLD are high at 34–74% (8).

There are strong associations of other metabolic risk factors with NAFLD. Rates of NAFLD and NASH are 69.2% and 72.1% in dyslipidaemia, 39.3% and 68.0% in hypertension and metabolic syndrome is present in 42.5% and 70.7% respectively (232).

3.5.2.4 Trends

The prevalence of NAFLD has been increasing in line with the trends in obesity and metabolic disease. The National Health and Nutrition Examination Survey (NHANES) conducted in the United States demonstrates increases in NAFLD from 5.5% in 1998-1994 to 11% in 2005-2008 (**Figure 3.7**) (238). Paediatric NAFLD increased from about 3.9% two decades ago to 10.7% in 2007-2010 ($p<0.001$). Of note, the diagnosis of NAFLD in these studies was made using serum aminotransferases, which can lead to a misclassification and underestimate the true prevalence.

Figure 3.7: Prevalence rates of chronic liver disease (CLD) in the United States National Health and Nutrition Examination Survey (NHANES), showing rising nonalcoholic fatty liver disease (black), with stability of other causes of CLD (238).



CLD – chronic liver disease; ALD – alcoholic liver disease; CH-B – chronic viral hepatitis B; HCV(+) – hepatitis C virus; NAFLD – nonalcoholic fatty liver disease. From Younossi Z et al. Changes in the prevalence of the most common causes of chronic liver diseases in the United States from 1988 to 2008. Clin Gastroenterol Hepatol. 2011;9(6):524-530.

3.5.3 **Histopathology**

There is a wide spectrum of histopathological changes encompassed within the term "NAFLD". This ranges from the relatively benign simple steatosis to the more severe form, nonalcoholic steatohepatitis (NASH), and can result in progressive fibrosis and cirrhosis.

It is important to note that the diagnostic criteria for NASH has undergone substantial change over the last three decades and is still evolving. In the 1990s and early 2000s, there was significant variation in the criteria for NASH used in studies. Controversy and debate still occur around the appropriate weighting of histological features. The two main histological grading systems are described below (239, 240).

3.5.3.1 Clinical Research Network NAFLD activity score (NAS)

In 2005, due to the lack of standardised reporting for NAFLD, the Clinical Research Network develop a scoring system (240). The main purpose of the score was for use in clinical trials, to enable systematic and standardised reporting of histological severity, and documentation of changes in NAFLD within individuals (240). The result was the creation of the Clinical Research Network (CRN) NAFLD Activity Score (NAS). The final components used in the score were steatosis, lobular inflammation and ballooning.

Steatosis describes the accumulation of fat within the liver. The severity of steatosis is determined by estimating the proportion of hepatocytes containing cytoplasmic fat droplets visible under light microscopy (241). The NAS grades steatosis from 0 to 3, with S0 being <5% steatosis (not NAFLD), S1 being 5-33% steatosis, S2 being 34-66% steatosis and S3 being ≥67% steatosis. 'Simple steatosis' is the term often used to describe the accumulation of fat without any inflammation present.

Lobular inflammation is the presence of foci of two or more inflammatory cells within a liver lobule. The severity of lobular inflammation is graded from 0 to 3, with 0 being no lobular inflammation, 1 being <2 foci per 200x field, 2 being 2-4 foci per 200x field, and 3 being >4 foci per 200x field.

Finally, **hepatocyte ballooning** is the most characteristic features of steatohepatitis. This is characterised by a rounded shape and unusually enlarged, lightly stained cytoplasm on routine histology (cellular diameter >30 μm). The consensus in the histopathology community is that the diagnosis of NASH should include hepatocyte ballooning, in addition

to steatosis and inflammation (242). Hence, it remains a key component of existing grading systems of NAFLD activity.

The final NAS is the unweighted sum of the scores for steatosis (0-3), lobular inflammation (0-3) and ballooning (0-2), thus ranging from 0 to 8 (**Table 3.15**). A score of 0-2 is considered *very unlikely* to be diagnostic of NASH, a score of 3 or 4 is *equivocal* for NASH, and a score of 5 or more is considered *very likely* to be NASH (240). It should be noted that this score is not definitive for the diagnosis of NASH, and the authors have emphasised in this and subsequent publications, that the diagnosis of NASH depends on the pathologist's overall assessment rather than a score (243).

Table 3.15: The Clinical Research Network NAFLD activity scoring (NAS) system (240)

NAFLD Activity Score (0 – 8) Sum of scores for steatosis, lobular inflammation and hepatocyte ballooning		
Steatosis (0 – 3)	*0*	<5% hepatocytes involved
	1	5-33% hepatocytes involved
	2	33-66% hepatocytes involved
	3	>66% hepatocytes involved
Lobular inflammation (0 – 3)	*0*	None
	1	<2 foci per x200 field
	2	2-4 foci per x200 field
	3	>4 foci per x200 field
Hepatocyte ballooning (0 – 2)	*0*	None
	1	Few ballooned cells
	2	Many cells/prominent ballooning

Fibrosis

Fibrosis initially begins in the perisinusoidal space of zone 3 of the liver acinus, in a so-called chicken-wire pattern. With progression, zone 1 periportal fibrosis develops. Eventually portal triad and hepatic veins are connected by bridging fibrosis, and further collagen deposition and nodular regeneration leads to cirrhosis. Fibrosis is scored via the modified Brunt score, from 0 to 4 (**Table 3.16**) (240).

Table 3.16: The modified Brunt scoring system for fibrosis in NAFLD (240)

Fibrosis stage	Description
0	No fibrosis
1 *1a* *1b* *1c*	Perisinusoidal or periportal Mild, zone 3, perisinusoidal Moderate, zone 3, perisinusoidal Portal/ periportal fibrosis only
2	Perisinusoidal and portal/periportal fibrosis
3	Bridging fibrosis
4	Cirrhosis

SAF score

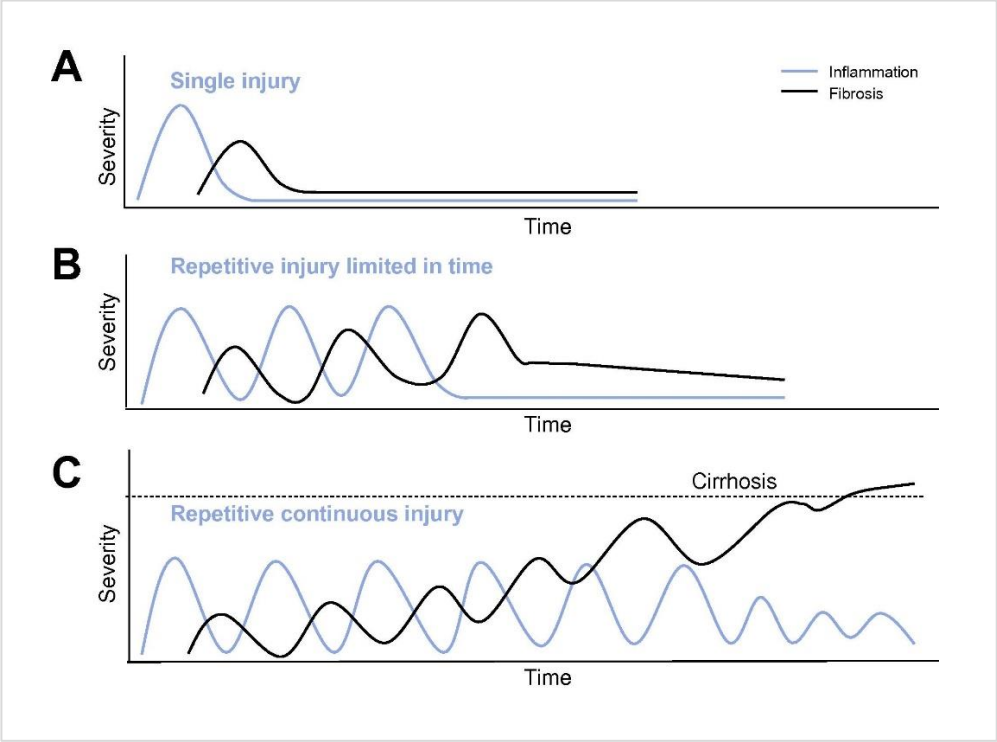
The more recent SAF (steatosis, activity, fibrosis) score by Bedossa *et al* (2012), was created to address some of the shortcomings of the NAS grading system (239). This included the ambiguity of NAS 3-4 (equivocal for NASH), and the absence of fibrosis. It combines a steatosis score (as per the NAS), activity score (inflammation and ballooning score) and fibrosis grade (as per the Kleiner fibrosis grades). The initial study describing the SAF shows promising results, with consistent classification of NASH, good correlation with alanine aminotransferase (ALT) and excellent interobserver agreement (κ=0.80). However, further validation is still required.

3.5.4 **Natural history and prognosis**

There is still considerable uncertainty about the natural history and prognosis of NAFLD. Detailed study of the progression of NAFLD is difficult, with few studies examining the histological evolution over time. This is largely due to the ethics and practicality of performing serial liver biopsies.

Despite its growing prevalence, many patients will continue along a benign course and never develop significant liver disease (244). However, injury or "hits" on the liver, including insulin resistance, ongoing obesity and its sequelae, altered intestinal microbiome, oxidative stress and lipotoxicity can activate intrahepatic inflammatory cascades. Whilst a few hits may result in complete healing with no real sequelae, repeated injury can activate fibrogenesis, and may ultimately result in cirrhosis (**Figure 3.8**) (245). Similar to other causes of liver cirrhosis, NAFLD can lead to hepatocellular carcinoma (HCC) and liver transplantation.

Figure 3.8: Fibrosis progression developing as a result of wound healing due to repeated episodes of acute inflammation (245).



From Schuppan D et al. Determinants of fibrosis progression and regression in NASH. J Hepatol. 2018; 68:238-250.

3.5.4.1 Histological factors associated with prognosis

Fibrosis

Fibrosis has the strongest link to progression of NAFLD. Recent evidence suggests that fibrosis stage is the only histological feature associated with liver related morbidity, transplantation and long-term mortality (246, 247). In a study by Angulo *et al* in 2015 (246), even with Stage 1 fibrosis carried a significantly increased risk of death or liver transplantation, with a hazard ratio (HR) of 1.88 (1.28-2.77). Risk increased with increasing stage of fibrosis. These findings were corroborated by a study by Ekstedt *et al* (247), involving up to 33 years follow-up in 229 participants with biopsy-proven NAFLD. They also reported that Stage 3 and Stage 4 fibrosis was the most important feature that predicted overall mortality (HR 1.29, p=0.020).

Nonalcoholic steatohepatitis (NASH)

There is evidence to suggest that NASH plays an important predictive role in the natural history of NAFLD. In 1999, Matteoni *et al* was the first to describe the progression of NASH to cirrhosis, with a 22% prevalence at 8.3 years follow-up in those with NASH only at

baseline (248). Multiple subsequent studies have shown the increased risk of progression in the setting of hepatocyte ballooning and NASH (249, 250). A systematic review by Musso *et al* reports a progressive fibrosis prevalence of 25-30% at 4 years, and 50% over 6 years in patients with NASH at baseline.

Steatosis

The importance of simple steatosis in NAFLD is unclear, with conflicting results in the literature. Initial studies suggested that steatosis with or without inflammatory change, will usually follow a benign cause with low risk of progression to either NASH or fibrosis and cirrhosis (248, 250, 251).

However, some evidence suggests that simple steatosis can progress, and potentially at a similar rate (249, 252). A cohort study by McPherson *et al* (252) studied the progression in 108 NAFLD patients with serial biopsies, and showed equivalent rates of fibrosis progression between simple steatosis and NASH (37% vs 43%, p=0.65). Consistent with the known contribution of insulin resistance to NAFLD pathogenesis, diabetics were more likely to have progression (252). A meta-analysis in 2015 of 11 cohort studies comprising 411 patients with paired liver biopsies showed those with simple steatosis still progressed, but the rate of progression was one fibrosis stage every 14.3 years, compared to double the rate (one fibrosis stage per 7.1 years) for those with NASH (249).

3.5.4.2 Clinical outcomes

Cirrhosis and liver transplantation

The development of cirrhosis is highly dependent on initial histological findings, with the presence of fibrosis and, less so, NASH, being the most predictive for progression (as discussed above). However, the exact rate of progression to cirrhosis is difficult to ascertain, due to the uncertainties in population prevalence and difficulties with non-invasive disease monitoring.

Liver transplantation is an indication of the importance of NASH and fibrosis, and its contribution to the burden of end-stage liver failure. In the United States, the percentage of liver transplantation patients with NAFLD-related cirrhosis as a primary or secondary indication rose from 1.2% in 2001 to 9.7% in 2009, making it the third most common indication for transplantation (253). This trend, together with the advances that have made

remission of viral hepatitis C possible (254), means that NAFLD is projected to become the primary reason for liver transplantation in the next decade.

Hepatocellular carcinoma

Similar to other causes of cirrhosis, NAFLD predisposes to hepatocellular carcinoma through the formation of cirrhosis (2, 255). With 33 years follow-up, Ekstedt *et al* reported a 5% incidence of HCC in NAFLD patients, correlating to HR 6.55 (95% CI: 2.14-20.03, p=0.001) (247).

Metabolic and cardiovascular disease

NAFLD is recognised as a key determinant of metabolic health and multisystem disorders (256, 257).

Cardiovascular disease is the leading cause of death in individuals with NAFLD (258). Ultrasound-detected steatosis (~30% liver steatosis) has been independently associated with atherosclerosis and endothelial dysfunction (258). A systematic review by Oni *et al* of 27 studies showed an independent association of NAFLD with increased carotid intima-media thickness, coronary artery calcification, endothelial dysfunction and arterial stiffness (259). These are all established factors increasing risk for multiple cardiovascular disease outcomes (260, 261). NAFLD is associated with a hazard ratio of 1.55 (p=0.01) for development of cardiovascular disease compared to reference populations (247).

Liver steatosis also predicts the development of T2DM (244, 262) and is associated with worse diabetic complications (6).

Mortality

The overall and liver specific mortality among patients with NAFLD is consistently higher than that of the general population. In the NHANES III, Ong *et al* (263) showed a significantly higher overall mortality (HR 1.038, p<0.001), after adjustment for BMI, hypertension, diabetes, social and demographic variables. Liver-related mortality was substantially higher than the general population (HR 9.32, p<0.001). In a population based setting in Minnesota, the standardised mortality ratio was 1.34 (p=0.03) compared to the general population over a mean follow-up of 7.6 years (3192 person-years) (2). Overall, a meta-analysis by Musso *et al* in 2011 showed the pooled overall mortality from six studies to be an OR 1.40 (95% CI: 1.23-1.60, p<0.001) (264).

In population-based studies, liver-related death was the third most common cause of death (13%), following malignancy (28%) and ischaemic heart disease (25%). Liver-related mortality ranks 11th in the general population (263).

3.6 Diagnosis of nonalcoholic fatty liver disease

Despite its high prevalence, the diagnosis of NAFLD is usually made incidentally, as most patients are asymptomatic. The only reliable means of diagnosing and grading NAFLD is by liver biopsy. However, liver biopsy has various drawbacks (227), and cannot, therefore, be performed for all patients at risk of NAFLD.

As a result, there has recently been substantial interest in development of non-invasive tests to accurately diagnose NAFLD. Non-invasive tests may be grouped according to their detection of (1) steatosis, (2) inflammation/NASH and (3) fibrosis. Because of the greater prognostic implications of NASH and progressive fibrosis, the key issues are to accurately differentiate NASH from normal liver or simple steatosis, and to accurately staging of fibrosis (265).

Non-invasive methods for assessment of NAFLD are being used with increasing frequency in clinical practice. In obese cohort, the current dilemma is in appropriate interpretation of their results, due to variable validation and differences in accuracy and feasibility.

3.6.1 **Liver biopsy**

Liver biopsy remains the gold standard for diagnosing nonalcoholic fatty liver disease (266). It is the only reliable means of staging the severity of injury, particularly the presence of inflammation or fibrosis.

3.6.1.1 Liver biopsy - An imperfect gold standard

Liver biopsy itself is an imperfect gold standard (227). Firstly, it is an invasive procedure with various risks. Up to 84% of patients have been reported to experience at least mild pain and discomfort (267). The most common severe complication after liver biopsy is bleeding, occurring in 1 in 2,500 to 1 in 10,000 percutaneous procedures. Other complications include pneumothorax, haemothorax, perforation of hollow viscus, haemobilia, bilious peritonitis, infection and neuralgia. Mortality after liver biopsy is a rare, but possible occurrence (227).

The quality of the liver tissue obtained from the biopsy is essential for accurate diagnosis and staging by histopathology. For accurate assessment, the sample should have at least 11 complete portal tracts. Studies looking at biopsy adequacy have shown biopsies ≥1.5cm long

have a higher yield for diagnosing definitive NASH and lower variability in NAFLD fibrosis stage (268).

Sampling error presents an additional challenge. A single core biopsy represents only 1 in 50,000-65,000 of the liver, a large organ where disease may be heterogeneously spread. Ratziu *et al* has shown that between two liver biopsy samples, the relative degree of fibrosis and inflammation can vary significantly (269). Reports also suggest histology and stage may vary between lobes of liver (270). Magnetic resonance imaging (MRI) studies have demonstrated significant variation in fatty deposition and sparing throughout the liver (271), however few studies have thus far studied this variability in NAFLD.

Interpretation of the liver specimens yields inter- and intra- observer variability, which poses a challenge in the diagnosis of NAFLD. Although interpretation of fatty change and fibrosis showed good inter-observer agreement, assessment of inflammatory change shows significant inconsistency (κ =0.33) (272).

Finally, there are economic considerations to performing liver biopsies, which are a relatively costly investigation. Performing a biopsy for all patients with suspected NAFLD would be prohibitively expensive and time-consuming (273).

3.6.2 Clinical features of NAFLD

Most patients with NAFLD are asymptomatic. Some patients experience non-specific symptoms, such as malaise or vague upper quadrant pain from hepatomegaly (274). In most patients, it is more likely that liver disease will become apparent incidentally from persistently elevated aminotransferases, abdominal imaging suggesting steatosis, or abnormal intraoperative appearances.

Physical examination may reveal hepatomegaly due to fatty infiltration, but this finding has been shown to be very variable in NAFLD. Those with severe disease may have signs of end stage liver failure and cirrhosis, such as bruising, varices, ascites, jaundice and encephalopathy (275).

3.6.3 Non-invasive tests

There are two broad types of non-invasive diagnostic tests – serum biomarkers and imaging techniques. Each test has variable validation, and often has been developed within a specific

population. An overview of the more widely used tests is seen in **Table 3.17**, divided into the components of NAFLD that they detect. Details of common biochemical tests, their algorithms and thresholds are seen in **Table 3.18**.

Table 3.17: Available and more commonly used non-invasive tests.

Component of NAFLD	**Serum biomarker**	**Imaging technique**
General liver injury	ALT AST:ALT ratio (AAR) AST to platelet ratio index (APRI)	Ultrasound
Steatosis	SteatoTest Fatty liver index (FLI) Lipid accumulation product (LAP) Hepatitis Steatosis Index	Ultrasound Controlled attenuation parameter (CAP) Magnetic resonance spectroscopy (MRS)
Inflammation/ NASH	CK-18 NashTest oxNASH NASH score Palekar score NASH diagnostic score	Diffuse weighted magnetic resonance imaging (MRI) Computed tomography perfusion
Fibrosis	BARD NAFLD fibrosis score (NFS) Fibrosis-4 index (FIB-4) AAR APRI Fibrometer NAFLD Fibrotest Hepascore Enhanced liver fibrosis (ELF) score	Transient elastography (TE) Acoustic radiation force index (ARFI) Shear wave elastography (SWE) Magnetic resonance elastography (MRE)

AST – aspartate aminotransferase; ALT – alanine aminotransferase

3.6.3.1 General liver damage and exclusion of other liver disease

Alanine aminotransferase

Alanine aminotransferase (ALT) is part of the standard 'liver function test' in clinical practice. It has a modestly good ability to predict the general presence of NAFLD in the absence of other liver disease. It is commonly used in clinical practice to monitor progression of disease (276, 277) or determine prevalence in large population studies (278).

The reference ranges for ALT have been investigated and updated by various groups (229, 279). When initial normal limits were defined in the 1980s, the cohort was not screened for

viral hepatitis C nor NAFLD. Hence, the standard laboratory reference ranges included patients with hepatitis C viraemia and NAFLD. This resulted in falsely high reference ranges. The updated reference ranges have been calculated after excluding these and other causes of liver disease. Wu *et al* (279) and Prati *et al* (229) suggested upper limit reference ranges of 21-30 IU/L and 17-19 IU/L for men and women respectively. These reference ranges are associated with higher sensitivity (76.3%) and specificity (88.5%) for viral hepatitis C and NAFLD, and subjects with this ALT category have lower rates of liver-related mortality than those above these levels (280).

The use of ALT for diagnosis or monitoring of liver disease is contentious, with some studies emphasizing its relative weakness in comparison to liver biopsy (13). Despite this, liver biopsy or specialised tests are not always practical due to inherent risks, costs and logistical burden (227). Serum ALT, although imperfect, may be a good proxy to regularly monitor disease. It has been considered a reasonable marker for hepatocellular injury and inflammation in NAFLD after exclusion of other causes, with modestly good ability in predicting NASH (AUROC 0.60-0.81) (277).

Exclusion of other liver disease

Patients should undergo a thorough alcohol history to ascertain past or current excessive alcohol intake. The guidelines for alcohol limits are <210g/week for men and <140g/week for women. A thorough history should be taken to exclude other aetiologies including viral hepatitis risk factors, medication use, use of parenteral nutrition, family history of genetic or other diseases (3).

Blood tests should exclude viral hepatitis B and C, autoimmune hepatitis, Wilson's disease, haemochromatosis, thyroid disease and coeliac disease. An elevated serum ferritin or iron should prompt genetic testing for human factors engineering (HFE) mutations (3).

3.6.3.2 Steatosis

Blood tests: Steatosis

There are five steatosis tests more commonly used: SteatoTest, Fatty Liver Index, NAFLD liver fat score, Lipid Accumulation Product and the Hepatic Steatosis Index. However, they have not gained much traction in clinical practice, for a number of reasons. Firstly, they do not add substantial value to clinical information already available. Secondly, they are often

unable to distinguish degree of steatosis present. Finally, the presence of bland steatosis is more widely thought of as having a benign course in terms of liver disease progression (251), with development of inflammation and fibrosis tests taking precedence.

SteatoTest

SteatoTest is a proprietary score that combines α2-macroglobulin, haptoglobin, apolipoprotein A1, GGT, bilirubin and ALT, with the metabolic factors including BMI, cholesterol, triglycerides and glucose. It is then adjusted for age and gender (281). Limited studies show a sensitivity of 85%, and specificity of 80%, and an AUROC of 0.80 for detection of >30% steatosis (281, 282).

Fatty Liver Index (FLI)

The Fatty Liver Index was developed from the Dionysos Nutrition and Liver Study in 2006. Notably, NAFLD was diagnosed by ultrasound rather than biopsy (283). The final FLI algorithm is based on triglyceride level, BMI, GGT and waist circumference. This gives a score of 0-100, where <30 is low risk for NAFLD (negative likelihood ratio 0.2) and ≥60 is high risk (positive likelihood ratio 4.3). Independent validation of the FLI showed an AUROC of 0.83 for the presence of any steatosis (284), however detection of steatosis >33% showed a poor AUROC (0.65) and this score was unable to differentiate severe steatosis.

NAFLD liver fat score

The NAFLD liver fat score is based on the diagnosis of metabolic syndrome, type II diabetes, fasting insulin, AST and the AST/ALT ratio. In an internal validation set, AUROC was 0.860, and a score of -0.640 predicts increased steatosis with sensitivity 86% and specificity 71% (285).

Lipid accumulation product (LAP)

The LAP uses three variables: waist circumference, triglycerides and gender (286). It was developed as a continuous marker of risk for lipid accumulation for monitoring disease (287). There are few validation studies for its use in NAFLD.

Hepatitis Steatosis Index (HSI)

The HSI consists of AST/ALT ratio, BMI and diabetes (288). Using ultrasound diagnosed steatosis, the AUROC was 0.812, with a value of >30 correlating with a sensitivity of 93.1%, and ≤36 correlating with a specificity of 92.4%.

Imaging tests: Steatosis

Ultrasound and computed tomography (CT)

Ultrasound is usually the first line investigation of hepatic steatosis and pathology. It is effective at diagnosing steatosis above 33%, however sensitivity decreases with lower steatosis levels. Computed tomography (CT) often incidentally diagnoses steatosis and hepatomegaly, however this is not routinely used as an initial assessment tool for steatosis (289).

Controlled attenuation parameter (CAP)

Controlled attenuation parameter (CAP) measures the degree of ultrasound attenuation caused by hepatic fat, and converts it into a measurement of steatosis. It is performed simultaneously with transient elastography (see **3.6.3.4 Fibrosis**). Studies in hepatitis C cohorts show an AUROC of 0.80, 0.86 and 0.88 for mild, moderate and severe steatosis (290). The diagnostic accuracy in patients with NAFLD has not been well studied.

When compared to other measures of steatosis, CAP had better sensitivity than serum steatosis tests (FLI and SteatoTest) (291). Larger studies of CAP on NAFLD and mixed liver disease show AUROC of 0.79-0.97 for detection of mild steatosis, 0.70-0.86 for detection of moderate steatosis and 0.66-0.84 for severe steatosis (292, 293). In subgroups of exclusively obese patients, the reported AUROC decreases substantially, to 0.92, 0.64 and 0.58 for mild, moderate and severe steatosis (292). This could be related to issues with feasibility and imaging artefact (see **Section 3.6.4 – Non-invasive tests in the obese**).

Magnetic resonance spectroscopy (MRS)

The use of ^{1}H-magnetic resonance spectroscopy (MRS) for quantification of liver steatosis was first described in 1995 by Longo *et al* (294). Several sequences have been described over the years, including single-voxel spectroscopy (SVS), point resolved spectroscopy (PRESS) or stimulated-echo acquisition mode (STEAM) sequences (295), with many more described.

Excellent accuracy and correlation with histopathology is reported with the use of MRS. Correlation ranges from r 0.819-0.890 ($p<0.001$), with AUROC between 0.930-0.981 ($p<0.001$) in various small to medium sized studies (296, 297). Few studies have reported on failure rates of MR techniques, particularly associated with obesity.

3.6.3.3 Inflammation

Blood tests: Inflammation

Cytokeratin-18 (CK-18)

Cytokeratin-18 is a marker of hepatocyte apoptosis and is one of the most widely studied markers of nonalcoholic steatohepatitis (NASH). It is used as a stand-alone test, or incorporated in other predictive algorithms. It was originally described in 2006 in a study of 399 patients with suspected NAFLD. The AUROC, sensitivity and specificity for differentiation of NASH was promisingly high with a threshold value of 395 U/L (298). A small validation study of 139 patients showed similar results, with AUROC 0.83, sensitivity 75% and specificity 81%. The threshold value, however, was lower, at 250 U/L (299). Conflicting results have been shown since (300, 301). A meta-analysis in 2014 by Kwok *et al* (302) showed a pooled sensitivity of 66% and specificity of 82% using a "best" cut-off, which ranged widely from 121.6-338.0 U/L. The heterogeneity of thresholds used makes interpretation of CK-18 difficult in clinical settings.

NashTest

NashTest is one of a family of proprietary formulae by Biopredictive (Paris, France), which includes 13 variables (age, sex, height, weight, triglycerides, cholesterol, α2-macroglobulin, apolipoprotein A1, haptoglobin, GGT, ALT, AST and bilirubin) (303). The initial study yielded AUROC of 0.79, 0.69 and 0.83 for the diagnosis of NASH, borderline NASH and no NASH respectively, in the validation cohort. Whilst specificity for NASH was good (94%), sensitivity was poor (33%). Further validation studies revealed similar diagnostic accuracy (sensitivity 14.1%, specificity 95.8%), which compared unfavourably to other diagnostic tests (282).

ActiTest

ActiTest is one of a family of proprietary formulae by Biopredictive (Paris, France), combining GGT, bilirubin, α2-macroglobulin, apolipoprotein A1, haptoglobin and ALT. Whilst NASHtest was created specifically for metabolic liver disease, ActiTest was initially developed for any hepatitis. Despite this, it has shown comparatively better results to NASHtest in some cohorts (282).

oxNASH

Oxidative stress is one of the known mechanisms involved in the development of NASH. The oxNASH score combines clinical and biochemical variables (age, body mass index, and AST) with results from mass spectrometric measurements of oxidative stress and circulating lipid peroxidation products (13-hydroxyl-octadecadienoic acid/linoleic acid ratio (13-HODE/LA ratio)). Internal validation of this score showed good results (AUROC 0.83, 95% CI: 0.73, 0.93) (304). There was reasonable sensitivity of 81% with a low threshold of 55, and specificity of 97% with a high threshold of 73. There have been minimal external validation studies for oxNASH.

Other scores

Other scores developed include the NASH score (PNPLA3 genotype, AST and fasting insulin) (305), HAIR (hypertension, high ALT, and insulin resistance) (9), Nice model (ALT, CK-18, presence of the metabolic syndrome), NASH diagnostics (CK-18, adiponectin, resistin) (306), Palekar score (age, gender, AST, BMI, AAR, hyaluronic acid) (307). However, many of these scores were developed in small and highly selected populations, and have not been externally validated.

Imaging tests: Inflammation

Experimental studies have explored different imaging techniques in differentiating simple steatosis from NASH. Diffusion weighted MRI has previously shown some differences between non-NASH and NASH patients. Perfusion CT scans have also been attempted. However, no studies have shown any definitive results, and none of these studies have been properly validated (308).

3.6.3.4 Fibrosis

Blood tests: Fibrosis

The common fibrosis serum marker panels can be classified as either ‘simple’ or ‘complex’ composite scores. Simple composite scores are based on standard laboratory and clinical factors. Complex composite scores consist of a range of biomarkers that directly measure fibrogenesis and fibrinolysis, such as hyaluronic acid, tissue inhibitor of matrix metalloproteinase 1 (TIMP1) and procollagen III amino-terminal peptide (PIIINP) (309).

Simple composite scores include the NAFLD fibrosis score, BARD index, Fibrosis-4 (FIB-4) score, Forn index and the Fibrometer NAFLD. Complex scores include the FibroTest, Hepascore and Enhanced Liver Fibrosis score (ELF).

NAFLD fibrosis score

The NAFLD fibrosis score is one of the most studied and validated tests for NAFLD-related fibrosis. It was created by Angulo *et al* in 2007 for detection of advanced fibrosis (≥F3) in NAFLD (310). It is a composite of age, BMI, diabetes status, AST to ALT ratio, platelet count and albumin, with a high and low cut-off. Measures of diagnostic accuracy vary significantly between studies, with AUROC 0.615-0.850 for detection of ≥F3 (311).

BARD index

In 2008, Harrison *et al* (312) formulated the BARD index as a simple means of excluding ≥F3 in NAFLD. A BMI ≥28, AST/ALT ratio ≥0.8 and positive diabetes status were assigned a score of 1 or 2, with a total score of ≥2 was determined to be high risk. There are conflicting reports on its accuracy, with some studies show good accuracy (312), whilst others show poor performance compared to complex scores (313).

FIB4 score

The FIB4 score was developed initially for diagnosis of Ishak fibrosis stage 4-6 in patients with HIV and hepatitis C virus coinfection (314). It combines age, aminotransferase levels and platelet levels. It has since been validated in NAFLD specific populations, predominantly for detection of ≥F3 (313, 315).

Fibrometer

Fibrometer is a proprietary panel of markers used for diagnosing hepatic fibrosis, and has previously shown good accuracy for staging NAFLD-related fibrosis in general populations (316). It combines platelet level, aminotransferase levels, ferritin, glucose, age and weight.

Enhanced Liver Fibrosis score

The Enhanced Liver Fibrosis (ELF) score is widely available and utilised test for calculation of liver fibrosis. It is calculated from tissue inhibitor of matrix metalloproteinase 1 (TIMP-1), hyaluronic acid (HA) and aminoterminal peptide of pro-collagen III (PIIINP) levels (317). Validation studies show good accuracy for ELF (AUROC 0.84, 0.93 and 0.98 for any, moderate and severe fibrosis) (317, 318), however further studies are required to establish this definitively.

Fibrotest

Fibrotest is a panel of biochemical markers developed by BioPredictive Paris, incorporating α_2-macroglobulin, apolipoprotein A1, haptoglobin, total bilirubin and GGT to create a risk estimate for advanced fibrosis (319). Studies have shown good diagnostic accuracy of the FibroTest (AUROC 0.75-0.92) (282, 313, 319), however, it has not been widely validated, possibly due to the proprietary nature of the test.

Hepascore

The Hepascore was developed in 2005, initially for viral hepatitis C (320). It is a composite score comprising age, gender, bilirubin, GGT, HA and α_2-macroglobulin. Although it has shown good accuracy, few studies have validated its use in NAFLD (313).

Imaging tests: Fibrosis

Elastography techniques

Elastography detects the 'hardness' or elasticity of a tissue, by measuring the velocity of a vibration wave generated on the skin as it travels through the liver (323). The most commonly used elastography techniques are ultrasound-based, and include transient elastography (FibroScan, Echosens, Paris, France), shear wave elastography (SWE) (Aixplorer SuperSonic Imagine S.A., Aix-en-Provence, France), and acoustic radiation force impulse imaging (ARFI) (point SWE, Virtual Touch Quantification, Siemens, Germany).

Transient elastography (TE, Fibroscan^{TM}) is now a well-established technique used in clinical practice for the management of patients with liver fibrosis (323). A reading is taken over an area at 2.5-6.5cm depth from the skin. More recently, the XL probe has been developed, particularly for overweight and obese individuals, which reads at a depth of 3.5-7.5cm. A reliable TE reading is one that has a minimum of 10 valid readings, with ≥60% success rate, and an interquartile range of ≤30%. The liver stiffness measurements (LSM) are expressed in kilopascals (kPa).

Transient elastography has been best studied in viral and alcoholic hepatitis, however, validations studies in NAFLD have been conducted for over 10 years. A meta-analysis by Kwok *et al* (302) showed overall favourable results (pooled sensitivity 85%, specificity 85% for F3-4 fibrosis), but with reasonably high variability (sensitivity 65-100%, specificity 75-97%, AUROC 0.76-0.98) and a wide variety of threshold values used (LSM 8.0-10.4kPa).

Table 3.18: Biochemical tests and panels for detection of components of NAFLD, with algorithms and standard threshold levels.

Test	Description	Thresholds
FIBROSIS		
NAFLD fibrosis score (NFS) (310)	-1.675 + 0.037 x **age** [years] + 0.094 x **BMI** + 1.13 x **diabetes status** + 0.99 x **AST/ALT** – 0.013 x **platelet** [10^9/L] – 0.66 x **albumin** [g/dl]	Detection of F3-F4 < -1.455: Low risk -1.455–0.676: Indeterminate ≥ 0.676: High risk
BARD score (312)	**BMI** ≥ 28 = 1 **AST/ALT ratio** ≥ 0.8 = 2 **Diabetes status** = 1	Detection of F3-F4 ≥2: High risk
FIB4 (315)	(**Age** [years] x **AST** [U/L]) / (√ **ALT** [U/L] x **platelet** [10^9/L])	Detection of F3-F4 <1.3: Low risk 1.3-3.25: Indeterminate >3.25: High risk
Forn index	7.811-3.131 x $\log_e$(**platelet** [10^9/L]) + 0.781 x $\log_e$(**GGT** [U/L]) + 3.467 x $\log_e$(**age** [years]) – 0.014 x **cholesterol** [mg/dl]	Detection of F3-F4 <4.2: Low risk 4.2-6.9: Indeterminate >6.9: High risk
FibroMeter (321)	Proprietary test combining **age**, **weight**, **fasting glucose** level, **ALT**, **AST**, **ferritin**, **platelet count**.	
Enhanced liver fibrosis (ELF) score (318)	Combination of **age**, **hyaluronic acid** (HA), amino-terminal propeptide of type III collagen (**PIINP**) and tissue inhibitor of matrix metalloproteinase 1 (**TIMP-1**)	
Fibrotest (322)	Proprietary test combining **α2-macroglobulin**, **haptoglobin**, **apolipoprotein A1**, **GGT**, **bilirubin** and **ALT**.	
HepaScore (320)	Proprietary test combining **age**, **gender**, **bilirubin**, **GGT**, **α2-macroglobulin** and **hyaluronic acid** (HA)	
STEATOSIS		
SteatoTest (281)	Proprietary test combining components of the FibroTest-Actitest (**α2-macroglobluin**, **haptoglobin**, **apolipoprotein A1**, **GGT**, **bilirubin**, **ALT**), **BMI**, **cholesterol**, **triglycerides** and **glucose**, then adjusted for **age** and **gender**.	<0.3: Low risk 0.3-0.7: Indeterminate >0.7: High risk
Fatty Liver Index (283)	e ^ (0.953 x loge (**triglycerides**) + 0.139 x **BMI** + 0.718 x loge (**GGT**) + 0.053 x **waist circumference** -15.745) / (1+e 0.953 x log e (**triglycerides**) + 0.139 x **BMI** + 0.718 x loge (**GGT**) + 0.053 x **waist circumference** -15.745)) x 100	<30: Low risk 30-60: Indeterminate ≥60: High risk
NAFLD liver fat score (285)	-2.87 + 1.18 x **metabolic syndrome** (yes=1, no=0) + 0.45 x **T2DM** (yes=2, no=0) + 0.15 x **insulin** [mU/L] + 0.04 x **AST** [U/L] – 0.94 **AST/ALT**	>-0.640: High risk
Lipid accumulation product index (286)	Men: (**WC** [cm] - 65) x (**triglycerides** [mmol/L]) Women: (**WC** [cm] – 58) x (**triglycerides** [mmol/L])	Continuous marker of liver steatosis.
Hepatic steatosis index (288)	8 x (**ALT/AST ratio**) + **BMI** + 2 x **gender** (male=0, female=1) + 2 x **diabetes** (yes=1, no=0).	<30.0: Low risk 30-36: Indeterminate ≥36: High risk
INFLAMMATION		
Cytokeratin-18 (298)	Hepatocyte apoptosis marker.	"Best" cut-off: 121.6-338.0 U/L (302)
NashTest (303)	Proprietary formula by Biopredictive (Paris, France) using 12 variables.	
ActiTest	Proprietary formula by Biopredictive (Paris, France) using 6 variables: GGT, bilirubin, α2-macroglobulin, apolipoprotein A1, haptoglobin and ALT.	
oxNASH (304)	Combination of **13-hydroxyl-octadecadienoic acid/linoleic acid ratio**, **age**, **BMI** and **AST**.	

Magnetic resonance imaging (MRI) techniques

Magnetic resonance imaging (MRI) based techniques have been developed for detection of liver fibrosis. Two main techniques are magnetic resonance elastography (MRE) (324) and T1 mapping (297).

MR elastography uses an external acoustic driver, placed on the body over the liver, to generate a vibration that is recorded by MRI. These data are processed by specialised software and interpreted as a stiffness reading (325). Preliminary studies of mixed aetiology cohorts have shown that MRE is useful for differentiating various stages of fibrosis, and may detect fibrosis prior to other imaging (326). However, these studies need further validation, to assess specific aetiologies and assess confounding factors such as inflammatory change.

Banerjee *et al* are currently investigating the diagnostic accuracy of T1 imaging using a shortened Modified Look Locker Inversion (shMOLLI) sequence (297). Preliminary studies have shown excellent correlation with histological assessments of fibrosis (r 0.68, $p<0.001$) and AUROC of 0.94 for detection of any (F1-4) fibrosis. Further validation with larger cohorts is currently underway by this group.

3.6.4 **Non-invasive tests in the obese**

Whilst many of the common diagnostic techniques have been widely tested in general NAFLD populations, few studies have focused on the high-risk obese population. Evidence suggests morbid obesity represents a very different metabolic, biochemical and clinical state to normal weight populations (150). The inherent baseline differences in an exclusively obese cohort can affect the calculation of these scores significantly, and therefore their accuracy. For example, many of these scores incorporate BMI into their algorithms, which can substantially skew the results in the morbidly obese.

Development and validation studies are often based in highly specialised populations. Many studies include populations selected due to abnormal liver function tests (LFT), which excludes the known significant proportion of NAFLD patients who have LFTs within the normal range (229). Similarly, many are performed in hepatology specialist referral centres, where patients are pre-selected based on prior tests or having known risk of NAFLD. Therefore, these study cohorts often represent a population with higher prevalence of more severe disease, which can affect their generalisability and accuracy in other populations.

Furthermore, body habitus is a particularly important consideration when the feasibility of imaging techniques is assessed. Ultrasound and elastography techniques are often limited by skin-to-liver distance, as probes have only a limited depth of view (3.5cm for transient elastography XL-probe) (327). Studies on general NAFLD populations often cite significant failure rates, related to patient BMI (327, 328).

Magnetic resonance imaging is particularly limited by body mass index. Although MRI often has excellent accuracy and provides valuable structural information, this has always been offset by the practicalities of imaging in obesity (17). Diagnostic and research MRI machines have a weight limit of 250kg, but more pertinent, a maximum aperture diameter of up to 70cm. Furthermore, patients with severe obesity often suffer from back pain and respiratory problems, which restrict their ability to lie supine for prolonged periods. When images can be obtained, image quality can be compromised due to increased sound-to-noise ratio and artefact. These factors clearly restrict the use of MRI in morbid obesity.

3.7 Treatment of nonalcoholic fatty liver disease

There is no single therapeutic approach that effectively treats nonalcoholic fatty liver disease. Management is often multifaceted, treating both liver disease and underlying metabolic comorbidities, including obesity, diabetes, dyslipidaemia and type II diabetes. Modes of treatment are: (1) lifestyle modification, (2) medical management of metabolic risk factors, (3) targeted pharmacological therapy, (4) surgical weight loss strategies and (5) management of end stage disease.

This section will briefly describe non-operative management strategies, with operative management strategies discussed in **Chapter 3.9 – Bariatric surgery, NAFLD and metabolic disease**.

3.7.1 Lifestyle modification

Lifestyle modification is generally employed irrespective of disease severity.

3.7.1.1 Diet

There is, thus far, no optimal diet for NAFLD. General recommendations suggest a diet with restricted calories designed to drive weight loss and improvement in comorbidities, with guidelines taken from obesity management (329). A review in 2007 of macronutrient components and popular diets reported that although data is still lacking, avoidance of saturated fats, simple carbohydrates and sweetened drinks are generally recommended (330). High monounsaturated fatty acids (MUFA) and n-3 polyunsaturated fatty acids (PUFA), together with fruit, vegetables, high fibre and low-GI foods are advocated in NAFLD (331).

Coffee consumption and caffeine has been associated with reduction in hepatic fibrosis of all causes. Recent studies have linked the consumption of unsweetened, unfiltered caffeinated coffee with decreased risk of NASH and fibrosis, after controlling for weight (332). There are no prospective trials currently available. Effects could be mediated by anti-inflammatory properties, or by reduction in serum cholesterol levels (332).

3.7.1.2 Exercise

There is substantial epidemiological evidence on the health benefits of physical activity (333). Orci *et al* showed in a meta-analysis of 28 studies that physical activity, independent of diet and weight loss, was associated with improvements as measured by intrahepatic lipid content and aminotransferase levels (329). Those with a higher BMI had greater benefit with physical activity. The key physiological mechanism is likely improvement in muscle mass and systemic as well as hepatic insulin resistance in the obese (334).

3.7.2 Medical management of metabolic risk factors

Individuals diagnosed with NAFLD are at greater risk of developing metabolic comorbidities, and thus should be regularly screened for these disorders. As metabolic diseases, such as insulin resistance and hypertension, may additionally impact on the progression of NAFLD, diagnosis is essential for adequate control of these risk factors.

3.7.2.1 Type II diabetes mellitus (T2DM)

All patients diagnosed with T2DM are encouraged to undertake lifestyle interventions. Anti-diabetic medications have been found to treat both T2DM and NAFLD (see **Section 3.7.3 - Pharmacological management**).

3.7.2.2 Hypertension

Over 70% of patients with NAFLD have hypertension (335). Currently, there are no established antihypertensive regimes known to specifically prevent progression of NAFLD (3). Current guidelines recommend aggressive treatment of all cardiovascular risk factors, due to the strong link between NAFLD and cardiovascular mortality.

Some studies suggest that the renin-angiotensin-aldosterone system contributes to liver fibrosis in animal models, and blocking this system is associated with improvement in fibrosis (336). An intervention study in 54 NASH patients with hypertension showed that telmisartan 20mg reduced aminotransferase levels, decreased NAS grading and fibrosis (337). They are known to reduce the incidence of T2DM. Therefore, angiotensin converting enzyme inhibitors (ACEi) or angiotensin receptor blockers (ARB) potentially have additional benefits for patients with hypertension and concurrent NAFLD.

3.7.2.3 Dyslipidaemia

Statins are first line treatment for hypercholesterolaemia in NAFLD, as primary prevention for cardiovascular disease. Although they are known to increase aminotransferase levels, they can be safely used in NAFLD (3). Longer-term statin therapy is associated with improvements in liver function test abnormalities due to NAFLD (338).

3.7.3 Pharmacological management

Despite significant research into possible drugs that may treat NAFLD, there are currently no optimal pharmacological options. Risks accompany many direct drug therapies, and therefore are restricted to those with biopsy-proven NASH and fibrosis (3). Current pharmacological therapies for NAFLD have two approaches: (1) drugs directed at weight loss with secondary improvements in NAFLD and (2) NAFLD-directed pharmacotherapy.

3.7.3.1 Metformin

Metformin is a biguanide anti-diabetic medication, which acts by decreasing hepatic glucose production, decreasing gastrointestinal absorption of glucose and increasing peripheral glucose uptake. A systematic review in 2013 pooling data from nine studies with 417 patients showed improvements in aminotransferase levels, weight and insulin resistance, but no histological response to treatment with metformin (339). Although metformin is not recommended as a specific therapy for NAFLD, it can be used as a possible adjunct therapy in diabetic patients with NASH. It is furthermore associated with weight loss, which can be beneficial in NAFLD (3).

3.7.3.2 Pioglitazone

Pioglitazone is a thiazolidinedione, which acts on peroxisome proliferator-activated receptors (PPAR-γ and PPAR-α) to alter transcription of genes involved in carbohydrate and lipid metabolism, improving insulin sensitivity and adipose tissue dysfunction in T2DM and obesity (340). Variable outcomes have been achieved with pioglitazone, however most studies show improvement in elements of NAFLD (341, 342).

The PIVENS study was a multicentre randomised placebo-controlled trial of 247 patients in 2010 that compared pioglitazone, vitamin E and placebo. Pioglitazone showed improvements

in aminotransferases ($p<0.001$), and histological components such as ballooning ($p=0.004$) and steatosis ($p<0.001$) compared to placebo. However, it did not show a significant difference in the rates of improvements of NASH (34% vs 19%, $p=0.04$, with *a priori* significance calculated at $p=0.025$ due to two primary comparisons), defined by an improvement of ≥2 NAS points. There was, however, a difference in *proportion* of NASH resolution (47% vs 21%, $p<0.001$). There was no improvement in fibrosis score seen ($p=0.12$) (341). A meta-analysis in 2012 confirms improvement in inflammation, and a lesser improvement in fibrosis (343).

There are some adverse effects with pioglitazone that restrict its wider use, including weight gain (2.5-4.7kg at over 12-36 months duration (3, 342)), increased risk of congestive cardiac failure, bladder cancer and reduced bone density (329). However, its use in type II diabetes is accompanied by reductions in mortality, myocardial infarction and stroke.

Current guidelines recommend the use of pioglitazone in those with biopsy proven NASH, with or without diabetes, after a thorough discussion on risks and benefits (3, 344).

3.7.3.3 Glucagon-like peptide-1 analogues

Glucagon-like peptide-1 (GLP-1) analogues include liraglutide or exenatide. They increase insulin sensitivity, inhibit gastric emptying and increases satiety (345). Through control of glycaemic index and weight reduction, evidence from a meta-analysis of phase III studies show that 26 weeks of 1.8mg liraglutide was associated with improved aminotransferases, and a trend towards steatosis improvement. Risks include pancreatitis, and an increased risk of pancreatic cancer (346). With the current evidence available, it is premature to consider GLP-1 analogues for treatment of NAFLD or NASH (3, 344).

3.7.3.4 Vitamin E

Vitamin E is an antioxidant that has shown benefit in NASH in diabetic patients. It prevents free radical formation and propagation. The PIVENS study showed significant histological improvement with vitamin E therapy compared to placebo, and superiority over pioglitazone (341). The trial dose was 800 IU/L daily over 96 weeks. Multiple other studies have been performed, and show that vitamin E decreases aminotransferase levels, improves steatosis,

inflammation and NASH, but has no effect on fibrosis (3). Long-term use of vitamin E has been associated with increased risk of haemorrhagic stroke, and prostate cancer (347, 348).

The AASLD Guidelines suggest use of Vitamin E in non-diabetic patients with biopsy-proven NASH, after thorough discussion regarding risks and benefits (3).

3.7.4 **Weight loss**

Weight loss is a powerful means of tackling NAFLD. In this section, the effects of weight loss, by non-operative methods, are presented. The effects of bariatric surgery on NAFLD will be covered in **Chapter 3.9 – Bariatric surgery, NAFLD and metabolic disease**.

3.7.4.1 Weight loss through lifestyle modification

Evidence suggests that weight loss, through any form of lifestyle changes, is associated with improvements in hepatic steatosis and inflammation. A randomised trial by Promrot *et al* showed that weight loss >7% by any method, was associated with significant improvements in inflammation. A larger prospective study of 293 patients with histologically proven NASH showed that weight loss over 52 weeks with lifestyle changes were associated with significant reductions in liver disease. Resolution in NASH occurred in 25%, with 19% showing fibrosis regression. These changes were independently associated with weight loss, with more NASH resolution in those who achieved ≥5% weight loss. Greater weight loss of ≥10% resulted in 90% NASH resolution and 45% fibrosis regression (349). The exact amount of weight loss and factors associated with improvement is unknown, with improvements seen between 7-9% TBWL in trials on lifestyle modification (350). Effects of lifestyle-driven weight loss on NAFLD in morbidly obese patients have not been explored.

The hepatic changes with weight loss can be rapid. A study of pre-operative weight loss by very low-calorie diet (VLCD) showed a 28.7% reduction in liver volume over twelve weeks, as assessed by MRI. This was directly related to weight loss ($r=0.54$, $p=0.001$) and initial liver volume ($r=0.43$, $p=0.015$), and 80% of the total reduction occurred within the first two weeks. There was a concomitant reduction in ALT from 40.6 to 32.8 U/L ($p=0.05$) (180).

The sustainability of lifestyle-induced weight loss for NAFLD is limited to the relative short term. Long term results are not known, and changes in hepatic health with subsequent weight gain have not been studied.

3.7.4.2 Orlistat for weight loss in NAFLD

Two randomised control trials have assessed the efficacy of orlistat in reversal of NAFLD, with conflicting results. One study in 2006 found improvements in aminotransferases and steatosis with orlistat (351). However, a subsequent study showed no independent change in histology compared to placebo in the setting of equivalent weight loss (352). Regardless, orlistat can induce significant weight loss in those who have not been successful with lifestyle modifications alone.

3.8 Pathophysiology of nonalcoholic fatty liver disease

The pathogenesis of NAFLD, and the factors that drive progression to steatohepatitis, are complex and not completely understood. It likely involves a combination of factors, including over-nutrition, insulin resistance, genetic predisposition, meta-inflammation, lipotoxicity, innate immunity and gut microbiota. Obesity substantially contributes to many of these pathophysiological mechanisms, and drives NAFLD development and progression.

There is significant research interest in the pathophysiology of NAFLD and NASH, with the aim of helping to improve our understanding, focusing preventative strategies and assisting in identification of new therapeutic targets.

3.8.1 Hepatic lipid homeostasis and steatosis formation

3.8.1.1 Liver lipid homeostasis

Normal lipid metabolism in the liver involves a balance of three mechanisms: (1) increase in free fatty acids (FFA), via uptake from peripheries or *de novo* lipogenesis, (2) disposal of FFA, via triglyceride (TG) formation or β-oxidation and (3) export from the liver, via TG in very low-density lipoproteins (VLDL). Hepatic steatosis results when there is an imbalance in these elements (353).

Free fatty acids in the liver are derived from three main sources – dietary FFA, circulating FFA influx and de novo lipogenesis (**Figure 3.9**). The contribution of FFA by each of these sources has been studied using a multiple-stable-isotope tracer approach (354). Donnelly *et al* demonstrated that most intrahepatic fat comes from adipose tissue FFA influx (60-80%), whereas 5-26% come from *de novo* lipogenesis and approximately 15% from dietary sources.

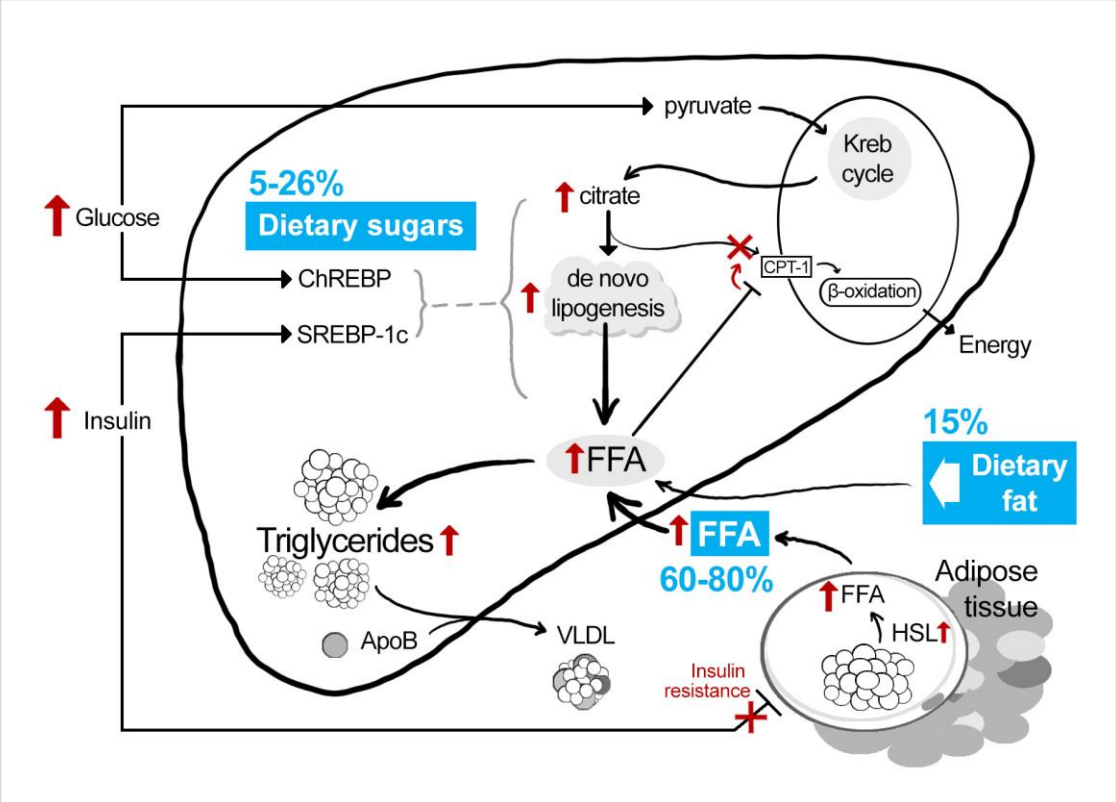
Once they enter the liver, FFAs are shuttled into various pathways. They can be transported into mitochondria via carnitine palmitoyl transferase-1 (CTP-1) and undergo β-oxidation to produce ATP. FFAs can also be converted into lipotoxic intermediates, such as diacylglycerol (DG), which promote hepatic insulin resistance. They may then be converted into TGs for storage or export via very low-density lipoproteins (VLDL). The excessive formation of TGs produces the classic histological hepatic steatosis associated with NAFLD (355).

3.8.1.2 Factors influencing steatosis formation

Any increase in supply of free fatty acids to the liver can promote steatosis formation. Dietary sources of both fats and sugars increase hepatic steatosis by increased delivery of TG directly via chylomicrons or increase in substrates for *de novo* lipogenesis. However, these sources combined deliver as little as 20% of the FFA flux into the liver, with up to 80% coming from circulating FFA (354).

Insulin resistance and glucose homeostasis heavily influence hepatic steatosis, via changes in FFA production, influx and export (**Figure 3.9**) (356).

Figure 3.9: Model of lipid flux through the liver showing main sources of free fatty acids to the liver (shaded in blue) – de novo lipogenesis (DNL) from dietary and circulating sugars, dietary sources in the form of chylomicrons, and circulating free fatty acid influx (354). Metabolic changes in liver lipid metabolism that occur with insulin resistance indicated in red (356).



FFA – free fatty acid; ChREBP – carbohydrate responsive element-binding protein; SREBP-1c – sterol regulatory element-binding protein 1c; CPT-1 – carnitine palmitoyltransferase 1; HSL – hormone sensitive lipase; VLDL – very low density lipoprotein; ApoB – apolipoprotein B
Adapted from Browning JD, Horton JD. Molecular mediators of hepatic steatosis and liver injury. J Clin Invest. 2004;114:147-152.

Firstly, insulin resistance in adipocytes increases the rates of lipolysis, via increased hormone-sensitive lipase (HSL) activity, thereby increased peripheral FFA production and circulation. Recent evidence also shows that insulin increases the expression of FFA uptake transporter cluster differentiation protein-36 (CD36), thereby inherently increasing peripheral FFA uptake in the liver. Fatty acid transporter CD36 may also be influenced by various factors such as adiponectin and dietary fatty acids (357).

Secondly, hyperinsulinaemia and hyperglycaemia both drive lipid accumulation in the liver via their respective transcription factors, sterol regulatory element-binding protein 1c (SREBP-1c) and carbohydrate-responsive element-binding protein (ChREBP) (356). Both SREBP-1c and ChREBP increase *de novo* lipogenesis (DNL), converting glucose to fatty acids. Increased *de novo* lipogenesis inhibits β-oxidation of fatty acids, via malonyl-CoA, which blocks the transport of fatty acid into the mitochondria (358).

Alterations in lipid export have also been linked to hepatic steatosis. Hyperinsulinaemia suppresses VLDL secretion (359). In obese patients, NASH has been linked with decreased VLDL production, as well as dysfunctional VLDL synthesis (360).

Overall, insulin resistance plays a vital role in the development of hepatic steatosis, by increasing external flux of FFA, increasing *de novo* lipogenesis, and then promoting preferential conversion of synthesised FFA into triglycerides over β-oxidation (356).

3.8.2 Pathophysiological mechanisms for steatohepatitis

Steatosis was initially considered to be the "first hit" in the pathogenesis of NAFLD (361). However, more recently, steatosis has been viewed as an epiphenomenon that reflects the changes in FFA handling and cellular stress, rather than the pathogenic process itself (355).

In fact, the partitioning of lipids into relatively inert intracellular triglyceride stores (seen as hepatic steatosis in biopsies) is an early adaptive response that protects from lipotoxicity of other lipid intermediates. This was astutely demonstrated in a NAFLD mouse model by Yamaguchi *et al*, where the conversion of FFA to triglycerides was suppressed by silencing the enzyme diacylglycerol acyltransferase 2 (DCAT2). Whilst this decreased triglyceride production and histological steatosis, there was significant increase in hepatic free fatty acids, necroinflammation, and fibrosis (362). This suggests a role for non-triglyceride lipids in the pathogenesis of progressive NASH and fibrosis.

3.8.2.1 Lipotoxicity

Lipotoxicity is the term used to describe cellular dysfunction and damage caused by an excess of lipid metabolites in non-adipose tissue (107). Adipose tissue is specifically designed for effective storage and management of lipids. However, ectopic fat deposition can occur when these stores are saturated or metabolism is altered, in the setting of over-nutrition and insulin resistance.

In the liver, various lipid metabolites have now been linked with hepatic apoptosis and liver injury. **Section 3.8.5 - Lipidomics** and **Section 3.8.6 – Lipidomics in NAFLD** further discusses lipidomics and current evidence regarding lipotoxicity in NAFLD.

3.8.2.2 Cytokines

Proinflammatory cytokines, from peripheral and hepatic sources, have a major role in systemic and local inflammation. This subsequently influences the development of NAFLD and insulin resistance. The most commonly studied cytokines include tumour necrosis factor alpha (TNFα), interleukin 6 (IL-6) and interleukin 1 (IL-1).

Interleukin 6 (IL-6)

Interleukin 6 (IL-6) is a proinflammatory cytokine secreted by adipocytes, hepatocytes, immune cells and endothelial cells. IL-6 binds to the IL-6 receptor (IL-6R), leading to activation of the tyrosine kinase Janus kinase (JAK)-1. This leads to activation of several intracellular signalling pathways, including the mitogen-activated protein (MAP) kinase and phosphoinositide 3 (PI3) kinase pathways, which activate the signal transducer and activators of transcription (STAT1 and STAT3) pathways (363).

Under normal conditions, IL-6 levels are very low (1-5 pg/ml). Both obesity and NAFLD have been associated with increased levels of IL-6 (363-365). In obesity, visceral adipose tissue produces larger amounts of IL-6 compared to subcutaneous adipose tissue. Within the liver, accumulation of free fatty acids (FFA) upregulate the production of pro-inflammatory cytokines, including IL-6 (363).

The role of IL-6 in NAFLD development is somewhat controversial. Robust evidence suggests that IL-6 promotes insulin resistance via several pathways, thereby influencing the development of NAFLD. Within the liver, many studies confirm the correlation of IL-6 with

NAFLD presence. Its relationship with progression to NASH or fibrosis is uncertain. IL-6 plays a hepatoprotective and hepatoproliferative role (365). However, IL-6 is also a major driver of the development of liver inflammation and hepatocellular carcinoma, with levels corresponding to increased risk (363).

The use of serum IL-6 as an independent predictor of NAFLD has not been successful, although combination with other markers, such as CK-18 and adiponectin, has reasonable results (364, 365).

Tumour necrosis factor alpha (TNF-α)

Evidence suggests that TNFα affects all stages of NAFLD development, from liver steatosis to necroinflammation and fibrosis (365). TNFα is a proinflammatory cytokine secreted by adipose tissue macrophages, hepatocytes, and Kupffer cells as a chronic inflammatory response. It has both hepatic and systemic effects.

Systemic TNFα increases insulin resistance by decreasing translocation of glucose transporter-4 (GLUT4) to the plasma membrane, thereby impairing peripheral glucose uptake. It also increases serum FFA by stimulating hormone sensitive lipase (HSL). The overall effect is increased FFA flux into the liver and promotion of steatosis (365).

Within the liver, TNFα levels are increased via activation of nuclear factor-κB (NF-κB) in response to accumulation of lipids. Kupffer cells also produce TNFα in response to bacterial endotoxins, a mechanism that has been linked with NAFLD pathogenesis (365).

This increased in hepatic TNFα has several actions. Firstly, it induces hepatic insulin resistance and activates sterol regulatory element-binding protein 1c (SREBP-1c), leading to increased *de novo* lipogenesis. It also activates cytosolic sphingomyelinase, which produces lipotoxic ceramides that can further impair insulin signalling and produce reactive oxygen species (ROS). Increased ROS acts as a positive feedback loop, further enhancing TNFα production. Increased TNFα and ROS can increase mitochondrial permeability, therefore causing inflammation and hepatocyte death (364, 365).

Treatment with pentoxifylline, a TNFα inhibitor, reduces liver enzymes and serum TNFα, and improves insulin resistance. Ultimately, it is associated with improved steatosis, fibrosis and lobular inflammation (365).

Interleukin 1 (IL-1)

Cytokines in the interleukin-1 family are secreted by macrophages, endothelial cells and fibroblasts. This family of cytokines are divided into proinflammatory (IL-1β and IL-18) and anti-inflammatory (IL-1Ra).

IL-1β is most commonly investigated in the pathogenesis of NAFLD. Both Kupffer cells and macrophages are key producers of IL-1β, via NF-κB. Various *in vivo* and *in vitro* studies demonstrate its role in the development of insulin resistance associated with NAFLD. Elevated levels of IL-1β promote hepatic lipid accumulation and fibrosis. Inhibition of IL-1β can decrease insulin resistance and ameliorate hyperglycaemia (364).

3.8.2.3 Adipokines

Adipokines, particularly adiponectin and leptin, have been linked with NAFLD pathogenesis. Low levels of adiponectin are found in obesity, and have been linked with the development of insulin resistance, type II diabetes and NAFLD (365). Adiponectin has protective effects on the liver, via anti-inflammatory effects of IL-10, suppression of TNF-α and suppression of Kupffer cells and hepatic stellate cells. Leptin concentrations are higher in obesity, and dependent on the level of adiposity. Leptin has two effects on NAFLD. Firstly, it promotes insulin resistance, and thereby drives liver steatosis. Secondly, leptin regulates hepatic stellate cells (HSC), and therefore has been linked to the development of hepatic fibrosis (365).

3.8.2.4 Oxidative stress

The primary source of ROS in hepatocytes is via oxidation of fatty acids. Normally, FFAs undergo β-oxidation via a "safe" mechanism. With an overload of FFAs, increased oxidation occurs via minor pathways, including β-oxidation in peroxisomes, and cytochrome P450-4A and P450-2A1 mediated ω-oxidation in the ER. Overuse of these minor pathways result in increased ROS production (354). This results in damage to nuclear and mitochondrial damage, phospholipid membrane disruption and production of pro-inflammatory cytokines.

3.8.2.5 Endoplasmic reticulum stress

The endoplasmic reticulum (ER) is highly sensitive to lipids. Excess FFA accumulation can precipitate accumulation of misfolded or unfolded proteins, precipitating the so-called

unfolded protein response (UPR). The UPR instigates a host of responses to re-establish cellular homeostasis, including cell cycle arrest and attenuation of protein synthesis. Failure of these compensatory mechanisms can lead to activation of inflammatory cascades and ROS production. This ultimately leads to organelle and cell death (366). UPR activation has been shown in human biopsies of NAFLD and NASH (367).

3.8.2.6 Immunometabolism

Kupffer cells are the best studied immune cells in the pathogenesis of NAFLD. Kupffer cells are activated by a variety of factors, including increased cholesterol uptake to the liver, saturated fatty acids, lipopolysaccharide (LPS) and endotoxins from the microbiota, and ROS. Activation triggers the production of tumour necrosis factor-α (TNFα) and interleukin-1β (IL-1β). Furthermore, functional Kupffer cells can recruit immune cells, such as monocytes and natural killer cells, which have been implicated in the inflammatory response. Reduction in Kupffer cells in NASH models attenuates severity of disease (368).

3.8.2.7 Genetic predisposition

Genome-wide association studies (GWAS) have shown that the heritable susceptibility accounts for 30-50% of relative risk for NAFLD. However, complex traits such as NAFLD are a result of complex interactions between environment, modifiers and genetic predisposition (369). There are only a few distinct genes that have been associated with NAFLD and NASH. Of these, the most widely validated is the PNPLA3 gene.

The PNPLA3 gene is located on chromosome 22 and codes for a 481-amino acid protein related to adipose triglyceride lipase. It has been consistently validated as a modifier of NAFLD pathogenesis and progression to NASH (235). Multiple studies have associated PNPLA3 with increased triglyceride accumulation and raised biochemical markers of liver damage. The rs738409 (Ile148Met) variant is associated with steatohepatitis and fibrosis, and may be associated with development of hepatocellular carcinoma (HCC) (355).

3.8.2.8 Gut microbiota

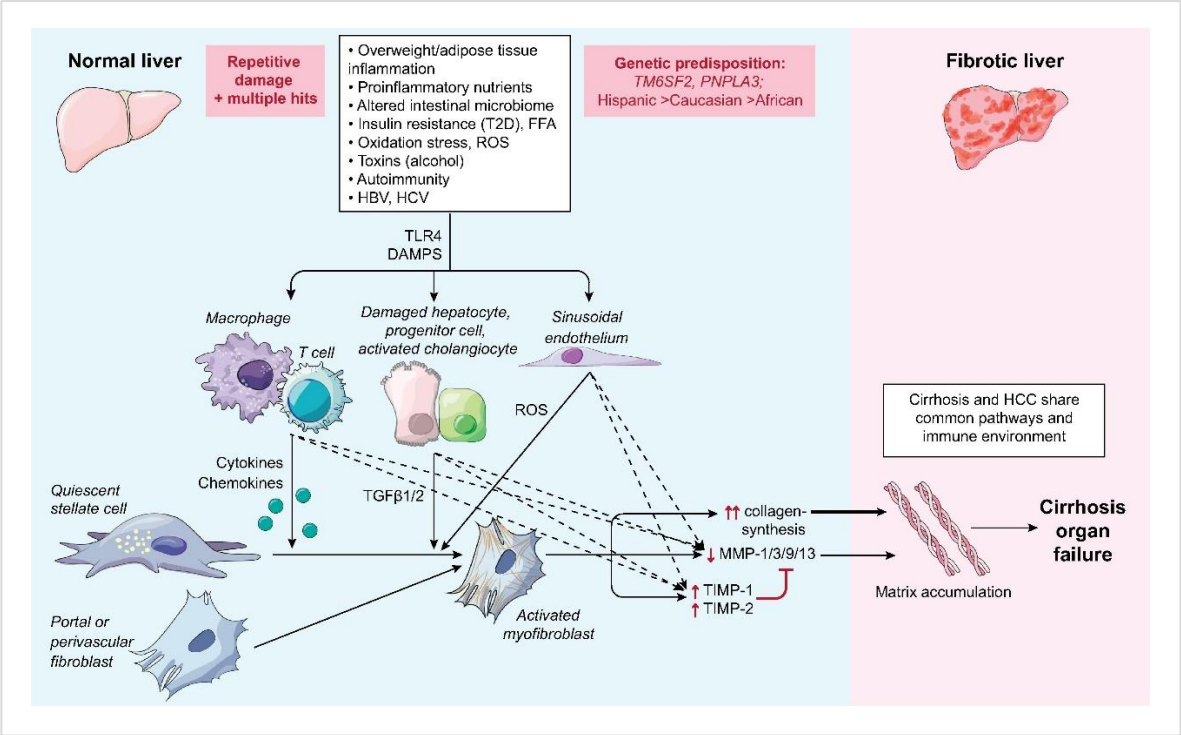
It is well established that the gut microbiota has a significant effect on metabolic function and inflammatory disease. In NAFLD, the gut microbiota promotes liver steatosis and progression to NASH by several mechanisms. These include the promotion of obesity,

activation of the immune system via LPS and toll-like receptor 4 (TLR-4) signalling, increased gut permeability, regulation of bile acid metabolism with alteration of FXR signalling, and endogenous ethanol production (370).

3.8.3 **Development of fibrosis**

Fibrosis develops on a background of chronic or repeated inflammation, which activates various cellular players, stimulates excessive collagen production, and inhibits physiological extracellular matrix (ECM) removal by proteolytic enzymes (**Figure 3.10**).

Figure 3.10: Fibrogenesis pathway in NAFLD, showing stimulation of immune cells, hepatocytes and sinusoidal epithelial cells by noxious stimuli. This results in activation of myofibroblasts, promotion of fibrogenesis and inhibition of fibrolytic pathways (245).



T2D – type 2 diabetes mellitus; FFA – free fatty acid; ROS – reactive oxygen species; HBV – hepatitis B virus; HCV – hepatitis C virus; TLR4 – toll-like receptor 4; DAMP – damage associated molecular patterns; TGFβ1/2 – transforming growth factor β1/2; ROS – reactive oxygen species; TIMP – tissue inhibitor of metalloproteinases; MMP – matrix metalloproteinase; HCC – hepatocellular carcinoma. From Schuppan D et al. Determinants of fibrosis progression and regression in NASH. J Hepatol. 2018;68:238-250.

Myofibroblasts (MF) are the key cell type responsible for ECM production. These are derived from either hepatic stellate cells (HSC) or portal/perivascular fibroblasts. Conversion of HSC and portal fibroblasts into activated MF cells is influenced by apoptosis of hepatocytes, hepatic parenchymal cells and a variety of toxic stimuli, such as systemic

inflammatory change, endotoxaemia, oxidative stress and lipotoxicity. These noxious stimuli also activate sinusoidal epithelial cells and immune cells such as macrophages and T-cells, which play a role in fibrogenesis (245).

Activated MF cells promote collagen synthesis, with collagen type I being the predominant ECM component in fibrosis. Damaged hepatocytes, activated macrophages, T-cells and sinusoidal epithelial cells inhibit production of fibrolytic matrix metalloproteinases (MMPs) and increase tissue inhibitor of metalloproteinases (TIMP), further promoting fibrogenesis (245).

Abnormal differentiation of hepatic progenitor cells can also promote fibrosis development. Progenitor cells respond to hepatocyte damage, with the aim of replenishing hepatocytes. Under conditions of chronic inflammation and oxidative stress, progenitor cells will differentiate into more stress resistant fibrogenic cholangiocytes, characterised by CK7 and CK19 (371). These produce a variety of fibrogenic mediators, including transforming growth factors (TGF β1 and β2) and platelet derived growth factors (PDGF-BB).

Acute injury of Kupffer cells results in recruitment of additional innate immune cells, including systemic and hepatic macrophages and monocytes. These immune cells play a role in both fibrogenesis and fibrolysis, depending on stimulation, with repeated inflammation and recovery promoting progressive fibrosis (245).

Overall, repeated or chronic stimuli are required to stimulate excessive collagen production, inhibit normal fibrolytic pathways and ultimately, promote fibrosis progression.

3.8.4 **Summary of pathophysiological links between obesity and NAFLD**

Various aspects of the pathophysiology of NAFLD reviewed above are fuelled directly and indirectly by obesity. These are summarised in **Table 3.19**. The basis behind management of obesity as a treatment for NAFLD is the reversal of these obesity-related factors that drive NAFLD.

Table 3.19: Factors that link obesity with the pathogenesis of NAFLD

Obesity-related factor	Impact on NAFLD pathogenesis
Insulin resistance	Increased **lipolysis**: o increased FFA flux to liver **Hyperinsulinaemia**: o increased *de novo* lipogenesis (DNL) via SREBP-1c o increased FFA uptake via CD-36 upregulation o suppression of VLDL secretion and dysfunctional synthesis **Hyperglycaemia**: o increased DNL via ChREBP Preferential **synthesis of TG** over β-oxidation
Adipokines	Decreased **adiponectin:** o increased β-cell dysfunction and insulin resistance o decreased protective effects of adiponectin on liver injury Increased **leptin:** o increased insulin resistance o regulates hepatic stellate cells, and has links with fibrosis development
Adipose tissue inflammation	Increased **IL-6** from visceral fat stores: o promotes insulin resistance o increased hepatic inflammation o increased HCC risk Increased **TNFα** from adipose tissue macrophages: o promotes hepatic insulin resistance o activates SREBP-1c o activates cytosolic sphingomyelinase, producing ceramides that further impair insulin resistance and produce ROS o increased mitochondrial permeability, resulting in inflammation and hepatocyte death Increased **IL-1** from adipose tissue macrophages: o increased insulin resistance o hepatic lipid accumulation and fibrosis
Over-nutrition	Increased **dietary fat** delivery to liver Increased **ectopic fat** deposition

FFA – free fatty acids; TG – triglycerides; DNL – de novo lipogenesis; SREBP-1c – sterol regulatory element-binding protein 1c; ChREBP – carbohydrate-responsive element-binding protein; HCC – hepatocellular carcinoma; ROS – reactive oxygen species

3.8.5 **Lipidomics**

Lipids are a broad and heterogenous range of small molecules that dissolve in non-polar solvents. They are associated with nearly all biological processes, and serve a wide variety of functions in energy storage (e.g. triglycerides), cellular membrane structure (membrane phospholipid bilayer), emulsification (e.g. bile acids), and messenger molecules (e.g. steroid hormones) (372).

Lipidomics is the identification and quantification of lipids within biological systems. The field of lipidomics has rapidly expanded, driven by two broad themes. Firstly, ongoing technological advances in mass spectrometry and chromatography have vastly improved our

ability to observe and analyse detailed lipid changes. It is now possible to quantify very low levels of lipids, increasing numbers of lipid species, subspecies and isomers, and take broad snapshots of hundreds of lipids quickly and efficiently.

Secondly, lipidomics is now being recognised as a crucial component, along with genomics, proteomics and other metabolic studies, in gaining a broad and integrated understanding of biological systems and disease processes. Emerging research has shed light on the importance of lipid-lipid and lipid-protein interactions, and revealed the active role of lipids in biological processes (373).

Therefore, lipidomics is a powerful and growing field in basic and translational research, allowing us insights into the biological function of health and disease.

3.8.5.1 Lipidomic analysis methods

There are many different approaches to lipidomic analyses. However, mass-spectrometry based lipidomic techniques are the most powerful for achieving detailed analysis at a molecular lipid species level. There are two main approaches – direct infusion analysis (shotgun lipidomics) on high resolution instruments, or liquid chromatography mass spectrometry-based lipidomics, which each have their pros and cons (374, 375).

Direct infusion allows continuous infusion of samples, and thereby identification and quantification of lipid species in a consistent environment, leading to better accuracy. However, this technique is more susceptible to extraneous salts, and so lipid extraction is more demanding.

Liquid chromatography techniques add an additional dimension of separation of lipids. It is advantageous in that it avoids complexities arising from isomeric (same composition, different structure), isotopic (same composition and structure, different mass) and isobaric (same nominal mass, different composition) lipid species, as they can usually be separated chromatographically. This, however, increase analysis time, and requires experiments to be performed with chromatographic timescale (374, 375).

Raw data processing

Raw data is firstly normalised to internal standards, to provide quantitative measures of each lipid. As most of the data is right skewed, lipid species concentrations then require

logarithmic transformation to achieve normal distribution. As the concentration of lipids can vary by several orders of magnitude, concentrations are usually expressed as multiples of interquartile range (IQR) or standard deviation (SD). This facilitates ease of interpretation in subsequent associated studies (374, 375).

Data analysis

For associations between lipidomic data and clinical outcomes, logistic or linear regression is the preferred method of analysis. Confounding factors can be adjusted for in these analyses.

Correction for multiple comparisons is essential for any lipidomic dataset. There are two approaches to this – controlling the false discovery rate (FDR, Benjamini-Hochberg) or controlling the family-wise error rate (FWER, Bonferroni method). FWER controls the probability of false positive results (Type I error), and the FDR method controls for the expected proportion of false positives. Due to the often small sample sizes in lipidomic studies, and collinear nature of lipidomic data, the FDR method is used over the more stringent FWER (374).

Techniques such as unsupervised clustering approaches (e.g. hierarchical clustering) may be used, but typically result in clustering into classes. Data-driven dimensionality reduction approaches such as principal component analysis (PCA) or partial least square scores can also be utilised to reduce data. However, the results can be difficult to interpret, as they may cluster lipid species into groups with no clear biological meaning (374).

3.8.5.2 Lipid classes

In 2005, the International Lipid Classification and Nomenclature Committee was established by the LIPID MAPS Consortium. The aim was to develop a universal and comprehensive system for lipid description and classification, based on chemical and biochemical principles, to allow integration of information (376).

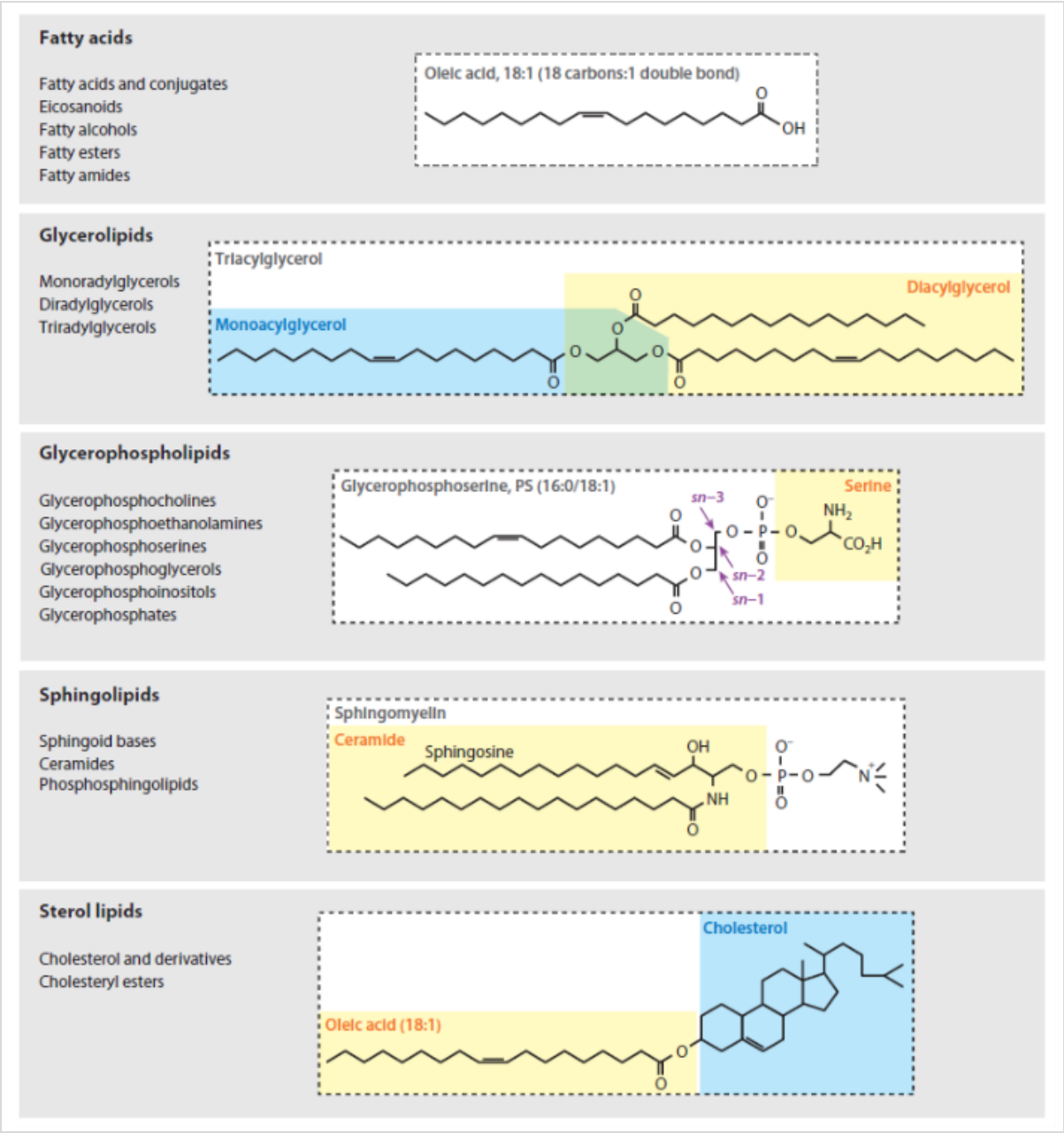
There are two fundamental building blocks for lipids – ketoacyl groups and isoprolene groups. The LIPID MAPS classification is based upon this, with lipids divided into eight categories (377):

- Ketoacyl groups (6): Fatty acyls, glycerolipids, glycerophospholipids, sphingolipids, saccharolipids and polyketides.

- Isoprene groups (2): Sterol lipids and prenol lipids.

Saccharolipids and polyketides are found only in plants and bacteria. Therefore, the six main categories of mammalian lipids are: fatty acyls, glycerolipids (i.e. triacylglycerol), glycerophospholipids, sphingolipids, sterols (such as cholesterol esters) and phenols (**Figure 3.11**).

Figure 3.11: Common lipid classes with structures represented (375).

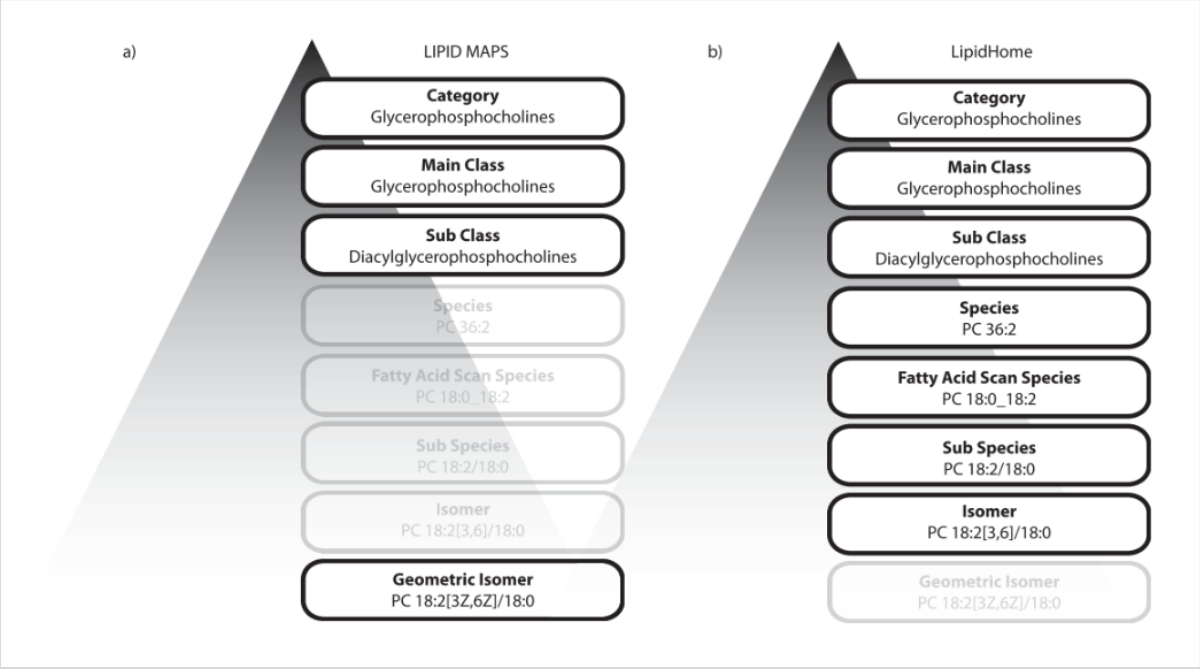


From Blanksby SJ, Mitchell TW. Advances in mass spectrometry for lipidomics. Annu Rev Anal Chem. 2010;3:433-465.

Nomenclature

Beyond major lipid classes, the LIPID MAPS hierarchy classifies lipids into *Classes*, *Subclasses* and then its detailed *Geometric Isomer*. Further classification systems, such as LipidHome, are based on the LIPID MAPS terminology, but provide structural detail between *Subclass* and *Geometric Isomer* (**Figure 3.12**). *Species* identifies the total number of carbons and double bonds within the lipid (e.g. PC(36:2) being a phosphatidylcholine with 36 carbon atoms and two double bonds). *Fatty acid scan species* identifies the fatty acids within the lipid, but not their position (e.g. PC(18:0_18:2) being a phosphatidylcholine with one 18 carbon saturated fatty acid and one 18 carbon polyunsaturated fatty acid). *Sub species* identifies the position, or stereospecific numbering (sn), of each of the fatty acids (e.g. PC(18:2/18:0)) on the glycerol backbone. *Isomer* information refers to the position of the double bond within each fatty acid chain (e.g. PC(18:2[3,6]/18:0)). Achieving characterisation to the *Isomer* level is uncommon and limited to a few laboratories with specific research interests.

Figure 3.12: Structural hierarchy of lipid records, showing (a) LIPID MAPS classification, and (b) LipidHome classification, with further detail from the subclass to isomer level (378).



From Cummings DE et al. Ghrelin and energy balance: focus on current controversies. Current Drug Targets. 2005;6:153-169.

Fatty acids

Fatty acids are a diverse group of molecules, containing a carboxyl group (COOH) with a hydrocarbon chain. They are classified according to the number of carbons (typically 4-24 carbons) and amount of double bonds (**Table 3.20**). Saturated fatty acids (SFA) have no double bonds and maximal hydrogen atoms per carbon atom, giving them a more linear structure. Monounsaturated (MUFA) and polyunsaturated fatty acids (PUFA) have one or more double bonds present, creating a bend in the hydrocarbon chain. Both length and saturation affect the structure and function of fatty acids and molecules containing fatty acids, and are important considerations in disease processes.

Table 3.20: Common saturated and unsaturated fatty acids (376)

Common name	Structure	Carbons : Double bonds
Saturated fatty acids		
Palmitic acid	$CH_3(CH_2)_{14}COOH$	16:0
Stearic acid	$CH_3(CH_2)_{16}COOH$	18:0
Arachidic acid	$CH_3(CH_2)_{18}COOH$	20:0
Unsaturated fatty acids		
Palmitoleic acid	$CH_3(CH_2)_5\mathbf{CH=CH}(CH_2)_7COOH$	16:1
Oleic acid	$CH_3(CH_2)_7\mathbf{CH=CH}(CH_2)_7COOH$ (*cis*-Δ^9)	18:1
Elaidic acid	$CH_3(CH_2)_7\mathbf{CH=CH}(CH_2)_7COOH$ (*trans*-Δ^9)	18:1
Linoleic acid	$CH_3(CH_2)_4\mathbf{CH=CH}CH_2\mathbf{CH=CH}(CH_2)_7COOH$	18:2
Arachidonic acid	$CH_3(CH_2)_4\mathbf{CH=CH}CH_2\ \mathbf{CH=CH}CH_2\ \mathbf{CH=CH}$ $CH_2\mathbf{CH=CH}\ (CH_2)_3COOH$ (*cis cis*-$\Delta^5\Delta^8\Delta^{11}\Delta^{14}$)	20:4

Fatty acids are highly metabolically active. Adipose tissue is the main source of free fatty acids, with composition closely related to dietary fatty acid composition (379). Normally, fatty acid release is governed by strict regulatory mechanisms, however in metabolic disease and insulin resistance, increased lipolysis leads to excessive free fatty acid release (380).

Sphingolipids

Sphingolipids are a diverse class of lipids, characterised by their 18-carbon amino-alcohol backbone, and typically found as a structural component of biological membranes. They are present in relatively low abundance, compared to other lipid types, but have a dynamic role in cell signalling, proliferation and apoptosis (381).

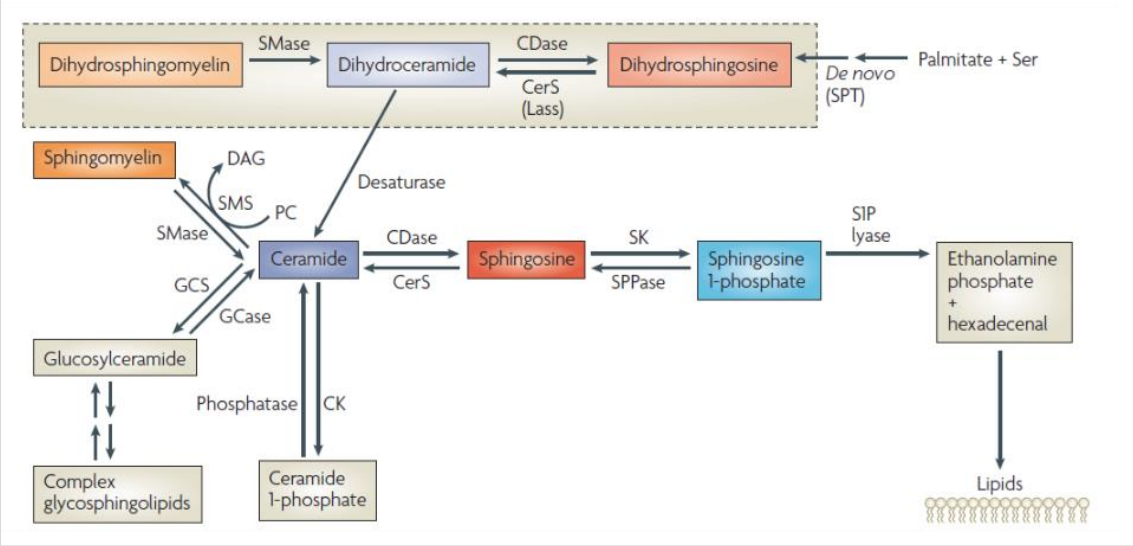
Modification of this basic structure gives rise to a broad range of bioactive sphingolipids that influence membrane biology and cell function. Prominent sphingolipids include ceramides,

sphingomyelins, gangliosides and complex sphingolipids. Despite their diversity, sphingolipids share a common synthetic and catabolic pathway (**Figure 3.13**) (382).

Sphingolipid metabolism

Sphingolipids are not absorbed appreciably from dietary sources but are synthesised *de novo* from palmitate and serine. This is a four-step process catalysed by several enzymes, ultimately leading to the conversion of dihydroceramide into ceramide. However, the rate-limiting step is the first, involving serine palmitoyl-CoA transferase (SPT), which has a high specificity for palmitoyl-CoA (382).

Figure 3.13: Sphingolipid metabolism, showing synthesis into dihydrosphingosine to catabolism. This also depicts the central role that ceramides play in the metabolism of sphingolipids (383).



From Hannun YA, Obeid LM. Principles of bioactive lipid signalling: lessons from sphingolipids. Nat Rev Mol Cell Biol. 2008;9:139-150.

Ceramides play a central role in sphingolipid metabolism. Pathways leading from ceramides include: (1) phosphorylation to create ceramide-1-phosphate, (2) glycosylation by glucosyl or galactosyl ceramide synthase to create glucosylceramides and complex sphingolipids, (3) addition of a phosphocholine headgroup from phosphatidylcholine (PC) via sphingomyelin (SM) synthases to create sphingomyelin, and diacylglycerol (DG) as a product of PC. Ceramide can be regenerated from any of these pathways (382).

Ceramides may also be catabolised by ceramidases (CDase) to form sphingosine. From this point, sphingosines can either be ‘salvaged’ back into the sphingolipid pathways to create ceramide again, or phosphorylated by sphingosine kinases (SK1 or SK2) to create

sphingosine-1-phosphate (S1P). This can either be dephosphorylated to create sphingosine again, or broken down further by S1P lyase, that irreversibly cleaves S1P into ethanolamine phosphate and hexadecenal (383).

Glycerolipids

Glycerolipids consist of a glycerol backbone with at least one fatty acid chain. The three most common glycerolipids are named according to the number of fatty acids – monoacylglycerol (MG), diacylglycerol or diglyceride (DG), and triacylglycerol or triglyceride (TG).

These prominent glycerolipids are formed via the glycerolipid/free fatty acid (GL/FFA) cycle. This cycle combines FFA with glycerol to form TG, DG or MG and various intermediaries. Importantly, DG and phosphatidic acid (PA) may be used in pathways for synthesis of other lipids (384).

Glycerolipids play an essential role in energy storage and release. However, more recent investigation suggests it plays an integral part in metabolic signalling, via the glycerolipid (GL)/free fatty acid (FFA) cycle and safe storage of FFAs (384).

Glycerophospholipids

The basic structure of glycerophospholipids are a glycerol backbone with two fatty acid chains and a phosphate head. Glycerophospholipid synthesis uses diacylglycerol as a substrate, adding various head groups to create different classes of lipids. Of the glycerophospholipids, phosphatidylcholine (PC) and phosphatidylethanolamine (PE) are the most abundant in humans, and provides the majority of lipids for the membrane bilayer.

Phosphatidylcholine (PC) is synthesised via the cytidine 5'-diphosphate (CDP)-choline pathway, which attaches a choline to a diacylglycerol. PC is also synthesised through conversion of PE to PC by methylation of the choline head group, catalysed by phosphatidylethanolamine N-methyltransferase (PEMT). Phosphatidylethanolamine is synthesised by the CDP-ethanolamine pathway, which attaches ethanolamine to DG. It may also be synthesised from phosphatidylserine, from within the mitochondria. Both PC and PE are converted to lysophosphatidylcholine (LPC) and lysophosphatidylethanolamine (LPE) respectively, by lipoprotein-associated phospholipase A2 (Lp-PLA2).

Phosphatidylcholine and phosphatidylethanolamine are major plasma membrane lipids. They are represented asymmetrically on internal and external membrane layer, with PC having greater concentration on the external membrane layer, with higher PE concentration internally. Changes to the ratio of PE to PC can alter the membrane potential and permeability to proteins and cytokines. Membrane composition can also alter binding of ions, and protein function (385).

Murine studies blocking the production of PE cause accumulation of DG and TG within tissues, but the retention of insulin sensitivity and oxidative capacity. This suggests that PE influences insulin sensitivity, rather than DG or TG.

Sterols

Sterols are a complex molecule with four interlocking carbon rings, three of which are six sided and the fourth which is five sided. The most common mammalian sterol is cholesterol. Cholesterol is derived either from the diet, or synthesised *de novo* in a complex series of enzymatic reactions.

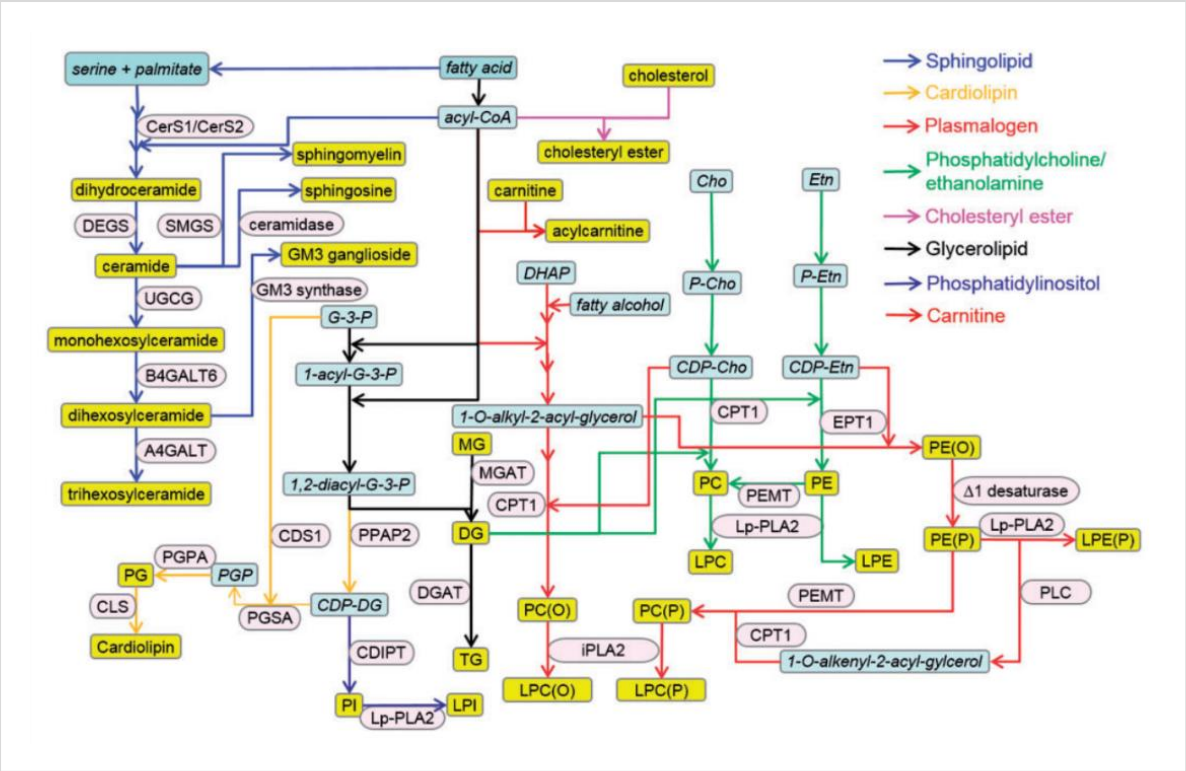
For more effective transport and storage, cholesterol is converted to cholesterol esters. Cholesterol esters have a long chain fatty acid attached to its hydroxyl group, making it far less polar. This occurs mainly via a process that ultimately transfers a fatty acid from phosphatidylcholine (PC) by the enzyme lecithin:cholesterol acyl transferase (LCAT), creating 1-acyl lysophosphatidylcholine as a by-product. Due to this, the majority of cholesterol esters contain polyunsaturated fatty acids, typical of PC.

Cholesterol plays an important structural role in phospholipid bilayer membrane, and influences the function of proteins within the membrane. It has an important role in cell signalling, lipid metabolism, and interactions with other bioactive lipids. Furthermore, it serves as the precursor for bile acids, many vitamins and steroid hormones.

Common lipid pathways

Figure 3.14 shows the complex interconnectedness of lipid species. This demonstrates the connection between sphingolipids, glycerolipids, particularly DG, and phospholipids (374).

Figure 3.14: Partial metabolic map of lipid classes and subclasses commonly measured in lipidomic studies (yellow boxes), intermediate metabolites not normally measured in lipidomic studies (italicised blue boxes) together with major enzymes (pink ovals). This demonstrates the complex interconnectedness of lipid classes and subclasses (374).



From Mundra PA et al. Lipidomic analysis in epidemiology [Review]. Int J Epidemiol. 2016;45(5)1329-1338.

3.8.6 **Lipotoxicity in NAFLD**

The majority of hepatic lipids, and the content of lipid vacuoles, are triglycerides. However, many other lipid types accumulate in the liver in the setting of steatosis, including free fatty acids (FFAs), diacylglycerol, free cholesterol, cholesterol esters, sphingolipids and phospholipids. As demonstrated in the pathophysiology section above, FFAs and lipid intermediates are closely interrelated with various pathogenic pathways, including cytokine production, oxidative stress, insulin resistance and reactive oxygen species. Understanding the specific lipidomic changes that occur in steatosis can help to determine the particular lipid species involved in inflammatory change.

Most studies analysing changes in NAFLD and NASH are based on animal models or cell line experiments. However, some studies are based on human cohorts (**Table 3.21**). The current evidence around lipidomic changes in NAFLD are summarised in the sections below.

Table 3.21: Key studies in human NAFLD analysing lipidomic changes.

Author	Study details	Tissue type			Lipid classes/ subclasses examined	Findings
		Liver	Blood	Fat		
Puri, 2007 (386)	Controls (n=9) NAFLD (n=9) NASH (n=9)	Y			FFA, DG, TG, FC, CE, PL	Increased TG, DG and FC, but FFA unaltered. Increase in TG:DG ratio with increasing severity of NAFLD. Decreased total PC in NAFLD. Increased n-6:n-3 FFA ratio in NASH.
Puri, 2009 (387)	Controls (n=50) NAFLD (n=25) NASH (n=50)		Y		FFA, DG, TG, FC, CE, PL, SL	Increased DG, TG. Detailed examination of changes in FFA chains. Increased MUFA vs SFA.
Yamada, 2014 (388)	SS (n=63) NASH (n=40)	Y			FFA	Increased MUFA in NASH (C16:1n7/C16:0 ratio). Increased C18:0/C16:0 ratio with increased steatosis score.
Gorden, 2011 (389)	Normal (n=12) NAFLD (n=17) Cirrhosis (n=9)	Y			DG, PL	Increased DG. Analysed differences in fatty acid composition of DG and PL species, showing increase in MUFA and SFA compared to PUFA.
Gorden, 2015 (390)	Normal (n=31) SS (n=17) NASH (n=20) Cirrhosis (n=20)	Y	Y		FFA, DG, TG, PL, SL, sterols,	Increased TG in steatosis/NASH. Decreased DG and CE in NASH compared to steatosis. Increased PUFA LPEs in steatosis vs normal. Increased short and saturated fatty acids in NASH, lower PUFA. Increased specific DG, TG, CE, PE, PC, PI, LPC, SL in serum with NASH.
Luukkonen, 2016 (391)	NAFLD (n=125), divided into HOMA-IR and PNPLA3 cohorts	Y			DG, TG, PL, SL	Higher SFA ceramide species and higher SFA in metabolic NAFLD.
Loomba, 2014 (392)	Control (n=10) NAFL (n=10) NASH (n=9)		Y		FFA	Top biomarkers for differentiating NAFL from NASH 11,12-dihydro-eicosatrienoic acid, 13,14-dihydro-14-ketoprostaglandin D_2, 20-carboxy arachidonic acid.
Kotronen, 2010 (393)	Obese patients (n=8)	Y	Y	Y	FFA, PL, MG, DG, TG, SL	Comparison of lipid content in each depot. Increased ceramide, sphingomyelin, PE, LPC in liver than fat. Serum levels correlated to liver levels of SCD1 activity.
Kolak, 2007 (394)	Non-diabetic healthy obese women (n=20)			Y	SL, PL, TG	MRI to quantify liver fat. TG and ceramides increase with liver fat content.
Anjani, 2015 (395)	No NASH (n=24) NASH (n=22)		Y	Y	Portal blood, systemic blood, adipose tissue	Increased systemic PC, PE, PI, PG, LPC and ceramides. Portal PG and PE were increased. Minor changes in visceral adipose tissue efflux.

NAFL – nonalcoholic fatty liver; SS – simple steatosis; NASH – nonalcoholic steatohepatitis; FFA – free fatty acid; DG – diacylglycerol; TG – triacylglycerol; FC – free cholesterol; CE – cholesterol ester; MUFA – monounsaturated fatty acid; SFA – saturated fatty acid; PL – glycerophospholipids; SL – sphingolipids; PUFA – polyunsaturated fatty acids.

3.8.6.1 Role of triglycerides in NAFLD

The liver stores lipids predominantly in the form of triglycerides (TG) (356). Evidence shows that neutral triglycerides are likely the least toxic form of lipid surplus, as they can be stored efficiently and safely. Storage of excess lipids as triglycerides may, in fact, help to protect against metabolic trauma by sequestering more toxic metabolites. This has been demonstrated in cell line models of lipotoxicity, where supplementation of oleic acid (a

monounsaturated fatty acid (MUFA)) is rapidly incorporated into triglycerides, causing significant steatosis, but minimal apoptosis. However, palmitic acid (a saturated fatty acid (SFA)), which is poorly incorporated into triglycerides, leads to less steatosis, but increased FFA flux and hepatocyte lipoapoptosis (396). Mice models that increase diacylglycerol (DG) conversion to TG through overexpression of the enzyme diacylglycerol acyltransferase 2 (DGAT2), have excessive liver steatosis with little to no concurrent metabolic sequelae of lipotoxicity or insulin resistance (397). Furthermore, blocking the actions of DGAT2 results in decreased steatosis, but increased oxidative stress, apoptosis and worsening inflammation and fibrosis (362). Therefore, evidence suggests that the incorporation of excess FFA into neutral triglycerides is a protective mechanism, which stalls progression to NASH by lipotoxicity.

Although triglycerides seem to play a more protective role in hepatic steatosis, as the most abundant lipid, it is a good measure of overall lipid excess, and may be a surrogate measure for excess of other toxic lipids. Additionally, it is often an indication of metabolic disturbance and insulin resistance, which can precipitate inflammatory change independently.

3.8.6.2 Diacylglycerol (DG)

Diacylglycerol (DG) is an activating ligand for many types of protein kinase C reactions, essential for a diverse range of cellular signalling. DG also plays a role in metabolism, as the substrate for the formation of triglycerides and phospholipids. Whilst some studies have found correlations between DG and hepatic damage (398), further studies have shown similar levels in NAFLD and NASH, although both higher than normal liver (386). Some suspect increases in DG are just reflective of lipid flux, and further study is needed to elucidate the role of DG in lipotoxic injury.

3.8.6.3 Sphingolipids

Ceramides

Ceramide production

Ceramides are a subset of the sphingolipid family. Whilst ceramides are synthesised around the body, the liver is the major site of ceramide production (399). The pathways for ceramide synthesis and breakdown are described in Chapter 5.8.4.2. Sphingolipids. However, there are

three main pathways of ceramide synthesis: (1) *de novo* synthesis from palmitoyl-CoA and serine, via serine palmitoyltransferase (SPT), (2) sphingomyelinase (SMase) pathway from sphingomyelin, and (3) a salvage pathway from sphingosines.

Ceramide production is highly dependent on supply of long-chain saturated fats, which is the first and rate-limiting step in *de novo* ceramide synthesis (400). Ceramide production is, thus, increased in states of obesity, insulin resistance or excessive dietary intake of saturated fatty acids. Additionally, ceramides can be created by the backdown of sphingomyelin through the SMase pathway. This pathway is a rapid means of ceramide production, triggered by Fas, TNF receptors or ROS formation (401).

The majority of plasma ceramides are likely derived from liver. Evidence suggests that 75% of ceramides are bound to VLDL or LDL, and ceramides secreted by hepatocytes in experimental settings are mainly contained in VLDL (402).

Role of ceramides in insulin resistance and inflammation

Substantial evidence now demonstrations the role of ceramides in insulin resistance and lipotoxicity (385, 401). They increase insulin resistance by inhibiting insulin-dependent glucose uptake, GLUT4 translocation to cell membranes, and glycogen synthesis (381, 401). Ceramides are involved in both intrinsic and extrinsic apoptosis, with studies showing increased programmed cell death after treatment with ceramide, or agents promoting ceramide accumulation (383, 401). Ceramides have been linked with proinflammatory cytokines such as IL-6, IL-1 and TNFα, which increase mitochondrial ROS synthesis (399). Furthermore, experimental models inhibiting ceramide *de novo* synthesis results in better insulin sensitivity, less atherosclerosis, reduced inflammation and reduced liver steatosis (403). These data indicate that ceramides likely represent a pathway that links excessive free fatty acids with increased insulin resistance and inflammation.

The characteristics within ceramides have recently been studied, showing importance of fatty acid side-chains. Ceramides comprising long side chains (C16:0 or C18:0) had increased insulin resistance and liver steatosis compared to very long chains (C24:0 or C24:1) (404, 405). Additionally, the presence of saturated fatty acid side-chains in ceramides has been linked with insulin resistance in humans (391).

Ceramides in human NAFLD

The link between ceramides and NAFLD is not clearly understood. There are few data describing the link between ceramides and NAFLD in humans, with some controversy regarding their role.

Luukkonen *et al* have found increased liver ceramide levels in those with metabolically-driven NAFLD associated with high HOMA-IR (391). Gorden *et al* analysed increasingly severe NAFLD, and found increases in serum ceramide and dihydroceramides, and increases in liver dihydroceramides with NASH (390). As dihydroceramides typically reflect increases in *de novo* ceramide synthesis, this likely indicates upregulation of ceramide synthesis in NASH. Furthermore, studies have significantly correlated adipose tissue ceramides with liver steatosis and NAFLD (394). Both lifestyle and surgical weight loss are associated with decreases in serum ceramide levels, and reductions in ceramide gene expression in the liver (406, 407).

Conflicting evidence has shown little differences in ceramide concentrations in normal compared to NAFLD patients (285). Experimental models have found that saturated fatty acid induced insulin resistance is independent of ceramide synthesis (408). Additionally, others have also found saturated fatty acid induced ER stress and inflammation were also ceramide independent (409, 410). The exact reasons for the differences in results is unknown.

3.8.6.4 Glycerophospholipids

Phosphatidylcholine to phosphatidylethanolamine ratio (PC:PE)

Decreased PC:PE ratio is seen in the liver of patients with NASH (411). This has been shown in numerous mice models that modulate PC and PE concentrations through knockout of various enzymes. Decrease in PC relative to PE increases membrane permeability and can lead to tissue damage. Mice deficient in PC develop NASH and liver failure, and increases in PC:PE ratio then restores membrane integrity and prevents NASH despite lipid accumulation (411).

Lysophosphatidylcholine (LPC)

Evidence suggests that increased lysophosphatidylcholine (LPC) levels are associated with FFA-induced liver damage and insulin resistance. Increasing hepatic levels of LPC were found in patients with increasing severity of NAFLD (412). This was also found in MCD and

high fat murine models of NASH (413). Inhibitors of PLA2, the enzyme converting phosphatidylcholine (PC) to lysophosphatidylcholine (LPC), ameliorated cellular toxicity, even in the setting of FFA accumulation (412). Therefore, increased LPC is instrumental in FFA-induced liver damage.

3.8.6.5 Free fatty acids

Patients with NASH have higher levels of circulating free fatty acids (387), therefore increasing the flux of fatty acids through the liver. These can both passively enter hepatocytes, or be actively transported via fatty acid transport protein (FATP) or fatty acid translocase (FAT/CD36). Fatty acids are driven down numerous metabolic pathways, including conversion to DG, then TG, as the safest form of lipid storage.

Excess free fatty acids (FFA) accumulation in non-adipocyte cells can cause damage, dysfunction and trigger apoptosis (lipoapoptosis) (396). Below, are some more established pathways by which FFAs can influence cellular damage – hepatic lipid partitioning, death receptors, mitochondrial-lysosomal pathway, and endoplasmic reticulum (ER) stress.

Hepatic lipid partitioning

The type of FFA in liver, termed hepatic lipid partitioning, is especially important, with monounsaturated fatty acids (MUFA) being highly incorporated and stored as more benign triglycerides, and saturated fatty acids (SFA) being associated with increased apoptotic damage. Li *et al* studied the role of stearoyl-CoA desaturase-1 (SCD1), the enzyme that converts saturated to unsaturated fatty acids. By inhibiting SCD1, they observed a decrease in the MUFA to SFA ratio, and an accompanying increase in the rate of SFA-induced apoptosis (414).

Fatty acid changes in human NAFLD and NASH corroborate findings in animal studies. Yamada *et al* profiled the changes in fatty acids with development of NASH, and demonstrated changes consistent with an overall increase in palmitic acid (C16:0) (a saturated fatty acid) in NASH (388).

Death receptors

Apoptosis is triggered either by extrinsic signalling mediated by death receptors (Fas, TNF, TNF-related apoptosis inducing ligand (TRAIL)), or the intrinsic pathway (organelle based).

Excessive free fatty acids can upregulate the expression of death receptors in hepatocytes, thereby increasing sensitivity to apoptotic signals (415). In human NASH, increased expression of Fas and death receptors have been demonstrated (416).

Mitochondrial-lysosomal pathway

Mitochondria play a vital role in cell death via intrinsic apoptotic pathways. Cell line studies show that saturation of hepatocytes with FFAs increases mitochondrial dysfunction, mitochondrial membrane permeability, reactive oxygen species (ROS) production, ultimately triggering cellular damage (417). Lysosomal permeability is also increased. In experimental studies, the major lysosomal cysteine protease, cathepsin B, is released in response to FFA application to hepatocytes. Cytoplasmic concentrations of cathepsin B are increased in human NAFLD (410).

Endoplasmic reticulum (ER) stress

The accumulation of SFA can increase the ER stress response, and subsequently increase mitochondrial-dependent apoptotic cell death (418). This has been shown in human NASH, which shows signs of UPR activation, ER stress and activation of JNK (367).

3.8.6.6 Free cholesterol

There are several studies that have shown progressive increases in serum and hepatic free cholesterol with progressive NAFLD and NASH (386, 387). Simultaneous increases in the cholesterol synthesis enzyme, HMC CoA reductase, can be seen in NAFLD and NASH, and correlate with severity of disease. Other enzymes central to the production of cholesterol, such as SREBP-2 and StAR (steroidogenic acute regulatory protein, a mitochondrial-cholesterol transporter) are also elevated in those with NASH compared to simple steatosis (419).

Data suggests that free cholesterol sensitises hepatocytes to inflammation and death-receptor mediated apoptosis. In rats fed choline-deficient diet (to increase hepatic triglyceride) or 2% cholesterol and sodium cholate diet (to increase cholesterol levels), only the animals with increased cholesterol showed increased apoptosis and ROS formation after TNF treatment (420).

In summary, current evidence suggests a link between the lipidomic profile of liver and development of NAFLD and NASH. Most studies involve animal models of NAFLD, however there is growing evidence within human studies that corroborate these mechanisms. These studies suggest that fatty acid characteristics, such as increased mono-unsaturated and saturated fatty acids, as well as ceramide and glycerophospholipid characteristics are associated with development of NASH. However, further studies of larger cohorts are needed to confirm these findings and determine their clinical relevance and utility.

3.9 Bariatric surgery, NAFLD and metabolic disease

A simple observational study by Walter Pories in 1995 is often credited with heralding the dawn of metabolic surgery and drawing the gaze of the medical community to the benefits of bariatric surgery beyond weight loss. That sentinel publication was entitled: "*Who would have thought it? An operation proves to be the most effective therapy for adult-onset diabetes mellitus*" (421). It has since been cited over 2640 times and was instrumental in the growth of bariatric and metabolic surgery that followed the introduction of laparoscopic surgery.

Since then, our knowledge around the further metabolic and cardiovascular benefits of bariatric surgery has grown exponentially. This chapter summarises our current understanding of the increasingly important role that bariatric surgery and bariatric clinicians play in NAFLD and metabolic disease.

3.9.1 Landmark studies

Over the last couple of decades, innumerable publications have looked at the weight loss, metabolic benefits and complications from bariatric surgery. The major systematic reviews, meta-analyses and large studies are listed in **Table 3.22** and discussed below.

3.9.1.1 Systematic reviews and randomised controlled trials

Various systematic reviews and meta-analyses have examined weight loss and other outcomes after bariatric surgery. Gloy *et al* reported on all available randomised controlled trials (RCT) of bariatric surgical vs non-surgical patients up to December 2012. This included 11 studies of 796 participants, with quality pooled follow-up data at two years post-operative. Across the board, there was substantially greater weight loss in surgical groups correlating to significantly greater improvements in type II diabetes (T2DM), metabolic syndrome (MetS), quality of life (QOL) and use of medications (190). There were no perioperative deaths, however one patient had an anastomotic leak with significant in-hospital complications, and 8% required revision operations. There were greater rates of iron deficiency anaemia in the surgical group (15% vs 2%).

Table 3.22: Meta-analyses, randomised controlled trials and large studies of bariatric surgical outcomes.

Study or review	Study details	Weight loss or comorbidity outcome	Complications
Meta-analysis (2013) by Gloy *et al* (190)	Systematic review and meta-analysis of 11 RCT's (n=796) up to Dec 2012, comparing **surgical and non-surgical**. *Outcomes*: Weight loss, T2DM, metabolic syndrome, QOL, medicinal use, cholesterol profile, HTN	*Weight loss:* Surgical cohort lost 26kg more, compared to non-surgical (95% CI -31 to -21), p<0.001 *T2DM remission:* Surgery relative risk (RR) 22.1 (3.2-154.3, p=0.002) vs non-surgical, RR 5.3 (1.8-15.8, p=0.003)	15% vs 2% iron deficiency anaemia. 1.5% vs 3% cholecystitis. No perioperative deaths, cardiovascular events or deaths during follow-up. 1 leak (0.4%). 8% reoperation rate.
Meta-analysis (2014) by Chang et al (206)	Systematic review and meta-analysis of **bariatric surgical outcomes** in 164 studies of 37 RCT and 127 observational studies (OBS) from 2003-2012. Meta-analysis of 161,756 patients. *Outcomes*: Weight loss, complication, mortality.	*Weight loss:* 12-17 kg at 5 years (from 11 OBS). RCT - 60% EWL at 1 year, 71% EWL at 2 years, 57% EWL at 3 years. OBS - 46% EWL at 1 year, 64% EWL at 2 years, 67% EWL at 3 years. *T2DM remission:* 86-92%. *HTN remission*: 74-75%. *Dyslipidaemia:* 68-76%. *OSA: 90*-96%	0.08% and 0.31% pooled peri- and post-operative mortality for RCTs 0.22% and 0.35% for OBS. 17% complications for RCTs, and 10% for OBS. 7% reoperation rate for RCTs, 6% for OBS.
Systematic review (2014) by Puzziferri	Systematic review of 29 studies (n=7971) from 1946 to 2014 **of RYGB, SG or LAGB with ≥2 years follow-up**. *Outcomes*: Weight loss, T2DM, HTN, cholesterol profile,	*Weight loss*: 65.7% (RYGB), 45.0% (LAGB), 64.5% (SG). *T2DM remission*: 66.7% (RYGB), 28.6% (LAGB). *HTN remission*: 38.2% (RYGB), 17.4% (LAGB). *Dyslipidaemia*: 60.4% (RYGB), 22.7% (LAGB).	1% (RYGB) and 0.2% (LAGB) mortality RYGB: 1% incisional hernia, 1% internal hernia, 1% marginal ulcer, 2% anaemia, 2% B12 deficiency, 0.2% reoperation, <1% GI bleed. LAGB: 6% port revision, 5% prolapse, 1% erosion, 3% treatment failure, 2% removal, 1% oesophagitis
Systematic review (2011) by Meijer (422)	Systematic review of 9 studies (1 RCT, 8 OBS) reporting **T2DM reversal rates** after RYGB and LAGB. *Outcomes*: Reversal of T2DM, incident diabetes, long term morbidity and mortality	*Weight loss*: 41% EWL or 26kg (LAGB), 66% EWL or 50.5kg (RYGB) *T2DM remission*: 43-87% remission, 91-100% improvement. *Incident diabetes*: 24% vs 7% (surgery vs non-surgical, SOS trial only). *Long term morbidity and mortality:* T2DM related deaths decreased 92%, mortality reduced by 40%.	2.1% (open RYGB) and 0.2% (laparoscopic RYGB) 30-day mortality.
Swedish Obesity Study (SOS) (168, 423-428)	Prospective observational study of **bariatric surgical patients (n=2010) with matched controls (n=2037)**. Multiple sub-studies performed *Outcomes*: Weight loss, metabolic comorbidities, cardiovascular disease, cancer, overall mortality.	*Weight loss*: -23% (2 years), -17% (10 years), -16% (15 years) , -18% (20 years). *Diabetes remission:* 2 years, odds ratio (OR) 8.42, p<0.001; 10 years OR 3.45, p<0.001. *Overall mortality*: Hazard ratio (HR) = 0.71 (0.54-0.92), p=0.01 for surgery. *Incident diabetes:* HR 0.17, p<0.001. *Myocardial infarction:* HR 0.71, p=0.02. *Stroke:* HR 0.66, p=0.008. *Cancer:* Women - HR 0.58, p=0.0008, men – not significant.	0.25% vs 0.1% mortality 90-days after inclusion into study 14.5% complication over 90-day post-operative period. 2.9% 90-day return to theatre.
Longitudinal Assessment of Bariatric Surgery (LABS) (429)	Multicentre observational cohort study of **2458 primary bariatric surgical patients** recruited from 2006-2009 in 10 US hospitals, followed up at 6 and 12 months then annually. *Outcomes:* Weight loss, T2DM, cholesterol profile, HTN	*Weight loss*: RYGB - 41kg (31-52) or 31.5% (24.6-38.4%) TBWL; LAGB - 20kg (10-29) or 15.9% (7.9-23.0%) TBWL *T2DM partial remission*: 67.5% (RYGB), 28.5% (LAGB). *Incident diabetes:* 0.9% (RYGB), 3.2% (LAGB). *Dyslipidaemia remission:* 61.9% (RYGB), 27.1% (LAGB). *HTN remission:* 38.2% (RYGB), 17.4% (LAGB).	*Overall mortality*: 0.9% (RYGB), 0.8% (LAGB). *Revision surgery*: 0.3% (RYGB), 17.5% (LAGB)
Surgical Treatment and Medications Potentially Eradicate Diabetes Efficiently (STAMPEDE) trial (430)	**Randomised controlled trial** of medical therapy alone ***vs*** medical plus RYGB ***vs*** medical plus SG for **diabetes** (n=134) *Outcomes*: HbA1c<6.0% (primary), weight loss, HTN, lipids, renal func, eye, meds, adverse events, QOL	*Weight loss*: 23% (RYGB) vs 19% (SG) vs 5% (medical) TBWL. *HbA1c<6.0%*: 5% (medical only), 29% (RYGB, p=0.01 (unadjusted, 0.03 (adjusted), 0.08 (ITT)), 23% (SG, p=0.03, 0.07, 0.17). *Triglycerides:* -40% vs -29% vs -8%. *HDL:* 32% vs 30% vs 7%. *Use of insulin*: -35% vs -34% vs -13%. *QOL:* 17 vs 16 vs 0.3	1 reoperation reported.
Utah Obesity Study (431)	**Prospective comparative observational study health outcomes of RYGB** patients (n=420), vs obese patients seeking RYGB (n=415), vs control obese patients (n=321). *Outcomes*: Weight loss, HTN, lipids, diabetes, OSA, QOL.	*Weight loss*: 15.8% (RYGB) vs 0.16-0.7% (not seeking surgery) *T2DM resolution*: 80% in surgical group. *HTN and dyslipidaemia*: 40-50% in surgical group vs <1-14% in comparator groups.	2 deaths in surgical group. 2 deaths in total in comparator groups.

RCT – randomised controlled trial; T2DM – type II diabetes mellitus; OSA – obstructive sleep apnoea; QOL – quality of life; HTN – hypertension; OBS – observational studies; ITT – intention to treat; RYGB – Roux-en-Y gastric bypass; SG – sleeve gastrectomy; LAGB – laparoscopic adjustable gastric band; TBWL – total body weight loss; EWL – excess weight loss; Data presented as [value for surgical group] vs [value for non-surgical group] where not specified.

Chang *et al* performed a meta-analysis of all randomised and non-randomised studies up to 2012, examining bariatric surgical outcomes, with and without comparator groups (206). This again showed substantial weight loss of 46-60% excess weight loss (EWL) at 1 year, 64-71% EWL at 3 years and 57-67% EWL at 5 years. Corresponding remission in T2DM (86-92%), HTN (74-75%), dyslipidaemia (68-76%) and obstructive sleep apnoea (90-96%) were seen.

Puzziferri *et al* (2014) focused on comparing RYGB, SG and LAGB, focusing on 29 studies with 7971 patients with at least two years follow-up (432). RYGB had the greatest weight loss, at 65.7% EWL, with slightly lower weight loss for SG (64.5%) and lower weight loss for LAGB (45.0%). Whilst substantial long-term data on comorbidity remission was not available for SG, a comparison of T2DM, HTN, and dyslipidaemia remission between RYGB and LAGB showed substantially greater improvement in RYGB patients (**Table 3.22**).

Together, these reviews undoubtedly show the benefit of bariatric surgery for weight loss and metabolic disease, whilst highlighting some of the inherent risks involved.

3.9.1.2 Large prospective follow-up studies and long-term studies

Swedish Obesity Study (SOS)

The Swedish Obesity Study is one of the largest long-term bariatric surgery outcomes studies, initiated in 1987 (424). It is a non-randomised matched observational trial with 2010 bariatric surgical patients and 2037 matched controls of obese individuals who declined surgery. In this cohort, the vertical band gastroplasty was the most common operation performed (n=1369), followed by the gastric band (n=376), and gastric bypass (n=265). Most operations were performed as open operations (89%). This is in significant contrast to the method and variety of bariatric operations performed today, consisting mainly of laparoscopic gastric bypass and sleeve gastrectomy. The primary endpoint was overall mortality, with secondary endpoints being cardiovascular events, metabolic risk factors and T2DM.

The primary endpoint was reported in 2007, showing better mortality rates with after bariatric surgery (433). There were 129 deaths in the control group versus 101 deaths in the surgical group, giving an adjusted hazard ratio of 0.71 (95% CI 0.54-0.92, p=0.01). Most common cause of death was cancer, followed by myocardial infarction. Other outcomes of the SOS study have been reported in several papers, showing significant benefit of surgical versus

non-surgical management of obesity in areas such as weight loss, overall mortality, cardiovascular disease, cancer incidence, and diabetes (425, 427, 428, 433, 434).

Surgical Treatment and Medications Potentially Eradicate Diabetes Efficiently (STAMPEDE) trial

The STAMPEDE trial was a randomised controlled trial of 134 patients comparing the efficacy of surgery in addition to best medical therapy for the treatment of diabetes (430). There were three arms in this study: medical therapy alone versus medical therapy plus RYGB versus medical therapy plus SG . The primary outcome was achieving an HbA1c<6.0% with or without medication.

Body weight decrease was -5.3 vs 23.2 vs 18.6kg for medical therapy alone, RYGB and sleeve gastrectomy (p=0.003 for both surgical procedures vs medical therapy). When adjusted for multiple comparisons and using an intention to treat analysis, there was no significant difference in primary outcome (HbA1c<6.0%) between medical therapy and RYGB (5.3% vs 28.6%, p=0.08) or SG (5.3% vs 23.4%, p=0.17). However, this study found that both surgical procedures were superior to medical therapy alone for other measures of glycaemic control, including greater reduction in HbA1c, fasting blood glucose, and use of anti-diabetic medication. Significant improvement in cholesterol variables and quality of life measures were also seen in surgical groups.

Longitudinal Assessment of Bariatric Surgery (LABS-2)

The LABS-2 study was a prospective multicentre observational study that aimed to prospectively examine longer term outcomes of contemporaneous bariatric procedures. It was based in 10 United States hospital in geographically diverse areas (429). The study recruited bariatric surgical patients undergoing primary RYGB (n=1738) or LAGB (n=610) between 2006 to 2009, with follow-up at 6 months, 12 months then annually. Primary outcomes were weight loss, diabetes, hypertension and dyslipidaemia, reported initially at three years post-operatively.

Weight loss, and remission of T2DM, dyslipidaemia and hypertension were marked in both groups, however greater benefit in all domains were seen for RYGB.

Utah Obesity Study

The Utah Obesity Study was a single centre study comparing the outcomes of patients undergoing gastric bypass (n=420), with obese patients seeking but not undergoing gastric bypass (n=415), and a control group of obese patients from the community (n=321). The aim was to compare health outcomes in gastric bypass patients versus non-operative patients at two years.

Weight loss was substantial in the RYGB group, at 15.8% TBWL, compared to both comparator groups who had an average of <1% TBWL. Changes in blood pressure, glucose, insulin, HbA1c, cholesterol parameters and quality of life were substantial and all significantly greater in the surgical group compared to either of the non-operative groups. At 2 years, with 15.8% TBWL, systolic blood pressure decreased 12.9% from baseline, blood glucose decreased 14.9%, LDL decreased 19.0% and triglycerides decreased 68.9% (431).

Centre for Obesity Research and Education

O'Brien *et al* published their fifteen-year outcomes of 3227 laparoscopic adjustable gastric bands placed between 1994 and 2011 by two surgeons (191). Peak weight loss was seen at 3 years with 50.5% EWL, with 47% EWL (n=714) at 10 years and 47.2% EWL (n=54) at 15 years. Whilst there were no peri-operative deaths, nor deaths related to the procedure, 34.6% of study patients required at least one revision operation over the follow-up period.

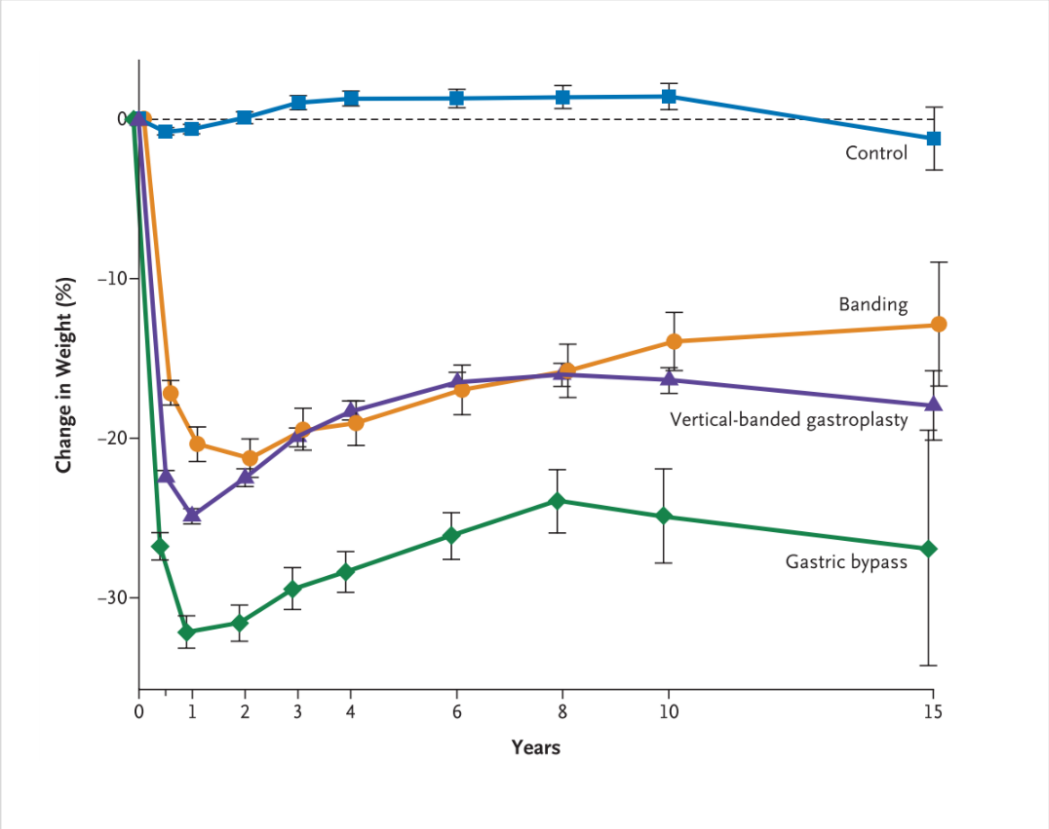
3.9.2 **Weight loss after bariatric surgery**

Most evidence suggests that maximal weight loss after bariatric surgery occurs within 1-2 years for gastric bypass and 2-3 years for gastric banding, and is substantially greater than conservative weight loss efforts (190). Various units of measurements have been used, however peak weight loss is approximately 20-35% TBWL (or 50-60% EWL), plateauing at approximately 15-30% TBWL at over 10 years (191, 206, 422, 424, 425, 427-429, 433, 434).

In studies comparing bariatric surgical procedures, weight loss appears to be better for RYGB and SG, compared to LAGB. The LABS-2 study showed a weight loss of 31.5% (24.6-38.4%) TBWL for gastric bypass vs 15.9% (7.9-23.0%) TBWL for LAGB. Long term weight loss outcomes in the Swedish Obesity Study show similar results, and foremost, show a direct comparison with control patients, demonstrating significant differences over 15 years (**Figure**

3.15). Similarly, the systematic review by Puzziferri *et al* demonstrated weight loss of 65.7% EWL for RYGB, 64.5% EWL for SG and 45.0% for LAGB (429).

Figure 3.15: Weight loss results from the Swedish Obesity Study, reported over 15 years, showing 27±12% TBWL for gastric bypass versus 13±14% for gastric banding. Comparison to control participants shows significantly greater weight loss in surgical participants (433).



From Sjostrom L et al. Effects of bariatric surgery on mortality in Swedish Obese Subjects. N Engl J Med. 2007; 357:741-752.

3.9.3 **Metabolic benefits after bariatric surgery**

Type II diabetes mellitus and insulin resistance

Bariatric surgery in obese individuals with T2DM results in significant weight loss accompanied by major improvements in glycaemic control (163). Remission of diabetes has been reported in up to 87% of patients (163, 422, 429, 431), with significant reductions in mean HbA1c and fasting blood glucose (163, 422, 432). The effect appears to be greater for RYGB than for LAGB (429, 432), with 67.5% RYGB patients achieving partial remission in the LABS-2 study, compared with 28.6% LAGB patients (429).

The Swedish Obesity Study examined rates of incident diabetes over up to 15 years comparing obese individuals having bariatric surgery versus usual weight loss strategies. There were significantly lower rates of T2DM developing in the bariatric surgery group with 6.8 vs 28.4 cases per 1000 person-years, corresponding to an adjusted hazard ratio with bariatric surgery of 0.17 ($p<0.001$) (427). Ultimately, a systematic review by Meijer *et al* in 2011 showed that deaths related to T2DM decrease by 92% after bariatric surgery (422).

Hypertension

Resolution in hypertension has been reported in up to 75% of patients undergoing bariatric surgical procedures (206). This varies with operation type, with the LABS study reporting 17.4% improvement in LAGB patients, compared to 38.2% after RYGB (429).

Dyslipidaemia

Weight loss after bariatric surgery is associated with improvements in cholesterol profile. Significant reductions in total cholesterol and triglyceride levels, and improvements in HDL levels are seen universally after bariatric surgery (**Table 3.23**) (426, 435, 436). The results for low density lipoprotein (LDL) vary (437), and this may be due to the changing subtypes of LDL instead of total LDL, particularly small dense LDL (438). Anatomy altering procedures, such as RYGB and BPD, appear to have a greater effect on cholesterol profile, and a more uniform beneficial effect on measurable LDL levels (435). Those undergoing LAGB, SG or lifestyle change show less significant effects on LDL levels (435).

Table 3.23: Studies reporting on specific changes in cholesterol profile with bariatric surgery.

Study	Weight loss	Total chol	Trig	HDL	LDL
Brolin, 2000 (439) n=651 **Gastroplasty, RYGB**	55% EWL 48 to 33 kg/m^2 F/U: 6 year	248-259 to 188-215 mg/dL	234-291 to 110-147 mg/dL	45-48 to 60-62 mg/dL	
Garcia-Marirrodriga, 2012 (440) n=114 **RYGB**	70% EWL F/U: 18 months	211 to172 mg/dL (p<0.001) -18.4%	123 to 70 mg/dL (p<0.001), -47.3%	53 to 63 mg/dL (p<0.001), +19.3% TC/HDL: 4.1 to 2.8 (p<0.001)	132 to 97 mg/dL (p<0.001) -26.7%
Wolf, 2007 (441) n=431 **Gastroplasty, LAGB**	Reported at 25% (n=406), 50% (n=323), 75% (n=140) and 100% (n=27) EWL	(50% EWL) Female: 199-204 to 211-204 mg/dL (p<0.01) Male: 206-232 to 203-210 mg/dL (p<0.001)	Female: 155-161 to 122-125 mg/dL (p<0.0001) Male: 220-303 to 136-149 (p<0.01)	Female: 42-44 to 46-49 mg/dL (p<0.0001) Male: 35 to 40 (p<0.01)	Female: 125-130 to 134-138 mg/dL (p<0.01) Male: 128-144 to 128-143 (n.s.)
Benaiges, 2012 (435) n=102 **SG, RYGB**	**RYGB** 45% TBWL 35 to 29 kg/m^2 F/U: 12 months	201 to 175 mg/dL (p<0.001) -11.3%	125 to 78 mg/dL (p<0.001) -28.9%	50 to 60 mg/dL (p<0.001) +22.2%	126 to 100 mg/dL (p<0.001) -18.5%
	SG 43.6% TBWL 34 to 29 kg/m^2 F/U: 12 months	192 to 196 mg/dL (p=0.231) +3.3%	120 to 84 mg/dL (p<0.001) -22.4%	48 to 64 mg/dL (p<0.001) +36.7%	119 to 115 mg/dL (p=0.220) -2.1%
Milone, 2015 (436) n=160 **SAGB, SG**	**SAGB** 36% TBWL F/U: 12 months	-17%	-35%	+19%	-21%
	SG 35% TBWL F/U: 12 months	-18%	-35%	+20%	-22%
To, 2012 (437) n=52 **SG**	51 to 38 kg/m^2 F/U: 12 months	201 to 205 mg/dL (n.s.) +4%	159 to 116 mg/dL (p<0.001) -43%	46 to 56 mg/dL (p<0.001) +9%	130 to 132 mg/dL (n.s.) +3%
Heffron, 2014 (438) n= 47 **LAGB**	35 to 28 kg/m^2 19.8% TBWL F/U: 5 years		154 to 103 mg/dL (p<0.01)	56 to 70 mg/dL (p<0.001)	124 to 117 mg/dL (n.s.)
Bonner, 2014 (442) n=230 **RYGB, SG, LAGB**	TBWL: 63% (RYGB), 54% (SG) and 40% (LAGB) F/U: 12 months			48.1 to 52.5 mg/dL (p=0.008)	99.4 to 95.2 mg/dL (p=0.44)

F/U – follow-up duration; TC – total cholesterol; HDL – high density lipoprotein; LDL – low density lipoprotein; RYGB – Roux-en-Y gastric bypass; EWL – excess weight loss; LAGB – laparoscopic adjustable gastric band; SG – sleeve gastrectomy; n.s. – not significant

3.9.4 **Role of bariatric surgery in NAFLD treatment**

As bariatric surgery improves all aspects of metabolic disease, it is not surprising that it can also significantly improve NAFLD, which is often considered the hepatic component of the metabolic syndrome.

Many hepatology societies have now incorporated guidelines on bariatric surgery in the management of NAFLD. Recommendations in favour of bariatric surgery for NAFLD are limited by the lack of Level I evidence (443). Growing evidence, however, suggests clear benefits for NAFLD with surgical weight loss.

3.9.4.1 Changes in steatosis and steatohepatitis

Studies of NAFLD in bariatric cohorts show significant reductions in NAFLD-related steatosis and steatohepatitis. The Lille Bariatric Cohort was a detailed study of 109 morbidly obese patients with biopsy-proven NASH. Repeat biopsies were performed for all patients one year after surgery, with an average reduction in BMI of 11.9kg/m^2. NASH resolved in 85.4% of cases, with significant reductions in NAS grading from 5 to 1 ($p<0.001$). Those with persistent NASH had lost significantly less weight than their counterparts, and had refractory insulin resistance (12).

These results are mirrored by several systematic reviews, showing benefits of bariatric surgery for NASH, components of NAFLD and aminotransferase levels (443-445). A meta-analysis by Mummadi *et al* in 2008 combined data from 15 studies with 766 paired liver biopsies (444). It showed significant reductions in steatosis (improvement or resolution in 91.6%), steatohepatitis (81.3%) and fibrosis (65.5%). The associated BMI decrease was substantial, from 43.9-56 kg/m^2 to 28.6-29 kg/m^2.

A Cochrane review in 2010 reviewed 21 cohort studies, again showing improvements in steatosis and inflammation. However, histological deterioration was seen in some studies, with deterioration in NAS grading in two studies. The criticism of this review was the significant heterogeneity between studies. Additionally, they did not fulfil the primary aim of the review, to assess randomised controlled trials, as none currently exist (443).

3.9.4.2 Changes in NAFLD-related fibrosis

Whilst steatosis and inflammation appear reversible, there have been inconsistent reports on the outcome of hepatic fibrosis after surgical weight loss. Systematic reviews show heterogeneity in fibrosis progression among studies. The Cochrane review by Chavez-Tapia *et al* in 2012 reported only six of 21 studies showing improvement in fibrosis, with an almost equal amount showing deterioration (443). Whilst studies such as the Lille Bariatric Cohort (n=109) showed improvements in liver fibrosis stage in 46.3% of patients (12), a prospective study by Mathurin *et al* of 351 obese patients reported increases in mean fibrosis score from 0.27 to 0.36 ($p<0.001$) after 5 years of weight loss (446). It is difficult to ascertain whether this is natural progression with time, or an adverse effect of weight loss surgery.

More recent studies have described fibrosis improvement and resolution after substantial weight loss following bariatric surgery. An example is a 2014 study by Taitano *et al*, which reported on 160 bariatric surgical patients who underwent routine intraoperative liver biopsies. Follow-up biopsies were performed during subsequent abdominal operations, and occurred at 31±26 months, after 62±22% excess weight loss. Grade 2 fibrosis resolved in 58% and Grade 3 fibrosis (bridging fibrosis) resolved in 29% after gastric bypass or banding. Deterioration occurred in a minority (28% and 13% of patients respectively) (447).

3.9.4.3 Differences between bariatric procedures

There is little evidence exploring the superiority of different bariatric surgical procedures for NAFLD. In a study of 381 patients undergoing biliopancreatic bypass, gastric bypass and gastric band surgery, there was no difference in histological improvement between procedure types (446).

Studies that have shown significant differences in NAFLD outcomes, have associated these differences with weight loss outcomes. In subgroup analysis comparing 70 gastric bypass patients with 32 gastric band patients, Lassailly *et al* showed higher rates of persistent NASH in gastric band patients (30.4% vs 7.6%, $p=0.015$). It is unclear if this is due to the lower weight loss achieved (decrease in BMI 6.4 vs 14.0, $p<0.0001$), or the procedure itself (12).

3.9.4.4 Possible detrimental effects of bariatric surgery

Very rapid weight loss of greater than 1.6kg per week may provoke hepatic fibrosis (448, 449). Friis *et al* monitored serum markers during fasting and showed a peak increase in liver function tests two weeks into rapid weight loss (448). Andersen *et al* found that in a group of 41 obese participants who had an average of 34kg weight loss via very low calorie diet, a slight increase in portal fibrosis was seen, significantly associated with faster weight loss. Mechanisms involved are theorised to be the rapid mobilisation and depletion of hepatic lipids and increase visceral free fatty acids, which can promote an inflammatory state (449).

3.9.5 Clinical aspects of nonalcoholic fatty liver disease in bariatric practice

Due to its high prevalence, NAFLD impacts heavily on bariatric surgeons and surgical practice. This section summarises the current guidelines, evidence and clinical aspects of NAFLD in the bariatric surgical population.

3.9.5.1 Current guidelines on nonalcoholic fatty liver disease and bariatric surgery

All current hepatology guidelines on NAFLD incorporate recommendations regarding bariatric surgery, but are unable to advocate for its use specifically for NAFLD.

As identified in the 2010 Cochrane Review by Chavez-Tapia (443), this is due to the current lack randomised controlled trials demonstrating benefit above best medical management of NAFLD. Despite this, many guidelines acknowledge the growing cohort and observational data that indicate the benefit of bariatric surgery in NAFLD. They recognize the benefits for obesity and diabetes, and the resultant improvements this may have on NAFLD (450). The American Association for the Study of Liver Disease (AASLD) guidelines review the existing literature on changes in NAFLD histology after bariatric surgery, outlining the benefits in steatosis and inflammation, as well as the mixed results on fibrosis (3).

Therefore, most state that bariatric surgery is an acceptable option for otherwise eligible obese individuals with NAFLD or NASH, particularly if patients are unresponsive to lifestyle or pharmacological weight loss measures. The specific recommendations by the major international NAFLD working groups are collated below in **Table 3.24**.

Table 3.24: Recommendations regarding bariatric surgery for NAFLD from current position papers and clinical practice guidelines issued by hepatology societies.

Society	Year	Recommendation
Asia Pacific Working Party on NAFLD	2007	"If patients are **obese and do not respond** to attempted lifestyle measures, they should be referred to centers specializing in obesity management. In those refractory to medical measures, **consideration should be given to bariatric surgery** or gastric ballooning." (344)
Italian Association for the Study of the Liver (AISF)	2010	"Although bariatric surgery is **not specifically indicated** in NAFLD, it may be **useful in morbidly obese patients**." (451)
Cochrane Review	2010	"The **lack of randomised clinical trials and quasi-randomised clinical studies** precludes us to assess the benefits and harms of bariatric surgery as a therapeutic approach for patients with NASH. Limitations of all other studies with inferior design **did not allow us to draw any unbiased conclusion on bariatric surgery for treatment of NASH.**" (443)
Chinese Association for the Study of Liver Disease (CASLD)	2011	"Upper gastrointestinal bariatric surgery might be **considered in patients with morbid obesity who do not respond to weight-reducing drug therapy,** unless the patient has liver failure or moderate or severe gastroesophageal varices (II-1)." (452)
World Gastroenterology Organisation (WGO) Global Guidelines	2012	"Weight loss (bariatric) surgery may be beneficial for patients with morbid obesity; again, this should be considered early, as most programs will decline such surgery for patients who are already cirrhotic. Limited studies have reported a dramatic improvement in liver disease, as well as other complications of metabolic syndrome/insulin resistance, following successful bariatric surgery." (453)
European Association for the Study of the Liver (EASL), Diabetes (EASD) and Obesity (EASO)	2016	"In patients unresponsive to lifestyle change and pharmacotherapy, **bariatric surgery is an option for reducing weight and metabolic complications**, with stable results in the long-term… By improving obesity and diabetes, bariatric (metabolic) surgery **reduces liver fat and is likely to reduce NASH progression**; prospective data have shown an improvement in all histological lesions of NASH, including fibrosis (B1 evidence)." (450)
American Association for the Study of Liver Disease (AASLD)	2017	"Foregut bariatric surgery can be **considered in otherwise eligible obese individuals** with NAFLD or NASH. It is **premature to consider foregut bariatric surgery an established option** to specifically treat NASH." (3)

3.9.5.2 Peri-operative considerations and safety

Bariatric surgery is an established treatment of obesity with an excellent safety profile (see **Chapter 3.4 – Overview of bariatric surgery**). There are no specific concerns regarding its safety in NAFLD, with the exception of those with established NAFLD-related cirrhosis (see **Section 3.8.5.3 – Unexpected cirrhosis**).

Pre-operative very low calorie diet (VLCD) has been recommended prior to bariatric surgery. Data shows that this reduces liver volume (180), and thereby improves surgical access. Improvements in perioperative metabolic status, perceived surgical difficulty, as well as 30 day complications have previously been reported in randomised controlled trials. Variable results have been seen in operating time, blood loss and intraoperative complications (454, 455).

3.9.5.3 Visual identification of NAFLD intraoperatively

Since the introduction of laparoscopic surgery, various studies have attempted to identify liver disease by visual inspection (**Table 3.25**) (9, 456-461). Whilst all studies employed different methodology, features generally include evidence of the components of fatty liver disease, and sequelae of chronic liver damage:

- *Steatosis*: Increased liver size, blunting of liver edge, yellowish or whitish discolouration
- *Inflammation:* Surface vascularity, redness, blunting of liver edge
- *Fibrosis:* Surface irregularity or nodularity, regenerative nodules, broken light reflux
- *Chronic liver disease:* Ascites, splenomegaly, increased portal vascularity, peritoneal changes, presence of focal lesions

The accuracy of visual inspection in diagnosing NAFLD differs between studies, likely due to differences in methodology and changes to histological reporting.

Earlier reports by Heit *et al* (456) and Jalan *et al* (457) showed excellent accuracy, particularly in the diagnosis of fibrosis and cirrhosis, with 100% sensitivity in both cases (n=10 and n=43). Inflammatory change was identified correctly in nearly 95% of cases.

More recent studies have had mixed results. Chiu *et al* (458) showed excellent correlation between steatosis and liver size (r 0.736, $p<0.001$), with weaker correlations between laparoscopic findings and inflammation (r 0.118-0.536) and fibrosis (r 0.263-0.545). Markers of diagnostic accuracy were not reported, and therefore the practical utility of these findings is unknown. Dolce *et al* (459) and Teixeira *et al* (460) used a simpler diagnostic schema, assessing size, colour, blunting of liver edge and surface greasiness in a binary manner. Both reported poor sensitivity and specificity of laparoscopic findings in distinguishing NASH, and therefore argue for mandatory liver biopsy in high-risk candidates.

Cirrhosis is more consistently identified by visual inspection alone. Poniachik *et al* showed a sensitivity of 98.3% and specificity of 83.0% for detection of cirrhosis, with only two patients (of 265) misdiagnosed as not having cirrhosis (462). In this same study, the authors reported 169 cases of "laparoscopically diagnosed cirrhosis", defined as those with features of significant nodularity and palpable liver hardness. In 54 of those cases (32.0%), there was no evidence of histological cirrhosis, and therefore the authors concluded that laparoscopic

diagnosis of cirrhosis was more sensitive that histology. Indeed, a previous study by Tameda *et al* found that laparoscopic findings, such as regenerative nodules and small lymphatic vesicles, are more predictive of cumulative survival than histological findings (463).

Table 3.25: Summary of studies assessing accuracy of macroscopic liver appearance for detection of NAFLD and liver disease

Study	Factors considered	Diagnostic accuracy	Overall
Heit 1978 (456) n=32	Colour, contour, size, consistency, abnormal vascular structures	Surface nodules (10 of 13 had cirrhosis, 3 had marked fibrosis, 100% specificity) Pale/yellow (12 of 19 had steatosis) Surface vascularity (19 of 24 had necroinflammatory change) Size and consistency did not predict any histological findings.	Good
Jalan 1995 (457) n=145	Assessment of chronic liver disease of any aetiology: Liver size, fatty change, vascularity of falciform, vascularity, redness, nodularity, broken light reflux	Fatty change: Sensitivity 96.4%, specificity 100% Fibrosis: 100%, 95% Inflammation: 94%, 95.7%	Good
Dixon 2001 (9) n=105	Score of 0-3 for each of: Size, fatty colour change, surface nodularity	No formal table of results. "Size of the liver at laparoscopy was an additional independent predictor of the level of steatosis" (no measure reported)	
Chiu 2008 (458) n=126	Assessed on a scale of 0-3: Colour, size, nodularity, liver margin, tumour, subcapsular neovascularity, spleen colour and size, varices of falciform ligament, ascites, peritoneal vessel dilatation	Significant Pearson correlation: Steatosis: Liver size (0.736), margin (0.364) and colour (0.548) Inflammation: Liver size (0.477), margin (0.536), colour (0.188), vascularity (0.330) and nodularity (0.473) Fibrosis: Liver size (0.263), nodularity (0.311), colour (0.545) and falciform varices (0.305)	Good
Dolce 2009 (459) n=108	Size (normal vs enlarged), tan speckling (yes vs no), blunting of edge (yes vs no)	Poor association between macroscopic features and histological features.	Poor
Teixeira 2009 (460) n=51	Size (normal vs enlarged), tan-speckling (yes vs no), blunting of edge (yes vs no), tactile greasy impression (yes vs no)	Poor sensitivity, specificity, PPV and NPV for NASH.	Poor
Yu 2009 (461) n=180	Size, colour, dullness of edge, texture of surface, capsular vascularity, lipid accumulation in falciform, greater omental thickening	No clear comparison of laparoscopic findings to histological findings (laparoscopy compared to sonography).	

PPV – positive predictive value; NPV – negative predictive value; NASH – nonalcoholic steatohepatitis

Several factors prevent translation of these results directly into clinical practice. Methodology varies significantly between studies, with little consensus on visual features of importance. Furthermore, these studies currently lack an assessment tool that is both easy to perform and accurate. Most studies list a constellation of visual features (457, 458, 461), with no guidelines for scoring. No study has assessed reliability of scoring between observers. The

two studies that developed clear and simple guidelines on visual features found poor correlations of their laparoscopic assessment with histological features (459, 460).

3.9.5.4 Unexpected cirrhosis

Generally, NAFLD does not increase the risk of perioperative complications for patients undergoing bariatric surgery (464). The exception is in cases of advanced disease, such as cirrhosis with portal hypertension or decompensated disease. In these patients, surgery can result in prolonged hospital admissions and high mortality. In a nationwide database study by Mosko *et al* in 2010, those with decompensated cirrhosis had a mortality rate of 16.3% (n=62), versus 0.9% (n=3,888) in compensated cirrhosis and 0.3% (n=670,095) in non-cirrhotic patients (p=0.0002) (465). Other factors associated with mortality included increasing age, comorbidities and operations at lower-volume centres.

Despite this, observational studies have shown reasonable perioperative outcomes for patients with compensated cirrhosis following bariatric surgery. Mortality in compensated NAFLD-related cirrhosis is substantially closer to that of non-cirrhotic patients (0.9%) (465). A recent systematic review of 122 patients with mainly Child-Pugh A cirrhosis showed 1.6% early and 2.45% late surgery-related mortality (466).

The appropriate type of bariatric surgery in obese individuals with cirrhosis has not been well investigated. Notably, in the systematic review by Jan *et al* (466), there was 0% surgical mortality among patients who underwent the gastric banding (n=15) or sleeve gastrectomy (n=41). Surgery-related mortality was 20% and 3.9% in BPD and RYGB groups. Liver decompensation occurred in 13.3%, 3.9% and 12.5% in BPD, RYGB and sleeve gastrectomy groups, with no decompensation in LAGB groups.

The AASLD Guidelines on NAFLD suggest that “in otherwise eligible patients with compensated NASH or cryptogenic cirrhosis, foregut bariatric surgery may be considered on a case-by-case basis by an experienced bariatric surgical program” (3).

3.9.5.5 Role of liver biopsy in bariatric surgery

Due to the increasing prevalence of NAFLD and NASH within the bariatric cohort, questions around the role of intraoperative liver biopsies during bariatric surgery have arisen.

The diagnosis of NAFLD is challenging in all patients, particularly in the setting of obesity. There are currently no reliable non-invasive tests for NAFLD or NASH (see **Chapter 3.6 - Diagnosis of nonalcoholic fatty liver disease**). A liver biopsy during a bariatric procedure is an opportune time to accurately screen individuals for substantial liver disease. The bariatric surgical population is a high-risk cohort, usually with severe obesity and multiple metabolic risk factors, known to increase the prevalence of NAFLD. The presence of NAFLD not only has an impact on liver-specific morbidity, but also impacts on cardiovascular and metabolic risk. An accurate diagnosis is vital for early treatment and initiation of preventative measures.

Intraoperative liver biopsies have the additional benefit of targeting areas of greatest abnormality, which has previously been shown to increase yield (467). These targeted biopsies may also be taken from multiple sites, sides and depths (both deep and superficial biopsies). Injury to other structures may be avoided by visual inspection, and post-procedure bleeding risk can be minimised by electrocautery and reinspection at the end of the case.

Therefore, the yield of a biopsy would theoretically be high, with little additional risks. As such, some clinicians have advocated for routine liver biopsies in this population.

However, routine use of liver biopsy in bariatric surgery have some drawbacks (**Table 3.26**), and currently has not been adopted in standard bariatric surgical practice. Several controversies underlie its routine use (**Table 3.27**).

Lack of evidence supporting screening programs

The benefits of screening in high-risk populations have not been established for NAFLD and current practice guidelines from the American Association for the Study of Liver Disease (AASLD) do not recommended routine screening in the setting of high risk populations (3). This is driven mainly by gaps in knowledge on natural history and treatment. There are no specific established management strategies for NAFLD. Thus, the results of liver histology (above other diagnostic measures) often does not substantially alter management.

Furthermore, cost-effectiveness has not been shown for NAFLD screening programs. A study by Corey *et al* showed that liver-related outcomes did improve in screening cohorts, however there was a marginal but significant, deficiency in cost-effectiveness (468). This was mainly attributed to deficiencies in therapy. It is predicted that with development of more effective and better tolerated therapies, routine screening may soon become cost-effective.

Guidelines currently recommend consideration of liver biopsy in select situations:

1. when diagnosis is unclear,
2. those at high risk of significant fibrosis, or
3. in clinical trials (469).

Otherwise, expert groups recommend "vigilance" and individualised approach to screening for chronic liver disease in high risk groups (3).

These arguments, however, ignore the bariatric surgical scenario, whereby additional risk and logistical burden is likely to be minimal. There are few data analysing efficacy and cost-effectiveness in operative cohorts. The AASLD touched briefly on bariatric and other surgical cohorts, stating that "greater consideration be given to liver biopsy in those coming for surgery for other procedures, particularly cholecystectomy and gastric banding" (469). These recommendations have been echoed by the Chinese Association for the Study of Liver Disease (452) and the Asia Pacific NAFLD Working Group (344).

Limited additional treatment options for NAFLD following bariatric surgery

The natural course of the disease is undoubtedly altered after bariatric surgery, with studies showing improvement in steatosis and NASH, and some showing improvement in fibrosis (see **Section 3.9.4 – Role of bariatric surgery in NAFLD treatment**). Due to the lack of effective therapy in addition to weight loss, some argue that there are minimal additional benefits of diagnosing NAFLD in bariatric surgical patients (470).

On the other hand, biopsies have the benefit of reliably determining the presence of NASH or fibrosis. This may change the nature and intensity of patient follow-up, including referral to speciality clinics and ongoing monitoring for complications of cirrhosis and liver failure. Additionally, more significant disease may be amenable to more aggressive pharmacological treatment in addition to expected post-operative weight loss. Therapies, such as pioglitazone and Vitamin E, have known long-term side effects, including cardiac failure, bladder and prostate cancer, and haemorrhagic stroke. As such, they are recommended only for those with biopsy-proven NASH (3).

Selective vs routine liver biopsy

Independent of cost and therapy concerns, there is variable evidence for and against routine versus selective liver biopsy for detection of NAFLD in bariatric patients.

Use of laparoscopic inspection of the liver has been suggested as a means of selecting patients for liver biopsy (see **Section 3.9.5.3 – Visual identification of NAFLD intraoperatively**) (458, 471). As previously discussed, factors such as surface texture, shape, size, colour, vascularity and nodularity potentially correlate with histological findings (458). This is particularly the case for frank cirrhosis, where visual inspection has shown great accuracy (457, 462). Other cues, such as pre-operative blood tests or imaging may further select patients for liver biopsy.

Conversely, others have criticised selective intraoperative liver biopsy by assessment of liver appearance as being inaccurate and unreliable (13, 459, 460). The Longitudinal Assessment of Bariatric Surgery (LABS) studies have suggested that lack of routine biopsies result in missed diagnosis in 86% of patients with NASH and 88% with advanced fibrosis (13).

Table 3.26: Pros and cons of routine liver biopsy

Liver biopsy during bariatric surgery	**Pros**	**Cons**
Prevalence	High yield investigation, based on current epidemiological data	Lower yield for significant disease (NASH, cirrhosis)
Technique	Improved technical proficiency	
Risk	Little additional risk	Risk of bleeding, increased pain, bile leak, damage to adjacent structures
Cost and logistics		Increased cost and logistical burden
Diagnosis	Gold standard – Best investigation to grade steatosis, NASH and fibrosis	Imperfect gold standard (e.g. sample and interobserver variability)
Treatment	New treatment regimes increasingly being developed.	No established therapy currently available, apart from weight loss
Research	Potential to improve research in NAFLD	
Prognosis	Surveillance protocols for significant cirrhosis.	Unknown implications for prognosis, particularly in the setting of weight loss

Table 3.27: Controversies in liver biopsies during bariatric surgery

Controversies in liver biopsies during bariatric surgery
• Given that there are no established therapies and no surveillance guidelines, do the benefits of a liver biopsy and early diagnosis of NAFLD outweigh the additional risks? • What is the role of routine vs selective liver biopsy? • What is the best technique for intraoperative liver biopsy?

Risk, cost and logistics

Whilst the additional risk intraoperative is small, they still exist and may be significant. These include bleeding, additional pain, bile leak and damage to adjacent structures (see **Section 3.6.1 – Liver biopsy**).

Additional costs include operative time, cost of the core biopsy needle, histopathological processing and assessment, as well as additional costs and logistics of subsequent investigations and management. Given the increasing number of bariatric operations performed worldwide, routine liver biopsy could add substantially to the logistical burden of processing tissue specimens. Clinical and cost benefits would have to be considered prior to establishing guidelines recommending routine intraoperative liver biopsy.

Due to these controversies, the role of liver biopsy in bariatric surgery has not been established. As effective therapies are developed, the benefits of early diagnosis of NAFLD may outweigh the risks and costs of liver biopsy, leading to recommendations guiding screening liver biopsy in the surgical setting for obese individuals.

3.9.5.6 Choice of liver biopsy technique: Wedge vs core biopsy

The intraoperative setting allows for variation in biopsy technique (wedge versus core) and relative ease in choosing biopsy location (left versus right, segments). Evidence suggests that parenchymal abnormalities are irregularly distributed, particularly in NAFLD (227, 271, 472). This has substantial implications for diagnosis, especially when considering that a single core liver biopsy represents only 1/50,000-65,000 of the liver (7).

Previous studies examining variation in liver biopsies in NAFLD have yielded conflicting results (**Table 3.28**) (269, 270, 473-478). Whilst concordant results have been reported, discrepancies in steatosis, inflammation and fibrosis have all been described. Wedge biopsy have previously been criticised for upgrading fibrosis stage (7). Studies range from as few as eight participants to 146 participants. Several of the larger studies examined post-mortem specimens, where biopsy samples vary significantly from the biopsies obtained in living patients. Therefore, scarce evidence currently guides our clinical practice, and guidelines on the ideal intraoperative biopsy technique have yet to be established.

Table 3.28: Summary of previous studies on variability between liver biopsies

Study	n=	Description	Results	Agreement Good	Poor
Abdi, 1979 (473)	118	Autopsy study. 3 right lobe cores vs routine autopsy section.	No kappa coefficient. Discrepancies in steatosis and fibrosis, including cirrhosis.		Steatosis Fibrosis
Merat, 2005 (474)	146	Autopsy study. Sections from left, right, caudate.	Good concordance in fibrosis, lobular and portal inflammation (κ 0.87, 0.83, 0.83). Steatosis and ballooning less uniform (κ 0.64, 0.57).	Fibrosis Inflammation	Steatosis Ballooning
Goldstein, 2005 (475)	46	Single core biopsy, examining differences between deep vs superficial ends of biopsy.	NAFLD has significant variation in fibrosis (2.3-3.7 fibrosis grades).		Fibrosis
Larson, 2007 (476)	43	Left and right intraoperative core biopsies (14G)	Good steatosis (κ 0.91), fibrosis (κ 0.96) and NAS (κ 0.83) concordance. Ballooning (κ 0.73) and inflammation (κ 0.58) less uniform.	Steatosis Fibrosis NAS	Ballooning Inflammation
Janiec, 2005 (477)	10	Left and right intraoperative core biopsies (16G)	Variation in fibrosis (30% of patients with differences in fibrosis grade). No differences in necroinflammation.	Necroinflam-mation	Fibrosis
Ratziu, 2005 (269)	51	Two right sided percutaneous biopsies.	No high agreement in any component. Steatosis (κ 0.64) and interface hepatitis (κ 0.78) reasonable agreement. Poor uniformity in inflammation (κ 0.13), fibrosis (κ 0.47), ballooning (κ 0.45).	Steatosis Interface hepatitis	Inflammation Fibrosis Ballooning
Merriman, 2006 (270)	41	Left and right intraoperative core biopsies. Examined inter-lobe variability and intra-observer variability.	Good concordance in steatosis (κ 0.88) and NAS classification (κ 0.89). Poor uniformity for inflammation (κ 0.32), ballooning (κ0.20) and fibrosis (κ 0.53). Similar results with intra-observer variability.	Steatosis NAS	Inflammation Ballooning Fibrosis
Rawlins, 2013 (478)	8	Left/right intraoperative core and wedge biopsies (4 biopsies per patient)	Very variable concordance, between wedge and core, and between sides of liver (κ 0.15-0.82).		

3.9.5.7 Recommended follow-up of incidentally diagnosed NAFLD during bariatric surgery

Incidentally identified NAFLD during bariatric surgery is not uncommon. There are no guidelines dedicated to the management of incidentally diagnosed NAFLD after bariatric surgery, and therefore management follows general NAFLD recommendations.

Diagnosis of NAFLD

A liver biopsy is currently the gold standard for diagnosing NAFLD, and therefore consideration of an intraoperative liver biopsy when NAFLD is suspected will help to establish a diagnosis and grade the severity of disease.

For those without a liver biopsy, investigations that aid diagnosis of hepatic steatosis (ultrasound, MR spectroscopy or CAP) together with an estimated of risk of steatohepatitis or fibrosis (NAFLD fibrosis score, FIB-4, transient elastography, presence of MetS or T2DM) may be used to determine the likelihood of having NAFLD. According to AASLD guidelines," liver biopsy should be considered in patients with NAFLD who are at increased risk of having steatohepatitis and/or advanced fibrosis" (3).

Post-operative investigations

In those with confirmed NAFLD, further examination and baseline tests should be performed with the purpose of:

- Identifying underlying metabolic and related disease,
- Excluding other aetiology of liver disease,
- Assessing the severity of NAFLD/NASH (**Table 3.29**) (3, 344, 450).

Table 3.29: Protocol for comprehensive evaluation of NAFLD (450)

Component	Aetiology	Investigation
History	*Alcohol-related*	Careful alcohol history (>20g daily or >140g weekly for men and >10g daily or >70g weekly for women)
	Medication related	Amiodarone, anticonvulsants, methotrexate, tamoxifen, oestrogens, corticosteroids, HIV therapy, perhexiline
	Metabolic disease	Personal or family history of diabetes, hypertension and cardiovascular disease
	Related diseases	Polycystic ovarian syndrome, obstructive sleep apnoea
Examination	*Anthropometrics*	Ongoing measurement of height, weight, BMI and waist circumference
	Blood pressure	
Metabolic screen	*Insulin resistance and diabetes*	Fasting blood glucose, HbA1c, oral glucose tolerance test (OGTT), fasting insulin for calculation of HOMA-IR
	Dyslipidaemia	Fasting cholesterol profile (total cholesterol, HDL, LDL, triglycerides)
Baseline blood tests	*Liver function tests*	Bilirubin, ALT, AST, GGT, albumin, globulin
	Haematological tests	Full blood count, including platelet count
	Coagulation screen	Prothrombin time
Exclusion of other liver disease	*Hepatitis B*	HbsAg, anti-HBs, anti-HBc
	Hepatitis C	Hepatitis C antibody
	Autoimmune hepatitis	Antinuclear antibody (ANA), anti-smooth muscle antibody (ASMA), antimitochondrial antibody (AMA)
	Haemochromatosis	Iron studies (C282Y HFE mutation if indicated)
	Thyroid disease	Thyroid function tests
	Coeliac disease	Coeliac antibodies (anti-transglutaminase antibody and/or anti-endomysial antibody)
	*Wilson disease**	Caeruloplasmin
	*a1 antitrypsin deficiency**	α1 antitrypsin (AAT)

**consider according to a priori probability or clinical investigation*

Treatment

Whilst weight loss is expected after bariatric surgery, ongoing lifestyle changes should be advised, including increasing physical activity. Patients should be counselled on avoiding heavy alcohol use, however there is insufficient data with regards to moderate alcohol intake and its impact on liver disease (3).

Due to its strong association with cardiovascular morbidity and mortality, patients with NAFLD should undergo aggressive modification of CVD risk factors (3).

Pharmacological therapies may be trialled in cases with biopsy-proven NASH, after full consideration of potential side effects of long term treatment (see **Section 3.7.3. - Pharmacological management**).

Follow-up

Follow-up is largely based on the histological severity of disease as outlined in **Table 3.30**.

Table 3.30: Recommended follow-up after diagnosis of NAFLD (451)

Disease severity	Risk	Recommendations
Simple steatosis	Likely benign course	"Low intensity" surveillance schedule* Consider repeat ultrasound or LFT in ~6 months, to assess improvement after weight change and optimization of comorbidities.
NASH	Risk of chronic liver disease, fibrosis and liver failure	Monitored as per patients with chronic viral hepatitis.* Referral to Specialist Hepatology or NAFLD service.
NASH-cirrhosis	Risk of HCC	Systematic follow-up as per patients with cirrhosis, with the aim of early diagnosis of HCC.* Referral to Specialist Hepatology or NAFLD service.
Any NAFLD	Metabolic and cardiovascular disease	6-12 monthly screening for common metabolic disorders (dependent on metabolic risk of patient and severity of liver disease).* Patients with NASH should undergo periodic non-invasive evaluation of their cardiovascular disease.*

LFT – liver function test; HCC – hepatocellular carcinoma
**Level III evidence: Evidence from non-randomised cohort studies with concurrent or historical controls or their metanalytic review.*

4 Summary of literature review and rationale for research directions: Matching knowledge gaps with research opportunity

This literature review summarised various clinical and pathophysiological aspects of obesity and nonalcoholic fatty liver disease (NAFLD). It described the significant changes that occur in severe and morbid obesity, including substantial clinical, biochemical, physical and physiological differences. Importantly, it highlighted the unique nature of NAFLD in the context of obesity and outlined the complex and interconnecting elements between both.

Obesity and NAFLD are increasing rapidly, with significant implications for morbidity and mortality. As such, they are now emerging and increasingly recognised public health problems. Yet research focusing on NAFLD in the setting of obesity and bariatric surgery is lacking. This is particularly in the context of bariatric surgery, where severe and morbid obesity is abundant. In this cohort, a substantial number of patients are at significant risk of NASH and NAFLD, as well as related metabolic disease.

4.1 Clinical need and research opportunity

4.1.1 **Research opportunity**

The premise for this research arose from two key observations from clinical bariatric surgical practice:

1. **Nonalcoholic fatty liver disease was an increasingly severe and prevalent disease process, seen in a substantial number of bariatric surgical patients**. NAFLD has been described as the hepatic component of the metabolic syndrome. Similar to the metabolic syndrome, signs of NAFLD are seen in the vast majority of bariatric surgical patients, and this impacts on everyday surgical practice and decision making. Yet compared to these other related metabolic diseases in obesity, there is a limited understanding of NAFLD across all domains, ranging from epidemiology, diagnostic modalities, treatment strategies and pathophysiology. This poses considerable difficulty in developing guidelines and management strategies for the obese and

bariatric surgical populations. This is in stark contrast to well-established guidelines endorsed by multiple societies on the role of bariatric surgery in type II diabetes.

2. **Bariatric surgery provides a unique opportunity to access patients at risk of NAFLD and treat those affected.** It is ideally placed to facilitate systematic investigation of NAFLD in the setting of obesity, and particularly, more severe forms of obesity. Bariatric surgery has the capacity to provide a well-characterised obese patient cohort, with relatively safe access to an accurate histological diagnosis of NAFLD and tissue samples. It also provides an opportunity to easily monitor the response of NAFLD to weight loss. Weight loss is currently recognised as one of the most effective treatments for NAFLD, although it is rarely used as a primary therapy.

By basing NAFLD research in a bariatric surgical cohort, we were able to capitalise on a highly valuable interface of clinical material, knowledge gap, and clinical need. We systematically addressed a broad range of clinical and pathophysiological questions regarding NAFLD and obesity, and closely examined related metabolic disorders.

4.1.2 Clinical relevance

Outcomes of this research could potentially impact upon:

- Patient care, through better stratification of disease risk, utility of diagnostic strategies, and use of weight loss and bariatric surgery as a treatment for NAFLD. These data are of great utility to primary care physicians, hepatologists, and bariatric surgeons.
- Our knowledge of the pathophysiology of human NAFLD, by adding to and extending current understanding of disease mechanisms. This could potentially be used to develop therapeutics or management strategies that prevent or reverse disease.
- Better understanding and application of existing diagnostic and treatment modalities for NAFLD for those affected by the more severe forms of obesity.
- Better informing surgeons on their approach to NAFLD, particularly in the development of guidelines towards diagnosing and managing NAFLD in their patient population.

4.2 Aims and research themes

The central goal of this thesis was to systematically investigate key areas of uncertainty in NAFLD and NASH in the setting of obesity, especially in bariatric surgical populations. Key knowledge gaps included its pathogenesis, associations with obesity, diagnosis, and management, specifically the effect of weight loss. The focus of this thesis was on severe and morbid obesity but extended to the super obese.

This thesis was written around four main research themes, with aims and studies that addressed key deficiencies in our understanding of NAFLD in the setting of severe obesity:

1. Current scope of the problem – NAFLD, obesity and metabolic disease
2. Challenges of diagnosing NAFLD in obesity
3. Impact of weight loss on NAFLD and related metabolic diseases
4. Developing an understanding of pathophysiological drivers of NAFLD in obesity

Research Theme 1: Current scope of the problem – NAFLD, obesity and metabolic disease

Knowledge gap

The current literature shows substantial variation in prevalence of NAFLD reported in obesity. This could be due to the influence of obesity-related factors on NAFLD, including obesity severity, insulin sensitivity, and adipose tissue inflammation, which has not been well explored.

Aims

As a prelude to more specific studies, this research theme studied the prevalence and severity of NAFLD in the bariatric surgical population, and its relationship with increasing adiposity, adipose tissue factors and metabolic disease. Our aims were:

- To prospectively measure the prevalence and severity of NAFLD in a group of severely and morbidly obese bariatric surgical patients;
- To determine the effect of increasing levels of obesity and metabolic health status on severity of NAFLD;
- To determine the relationship of adipose tissue meta-inflammation and the risk of more severe NAFLD;

- To determine whether serum meta-inflammation predicted severe NAFLD.

Studies

Study 1: Effect of body mass index, metabolic health and adipose tissue inflammation on the severity of nonalcoholic fatty liver disease in bariatric surgical patients: A prospective study

Research Theme 2: Challenges of diagnosing NAFLD in obesity

Knowledge gap

Current diagnostic tests for NAFLD have been developed and validated in general NAFLD populations. Few studies have evaluated their feasibility and diagnostic accuracy in exclusively obese populations. This is particularly important, as obesity can significantly affect clinical, biochemical and physical characteristics, and therefore alter diagnostic accuracy. Furthermore, in many stratification tools, obesity itself is assigned significance in apportioning risk of NAFLD, such that all obese individuals are considered high risk.

Aims

This research theme investigated current methods for diagnosing NAFLD in an exclusively obese population, validated their use in obesity and determined factors that improved diagnostic accuracy. Specifically, we aimed:

- To systematically review the current evidence for common non-invasive tests for NAFLD-related fibrosis in obesity;
- To validate established fibrosis risk scores in an obese population, and determine simple methods for improving accuracy in this population;
- To determine the diagnostic accuracy of new non-invasive blood tests and imaging tests for detection of NAFLD in obesity;
- To develop a simple intraoperative assessment score to identify NAFLD and assist with stratifying patients for intraoperative liver biopsy;
- To study the differences in NAFLD grading with core versus wedge biopsy.

Studies

Study 2: Systematic review and meta-analysis: Non-invasive detection of nonalcoholic fatty liver disease related fibrosis in the obese

Study 3: Modified thresholds for fibrosis risk scores in nonalcoholic fatty liver disease are necessary in the obese

Study 4: Evaluating feasibility and accuracy of non-invasive tests for nonalcoholic fatty liver disease in obese patients

Study 5: Visual liver score to stratify nonalcoholic steatohepatitis risk and determine selective intraoperative liver biopsy in obesity

Study 6: Evaluating the histological variability of nonalcoholic fatty liver disease in obesity

Research Theme 3: Impact of weight loss on NAFLD and related metabolic diseases

Knowledge gap

Bariatric surgery reliably provides substantial long-term weight loss, with associated improvements in metabolic risk factors. There is currently a limited understanding of the patterns of improvement with incremental weight loss in obesity, the effects of modest weight loss on disease and therefore weight loss goals for meaningful metabolic gains.

Aims

This research theme investigated the effect of weight loss on NAFLD and related metabolic disorders in an obese population. We aimed to determine target weight loss goals for meaningful improvements. Specifically, we aimed:

- To study the improvement in NAFLD with weight loss;
- To determine the effects of weight loss on the metabolic syndrome and its components;
- To study the patterns of improvement in lipid variables with weight loss.

Studies

Study 7: Effects of bariatric surgery on liver function tests in patients with nonalcoholic fatty liver disease

Study 8: Weight loss after laparoscopic adjustable gastric band and resolution of the metabolic syndrome and its components

Study 9: Detailed description of change in serum cholesterol profile with incremental weight loss after restrictive bariatric surgery

Research Theme 4: Developing an understanding of pathophysiological drivers of NAFLD in obesity

Knowledge gap

NAFLD is characterised by excessive lipid accumulation within the liver. Lipotoxicity is the term used to describe cellular damage due to excess lipid content. There is increasing evidence on the role of lipotoxicity in the pathogenesis of disease processes, such as metabolic disease and NAFLD.

Lipidomics is an advancing field with great potential to capture the vast lipid changes that occur with NAFLD, and correlate these with disease.

Aims

Using state-of-the-art lipidomic analysis, we aimed to investigate the diverse changes in lipid profile that occur in human NAFLD, and how this may be related to disease progression in a large sample size of well characterised obese patients. Specifically, we aimed:

- To characterise the hepatic lipid profile associated with increasing NAFLD severity and the presence of NASH, and to identify specific lipids associated with disease severity;
- To explore the relationship between increasing NAFLD severity with the changes in lipid profile in visceral adipose tissue (VAT), subcutaneous adipose tissue (SAT) and plasma;
- To explore the relationship of the plasma lipid profile to the lipidome of liver, and therefore to investigate the utility of plasma lipid levels as an indication of liver disease.

Studies

Study 10: Lipidomic analysis of nonalcoholic fatty liver disease (NAFLD) in morbid obesity: Alterations in liver lipid profile and parallel serum changes with progressive disease

Through this series of studies, this thesis aimed to provide a better understanding of the clinical and pathophysiological aspects of NAFLD and metabolic disease in the setting of severe and morbid obesity, and particularly in the bariatric surgical population.

5 Research methods

Three major research methods were used:

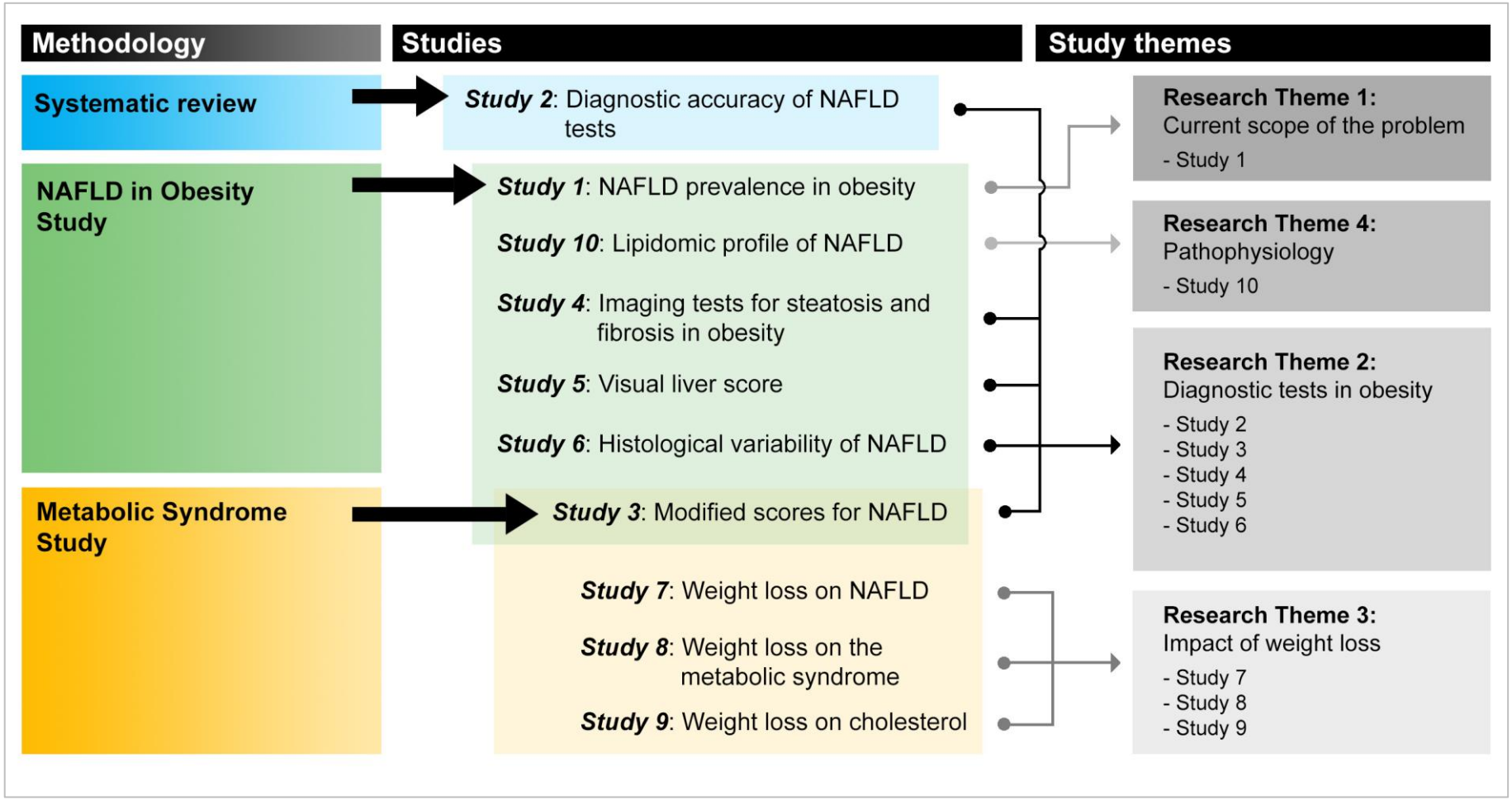
1. *Systematic review and meta-analysis:* Using PRISMA guidelines, we conducted a systematic review and meta-analysis to investigate the current evidence on non-invasive tests for NAFLD-related fibrosis in obesity.

2. *Prospective study of NAFLD in an obese bariatric surgical cohort*: This was a prospectively conducted study of obese patients at risk of NAFLD. All eligible patients were recruited between June 2015 to November 2016. This study involved collection of clinical, biochemical, tissue and radiological data. This study was conceived and conducted in its entirety during the PhD tenure and was the central research undertaking.

3. *The Metabolic Syndrome Study*: This was a prospectively conducted follow-up study of patients with the metabolic syndrome who underwent laparoscopic adjustable gastric banding from 2009-2010. This study collected repeated measures of clinical and biochemical data over two years. Clinical data and tissue were collected to service future endeavours such as this.

The associated methodologies for each study within this thesis are shown in **Figure 5.1**. The general methods used are detailed within this section, with specific methods detailed within each chapter.

Figure 5.1: Methodologies, associated studies and research themes.



NAFLD – nonalcoholic fatty liver disease

5.1 Systematic review and meta-analysis

5.1.1 Study selection and quality assessment

This was a systematic review and meta-analysis of the current evidence around non-invasive diagnosis of NAFLD-related fibrosis in obese populations. This study was conducted in accordance with the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines (479). Articles were identified by two independent reviewers by searching MEDLINE (via Ovid, 1946 to November 2016), EMBASE (1974 to November 2016), Science Citation Index (until November 2016), and the Cochrane Library (until November 2016).

Data extraction and quality assessment was performed by two independent reviewers according to the QUADAS-2 assessment tool (480). All disputes were resolved by consensus.

5.1.2 Meta-analyses

The weighted mean pooled sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), diagnostic odds ratio (DOR), and their 95% confidence intervals (CIs) were calculated using the DerSimonian and Laird random effects model. Summary receiver operator characteristic (SROC) curves were fitted using the Moses-Shapiro-Littenberg method, and the area under the receiver operator characteristic curve (AUROC), Q* index, and their respective standard errors were estimated. Heterogeneity was quantified using the I^2 index, I^2 values of 25, 50 and 75% corresponded to low, moderate and high degrees of heterogeneity respectively. Publication bias was assessed via Deeks funnel plots and associated regression test of asymmetry.

Full meta-analyses methods are found in **Chapter 6**.

5.2 NAFLD in obesity study

This was a prospective cohort study of bariatric surgery patients recruited between June 2015 and November 2016. The overall aim of this study was to investigate various aspects of epidemiology, diagnosis and pathophysiology of NAFLD in obese individuals.

All participants provided informed consent to participate in this study. Ethics approval was obtained from the Alfred (ref.195/15), Avenue (ref.190) and Cabrini (ref. 09-31-08-15) Human Research Ethics Committee. This study was registered with the Australian Clinical Trials Register (ACTRN12615000875505).

5.2.1 Subject recruitment

All patients who were undergoing bariatric surgery with participating surgeons at The Alfred Hospital, The Avenue Hospital and Cabrini Health, and who fit inclusion criteria were invited to participate.

5.2.1.1 Inclusion and exclusion criteria

Inclusion criteria: All patients undergoing any bariatric surgery were considered. Patients with likely NAFLD were approached for inclusion. This included any of: (1) hepatic steatosis on ultrasound or other imaging, (2) elevated transient elastography reading > 7kPa, and/or (3) abnormal LFTs: GGT greater than the upper limit of normal (ULN), and/or ALT or AST greater than *half* ULN.

Exclusion criteria: Age <18 years; refusal or inability to give informed consent to participate in the study; current or past excessive ETOH use (>210g/week males, >140g/week females); other causes of chronic liver disease and/or hepatic steatosis (Wilson's disease (caeruloplasmin levels), alpha-1-antitrypsin deficiency (α-1 antitrypsin levels), viral hepatitis (Hepatitis B and C serology), primary biliary cirrhosis (antimitochondrial antibody), autoimmune hepatitis (immunoglobulins and anti-SM antibody (F-actin)), genetic iron overload (3-4+ stainable iron on previous liver biopsy or negative C282Y +/+ or C282Y/H63D compound heterozygote), HIV (HIV serology), hypo- or hyperthyroidism (TSH), coeliac disease (anti-tissue transglutaminase antibodies, or HLA-DQ2/8)); recent (within 3 months of screening visit) or concomitant use of agent known to cause hepatic steatosis (corticosteroids, amiodarone, methotrexate, tamoxifen, tetracycline, high dose

oestrogens (standard HRT and OCP doses allowed), valproic acid) or current enrolment in a drug trial.

5.2.2 Interventions and outcome measures

All patients had their scheduled bariatric surgery as planned. Patients underwent pre-operative management, including very low calorie diet (VLCD), as per individual surgeon preference. Patients underwent a full medical history and physical examination on the day of surgery. The overall study design is seen in **Figure 5.2**.

Tests performed in this study included:

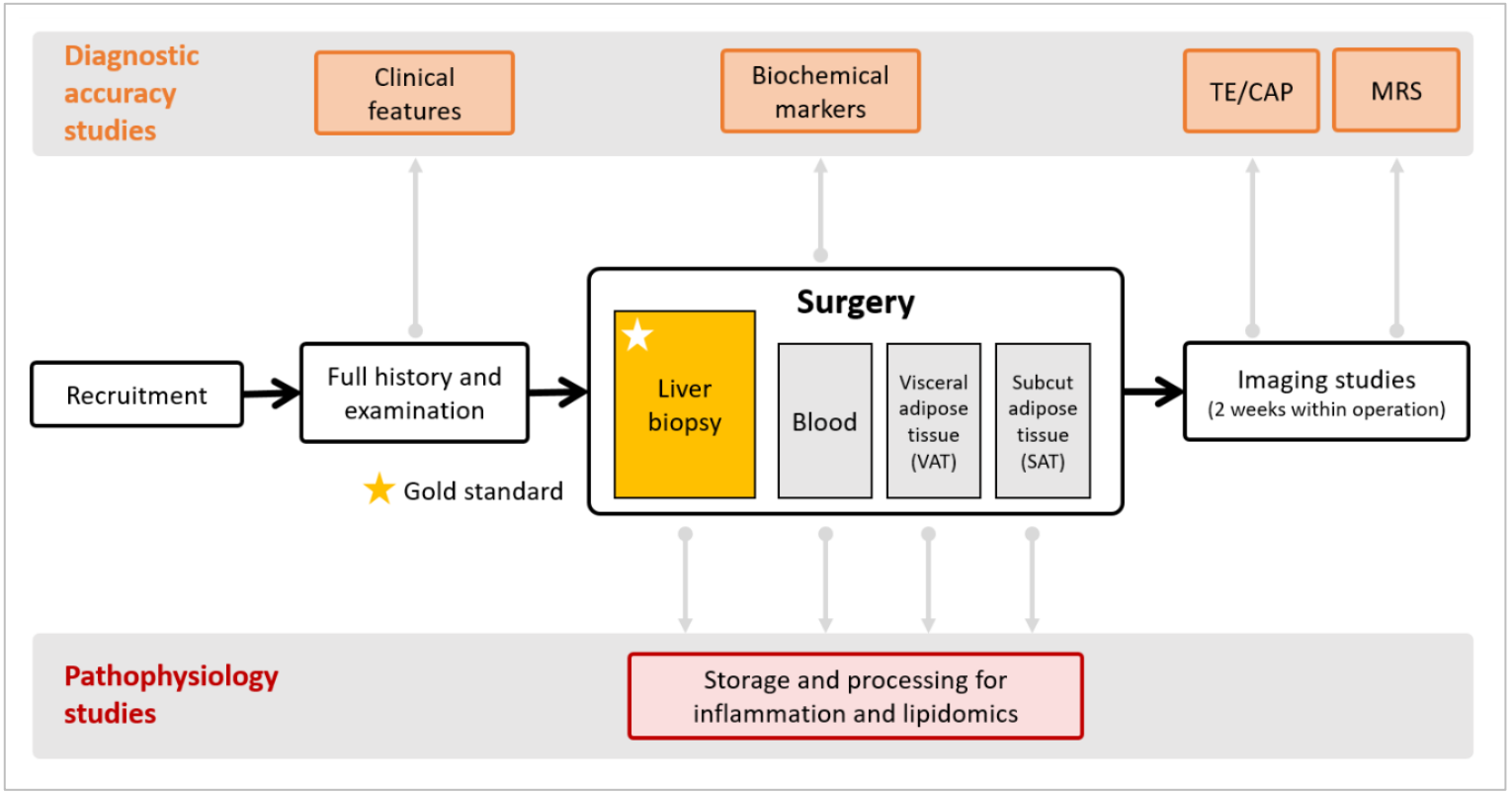
- Blood tests:
    - Standard biochemistry and metabolic parameters
    - Liver disease screening
    - Plasma for storage and further analysis
- Intraoperative specimens:
    - Liver biopsy (core and wedge)
    - Visceral (VAT) and abdominal subcutaneous adipose tissue (SAT) biopsy
- Imaging:
    - Transient elastography (TE) and controlled attenuation parameter (CAP)
    - Magnetic resonance spectroscopy (MRS) (sub-study, n=35)

5.2.2.1 Blood tests

Blood samples (50ml) were taken on induction of anaesthesia. All blood tests were sent to The Alfred Pathology Service for standardised measurement of biochemical and metabolic variables. The full schedule of blood tests is shown in **Table 5.1**. These results were also used to calculate risk scores as detailed in **Chapter 3.6 – Diagnosis of nonalcoholic fatty liver disease**.

A 10ml blood sample was collected in a K_2EDTA tube. This was centrifuged for 10 minutes at 4000 RPM, plasma was collected, then stored at -80^{o}C.

Figure 5.2: NAFLD in Obesity study design



VAT – visceral adipose tissue; SAT – subcutaneous adipose tissue; TE – transient elastography; CAP – controlled attenuation parameter; MRS – magnetic resonance spectroscopy

Table 5.1: Blood tests performed on day of surgery, including liver function tests, metabolic screen and liver disease screen.

Schedule of study blood tests	
Liver function tests (ALT, AST, total bilirubin, albumin, ALP, GGT) Full blood examination (FBE) Urea, creatinine, eGFR Cholesterol profile (Total cholesterol, HDL, LDL and triglycerides) Fasting glucose, insulin, HbA1c, C-peptide Ferritin Thyroid function test	Iron studies Coagulation studies Vitamin B12, D Liver screen (viral hepatitis and HIV serology, caeruloplasmin, alpha 1 antitrypsin serology, IgG, antimitochondrial antibody, anti-tissue transglutaminase antibody, anti-smooth muscle antibody, antigliadin antibody)

5.2.2.2 Intraoperative specimens

Collection and initial processing

All tissue specimens were collected by the operating surgeon (**Figure 5.3**). A wedge biopsy, at least 1cm in depth (**Figure 5.3b, 5.3e**), was taken from the left lobe of liver, and two core biopsies from each lobe of the liver. This was done to obtain a global assessment of liver pathology, due to the known heterogeneity of the liver (227). All core biopsies and half of the wedge liver biopsy were formalin fixed (10% buffered formalin) and paraffin embedded, for histopathological assessment. The remaining section of liver was frozen in dry ice for storage at -80^{o}C.

Approximately 5-10mL of omental visceral adipose tissue (VAT) and abdominal subcutaneous adipose tissue (SAT) were collected (**Figure 5.3a, 5.3c-d**). These were divided, with half undergoing formalin fixation (10% buffered formalin) and paraffin embedding, and half frozen in dry ice then stored at -80^{o}C.

Liver histology

Paraffin embedded liver samples were sectioned and stained with hematoxylin and eosin (H&E), and Masson's trichrome. Liver biopsies were assessed by a single histopathologist, blinded to clinical information, according to the NASH CRN Scoring System (481) and fibrosis stage as described by Kleiner (240).

Figure 5.3: Specimens taken, showing (a) visceral adipose tissue biopsy technique, (b) liver wedge biopsy technique, (c) visceral adipose tissue, (d) subcutaneous adipose tissue and (e) wedge liver biopsy.

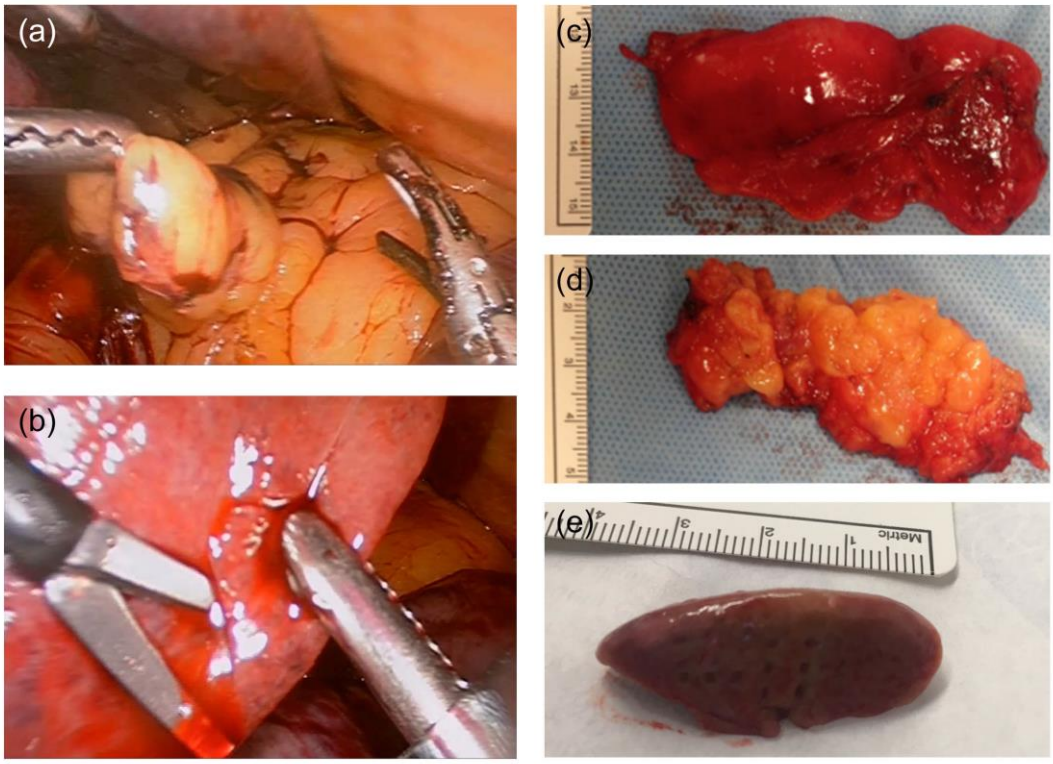


Image analysis of tissue

Liver and adipose tissue were sectioned and H&E stained. Image analysis was used to objectively quantify percentage area of liver steatosis, and adipose tissue cell size (Fiji, ImageJ, Madison, WI, USA) (**Figure 5.4**).(482)

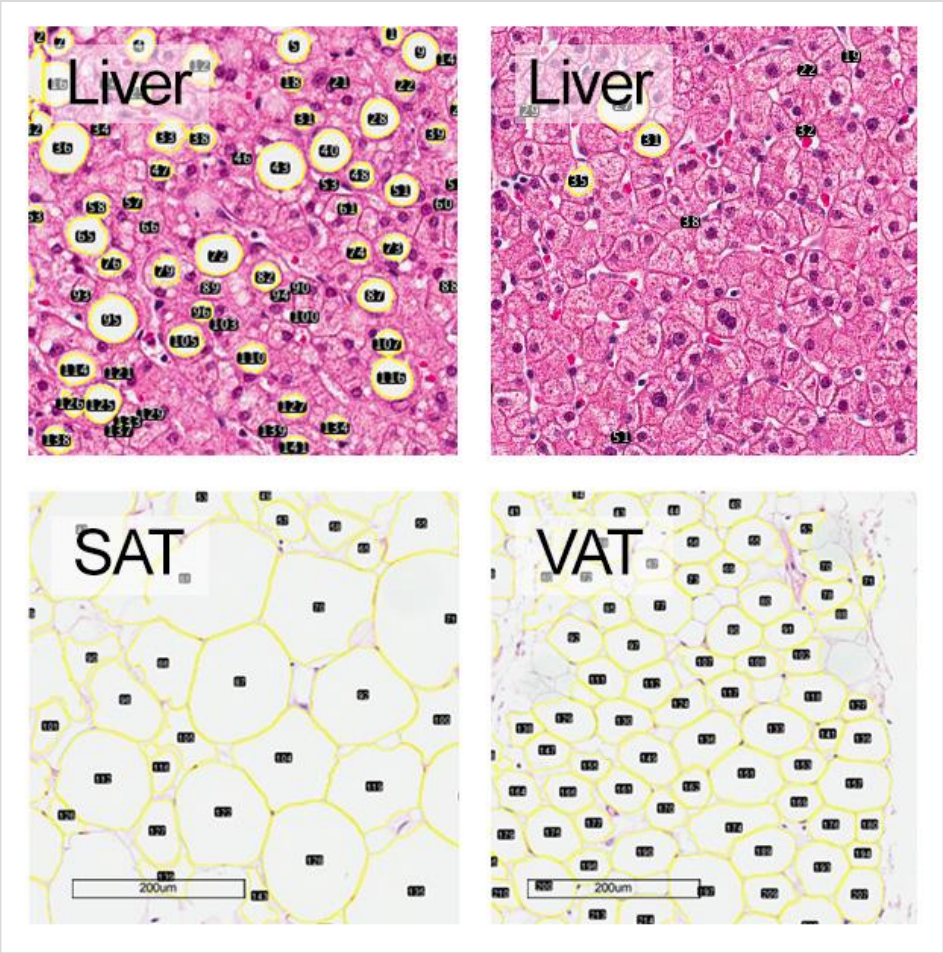
Biochemical analysis of frozen tissue

Frozen specimens (liver, VAT, SAT, plasma) were processed for measurement of cytokines and lipid profile (**Chapter 6**).

Plasma

Concentrations of the cytokines IL-1β, IL-6, IL-10 and TNF-α in plasma was determined by enzyme-linked immunosorbent assays (ELISA), using the Human IL-6, Human IL-1β, Human IL-10 and Human TNF-α ELISA kits (Invitrogen, Maryland, USA).

Figure 5.4: Image analysis (by Fiji ImageJ script) of liver H&E slides (above), determining total percentage of steatosis area, and adipose tissue (below, subcutaneous (SAT) and visceral adipose tissue (VAT)), determining average adipocyte cell area and maximal width.



Samples and standards were run in triplicates. Plasma and standards (100µL each) were added to wells on a 96-well plate, and incubated for 2 hours at room temperature. Samples were thoroughly aspirated and well washed 4 times. 100µL of biotinylated anti-IL-1β, anti-IL-6, anti-IL-10 or TNF-α antibody solution (Invitrogen, Maryland, USA) was added to each well and incubated for 1 hour at room temperature. Samples were again thoroughly aspirated and well washed 4 times. 100µL of Streptavidin-HRP Working Solution (Invitrogen, Maryland, USA) was added to each well and incubated at room temperature for 30 minutes. Samples were once more thoroughly aspirated and well washed 4 times. Finally, 100µL of Stabilised Chromogen (Invitrogen, Maryland, USA) and incubated for 30 minutes at room temperature in a dark room before Stop Solution (Invitrogen, Maryland, USA) was used to cease the reaction. Absorbance of each well was read at 450nm on a spectrophotometer (Implen, Munich, Germany).

Standard curves were constructed with a minimum acceptable coefficient of determination (r^2) of 0.91. Sample absorbance of duplicates was averaged and plotted on standard curves to determine cytokine concentration in pg/ml.

Tissue: mRNA extraction

Liver and adipose tissue was thawed on ice, then homogenised for 2 minutes at 50/second in 1mL of TRI Reagent (Sigma-Aldrich, St Louis, USA). 200µl chloroform was vigorously mixed to the homogenate for 15 seconds and incubated for three minutes at room temperature. This was centrifuged at 12,000G at 4^{o}C for 15 minutes. The aqueous top phase was transferred and stored in separate fresh tubes. 500µl isopropanol was mixed in, and then this was incubated at room temperature for 10 minutes. Samples were centrifuged at 12,000G at 4^{o}C for 10 minutes. The supernatant was discarded leaving the RNA pellet. 1mL of 100% ethanol was added, and the sample vortexed to dislodge the pellet. The sample was then centrifuged at 10,000G at 4^{o}C for 15 minutes. The supernatant was discarded, and the samples left to air dry at room temperature. The pellet was suspended in 30µl of RNAase free water.

Samples were analysed on a GmbH nanophotometer (Implen, Munich, Germany). The concentration of samples was determined by absorbance at 260nm. RNA purity was assessed by the ratio of absorbance at 260nm/280nm, and excess ethanol was detected by 260nm/230nm absorbance ratio. DNAase was added to eliminate DNA contamination.

Tissue: Reverse Transcription to cDNA

1000µg mRNA was aliquoted from the samples. Nuclease free water was added to a total volume of 15µl. Reverse transcription of mRNA to cDNA was performed using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories, California USA). 4µL of iScript Reaction Mixture, and 1µL of iScript Reverse Transcriptase was added to each tube. A T100 Thermal Cycler (Bio-Rad Laboratories, California, USA) was used to heat the samples. Samples were heated at 25^{o}C for 5 minutes for priming, then for 20minutes at 46^{o}C to facilitate reverse transcription and at 95^{o}C for 1 minute to terminate the reaction.

Tissue: Quantitative real time polymerase chain reaction (qRT-PCR)

mRNA expression of interleukin 6 (IL-6), interleukin 1β (IL-1β) and C-C motif chemokine ligand 2 (CCL-2) was determined by qRT-PCR reactions. Initially a series of primers were tested for their efficiency at dilutions of 0.2, 0.1, 0.02, 0.01, once primers were determined to

be efficient the samples were run. Samples were run in triplicate on a 384 well plate. Firstly, a master mix was made; containing 5µl of Sybr Green 2x (Qiagen, Hilden, Germany) 0.5µL of Forward Primer (20mM) and 0.5µL of Reverse Primer (20mM). This was added in each well to 4µL of cDNA to a total of 10µL per well. The plate was centrifuged at 1000 rpm, for 1 minute at room temperature. Samples were placed in a thermal cycler, (CFX384 Touch™ Real-Time PCR Detection System) (Bio-Rad Laboratories, California, USA) to run the reaction. Samples were heated to 94ºC for 2 minutes for initial denaturation, followed by 40 cycles at 94ºC for 15 seconds for further denaturing, and at 60ºC for 1 minute for annealing, extension and for reading of the fluorescence. A housekeeping gene, HRPT was used to control for differences in cDNA loading. A critical threshold (C_T) method was used to calculate the relevant quantities of each transcript.

5.2.2.3 Imaging tests

All imaging tests were performed within 2 weeks of surgery. All assessors were blinded to clinical information.

Transient elastography and control attenuation parameter

Transient elastography (Fibroscan®, EchoSens, Paris) was performed in a fasting state in The Alfred Gastroenterology Department. Two experienced gastroenterologists (>2000 procedures each) performed the scans as per manufacturer's recommendations. A pre-procedure liver ultrasound was performed to locate the liver along the mid-axillary line, and to measure skin-to-liver capsule distance. TE was performed according to the standard protocol. As all patients were obese, an XL probe was used. Attempts were made to collect ≥10 valid liver stiffness measurements (LSM). Where no successful measures were obtained after 10 measurements, the test was considered unsuccessful. Variability was assessed via the ratio of the interquartile range (IQR) and median LSM measure (IQR:M ratio). Unreliable readings were considered to be those with at least one of the following: <10 valid acquisitions, <60% successful readings, or IQR:M≥0.30. Standard and optimal thresholds were calculated and used for assessment of diagnostic accuracy (302).

Magnetic resonance spectroscopy

Magnetic resonance spectroscopy (MRS) was performed at The Baker IDI. Hepatic triglyceride concentration was measured by ^{1}H-MRS. T_1-weighted imaging was performed

on a 3.0 Tesla whole-body system (Siemens Prisma) with image-guided localised ^{1}H-MRS. Area of interest was centred in the right lobe of the liver (3.0 x 2.0 x 2.0 cm voxel). Excitation water suppression was used to suppress water signal during data acquisition. Unsuppressed water spectra were acquired for use as the internal standard. Spectral data were post-processed using magnetic resonance user interface software (jMRUI version 4.0, EU Project).

5.3 Metabolic syndrome study

The Metabolic Syndrome Study was a prospective observational follow-up study of patients undergoing a primary laparoscopic adjustable gastric band (LAGB) procedure, who fulfilled the National Cholesterol Education Program (NCEP) Adult Treatment Panel III (ATP III) criteria for the metabolic syndrome (**Figure 5.5**). All participants provided informed written consent to participate. Ethical approval was obtained from the Avenue Ethics Committee (ref. no. 099). The trial was registered with the Australian Clinical Trials Register (ACTRN12610000049077).

Patients were recruited between April 2009 and March 2010. Close follow-up (1-3 monthly) to monitor changes in biochemical and clinical features was conducted over a two-year period. The overall aim of this study was to examine the effects of surgically induced weight loss on the metabolic syndrome and associated conditions.

5.3.1 Subject recruitment

A routine medical history, physical examination and biochemical screen was conducted prior to recruitment at the patient's first appointment at The Centre for Bariatric Surgery, to assess for fulfilment of the metabolic syndrome criteria. Patients who reach the criteria for metabolic syndrome were invited to participate in the study.

5.3.1.1 Inclusion and exclusion criteria

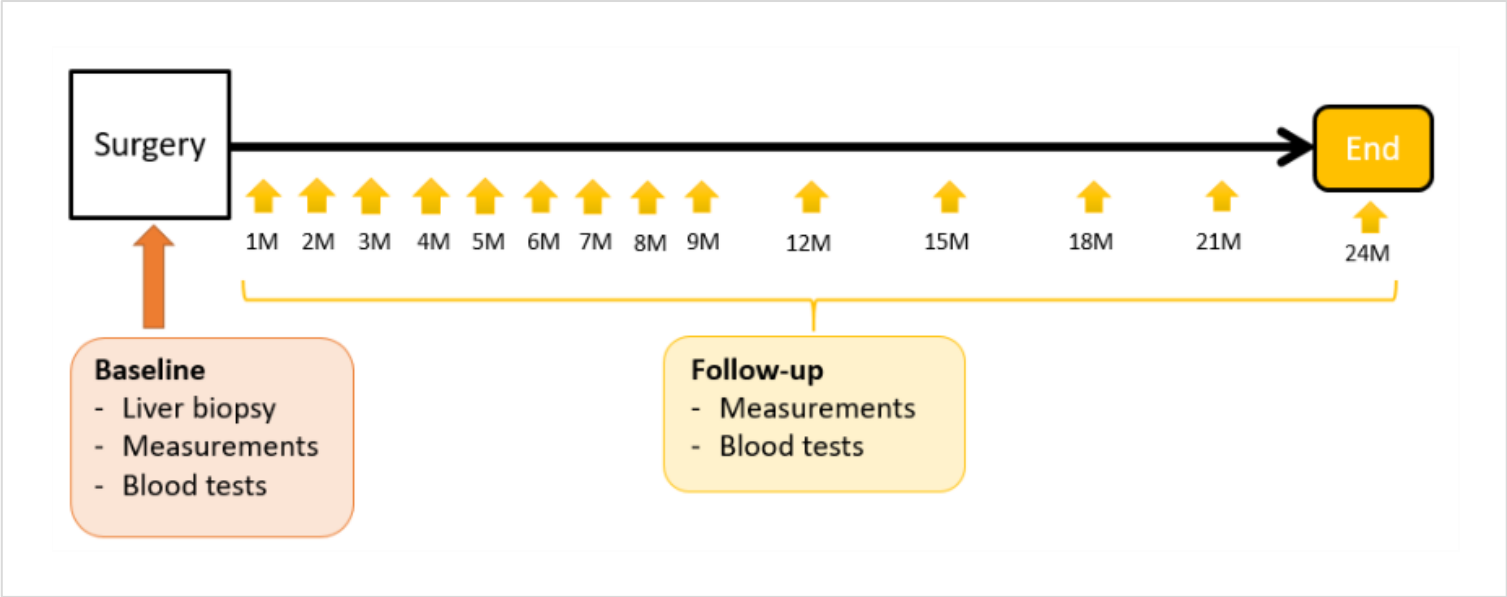
Inclusion criteria: Bariatric surgical patients were approached and included if they fulfilled all of the following: (1) metabolic syndrome according to ATPIII criteria, (2) scheduled for LAGB placement, (3) consented to close monitoring of their health changes and biochemistry over a two-year period, and (4) consented to an intra-operative liver biopsy.

Exclusion criteria: (1) Unable or unwilling to consent for the trial; (2) did not have the features of metabolic syndrome, and (3) unsuitable for LAGB surgery.

5.3.2 Interventions and outcome measures

All patients had their scheduled bariatric surgery as planned. Patients underwent pre-operative management, including very low calorie diet (VLCD), as per individual surgeon preference.

Figure 5.5: Metabolic Syndrome study design



5.3.2.1 Baseline measurements

On the day of surgery, the medical history and physical examination, including body anthropometrics, were repeated. Blood tests were taken from the intravenous cannula just prior to surgery. An intraoperative core liver biopsy was taken to identify and grade the severity of NAFLD.

5.3.2.2 Follow-up

Participants were followed-up every month until 9-months post-surgery, then 3-monthly thereafter until 24-months post-surgery. All blood tests and body measurements were repeated, to assess fulfilment of the metabolic syndrome criteria, and monitor progression of metabolic variables.

5.4 Data management

5.4.1 Data storage

All hard copies of data were held in a locked cabinet in the Centre for Obesity Research and Education (CORE) office, with full security cover. All relevant data was entered into a Microsoft Excel database. Electronic data was kept in a single group of folders on a secure drive in a password-protected computer in the CORE research office. This folder is backed up regularly by the data manager.

All patient information is retained in an identifiable or re-identifiable form.

5.4.2 General statistical analysis

Continuous variables were expressed as mean±standard deviation (SD) for parametric data, and median and interquartile range (IQR) for nonparametric data. Normality was assessed by Shapiro-Wilks test. Independent student t-test and one way ANOVA with post-hoc Bonferroni test was used for parametric data, and Mann Whitney U-test and Kruskal-Wallis test for non-parametric data. Categorical variables were expressed as numbers (with percentages). Pearson's chi-squared or Fisher's exact test were used for independent categorical variables.

Logistic and linear regression analyses were performed on binary categorical and continuous outcomes. Variables were removed by backward elimination method, using a criterion of $p<0.10$. Results are presented as IQR odds ratios (OR) with 95% CI for logistic regression, and beta coefficients with 95% CI for linear regressions.

A p-value ≤ 0.05 was considered statistically significant.

Data analysis was performed in Microsoft Excel (Microsoft Corporation, Redmond, WA, USA), SPSS v23 (SPSS Inc., Chicago, IL, USA), MATLAB (The MathWorks Inc., Natick, MA, USA) and Prism 7 (GraphPad Software Inc., La Jolla, CA, USA).

Research Theme 1

Current scope of the problem – NAFLD, obesity and metabolic disease

Study 1

Obesity undoubtedly contributes significant risk of NAFLD. This is evident through subgroup analysis in NAFLD epidemiology studies (8, 232), and a variety of animal and human pathophysiological studies that link obesity traits to NAFLD development (355). The accepted burden of NAFLD in obese populations is substantial at 71-98%, with rates of NASH of 25-56%.

Yet many of these studies are based in pre-selected populations, recruited after screening tests or from specialist referral centres. Therefore, these rates are often poorly representative of the bariatric surgical cohort. This is exemplified by studies in the bariatric surgical setting that report significant challenges in reaching these reported rates of disease (12, 13, 483, 484).

This chapter aimed to contribute to our understanding of the burden of NAFLD in the bariatric surgical population. By prospectively recruiting all eligible bariatric surgical patients, it intended to achieve a more representative sample of obese individuals.

Ultimately, this research theme aimed to better inform primary care and bariatric clinicians of the prevalence of NAFLD and NASH in obese patients, and link this to simple clinical risk factors that can aid in stratifying risk.

6 Effect of body mass index, metabolic health and adipose tissue inflammation on the severity of nonalcoholic fatty liver disease in bariatric surgical patients: A prospective study

6.1 Abstract

BACKGROUND: Nonalcoholic fatty liver disease (NAFLD), driven by the obesity epidemic, has become the most common form of liver disease. Despite this, there is controversy regarding the prevalence and severity of NAFLD in obesity. Obesity-related factors, such as increasing adiposity, metabolic disease and inflammation, may influence prevalence. We therefore prospectively measured NAFLD prevalence in obesity and studied factors associated with NAFLD.

METHODS: We recruited consecutive bariatric patients. Intraoperative liver biopsies were taken. Liver, adipose tissue and serum were collected to measure inflammation. Adipocyte cell size was measured. NAFLD severity was correlated to body mass index (BMI), metabolic health and adipose characteristics.

RESULTS: There were 216 participants; BMI 45.9±8.9kg/m^2, age 44.4±12.1years, 75.5% female. Overall NAFLD prevalence was 74.1%, with 17.1% having nonalcoholic steatohepatitis (NASH) and/or steatofibrosis. Odds of NASH/steatofibrosis increased independently with BMI category (odds ratio (OR) 2.28-3.46, all $p<0.05$) and metabolic disease (OR 3.79, $p=0.003$). These odds markedly increased when both super obesity (BMI>50) and metabolic disease were present (OR 9.71, $p<0.001$). NASH/steatofibrosis prevalence was significantly greater with diabetes, hypertension and dyslipidaemia. Although greater visceral adipocyte hypertrophy was evident in NASH/steatofibrosis, there was no significant association between adipose inflammation and NASH/steatofibrosis.

CONCLUSION: NAFLD remains endemic in obesity, however NASH/steatofibrosis are less common than previously reported. Worsening obesity and metabolic disease increase odds of NAFLD independently, with substantially compounded effect with

both. These observations may help with risk stratification in obese populations. We were unable to delineate clear associations between adipose inflammation and NASH/steatofibrosis in this obese population.

6.2 Introduction

Nonalcoholic fatty liver disease (NAFLD) is now the most common form of chronic liver disease (6). Increasing obesity and metabolic disease has fuelled rapidly increasing rates of NAFLD. The significance of NAFLD is that it may progress to its more severe form, nonalcoholic steatohepatitis (NASH) and steatofibrosis (485). Importantly, these more severe forms of NAFLD are most strongly associated with liver-related and overall mortality (247). Due to its close association with obesity, having a clear understanding of the clinical associations and pathophysiology of NASH and steatofibrosis is of substantial importance to bariatric surgeons.

Despite its importance, the precise prevalence of NASH in the presence of severe and morbid obesity remains unclear. This is largely due the paucity of robust non-invasive tests for NASH, which necessitates liver biopsy for accurate study of prevalence (311). Previous studies have reported high rates of NAFLD of up to 90% in obesity, with 25-56% NASH and 11% advanced fibrosis (10, 11, 486). In contrast, Lassailly *et al* (12) recently reported a NASH prevalence of only 7.3% in a well-documented series of 1489 consecutive bariatric surgical patients with routine intraoperative biopsies. Similarly, modest rates were found in the Longitudinal Assessment of Bariatric Surgery (LABS) Study, which reported 16.2% of patients with definitive NASH (13). The exact reasons for this wide variation in reported prevalence in obese populations is unclear, however differences in obesity-related characteristics may contribute.

Current understanding of NAFLD pathophysiology implicates obesity-related metabolic disease as a key mediator of NAFLD. Previous studies show that metabolic disease, including visceral obesity, dyslipidaemia, insulin resistance and hypertension, affects 42.5% of patients with NAFLD, and 70.7% of those with NASH(232). At the opposite end of the spectrum is the phenomenon of metabolically healthy obesity (MHO)(487). This term broadly describes the phenotype where clinical obesity is present, in the absence of metabolic consequences (488). Some argue that the MHO phenotype affords a similar metabolic risk profile to lean individuals (489, 490).

On a pathophysiological level, obesity-related inflammation, so called ‘meta-inflammation’, may influence the prevalence of NAFLD (102, 491). Meta-inflammation develops due to obesity-related changes in adipose tissue morphology such as adipocyte hypertrophy,

immune cell infiltrate, and adipokine production (102). This can disrupt metabolic pathways and drive insulin resistance and metabolic disease (491), and ultimately exacerbate NAFLD (102, 492). Both visceral (VAT) (493-496) and subcutaneous adipose tissue (SAT) (496, 497) inflammation have been implicated in liver damage. Furthermore, some studies show that these inflammatory changes may be reflected in serum (498), and therefore could potentially identify those at risk of NAFLD.

Improved understanding of the epidemiology of NASH in obesity would be of great clinical utility. Current uncertainty of prevalence and associations of NASH in bariatric surgical patients is accompanied by a wide variation in clinical approach. This was demonstrated by the LABS study, where biopsy rates varied widely between the 38 participating surgeons, from 94.1% down to no intraoperative biopsies (13). A better understanding of this cohort could improve guidelines on obesity-related liver disease, based on factors other than the presence or absence of obesity.

We aimed to study the prevalence of NAFLD and NASH in an obese population and describe obesity-associated factors that were associated with NAFLD and NASH. Specifically, we aimed to:

1) Prospectively measure the prevalence and severity of NAFLD in a group of obese bariatric surgical patients;
2) Determine the effect of increasing levels of obesity and metabolic health status on severity of NAFLD;
3) Determine whether adipose tissue or serum based inflammation and characteristics were associated with risk of more severe NAFLD.

We hypothesized that in a well-characterised prospectively recruited cohort, the overall prevalence of NASH and steatofibrosis would be lower than previously reported, but would increase with more severe obesity and metabolic disease. We further hypothesised that NASH and steatofibrosis would additionally be strongly correlated with the presence of adipose tissue inflammation and cellular hypertrophy.

6.3 Methods

All participants provided informed consent. Ethics approval was obtained (Alfred (195/15), Avenue (190) and Cabrini (09-31-08-15) Human Research Ethics Committees). This study was registered with the Australian Clinical Trials Register (ACTRN12615000875505).

6.3.1 Patients

We prospectively enrolled all eligible obese patients undergoing bariatric surgery in three metropolitan hospitals in Melbourne (Australia) between July 2015 and August 2017.

Inclusion criteria were: (1) age ≥18years, (2) BMI≥35kg/m^2, (3) alanine aminotransferase (ALT) or aspartate aminotransferase (AST) >0.5 times upper limit normal (ULN) (279). Patients were excluded if they had evidence of other liver disease, including viral hepatitis, medication-related, autoimmune, familial/genetic causes or a history of excessive alcohol use, as defined by the American Association for the Study of Liver Diseases (3).

6.3.2 Outcomes

6.3.2.1 Clinical and biochemical data

Medical history and examination were performed pre-operatively. Metabolic syndrome was defined as detailed below. Obstructive sleep apnoea was screened with the STOP-BANG questionnaire (499), and patients at risk were referred for polysomnography.

Fasting blood tests were taken prior to induction of anaesthesia. Serum was collected and frozen at -80^{o}C for cytokine analysis.

Participants were analysed in groups according to body mass index (BMI) categories: *obese* - BMI<40 kg/m^2; *morbidly obese* - BMI 40-50kg/m^2; *super obese* - BMI >50 kg/m^2.

6.3.2.2 Intraoperative biopsies

Intraoperative wedge liver biopsies, ≥1cm in depth, were taken for histology with a section frozen at -80^{o}C. A single pathologist graded the biopsies in a blinded manner, according to the Clinical Research Network (CRN) NAFLD activity score (NAS) (481) and Kleiner classification of liver fibrosis (240). For this study, we have classified NAFLD into the

following groups: (a) *Normal*: Steatosis ≤5%, no other abnormality; (b) *Non-NASH NAFLD*: Steatosis >5%, and does not reach criteria for NASH or steatofibrosis; (c) *NASH:* NASH by NAS criteria (NAS ≥5), with or without Stage 1 fibrosis (F1); and (d) *Steatofibrosis*: Stage 2-4 fibrosis (F2-4).

Visceral adipose tissue (VAT) and subcutaneous adipose tissue (SAT) were taken intraoperative, for histological examination and frozen for cytokine analysis.

Full details on tissue processing are found in **Appendix 1: Supplementary Methods**.

6.3.2.3 Metabolic disease

There are currently multiple published definitions for "metabolically healthy obesity" (MHO) (**Supplementary Table 6.1**) and metabolic syndrome (488). We have used a modified version of the National Cholesterol Education Program (NCEP) Adult Treatment Panel III (ATPIII) criteria (55), used previously for defining MHO (500). The criteria are: (a) Systolic blood pressure (SBP) >130mmHg, and/or diastolic blood pressure (DBP) >85mmHg; (b) Triglycerides ≥1.70mmol/L; (c) High density lipoprotein (HDL) <1.03mmol/L for males, <1.29mmol/L for females; and (d) Fasting blood sugar level (BSL) ≥5.6mmol/L.

Metabolically healthy obesity (MHO) is the absence of any of these criteria. *Borderline MHO* has been defined as fulfilment of only one of these criteria. *Metabolically abnormal obesity* (MAO) is defined as fulfilling two or more of these criteria.

6.3.2.4 Tissue and serum inflammation

Liver and adipose tissue were analysed by quantitative real-time polymerase chain reaction (qRT-PCR) to quantify mRNA expression of interleukin-6 (IL-6), interleukin-1β (IL-1β) and C-C motif chemokine ligand 2 (CCL2), as objective markers of inflammation. Serum cytokines were measured via enzyme-linked immunosorbent assay (ELISA). Full laboratory methods are found in the **Appendix 1: Supplementary Methods**.

6.3.2.5 Adipose tissue cell size

Image analysis was used to objectively quantify adipose tissue cell size (Fiji, ImageJ, Madison, WI, USA) (482).

6.3.3 **Statistical analysis**

Continuous variables were expressed as mean ± standard deviation (SD) for normally distributed data, and median and interquartile range (IQR) for non-normally distributed data. Independent student t-test and one-way ANOVA with post-hoc Bonferroni test, and Mann Whitney U-test and Kruskal-Wallis test were used where necessary. Categorical variables were expressed as numbers (with percentages). Pearson chi-squared or Fisher exact test were used for independent categorical variables. Logarithmic transformation (log_{10}) of qRT-PCR data was conducted prior to analysis as data was heavily skewed. Odds ratios were calculated as effect size, with 95% confidence intervals (CI).

Logistic and linear regression analyses were performed on binary categorical and continuous outcomes, to determine associations with NASH/steatofibrosis and inflammation. Covariates used included age, gender, metabolic health status, obstructive sleep apnoea and smoking status. Variables were removed by backward elimination, using a criterion of $p<0.05$.

A p-value ≤ 0.05 was considered statistically significant. Data analysis was performed in SPSS v23 (SPSS Inc., Chicago, IL, USA), and Prism 7 (GraphPad Software Inc., La Jolla, CA, USA).

6.4 Results

6.4.1 Patients

There were 216 consecutive eligible bariatric surgical patients (**Table 6.1**). Average age was 44.4±12.1 years, with 163 females (75.5%). The average body mass index (BMI) was 45.9±8.9 kg/m^2, corresponding to 58 (26.9%) with *BMI<40*, 106 (49.1%) with *BMI 40-50* and 52 (24.1%) with *BMI>50*.

Fifty-six participants had diabetes (26.0%), 99 had hypertension (46.0%) and 43 had pre-diagnosed dyslipidaemia (20.1%). Many participants had abnormal fasting lipid levels, with low HDL in 171 (79.2%) and high triglycerides in 86 (39.8%). Therefore, there were 18 (8.3%) participants who were metabolically healthy and obese (*MHO*), 73 (33.8%) *borderline MHO* and 125 (57.9%) were metabolically abnormal (*MAO*).

6.4.2 Prevalence of NAFLD and NASH

NAFLD (>5% steatosis) was present in 160 participants (74.1%). More severe disease (i.e. NASH or steatofibrosis) was found in 37 participants (17.1%), with NASH in 26 (12.0%) and steatofibrosis in 11 participants (5.1%) (**Supplementary Table 6.2**).

6.4.2.1 Factors affecting overall NAFLD prevalence

Any degree of NAFLD was present in similar proportions regardless of degree of obesity (**Table 6.2** and **Figure 6.1a**). Increased rates of NAFLD were seen in those with type II diabetes mellitus (T2DM) (85.7% vs 69.8%, p=0.020), however overall NAFLD was not affected by hypertension, dyslipidaemia or obstructive sleep apnoea (OSA). Neither number of metabolic risk factors, nor metabolic risk status, had significant impact on presence of any NAFLD in obese patients.

6.4.2.2 Factors affecting NASH/steatofibrosis prevalence

The presence of more significant disease, in the form of NASH/steatofibrosis, was significantly influenced by various obesity-related factors (**Table 6.2**). Increasing BMI category significantly affected rates of NASH/steatofibrosis (10.3% vs 15.1% vs 28.8%, p=0.027). Additionally, T2DM, hypertension and dyslipidaemia all affected NASH

prevalence rates. This was reflected in increasing rates of NASH/steatofibrosis with increasing numbers of metabolic risk factors, as well as increasing rates with worsening metabolic health status (5.6% vs 8.2% vs 24.0%, p=0.007).

6.4.2.3 Effect of obesity and metabolic disease on NASH/steatofibrosis risk

Table 6.3 shows the risk of severe progressive disease (NASH or steatofibrosis) according to BMI and metabolic health status groups. The *BMI>50* group had significantly higher risk of having severe progressive disease (NASH or steatofibrosis) compared to *BMI 40-50* (odds ratio (OR) 2.28, p=0.044), and *BMI<40* (OR 3.46, p=0.019). Metabolically abnormal obesity (MAO) had significantly increased odds of NASH/steatofibrosis compared to *borderline MHO* (OR 3.52, p=0.008), as well as a combined *MHO/borderline MHO* group (OR 3.79, p=0.003).

Odds of disease were substantially increased with combined risk from metabolic disease and high BMI. Those with *MAO* and *BMI>50* had a markedly increased OR of 9.71 for NASH/steatofibrosis (p<0.001), compared with healthier individuals (*MHO/borderline MHO* individuals with *BMI≤50*).

Multivariate analysis confirmed that the presence of *NASH/steatofibrosis* was significantly associated with both metabolic health status (β=1.44, p=0.002) and body mass index (β 0.061, p=0.002). Presence of *any fibrosis (F1-4)* and *steatofibrosis (F2-4)*, were associated with BMI (β 0.013, p<0.001 for *any fibrosis* and β 0.004, p=0.002 for *steatofibrosis*), but not metabolic health (**Supplementary Table 6.3**).

6.4.3 Adipose tissue and serum effects on NAFLD

6.4.3.1 Tissue characteristics

Analysis of liver IL-6 levels confirmed significantly increased inflammation in non-NASH NAFLD (log_{10} fold change: 0.035 vs 0.534, p=0.024) and NASH/steatofibrosis (0.035 vs 0.937, p=0.033) compared to normal liver (**Figure 6.2a**).

Neither visceral adipose tissue (VAT) or subcutaneous adipose tissue (SAT) cytokine mRNA expression correlated with NAFLD severity (**Figure 6.2c-d, Supplementary Figure 6.1-6.2**). Similarly, SAT cell size did not change with NAFLD severity. There were, however,

clear differences in VAT characteristics, with increased adipocyte size seen in non-NASH NAFLD (cell diameter 85.6µm vs 90.0µm, p=0.005), and NASH/steatofibrosis (85.6µm vs 92.3µm, p=0.003) compared to normal (**Figure 6.2e-f**).

Some of the differences seen in liver and adipose tissues between normal and NASH are demonstrated in **Figure 6.3**, showing increased liver steatosis and inflammation, and adipose tissue cell characteristics associated with NASH.

6.4.3.2 Serum inflammation

Serum cytokines (interleukin-6 (IL-6), interleukin-1β (IL-1β), interleukin-10 (IL-10) and tumour necrosis factor alpha (TNF-α)) were measured to compare differences in inflammation in NAFLD. There were no significant differences in serum IL-6 levels or other cytokines between NAFLD categories (**Figure 6.2b** and **Supplementary Figure 6.2**).

Full data for liver and fat mRNA expression and serum protein levels for IL-1β, CCL-2, IL-10) and TNF-α are provided in **Supplementary Figures 6.1-6.2**.

Table 6.1: Baseline characteristics of cohort, showing clinical and biochemical characteristics of all patients, and according to nonalcoholic fatty liver disease (NAFLD) status

Variables	All patients	NAFLD status			p-value
		Normal	*Non-NASH NAFL*	*NASH/ steatofibrosis*	
n=	216	56	123	37	
Age	44.4 ±12.1	43.8±13.7	44.5±11.6	44.8±11.1	0.899
Male gender	53 (24.5%)	10 (17.9%)	30 (24.4%)	13 (35.1%)	0.061
Waist circumference	125.3 ±21.1	120.1±16.7^	126±21.3	132±25.2^	**0.044**
Weight	129.0 ±29.8	120.7±23.2^	127.8±26.3$^{\#}$	145.4±41.9^$^{\#}$	**<0.001**
Body mass index	45.9 ±8.9	43.6±7.2^	45.5±7.8$^{\#}$	50.5±12.6^$^{\#}$	**0.001**
BMI <40	58 (26.9%)	20 (35.7%)	32 (26%)	6 (16.2%)	0.076
BMI 40-50	106 (49.1%)	28 (50%)	62 (50.4%)	16 (43.2%)	
BMI >50	52 (24.1%)	8 (14.3%)	29 (23.6%)	15 (40.5%)	
METABOLIC DISEASE STATUS					
Type II diabetes mellitus	56 (26.0%)	8 (14.3%)	31 (25.2%)	17 (47.2%)	**0.020**
Hypertension	99 (46.0%)	26 (46.4%)	51 (41.5%)	22 (61.1%)	0.782
Pre-diagnosed dyslipidaemia	43 (20.1%)	14 (25.5%)	17 (13.8%)	12 (33.3%)	0.164
Obstructive sleep apnea	64 (29.6%)	14 (25%)	37 (30.1%)	13 (35.1%)	0.557
Metabolic syndrome parameters					
Waist circumference (male >102cm, female>88cm)	216 (100%)	56 (100%)	123 (100%)	37 (100%)	-
Triglyceride level (>1.7mmol/L)	86 (39.8%)	16 (28.6%)	47 (38.2%)	23 (62.2%)	0.063
High density lipoprotein (male >1.03, female >1.29)	171 (79.2%)	44 (78.6%)	94 (76.4%)	33 (89.2%)	0.885
Fasting BSL (<7)	73 (33.8%)	12 (21.4%)	39 (31.7%)	22 (59.5%)	**0.015**
Blood pressure (>135/80mmHg)	101 (46.8%)	27 (48.2%)	50 (40.7%)	24 (64.9%)	0.652
Metabolic health status					
MHO	18 (8.3%)	4 (7.1%)	13 (10.6%)	1 (2.7%)	0.553
Borderline MHO	73 (33.8%)	23 (41.1%)	44 (35.8%)	6 (16.2%)	
MAO	125 (57.9%)	29 (51.8%)	66 (53.7%)	30 (81.1%)	
BIOCHEMICAL VARIABLES					
Fasting glucose	6.1±2.5	5.4±1.1	6±2.3	7.1±4	**0.007**
Albumin	36.4±4.1	36±5^	36±4	38±4^	**0.027**
Bilirubin	9.5±5.1	9±5	9±5	11±6	0.098
ALT	33 (24-50)	25 (17 - 35)	33 (26 - 51)	46 (36 - 70)	**<0.001***
AST	27 (21-35)	22 (17 - 28)	27 (22 - 35)	34 (27 - 51)	**<0.001***
GGT	33 (20-45)	23 (18 - 37)	34 (21 - 42)	44 (34 - 88)	**<0.001***
ALP	73.9±25.1	72±21^	71±20$^{\#}$	86±39^$^{\#}$	**0.007**
Total cholesterol	4.2±1	4.1±1.1	4.1±1	4.3±1.1	0.639
HDL	1.0±0.3	1.1±0.3	1±0.3	0.9±0.2	0.072
LDL	2.5±0.8	2.5±0.9	2.4±0.9	2.5±0.8	0.751
Triglyceride	1.5±0.7	1.2±0.5^$^{\#}$	1.6±0.7^	1.9±0.8$^{\#}$	**<0.001**
HbA1c	5.7 (5.4-6.3)	5.6 (5.3 - 5.9)	5.7 (5.4 - 6.35)	6.35 (5.6 - 8.15)	**<0.001***
C-peptide	795 (588-1119)	646 (481 - 911)	810 (588 - 1119)	966 (706 - 1850)	**<0.001***
Insulin	7.6 (4.7-13.9)	5.5 (3.6 - 10.9)	7.2 (4.8 - 12.4)	12.3 (7.7 - 25.6)	**<0.001***
HOMA2 IR	1.0 (0.6-1.9)	0.76 (0.5 - 1.6)	0.95 (0.6 - 1.7)	1.91 (1.1 - 3.6)	**<0.001***
Haemoglobin	132.7±12.8	129.8±12	133.3±13	136±13.2	0.086
White cell count	7.5±2.3	7.1±2.3	7.5±2.3	7.8±2.5	0.315
Platelet	239.8±57.6	230±64	245±56	240±50	0.310

*Data shown as mean±standard deviation, median (interquartile range) and number (percentage). Independent student t-test and ANOVA used, unless specified. *Mann-Whitney U-test or Kruskal-Wallis test. $^{+}$Significant difference between all pairs. ^ or $^{\#}$ Significant difference in pairs. BMI – body mass index; MHO – metabolically healthy obese; MAO – metabolically abnormal obese; BSL – blood sugar level*

Table 6.2: Factors significantly related to more significant disease (NASH and steatofibrosis)

Variable		Any NAFLD	p-value	NASH/steatofibrosis	p-value
BMI category					
	<40 kg/m^2	38 (65.5%)	0.073	6 (10.3%)	**0.027**
	40-50 kg/m^2	78 (73.6%)		16 (15.1%)	
	>50 kg/m^2	44 (84.6%)		15 (28.8%)	
Individual metabolic risk factors					
T2DM	No	111 (69.8%)	**0.020**	19 (11.9%)	**0.002**
	Yes	48 (85.7%)		17 (30.4%)	
Hypertension	No	86 (74.1%)	0.947	14 (12.1%)	**0.047**
	Yes	73 (73.7%)		22 (22.2%)	
Dyslipidaemia	No	130 (76.0%)	0.250	24 (14.0%)	**0.030**
	Yes	29 (67.4%)		12 (27.9%)	
OSA	No	110 (72.4%)	0.378	24 (15.8%)	0.420
	Yes	50 (78.1%)		13 (20.3%)	
Metabolic syndrome score (ATP III)					
	1	14 (77.8%)	0.171	1 (5.6%)	**<0.001**
	2	50 (68.5%)		6 (8.2%)	
	3	31 (66.0%)		5 (10.6%)	
	4	40 (83.3%)		14 (29.2%)	
	5	25 (83.3%)		11 (36.7%)	
Metabolic health status					
	MHO	14 (77.8%)	0.407	1 (5.6%)	**0.007**
	Borderline MHO	50 (68.5%)		6 (8.2%)	
	MAO	96 (76.8%)		30 (24.0%)	

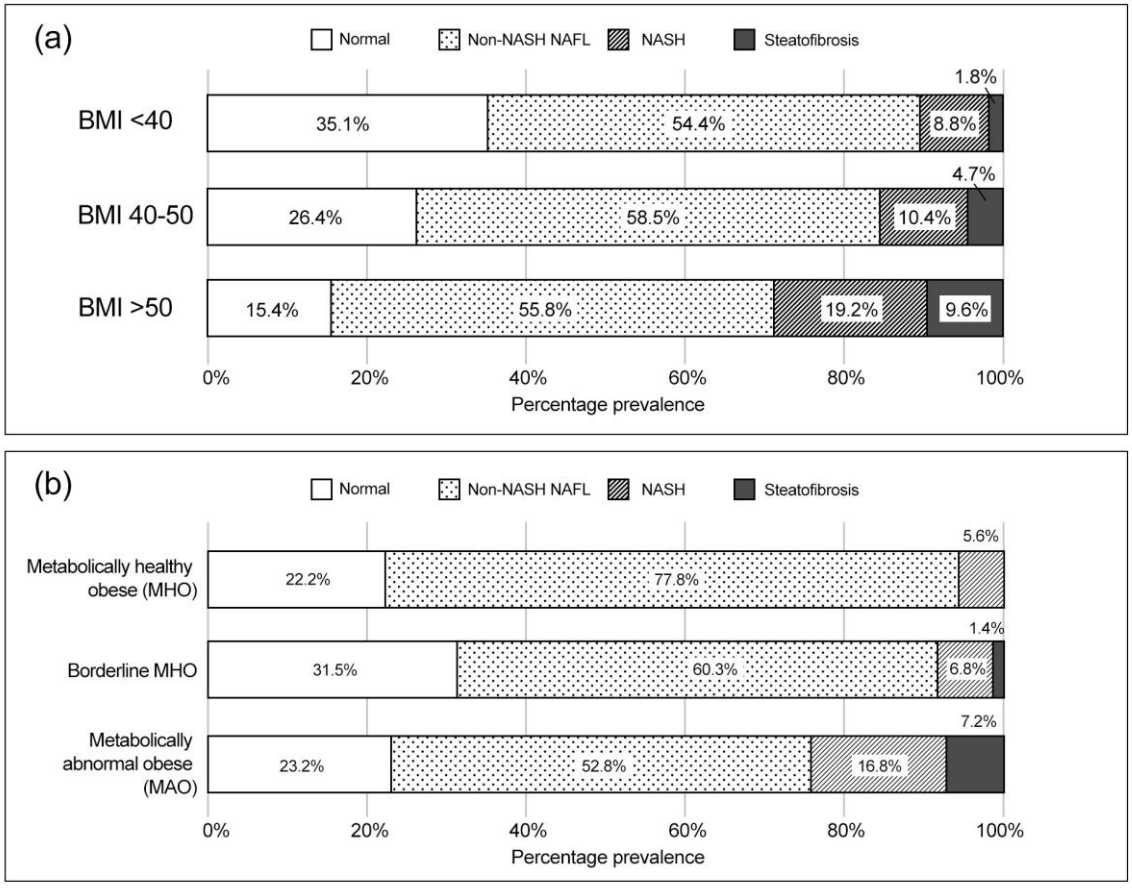
Percentage prevalence with number of participants in brackets. Significance testing by Chi-squared test. BMI – body mass index; T2DM – type II diabetes mellitus; OSA – obstructive sleep apnoea; ATP III – Adult treatment panel III; MHO – metabolically healthy obese; MAO – metabolically abnormal obese

Table 6.3: Odds ratio of having significant disease (NASH or steatofibrosis) in patients with differing body mass index, metabolic health status and a combination of both.

Variables		**Odds ratio (OR) (95% CI)**	**p-value**
Body mass index (BMI)			
BMI <40*	BMI 40-50	1.51 (0.56-4.10)	0.418
BMI <40*	BMI >50	3.46 (1.22-9.72)	**0.019**
BMI 40-50*	BMI >50	2.28 (1.02-5.08)	**0.044**
Metabolic health status			
MHO*	Borderline MHO	1.52 (0.17-13.5)	0.706
MHO*	MAO	5.37 (0.69-42.0)	0.110
Borderline*	MAO	3.52 (1.39-8.94)	**0.008**
MHO/borderline*	MAO	3.79 (1.58-9.08)	**0.003**
Metabolic health status and body mass index (BMI)			
MHO/borderline + BMI≤50*	MHO/borderline + BMI>50	3.19 (0.65-15.7)	0.154
	MAO + BMI≤50	3.40 (0.89-12.9)	0.073
	MAO + BMI>50	9.71 (2.83-33.3)	**<0.001**

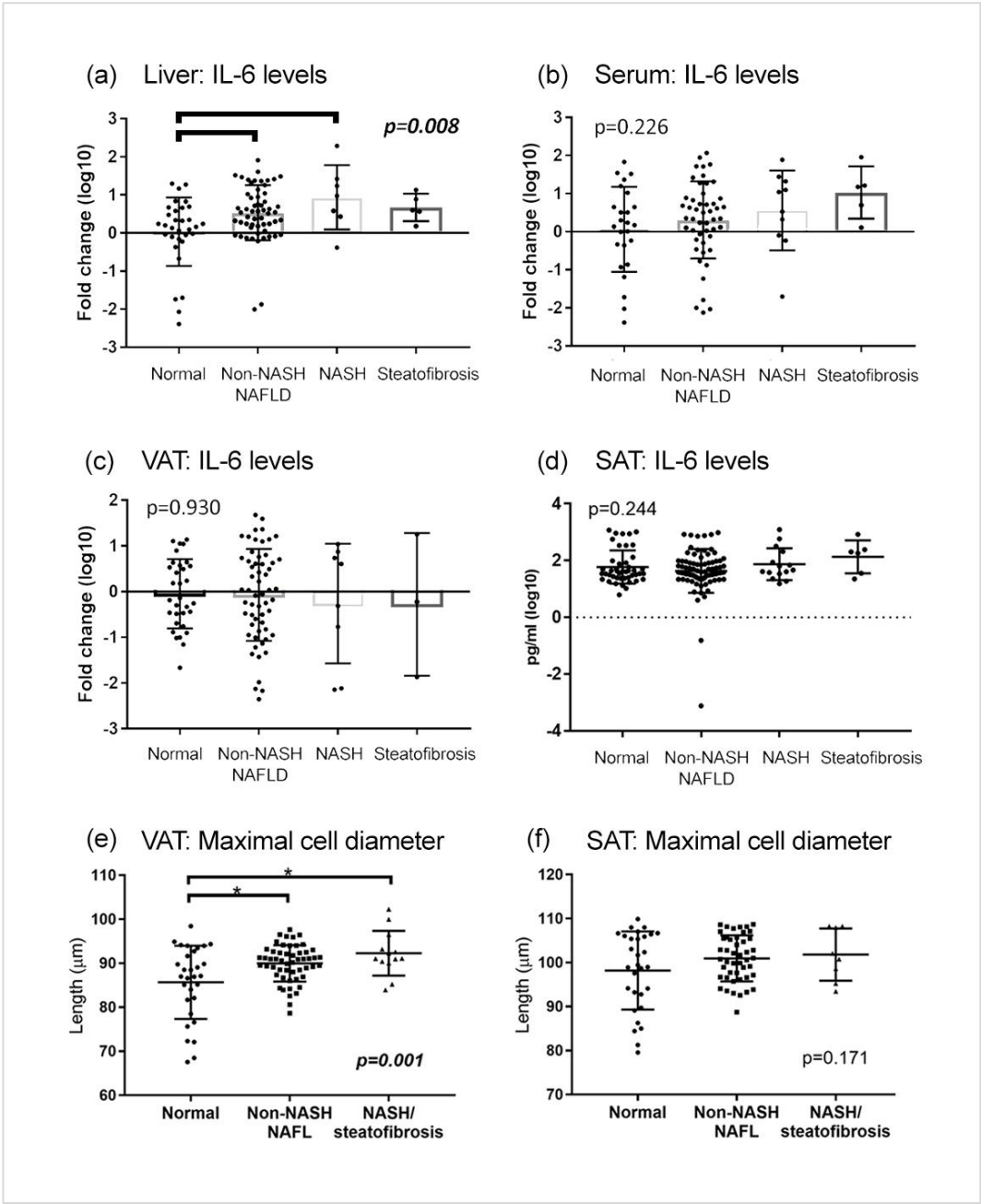
**reference group*
NASH – nonalcoholic steatohepatitis; BMI – body mass index; MHO – metabolically healthy obesity; MAO – metabolically abnormal obesity

Figure 6.1: (a) Prevalence and severity of NAFLD with Class I (BMI 30-40), Class II (BMI 40-50) and Class III obesity (BMI >50), and (b) prevalence and severity of NAFLD with metabolic health status.



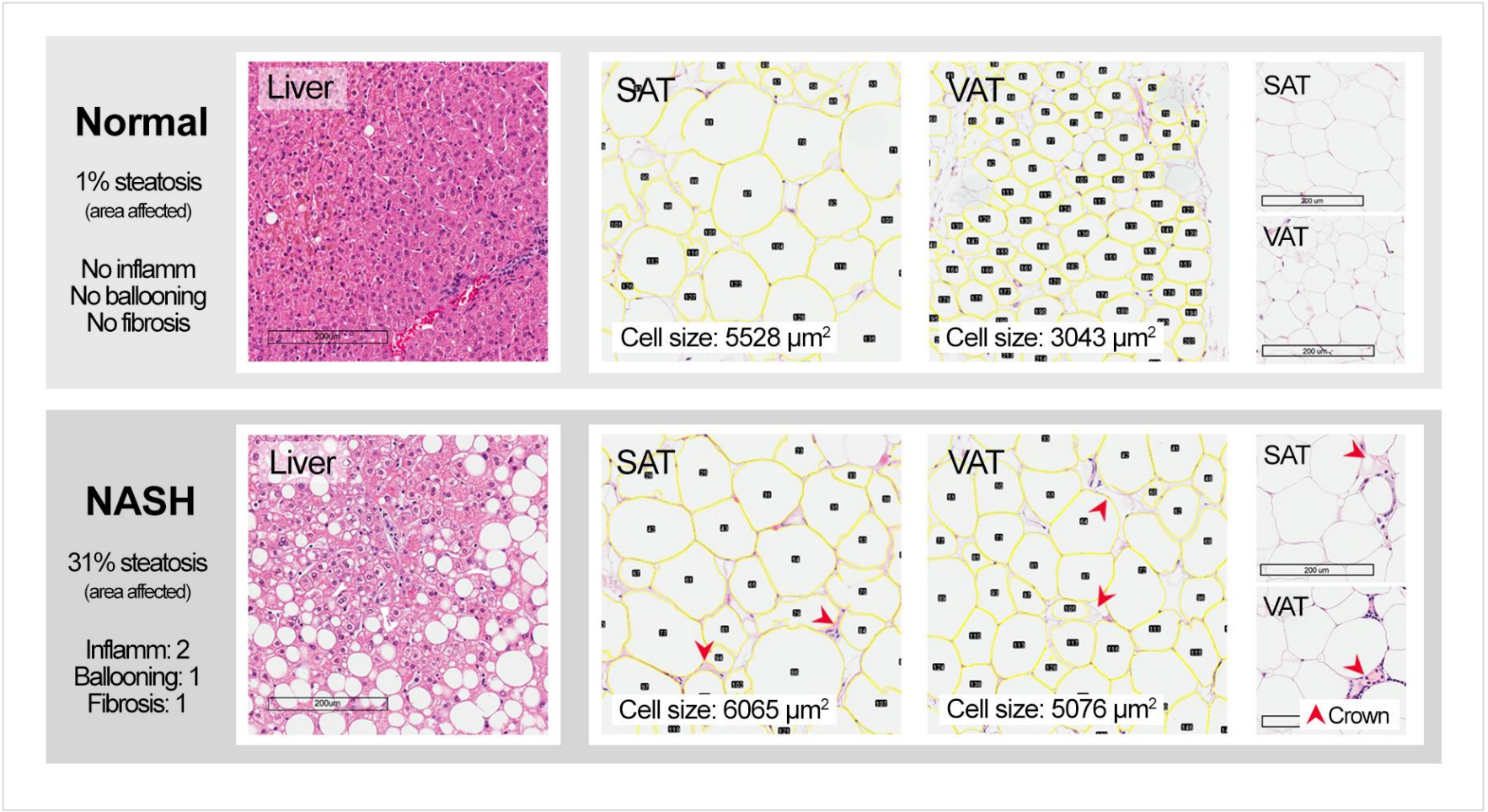
BMI – body mass index; NASH – nonalcoholic steatohepatitis; non-NASH NAFL – nonalcoholic fatty liver disease but not NASH; MHO – metabolically healthy obese; MAO – metabolically abnormal obese

Figure 6.2: Changes in interleukin 6 (IL-6) in (a) liver, (b) serum, (c) visceral adipose tissue (VAT) and (d) subcutaneous adipose tissue (SAT), and changes adipocyte characteristics in (e-f) visceral adipose tissue.



SAT – subcutaneous adipose tissue; VAT – visceral adipose tissue; IL-6 – interleukin-6; NASH – nonalcoholic steatohepatitis; NAFLD – nonalcoholic fatty liver disease

Figure 6.3: Liver and adipose tissue characteristics in a patient with normal liver histology versus nonalcoholic steatohepatitis (NASH). This shows increased levels of steatosis and inflammation on liver histology, and visceral adipose tissue cell hypertrophy, associated with signs of adipocyte death ("crown-like" structures).



6.5 Discussion

In this prospective bariatric surgical cohort with histologically-defined NAFLD, we have shown that liver steatosis is exceedingly common in severely obese patients, with around three quarters of individuals affected. However, despite significant metabolic disease burden and substantial obesity in this population, strikingly lower overall rates of NASH (12.0%) and steatofibrosis (5.1%) were observed, in comparison to previously published data. Both increasing body mass index (BMI) and metabolic health status independently affected the risk of having NASH/steatofibrosis (OR 2.3-3.8). The risk was markedly increased when both were present, with almost ten times the odds of NASH/steatofibrosis (OR 9.7, $p<0.001$). We were unable to demonstrate that adipose tissue inflammation mediated risk of NASH/steatofibrosis, however differences in adipocyte size may indicate underlying contribution of VAT to liver disease.

One possible explanation for the surprisingly lower rates NASH and steatofibrosis is the inherent differences in study setting, design and recruitment strategy. Although our results differed from some previous reports (10, 11, 486), our rates were similar to those reported by Lassailly *et al* (12), with a NASH rate of 7.3% in a cohort of bariatric surgical patients who similarly had routine liver biopsies. Many other studies with histologically-defined NAFLD have enrolled pre-selected patients via liver clinics or with substantially abnormal aminotransferases (8, 10, 11, 486). Such selection bias would increase the prevalence of severe disease. By including consecutive bariatric surgical patients in a prospectively registered clinical trial, our population more likely reflects the general obese population, and this may account for the lower, but potentially more representative, prevalence of severe disease.

Several observations can be made from these data regarding the influence of obesity and metabolic disease on NAFLD severity. Firstly, any degree of NAFLD (i.e. >5% steatosis) appears to be widespread in obesity, and not significantly affected by degree of obesity or metabolic risk factors. The majority of these patients have simple steatosis, without inflammation or fibrosis. Evidence suggests that simple steatosis has very little hepatic sequelae, and the clinical importance of identifying simple steatosis in obesity is not clear (248, 250).

More significant disease, such as NASH and/or steatofibrosis, is substantially and independently influenced by both obesity severity and metabolic disease. The odds for NASH/steatofibrosis are more than threefold with greater BMI, and nearly fourfold for metabolically abnormal individuals. Being both metabolically unhealthy as well as being super obese (BMI>50) compounds this risk, with a 10-fold increased risk of having NASH/steatofibrosis. This is of significant importance, as NASH and fibrosis are associated with progression of liver disease and liver-related mortality (246, 247, 264).

The effects of obesity and obesity-related metabolic disease may contribute to the wide variation in reported prevalence of NASH and steatofibrosis within obese populations. Whilst these factors have been associated with NAFLD and NASH (232), the impact of degrees of obesity and metabolic disease severity has not previously been assessed in a prospective histologically-defined cohort. This knowledge is vital for stratifying NASH risk, particularly in exclusively obese and bariatric surgical cohorts.

We did not find a convincing correlation between adipose tissue or systemic inflammation and NAFLD. This is in contrast to other groups, who have shown that increased VAT cytokine expression and adipose tissue macrophages are associated with liver inflammation and damage (493, 495, 496). We did find that VAT cellular hypertrophy was evident in states of NASH as well as non-NASH NAFLD, compared to normal. Cellular hypertrophy occurs when adipose tissue depots stored more lipids (102) and may also reflect a reduced capacity for adipogenesis, that is, the production of new adipocytes. These changes may lead to venous drainage of lipotoxic by-products and adipokines into the portal circulation, although direct support for this hypothesis is currently lacking. Overall, the lack of strong associations between adipose inflammation and NAFLD likely indicates the complexities that underlie NAFLD development and progression in obesity (355).

These data have substantial clinical application. It highlights the burden of NAFLD in the bariatric surgical population and reinforces the need to consider the possibility of undiagnosed liver disease and counsel patients appropriately. This study emphasises the importance of obesity severity and obesity-related factors in determining likely risk of more significant disease. Factors such as increased BMI, particularly above 50 kg/m^2 and those with metabolic syndrome, independently contribute to a greater risk. A combination of both risk factors should alert clinicians to the greatly increased odds of disease, and therefore the necessity for pre-operative work-up and consideration of intraoperative liver biopsy.

A significant strength of this study was the large number of well-documented patients, and the prospective nature of the study. Additionally, we have used the gold standard, liver biopsy to accurately characterise NAFLD, and correlated this histological assessment with clinical and inflammation data. Finally, we focused on the effects of increasing BMI and degrees of metabolic disease on NAFLD. Existing studies often comment on obesity in general, but few stratify by increasing obesity severity. These data are particularly important to bariatric surgeons and physicians, where the patient population is exclusively obese and vary by obesity class, rather than presence or absence of obesity.

This study has some drawbacks that warrant discussion. Firstly, the prevalence of NASH and steatofibrosis was lower than anticipated. This, in itself, is a noteworthy observation, but has diminished our statistical power to find associations with severe disease. Secondly, there was a low prevalence of metabolically healthy obese individuals, compared to the metabolically abnormal and borderline patients. This a potential cause for the lack of statistically significant differences between MHO cohort alone, and borderline or MAO patients. A larger study that captures more MHO patients would strengthen this analysis and could provide adequate power to demonstrate any significant differences in this group. Thirdly, we did not stratify for body composition. In particular, visceral fat deposition has been associated with increased metabolic disease, and specific measurement of this depot could be the focus of a future endeavour. Finally, we have targeted a few of the most prominent inflammatory markers associated with meta-inflammation. Future studies could analyse a wider range of cytokines.

In conclusion, this study provides significant insights into the impact of obesity, metabolic disease and inflammation on NAFLD in the obese. Whilst NAFLD was found to be highly prevalent in obesity, more severe NAFLD (i.e. NASH and steatofibrosis) is less common than previously reported. Increasing obesity and metabolic disease both independently increase the risk of NASH and steatofibrosis, with a substantially compounded effect when both are present. Whilst previous studies have shown significant correlation of inflammation with NAFLD, we failed to demonstrate this relationship, which could be due to underlying complexities in this interaction. Clinicians should be aware that increasing obesity

independently increases the risk of more severe NAFLD, and the additional presence of metabolic disease should raise suspicion for NASH and fibrosis.

Research Theme 2

Challenges of diagnosing NAFLD in obesity

Studies 2-6

Unlike other obesity-related metabolic disorders such as diabetes or dyslipidaemia, there are no reliable diagnostic tests for NAFLD and NASH in obesity. Whilst tests exist for NAFLD, many have not been validated for use in exclusively obese cohorts. Yet obesity is associated with clinical, biochemical and physiological changes that can affect diagnostic accuracy and feasibility (17, 327).

Many bariatric surgical patients currently go without screening or diagnosis. This is the case even after surgery, when intraoperative liver biopsy may have been performed with relative ease. Therefore, the opportunity to diagnosis, and subsequently manage and monitor disease is missed.

Identifying those with NAFLD and NASH is important for several reasons. Firstly, NAFLD is a key determinant of metabolic health. The presence of NAFLD predicts the development of type II diabetes mellitus, and is an independent risk factor for atherosclerosis and cardiovascular disease (244). Secondly, detection of early stages of NAFLD allows institution of simple measures that can prevent progression (180). Finally, as with other liver disease, identification of significant NASH and fibrosis is the first step in providing specialist hepatology care and ongoing monitoring for complications of liver failure and cirrhosis.

This research theme aimed to evaluate the accuracy of diagnostic techniques for NAFLD in obese populations and determine methods to improve risk stratification. The overall aim is to enhance our ability to identify NAFLD in a bariatric surgical cohort.

7 Systematic review and meta-analysis: Non-invasive detection of nonalcoholic fatty liver disease related fibrosis in the obese

7.1 Abstract

BACKGROUND: Nonalcoholic fatty liver disease (NAFLD) is a significant disease burden in obesity. Liver fibrosis is an important prognostic factor in NAFLD, and detection is vital. The pathophysiological changes of obesity can alter the accuracy of non-invasive NAFLD tests. We aimed to review current evidence for common non-invasive tests for NAFLD-related fibrosis in obesity.

METHODS: We systematically searched for studies assessing the diagnostic accuracy of 11 biomarker panels and elastography techniques for NAFLD-related fibrosis in obesity. Meta-analyses were performed where possible.

RESULTS: Thirty-eight studies were identified assessing the selected tests in obese populations. Simple biomarker panels (e.g. NAFLD fibrosis score (NFS)) were the most validated. Evidence showed better accuracy of complex biomarker panels (NFS: SROC 0.795-0.813 vs. ELF: SROC 0.962), however these were poorly validated in obesity. Elastography techniques were better studied, and had high diagnostic accuracy (transient elastography: SROC 0.859; magnetic resonance elastography: SROC 0.965), but were limited by BMI-dependent failure. Limited evidence was found to validate the accuracy of any test in exclusively obese populations.

CONCLUSION: In obese subjects, complex biomarker panels and elastography have reasonable to good accuracy for NAFLD-related fibrosis, however these methods have not been well validated. Further study in this high-risk population is needed.

7.2 Introduction

Obesity, especially coupled with insulin resistance, is a significant risk factor for the development and progression of nonalcoholic fatty liver disease (NAFLD). Therefore, it is not surprising that NAFLD is endemic in obese populations, affecting up to 90% of patients with a body mass index (BMI) greater than 30kg/m^2 (8). The more severe form, non-alcoholic steatohepatitis (NASH), is present in 36-67% of patients with obesity, and advanced fibrosis in up to 30% (232, 501).

Liver fibrosis, in particular, is an important prognostic factor for patients with NAFLD. Fibrosis stage, regardless of steatosis or inflammation status, has been shown to be the only independent histological variable associated with overall and liver-related mortality (246). Even early fibrosis (F1) has been significantly associated with death or liver transplantation (hazard ratio 1.88) (246).

Liver biopsy remains the gold standard for diagnosing non-alcoholic fatty liver disease, and staging fibrosis (227). However, it is an invasive procedure with inherent risks including up to 0.1% incidence of significant complications, and 0.01% risk of mortality. Moreover, other major limitations of liver biopsy include inter-observer variability, sampling error, and cost (227). These factors make it a poor screening tool for the sizeable at-risk population.

Non-invasive methods for assessment of liver fibrosis have been used with increasing frequency in clinical practice. The two categories of commonly utilised diagnostic techniques are those based on serum markers and those based on elastography (**Section 3.6.3 – Non-invasive tests**) (502). Most of these modalities were developed and validated primarily in studies of viral hepatitis patients, however disease specific biomarkers have also been trialled in NAFLD with varying success.

Whilst many of the common diagnostic techniques have been widely tested in general NAFLD populations, few studies have focused on the high risk, yet unselected, obese population. This is important, as morbid obesity represents a very different metabolic, biochemical and physical state to general populations (503). In addition, BMI is a particularly important consideration when the feasibility of elastography techniques is assessed, as the accuracy and success rates are lower in the obese (327). Furthermore, studies often include populations selected due to abnormal serum aminotransferase levels, which excludes the

known significant proportion of patients with liver disease who have aminotransferase levels within the normal range (229).

The objective of this systematic review and meta-analysis was to gather and evaluate the current evidence available on the accuracy of non-invasive techniques for the diagnosis of NAFLD-related fibrosis, focusing on the obese. A description of all chosen tests can be found in the **Section 3.6.3 – Non-invasive tests**. We aimed to assess tests that are more commonly used in clinical practice, particularly the NAFLD fibrosis score (NFS), Enhanced Liver Fibrosis score (ELF), Transient Elastography (TE) and Magnetic Resonance Elastography (MRE) (502).

7.3 Methods

For this systematic review, we focused on the most commonly available and widely used non-invasive tests (502) including: 1. Biomarker panels: NAFLD fibrosis score (NFS), enhanced liver fibrosis (ELF) score, BARD score, FIB4 score, FibroMeter, Fibrotest, Hepascore; and 2. Elastography techniques: Transient elastography (TE), magnetic resonance elastography (MRE), shearwave elastography (SWE), acoustic radiation force impulse (ARFI).

7.3.1 Search methodology

Two independent reviewers (GO, SM) performed a comprehensive literature search to identify studies on non-invasive diagnosis of NAFLD-related fibrosis in obese patients, according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) statement for the conduct of meta-analyses of observational studies. We searched MEDLINE (1946 to November 2016), EMBASE (1974 to November 2016), Science Citation Index (until November 2016), and the Cochrane Library (until November 2016). Our primary search strategy comprised variations of text words as well as MeSH terms for each test of interest, NAFLD (*nonalcoholic fatty liver disease, nonalcoholic steatohepatitis*) AND liver biopsy (*biopsy, histology, histopathology*). The search and study selection were limited to English language. An additional MEDLINE search was performed for existing systematic reviews. Manual searching of reference lists from reviews, as well as references from selected primary studies was performed to identify additional studies.

7.3.2 Study selection and eligibility criteria

For the systematic review studies had to meet all of the following inclusion criteria: a) liver biopsy as the reference; b) assessment of the index test against reference standard; c) ≥18 years old; 4) ≥20 participants with NAFLD; d) published full-length manuscripts; and e) focus on obesity, with either i) recruitment of patients with BMI≥ 30kg/m^2 ("obese-only" study)**,** or ii) studies with an average BMI ≥30kg/m^2 ("average-obese" study). Exclusion criteria were: a) non-English based publications; and b) animal studies. Both prospective and retrospective studies were included. Studies with multiple aetiologies were accepted if data were separable. Additionally, studies including patients with and without liver biopsy, but where data for patients with liver biopsies were separable, were included.

7.3.3 Data extraction and meta-analysis

Both reviewers performed data extraction independently. Any disputes were resolved through consensus. Information extracted included study data (year, country, design, study size), demographic data, recruitment setting including selection from exclusively obese ("obese only") populations, prevalence of fibrosis, and the index tests and their details. Measures of diagnostic accuracy were extracted for each index test, each level of fibrosis diagnosed, and each threshold used. The studies included in the systematic review were scrutinised, and 2x2 tables and the area under the receiver operator curve (AUROC) for each index test were extracted. Where 2x2 tables could not be formed from published information, efforts were made to contact the relevant corresponding authors for study-level data. Corresponding authors were contacted three times via email and if these efforts failed, the studies were excluded from the meta-analysis.

7.3.4 Quality assessment

Review-specific criteria were developed for the QUADAS-2 tool for quality assessment of diagnostic accuracy studies (**Supplementary Table 7.1**). Two reviewers independently assessed the quality of included studies.

7.3.5 Data analysis

The weighted mean pooled sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), diagnostic odds ratio (DOR), and their 95% confidence intervals (CIs) were calculated using the DerSimonian and Laird random effects model because of

anticipated heterogeneity. Random effects modelling takes into account both within-study and between-study variation. To correct for any continuity errors, 0.5 was added to all cells with a frequency of 0 in order to calculate the pooled estimates using the Yates correction method (504). Summary receiver operating characteristics (SROC) curves were fitted using the Moses-Shapiro-Littenberg method, and the area under the receiver operator characteristic curve (AUROC), Q* index, and their respective standard errors were estimated. The Spearman's correlation coefficient was calculated to assess for threshold effect, and variability between individual studies was evaluated by plotting the diagnostic accuracy estimates on a forest plot. Heterogeneity was quantified using the I^2 index, I^2 values of 25, 50 and 75% corresponded to low, moderate and high degrees of heterogeneity respectively. Publication bias was assessed via Deeks funnel plots and associated regression test of asymmetry (505). Statistical analyses were performed using Meta-Disc (version 1.4, Unit of Clinical Biostatics, Ramon y Cajal Hospital, Madrid, Spain), GraphPad Prism (version 6.0, GraphPad Software, San Diego, CA), and Microsoft Excel (version 14.2.0, Microsoft, 2011).

7.4 Results

7.4.1 Literature search

Using the search strategy as described, we identified 1505 studies. After removal of duplicates, we screened titles and abstracts of 1084 studies. Ninety full text articles were assessed for inclusion by the review criteria specified. Fifty-two studies were excluded due to inseparable data from mixed aetiology studies (n=9), and having a mean study BMI<30kg/m^2 (n=43). Therefore, thirty-eight studies were included in the systematic review (**Figure 7.1**).

7.4.2 Study characteristics

The characteristics of the included studies are shown in **Table 7.1**. Study size varied from 28 to 827 patients. Most studies recruited from Liver disease setting ("*average-obese"* study), with only twelve studies recruiting specifically from settings where only obese patients are treated ("*obese-only"* study). These *obese-only* settings included Bariatric or Obesity clinics (327, 483, 484, 503, 506-513). Demographics and baseline characteristics of *obese-only* cohorts were substantially different from *average-obese* cohorts (**Table 7.2**). *Obese-only*

cohorts were larger, generally younger, with a higher proportion of females, lower rates of advanced fibrosis and lower average ALT levels.

The number of studies that assessed each test varied, from the most validated test with 22 studies (NAFLD fibrosis score) to only one study (SWE) (**Supplementary Table 7.2a**). There were far fewer *obese-only* studies (**Supplementary Table 7.2b**). Cut-off histological fibrosis level varied, with assessment of *any fibrosis* (F1-4), *significant fibrosis* (F2-4), *advanced fibrosis* (F3-4), and *cirrhosis* (F4). The most commonly assessed fibrosis grade was *advanced fibrosis* (F3-4).

There were two pairs of studies with a proportion of patient overlap (514-517), however with some data examining different diagnostic techniques. These were included in the systematic review, but data for similar tests and fibrosis levels were excluded in the meta-analysis.

Publication bias was assessed with Deeks' funnel plots and associated regression test for asymmetry (505), for tests assessing seven or more studies (**Supplementary Figure 7.1**). This showed the potential for publication bias in some tests including the BARD (p=0.009), FIB4 (p=0.016) and transient elastography (p=0.046).

7.4.2.1 Patient recruitment and selection

The included studies consisted of 22 prospective studies, 14 retrospective studies, one mixed prospective/retrospective recruitment and one with unclear recruitment (**Table 7.1**).

Twenty-three studies assessed diagnostic accuracy in a population with confirmed histological diagnosis of NAFLD, excluding patients with <5% steatosis. Fifteen studies (317, 319, 327, 484, 503, 507, 510-513, 518-522) included all recruited patients in the final analysis, regardless of histological NAFLD diagnosis. Nine of these recruited patients from *obese-only* cohorts. Five bariatric surgical studies reported liver biopsies on all bariatric surgical patients (484, 503, 507, 511, 513). In all studies, one or two dedicated pathologists scored all liver biopsies. All studies reported using the NASH Clinical Research Network histological criteria and Kleiner or Brunt classification of fibrosis. Common indications for inclusion in many studies were the use of abnormal aminotransferase levels or ultrasound scans for referral to the recruitment setting. All studies reported the exclusion of other causes of liver disease prior to diagnosis of NAFLD. Additional study exclusions were prolonged time interval between biopsy and diagnostic test, or poor quality tests.

7.4.2.2 Methodological quality and risk of bias in included studies

Quality assessment via the QUADAS-2 criteria is seen in **Figure 7.2**. Studies not recruited from an *obese-only* population were considered to be of high applicability concern due to inclusion of non-obese patients within the final analysis. Additionally, a significant number of studies used non-conventional threshold values that were not pre-specified nor validated (**Supplementary Figure 7.2-7.5**), creating substantial variation in test application between studies. Blinding was specified in 22 studies (313, 317, 319, 327, 484, 503, 506-508, 510, 511, 514, 517-520, 522-527)

7.4.3 Diagnostic test accuracy for all included studies

Using available 2x2 tables, forest plots for diagnostic accuracy were created (**Supplementary Figure 7.2-7.5**), with pooled meta-analysis results are reported in **Table 7.3** (sensitivity, specificity and diagnostic odds ratio (DOR)) and **Supplementary Table 7.3a-b** (positive (LR+) and negative likelihood ratios (LR-)). Summary receiver operator characteristic curves (SROC) shown in **Figure 7.3**, **Figure 7.4** and **Supplementary Figure 7.6**.

The most commonly used and widely available simple biomarker (NAFLD fibrosis score), complex biomarker (ELF) and elastography techniques (TE, MRE) are discussed below. Similarly, the greatest evidence was available for distinguishing ≥F3 fibrosis (advanced fibrosis), and are discussed in greater detail below. Full details for all other tests and for all other levels of fibrosis are available in the **Appendix 2: Supplementary Text**.

7.4.3.1 Biomarker panels

NAFLD fibrosis score (NFS)

Twenty-two studies determined the diagnostic accuracy of the NAFLD fibrosis score in obese populations. For ≥F3, the AUROC ranged from 0.615-0.850, with sensitivity 23-100% for a low threshold (NFS<-1.455, -1.31 or unstated), and specificity 31-100% for a high threshold (NFS>0.676 or unstated). For studies reporting dual thresholds, the indeterminate fraction varied from 13% (523) to 52% (510), with an average of 34.5%. The pooled sensitivity and specificity for a high threshold were 60.2% (56.7-63.7%) and 92.4% (91.1-

93.5%) (**Table 7.2**). For a low threshold, the sensitivity and specificity were 78.6% (75.5-81.4%) and 62.2% (60.3-64.0%). The heterogeneity for these pooled values was considerable (I^2: 45.5-94.5%). The pooled area under the SROC curve was 0.813 (standard error 0.053) and 0.795 (0.020) for high and low threshold respectively (**Figure 7.3**).

The results for ≥F1, ≥F2 and F4, corresponding to mild fibrosis, significant fibrosis and cirrhosis respectively, are seen in **Table 7.3**.

Enhanced Liver Fibrosis (ELF)

Three studies assessed the diagnostic accuracy of the ELF in the obese. For ≥F3, two studies showed an AUROC 0.900-0.973, sensitivity 80-93% and specificity 90-97%. The thresholds used differed significantly (-3.37 to 0.358). Pooled sensitivity and specificity were 84.8% (75.0-91.9%) and 91.6% (87.1-94.8%) (**Table 7.3**). The SROC was 0.962 (0.040), with good consistency in diagnostic accuracy between these two studies (I^2: 14.8-31.8%) (**Figure 7.4**). Pooled results for ≥F1 and ≥F2 are seen in **Table 7.3**.

7.4.3.2 Elastography modalities

Transient elastography (TE)

Nine studies examined transient elastography in the obese with NAFLD. For ≥F3, AUROC was 0.831-0.938, with sensitivity 57-100% and specificity 61-90%. The threshold values used varied significantly from 7.6-12.5kPa. Pooled sensitivity, specificity and DOR were 82.7% (78.7-86.2%), 72.1% (68.9-75.2%) and 16.6 (10.1-27.3). The SROC was 0.859 (0.017) with moderate to high heterogeneity (I^2: 32.5-89.6%) (**Figure 7.4**). There were three studies assessing detection of F4 showing good accuracy, with AUROC 0.870-0.950, sensitivity 65-100% and specificity 76–91% (threshold 7.9-16.1). Pooled sensitivity and specificity were 81.8% (72.2-89.2%) and 78.3% (74.5-81.8%). The pooled results for ≥F2 are seen in **Table 7.3**.

Failure rate related to obesity was reported in seven studies, with the remainder excluding patients based on failed readings (**Supplementary Table 7.4**). Myers *et al* reported an overall low failure rate of 1.1% in a mixed aetiology cohort. In this study, failure rates related to BMI, being 4.9% with a BMI ≥40kg/m^2. Naveau *et al* reported a substantially higher failure rate of 11.1%. Patients with failed readings were significantly larger than those with successful readings (47.5 vs. 42.0, $p<0.005$). Both studies utilised the XL probe. The

difference in failure rate could be accounted for by the difference in average study BMI (30.0 vs. 42.3 kg/m^2).

Magnetic resonance elastography (MRE)

Four studies assessed the diagnostic accuracy of MRE. For ≥F3, the AUROC was 0.924–0.957. Variable threshold values (3.62–4.15kPa) were used to calculate a sensitivity 82–95% and specificity 89–93%. The pooled SROC was 0.965 (0.015) (**Figure 7.4**). Pooled sensitivity and specificity were 87.0% (79.2-92.7%) and 91.5% (88.3-94.1%) giving it excellent diagnostic accuracy for detection of advanced fibrosis, with little heterogeneity (I^2 0.0-22.7%). The mean BMI in these studies were mainly in the low thirties (31.7-34.8 kg/m^2). Pooled data for ≥F1, ≥F2 and ≥F4 are seen in **Table 7.3**.

7.4.4 Subgroup analysis of obese-only studies

Only 12 studies done in exclusively obese cohorts (**Supplementary Table 7.2b**). Pooled analyses for *obese-only* studies were not performed, due to low study numbers. Fibrometer, Hepascore, SWE and MRE were not assessed by any *obese-only* studies.

NAFLD fibrosis score (NFS)

The AUROC for differentiating ≥F3 was available for six studies, ranging from 0.615–0.776. Using a high threshold, specificity and sensitivity varied significantly, from 33–98% and 20–91% respectively. A low threshold had sensitivity 87–100% and specificity 24–80%.

ELF

Only one study (n=41) reported the diagnostic accuracy for ELF at a level of ≥F2. An optimum cut-off of 9.920 was used, producing a sensitivity of 100% and specificity of 87%.

Transient elastography

Three studies assessed the diagnostic accuracy of TE, however one study had a 2.4% prevalence of ≥F2 fibrosis (1 of 41 patients). The two remaining studies had an AUROC of 0.850-0.900, sensitivity 100% and specificity 74% for ≥F3 fibrosis (threshold 7.6kPa). For ≥F2, the AUROC was 0.810–0.850, with sensitivity 73–81% and specificity 65–78% (threshold 6.4–7.6kPa).

Table 7.1: Characteristics of included studies.

	Setting	Study type	Country	n=	Age	%male	BMI	%F3/4	ALT	Tests
Adams 2011 (313)	Liver clinic	Prospective	Australia, Italy	242	46.8±12.4	60.3%	30.2	21.9%	66.5	BARD, FIB4, Hepascore, Fibrotest
Angulo 2007 (310)	Liver clinic	Prospective	US, Italy, UK, Australia	733	47.7±13.2	53%	32.2	27.2%	87±72	NFS
Aykut 2014 (528)	Liver clinic	Prospective	Turkey	88	46±9	56.8%	30.3	30.7%	84±56	NFS, Fibrotest, TE
Boursier 2016 (526)	Liver clinic	Prospective	France	452	55.9±12	60%	31.1	38.0%	68±39	NFS, BARD, FIB4, Hepascore, Fibrotest, Fibrometer, TE
Cassinotto 2016 (520)	Liver clinic	Prospective	France	291	56.7±12	59.1%	32.1	43.3%	71.2±50.7	TE, ARFI
Cui 2015^ (517)	Liver clinic	Prospective	USA	102	51.3±14	41.2%	31.7	18.6%	58.0±56.1	NFS, BARD, FIB4, MRE
Cui 2016 (514)	Liver clinic	Prospective	USA	125	48.9±15.4	45.6%	31.8	16.8%	56.4±51.8	ARFI, MRE
Demir 2013 (523)	Liver clinic	Prospective	Italy	267	43.8±21.1	47.2%	37	8.2%	56.6±55.2	NFS, BARD, FIB4
Dincses 2015 (529)	Liver clinic	Retrospective	Turkey	52	45±9	57.7%	30.8	19.2%	89±58	NFS, Fibrometer, TE
Dvorak 2014 (519)	Liver clinic	Prospective	Turkey	56	48.9±14.9	70.5%	31.2	30.4%	135±96	NFS, BARD, FIB4, ELF
Ergelen 2015 (521)	Liver clinic	Prospective	Turkey	87	45.8±9	49.4%	30.6	21.8%	77.8±56.1	TE
Ergelen 2016 (527)	Liver clinic	Prospective	Turkey	63	47.1±8.4	61.9%	30.4	50.8% (≥F2)	-	TE
*Francque 2012 (483)	Obesity clinic	Prospective	Belgium	313	43.5±12.7	28.6%	38.6	7.2%	43.3±22.1	NFS, BARD, FIB4
Guha 2008 (317)	Liver clinic	Prospective	UK	196	48.7±12.5	64%	32.4	23%	77.3±57.8	ELF
Harrison 2008 (312)	Liver clinic	Retrospective	USA	827	49 (17-95)	49%	33	30%	69	NFS, BARD
*Karlas 2015 (511)	Bariatric surgical	Prospective	Germany	41	45.7±10.2	32%	46.8	2% (≥F2)	-	ELF, TE, ARFI
Kim 2013 (522)	Liver clinic	Retrospective	USA	142	52.8±12.8	40.3%	34.8	32.4%	70.6±61.0	NFS, FIB4, BARD, MRE
Kruger 2011 (530)	Liver clinic	Uncertain	South Afr	111	52 (50-54)	27%	35	17%	-	NFS
*Lassailly 2011 (484)	Bariatric surgical	Prospective	USA	288	41.6 ±12.8	23.6%	48.6	2.4%	34±23	Fibrotest
Lee 2013 (531)	Liver clinic	Retrospective	USA	107	48.9 (40.9–50)	38.3%	35.9	31.8%	63 (29-105)	BARD
Loomba 2014 (524)	Liver clinic	Prospective	USA	117	50.1±13.4	43.6%	32.4	18.8%	66.3±54.4	MRE
McPherson 2010^ (516)	Liver clinic	Prospective	UK	145	49.3	61%	35	19%	94±63	NFS, BARD, FIB4
McPherson 2013 (515)	Liver clinic	Retrospective	UK	305	51±12	56%, 63%	35	24%, 17%	28±9, 95±62	NFS, BARD, FIB4
*Myers 2012 (506)	Liver clinic, obese only	Prospective	Canada	75	50 (43-57)	63%	30	29.3%	55 (36–87)	TE
*Nassif 2016 (512)	Bariatric surgical	Retrospective	Brazil	298	40.1	11.1%	43.6	91.5% (≥F1)	-	BARD
*Naveau 2014 (327)	Bariatric surgical	Prospective	France	100	42.5 (SEM 0.5)	19%	42.3	9%	38±29.5	TE
*Ooi 2016 (503)	Bariatric surgical	Prospective	Australia	101	49 (37-54.5)	33.7%	41.9	3.0%	32 (22-42)	NFS, BARD, FIB4
Palmeri 2011 (532)	Liver clinic	Mix	USA	135	-	37.8%	>30	29.6%	-	ARFI
*Pimentel 2010 (507)	Bariatric surgical	Retrospective	Brazil	158	36±10	22.8%	41	14%	55 (36–87)	NFS
*Praveenraj 2016 (513)	Bariatric surgical	Prospective	India	28	48.3	-	49.6	28.6%	21.9	ARFI
*Qureshi 2008 (508)	RYGB patients	Retrospective	USA	331	40.5±9	17%	48.5	13.6%	29.1±15.7	NFS
Ratziu 2006 (319)	Liver, inpatient	Prospective	France	267	51.2	58%	>27	18.8%	71±3, 79±5	Fibrotest
*Rodriguez 2009 (509)	Bariatric surgical	Retrospective	France	88	40.6±11.3	NA	52.7	5.5%	34.3±28.8	NFS
Ruffillo 2011 (533)	Liver clinic	Retrospective	Argentina	138	49 (38–57)	48.6%	30.3	26.8%	69 (50-96.5)	NFS, BARD
Shah 2009 (525)	Liver clinic	Retrospective	USA	541	48±12	40%	34	23.1%	57 (35-83), 68 (47-103)	NFS, BARD, FIB4
Siddiqui 2016 (534)	Liver clinic	Retrospective	USA	145	52.9±11.7	36.7%	35.8	35.2%	80.7±53.3	NFS, BARD, FIB4, Fibrometer
*Simo 2014 (510)	RYGB patients	Retrospective	USA	225	43.2±9.6	14.7%	44.6	6.6%	31.2±24.3	NFS
Subasi 2015 (315)	Liver clinic	Retrospective	Turkey	142	45±9	52.8%	30.9	21.1%	91±61	NFS, BARD, FIB4, Fibrometer

**Obese-only study. ^Some data not included in meta-analyses due to patient overlap with subsequent study. NS – not stated; NFS – NAFLD fibrosis score, SEM – standard error of the mean; ELF – enhanced liver fibrosis score; TE – transient elastography; MRE – magnetic resonance elastography; ARFI – acoustic radiation force impulse; RYGB – Roux-en-Y gastric bypass*

Table 7.2: Comparison of obesity-only studies and studies recruiting from liver disease settings

	"Average-obese" study (e.g. Liver clinic)	**"Obese-only" study** (e.g. Obesity clinic)	p-value
n=	26	12	
Age	49.3±3.1 years	43.3±4.0 years	<0.001
Male gender	51.1±10.3%	26.6±14.0%	<0.001
Body mass index	32.4±2.3 kg/m^2	44.0±5.7 kg/m^2	<0.001
Advanced fibrosis (F3-F4)	25.7±9.0%	11.0±9.3%	<0.001
Alanine aminotransferase (ALT)	74.2±19.4 IU/L	37.4±10.3 IU/L	<0.001

Data represented as mean±standard deviation unless otherwise specified. p-values calculated using independent Student t-test.

Table 7.3: Meta-analysis with pooled sensitivity, specificity and diagnostic odds ratio (DOR), and summary receiver operator characteristic (SROC) curve for detection of each level of fibrosis

	Studies (n_{total}=)	SROC	Sensitivity		Specificity		DOR	
			Pooled	Heterogeneity	Pooled	Heterogeneity	Pooled	Heterogeneity
F1-4								
NFS (low threshold)	2 (432)	-	0.489 (0.432-0.546)	I^2=99.2%	0.816 (0.748-0.872)	I^2=97.4%	6.93 (3.84-12.53)	I^2=0.0%
NFS (high threshold)	2 (476)	-	0.766 (0.712-0.815)	I^2=0.0%	0.487 (0.406-0.569)	I^2=0.0%	3.10 (2.03-4.73)	I^2=0.0%
FIB4	2 (246)	-	0.398 (0.324-0.475)	I^2=98.0%	0.907 (0.817-0.962)	I^2=0.0%	3.75 (0.14-102.77)	I^2=92.4%
BARD	3 (504)	0.607 ±0.114	0.736 0.668-0.796)	I^2=76.5%	0.362 (0.309-0.418)	I^2=81.3%	2.80 (1.44-5.43)	I^2=23.6%
MRE	2 (242)	-	0.514 (0.430-0.597)	I^2=63.9%	0.906 (0.830-0.956)	I^2=0.0%	10.51 (4.909-22.513)	I^2=0.0%
F2-4								
NFS (low threshold)	3 (471)	0.768 c±0.035	0.729 (0.652-0.797)	I^2=87.6%	0.509 (0.453-0.566)	I^2=96.2%	5.18 (2.91-9.23)	I^2=16.2%
NFS (high threshold)	2 (432)	-	0.351 (0.264-0.446)	I^2=90.5%	0.881 (0.840-0.914)	I^2=0.0%	2.26 (0.33-15.37)	I^2=80.6%
BARD	2 (343)	-	0.508 (0.416-0.601)	I^2=88.9%	0.574 (0.506-0.640)	I^2=96.5%	1.87 (1.16-3.03)	I^2=0.0%
FIB4	2 (343)	-	0.450 (0.359-0.543)	I^2=95.7%	0.919 (0.875-0.951)	I^2=93.9%	8.57 (4.59-15.97)	I^2=0.0%
Fibrotest (low threshold)	2 (509)	0.827 ±0.029	0.667 (0.590-0.737)	I^2=74.8%	0.751 (0.701-0.796)	I^2=0.0%	7.23 (3.02-17.29)	I^2=72.4%
Fibrotest (high threshold)	2 (555)	0.729 ±0.407	0.132 (0.070-0.219)	I^2=4.1%	0.989 (0.975-0.996)	I^2=36.3%	9.82 (3.28-29.39)	I^2=0.0%
ELF	2 (231)	-	0.709 (0.596-0.806)	I^2=0.0%	0.816 (0.745-0.874)	I^2=0.0%	9.71 (5.04-18.68)	I^2=0.0%
TE	8 (709)	0.851 ±0.017	0.758 (0.718-0.795)	I^2=85.5%	0.773 (0.730–0.813)	I^2=88.6%	13.90 (8.44-22.88)	I^2=39.9%
ARFI	3 (398)	0.837 ±0.024	0.740 (0.691-0.785)	I^2=94.1%	0.681 (0.622-0.737)	I^2=94.8%	10.41 (6.49-16.68)	I^2=0.0%
MRE	2 (242)	-	0.647 (0.522-0.759)	I^2=0.0%	0.937 (0.890-0.968)	I^2=22.7%	26.42 (11.19-62.38)	I^2=14.0%
F3-4								
NFS (low threshold)	17 (3388)	0.795 ±0.020	0.786 (0.755-0.814)	I^2=60.2%	0.622 (0.603-0.640)	I^2=94.4%	7.47 (5.47-10.20)	I^2=45.5%
NFS (high threshold)	13 (2733)	0.813 ±0.053	0.602 (0.567-0.637)	I^2=93.2%	0.924 (0.911-0.935)	I^2=94.4%	14.42 (7.81-26.61)	I^2=71.1%
BARD	13 (2804)	0.725 ±0.016	0.732 (0.697–0.765)	I^2=83.2%	0.578 (0.556–0.599)	I^2=88.3%	4.35 (3.52–5.37)	I^2=0.2%
FIB4 (low threshold)	10 (2202)	0.831 ±0.015	0.766 (0.729-0.800)	I^2=0.0%	0.726 (0.704-0.748)	I^2=86.0%	10.17 (7.33-14.09)	I^2=37.3%
FIB4 (high threshold)	7 (1392)	0.770 ±0.052	0.360 (0.307-0.417)	I^2=71.9%	0.950 (0.936-0.962)	I^2=92.6%	13.25 (8.77-20.03)	I^2=0.0%
Fibrometer	4 (827)	0.774 ±0.092	0.676 (0.621-0.728)	I^2=94.2%	0.691 (0.649-0.731)	I^2=87.0%	4.89 (1.71-14.04)	I^2=87.1%
Hepascore	2 (694)	-	0.693 (0.629-0.753)	I^2=21.0%	0.793 (0.754-0.829)	I^2=78.1%	9.83 (4.07-23.77)	I^2=77.2%
Fibrotest (low threshold)	2 (719)	0.359 ±0.244	0.832 (0.774-0.880)	I^2=38.2%	0.630 (0.587-0.672)	I^2=81.0%	12.02 (3.56-40.58)	I^2=62.1%
Fibrotest (high threshold)	2 (509)	0.847 ±0.062	0.461 (0.354-0.570)	I^2=82.1%	0.943 (0.916-0.963)	I^2=84.6%	14.03 (7.53-26.12)	I^2=0.0%
ELF	2 (248)	0.962 ±0.040	0.848 (0.750–0.919)	I^2=14.8%	0.916 (0.871–0.948)	I^2=31.8%	84.86 (20.23–356.0)	I^2=46.7%
TE	6 (1002)	0.859 ±0.017	0827 (0.787-0.862)	I^2=89.0%	0.721 (0.689-0.752)	I^2=89.6%	16.65 (10.14-27.32)	I^2=32.5%
ARFI	3 (496)	0.902 ±0.035	0.789 (0.733-0.839)	I^2 = 91.5%	0.784 (0.745-0.820)	I^2=92.4%	24.25 (10.52-55.93)	I^2=62.4%
MRE	4 (486)	0.965 ±0.015	0.870 (0.792-0.927)	I^2 = 0.0%	0.915 (0.883-0.941)	I^2=0.0%	72.03 (35.62-145.6)	I^2=0.0%
F4								
TE	3 (386)	0.891 ±0.024	0.818 (0.722–0.892)	I^2=79.5%	0.783 (0.745–0.818)	I^2=94.2%	20.51 (10.69-39.38)	I^2=0.0%
ARFI	2 (361)	0.878 ±0.057	0.678 (0.569-0.774)	I^2=90.3%	0.818 (0.781-850)	I^2=95.7%	14.61 (5.22-40.95)	I^2=59.6%
MRE	2 (242)	-	0.789 (0.544-0.939)	I^2=5.7%	0.919 (0.875-0.951)	I^2=0.0%	41.26 (11.78-144.5)	I^2=0.0%

SROC – summary receiver operator characteristic curve; DOR – diagnostic odds ratio; NFS – NAFLD fibrosis score, TE – transient elastography, ELF – enhanced liver fibrosis score; MRE – magnetic resonance elastography.

Figure 7.1: Study selection flowchart.

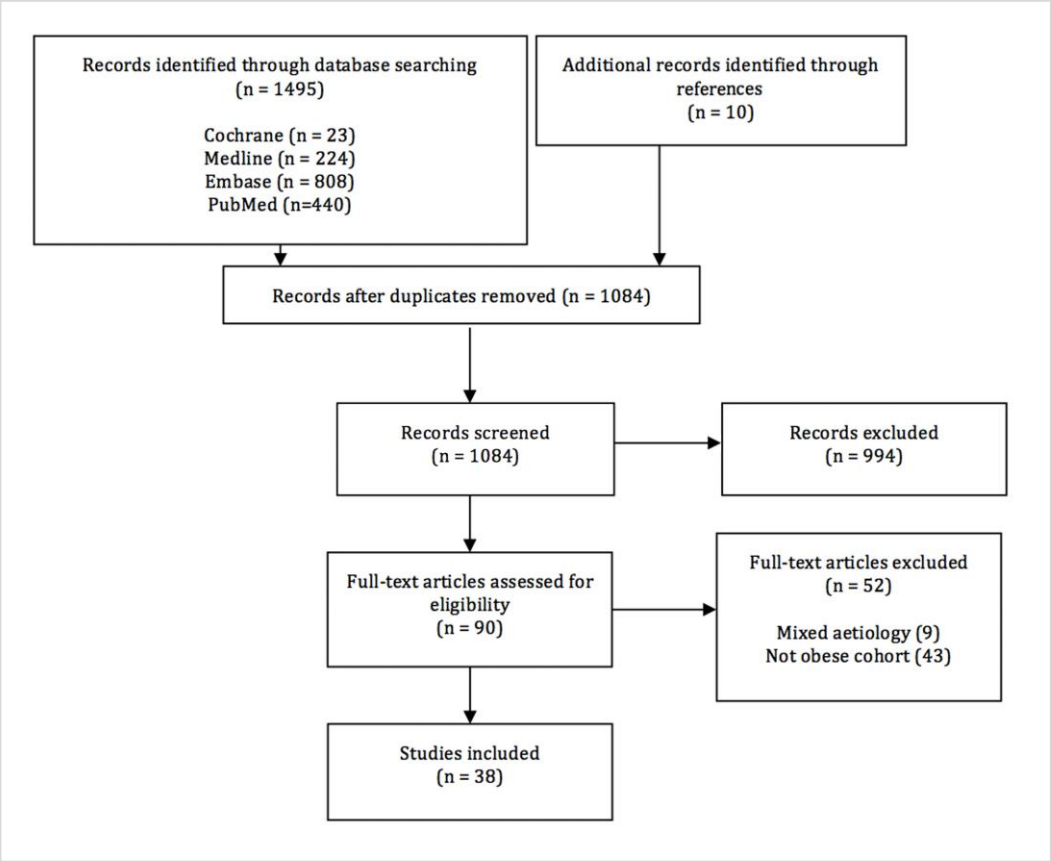


Figure 7.2: QUADAS-2 quality assessment of included studies.

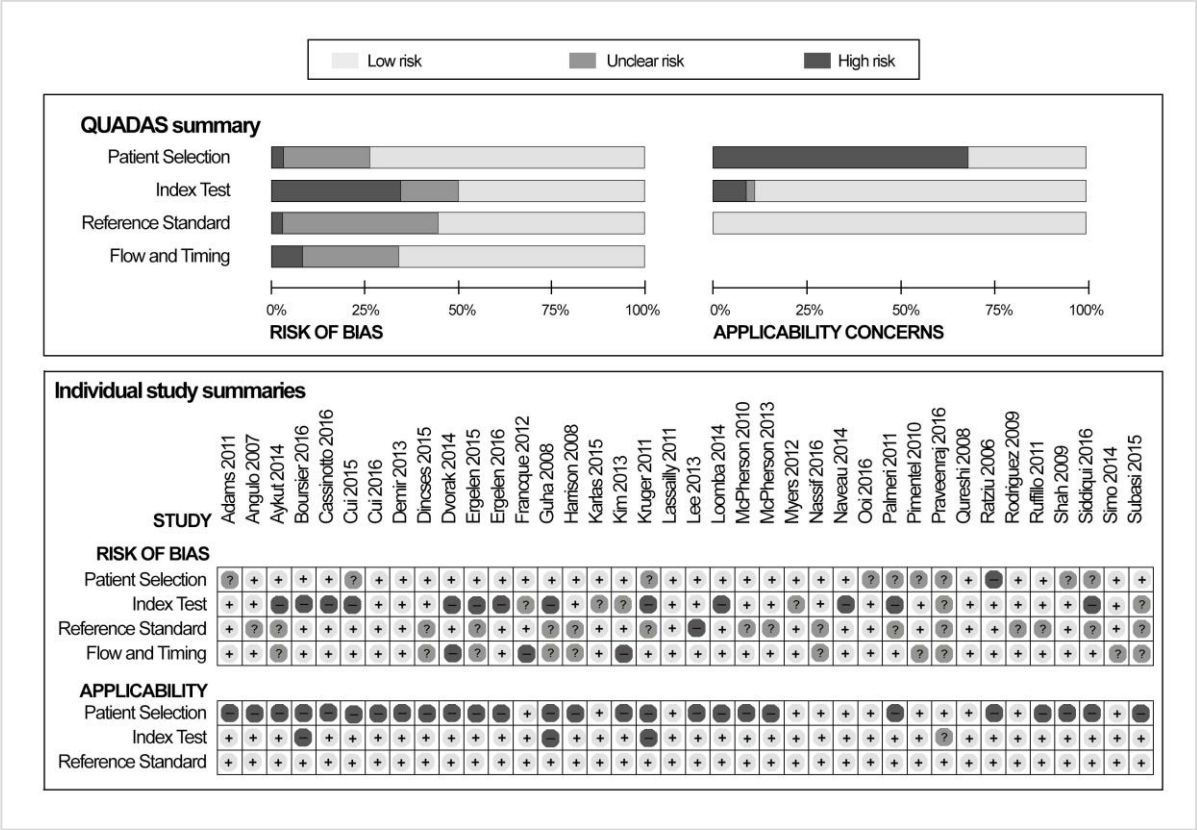
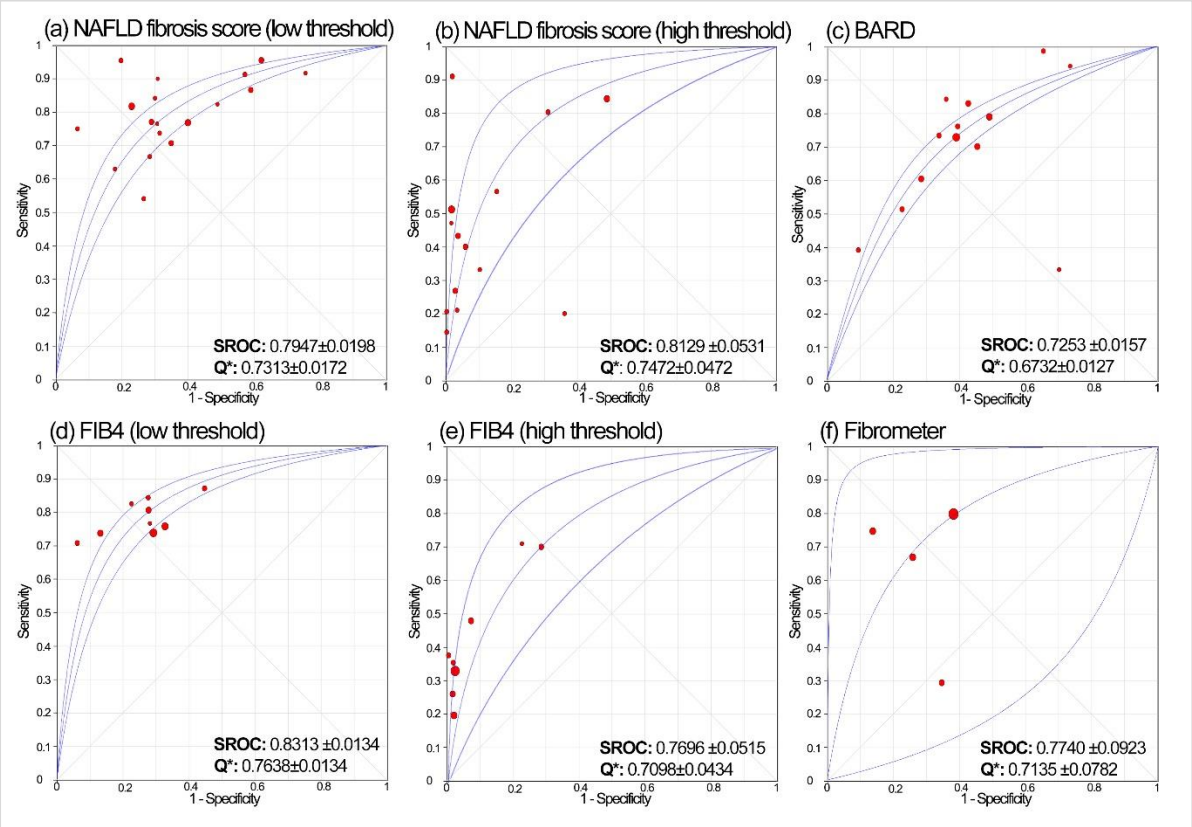
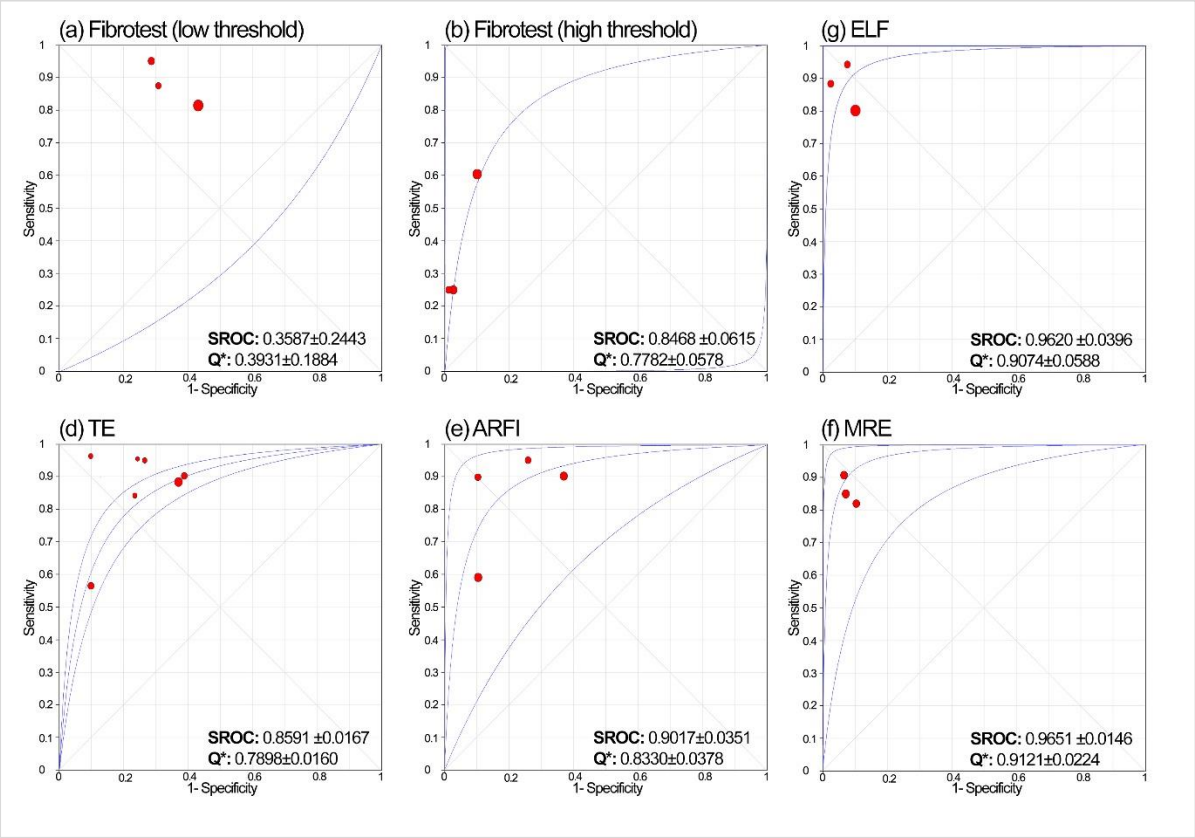


Figure 7.3: Summary receiver operator curves for detection of F3-4 fibrosis with simple composite serum panels.



SROC – summary receiver operator characteristic curve

Figure 7.4: Summary receiver operator curves for detection of F3-4 fibrosis with complex composite serum panels and elastography techniques.



SROC – summary receiver operator characteristic curve

7.5 Discussion

The aim of our systematic review was to evaluate the accuracy of commonly used non-invasive diagnostic tests for NAFLD-related fibrosis in the high-risk obese population. With limited evidence available, complex composite scores comprising fibrosis biomarkers (such as the ELF) appeared more accurate than simple composite scores (such as the NFS). Elastography techniques had the best and most consistent accuracy, but were limited by failure rates dependent on BMI. Overall, we found that non-invasive tests were not well validated in the obese, with limited studies and high heterogeneity.

Whilst there have been previous reviews on non-invasive tests for NAFLD-related fibrosis (226, 302), there are few meta-analyses published. Specifically, there is limited review of the evidence in the morbidly obese populations, where NAFLD is endemic (501, 535). A meta-analysis on TE by Kwok *et al* reported reasonable accuracy for NAFLD-related fibrosis, but included studies with significant proportions of non-obese individuals (302). A focus on the use of XL probes and failure rates were not included, but are pertinent considerations in high BMI populations.

There are few studies that cover the gap in knowledge about applicability of these tests in the obese. Our systematic review identified only 38 studies assessing the most common and clinically available diagnostic tests. There was significant heterogeneity of results, and the suggestion of possible publication bias. There was substantially less evidence when assessing an *obese-only* population, with only twelve such studies available. Notably, the diagnostic accuracy of non-invasive tests was not as favourable in *obese-only* cohorts. Importantly, there were significant differences between *obese-only* and *average-obese/liver clinic* studies, particularly in terms of body mass index and ALT levels. This raises the possibility of two distinct groups with differential hepatic risk profiles. As many serum-based scores are derived from the liver clinic setting, it is possible that currently available studies may not adequately reflect the accuracy of these tests in a general morbidly obese population (503).

Of the serum-based diagnostic tests, we found that complex scores show better accuracy than simple scores. Previous individual studies support these findings in general NAFLD populations (313, 526, 529). In our meta-analysis, both the NFS and BARD had average, and often poor, pooled sensitivities and specificities, with high heterogeneity. Furthermore, 35% of patients had indeterminate NFS scores, which considerably affects the clinical efficacy of

this scoring system. The assessment of ELF was more favourable and less variable. Certainly, ELF is now used with increasing confidence in many centres, with 2015 NICE guidelines on NAFLD stating that ELF was "the most cost-effective and the most appropriate test for advanced fibrosis in adults with NAFLD" (536). However, consideration should be given to the level of evidence available for the ELF in obese populations, with only two such studies found. Additionally, both studies used variable optimal thresholds to calculate diagnostic accuracy. Further validation of complex serum scores in the obese is necessary before these findings can be confirmed with confidence.

Elastography techniques showed consistently good diagnostic accuracy. The SROC for TE, ARFI and MRE for all levels of fibrosis ranged from 0.851-0.965. Furthermore, pooled sensitivity and specificity of TE and MRE were good to excellent, with little heterogeneity. These results are consistent with previous data suggesting that elastography has advantages over serum-based predictive scores (526, 528). However, one of the current major difficulties with these techniques, and a barrier to use in clinical practice, is the heterogeneity of cut-off points quoted in the literature. This variation can be seen in **Supplementary Figures 7.2-7.5**, showing anywhere from mild variation in MRE thresholds (3.62-4.15kPa) to significant variation in TE (7.6-12.5kPa). These differences may potentially be influenced by the levels of obesity and variation in skin-to-capsule distance, which are known to affect other elastography-based techniques (537, 538).

The success of elastography techniques was limited by patient size. Naveau *et al* found that the 26 patients with unreliable or unfeasible readings had a significantly higher BMI (47 vs. 42, $p<0.005$) (327). Other elastography studies, including those utilizing ARFI and SWE (511, 517, 520) consistently report similar difficulties with increasing BMI (**Supplementary Table 7.4**). Myers *et al* reported a failure rate consistent with BMI, with over half of patients with BMI≥40 having unsuccessful readings with the M probe. This was partially mitigated by the XL probe, decreasing this to a 5% failure rate in this population (506). The XL probe is relatively new and has been designed specifically for larger subjects. There are currently few published studies on the diagnostic accuracy of TE using this probe in the obese (**Supplementary Table 7.4**). Further investigation into the impact of the XL probe on feasibility and accuracy could significantly increase the applicability of TE in an obese cohort.

The accuracy and structural information obtained by MRI has always been offset by the practicalities of imaging in obesity (17). Diagnostic and research MRI machines have a weight limit of 250kg, but more importantly, a maximum aperture diameter of up to 70cm. In addition, image quality can be compromised due to increased sound-to-noise ratio and artefact. This clearly restricts its use in morbid obesity.

The evaluation of non-invasive diagnostic tests for NAFLD, particularly in the obese cohort, is becoming increasingly important. With the rising rates of obesity, metabolic disease and deleterious lifestyle choices, NAFLD and steatofibrosis is becoming an increasingly common cause of end-stage liver disease and liver transplantation (1). Viable treatment options are now available for all stages of NAFLD, and monitoring for liver failure and hepatocellular carcinoma is essential for those with more significant fibrosis. Weight loss is central to treatment of NAFLD in the morbidly obese, with persuasive evidence of regression in disease with weight loss, particularly in those with less severe disease progression (443, 539). Effective and durable weight loss, however, is a notoriously challenging endeavour (21). Lifestyle changes alone are often ineffective in the long term (426), and significant sustained weight loss requires a multidisciplinary step-up approach involving allied health, pharmacology and occasionally, surgery (21). The current lack of reliable, safe and practical diagnostic test presents a significant barrier to initiating this pathway of treatment for NAFLD in the setting of obesity.

There are several strengths of this systematic review. Firstly, our focus on the obese population provides a comprehensive summary of the evidence for this high-risk population. Increasing rates of morbid obesity now affect all medical fields, from primary practice to tertiary referral centres. Importantly, this review has identified the paucity of evidence available in this domain. Diagnosis of significant NAFLD in the obese in the absence of liver biopsy remains challenging, and further research is required. Secondly, this review covers the most commonly used non-invasive methods of detecting fibrosis, allowing for comparison of results. Lastly, we have chosen to focus on studies with liver biopsy as the reference standard. Although there are drawbacks to liver biopsy (227), it is the accepted gold standard that allows a standardised assessment of NAFLD-related fibrosis.

This review has some limitations that warrant discussion. Firstly, there are a variety of thresholds used across the studies for each test (**Supplementary Figure 7.2-7.5**). Whilst long established scores (e.g. the NFS and BARD) are more standardised, newer tests are more

variable, with studies often report diagnostic accuracy based on optimal thresholds. Unfortunately, considerable variability in threshold values does not allow us to draw practical recommendations from our meta-analysis regarding the best application and interpretation of tests. Secondly, the inclusion of studies based on average BMI≥30 has meant that a proportion of data from the meta-analyses includes results from non-obese individuals. These studies include a proportion of non-obese participants, and thus may not completely represent results in an obese cohort. Endeavours to obtain study-level data have been unsuccessful, with poor responses for clarification of study data. Additionally, no studies published analyses of the differences between obese and non-obese subgroups within their studies. There are only twelve studies that have recruited from obese-only populations, with insufficient data for meaningful pooled analyses. Thirdly, although we have assessed publication bias, funnel plots and tests for publication bias have weaknesses in the setting of highly variable results, and may be influenced by type of population and study quality (505, 540). Since variability in test accuracy is expected in diagnostic accuracy studies, the Cochrane Handbook as warned against interpreting statistical evidence of funnel plot asymmetry as necessarily implying publication bias (540). Lastly, we have focused on full-length publications and excluded abstracts, non-English articles, letters, editorials and grey literature for practical reasons, particularly in relation to having access to sufficient data on diagnostic test accuracy. This may ultimately have had some effect on the results.

In conclusion, this systematic review and meta-analysis focused on the accuracy of established and widely used non-invasive methods in diagnosing NAFLD-related fibrosis in the obese. Complex serum scores, particularly the ELF, had better accuracy compared to simple composite scores, but significantly less validation. Elastography techniques showed the highest accuracy, however, studies report failure rates directly related to BMI. Overall, there is currently limited evidence for non-invasive tests for NAFLD-related fibrosis in the obese, and further studies should be performed to establish their accuracy in this high-risk population.

8 Modified thresholds for fibrosis risk scores in nonalcoholic fatty liver disease are necessary in the obese

8.1 Abstract

BACKGROUND: Obesity and its related comorbidities are significant risk factors for nonalcoholic fatty liver disease (NAFLD). Liver fibrosis is the major determinant of long-term outcomes in NAFLD. A non-invasive tool that accurately identifies obese patients at elevated risk of liver fibrosis would be of significant value. Fibrosis risk scores in patients with NAFLD have been proposed but have not been validated in obese populations. We aimed to validate established simple fibrosis scores in bariatric surgical patients.

METHOD: We conducted a prospective study of 107 consecutive high-risk obese patients undergoing primary bariatric surgery. Proposed fibrosis scores (NAFLD fibrosis score, BARD, FIB-4, Forn and AST-to-platelet ratio index) were calculated and compared hepatic fibrosis determined by histology of intraoperative liver biopsies. Accuracy was determined, and fibrosis score thresholds were optimised. These modified thresholds were then validated in an independent bariatric surgical population.

RESULTS: Liver biopsies were available in 101 patients. Sixty-eight patients had some degree of fibrosis, with 23 patients (23%) having significant fibrosis (F2-F4). The Forn score best predicted significant fibrosis (AUROC 0.724, p=0.001). With standard thresholds, the sensitivity for the Forn score for identification of significant fibrosis (F2-4) was 0%. Using modified thresholds of 3.5, the sensitivity and negative predictive value increased to 85.7% and 94.7%. This threshold was applied to an independent validation cohort with good accuracy.

CONCLUSION: Fibrosis risk scores using simple markers have moderate success at delineating obese patients with significant NAFLD-related fibrosis. Thresholds, however, need to be lowered to maximise diagnostic accuracy in this cohort.

8.2 Introduction

Obesity is a significant risk factor for nonalcoholic fatty liver disease (NAFLD). Together with commonly associated metabolic disorders such as insulin resistance and lipid abnormalities, obesity can result in hepatic dysfunction. Nearly 90% of the obese have hepatic steatosis (541). Nonalcoholic steatohepatitis (NASH) and advanced fibrosis, although less common, are more clinically important, and have a prevalence of 36-67% and 12-17% respectively in obese populations (472, 501, 542). Liver fibrosis, in particular, has recently been shown to be the most important prognostic marker. Angulo *et al* (246) showed that even mild (F1) fibrosis is associated with an increased risk of liver transplantation and mortality (hazard ratio 2.07). This risk increases with fibrosis stage, and is independent of inflammation (246, 247). Therefore, the assessment of fibrosis in the NAFLD population is a priority.

The diagnosis of NAFLD in the obese is usually made incidentally, as the majority of patients are asymptomatic (7, 13, 543). Liver biopsy is the current gold standard for diagnosing NAFLD, and the only reliable means of staging fibrosis. However, liver biopsy is an impractical tool for population-based screening (273). Considering the importance of fibrosis assessment, a series of non-invasive fibrosis risk scores composed of routinely available measures have been developed for use in primary care (9, 544, 545). Such scores include the aspartate aminotransferase (AST) to platelet ratio index (APRI), the Fibrosis-4 (FIB-4) score and the Forn index. Although not initially designed for NAFLD, various studies demonstrate good accuracy in NAFLD with area under the receiver operator characteristic curve (AUROC) values up to 0.980 in the general NAFLD population (315, 530, 546).

Two simple scores specific to NAFLD-related fibrosis have also been developed. The NAFLD fibrosis score (NFS) and BARD score both incorporate body mass index (BMI) and diabetes status into their formula (310, 312, 547). Independent studies show modest to good accuracy of these scores (AUROC 0.628–0.850 (315, 522, 525, 533) and 0.601–0.816 (517, 518, 523) respectively for F3-4 fibrosis). However, these validation studies have generally been performed in patients with an established or suspected diagnosis of NAFLD with subjects sourced from populations with abnormal liver function tests (LFT). Many studies have also been at least partially retrospective in nature (310, 312, 315, 525, 533, 547).

The performance of fibrosis risk scores has not been well validated in obese patients, despite obesity having a prevalence of over 25% in many Western countries. Obese patients represent an unselected, yet high-risk group for NAFLD and liver fibrosis. The ability to accurately predict fibrosis in obese patients would aid physicians in identifying those likely to derive direct benefit from weight loss surgery (12).

Providing a clinical tool to help surgeons stratify the risk of liver fibrosis would aid in determining the appropriateness of intra-operative liver biopsy. Factors such as intra-operative liver appearance and pre-operative liver function tests have been shown to be poor predictors of NAFLD (548). Data from the Longitudinal Assessment of Bariatric Surgery (LABS) study showed that only a small proportion of bariatric surgical patients have a liver biopsy performed, but highlights that a large proportion of liver disease currently goes undiagnosed (13). If a simple and reliable risk score were available, this would be of great clinical value in the bariatric population.

Our goal was to determine whether established simple liver fibrosis scores were accurate in obese patients. We aimed to determine the accuracy of different risk scoring systems at predicting either *advanced fibrosis* (F3-4), *significant fibrosis* (F2-4) or the presence of *any fibrosis* (F1-4). We also aimed to determine the comparative accuracy of different scores and identify whether simple modifications would result in improved performance.

8.3 Methods

We conducted a prospective study comparing simple fibrosis risk scores to liver biopsy findings in obese patients with metabolic syndrome undergoing bariatric surgery. All participants provided informed written consent to participate in the study, which was conducted in accordance with the Declaration of Helsinki. The Avenue (HREC no.099) and The Alfred Human Research Ethics Committee (HREC no.195/15) approved the study. The study was registered in the Australian Clinical Trials Registry (ACTRN12610000049077 and ACTRN12615000875505).

8.3.1 Patients

A training cohort consisted of consecutive patients undergoing a primary bariatric procedure recruited from The Avenue Hospital, Melbourne, Australia, between April 2009 and March 2010. Criteria for inclusion included patients age ≥18 years old with body mass index (BMI) >30 kg/m^2, who had metabolic syndrome as defined by the Adult Treatment Panel III (84). Subsequently, a validation cohort was recruited comprising consecutive patients undergoing bariatric surgery at The Alfred Hospital, Melbourne Australia, between June and December 2015.

Patients were excluded if they had any other liver disease including viral, medication-related, autoimmune, or familial/genetic, or had a history of excessive alcohol intake, as defined by the American Association for the Study of Liver Diseases (84).

8.3.2 Clinical and biochemical data collection

Patients underwent a complete medical history and physical examination within two weeks of surgery. Fasting blood tests were taken before surgery and analysed in accredited local laboratories.

Fibrosis scores were calculated using the algorithms in **Table 3.18** in **Section 3.6.3 - Non-invasive tests**.

8.3.3 Bariatric surgery and intraoperative liver biopsy

Bariatric surgical procedures were performed by five experienced bariatric surgeons (PEO, WAB, PRB, SS, AS). Intraoperative biopsies were taken from the left lobe of liver using a

14-16G Temno needle. Fibrosis was staged according to the Kleiner classification (240) by a single liver pathologist blinded to clinical information.

8.3.4 Statistical analysis

Data were analysed using IBM SPSS v.22 (SPSS Inc, Chicago, IL, USA) and Microsoft Excel® (Microsoft Corporation, Redmond, WA, USA).

Continuous variables were expressed as mean ± standard deviation (SD) for parametric data, and median ± interquartile range (IQR) for nonparametric data. Student t-test was used for parametric data, and Mann Whitney U-test for nonparametric data. Normality was assessed by Shapiro-Wilk test. Categorical variables were expressed as numbers (with percentages) and Pearson's chi-squared or Fisher's exact tests were used. A p-value ≤ 0.05 was considered statistically significant.

8.3.4.1 Fibrosis groups

Patients were analysed in groups according to *any fibrosis* (F1-4), *significant fibrosis* (F2-F4) and *advanced fibrosis* (F3-4).

8.3.4.2 Optimizing threshold values

The AUROC was calculated, as well as measures of diagnostic accuracy (sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and correctly classified (CC = true positive + true negative)) based on established thresholds (**Table 3.18** in **Section 3.6.3 - Non-invasive tests**).

Modified cut-off values for differentiating F2-4 and F3-4 disease were calculated by finding the highest Youden Index (sensitivity + specificity – 1) using coordinate points of the receiver operator characteristic (ROC) curve. This method was chosen as a compromise between optimization of sensitivity and specificity.

8.4 Results

8.4.1 **Patients**

One hundred and seven patients were recruited in the training cohort. Six patients were excluded due to newly diagnosed haemochromatosis (n=1), corticosteroid use (n=1) and technical difficulty during the operation (n=4), leaving a total of 101 patients. Baseline demographics are seen in **Table 8.1**.

Fifty-three patients were recruited into the validation cohort (**Table 8.2**). Significant differences between groups included the age, BMI, comorbidities and fibrosis stage distribution.

8.4.1.1 Baseline histology

The mean liver core length per sample was 15.4±4.8mm, with 14.7±6.2 portal tracts.

Liver fibrosis prevalence can be seen in Table 3. Three participants (3.0%) had *advanced fibrosis* (F3-4), 23 participants (22.8%) had *significant fibrosis* (F2-4) and 68 participants (67.3%) had some degree of fibrosis (F1-4).

Eleven patients (10.9%) had normal histology, with the remaining 89.1% having a histological diagnosis of NAFLD. Twenty-two (21.8%) patients had a NAS ≥ 5, considered to be diagnostic of NASH, whilst 37 patients (36.6%) had a borderline NAS of 3-4.

The accuracy of the scores was tested for differentiating *significant fibrosis* (F0-1 vs F2-4) and *any fibrosis* (F0 vs F1-4). Due to the low numbers of patients with *advanced fibrosis* (F3-4, n=3, 3.0%), data has been described, but no definitive conclusions could be made.

8.4.2 **Differentiating patients with significant fibrosis (F2-4)**

8.4.2.1 Baseline clinical and biochemical differences

Table 8.1 summaries the baseline characteristics for patients with *no/minimal fibrosis* and *significant fibrosis*. There were more males in the *significant fibrosis* group (52.2% vs 28.2%, p=0.033), and a higher proportion of diabetic patients (69.6% vs 24.4%, p<0.001).

8.4.2.2 Accuracy of non-invasive composite scores

The values for the Forn and FIB-4 scores were significantly higher in the *significant fibrosis* group (4.40 vs 3.39, p=0.001 and 0.95 vs 0.78, p=0.043) (**Table 8.1**). The AUROC for the Forn and FIB-4 scores had a statistically significant moderate ability for differentiating *significant fibrosis* (AUROC 0.724, p=0.001 and AUROC 0.640, p=0.043) (**Figure 8.1a**). The remaining scores had poor AUROC values (NFS 0.615, p=0.096, APRI 0.602, p=0.139 and BARD 0.581, p=0.238).

8.4.2.3 Risk stratification

Based on the standard threshold values (**Table 3.18** in **Section 3.6.3 - Non-invasive tests**), sensitivities were poor for all scores (**Table 8.3, Figure 8.2**). Negative predictive values and the correctly classified (CC) rates (true positives + true negatives) were reasonable. However, this may reflect the relatively high prevalence of F0-1 fibrosis.

8.4.2.4 Modification of thresholds

When thresholds were modified to optimise detection of *significant fibrosis*, they were significantly lower than those in the literature (**Table 8.3**). The Forn index threshold decreased from 6.9 to 3.5, and FIB-4 from 3.25 to 0.74.

Subsequently, a greater proportion of *significant fibrosis* was identified, resulting in a substantially improved sensitivity (78.2–82.6%). The negative predictive values were also improved for each score (85.3–93.1%).

The Forn index had the best overall profile, with sensitivity of 82.6%, specificity of 69.2% and NPV of 93.1%, with a reasonable CC rate maintained (n=73, 72.3%). It had the lowest number of false positives (n=24).

8.4.2.5 Validation cohort

The AUROC values in the validation cohort were good, ranging from 0.636 to 0.795 (**Figure 8.1b**). Again, the Forn index and FIB-4 had statistically significant AUROC values of 0.795 and 0.769 respectively.

When modified thresholds were applied to this independent obese cohort (**Table 4, Figure 8.2**), the sensitivities and NPV are comparable to those obtained in the training cohort (6 of 7 patients with *significant fibrosis* identified, sensitivity 85.7%). The 'correctly classified' rate, however, is low, mainly due to a low specificity (17.4–52.2%) and concurrent high false positive rate. The NPV achieved is above 85% in all scores using modified thresholds.

The Forn and FIB-4 scores had the best overall profile in the validation cohort.

8.4.3 Differentiating patients with no fibrosis (F0)

8.4.3.1 Diagnostic accuracy using simple measures

Baseline characteristics of F0 (n=33) vs F1-4 (n=68) are seen in **Table 8.1**. Simple markers of liver disease showed lower ALT (29 vs 35.5, p=0.017) in the *no fibrosis* group and a trend for lower AST (23 vs 26, p=0.053).

The Forn index was the only score to show a significantly lower score in the *no fibrosis* group (2.64 vs 4.05, p=0.003) (**Table 8.1**), and had the only statistically significant, albeit modest, AUROC (0.686, p=0.003) (**Figure 8.1a**).

8.4.3.2 Modification of cut-off points

Measures of diagnostic accuracy were poor when using standard thresholds (**Table 8.3**).

With modification (**Table 8.3, Figure 8.2**), the 'correctly classified' rate improved for all scores (68.3-74.3%). Improvements in NPV ranged from 53.8%, up to 100% for the APRI. However, all modified threshold scores were very low (APRI 0.13, Forn 2.4, FIB4 0.48 and NFS -2.467), and identified few of the F0 patients, with a high number of false positive cases (19–27 of 33 patients).

8.4.3.3 Validation cohort

The AUROC values obtained for the validation cohort for differentiating *any fibrosis* were not statistically significant for any scores (**Figure 8.1b**). When the modified thresholds were applied to the validation cohort, there were poor results with little ability to differentiate *no fibrosis* (**Table 8.2**).

8.4.4 **Differentiating patients with F3-4 fibrosis**

There were three patients with advanced fibrosis (F3-4) in the training cohort. Simple markers of liver function showed that patients with *advanced fibrosis* had a higher ALT (80 vs 32 IU/L, p=0.001) and AST (42 vs 26 IU/L, p<0.001), and lower platelet count (163 vs 278 $x10^9$, p=0.028).

The *advanced fibrosis* group had a higher APRI (0.24 vs 0.63, p=0.001), FIB-4 (0.81 vs 1.41, p=0.030) and Forn index (3.81 vs 5.74, p=0.030).

The best AUROC values were obtained from the APRI (0.966, p=0.006), followed by the FIB-4 (0.859, p=0.035) and Forn index (0.859, p=0.035) (**Supplementary Figure 8.1**). The NAFLD fibrosis score had a poor AUROC (0.615, p=0.498), and the BARD score had an AUROC value below 0.5 (0.287, p=0.210).

Based on standard thresholds, sensitivities were poor (**Supplementary Table 8.1, Supplementary Figure 8.2**). Optimization of thresholds for the APRI, Forn and FIB-4 scores produced lower thresholds, with all cases of F3-4 fibrosis (n=3) captured within a high-risk score (sensitivity 100%). However, the PPV was poor, ranging from 7.5-27.3%.

Applying these new thresholds to the validation cohort stratified the one patient with *advanced fibrosis* into a 'high-risk' category for all scores (sensitivity 100%). The APRI had a reasonable specificity (73.6%) and CC rate (75.5%) (**Supplementary Table 8.2, Supplementary Figure 8.2**).

Table 8.1: Baseline demographics and measurements for training cohort, and comparison of those with any fibrosis (F1-4) and significant fibrosis (F2-4).

	Training cohort	F0	F1-F4	p=	F0-1	F2-4	p=
n =	**101**	**33 (32.7%)**	**68 (67.3%)**		**78 (77.2%)**	**23 (22.8%)**	
Age	49.0 (37.0 – 54.5)	45 (34 - 52)	51 (41 - 56)	0.048†	48 (34 - 54)	52 (44 - 56)	0.063†
Male	34 (33.7%)	7 (21.2%)	27 (39.7%)	0.065	22 (28.2%)	12 (52.2%)	0.033
BMI	41.9 (39.0 – 46.5)	42.3 ± 5.3	43.2 ± 6.3	ns	43.4 ± 5.8	41.4 ± 6.6	ns
Height	166.0 (161.5 – 172.5)	165.6 ± 7.9	168.2 ± 9.0	ns	166.8 ± 8.8	169.3 ± 8.2	ns
Weight	118.8 (106.0 – 132.6)	115.9 ± 17.3	122.5 ± 20.7	ns	120.8 ± 19.4	118.7 ± 21.4	ns
Waist circumference	126.0 (117.3 – 135.0)	124.5 ± 13.0	127.6 ± 12.3	ns	127 ± 13	125 ± 11	ns
Neck circumference	44.0 (41.0 – 47.5)	42 (34 - 52)	51 (41 - 56)	0.007†	44 ± 5	45 ± 3	ns
Comorbidities							
Type II diabetes	35 (34.7%)	5 (15.2%)	30 (44.1%)	0.004	19 (24.4%)	16 (69.6%)	<0.001
IGT	30 (29.7%)	12 (36.4%)	18 (26.5%)	ns	27 (34.6%)	3 (13.0%)	0.068‡
Hypertension	80 (79.2%)	23 (69.7%)	57 (83.8%)	ns	60 (76.9%)	20 (87.0%)	ns‡
Hypercholesterolaemia	74 (73.3%)	23 (69.7%)	51 (75.0%)	ns	55 (70.5%)	19 (82.6%)	ns‡
Simple blood tests							
Albumin	44 (41 – 45.5)	43 ± 3.0	43.5 ± 4.1	ns	43.4 ± 3.8	43.2 ± 3.9	ns
Fasting glucose	6.0 (5.3 – 7.4)	5.6 (4.9 – 6.1)	6.4 (5.6 – 8.3)	0.002†	5.8 (5.1 – 6.7)	7.9 (6.0 – 10.3)	<0.001†
ALT	32 (22 – 42)	29 (18 - 35)	35.5 (23.6 – 45.5)	0.017†	31 (22 - 40)	37 (22 - 53)	ns†
AST	25 (21 – 31.5)	23 (19 - 28)	26 (22 - 33)	0.053†	25 (21 - 31)	26 (21 - 35)	ns†
GGT	24 (19 – 35.5)	23 (17 - 31)	25 (20 - 38)	ns †	24 (19 - 33)	25 (20 - 38)	ns†
ALP	72 (58.5 – 87)	68 (59 - 87)	76 (58 - 87)	ns †	75 ± 21	72 ± 19	ns
Bilirubin	8 (6-10)	8 (6 - 10)	8 (6 - 11)	ns †	8 (6 - 10)	8 (6 - 11)	ns†
Platelet	266 (229.5 – 312.3)	286 (249 - 315)	258 (225 - 299)	ns †	279 ± 68	249 ± 52	0.054†
Total cholesterol	4.5 (4.0 – 5.4)	4.86 ± 0.92	4.50 ± 1.08	ns	4.7 ± 1.0	4.4 ± 1.2	ns
Triglyceride	1.6 (1.2 – 2.1)	1.7 (1.3 – 2.3)	1.6 (1.2 – 2.0)	ns †	1.6 (1.3 – 2.1)	1.6 (1.1 – 1.9)	ns†
HDL	1.07 (0.93 – 1.24)	1.15 ± 0.26	1.06 ± 0.25	ns	1.11 ± 0.26	1.01 ± 0.20	0.083
LDL	2.75 (2.03 – 3.20)	2.86 ± 0.76	2.66 ± 0.98	ns	2.8 ± 0.8	2.6 ± 1.1	ns
Composite scores							
APRI	0.24 (0.18 – 0.31)	0.22 (0.16 – 0.27)	0.25 (0.20 – 0.33)	0.071†	0.23 (0.17 – 0.29)	0.26 (0.21 – 0.36)	ns†
Forn index	2.74 (2.51 – 4.68)	2.64 (2.10 – 3.76)	4.05 (2.98 – 4.89)	0.003†	3.39 ± 1.73	4.40 ± 0.96	0.001
FIB-4	0.79 (0.60 – 1.01)	0.73 (0.53 – 0.91)	0.83 (0.66 – 1.07)	0.092†	0.78 (0.59 – 0.97)	0.95 (0.74 – 1.07)	0.043†
NFS	-0.639 (- 1.684 – 0.129)	-1.19 (-1.98 – 0.06)	-0.51 (-1.42 – 0.21)	0.068†	-0.876 ± 1.391	-0.294 ± 1.145	0.071
BARD ≥2	71 (70.3%)	21 (63.6%)	50 (73.5%)	ns	52 (66.7%)	19 (82.6%)	ns‡

Values expressed in median (IQR), mean ± SD or numbers (percentages). Student t-test for parametric continuous variables, †Mann Whitney U test for nonparametric continuous variables, Pearson Chi-square for categorical with ≥5 participants and ‡Fisher's Exact Test with <5 participants.

Table 8.2: Training and validation cohorts compared.

n =	All patients 154	Training 101	Validation 53	p =
Demographics				
Age	47 (35 – 54)	49 (38 – 54)	43 (32 – 52)	0.047†
Male	50 (32.5%)	23 (33.7%)	16 (30.2%)	ns
BMI	43.3 (39.3 – 47.8)	41.9 (39.1 – 46.5)	46.6 (40.1 – 52.6)	0.007†
Weight	121.7 (106.8 -135.9)	118.8 (106.4 – 132.4)	129.2 (112.0 – 139.4)	0.022†
Comorbidities				
Type II diabetes	48 (31.2%)	35 (34.7%)	13 (24.5%)	ns
IGT	34 (22.1%)	30 (29.7%)	4 (7.5%)	0.002‡
Hypertension	104 (67.5%)	80 (79.2%)	24 (45.3%)	<0.001
Hypercholesterolaemia	84 (54.9%)	74 (73.3%)	10 (19.2%)	<0.001
Fibrosis grade				
F0	77 (50.0%)	33 (32.7%)	34 (64.2%)	
F1	57 (37.0%)	45 (44.6%)	12 (22.6%)	
F2	26 (16.9%)	20 (19.8%)	6 (11.3%)	0.006
F3	1 (0.6%)	1 (1.0%)	0	
F4	3 (1.9%)	2 (2.0%)	1 (1.9%)	
Fibrosis scores				
APRI	0.25 (0.19 – 0.35)	0.24 (0.18 – 0.30)	0.29 (0.20 – 0.41)	0.026†
Forn	3.84 ± 1.66	3.63 ± 1.64	4.25 ± 1.65	0.027
FIB4	0.79 (0.60 – 1.10)	0.79 (0.60 – 1.01)	0.79 (0.52 – 1.26)	ns†
NFS	-0.502 ± 1.410	-0.742 ± 1.356	-0.049 ± 1.410	0.003
BARD	102 (66.2%)	71 (70.3%)	31 (58.5%)	ns

Student t-test for parametric continuous variables, †Mann Whitney U test for nonparametric continuous variables, Pearson Chi-square for categorical with ≥5 participants and ‡Fisher's Exact Test with <5 participants.

Table 8.3: Diagnostic accuracy of scores for classifying significant fibrosis (top) and any fibrosis (bottom). Columns define diagnostic accuracy using standard threshold (left) and modified thresholds in the training cohort (right).

Training cohort (n = 101)		**Standard cut-off**				**Modified cut-off**			
		APRI	**Forn**	**FIB-4**	**NFS**	**APRI**	**Forn**	**FIB-4**	**NFS**
Significant fibrosis (F2-F4)	***Cut-off***	***>0.7***	***>6.9***	***>3.25***	***>0.676***	***>0.20***	***>3.5***	***>0.74***	***>-1.292***
	Correctly classified (CC)	74 (73.2%)	76 (75.3%)	79 (78.2%)	71 (70.3%)	50 (49.5%)	73 (72.3%)	55 (54.5%)	47 (46.5%)
	CC: F0-1	73	76	78	69	31	54	37	29
	CC: F2-4	1	0	1	2	19	19	18	18
	Sensitivity	4.3%	0%	4.3%	8.7%	82.6%	82.6%	78.3%	78.3%
	Specificity	93.6%	97.4%	100%	88.5%	39.2%	69.2%	47.4%	37.2%
	PPV	16.7%	0%	100%	18.2%	28.8%	44.2%	30.5%	26.9%
	NPV	76.8%	76.8%	78.0%	76.7%	88.6%	93.1%	88.1%	85.3%
Any fibrosis (F1-F4)	***Cut-off***	***<0.7***	***<4.2***	***<1.3***	***<-1.455***	***<0.13***	***<2.4***	***<0.48***	***<-2.467***
	Correctly classified (CC)	33 (32.7%)	55 (54.5%)	35 (34.7%)	66 (65.3%)	75 (74.3%)	73 (72.3%)	69 (68.3%)	69 (68.3%)
	CC: F0	32	26	29	14	7	14	7	6
	CC: F1-4	1	29	6	52	68	59	62	63
	Sensitivity	1.5%	42.6%	8.8%	76.5%	100%	86.8%	91.2%	92.6%
	Specificity	97.0%	83.9%	87.9%	42.4%	21.2%	42.4%	21.2%	18.2%
	PPV	50%	85.3%	60%	73.2%	72.3%	75.6%	70.5%	70%
	NPV	32.3%	40.0%	31.9%	46.7%	100%	60.9%	53.8%	54.5%

Table 8.4: Diagnostic accuracy of scores for classifying significant fibrosis (top) and any fibrosis (bottom) in validation cohort using modified thresholds for each score.

Validation cohort (n = 53)		**Modified cut-off**			
		APRI	**Forn**	**FIB-4**	**NFS**
Significant fibrosis (F2-4)	***Cut-off***	***>0.20***	***>3.5***	***>0.74***	***>-1.292***
	Correctly classified (CC)	19 (35.8%)	24 (45.3%)	30 (56.6%)	14 (26.4%)
	CC: F0-1	13	18	24	8
	CC: F2-4	6	6	6	6
	Sensitivity	85.7%	85.7%	85.7%	85.7%
	Specificity	28.3%	39.1%	52.2%	17.4%
	PPV	15.4%	17.6%	21.4%	13.5%
	NPV	92.9%	94.7%	96%	88.9%
Any fibrosis (F1-4)	***Cut-off***	***<0.13***	***<2.4***	***<0.48***	***<-2.467***
	Correctly classified (CC)	34 (64.1%)	22 (41.5%)	22 (41.5%)	21 (39.6%)
	CC: F0	33	6	6	2
	CC: F1-4	1	16	16	19
	Sensitivity	5.3%	84.2%	84.2%	100%
	Specificity	97.1%	17.6%	17.6%	5.9
	PPV	50%	36.4%	36.4%	37.3%
	NPV	64.7%	66.7%	66.7%	100%

Figure 8.1: (a) Receiver operator characteristic (ROC) curves for the APRI, FIB-4, Forn, and NAFLD fibrosis scores in the training cohort. Left *ROC curves for the best performing fibrosis risk scores for differentiating no fibrosis (F0) from any fibrosis (F1-4).* Right *ROC curves for differentiating significant fibrosis (F2-4). (b) Receiver operator characteristic (ROC) curves for the validation cohort.* Left *ROC curves for the best performing fibrosis risk scores for differentiating no fibrosis (F0) from any fibrosis (F1-4).* Right *ROC curves for differentiating significant fibrosis (F2-4).*

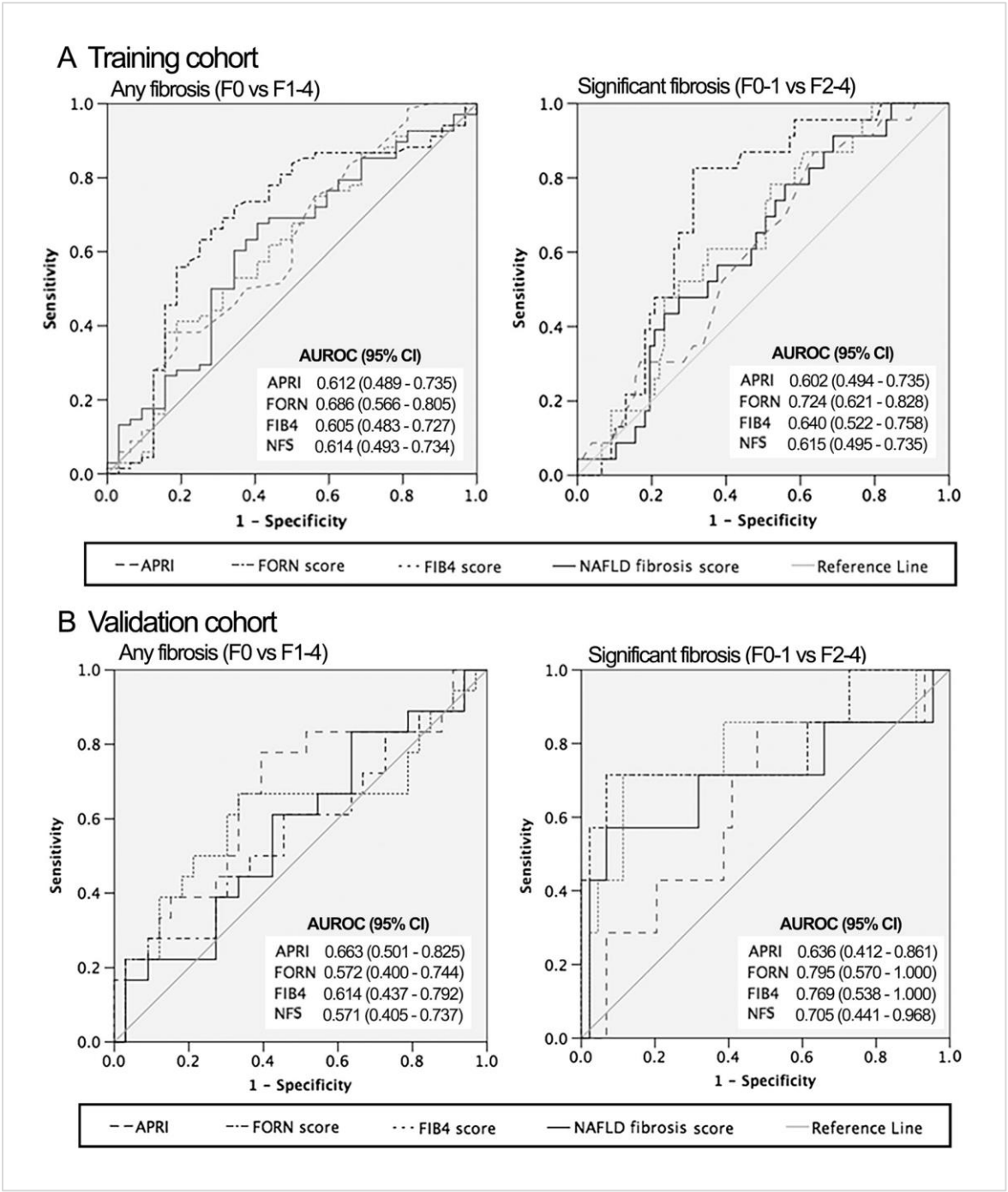
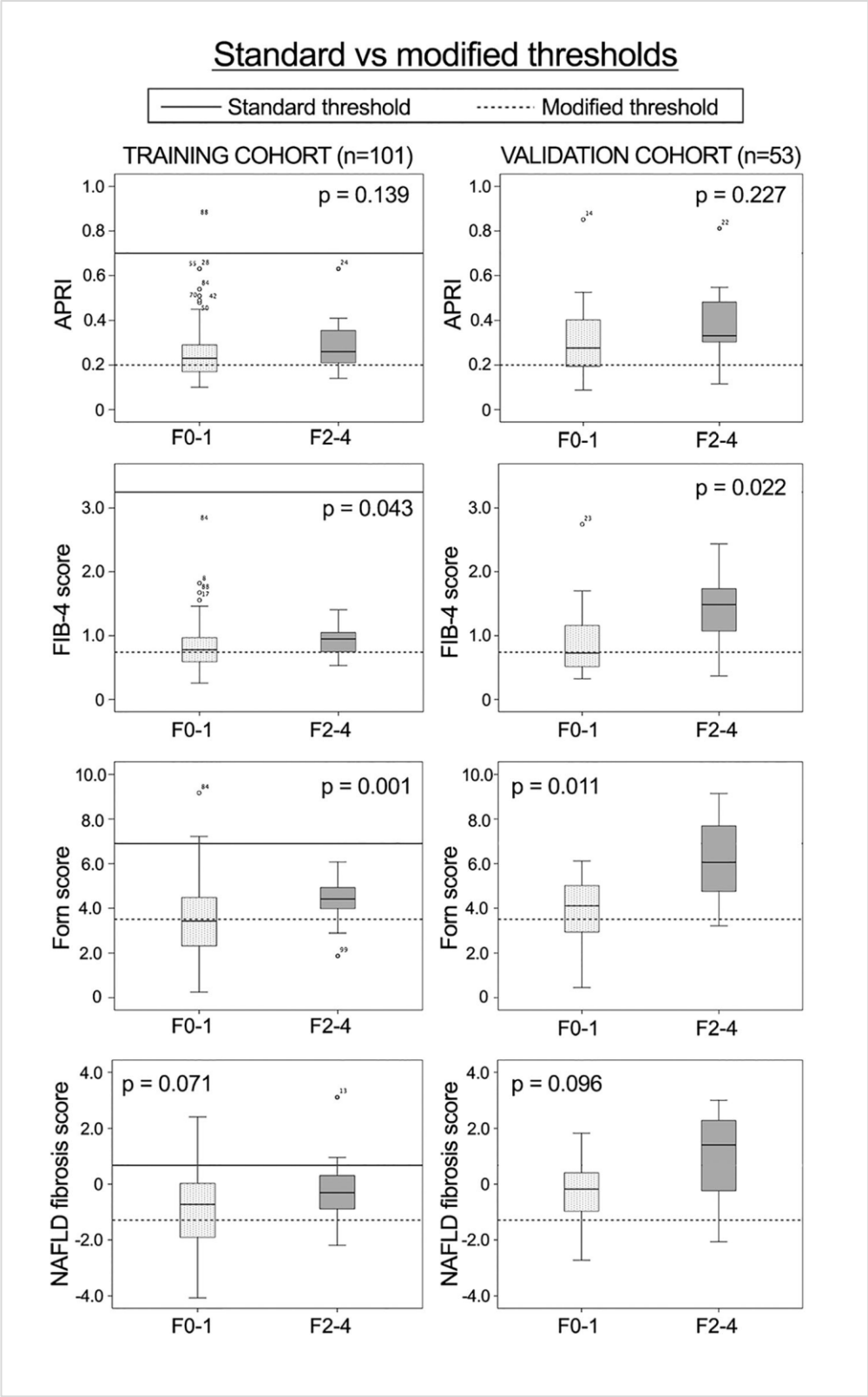


Figure 8.2: Box plots of training (left) and validation (right) cohorts, showing average values for the APRI, FIB-4, Forn and NAFLD fibrosis scores for F0-1 vs F2-4 (significant fibrosis). Standard cut-off (solid line) and modified cut-off (dotted line) are shown.



8.5 Discussion

We conducted a prospective study evaluating the utility of simple and convenient non-invasive scores in predicting NAFLD-related fibrosis in a cohort of obese and morbidly obese patients. We confirmed a high prevalence of NAFLD, although the rates of advanced fibrosis were low. An important finding was the very poor performance of all fibrosis risk scores in the obese, using standard thresholds. However, when thresholds were lowered, the Forn index predicted, with reasonable accuracy, the presence of significant fibrosis (F2-4). Importantly, a low-risk score using these modified thresholds could isolate a population who were very unlikely to have significant fibrosis, and who may confidently avoid a liver biopsy.

Our study primarily focused on obese patients with a high degree of metabolic disturbance. Obesity, in itself, has been well studied as a state of chronic inflammation that drives obesity-related insulin resistance and NAFLD (104, 549). Epidemiological studies support the implication of increased risk, with odds ratios of up to 4.4 for NAFLD in the setting of type II diabetes (9). These inherent differences in clinical, biochemical and cellular profiles associated with obesity may impact on the applicability of these fibrosis risk scores.

Our data confirms that simple predictive tools, as they were originally designed, are not adequately refined for the obese population. The sensitivities obtained are universally poor, ranging from 0–8.7%. This is in contrast to the good sensitivities and AUROC values obtained by other studies (310, 312, 517, 519, 523, 528). We believe this because these scores have been developed for maximal accuracy in a different population. Previous studies have recruited participants referred for investigation of altered LFTs, leading a higher prevalence of advanced disease and the exclusion of an important subgroup of patients with normal aminotransferase level yet abnormal histology. Original studies for the APRI, FIB-4 and NFS have average ALT levels between 2-3 times the upper limit of normal (>40 IU/L). The average ALT value in our study falls within these reference ranges, and over half of the patients with significant fibrosis had a normal ALT level. This likely explains the lower threshold required in our population of obese individuals, versus the higher thresholds required in the general hepatology cohort.

Simple modification of threshold values substantially improved their accuracy for differentiating F2-4 fibrosis. In particular, the Forn index performed the best in most domains, including a sensitivity of 82% and NPV of 93% in the training cohort. It had the

highest specificity of 69%, and correspondingly the lowest false positive rate, and had similar accuracy in the validation cohort. The FIB-4 was slightly less accurate, but may be more readily applied in clinical practice, as the algorithm is easier to calculate. These two scores notably use a varying combination of liver function tests, platelet count and age.

Scores that included BMI and diabetes were less diagnostic of fibrosis in this cohort. Given this population all had a high BMI, this measure is unlikely to contribute significantly in differentiating the presence of fibrosis. Additionally, given that insulin resistance is the hallmark of metabolic syndrome and closely related to obesity, the presence of diabetes could also be less useful. It is therefore not surprising that scores such as the NAFLD fibrosis score and BARD, which both utilise these parameters, showed poor diagnostic power.

Recent data from the prospectively conducted LABS study has highlighted that 86% of advanced fibrosis and 88.1% nonalcoholic steatohepatitis (NASH) goes undiagnosed in bariatric patients who are not biopsied (13). Consequently, it has been suggested that all patients undergoing bariatric surgery should undergo intra-operative liver biopsy. This study suggests that non-invasive scores and new thresholds may be a valuable tool to aid bariatric physicians and surgeons decide which obese patients are very unlikely to have significant fibrosis. A modified low-risk score may reasonably be used to exclude those with significant fibrosis. Its application may also extend to risk stratifying non-operative obese patients, who appear to have all the hallmarks of NAFLD, and where a percutaneous liver biopsy would otherwise have to be obtained.

The main limitation faced by this study was the lower rates of fibrosis. A possible explanation may be our recruitment of consecutive obese patients who are considered high-risk, but have not been preselected on the basis of a known diagnosis of NAFLD. This situation more accurately reflects the clinical scenario we see in primary care and bariatric practice, where the majority of patients reviewed will have clinical risk factors for NAFLD, but only a proportion of these patients will be affected by NAFLD-related fibrosis.

The low prevalence of advanced fibrosis (F3-4), in particular, does not allow for confident interpretation of these results. However, our data suggests that optimization of thresholds may have good accuracy for identification of advanced fibrosis (F3-4), particularly for the APRI and Forn index. A larger cohort is required to confirm this hypothesis.

Future studies should be aimed at better understanding the nature of NAFLD in obese patients. We need thorough validation of scores that are appropriate to the morbidly obese. In addition, investigation of further methods of predicting fibrosis in the obese would be valuable, so that morbidly obese patients may be appropriately referred for liver biopsies, as well as more easily monitored after diagnosis.

In conclusion, we found that the ability of existing risk scores to stratify fibrosis severity in the obese and morbidly obese was poor when using standard thresholds. By reducing thresholds of the Forn index to 3.5, we were able to reasonably differentiate those with significant fibrosis. With an NPV of 95%, a low Forn score could be used to identify morbidly obese patients who have all the clinically suspicious features of NAFLD, but who are unlikely to have significant histological fibrosis.

9 Evaluating feasibility and accuracy of non-invasive tests for nonalcoholic fatty liver disease in severe and morbid obesity

9.1 Abstract

INTRODUCTION: In obese individuals, non-alcoholic fatty liver disease (NAFLD) is common but often goes undiagnosed, and therefore untreated. The presence of significant fibrosis is a key determinant of NAFLD progression, and liver steatosis has substantial cardiovascular implications. We aimed to determine the diagnostic accuracy of common non-invasive diagnostic tests for steatosis and fibrosis in the obese.

METHODS: We recruited 182 severely and morbidly obese individuals undergoing bariatric surgery (age 44±12years, body mass index 45.1±8.3kg/m^2). Medical history, blood tests and liver biopsy were taken on the day of surgery. Serum steatosis and fibrosis scores were calculated. In a subgroup of patients, transient elastography with controlled attenuation parameter (TE/CAP) (n=82) and proton magnetic resonance spectroscopy (^{1}H-MRS) (n=49) were performed.

RESULTS: ^{1}H-MRS had excellent diagnostic accuracy for steatosis, with strong correlation to steatosis (r=0.647, p<0.001), good AUROC (0.852, p=0.001), sensitivity (81.3%) and specificity (87.5%). However, due to low feasibility in this cohort (65.3% success), this was substantially decreased with intention-to-diagnose analysis (sensitivity 50.0%, specificity 60.9%). CAP had good feasibility (80.5%), and performed better in intention-to-diagnose analysis (AUROC 0.688, sensitivity 84.8%, specificity 47.2%). Serum steatosis scores performed poorly, with comparable accuracy to ALT. For significant fibrosis, TE had the best accuracy (AUROC 0.903, p=0.007), which remained reasonable after intention-to-diagnose analysis (sensitivity 100%, specificity 59.0%). A combination approach using CAP with ALT for steatosis and TE with Forn index for fibrosis, yielded reasonable overall accuracy.

CONCLUSIONS: ^{1}H-MRS and TE/CAP had greatest accuracy for NAFLD-related steatosis and fibrosis. Failure rates in obesity significantly diminished diagnostic

ability. Use of a combination of serum and imaging tests improved overall feasibility of assessment and diagnostic accuracy in obese individuals.

9.2 Introduction

Nonalcoholic fatty liver disease is endemic in obesity, affecting up to 95% of obese individuals (7). Obesity and related metabolic disorders fuel the development of the more serious form of NAFLD, namely nonalcoholic steatohepatitis (NASH), as well as the development of liver fibrosis (6, 550). Therefore, it is not surprising that 25-56% of obese individuals have NASH, and 1-2% have cirrhosis (7). Recently, studies have shown that hepatic fibrosis is the only histological factor associated with progressive liver disease and liver related mortality in NAFLD patients (246, 247).

In addition to liver-related disease, NAFLD is now recognised as a key determinant of metabolic health and multisystem disorders (256, 257). Ultrasound-detected steatosis (~30% liver steatosis) has been independently associated with atherosclerosis and endothelial dysfunction (258). Liver steatosis also predicts the development of type 2 diabetes (T2DM) (244, 262) and is associated with worse diabetic complications (6). Subsequently, epidemiological studies have shown that cardiovascular disease is the leading cause of death in individuals with NAFLD (258).

Therefore, identification and grading of NAFLD-related steatosis and fibrosis is of considerable importance. This allows for accurate prognostication and risk assessment of associated liver and cardiovascular endpoints. Additionally, diagnosis allows the institution of management strategies, such as weight loss in the setting of obesity, that can slow progression, reverse or even resolve associated risks (12, 447).

Despite the systemic and liver-specific importance of NAFLD, the detection of NAFLD , particularly in the presence of severe obesity, remains challenging. Liver biopsy is currently the gold standard for diagnosing and grading NAFLD. However, due to risks, costs and various other drawbacks, it is an impractical screening tool for the large at-risk obese population (227). Non-invasive methods may provide a practical solution for detection of NAFLD, and are increasingly being used in clinical practice (265, 502). Common diagnostic methods can be broadly categorised into those based on serum markers and those that are image-based. Simple and commonly used tests of fibrosis include the NAFLD fibrosis score and transient elastography (TE), whilst tests such as computed attenuation parameter (CAP) and the NAFLD liver fat score have been used to grade steatosis.

Whilst many of these have been widely tested in general NAFLD cohorts, few studies have focused on severely and morbidly obese populations. These patients are both high-risk and often difficult to assess. Validation in obese cohorts is vital, as obesity represents a substantially different physical, biochemical and physiological environment (102). In particular, the feasibility and accuracy of imaging-based techniques can pose a significant challenge in morbid obesity (17).

The aim of this study was to assess the feasibility and diagnostic accuracy of serum and imaging tests for detecting NAFLD in an exclusively obese population. We focused on detection of fibrosis, as the key determinant of liver-related prognosis, and steatosis, as an important factor in cardiometabolic disease. We evaluated the performance of commonly-used and widely available tests, such as simple serum steatosis and fibrosis scores, transient elastography (TE) with controlled attenuation parameter (CAP) and proton magnetic resonance spectroscopy (^{1}H-MRS).

9.3 Methods

9.3.1.1 Patients

Between July 2015 and November 2016, we prospectively enrolled consecutive eligible severely and morbidly obese patients undergoing bariatric surgery in three metropolitan hospitals in Melbourne, Australia.

Inclusion criteria included: (1) age ≥18 years, (2) BMI≥35 kg/m^2, (3) alanine aminotransferase (ALT) or aspartate aminotransferase (AST) >0.5 times the upper limit normal (ULN) (equivalent to ALT 19IU/L for women and 21IU/L for men (229)), or gamma-glutamyl transferase (GGT)>ULN. All patients were elective bariatric surgical patients, with no acute illness or malignancy. Patients were excluded if they had any clinical or serological evidence of other liver disease, including viral hepatitis, medication-related, autoimmune, familial/genetic causes or a history of excessive alcohol use, as defined by the American Association for the Study of Liver Diseases (551). Patients who were unable or unwilling to attend additional appointments for transient elastography (TE) or magnetic resonance spectroscopy (^{1}H-MRS) within two weeks of surgery were still recruited, for assessment of blood tests.

All participants provided informed consent to participate in this study. Ethics approval was obtained from Alfred (195/15), Avenue (190) and Cabrini (09-31-08-15) Human Research Ethics Committee. This study was registered with the Australian Clinical Trials Register (ACTRN12615000875505).

9.3.1.2 Clinical and biochemical data

Patients underwent a complete medical history and physical examination preoperatively. Fasting blood tests were taken prior to induction of anaesthesia.

9.3.1.3 Bariatric surgery and intraoperative liver biopsy

Intraoperative wedge liver biopsies, at least 1cm in depth, were taken. A single pathologist graded the biopsies in a blinded manner, according to the NAFLD activity score (NAS)(481) and Kleiner classification of liver fibrosis.(240)

In this study, we focused on moderate steatosis (S2-3) and significant fibrosis (F2-4). In addition, all other levels of steatosis and fibrosis were analysed. Full details are presented in the **Appendix 4: Supplementary Materials**.

9.3.1.4 Assessment of fibrosis

Fibrosis serum scores

Fibrosis scores included the AST to ALT ratio (AAR), AST to platelet ratio index (APRI), NAFLD fibrosis score (NFS), BARD index, FIB4, and Forn index. These were calculated according to published algorithms (see **Table 3.18** in **Section 3.6.3 - Non-invasive tests**).

Transient elastography

Transient elastography (Fibroscan®, EchoSens, Paris) was performed in a fasting state on patients within two weeks of surgery. Two experienced gastroenterologists (>2000 procedures each) performed the scans as per manufacturer's recommendations. A pre-procedure liver ultrasound was performed to locate the liver along the mid-axillary line, and to measure skin-to-liver capsule distance. TE was performed according to the standard protocol. As all patients were obese, an XL probe was used. Attempts were made to collect ≥10 valid liver stiffness measurements (LSM). Where no successful measures were obtained after 10 measurements, the test was considered unsuccessful. Variability was assessed via the ratio of the interquartile range (IQR) and median LSM measure (IQR:M ratio). Unreliable readings were considered to be those with at least one of the following: <10 valid acquisitions, <60% successful readings, or IQR:M≥0.30. Standard and optimal thresholds were calculated and used for assessment of diagnostic accuracy (302).

9.3.1.5 Assessment of steatosis

Serum steatosis scores

Common steatosis scores were used to calculate risk of NAFLD-related liver steatosis (see **Table 3.18** in **Section 3.6.3 - Non-invasive tests**). These scores included the Fatty Liver Index (FLI), NAFLD liver fat score, Lipid Accumulation Product Index (LAP) and Hepatic Steatosis Index (HSI).

Controlled attenuation parameter

Controlled attenuation parameter (CAP) was measured by a transient elastography system (Fibroscan®, EchoSens, Paris), as described above. CAP measures ultrasonic attenuation of the liver at 3.5MHz, and expresses this in dB/m.

Magnetic resonance spectroscopy (^{1}H-MRS)

Hepatic triglyceride concentration was measured by ^{1}H-MRS. T_1-weighted imaging was performed on a 3.0 Tesla whole-body system (Siemens Prisma) with image-guided localised ^{1}H-MRS. Area of interest was centred in the right lobe of the liver (3.0x2.0x2.0cm voxel), by technicians blinded to patient disease status. Participants lay in a supine position within the MRI machine. Spectra were acquired as previously described (552). Excitation water suppression was used to suppress water signal during data acquisition. Unsuppressed water spectra were acquired for use as the internal standard. Spectral data were post-processed using magnetic resonance user interface software (jMRUI version 4.0, EU Project) by an experimenter blinded to clinical details, as detailed elsewhere (553).

9.3.1.6 Statistical analysis

Data were analysed using IBM SPSS v.22 (SPSS Inc., Chicago, IL, USA) and Microsoft Excel (Microsoft, Redmond, WA, USA). Continuous variables were expressed as mean±standard deviation (normal), and median±interquartile range (IQR) (skewed). Independent Student t-test and Mann-Whitney U-test were used. Multiple groups were compared with ANOVA, with post-hoc Bonferroni test. Categorical variables were expressed as numbers (percentage) and Pearson's chi-squared or Fisher's exact tests were used. Multivariable linear regression with backward elimination set at 5% probability of exclusion was used to find covariates significantly associated with outcomes.

Area under the receiver operator characteristic curves (AUROC) were constructed. A Youden index was used to find overall optimal thresholds. Sensitivity, specificity, positive predictive value (PPV), negative predictive values (NPV), positive likelihood ratio (LR+) and negative likelihood ratio (LR-) were calculated according to optimal and standard (previously published) thresholds. *Intention to diagnose (ITD)* analysis was performed, treating reading failures as false results.

Tests were combined in pairs, calculating diagnostic accuracy based on concordant results. The percentage of patients with discordant tests (indeterminate) and correctly classified (CC) patients (true positives and negatives) were calculated (**Supplementary Figure 9.1**).

A p-value <0.05 was considered statistically significant.

9.4 Results

9.4.1.1 Patients

One hundred and eighty-two obese patients undergoing bariatric surgery were recruited (**Table 9.1**). The average body mass index (BMI) was 45.1±8.3kg/m^2, average age was 44±12 years and there were 45 males (24.7%). There was a high prevalence of metabolic syndrome (61.0%) and type II diabetes mellitus (27.1%). A subset of patients was recruited for imaging tests (**Figure 9.1**), with 82 participants in the TE/CAP subgroup and 49 in the ^{1}H-MRS subgroup.

Histological prevalence of steatosis, NASH and fibrosis are shown in **Supplementary Figure 9.2**. There were 151 participants (83.0%) with histological NAFLD, with 81 participants (45.6%) having moderate steatosis (S2-3), and 7 (3.8%) having significant fibrosis (F2-4).

9.4.1.2 Feasibility of imaging studies

Feasibility of transient elastography (TE) and controlled attenuation parameter (CAP)

Eighty-two participants underwent TE/CAP, with successful readings obtained on 66 (80.5%) (**Table 9.2**). Patients with failed TE/CAP had higher BMI (56.9 vs. 44.7, p=0.001), waist circumference (160 vs. 129cm, p=0.001) and skin-to-liver capsule distance (43 vs. 30mm, p<0.001). Up to a BMI of 41 kg/m^2, all participants had a successful TE/CAP. Notably, patients with a BMI up to 74kg/m^2 had successful TE/CAP readings.

Feasibility of magnetic resonance spectroscopy (^{1}H-MRS)

Forty-nine participants participated in the ^{1}H-MRS component of the study. The overall feasibility rate was 65.3% (n=32) (**Table 9.2**). Reasons for unsuccessful scans were predominantly due to body habitus (n=9, BMI 43-69kg/m^2), claustrophobia (n=5), back pain (n=2) and previous injury with metal discovered on pre-scan screening (n=1). Successful scans were performed on patients up to BMI of 57.3kg/m^2.

9.4.1.3 Detecting moderate steatosis (S2-3)

All results for detection of moderate steatosis (S2-3) are discussed below. Results for detection of any steatosis (S1-3) and severe steatosis (S3) are found in **Appendix 4: Supplementary Materials (Supplementary Tables 9.2-9.6, Supplementary Figure 9.3)**.

Diagnostic accuracy of serum steatosis scores

Moderate steatosis was associated with significantly elevated ALT (29 vs 41, p<0.001) and AST (24 vs 30, p<0.001) (**Table 9.1, Supplementary Table 9.2**). Fatty liver index (FLI), NAFLD liver fat score (NLFS) and the lipid accumulation product index (LAP) showed significant increases with steatosis (p=0.022, 0.015 and 0.001 respectively).

ALT and AST had equivalent or better accuracy to serum steatosis scores, with area under the receiver operator characteristic (AUROC) curve values of 0.699 (p<0.001) and 0.703 (p<0.001) respectively (**Figure 9.2** and **Supplementary Table 9.3**). Sensitivity and specificity for all serum tests are seen in **Supplementary Table 9.4**.

Diagnostic accuracy of controlled attenuation parameter (CAP)

CAP readings were significantly higher in those with moderate steatosis (295 vs 338 dB/m, p=0.002) (**Table 9.1**). There was a weak but statistically significant correlation of CAP reading to percentage liver steatosis (r=0.389, p=0.003) (**Supplementary Figure 9.4**). The AUROC for CAP was 0.688 (p=0.007) (**Figure 9.2** and **Supplementary Table 9.3**), with low to moderate ability to distinguish moderate steatosis with optimised threshold of 285 dB/m (sensitivity 84.8%, specificity 47.2%, correctly classified 65.2%). When incorporating feasibility into an *intention to diagnose (ITD)* analysis, the diagnostic accuracy decreases further (sensitivity 75.7%, specificity 39.5%) (**Table 9.3**).

In addition to histological steatosis severity (β 19.7, p=0.007), baseline BMI (β 2.1, p=0.012) also significantly influenced CAP readings on multivariate analysis (**Supplementary Table 9.5**).

Diagnostic accuracy of magnetic resonance spectroscopy (^{1}H-MRS)

^{1}H-MRS measurements (in percentage hepatic triglyceride concentration (%HTC)) were significantly higher in those with moderate steatosis (3.6% vs 11.4%, p<0.001) (**Table 9.1**), with strong correlations with histological steatosis (r=0.647, p<0.001) (**Supplementary**

Figure 9.4). The AUROC for %HTC was very good for discrimination of moderate steatosis (AUROC 0.852, p=0.001) (**Figure 9.2** and **Supplementary Table 9.3**). Similarly, globally good sensitivities and specificities were obtained using optimal %HTC thresholds, with good biopsy saved rate (53.1%) and correctly classified rate (84.4%) (**Table 9.3**). However, accuracy decreased significantly with *intention to diagnose (ITD)* analysis due to failure rates (sensitivity 50.0%, specificity 59.1%).

Factors influencing %HTC were histological steatosis (β 3.51, p=0.009) and presence of hepatocyte ballooning (β 5.14, p=0.025) (**Supplementary Table 9.6**).

9.4.1.4 Detecting significant fibrosis (F2-4)

All results for detection of significant fibrosis (F2-4) are discussed below. The results for other levels of fibrosis are presented in the **Appendix 4: Supplementary Materials (Supplementary Tables 9.7-9.8** and **Supplementary Figures 9.5-9.6)**.

Diagnostic accuracy of serum fibrosis scores

Fibrosis risk scores showed moderate ability to differentiate significant fibrosis (**Table 9.1** and **Figure 9.2**). Area under the receiver operator characteristic (AUROC) curves showed good to excellent ability to differentiate significant fibrosis, with the best scores being the BARD (AUROC 0.823, p=0.030), Forn (AUROC 0.800, p=0.025), and APRI (AUROC 0.792, p=0.049) (**Figure 9.2** and **Supplementary Table 9.7**).

After calculating sensitivity and specificity using standard thresholds, Forn performed best, with sensitivity 42.9%, specificity 97.7%, and correctly classified rate of 95.5% (**Supplementary Table 9.8**).

Diagnostic accuracy of transient elastography (TE)

The AUROC for TE for diagnosis of significant fibrosis was excellent (0.903, p=0.007) (**Figure 9.2** and **Supplementary Table 9.7**). At an optimal threshold of 9.0kPa, a sensitivity of 100% and specificity of 74.2% was achieved, with correctly classified rate of 75.8%. This reduced slightly with an *intention to diagnose (ITD)* analysis (sensitivity 100%, specificity 59.0%) (**Supplementary Table 9.8**).

Factors influencing LSM readings included the level of histological fibrosis (β 3.947, p<0.001) and the skin-to-liver capsule distance (β 0.348, p<0.001) (**Supplementary Table 9.9**). Other variables, including weight and histological steatosis or inflammation, did not significantly affect LSM.

9.4.1.5 Combining imaging and serum tests

Imaging tests were paired with serum tests, to assess the diagnostic ability of combination approach (**Table 9.3** and **Supplementary Table 9.10-9.11**). Tests were combined using two positive, two negative tests or a serum result in the setting of a failed imaging to determine high or low risk patients, with an indeterminate fraction consisting of patients with discordant results (**Supplementary Figure 9.1**).

CAP and ^{1}H-MRS in combination with alanine aminotransferase (ALT)

Combination of CAP or ^{1}H-MRS with ALT partially mitigated the decreased diagnostic accuracy due to failure rates in an *intention to diagnose (ITD)* analysis (**Table 9.3**). CAP (≥285 dB/m) combined with ALT, had a reasonable sensitivity of 77.8%, with specificity of 56.0%. MRS (≥6.6%) and ALT had a sensitivity of 84.6% for detecting moderate steatosis, but a specificity of 26.1%. Importantly, concordant results for both CAP/ALT and ^{1}H-MRS/ALT resulted in an excellent negative predictive value and improved sensitivity.

Figure 9.3a shows the clinical pathway for use of CAP in combination with ALT, using a serum test initially, followed by CAP for those with elevated ALT.

Transient elastography in combination with serum scores

The combination of TE (≥9kPa) and Forn (≥6.9) substantially improved the positive predictive value (PPV) compared to TE alone, whilst maintaining a good sensitivity of 75% and correctly classified (CC) rate of 76.5% for detecting significant fibrosis (**Table 9.3**). The indeterminate fraction was 21.0% (n=17). Importantly, using TE and Forn to identify lower stages of fibrosis would confidently save biopsies in 59 patients (72.8%). Combination of TE with other scores was not as favourable.

Figure 9.3b demonstrates a practical use of Forn as an initial blood test, and TE as a follow-up imaging test for those with a high Forn index. This clinically applicable pathway has a

75% sensitivity, 98.7% NPV, and 60% PPV for F2-4 disease, with only 2 patients with discordant assessments.

Table 9.1: Baseline characteristics of patients, and subgroups according to steatosis and fibrosis level

	All participants n=182	**S0-1** n=99	**S2-3** n=83	**p-value**	**F0-1** n=175	**F2-4** n=7	**p-value**
Age	44±12	44±13	45±11	0.712	44±12	51±11	0.142
Male gender	45 (24.7%)	18 (18.2%)	27 (32.5%)	0.025	39 (22.3%)	6 (85.7%)	0.001*
Weight (kg)	126.6±28.4	123.1±26.7	130.8±30.0	0.073	125.3±26.6	160.6±49.4	0.108
Body mass index (kg/m^2)	45.1±8.3	44.6±8.2	45.6±8.4	0.432	44.9±8.0	49.3±14.4	0.166
Waist circumference (cm)	125±21	124±19	127±22	0.406	125±21	144±17	0.012
IFG or DM	49 (27.1%)	20 (20.4%)	29 (34.9%)	0.028	44 (25.3%)	5 (71.4%)	0.016*
HTN	81 (44.8%)	41 (41.8%)	40 (48.2%)	0.391	76 (43.7%)	5 (71.4%)	0.245*
Hypercholesterolaemia	35 (19.4%)	17 (17.5%)	18 (21.7%)	0.482	31 (17.9%)	4 (57.1%)	0.028*
ALT	33 (25-52)	29 (20-41)	41 (31-62)	<0.001^	33 (25-52)	42 (21-61)	0.766^
AST	27 (22-35)	24 (20-31)	30 (26-47)	<0.001^	27 (22-35)	34 (25-45)	0.101^
GGT	33 (21-42)	23 (18-38)	36 (31-49)	<0.001^	32 (20-42)	69 (42-105)	0.001^
ALP	70 (58-85)	74 (62-86)	66 (53-84)	0.073^	70 (58-85)	67 (50-104)	0.794^
AST:ALT ratio	0.86±0.30	0.89±0.32	0.82±0.27	0.147	0.85±0.28	1.04±0.53	0.084
Total cholesterol	4.1±1.0	4.0±1.0	4.2±0.9	0.228	4.1±1.0	3.4±1.1	0.074
HDL	0.9 (0.8-1.1)	1.0 (0.8-1.2)	0.9 (0.8-1.0)	0.007^	0.9 (0.8-1.1)	0.8 (0.7-1.0)	0.191^
LDL	2.4±0.8	2.4 ±0.9	2.5±0.8	0.474	2.4±0.8	1.8±0.8	0.041
Triglycerides	1.3 (1.0-1.8)	1.2 (0.9-1.5)	1.4 (1.1-2.1)	<0.001^	1.3 (1.0-1.8)	1.6 (1.4-2.0)	0.135^
Insulin	7.1 (4.2-12.2)	5.7 (3.7-10.9)	5.8 (5.5-6.7)	0.003^	6.7 (4.2-11.6)	14.5 (8.7-30.2)	0.009^
HbA1c	5.7 (5.4-6.1)	5.6 (5.4-6.0)	5.8 (5.5-6.7)	0.006^	5.7 (5.4-6.1)	6.6 (5.8-7.1)	0.061^
Fasting glucose	5.4 (4.8-6.3)	5.2 (4.8-6.0)	5.5 (4.9-6.6)	0.118^	5.3 (4.8-6.1)	6.5 (5.0-7.2)	0.208^
FIBROSIS RISK SCORES							
APRI	0.37±0.34	0.31±0.18	0.44±0.44	0.015	0.37±0.34	0.45±0.21	0.520
NFS	-0.179±1.318	-0.148±1.332	-0.214±1.309	0.740	-0.241±1.258	1.312±1.890	0.002
BARD	3 (1-3)	3 (1-3)	3 (1-3)	0.768^	3 (1-3)	4 (3-4)	0.004^
FIB4	0.175±0.106	0.955±0.502	1.089±0.660	0.127	0.171±0.103	0.264±0.152	0.023
Forn	5.02±0.79	4.21±1.47	4.30±1.58	0.699	4.98±0.75	5.96±1.14	0.001
TRANSIENT ELASTOGRAPHY							
Liver stiffness measure (LSM, in kPa)	9.0±5.8	8.1±5.1	9.9±6.5	0.210	8.3±4.6	20.1±13.0	0.167
Skin to capsule (mm)	32±9	32±10	33±7	0.726	32±9	35±7	0.719
LSM range (kPa)	2.8-37.8	2.8-23.5	3.7-37.8		2.8-23.5	9.1-37.8	
STEATOSIS SCORES							
Fatty liver index (FLI)	90.6±12.9	88.6±15.0	92.9±9.5	0.022	90.2±13.0	98.9±1.2	<0.001
Low (<30)	1 (0.6%)	1 (1.0%)	0	0.144*	1 (0.6%)	0	0.842*
Med (30-60)	7 (3.9%)	6 (6.3%)	1 (1.2%)		7 (4.1%)	0	
High (<60)	170 (95.5%)	89 (92.7%)	81 (98.8%)		163 (95.3%)	7 (100%)	
NAFLD liver fat score (NLFS)	0.621±3.284	0.045±3.644	1.286±2.683	0.015	0.582±3.327	1.643±1.592	0.439
Low (<-0.640)	58 (34.9%)	43 (48.3%)	15 (19.5%)	<0.001	57 (35.6%)	1 (16.7%)	0.666*
High (≥-0.640)	108 (65.1%)	46 (51.7%)	62 (80.5%)	*	103 (64.4%)	5 (83.3%)	
Lipid accumulation product (LAP)	98.2±59.2	83.7±39.3	115.3±72.9	0.001	96.6±59.3	137.0±41.5	0.077
Hepatic steatosis index (HSI)	53.9±8.7	53.9±8.8	54.0±8.6	0.937	53.7±8.1	59.4±17.8	0.429
CONTROLLED ATTENUATION PARAMETER							
CAP (dB/m)	316±60	295±61	338±50	0.002	315±60	329±49	0.661
CAP range	164-400	164-400	231-400		164-400	294-400	
MAGNETIC RESONANCE SPECTROSCOPY							
MRS (% triglyceride content)	6.2 (3.0-11.4)	3.6 (2.5-5.5)	11.4 (7.5-15.5)	<0.001^	6.2 (3.0-11.4)	-	-

*Expressed in mean ± standard deviation, median (interquartile range) or number (%). Independent student t-test and chi-squared test used unless specified. *Fisher exact test, ^Mann Whitney U-test. IFG – impaired fasting glucose; DM – diabetes mellitus; HTN – hypertension; ALT – alanine aminotransferase; AST – aspartate aminotransferase; GGT - gamma-glutamyl transferase; ALP – alanine phosphatase; HDL – high density lipoprotein; LDL – low density lipoprotein; APRI – AST to platelet ratio index; NFS – NAFLD fibrosis score; FIB-4 – Fibrosis-4 score*

Table 9.2: Characteristics of patients with successful, unreliable and unsuccessful transient elastography/controlled attenuation parameter readings

	TE / CAP				**MRS**		
	Successful n=66	**Unreliable** n=7	**Unsuccessful** n=9	**p-value**	**Successful** n=32	**Unsuccessful** n=17	**p-value**
% successful^	80.5%				65.3%		
Age	46 ±12	42 ±16	40 ±11	0.312	44±12	46±12	0.581
Male	21 (31.8%)	1 (14.3%)	1 (11.1%)		5 (15.6%)	11 (64.7%)	<0.001
BMI	44.7±8.6	46.4 ±3.8	56.9 ±9.7*, **	0.001	43.5±6.4	53.6±12.5	0.005
Weight	126.5 ± 30.6	129.1 ±18.3	153.6 ±27.4**	0.039	118.3 ±18.4	162.5±40.9	<0.001
Waist circumference	129 ±21	127 ±21	160 ±17*, **	<0.001	121.6±16.4	155.5±21.0	<0.001
Skin to capsule distance	30±8	36±7	43 ±9**	<0.001	-	-	-
Range	12-47	26-51	33-59		-	-	-
% over 40mm	8 (12.1%)	2 (28.6%)	4 (44.4%)	0.046	-	-	-

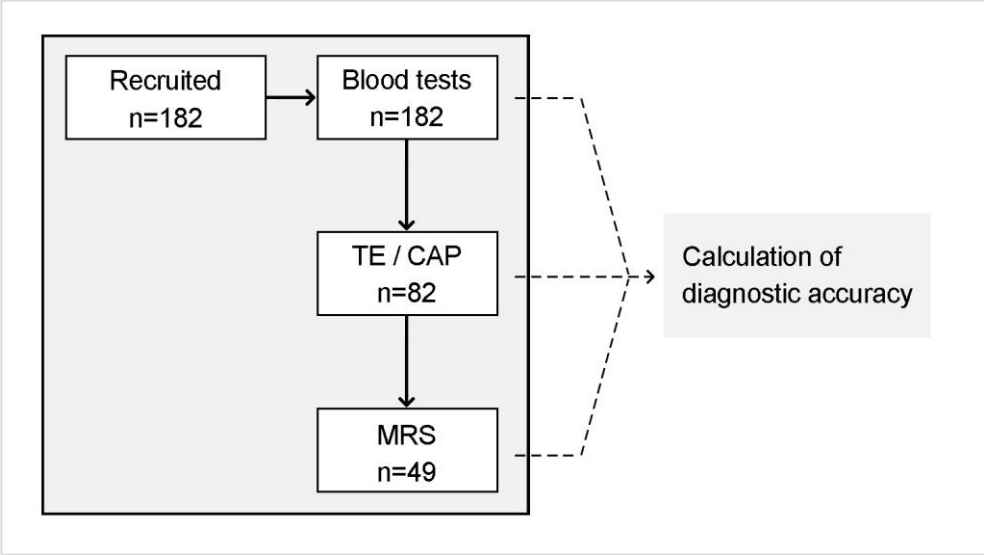
**statistically significant difference compared to unreliable, ** statistically significant difference compared to successful. ^difference in feasibility, p=0.007*

Table 9.3: Diagnostic accuracy of transient elastography (TE) in combination with tests, with intention to diagnose (ITD) analysis.

Combination of tests	Both tests positive^		Both tests negative^		Biopsies saved	Indeterminate/failed	Correctly classified
	Sensitivity	PPV	Specificity	NPV			
S2-3 (threshold CAP≥285 dB/m, MRS ≥6.6%)							
CAP alone	84.8%	59.6%	47.2%	77.3%	22 (31.9%)	-	45 (65.2%)
CAP alone (ITD)	75.7%	51.9%	39.5%	65.4%	22 (27.5%)	16 (20.0%)	45 (56.3%)
MRS alone	81.3%	86.7%	87.5%	82.4%	17 (53.1%)	-	27 (84.4%)
MRS alone (ITD)	50.0%	59.1%	60.9%	51.9%	17 (34.7%)	17 (34.7%)	27 (55.1%)
CAP + ALT	77.8%	56.0%	9.3%	100%	4 (5.0%)	25 (31.3%)	32 (40.5%)
MRS + ALT	84.6%	78.6%	26.1%	100%	6 (12.2%)	15 (30.6%)	28 (57.1%)
F2-4 (TE threshold ≥9kPa)							
TE alone	100%	20.0%	74.2%	100%	46 (55.7%)	-	50 (72.5%)
TE alone (ITD)	100%	11.1%	59.0%	100%	46 (56.1%)	16 (19.5%)	50 (60.9%)
TE+APRI	0	0	77.9%	100%	60 (74.1%)	20 (24.7%)	60 (74.1%)
TE+BARD	100%	18.2%	32.5%	100%	25 (30.9%)	34 (42.0%)	29 (35.8%)
TE+NFS (high)	75%	21.4%	61.5%	100%	48 (58.5%)	20 (24.4%)	51 (62.2%)
TE+FIB4 (low)	75%	27.3%	63.6%	100%	49 (60.5%)	21 (25.9%)	52 (64.2%)
TE+Forn (high)	75%	60%	75.5%	100%	59 (72.8%)	17 (21.0%)	62 (76.5%)

TE–transient elastography; APRI–AST to platelet ratio index; NFS–NAFLD fibrosis score; FIB4–Fibrosis-4 score; PPV–positive predictive value; NPV–negative predictive value.
^Both negative or both positive, or serum result in the absence of successful TE reading.

Figure 9.1: Recruitment of all patients, and substudy recruitment for assessment of imaging tests.



TE/CAP - Transient elastography and controlled attenuation parameter; ^{1}H-MRS – magnetic resonance spectroscopy

Figure 9.2: Area under receiver operator characteristic (AUROC) curves for identification of moderate steatosis (S2-3) with alanine aminotransferase (ALT), NAFLD fatty liver score (NLFS), controlled attenuated parameter (CAP) and magnetic resonance spectroscopy (^{1}H-MRS), and significant fibrosis (F2-4) with AST to platelet ratio index (APRI), NAFLD fibrosis score (NFS), BARD, fibrosis-4 score (FIB-4), Forn and transient elastography (TE).

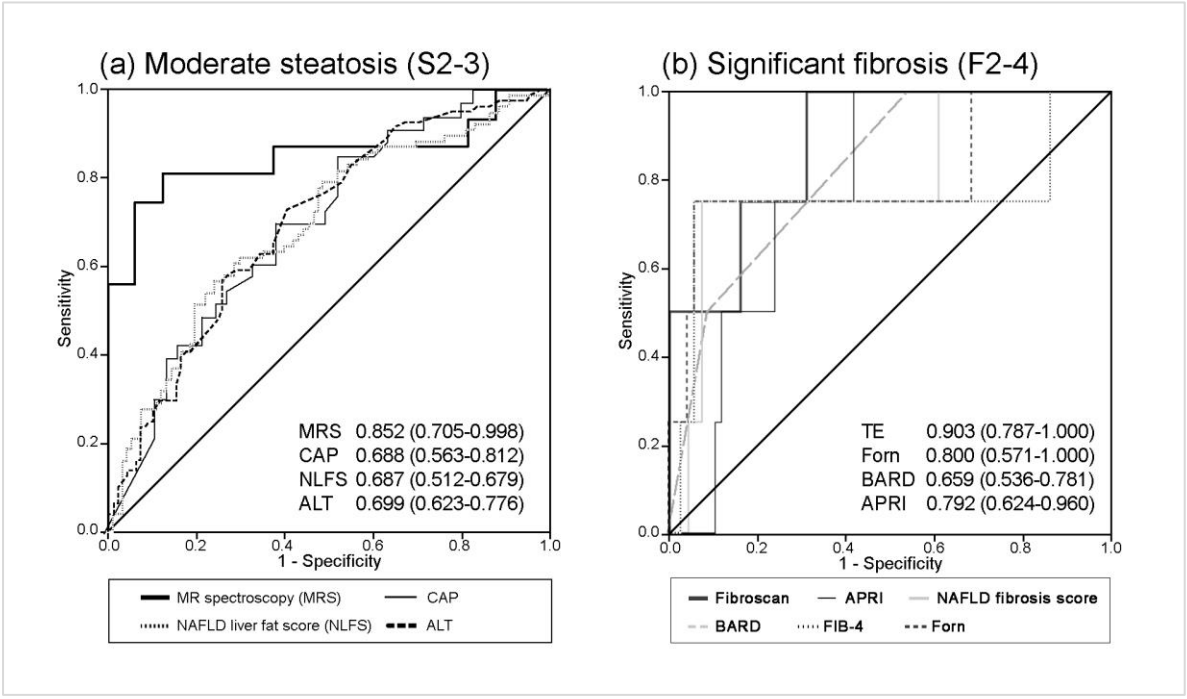
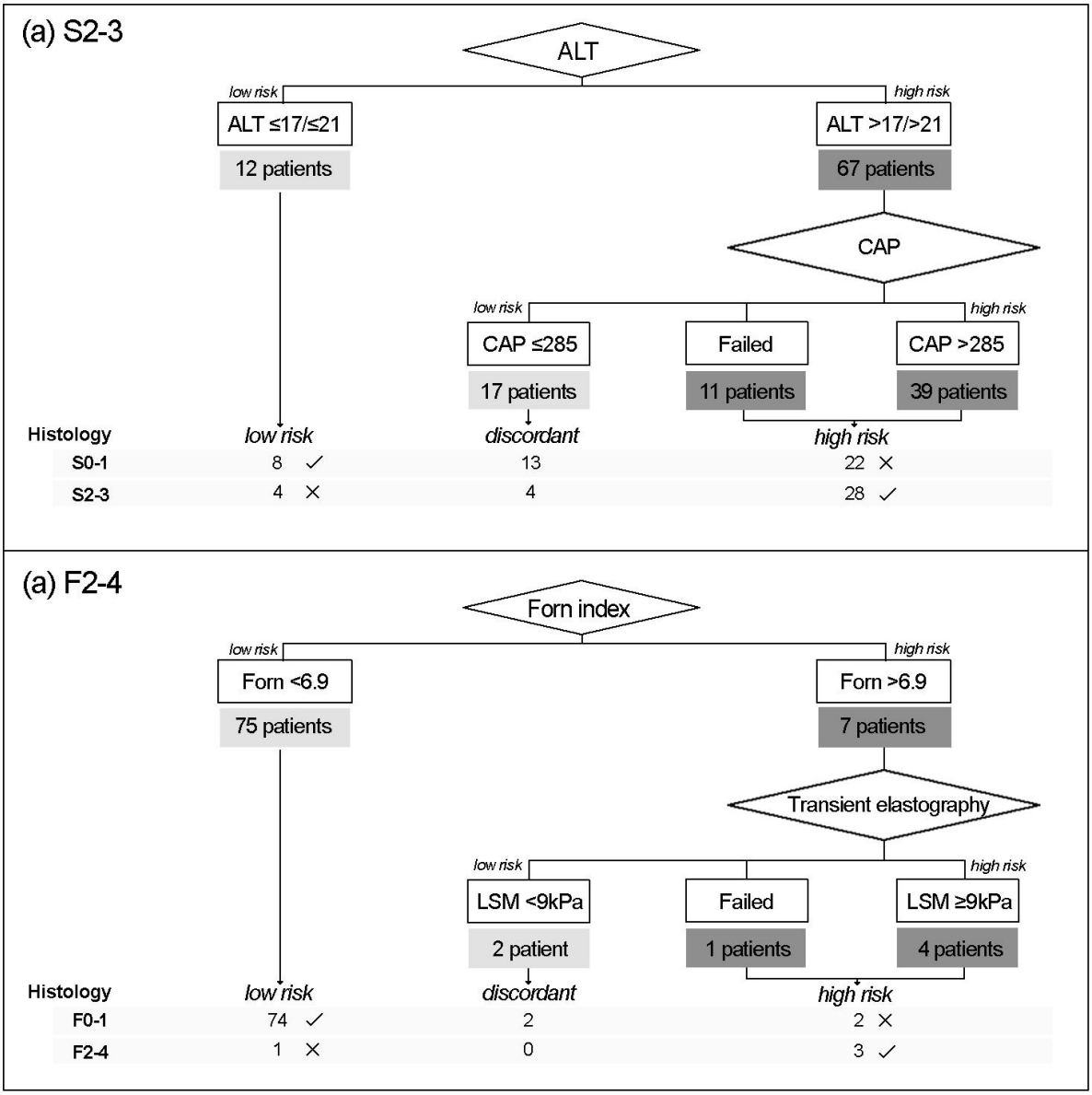


Figure 9.3: Flowchart showing practical application of ***(a)*** *combination of ALT and controlled attenuation parameter (CAP) for detection of moderate steatosis (S2-3) and* ***(b)*** *combination of Forn index then transient elastography (TE) to differentiate patients with significant fibrosis* ***(****F2-4). This figure includes only the subgroup of patients with CAP performed (n=79). Histological grading shown in grey box at bottom of flowcharts.*



9.5 Discussion

Liver steatosis and fibrosis are key determinants of cardiometabolic risk and liver-related morbidity in obesity-related nonalcoholic fatty liver disease. We examined the feasibility and diagnostic accuracy of common non-invasive tests for detection of NAFLD-related steatosis and fibrosis in an exclusively obese population. Due to the challenging nature of obesity and its increasing prevalence, identifying tests and diagnostic strategies that are feasible and accurate in obesity is a priority.

Overall, imaging tests provided the most accurate assessment of both steatosis and fibrosis, with proton magnetic resonance spectroscopy (^{1}H-MRS) having the greatest accuracy for moderate steatosis and transient elastography (TE) being best for significant fibrosis. A considerable drawback of imaging tests was the low success rate due to obesity, with failed readings in one fifth to one third of this obese cohort. This greatly diminished their diagnostic accuracy when using an *intention to diagnose* analysis. Algorithms combining serum markers with imaging tests increased overall diagnostic accuracy in obese individuals by circumventing issues with feasibility. In particular, the combination of TE with the Forn index for fibrosis, and CAP with ALT for steatosis, had reasonable overall accuracy. This would translate to a routine blood test and a rapid imaging study that could be a practical tool for implementation in current clinical practice.

We found that steatosis was most accurately quantified with magnetic resonance spectroscopy (^{1}H-MRS). When feasible, ^{1}H-MRS had excellent accuracy for moderate steatosis (S2-3) with a threshold of 6.6%, however significant failure rates substantially decreased its utility. This, together with inherent drawbacks such as cost, availability and time, make ^{1}H-MRS a somewhat impractical routine assessment tool for the obese population.

Consistent with previous studies (284), serum steatosis scores were not found to have adequate accuracy, particularly not above the use of ALT alone. A possible explanation for the poor performance of steatosis scores could be the heavy weighting of BMI and waist circumference measurements in their algorithms, which can distort the overall score when applied to a morbidly obese cohort.

Controlled attenuation parameter (CAP) combined with ALT provided a practical, cost-effective alternative for detection of moderate steatosis, with reasonable overall diagnostic accuracy and acceptable indeterminate rate (**Figure 9.3**). This could be a reasonable initial clinical algorithm for assessment of those at risk of steatosis.

Diagnosing hepatic steatosis is becoming increasingly relevant, due to growing evidence suggesting its significant and independent links with cardiovascular and metabolic outcomes. A systematic review by Oni *et al* of 27 studies showed evidence of independent association of NAFLD with increased carotid intima-media thickness, coronary artery calcification, endothelial dysfunction and arterial stiffness (259). These are all established factors increasing risk for multiple cardiovascular disease outcomes (260, 261). The level of hepatic steatosis associated with increased risk is not clear, however, we have chosen to focus on S2-3 steatosis, as the majority of evidence has been established around ultrasound-diagnosed NAFLD (259). Typically, ultrasound detects steatosis after approximately 30% of the liver is affected (228), consistent with S2-3 steatosis. In our study of exclusively obese participants, less than half (45%) had S2-3 steatosis, with over one third having mild (S1) simple steatosis only (37%). Therefore, developing and validating tests for detection of steatosis, particularly moderate steatosis, remains relevant. Like detection of diabetes or dyslipidaemia, this may help to stratify those at increased risk of cardiovascular and systemic disease.

For significant fibrosis, transient elastography (TE) had better diagnostic accuracy compared to serum markers (AUROC 0.903 vs 0.772-0.823). We found that a threshold of >9.0kPa had a sensitivity of 100% with specificity of 59.0%. There is currently a large variation of recommended thresholds for detection of significant fibrosis, from 6.4–7.7kPa (302). Naveau *et al* (327) used a threshold of 7.6kPa with sensitivity and specificity of 73% and 78% respectively, and Myers *et al* (506) used an even lower threshold of >6.4kPa. We found that higher thresholds of ≥9.0kPa provided better sensitivity and specificity in this obese cohort. Again, these populations differed in their average BMI (42 kg/m^2 and 30 kg/m^2), which can influence the optimal LSM thresholds.

Combining TE and serum markers, particularly the Forn index, improved the ability to define a subgroup of patients at very high or very low risk of fibrosis. Importantly, 59 patients without significant fibrosis may confidently avoid a liver biopsy with combined low-risk TE and Forn results (72.8% of patients).

Recent evidence suggests that fibrosis stage is the only histological feature associated with liver-related morbidity and transplantation (246, 247). A recent study by Angulo *et al* in 2015 showed that for even Stage 1 fibrosis, there is a hazard ratio (HR) of 1.88 for death or liver transplantation, with increasing risk with more advanced fibrosis (246). In contrast, NAFLD activity score, which integrates histological steatosis and inflammation, and forms the basis for NASH diagnosis, was found to be unrelated to disease progression. This was confirmed with a study by Ekstedt *et al*, which also reported that Stage 3 and Stage 4 fibrosis, regardless of presence of inflammation, was the most important feature that predicted overall mortality (247). Therefore, it is imperative that patients with any degree of fibrosis are identified, for further monitoring and treatment.

Failure rates with imaging techniques in this obese cohort were comparatively high. Magnetic resonance spectroscopy had failure rates of 34.7%, and TE/CAP had failure rates around 20%. This is substantially higher than previously reported (327, 506, 526), but may be explained by the higher BMI distribution in our cohort. Despite this, many patients were effectively assessed, with 100% success up to a BMI of 41 kg/m^2, as well as successful readings on patients with BMI up to 74.0 kg/m^2. This is likely due to variations in skin-to-capsule distance, which is substantially influenced by body habitus and fat deposition patterns, independent of BMI or weight. Use of B-mode ultrasound imaging can also optimise probe position, which may have assisted in achieving successful readings in some obese patients. Hence, BMI alone should not dissuade attempts to assess patients via TE/CAP.

Detection of NAFLD in obesity is particularly relevant due to increasing evidence of successful treatment. Weight loss in the setting of obesity has shown promise in remitting NAFLD. A Cochrane review from 2010 reported eighteen cohort studies showing significant benefit for steatosis following bariatric surgery (443). The evidence for fibrosis resolution was weak, with six studies showing improvement and four showing deterioration. However, more recent evidence, including a systematic review and meta-analysis in 2015, reported improvement in fibrosis after substantial weight loss up to five years after surgery (12, 445, 447). Pharmacological treatment options, such as insulin sensitisers and Vitamin E, are also being developed with evidence of benefit (536). The implications for cardiovascular risk after treatment of NAFLD have not yet been established. However, like other cardiovascular disease risk factors, such as diabetes and hypercholesterolaemia, the effects of management

of NAFLD should be explored. As treatment options grow, and accurate identification of individuals with NAFLD will become increasingly important.

This study differs from previous studies in several ways. Firstly, we have focused on severe and morbidly obese subjects, as a high-risk group for NAFLD. Differences between normal weight and obese individuals extend beyond body measurements, but include significant biochemical, hormonal and inflammatory changes (102). Failure of imaging assessments are common in severe obesity, with known difficulties in acquiring images, as well as inaccuracies from artefact or systematic error due to tissue depth (17). Standard MRI machines have a weight limit of 250kg, but more importantly, a maximum aperture diameter of up to 70cm. TE/CAP also has an optimal reading depth of between 35-75mm, making assessment of many obese patients unfeasible (323). With the rising prevalence and severity of obesity, there is a growing need to tailor effective assessment and management strategies for obese individuals.

Secondly, we have assessed various relatively common diagnostic modalities within this cohort, with comparison to the liver histology as the gold standard. By doing so, we have been able to make a direct comparison of their relative accuracy, and may therefore help develop practical recommendations for clinical practice. Our study was also unique in that we have undertaken a prospective evaluation in consecutive obese patients with NAFLD, rather than those pre-selected for the fibrosis risk. This is of considerable clinical relevance if accurate risk stratification is to be achieved.

The principal limitation in this study was the low rates of significant and advanced fibrosis. The incidence of NASH and fibrosis is lower than that reported in the literature (7, 8). This was surprising, especially given the degree of obesity and prevalence of comorbid conditions. A possible explanation for this may be the recruitment of consecutive bariatric surgical patients rather than a pre-screened cohort of patients from a liver disease setting, which often have an inherent selection bias. Other studies recruiting consecutively from obese or bariatric cohorts have had similar experiences (460, 483, 484, 554). These quoted a prevalence of significant fibrosis of 6.9-19.5% and 13.7-23.8% NASH. This is more likely to represent a true reflection of obese populations that is seen regularly in non-specialised areas. Additionally, bariatric surgical patients are usually younger than the average population, and potentially also self-selected as a more health conscious group. Although diagnostic accuracy of fibrosis tests and combinations were promising in our obese cohort, due to low rates of

significant fibrosis, we make any strong recommendations from these data alone. Future study with higher levels of fibrosis will be required to validate these findings.

We examined the diagnostic accuracy of non-invasive imaging and serum markers for the detection of NAFLD-related steatosis and fibrosis in an obese cohort. Imaging tests, including TE, CAP and ^{1}H-MRS had the greatest accuracy, however failure rates in the setting of obesity were a significant barrier. A combination approach, using best serum markers to augment imaging assessment, improved diagnostic ability. This could provide an easy and practical approach to assessing obese individuals in current clinical practice. However, overall, these data highlight the need for improved diagnostic, screening and monitoring tools for NAFLD in the obese.

10 Visual liver score to stratify nonalcoholic steatohepatitis risk and determine selective intraoperative liver biopsy in obesity

10.1 Abstract

INTRODUCTION: Nonalcoholic fatty liver disease (NAFLD) and its progressive form, nonalcoholic steatohepatitis (NASH), are endemic in obesity. We aimed to evaluate the diagnostic accuracy and reproducibility of a simple intraoperative visual liver score to stratify the risk of NASH and NAFLD in obesity, and determine the need for liver biopsy.

METHODS: This is a prospective cohort study of obese adults undergoing bariatric surgery. The surgical team used a visual liver score to evaluate liver colour, size, and surface. This was compared to histology from an intraoperative liver biopsy.

RESULTS: There were 152 participants, age 44.6±12years, BMI 45±8.3kg/m^2. Prevalence of NAFLD was 70.4%, with 12.1% NASH and 26.4% borderline NASH. Single visual components were not as accurate as the total composite score. Steatosis was most accurately identified (AUROC 0.855, $p<0.001$). NASH was identified with moderate accuracy (AUROC 0.746, $p=0.001$), with sensitivity 75% for a score ≥2. Stratification into low (≤1) and high-risk (≥4) visual scores accurately identified patients who should or should not have an intraoperative biopsy. Most patients with a normal-appearing liver did not have disease (94.4%). The structured visual assessment was quick and interobserver agreement was reasonable ($\kappa=0.53$, $p<0.001$).

CONCLUSIONS: A simple, structured tool based on liver appearance can be a useful and reliable tool for NAFLD risk stratification, and identification of patients who would most and least benefit from a biopsy. A normal liver appearance reliably excludes significant liver disease, avoiding the need for liver biopsy in patients otherwise at high clinical risk of NAFLD and NASH.

10.2 Introduction

Nonalcoholic fatty liver disease (NAFLD) and its more advanced manifestation, nonalcoholic steatohepatitis (NASH), are of increasing relevance to general and bariatric surgeons. Whilst the prevalence of NAFLD in the general population ranges from 2.8-53%, up to 70-88% of obese individuals have NAFLD, with 33-56% having nonalcoholic steatohepatitis (NASH) (555). Diagnosing NAFLD, especially in obesity, is challenging, as there are no obvious early signs or any reliable non-invasive diagnostic test (3, 503). Yet, identification of these patients is vital, to institute appropriate treatment and surveillance for prevention of disease progression and liver failure (3).

Due to the significant prevalence of NAFLD and the relative safety of intraoperative liver biopsy (IOLB), routine biopsy in bariatric and morbidly obese patients during abdominal procedures has previously been advocated (13, 460, 548). The Longitudinal Assessment of Bariatric Surgery (LABS) studies have suggested that lack of routine biopsies result in missed diagnosis in 86% of patients with NASH and 88% with advanced fibrosis (13).

However, even in these studies, the rates of advanced NAFLD were low, resulting in a significant proportion of normal biopsies being performed. Furthermore, the risks, costs and additional operative time of the procedure remain pertinent (470), making it an impractical strategy for all obese patients undergoing abdominal surgery. These factors contribute to why most bariatric surgeons are not performing routine liver biopsy (470).

Incidental diffuse liver abnormalities found during bariatric surgery are common. However, the significance of visual abnormalities of the liver and its relationship to NAFLD is not completely understood. There are few and conflicting data around the accuracy of visual assessment for discriminating liver disease. Some studies have suggested substantial correlation of laparoscopic inspection of the liver to histological diagnosis (458). Conversely, attempts to perform selective IOLB by assessment of liver appearance have been criticised as inaccurate and unreliable (13, 459, 460). Methodology varies significantly between studies, and there is currently minimal evidence that guides selection of patients for IOLB in the setting of suspected NAFLD intraoperatively, particularly to discern NASH or fibrosis.

In this study, we hypothesise that a simple structured method of assessing liver abnormality can standardise practice and more accurately select patients for intraoperative liver biopsy

(IOLB). Importantly, we hypothesise that visual cues may be used to safely and reliably avoid a liver biopsy in patients with otherwise high-risk clinical features. We aimed to examine the diagnostic accuracy and reproducibility of a standardised visual liver scoring (VLS) system for intraoperative identification of NASH and fibrosis in an obese cohort undergoing bariatric surgery. Ultimately, we aimed to assess the utility of this score to more accurately select obese patients for intraoperative liver biopsy.

10.3 Methods

All participants provided informed consent to participate in this study. Ethics approval was obtained from The Alfred Ethics Committee (ref. no. 195/15), The Avenue Ethics Committee (ref. no. 190) and Cabrini Human Research Ethics Committee (ref. no. 09-31-08-15). This study was registered with the Australian Clinical Trials Register (ACTRN12615000875505).

10.3.1.1 Patients

This was a prospective study of consecutive eligible obese and morbidly obese patients who underwent a bariatric surgical procedure between 2015 and 2016 in three metropolitan hospitals in Melbourne, Australia, to investigate nonalcoholic fatty liver disease (NAFLD) in the obese.

Inclusion criteria in the study included: (1) age ≥18 years, (2) undergoing a bariatric surgical procedure, (3) BMI≥35 kg/m^2, (4) alanine aminotransferase (ALT) or aspartate aminotransferase (AST) > half upper limit normal (ULN), or gamma-glutamyl transferase (GGT) > ULN. Patients were excluded if they had evidence of any other liver disease, including viral, medication-related, autoimmune, familial/genetic causes or a current or past history of excessive alcohol use, defined as per the American Association for the Study of Liver Diseases (3).

10.3.1.2 Clinical and biochemical data

Patients underwent a complete medical history and physical examination in clinic prior to surgery. Weight and measurements were repeated on the day of operation. Fasting blood tests were taken prior to induction.

10.3.1.3 Bariatric surgery and intraoperative liver biopsy

Intraoperative core and wedge (1cm^3) liver biopsies were taken from each patient from the left lobe of liver. A single experienced pathologist graded the biopsies in a blinded manner, according to the NAFLD activity score (NAS) (481) and Kleiner classification of liver fibrosis (240). The NAFLD activity score is a composite score from 0-8 based on steatosis severity (0-3), inflammation severity (0-3) and presence of hepatocyte ballooning (0-2).

A total NAS score of ≥5 is diagnostic for NASH, NAS 3-4 is equivocal for NASH and NAS 0-2 is not diagnostic of NASH. Of note, this scoring system was developed primarily for research purposes, and not strictly for clinical diagnosis of NASH (243). Fibrosis was staged from F0-4, with F1 being perisinusoidal or periportal fibrosis, F2 being perisinusoidal and portal/periportal fibrosis, F3 being bridging fibrosis and F4 being cirrhosis.

10.3.1.4 Visual liver scores

A visual liver score (VLS) was developed, based on previous criteria with modifications based on other assessment criteria (9, 457, 458, 460). The liver appearance was scored in the categories of *colour* (0-2), *size* (0-3) and *surface* nodularity (0-3), with a *total* score calculated by the sum of these categories. The VLS was developed to be simple, easy to learn and easy to calculate. The grading system is seen in **Table 10.1**, with **Figure 10.1** showing example of *total* VLS scores and associated histological findings in four study patients.

Six experienced bariatric surgeons participated in this study. During the operation, the operating surgeon was asked to score the liver according to the scoring system provided. In addition, a subjective *overall impression* on presence or absence of liver abnormality was elicited, with 0 for 'likely normal', 1 for 'unsure/equivocal' and 2 for 'likely abnormal'.

An independent sample of laparoscopy videos showing the liver in detail was used to quantify the overall level of agreement across raters.

10.3.1.5 Statistical analysis

Data were analysed using IBM SPSS v.23 (SPSS Inc., Chicago, IL, USA) and Microsoft Excel (Microsoft Corporation, Redmond, WA, USA).

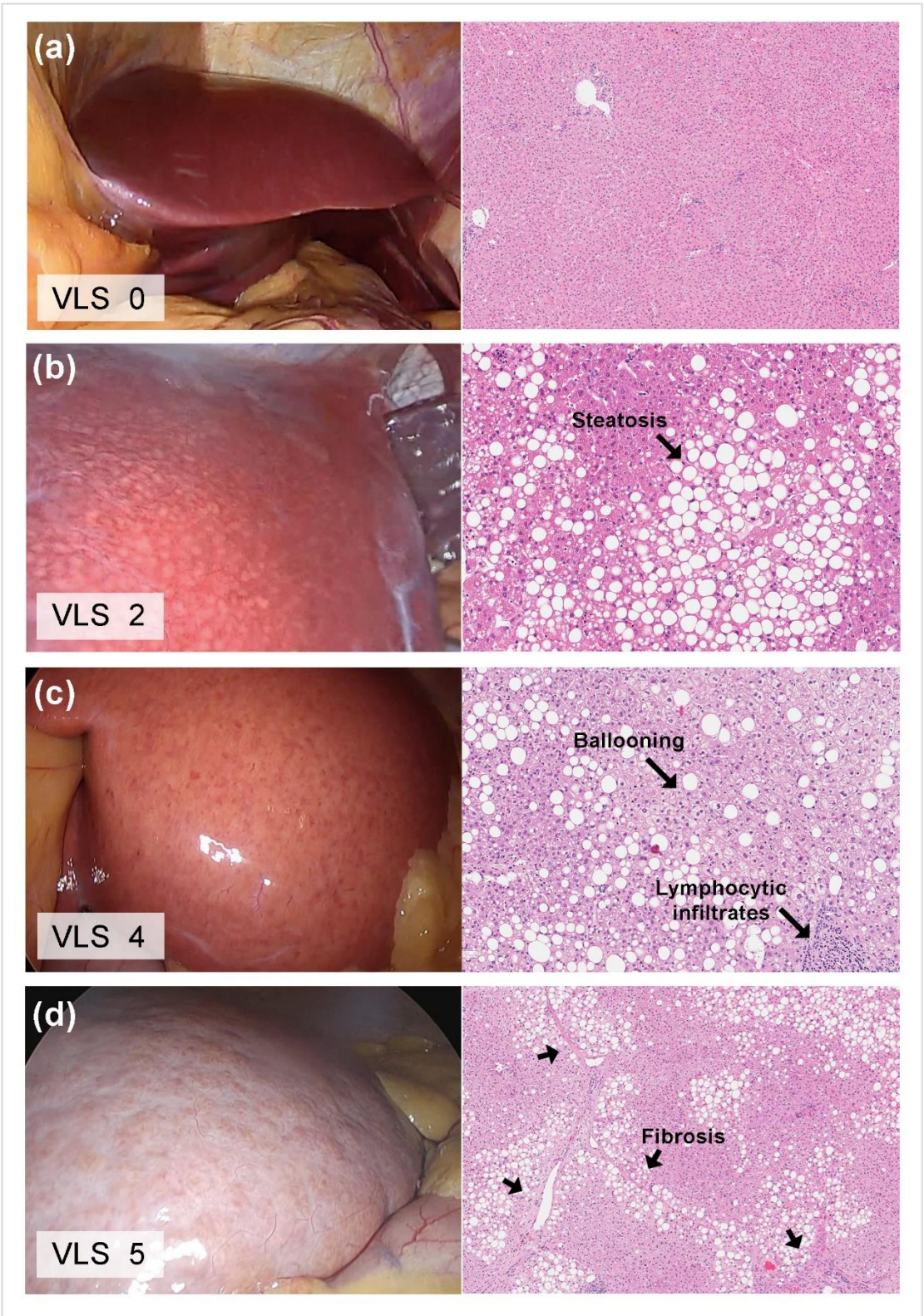
Continuous variables were expressed as mean±standard deviation (SD) for parametric data, and median±interquartile range (IQR) for nonparametric data. Independent student *t*-test was used for parametric data and Mann-Whitney *U*-test for nonparametric data. Normality was assessed by the Shapiro-Wilk test. Categorical variables were expressed as numbers (with percentages) and Pearson chi-squared or Fisher's exact tests were used. Kappa statistic was used for interobserver agreement. A p-value <0.05 was considered statistically significant.

Patients were analysed according to presence of steatosis, NASH, and fibrosis, particularly focusing on significant steatosis (S2-3), NASH (NAS≥5) and significant fibrosis (F2-4). Sensitivity and specificity were calculated for a range of VLS and overall impression scores. A Youden index was calculated to determine optimum thresholds. A low and high threshold was also determined for use in a diagnostic tool, to divide patients optimally into low, intermediate and high-risk groups. An area under the receiver operator characteristic (AUROC) curve was created for each of the visual liver scale categories, *total* VLS and *overall impression* score.

Table 10.1: Intraoperative visual liver scoring (VLS) system, incorporating scores for colour, size and surface, with total VLS score as a sum of these components scores.

Grade	Description	Details
Colour component		
0	Normal colour	Tan colour.
1	Moderate steatosis	Sparse to moderate spotty yellow.
2	Significant steatosis	Mostly homogenous yellow colour.
Size component		
0	Normal size	Sharp liver edge, no gross enlargement.
1	Mild enlargement	Blunted liver edge, minimal liver enlargement.
2	Moderate enlargement	Blunted liver edge with moderate liver enlargement, some difficulty with retraction due to size.
3	Severe enlargement	Significant liver enlargement, impeding operation (oesophagogastric/bariatric).
Surface component		
0	Smooth surface	Smooth surface, clear light reflex.
1	Mild nodularity	Fine rough surface, fine spotty light reflex.
2	Prominent nodularity	Rough surface, coarsely rough spotty light reflex.
3	Cirrhotic	Nodular liver.
Total score **(0-8) calculated by sum of colour, size and surface scores.**		

Figure 10.1: Examples of visual liver score (VLS) total scores for patients, and comparison with histological findings.



10.4 Results

10.4.1 **Patients**

One hundred and fifty-two obese patients undergoing bariatric surgery were recruited. Baseline characteristics are shown in **Supplementary Table 10.1**. The average age was 44.9±11.8 years with a female predominance (76.3%). The average BMI was 45.0±8.2 kg/m^2.

There were 107 with histologically diagnosed NAFLD (70.4%). Significant steatosis (S2-3) was seen in 61 patients (40.1%) and significant fibrosis (F2-4) was seen in 6 patients (4.0%). There were 20 patients with definitive NASH (NAS ≥5) (13.2%) and 34 patients with features equivocal for NASH (NAS 3-4) (22.4%).

Patients with NASH were significantly heavier (BMI 48.5±11.2 vs 44.5±7.5, p=0.041), with more insulin resistance (HOMA2-IR 2.4±1.8 vs 1.2±1.1, p=0.011), and higher triglyceride levels (2.0±0.8 vs 1.4±0.7, p=0.003) and liver function tests (ALT 67.2±44.1 vs 43.4±41.9, p=0.026 and GGT 42 (31-57) vs 31 (19-41), p=0.003) (**Supplementary Table 10.1**).

10.4.2 **Visual liver scores and assessments**

The distribution of visual liver score (VLS) components and other visual assessment criteria according to histological severity are seen in **Table 10.2**.

There were significant differences in *colour* scores for those with significant steatosis (S2-3) and NASH. Significant differences in *size* score were seen for steatosis, NASH and fibrosis, and differences in *surface* nodularity were seen in significant fibrosis (**Supplementary Table 10.1**).

The *total* VLS score, consisting of a sum total of *colour*, *size* and *surface* scores, was significantly different in those with significant steatosis (0.87±1.19 vs. 2.43±1.73, p<0.05), NASH (3.05±1.99 vs 1.26±1.42, p=0.001), and significant fibrosis (1.40±1.53 vs 3.83±2.14, p<0.05). Similarly, the *overall impression* score was significantly higher for patients with significant steatosis, NASH, and significant fibrosis (**Table 10.2**).

10.4.2.1 Diagnostic accuracy

The area under the receiver operator characteristic (AUROC) curves for visual appearance in differentiating steatosis, NASH and fibrosis are shown in **Figure 10.2** and **Supplementary Table 10.3a-b**.

The sensitivity and specificity for a variety of threshold values of the *total* VLS and *overall impression* scores are shown in **Table 10.3a**. An assessment of the proposed VLS risk tool, stratifying patients into low, intermediate and high-risk cohorts, is shown in **Table 10.3b**.

Detecting steatosis

Of all the histological components of NAFLD, significant steatosis was most easily detected with visual cues (**Figure 10.2** and **Supplementary Table 10.3a**). Of the individual components, *colour* score best predicted the presence of significant steatosis (S2-3), with AUROC 0.733 (0.649-0.817, $p<0.05$). The *total* VLS (AUROC 0.767 (0.689-0.847), $p<0.05$) performed better than any individual component and the *overall impression* score (AUROC 0.759 (0.678-0.841), $p<0.05$) (**Figure 10.2**).

An optimised threshold of ≥2 for *total VLS* correctly classified 75.0% of patients according to presence of significant steatosis, with sensitivity 70.5% and specificity 78.0% (**Table 10.3a**). Using dual thresholds, a high threshold VLS≥4 had a positive predictive value (PPV) of 79.2%, and a low threshold VLS≤1 had a negative predictive value of 79.8%. There were 39 patients (25.7%) with an intermediate score of VLS 2-3.

Identifying NASH (NAS ≥5)

The *total* VLS score best predicted the presence of NASH (NAS≥5), with reasonable diagnostic accuracy (AUROC 0.746 (0.616-0.876), $p=0.001$), and better accuracy than any individual component (AUROC from 0.546 to 0.728). The *overall impression* score showed a similar diagnostic accuracy to the *total* VLS (**Figure 10.2**).

An optimal *total* VLS score of ≥2 gave a sensitivity of 70.5%, specificity of 78.0% and correctly classified rate of 75.0% for NASH (**Table 10.3a**). Using dual thresholds, the NPV for a low threshold score (VLS≤1) was excellent at 94.4%, corresponding to 5 patients with NASH misclassified (**Table 10.3b**). Twenty-four patients had a high VLS≥4, however only 10 of these patients had histological NASH (PPV 41.7%), giving a high false positive rate.

Identifying fibrosis

The *total* VLS was able to distinguish significant fibrosis (F2-4) with good accuracy (AUROC 0.841 (0.716-0.966), p=0.005), and performed better than individual VLS components alone (AUROC from 0.637 to 0.818) and the *overall impression* score (AUROC 0.797 (0.682-0.911), p=0.014) (**Figure 10.2**). Whilst *surface* score had poor diagnostic accuracy for detection of NASH and milder fibrosis, the AUROC for detection of significant fibrosis was 0.818 (p=0.008). An optimal *total* VLS ≥2 had a sensitivity of 100% and specificity of 61.0%. Negative predictive value for low-risk VLS≤1 was 100%, showing an excellent ability to exclude significant fibrosis accurately. A PPV of 12.5% with a high-risk VLS≥4 meant that 21 patients without significant fibrosis have a falsely high score.

Interobserver agreement

Blinded rating of thirty-three independent laparoscopy videos showed variable agreement among five participating surgeons. The *total VLS* score had modest agreement between the five surgeons (κ=0.53, p<0.05) (**Supplementary Table 10.4a**). Paired agreement between the five surgeons was variable (κ from 0.39 to 0.90) (**Supplementary Table 10.4b**). There was poor to average agreement on individual components of disease (colour: κ=0.46, p<0.05; size: κ=0.42, p<0.05; surface: κ=0.45, p<0.05), and the *overall impression* score (colour: κ=0.38, p<0.05). Assessment of the VLS took an average of 15.7±6.8 seconds per case viewed via video.

Table 10.2: Characterisation of the study population by visual liver score (VLS). Differences in scores according to (a) significant steatosis (S2-3), (b) NASH diagnosis as per the NAFLD activity score histological criteria, (c) presence of significant fibrosis (F1-4).

Variable			All patients	Significant steatosis (S2-3)			NASH (NAS ≥5)			Significant fibrosis (F2-4)		
				S0-1	S2-3	p-value	Not NASH	NASH	p-value	F0-1	F2-4	p-value
			n=152				n=132 (86.8%)	n=20 (13.2%)		n=146 (96.0%)	n=6 (4.0%)	
VLS: Colour			0.61 ±0.77	0.22±0.62	1.02±0.81	<0.001	0.52±0.73	1.20±0.83	<0.001	0.59±0.77	1.00±0.89	0.203
	Score	0	87 (57.2%)	68 (61.2%)	19 (15.4%)	<0.001	82 (62.1%)	5 (25.0%)	0.001	85 (58.2%	2 (33.3%)	0.438
		1	38 (25.0%)	16 (25.9%)	22 (15.4%)		32 (24.2%)	6 (30.0%)		36 (24.7%)	2 (33.3%)	
		2	27 (17.8%)	7 (12.9%)	20 (69.2%)		18 (13.6%)	9 (45.0%)		25 (17.1%)	2 (33.3%)	
VLS: Size			0.78 ±0.84	0.48±0.64	1.21±0.91	<0.001	0.66±0.74	1.55±1.05	0.001	0.75±0.82	1.50±1.05	0.031
	Score	0	68 (44.7%)	53 (58.2%)	15 (24.6%)	<0.001	64 (48.5%)	4 (20.0%)	<0.001	67 (45.9%)	1 (16.7%)	0.157
		1	56 (36.8%)	33 (36.3%)	23 (37.7%)		51 (38.6%)	5 (25.0%)		54 (37.0%)	2 (33.3%)	
		2	22 (14.5%)	4 (4.4%)	18 (29.5%)		15 (11.4%)	7 (35.0%)		20 (13.7%)	2 (33.3%)	
		3	6 (3.9%)	1 (1.1%)	5 (8.2%)		2 (1.5%)	4 (20.0%)		5 (3.4%)	1 (16.7%)	
VLS: Surface			0.11 ±0.41	0.05±0.23	0.20±0.57	0.070	0.08±0.35	0.30±0.66	0.164	0.06 ±0.24	1.33±1.21	0.050
	Score	0	139 (91.4%)	86 (94.5%)	53 (86.9%)	0.164	123 (93.2%)	16 (80.0%)	0.003	137 (93.8%)	2 (33.3%)	<0.001
		1	10 (6.6%)	5 (5.5%)	5 (8.2%)		8 (6.1%)	2 (10.0%)		9 (6.2%)	1 (16.7%)	
		2	2 (1.3%)	-	2 (3.3%)		0	2 (10.0%)		0	2 (33.3%)	
		3	1 (0.7%)	-	1 (1.6%)		1 (0.8%)	0		0	1 (16.7%)	
Total VLS score			1.49 ±1.62	0.87±1.19	2.43±1.73	<0.001	1.26±1.42	3.05±1.99	0.001	1.40±1.53	3.83±2.14	<0.001
Completely normal (VLS score of 0)			58 (38.2%)	63 (69.2%)	16 (26.2%)	<0.001	55 (41.7%)	3 (15.0%)	0.002	58 (39.7%)	0	0.011^
VLS grade	0-1 (low risk)		89 (58.6%)	71 (78.0%)	18 (29.5%)		84 (63.6%)	5 (25.0%)		89 (61.0%)	0	
	2-3 (intermediate)		39 (25.7%)	15 (16.5%)	24 (39.3%)		34 (25.8%)	5 (25.0%)		36 (24.7%)	3 (50.0%)	
	≥4 (high risk)		24 (15.8%)	5 (5.5%)	19 (31.1%)		14 (10.6%)	10 (50%)		21 (14.4%)	3 (50.0%)	
Overall impression			0.8 ±0.9	0.41±0.67	1.26±0.85	<0.001	0.6±0.8	1.5±0.8	<0.001	0.71±0.85	1.67±0.52	0.005
	Normal		79 (52.0%)	63 (69.2%)	16 (26.2%)	<0.001	75 (56.8%)	4 (20%)	<0.001	79 (54.1%)	0	0.025
	Equivocal		32 (21.1%)	19 (20.9%)	13 (21.3%)		30 (22.7%)	2 (10%)		30 (20.5%)	2 (33.3%)	
	Abnormal		41 (27.0%)	9 (9.9%)	32 (52.5%)		27 (20.5%)	14 (70%)		37 (25.3%)	4 (66.7%)	
Biopsy?	No		131 (86.2%)	85 (93.4%)	46 (75.4%)	0.003	118 (89.4%)	13 (65.0%)	0.003	129 (88.4%)	2 (33.3%)	0.003^
	Yes		21 (13.8%)	6 (6.6%)	15 (24.6%)		14 (10.6%)	7 (35.0%)		17 (11.6%)	4 (66.7%)	

*Expressed as mean±standard deviation and number (percentage). Student t-test used for continuous variables, and chi-squared test used for categorical variables unless otherwise stated. *Mann Whitney U-test. ^Fisher exact test*

Table 10.3a: Diagnostic accuracy of various thresholds for visual liver scores and overall visual impression scores

		Sensitivity	Specificity	PPV	NPV	CC	Correct	Avoid Bx
Significant steatosis (S2-3), n=61								
VLS	≥1	82.0%	51.6%	53.2%	81.0%	63.8%	97	58
	≥2	70.5%	78.0%	68.3%	79.8%	75.0%	114	89
	≥3	42.6%	91.2%	76.5%	70.3%	71.7%	109	118
	≥4	31.1%	94.5%	79.2%	67.2%	69.1%	105	128
Overall impression	Equivocal/abnormal	73.8%	69.2%	61.6%	79.7%	71.1%	108	79
	Abnormal	52.5%	90.1%	78.0%	73.9%	75.0%	114	111
NASH (NAS≥5), n=20								
VLS	≥1	85.0%	41.7%	18.1%	94.8%	47.4%	55	58
	≥2	75.0%	63.6%	23.8%	94.4%	65.1%	84	89
	≥3	60.0%	83.3%	35.3%	93.2%	80.3%	110	118
	≥4	50.0%	89.4%	41.7%	92.2%	84.2%	128	128
Overall impression	Equivocal/abnormal	80.0%	56.8%	21.9%	94.9%	59.9%	75	79
	Abnormal	70.0%	79.5%	34.1%	94.6%	78.3%	105	111
Significant fibrosis (F2-4), n=6								
VLS	≥1	100%	39.7%	6.4%	100%	42.1%	58	58
	≥2	100%	61.0%	9.5%	100%	62.5%	89	89
	≥3	50.0%	78.8%	8.8%	97.5%	77.6%	115	118
	≥4	50.0%	85.6%	12.5%	97.7%	84.2%	128	128
Overall impression	Equivocal/abnormal	100%	54.1%	8.2%	100%	55.9%	79	79
	Abnormal	66.7%	74.7%	9.8%	98.2%	74.3%	109	111

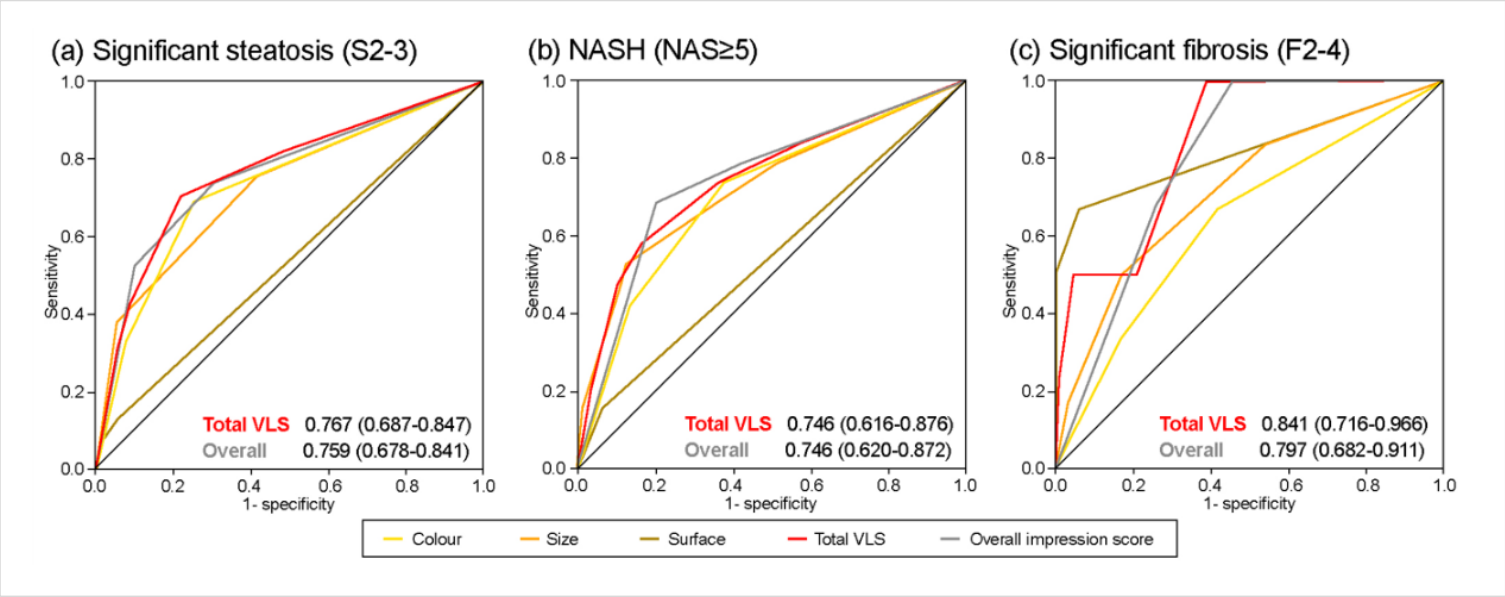
VLS – visual liver score; PPV – positive predictive value; NPV – negative predictive value; CC – correctly classified rate.

Table 10.3b: Diagnostic accuracy using dual thresholds – low, intermediate and high risk scores – for significant steatosis, NASH and significant fibrosis.

	Total	Low risk VLS ≤1	Intermediate VLS 2-3	High risk VLS ≥4	Diagnostic utility	
		n=89 (58.6%)	n=39 (25.7%)	n=24 (15.8%)	Low cut-off	High cut-off
Significant steatosis (S2-3)						
S0-1	91	71	15	5	Sens: 70.5%	Spec: 94.5%
S2-3	61	18	24	19	NPV: 79.8%	PPV: 79.2%
NASH (NAS≥5)						
NAS <5	132	84	34	14	Sens: 75.0%	Spec: 89.4%
NAS ≥5	20	5	5	10	NPV: 94.4%	PPV: 41.7%
Significant fibrosis (F2-4)						
F0-1	146	89	36	21	Sens: 100%	Spec: 85.6%
F2-4	6	0	3	3	NPV: 100%	PPV: 12.5%

VLS – visual liver score; Sens – sensitivity; Spec – specificity; NPV – negative predictive value; PPV – positive predictive value; NAS – NAFLD activity score

Figure 10.2: Area under the receiver operator characteristic (AUROC) curves of visual assessment tools for detection of (a) significant steatosis, (b) NASH, and (c) significant fibrosis.



10.5 Discussion

In this study, we developed a standardised visual liver score (VLS) for prediction of NAFLD and NASH in obese patients undergoing laparoscopic surgery. It provides a simple, accurate and reproducible framework for assessment of diffuse liver abnormalities incidentally found during an operation. Performance of the composite structured score was far more reliable than use of individual components or a more subjective assessment. Importantly, this visual liver score reliably stratifies patients into low, intermediate and high-risk for NAFLD and NASH, providing guidance on the benefit of performing intraoperative liver biopsy.

In clinical practice, a low-risk score (VLS≤1) would indicate a biopsy is unnecessary (NPV 79.8-100%), even with high-risk clinical features such as morbid obesity. Conversely, an intraoperative liver biopsy can be recommended in those with a high-risk score (VLS≥4), and should be considered for those with an intermediate score (VLS 2-3). The sensitivity of an intermediate to high-risk score is excellent for detection of NASH and fibrosis, despite lower positive predictive values. Together with the relative ease and safety of intraoperative liver biopsy, this approach offers a reasonable risk-benefit ratio.

Our findings lead us to a different conclusion to other studies where routine intraoperative liver biopsy (IOLB) has been recommended. Proponents of routine IOLB argue that the substantial prevalence of NASH, especially in this high-risk bariatric cohort, mandates a biopsy in all patients, particularly in the relatively safe conditions that laparoscopic surgery affords. The Longitudinal Assessment of Bariatric Surgery (LABS) studies, and other subsequent observations suggested that not performing routine IOLB on bariatric patients resulted in significant missed diagnosis (13, 459, 460). However, our study and previous studies in bariatric surgical cohorts (460, 503) including the LABS study (13), also found that a substantial proportion do not have clinically significant NAFLD, and instituting routine IOLB would result in considerable numbers of unnecessary biopsies. A liberal selective approach to intraoperative liver biopsy with this structured intraoperative visual assessment provides a convenient and accurate method to avoid biopsies in the large proportion of patients who are unlikely to have any benefit.

The use of a systematic scoring system such as the VLS has substantial advantages over an individual *overall impression* by individual surgeons. Firstly, we have shown that this standardised scoring system has higher accuracy. The total VLS showed reasonable

sensitivity for identification of significant steatosis and NASH, and an excellent ability for identifying significant fibrosis (F2-4). Furthermore, risk stratification with the VLS allows for a level of certainty for the majority of patients. Notably, patients with a low-risk VLS (≤1) can avoid an unnecessary biopsy. In clinical practice, this would translate to 89 patients without significant fibrosis (58.6%) who could avoid an unnecessary biopsy in this cohort. Secondly, this system was more consistent among users, with higher interobserver agreement than the subjective *overall impression* score (κ 0.53 vs 0.38). This demonstrates the importance of a systematic approach to assessment of visual cues. The VLS is also simple to learn, quick to assess and easy to calculate during an operation.

In this cohort, many patients who had a considerably abnormal appearing liver (VLS≥4) did not have NASH or significant fibrosis. The positive predictive values were low, at 41.7% for NASH and 12.5% for significant fibrosis. The high rate of false positive patient likely represents those with simple steatosis rather than more advanced disease, which can significantly enlarge the liver and change its appearance. Nonetheless, the ability to identify significant steatosis (PPV 79.2% for VLS≥4) remains clinically important, as steatosis can progress to more severe disease, albeit at a slower rate (249, 252). Additionally, patients with any grade of NAFLD, including steatosis only, have been shown to have worse overall and liver-related mortality (2, 263).

Nonalcoholic fatty liver disease is projected to become the leading cause of cirrhosis, liver failure and transplantation in the near future (1). Despite its growing prevalence and increasing burden, the diagnosis of NAFLD is often made incidentally, as most patients are asymptomatic. Non-invasive techniques are not adequately reliable (229, 503, 556), and inherent risks and drawbacks of liver biopsy means that it cannot be used as a diagnostic test for the large number of at-risk patients (227). Patients with morbid obesity, usually accompanied by significant metabolic disease and compounded by adverse dietary and lifestyle factors, are one such high-risk population.

Identification and accurate grading of patients with NAFLD is essential to institute effective and appropriate treatment strategies according to disease severity (329). Weight loss and control of metabolic disease are central to treatment for all patients with NAFLD (3). Reasonable weight loss goals of 10-15% total body weight loss should be targeted for improvement in NAFLD (554) and the metabolic risk factors (557). Patients with NASH or fibrosis may be considered for pharmacotherapy, such as Vitamin E or thiazolidinediones (3).

More advanced disease, such as cirrhosis, require surveillance for HCC and potentially management of liver failure.

This study has some limitations that warrant discussion. Firstly, there were lower than expected rates of NASH (13.2%), and low rates of fibrosis (23.7%), particularly significant (F2-4) fibrosis (3.9%), compared to published epidemiological evidence (8). This may be due to the recruitment of consecutive obese patients who are considered high risk, but have not been preselected based on a known or suspected diagnosis of NAFLD. This has been seen in other studies that recruit from bariatric or obese populations for the study of NAFLD (13, 460, 483, 484). Secondly, this visual liver score has been developed based on scores used by previous studies (9, 458-460) and discussions with surgeons. A multitude of variables have previously been used, including tactile impression, blanching, greasy surface, congestion, surface vascularity, light reflux, vascularity of the falciform ligament and splenic congestion (458-460). Future endeavours should examine these variables to create an optimised tool for NAFLD diagnosis. Thirdly, we have focused on bariatric patients in this study, which may limit its applicability to the general population and normal-weight populations. Finally, this visual liver scoring system has not been validated independently, and further study should focus on external validation in a larger cohort.

In conclusion, we recommend that surgeons adopt a systematic approach to assessment of liver appearance and consideration of intraoperative liver biopsy. The visual liver score (VLS) can reliably exclude significant NAFLD, therefore removing routine biopsy as a requisite in morbidly obese populations. It has reasonable sensitivity for identification of disease, with fair interobserver agreement, and importantly, it is simple to learn and quick to apply intraoperatively. Use of this simplified tool aids in stratifying the risk of NAFLD, NASH and fibrosis, and can be a valuable adjunct to clinical practice.

11 Evaluation of the histologic variability of nonalcoholic fatty liver disease

11.1 Abstract

PURPOSE: Liver biopsy remains the gold standard for characterizing nonalcoholic fatty liver disease (NAFLD). Liver heterogeneity, sampling and interobserver variability can affect the reliability of results. This study aimed to compare histological variability of intraoperative wedge and core liver biopsies in bariatric patients, to better inform clinicians on biopsy method and guide interpretation of results.

MATERIALS AND METHODS: We prospectively recruited bariatric surgical patients. Intraoperative left and right core biopsies and a left wedge biopsy was taken. Agreement of histological findings between biopsy sites and between pathologists was evaluated.

RESULTS: There were 91 participants (72.2% female), mean age 46.8years, body mass index 45.9kg/m^2. There was no significant pattern for up- or down-grading disease dependent on biopsy technique (core vs wedge). Good agreement was seen in presence of NAFLD and NASH (κ=0.609-0.865, $p<0.001$). Individual components showed less concordance (κ=0.223-0.656, $p<0.01$), with fibrosis showing particularly poor agreement between biopsy sites (κ=0.223-0.496, $p<0.01$). Discordant diagnoses of clinically important histological variables were seen in 12.0-22.8%. Interobserver variability of NASH diagnosis was significant (prevalence 13.5% vs 27.3%, p=0.004).

CONCLUSION: Overall diagnosis of NAFLD or NASH showed good agreement between biopsy types, but components, particularly fibrosis stage, varied significantly. Neither wedge nor core biopsy were shown to better assess NAFLD severity. When assessing NAFLD, surgeons should be aware of this variation and consider at least two biopsy sites for better overall assessment. These data have important implications in NAFLD fibrosis assessment and are relevant in the interpretation of histological efficacy of clinical investigational therapies.

11.2 Introduction

Nonalcoholic fatty liver disease (NAFLD) is common in obesity, affecting up to 71-98% of obese individuals (7, 8). Up to 56% have the more severe form, nonalcoholic steatohepatitis (NASH), which can progress to fibrosis, cirrhosis and ultimately, liver failure (7, 12, 245). The bariatric surgical cohort bears a particularly high prevalence of disease, owing to substantial levels of obesity and often multiple comorbid conditions, particularly diabetes.

Liver biopsy is currently the best standard for diagnosing and grading NAFLD (7). However, in nearly all liver disease, especially NAFLD, parenchymal abnormalities are irregularly distributed (227). Magnetic resonance imaging (MRI) studies have demonstrated significant variation in fatty deposition and sparing throughout the liver (271). This has substantial implications for diagnosis, especially when considering that a single core liver biopsy represents only 1/50,000-65,000 of the liver (7).

The assessment of intrahepatic heterogeneity in NAFLD has not been well established. Previous studies examining variation in liver biopsies in NAFLD have yielded conflicting results (see **Table 3.28** in **Section 3.9.5.6 – Choice of liver biopsy technique**) (269, 270, 473-478). Some studies showing significant variation in steatosis (474), and others report discordance in fibrosis (270, 475, 477) and inflammation (270, 476), with study populations ranging from as few as eight participants to 146 participants. Notably, two of the larger studies examined histology of deep sections taken post-mortem (473, 474), and therefore, these wedge biopsies will not necessarily reflect the peri-capsular wedge biopsies obtained on patients pre-mortem. Furthermore, the differences between wedge and core biopsies has not been well established, with only one study by Rawlins *et al* examining this variation in 8 participants (478).

Therefore, scarce evidence currently guides our clinical practice regarding the best choice of biopsy technique for NAFLD, particularly in obese and bariatric surgical patients where disease prevalence is high, and the opportunity to perform core and wedge biopsies under vision exists. Investigation into the degree of sampling error is essential to quantify variation, inform choice of biopsy method and guide interpretation of results.

In this study, we aimed to assess the variation in histological NAFLD between methods of intraoperative biopsy (wedge vs core biopsy) and lobe of liver (right vs left lobe), to

investigate the impact of biopsy type and location on NAFLD diagnosis and staging. Additionally, we aimed to assess agreement in NAFLD scoring between biopsy locations and between histopathologists. We hypothesise that variability and heterogeneity exist within NAFLD, which is an important consideration when interpreting liver biopsies in this disease. Ultimately, we hope this study may better inform clinicians on the most appropriate liver biopsy method for more accurate identification and monitoring of NAFLD.

11.3 Methods

Between June 2015 and November 2016, we prospectively recruited obese individuals undergoing bariatric surgery, who were at risk of NAFLD. All participants provided informed consent to participate in this study. Ethics approval was obtained from The Alfred Human Research Ethics Committee (HREC) (no. 195/15), Avenue HREC (no. 190) and Cabrini HREC (no. 09-31-08-15). This study was registered with the Australian Clinical Trials Register (ACTRN12615000875505).

11.3.1 **Participants**

Inclusion criteria included: (1) age ≥18 years, (2) BMI≥35 kg/m^2, (3) alanine aminotransferase (ALT) or aspartate aminotransferase (AST) > 1.5 times upper limit normal (ULN), or gamma-glutamyl transferase (GGT) >ULN. Patients are excluded if they had evidence of any other liver disease, including viral, medication-related, autoimmune, familial/genetic causes and/or a history of excessive alcohol use, defined by the American Association for the Study of Liver Diseases (3).

11.3.2 **Outcomes**

Intraoperative core liver biopsies were taken from the left and right lobes of liver (two 16-18G cores from each lobe, Bard Max-Core® Biopsy Instrument, Covington, GA, USA), and one wedge biopsy (at least 1cm in depth) was taken from the left lobe of liver. Biopsies with less than 11 portal tracts or less than 15mm in length were considered inadequate (227). Individual biopsies were graded by one histopathologist, blinded to clinical data, patient and site from which the biopsy was taken. A second histopathologist assessed each set of biopsies and gave an overall grade for each patient, again blinded to clinical data and prior histopathologist's opinion.

NAFLD was defined as greater than 5% hepatic steatosis. The NAFLD activity score (NAS) (481) and the Kleiner classification of liver fibrosis (240) were used to report liver histology. An NAS score of 0-2 is "not NASH", 3-4 is "equivocal for NASH" and NAS≥5 is defined as NASH. Pathologists did not only grade the disease, but gave a global diagnosis, semi-quantitated individual features of activity and staged fibrosis in each sample. For this study, F2-4 fibrosis was considered "significant fibrosis".

11.3.3 **Statistical analysis**

Agreement between liver biopsy sites (i.e. right versus left lobe) and biopsy methods (i.e. Wedge versus core) for each of the components of disease (e.g. steatosis, fibrosis, NASH) was evaluated kappa coefficients (κ) with 95 % confidence interval. Interobserver agreement was assessed between pathologists scores. A $\kappa < 0.4$ was considered minimal agreeance, with κ 0.4-0.6 being weak agreeance, κ 0.6-0.8 being moderate, and $\kappa > 0.8$ being strong.

Continuous variables were expressed as mean±standard deviation (SD) for parametric data, and median and interquartile range (IQR) for nonparametric data. Paired sample t-test, and Wilcoxon signed-rank test were used. Categorical variables were expressed as numbers (with percentages). McNemar's test were used for paired categorical variables. A p-value ≤ 0.05 was considered statistically significant.

Data analysis was performed in Microsoft Excel SPSS v23 (SPSS Inc., Chicago, IL, USA).

11.4 Results

11.4.1 **Participants**

There were 91 participants recruited, with mean age 46.8±12.0 years, mean BMI 45.9±9.4 kg/m^2 and a female predominance (n=65, 72.2%) (**Table 11.1**).

Of these patients, adequate left sided core biopsies were available in 89 participants, right sided core biopsies in 78 participants and wedge biopsies in 90 participants. Average length of all core biopsies was 23.4±8.2mm, with no significant difference between left and right core biopsy lengths (22.6 vs 22.4mm, p=0.146). Reasons for biopsy loss were lack of access intraoperatively and inadequate tissue sampling (i.e. at least one or more of <11 portal tracts, <15mm length, significant diathermy artefact).

There was one complication in the study from a right sided core biopsy specimen, which perforated the gallbladder, necessitating a cholecystectomy.

A summary of histopathological scores are shown in **Table 11.2a**. There were no statistically significant differences in scores for any component of NAFLD, between the biopsy types.

11.4.2 **Sampling variability**

The level of agreement between biopsy types is seen in **Figure 11.1a** with a direct comparison of fibrosis scores seen in **Supplementary Table 11.1a-b**.

Good concordance was seen between core biopsies on left and right sides. The overall diagnosis of NAFLD (>5% steatosis) and NASH diagnosed by the pathologist had excellent agreement between right and left core biopsies (κ 0.865 and 0.838 respectively). Reasonable concordance was seen in separate histological components of NAFLD, including steatosis grade, inflammation grade, and any presence of inflammation or ballooning (κ 0.613-0.720, p<0.001). Fibrosis appeared more variable between lobes, with weak concordance for fibrosis stage (κ 0.476, p<0.001) and presence of significant fibrosis (F2-4) (κ 0.496, p<0.001).

The comparison of wedge to core biopsies showed less agreement. The best agreement was in diagnosis of NAFLD, NASH diagnosis by pathologist and NASH diagnosis by NAS criteria (κ 0.609-0.664, p<0.001). Moderate agreement was also seen in steatosis and any inflammation/ballooning between left core and wedge biopsies (κ 0.604-0.638, p<0.001), and

NAS criteria between right core and left wedge biopsy (κ 0.637, p<0.001). Poor to weak agreement was seen in all other histopathological domains, including inflammation and ballooning grade, and importantly, fibrosis stage (κ 0.223-0.479, p<0.05).

In practice, these results translate to considerable rates of discordant diagnoses for clinically important variables (**Table 11.2b**). Presence of significant fibrosis was diagnosed on at least one, but not all, biopsies in 14 patients (15.2%). For NASH, 21 patients (22.8%) had differing NASH status, dependent on biopsy location. This variability could substantially alter our interpretation of disease severity, which will ultimately affect management recommendations and strategies.

11.4.3 **Interobserver variability**

There were significant differences in steatosis, ballooning and fibrosis between observers (**Table 11.2a**). Subsequently, differences in total NAS and NAS classification were seen, with over double the rate of NASH (13.5% vs 27.3%, p=0.004).

There was good agreement regarding the diagnosis of NAFLD (between wedge biopsies: κ 0.714, p<0.001), with moderate agreement about steatosis grade (κ 0.422-0.493, p<0.001) (**Figure 11.1b**). However, poor agreement was seen for the diagnosis of NASH (κ 0.273-0.404, p<0.001), and for components of NAFLD, including inflammation, ballooning and fibrosis stage (κ 0.115-0.345, p<0.05).

Table 11.1: Baseline population characteristics

Variable	
Age	46.8±12.0 years
Gender	65 (72.2%) female
Weight	130.0±32.7 kg
Body mass index	45.9±9.4 kg/m^2
Type II diabetes	25 (27.8%)
Hypertension	51 (56.7%)
Dyslipidaemia	21 (23.3%)
Bilirubin	10.4±5.9
ALT	31.5 (22.5-53.0) IU/L
AST	27.5 (20.5-34.5) IU/L
GGT	35.0 (20.0-45.0) IU/L
HbA1c	6.12±1.18%
Fasting glucose	5.6 (4.9-6.9) mmol/L
Total cholesterol	3.96±1.01 mmol/L
HDL	0.96±0.26 mmol/L
LDL	2.32±0.90 mmol/L
Triglycerides	1.53±0.76 mmol/L

ALT – alanine aminotransferase; AST – aspartate aminotransferase; GGT – gamma glutamyltransferase; HbA1c – glycosylated haemoglobin A1c; HDL – high density lipoprotein; LDL – low density lipoprotein.

Table 11.2a: Comparison of scores for each depot and between pathologists

Variable		Left core	Right core	Left wedge	p-value			2nd path overall score	p-value		
					Left v Right	Left v Wedge	Right v Wedge		2nd vs Left	2nd v Right	2nd v Wedge
Steatosis		1.52±1.05	1.47±0.99	1.49±1.09	0.698	1.000	0.854	1.21±0.91	**<0.001**	**<0.001**	**<0.001**
	S0	15 (16.9%)	14 (17.9%)	19 (21.3%)				23 (25.8%)			
	S1	35 (39.3%)	27 (34.6%)	29 (32.6%)				30 (33.7%)			
	S2	17 (19.1%)	23 (29.5%)	19 (21.3%)				30 (33.7%)			
	S3	22 (24.7%)	14 (17.9%)	22 (24.7%)				6 (6.7%)			
Inflammation		0.57±0.75	0.53±0.7	0.57±0.72	0.070	0.877	0.531	0.49±0.57	0.365	0.625	0.304
	0	51 (57.3%)	46 (59.0%)	49 (55.7%)				48 (53.9%)			
	1	26 (29.2%)	23 (29.5%)	29 (33.0%)				38 (42.7%)			
	2	11 (12.4%)	9 (11.5%)	9 (10.2%)				3 (3.4%)			
	3	1 (1.1%)	0	1 (1.1%)				0			
Ballooning		0.69±0.76	0.62±0.67	0.7±0.75	0.163	0.496	0.276	0.45±0.67	**0.005**	**0.047**	**<0.001**
	0	44 (49.4%)	38 (48.7%)	41 (46.6%)				58 (65.2%)			
	1	29 (32.6%)	32 (41.0%)	32 (36.4%)				22 (24.7%)			
	2	16 (18.0%)	8 (10.3%)	15 (17.0%)				9 (10.1%)			
Fibrosis		0.49±0.94	0.54±0.83	0.47±0.8	0.615	0.783	0.880	0.30±0.63	**0.026**	**0.010**	**0.013**
	0	64 (71.9%)	49 (62.8%)	59 (67.0%)				68 (76.4%)			
	1	14 (15.7%)	20 (25.6%)	21 (23.9%)				17 (19.1%)			
	2	4 (4.5%)	5 (6.4%)	5 (5.7%)				2 (2.2%)			
	3	6 (6.7%)	4 (5.1%)	2 (2.3%)				2 (2.2%)			
	4	1 (1.1%)	0	1 (1.1%)				0			
Total NAS		2.75±2.3	2.63±2.12	2.69±2.36	0.167	0.872	0.765	2.15±1.80	**0.003**	**0.002**	**<0.001**
NAFLD by definition	*Not NAFLD*	15 (16.9%)	14 (17.9%)	19 (21.4%)	1.000	0.508	0.508	23 (25.8%)	**0.039**	0.057	0.180
	NAFLD	74 (83.1%)	64 (82.1%)	70 (78.7%)				66 (74.2%)			
Pathologist defined NASH	*Not NASH*	52 (58.4%)	46 (59%)	49 (56.3%)	0.688	0.804	1.000	-	-	-	-
	NASH	37 (41.6%)	32 (41%)	38 (43.7%)				-			
Significant fibrosis	*F0-F1*	78 (87.6%)	69 (88.5%)	80 (90.9%)	1.000	1.000	1.000	85 (95.5%)	0.065	0.180	0.219
	F2-F4	11 (12.4%)	9 (11.5%)	8 (9.1%)				4 (4.5%)			
NAS classification	*NAS 0-2*	46 (51.7%)	41 (52.6%)	45 (51.1%)	0.289	0.774	0.227	56 (62.9%)	**0.027**	0.077	**0.004**
	NAS 3-4	20 (22.5%)	20 (25.6%)	19 (21.6%)				21 (23.6%)			
	NAS ≥5	23 (25.8%)	17 (21.8%)	24 (27.3%)				12 (13.5%)			

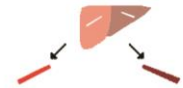
L v R – Left core vs right core; L v W – Left core vs left wedge; R v W – Right core vs left wedge

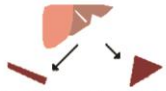
Table 11.2b: Concordant assessments between biopsy sites

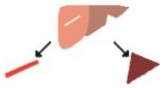
Histological variable	Patients with same diagnosis between all biopsy sites	Number with discordant results
NAFLD (steatosis >5%)	79	11 (12.0%)
NASH (pathologist)	70	21 (22.8%)
NASH (defined by NAS)	74	16 (17.4%)
Significant fibrosis (F2-4)	76	14 (15.2%)

Figure 11.1: (a) Sampling variability between (left) left core vs right core, (middle) left core vs left wedge and (right) right core vs left wedge, (b) interobserver variability between scoring for each liver biopsy compared to 2nd pathologist overall score.

(a) Sample variability







Overall diagnosis	Right core vs Left core kappa	Right core vs Left core p-value	Left core vs Left wedge kappa	Left core vs Left wedge p-value	Right core vs Left wedge kappa	Right core vs Left wedge p-value
Diagnosis of NAFLD	0.865	<0.001	0.664	<0.001	0.637	<0.001
NASH by pathologist	0.838	<0.001	0.615	<0.001	0.644	<0.001
NASH by NAS (≥5)	0.720	<0.001	0.644	<0.001	0.609	<0.001
Individual components	*kappa*	*p-value*	*kappa*	*p-value*	*kappa*	*p-value*
Steatosis grade	0.642	<0.001	0.638	<0.001	0.545	<0.001
Inflammation grade	0.656	<0.001	0.386	<0.001	0.458	<0.001
Ballooning grade	0.526	<0.001	0.474	<0.001	0.479	<0.001
Any inflammation/balloon	0.613	<0.001	0.604	<0.001	0.585	<0.001
NAS criteria	0.662	<0.001	0.543	<0.001	0.637	<0.001
Fibrosis stage	0.476	<0.001	0.223	0.004	0.284	<0.001
Significant fibrosis (F2-F4)	0.496	<0.001	0.413	0.001	0.335	<0.001

(b) Interobserver variability

Overall diagnosis	Left core vs 2nd Path kappa	Left core vs 2nd Path p-value	Right core vs 2nd Path kappa	Right core vs 2nd Path p-value	Left wedge vs 2nd Path kappa	Left wedge vs 2nd Path p-value
Diagnosis of NAFLD	0.603	<0.001	0.478	<0.001	0.714	<0.001
NASH by NAS (≥5)	0.273	<0.001	0.341	<0.001	0.404	<0.001
Individual components	*kappa*	*p-value*	*kappa*	*p-value*	*kappa*	*p-value*
Steatosis grade	0.455	<0.001	0.422	<0.001	0.493	<0.001
Inflammation grade	0.180	0.031	0.258	0.005	0.179	0.042
Ballooning grade	0.302	<0.001	0.287	0.001	0.345	<0.001
Any inflammation/balloon	0.117	0.050	0.256	<0.001	0.285	<0.001
NAS criteria	0.115	0.007	0.176	<0.001	0.285	<0.001
Fibrosis stage	0.247	0.002	0.291	0.001	0.265	0.001
Significant fibrosis (F2-F4)	0.215	0.019	0.253	0.015	0.467	<0.001

11.5 Discussion

In this cohort of obese individuals, we found good concordance for the overall diagnosis of NAFLD and NASH (κ 0.609-0.865) between all biopsies. In comparison, individual components such as steatosis, inflammation and ballooning showed only moderate to weak concordance between biopsy types (κ 0.386-0.656). Liver fibrosis showed especially poor agreement (κ 0.223-0.496). This translated to a 12.0-22.8% rate of discordant diagnoses of clinically significant histological variables. There was no clear tendency for up- or down-grading of NAFLD severity, based on core versus wedge biopsy. Interobserver agreement between pathologists was poor for all aspects of NAFLD grading.

There are several important learning points to gain from our study findings. Firstly, there is clear variation in NAFLD severity between biopsy sites within individual patients. This has implications for diagnosis, management and evaluation of treatment efficacy. This is particularly important for assessment of fibrosis stage, which has the greatest impact on NAFLD prognosis (246, 247), and yet shows the greatest variation between biopsies. Such substantial sampling variability can affect our ability to confidently assess severity of NAFLD, as well as the efficacy of NAFLD treatments in clinical practice and, importantly, in a trial setting.

Some strategies may help to decrease the impact of this variability. In an operative setting, consideration should be made towards at least two sites or methods of liver biopsy. This may more reliably capture severe disease represented heterogeneously within the liver. Furthermore, at least one intraoperative right-sided core biopsy should be considered. This could improve consistency and minimise inherent intrahepatic variability, when comparing with any future conventional right-sided percutaneous core biopsies.

Secondly, substantial interobserver variability is a key consideration in histological examination of NAFLD. This study shows poor interobserver agreement in all components, with exception for the overall diagnosis of NAFLD (steatosis ≥5%). In particular, fibrosis stage and presence or absence of significant fibrosis (F2-4) showed substantial variability (κ 0.215-0.467). Previous studies have similarly reported discrepancies in interpretation of histological features of NAFLD (κ 0.33-0.64) (272, 558, 559). In a clinical setting, this can create ambiguity regarding diagnosis and efficacy of therapies aimed at decreasing steatofibrosis.

With standardization and training, some studies show moderately good agreement (240, 272, 273, 560-562). Additional strategies that may mitigate interobserver variability include obtaining a consensus diagnosis by two specialist histopathologists, similar to other gastrointestinal disease processes such as Barrett's oesophagus (563). Furthermore, reassessment of past histological specimens with new specimens could also reduce the influence of inter- and intra-observer factors on assessment of NAFLD progression or resolution.

Wedge biopsies remain a viable method for assessment of liver histology. While we found agreement is low compared to either core biopsies, there is no clear up- or down-grading of NAFLD components seen with wedge biopsies. Notably, contrary to previous teaching (227, 564), we did not find that fibrosis grade or presence of significant fibrosis were increased in wedge biopsy specimens. This may be due to the collection of wedge biopsies greater than 1cm in depth (565). An additional benefit of wedge biopsy is the larger biopsy size obtained compared to a core biopsy. This facilitates adequate tissue collection for histological examination, and importantly, is a means of collecting specimens for NAFLD research.

The within-liver variation observed in this study is not surprising, given the size of the organ, its anatomy and complex function. Vascular anatomical variations are thought to influence fat deposition and sparing. For example, portal blood supply is theorized to contribute to geographic fat distribution, with the superior mesenteric vein, containing lipogenic alimentary products, preferentially distributed to the right lobe of liver (566). Moreover, smaller variations in capsular, peribiliary, cystic veins and right gastric veins can cause local haemodynamic abnormalities that may also affect fat deposition. Biliary duct anatomy and pathology have also been theorised to contribute to disease distribution (564). Greater sampling of various areas in the liver may help to better capture these variations, to more accurately detect clinically significant NAFLD.

Differential risk profiles accompany wedge and core biopsies performed intraoperatively. Bleeding risk accompanies both methods of biopsy, with potentially greater risk with wedge biopsy due to larger raw surface area. Wedge biopsies may also potentially injure peripheral biliary radicles. However, needle biopsy has the potential to damage deep or adjacent organs and structures, including major blood vessels, biliary ducts, gallbladder, and diaphragm. Additionally, tissue size obtained with needle biopsy may be compromised, and multiple

biopsies are often required for adequate assessment (227). Therefore, there is no clear choice of biopsy method based on risk profile alone.

This study has several strengths. To our knowledge, it is the largest study evaluating the liver heterogeneity of NAFLD within a live patient cohort. In contrast to autopsy studies, this present study uses practical and safe liver biopsy techniques, and therefore histological specimens obtained are representative of those from a clinical setting. Secondly, we aimed to compare core and wedge biopsy techniques. Both are viable intraoperative options, associated with their own risk and benefit profile, however data into the impact of each method on histological grade has been lacking. Finally, we have focused on NAFLD exclusively, due to the increasing burden of disease, particularly in obese cohorts. A laparoscopic procedure is an opportune time to consider diagnosis of NAFLD in the high risk bariatric surgical population. These data can help bariatric clinicians and surgeons make more informed decisions regarding biopsy method technique.

There are a few drawbacks that warrant discussion. Firstly, whilst we have taken biopsies from multiple sites, we have picked different lobes, without investigating differences within liver lobes or between liver segments. Investigation into segmental differences in the liver, and variation between wedge biopsies could lead to further insights into the variability of NAFLD. Secondly, the use of bariatric surgical patients may limit the applicability of these data to more general populations, particularly those undergoing percutaneously obtained biopsies. Thirdly, there have been substantial advancement in imaging techniques, such as magnetic resonance imaging (MRI), for assessing NAFLD (297, 567). Compared to biopsies, imaging techniques can characterise the liver in its entirety. In a heterogenous disease such as NAFLD, this can potentially be used to both assess liver disease, as well as target liver biopsies. Future studies could incorporate these imaging techniques with histological assessment.

In conclusion, although we have demonstrated good agreement in overall presence of NASH and NAFLD between biopsies, sample agreement was reduced and interobserver agreement was poor for individual NAFLD components. In particular, fibrosis shows significant variation between biopsy locations and observers. No systematic up- or down-grading of

disease severity was observed with either core or wedge biopsies, and hence wedge biopsy remains a valid means of NAFLD assessment. When possible, consideration should be given towards multiple liver biopsies, to more accurately capture disease severity. These findings can assist in informing interpretation of NAFLD histological results, and further highlights the need to develop improved strategies to more comprehensively assess and diagnose NAFLD.

Research Theme 3

Impact of weight loss on NAFLD and related metabolic diseases

Studies 6-9

Weight loss is one of the most effective therapies for NAFLD and metabolic disease. Substantial evidence suggests that significant weight loss, up to 20-30% TBWL, results improvement in diabetes, NAFLD, and other obesity-related disorders (163, 443-445). However, we do not have a good understanding of the effects of more modest weight loss, or the ideal threshold weight for meaningful metabolic and hepatic improvement.

Knowing the effects of incremental weight loss on NAFLD and metabolic disease has several benefits. Firstly, it can assist in setting realistic patient expectations regarding weight loss benefits. Secondly, it is beneficial for pre-treatment goal setting for both patients and clinicians. Finally, the ability to track expected metabolic improvements against weight loss can also assist in monitoring progress, identifying those who do not respond conventionally and implementing alternate strategies when required.

By closely monitoring patients undergoing weight loss after LAGB insertion, these following studies document the effects of progressive weight loss on NAFLD and metabolic disease. The overarching aims were to determine key weight loss goals for significant improvement in NAFLD and metabolic health, and examine the patterns of improvement.

12 Effects of bariatric surgery on liver function tests in patients with nonalcoholic fatty liver disease

12.1 Abstract

OBJECTIVES: Nonalcoholic fatty liver disease (NAFLD) affects over 80% of obese patients, and is fuelled by the metabolic syndrome. Weight loss is strongly advocated as a central treatment for NAFLD, and has been shown to induce histological improvement. We aimed to define the patterns of improvement in NAFLD with weight loss, and determine target weight goals for NAFLD resolution.

METHODS: A prospective study of 84 morbidly obese patients with NAFLD undergoing bariatric surgery was conducted. Intraoperative liver biopsies were taken. Monthly follow-up, including blood tests and measurements, was performed. We monitored improvements in NAFLD by monthly alanine aminotransferase (ALT) and gamma glutamyltransferase (GGT) levels over one year.

RESULTS: There was rapid improvement in ALT, particularly in the first six months following surgery. A significant decrease in ALT was seen at two months (35 vs 27 IU/L, $p<0.001$), corresponding to a percentage total body weight loss (%TBWL) of 6.4% (4.2–8.2). In multivariate analysis, a %TBWL of 10-15% was significantly related to ALT resolution (odds ratio 2.49, $p=0.005$). Triglyceride levels (odds ratio 0.59, $p=0.021$) and baseline NAFLD activity score (odds ratio 0.28, $p<0.001$) were also significantly related to ALT resolution. Improvements in ALT occurred prior to metabolic improvement, and well before traditional ideal weight goals were reached.

CONCLUSION: Improvements in NAFLD occurred rapidly after bariatric surgery and were closely related to weight loss and metabolic factors. A 10-15% reduction in body weight is an appropriate target to achieve substantial improvement in ALT levels.

12.2 Introduction

Over 80% of individuals with obesity have non-alcoholic fatty liver disease (NAFLD), with 15-56% having the more severe non-alcoholic steatohepatitis (NASH) and 2-4% having cirrhosis (8, 278). Obesity, together with commonly associated metabolic disorders including type II diabetes, drives hepatic steatosis as well as progression to inflammation and fibrosis (6). The significance of NAFLD relates to the associated increased all-cause and liver-related mortality (276).

Weight loss is advocated as a central treatment for NAFLD. Bariatric surgery has been shown to reliably provide substantial weight loss in the range of 20-25% total body weight loss (TBWL), with associated improvements in insulin resistance, NAFLD and overall mortality, in morbidly obese patients (568-572). Paired liver biopsy studies have shown reduction in all histological components of NAFLD after bariatric surgery, including reversal of fibrosis (447, 573, 574). However, the follow-up for these studies are longer-term, with intervals of up to ten years and average excess weight loss (EWL) of over 50% (447, 569, 570, 573, 574).

Whilst it is established that NAFLD and NASH both improve with substantial weight loss following bariatric surgery, there is limited understanding of the time course of these significant improvements post-surgery. Defining the patterns of improvement in liver chemistries with weight loss could inform weight loss targets that focus on maximum health benefit, rather than arbitrarily defined ideal body weight. This information may not only inform surgical weight loss, but also weight loss goals for conservative or pharmacological programs, which often do not achieve or sustain such substantial weight loss outcomes as seen in these surgical studies. This would be of great value to treating clinicians, as realistic goal setting is critical to successful weight loss programs.

We hypothesised that rapid improvements would be observed in obese and morbidly obese patients with weight loss following bariatric surgery. Similar to other metabolic conditions such as insulin resistance, we propose that significant improvements likely occur before peak weight loss goals are achieved. We further hypothesise that improvements in NAFLD are closely related to markers of the metabolic syndrome.

By measuring alanine aminotransferase (ALT) and gamma-glutamyl transferase (GGT) as surrogate markers for NAFLD, we were able to closely evaluate the response of NAFLD to

weight loss on a monthly basis. Both these markers have a modestly good ability to predict NAFLD in the absence of other liver disease, and are often used in clinical practice to monitor progression of disease (276, 277, 447, 575). In this study, we aimed to determine the amount of weight loss required to produce a significant improvement in NAFLD.

12.3 Methods

All participants provided informed written consent to participate in the study. The Avenue Human Research Ethics Committee approved the study. This study was registered in the Australian Clinical Trials Registry (ACTRN12610000049077). Data was collected prospectively and stored in a dedicated Access Database® (Microsoft Corporation, Seattle, WA, USA).

12.3.1.1 Patients

Consecutive patients with the metabolic syndrome undergoing a primary bariatric procedure were recruited prospectively from The Avenue Hospital between April 2009 and March 2010. All procedures were laparoscopic adjustable gastric bands (LAGB), and all patients underwent a two-week pre-operative very low calorie diet regime.

Criteria for inclusion in the study included age ≥18 years old, with a BMI >30 kg/m^2, undergoing a primary bariatric procedure and who reached criteria for metabolic syndrome as defined by the Adult Treatment Panel III (84).

Exclusion criteria were: (a) present or past excessive alcohol intake, defined as per guidelines of the American Association for the Study of Liver Diseases (3), (b) other cause of liver disease (viral hepatitis, haemochromatosis, autoimmune hepatitis, medications) or (c) contraindication to bariatric surgery. Alcohol intake was routinely assessed during the twelve-month follow-up period, to ensure that consumption levels had not changed significantly during the study.

12.3.1.2 Clinical and biochemical data collection

Patients underwent a complete medical history and examination within two weeks of surgery. Comorbid illnesses and medications were recorded on a standardised questionnaire. Body mass index (BMI) was categorised using the World Health Organization classification, with a BMI>30kg/m^2 defined as obesity (21). Percentage total body weight loss (%TWBL) was calculated by percentage weight loss from baseline. Fasting blood tests were taken on the same day as the surgery.

12.3.1.3 Bariatric surgery and intraoperative liver biopsy

Primary laparoscopic adjustable gastric banding procedures were performed on all included patients by five experienced bariatric surgeons (POB, WAB, PRB, SS, AS).

Intraoperative liver biopsies were taken from the left lobe of liver using a 14G Temno needle (Allegiance; Health Care Corp, McGraw Park, IL) prior to commencing the bariatric procedure. Liver disease was scored according to the NAFLD activity score (NAS) Clinical Research Network criteria and Kleiner fibrosis score by a single experienced liver pathologist (PSB) blinded to clinical information.

The NAS is divided into three categories: an NAS score of ≤2 is considered *not diagnostic of NASH*, a score of 3-4 is considered *borderline*, and ≥5 is considered *probable NASH* (240). Of note, this grading is used as a guide and objective research tool, with NASH diagnosis remaining dependent on histopathological assessment (481).

12.3.1.4 Follow-up

Patients were reviewed every month for nine months and then at twelve months, with repeat measurements taken at every visit, including fasting blood tests.

12.3.1.5 Definition of improvement and normalisation in aminotransferases

We used updated ALT values developed by Prati *et al* for analysis (229). We considered the upper limit of normal for ALT was 30 IU/L for men and 19 IU/L for women. These reference ranges are associated with higher sensitivity for NAFLD, and subjects within this ALT category have lower rates of liver related mortality than those above these levels (280, 481). The upper limit of normal reference levels for GGT has been taken as 50 IU/L for males and 35 IU/L for females (576).

12.3.1.6 Statistical Method

Continuous variables were expressed as mean ± standard deviation (SD) for parametric data, and median and interquartile range (IQR) for nonparametric data. Normality was assessed by Shapiro-Wilks test. Student t-test and one-way ANOVA with post-hoc Bonferroni test was used for parametric data, and Mann Whitney U-test, Wilcoxon signed-rank test and Kruskal-

Wallis test for non-parametric data. Categorical variables were expressed as numbers (with percentages). Pearson's chi-squared or Fisher's exact test was used for categorical variables. A p-value ≤ 0.05 was considered statistically significant. Simple linear regression analysis was used for relationship between blood tests and histological scoring.

To assess for relationship between independent variables with repeated measures, we used the Generalised Estimating Equation (GEE) models. The primary outcome was resolution of ALT at each follow-up time point. We formulated a binomial distribution along with a logit link function and an exchangeable correlation structure for the GEE model. Odds ratios and 95% confidence intervals were presented as measures of effect size. For multivariate analysis, we started with the most significant variable identified in the univariate analysis, and added the next most significant variable sequentially and examined for improvement in model fit using the likelihood ratio test.

Data analysis was performed in Stata V13.0 (Stata Corp, College Station, TX, USA).

12.4 Results

12.4.1 **Participants**

One hundred and seven patients were recruited into the study. Six patients were excluded due to newly diagnosed haemochromatosis (n=1), corticosteroid use (n=1) and technical difficulty during the operation precluding liver biopsy (n=4). Five patients had normal liver histology (NAFLD prevalence 96 of 101 participants, 95.0%). Nine patients were lost to follow-up and three patients became pregnant within the first year and were excluded from follow-up analysis. Therefore, 84 participants (78.5% of 107 patients initially recruited) were included in the final analysis. No significant complications were seen in this cohort within twelve months. Baseline characteristics are shown in **Table 12.1**.

12.4.1.1 Baseline histology

By the NAS histological grading scores, there were 31 patients (36.9%) with NAS 0–2, 33 patients (39.3%) with NAS 3-4 and 20 patients (23.8%) with NAS≥5. The breakdown of these scores into steatosis, inflammation and ballooning grades can be seen in **Table 12.1**.

Baseline demographics are similar between NAS category groups, with the exception of higher rates of hypertension in the NAS 3-4 and NAS≥5 cohorts (p=0.013). The NAS groups differ significantly in ALT (p<0.001) and AST levels (p=0.017).

12.4.1.2 Overall changes at twelve months

Table 12.2 shows the differences in variables at baseline and at 12 months. The BMI at baseline was 41.5±5.9 kg/m^2, which decreased to 35.4±6.2 kg/m^2 at 12 months (p<0.001), corresponding to a %TBWL of 15.0±7.5%. This weight loss has been maintained at 15.3±8.9% TBWL at last clinical follow-up in 2015-2016.

At 12 months follow-up, there was significant improvement in all aminotransferases (p<0.001). In addition, there was significant improvement in metabolic factors, such as BSL (p=0.004), cholesterol levels (p<0.001) and hsCRP (p<0.001).

12.4.2 **Relationship between ALT and GGT with histological scores**

A moderate and significant correlation of ALT level to NAS score was seen (Spearman ρ=0.461, p<0.001). Baseline ALT showed a significant difference between groups based on NAS category. The average ALT levels of NAS 0-2, NAS 3-4 and NAS ≥5 groups were 26 (20–35) vs 36 (32–43) vs 42 (37.5–57.8) IU/L at baseline (p<0.001).

The GGT levels showed less correlation with NAS histological category in our cohort (Spearman ρ=0.215, p=0.051). As such, the focus of subsequent generalised estimating equation (GEE) analysis is based on ALT levels.

12.4.3 **Changes in ALT and GGT with weight loss**

12.4.3.1 Rapid improvement in ALT and GGT

At baseline, the median ALT and GGT for the cohort was 35 IU/L (26–44) and 25 IU/L (20-38) respectively. Both these markers, but ALT more so, showed rapid improvement over 12-months, particularly in the first six months after surgery (**Figure 12.1a**). A statistically significant decrease in the ALT and GGT was observed by two month post-operatively (ALT 35 vs 27, p<0.001 and GGT 25 vs 22.5, p=0.001). The corresponding %TBWL during this time was 6.4% (4.2–8.2) with BMI 38.2 kg/m^2 (34.9–42.1).

12.4.3.2 ALT and GGT levels closely correlate with weight loss

ALT and GGT levels decreased with increasing weight loss. **Figure 12.1b** shows their change at each 5% TBWL interval.

12.4.3.3 ALT improves for all NAS categories

Figure 12.2 shows the initial ALT levels at baseline per NAS category, and subsequent changes in average levels over 12 months. The NAS≥5 cohort had the highest baseline ALT level (42 IU/L (37.5–57.8)). During follow-up, the ALT levels for all NAS categories decreased, but more so in the NAS ≥5 group, such that there was no significant difference in average ALT level by nine months post-operatively (p=0.065).

12.4.3.4 Patients achieving complete ALT normalisation

Irrespective of baseline ALT, the majority of patients had an improvement in ALT by 12 months (n=78, 92.9% response rate) (**Supplementary Figure 12.1**).

Using the updated normal range for ALT, ten patients (11.9%) had a normal ALT level at baseline. Of the remaining 74 patients with an elevated ALT at baseline, 49 patients had normalised by 12-months (58.3%), whereas 25 had persistently abnormal ALT (29.8%).

12.4.3.5 Weight loss associated with complete ALT normalisation

In univariate analysis, ALT normalisation was associated with both weight loss (%TBWL) and absolute BMI. A 5% TBWL was associated with a 2.57-fold increased likelihood of ALT normalisation (OR 2.57, 95% CI 1.86, 3.53; p<0.001). The odds of resolution increased with increasing TBWL, up to OR 6.36 with >15% TBWL (95% CI 4.25, 9.53; p<0.001). A similar, inverse relationship is seen with BMI category, with decreasing odds of resolution as BMI increases (**Table 12.3a**).

When multivariate analysis was performed, incorporating both measures of weight (BMI and TBWL) as well as metabolic variables, only weight loss (%TBWL), and not absolute BMI, was significantly associated with ALT normalisation. There was a statistically significant increased likelihood of normalisation after 10-15% TBWL was achieved (OR 2.49, 95% CI 1.31, 4.73; p=0.005) (**Table 12.3b**).

12.4.4 **Associations with improvements in ALT levels**

12.4.4.1 Metabolic variables associated with ALT normalisation

Over twelve months, complete ALT resolution (≤30 IU/L for males and ≤19 IU/L for females) was significantly associated with greater improvements in multiple metabolic markers (**Table 12.3**).

In univariate analysis, decreasing waist circumference (OR 0.94, p<0.001), decreasing triglyceride level (OR 0.42, p<0.001) and male gender (OR 2.83, p=0.001) were significantly associated with normalisation of ALT (**Table 12.3a**).

In multivariate analysis (**Table 12.3b**), male gender (OR 30.40, p<0.001), triglyceride level (OR 0.59, p=0.021) and baseline total NAS grade (OR 0.28, p<0.001) are significantly associated with ALT normalisation.

12.4.4.2 Improvement in ALT preceded improvement other metabolic factors

The percentage change in metabolic variables was compared with ALT and GGT change over time (**Figure 12.3**). Metabolic variables, specifically HDL, triglycerides and BSL, show progressive improvement throughout the twelve-month post-operative period. Compared to the other variables, the changes in ALT are rapid, reaching maximal improvement by six months. The changes in GGT were less marked, however also plateaued at six months.

Of note, weight loss continues throughout the 12-month follow-up period, demonstrating that maximal ALT improvement likely occurs before maximal weight loss outcomes after bariatric surgery, and well before ideal body weight is reached.

Table 12.1: Baseline demographics of study cohort, and comparison between NAFLD activity score (NAS) categories.

			NAS category			
Variable		**All patients**	**NAS 0-2**	**NAS 3-4**	**NAS≥5**	**p =**
n =		84	31 (36.9%)	33 (39.3%)	20 (23.8%)	-
Age		51 (42 – 55)	49 (38 – 55)	50 (45 – 55)	52 (43 – 56)	NS†
Male		29 (34.5%)	10	12	7	NS
BMI		41.5 ± 5.9	41.2 ± 6.3	40.2 ± 4.3	44.0 ± 6.9	0.065
%TBWL at 12 months		15.0 ± 7.5	15.7 ± 8.9	13.9 ± 6.8	15.7 ± 6.4	NS
Waist circumference		120.3 ± 12.6	118.9 ± 15.3	120.0 ± 10.8	123.1 ± 10.8	NS
Hip circumference		133.1 ± 13.9	132.3 ± 13.3	130.9 ± 12.4	139.1 ± 17.3	NS
Neck circumference		45 ± 4	44 ± 5	45 ± 4	45 ± 4	NS
COMORBIDITIES						
Type II diabetes		31 (36.9%)	8 (25.8%)	15 (45.5%)	8 (40.0%)	NS
Medication: Oral hypoglycemics only		18 (21.4%)	4 (12.9%)	11 (33.3%)	3 (15.0%)	NS
Medication: Insulin		13 (15.5%)	4 (12.9%)	4 (12.1%)	5 (25.0%)	NS
Impaired glucose tolerance		24 (28.6%)	11 (35.5%)	9 (27.3%)	4 (20.0%)	NS
Hypertension		66 (78.6%)	19 (61.3%)	29 (87.9%)	18 (90.0%)	0.013
Medication: Antihypertensives		44 (52.4%)	12 (38.7%)	21 (63.6%)	11 (55.0%)	NS
High cholesterol		63 (75.0%)	20 (64.5%)	27 (81.8%)	16 (80.0%)	NS
Medication: Cholesterol lowering		31 (36.9%)	11 (35.5%)	14 (42.4%)	6 (30.0%)	NS
BIOCHEMISTRY						
ALT		35 (26 – 44)	26 (20 – 35)	36 (32 – 43)	42 (37 – 58)	<0.001†
ALT resolution at 12 months		59 (70.2%)	27 (87.1%)	22 (66.7%)	10 (50%)	0.076
AST		26 (22 – 32)	22 (20 – 28)	26 (22 – 31)	30 (25 – 42)	0.017†
GGT		25 (20 – 38)	22 (18 – 28)	25 (22 – 38)	27 (20 – 42)	NS†
ALP		74 ± 21	75 ± 19	74 ± 21	72 ± 24	NS
Bilirubin		9 ± 3	8 ± 3	10 ± 4	7 ± 2	0.002
Albumin		43 ± 4	43 ± 3	44 ± 4	43 ± 5	NS
Fasting glucose		6.1 (5.3 – 7.5)	5.9 (5.2 – 6.4)	6.9 (5.7 – 9.1)	6.4 (5.5 – 8.3)	0.087†
Insulin		20.9 (12.2-33.3)	18.5 (9.9 – 35.4)	20.0 (10.9-33.2)	23.9 (16.3-33.2)	NS†
Platelet		273 ± 67	297 ± 69	259 ± 55	274 ± 78	NS
Total cholesterol		4.4 (3.9 – 5.4)	4.0 (3.7 – 5.1)	4.6 (3.9 – 5.4)	4.7 (4.2 – 5.5)	NS†
Triglyceride level		1.7 (1.3 – 2.1)	1.5 (1.2 – 1.8)	1.8 (1.4 – 2.3)	1.5 (1.1 – 2.1)	NS†
HDL		1.07 ± 0.26	1.10 ± 0.26	1.10 ± 0.25	0.98 ± 0.26	NS
LDL		2.7 ± 0.9	2.5 ± 1.0	2.6 ± 0.8	3.1 ± 0.9	0.063
hsCRP		4.2 (2.6 – 8.1)	4.1 (2.5 – 8.1)	4.0 (2.7 – 6.6)	5.7 (2.6 – 9.6)	NS†
HISTOLOGY						
Steatosis	1	34 (40.5%)	30(96.8%)	4 (12.1%)	0	<0.001
	2	21 (25.0%)	1 (3.2%)	18 (54.5%)	2 (10.0%)	
	3	29 (34.5%)	0	11 (33.3%)	18 (90.0%)	
Inflammation	0	14 (16.7%)	12 (38.7%)	2 (6.1%)	0	<0.001
	1	49 (58.3%)	19 (61.3%)	28 (84.8%)	2 (10.0%)	
	2	19 (22.6%)	0	3 (9.1%)	16 (80.0%)	
	3	2 (2.4%)	0	0	2 (10.0%)	
Ballooning	0	74 (88.1%)	31 (100%)	30 (90.9%)	13 (65.0%)	0.001
	1	10 (11.9%)	0	3 (9.1%)	7 (35.0%)	
	2	0	0	0	0	
Fibrosis	0	22 (26.2%)	11 (35.5%)	7 (21.2%)	4 (20.0%)	NS
	1	40 (47.6%)	17 (54.8%)	13 (39.4%)	10 (50.0%)	
	2	19 (22.6%)	3 (9.7%)	12 (36.4%)	4 (20.0%	
	3	1 (1.2%)	0	0	1 (5.0%)	
	4	2 (2.4%)	0	1 (3.0%)	1 (5.0%)	

Values represented as mean ± SD or median (IQR). Significance testing with one-way ANOVA for continuous or Chi-squared for categorical variables, unless otherwise stated. †Kruskal-Wallis test

Table 12.2: Baseline and 12 month measures for all patients.

Variables	Baseline	12 months	p =
BMI	41.5 ± 5.9	35.4 ± 6.2	<0.001
%TBWL	-	15.0 ± 7.5	-
Waist circumference	120.3 ± 12.6	109.5 ± 14.6	<0.001
ALT	35 (26 – 44)	20 (15 – 26)	<0.001†
ALT within normal limits	10 (11.9%)	59 (70.2%)	<0.001‡
AST	26 (22 – 32)	19 (16 – 22)	<0.001†
GGT	25 (20 – 38)	20 (16 – 30)	<0.001†
ALP	74 ± 21	69 ± 18	<0.001
Bilirubin	9 ± 3	10 ± 4	0.075
Albumin	43 ± 4	44 ± 3	NS
Fasting glucose	6.1 (5.3 – 7.5)	5.6 (5.0 – 7.0)	0.004†
Insulin	20.9 (12.2 – 33.3)	11.0 (7.0 – 16.6)	<0.001†
Platelet	273 ± 67	263 ± 60	NS
Total cholesterol	4.4 (3.9 – 5.4)	5.0 (4.1 – 5.6)	<0.001†
Triglyceride level	1.7 (1.3 – 2.1)	1.3 (1.0 – 1.5)	<0.001†
HDL	1.07 ± 0.26	1.41 ± 0.34	<0.001
LDL	2.7 ± 0.9	2.7 ± 0.9	<0.001
hsCRP	4.2 (2.6 – 8.1)	2.1 (1.2 – 5.8)	<0.001†
Medication use			
Any cholesterol lowering	31 (36.9%)	27 (32.1%)	NS
Any antihypertensives	44 (52.4%)	42 (50.0%)	NS
Any antidiabetic	31 (36.9%)	29 (34.5%)	NS

Values represented as mean ± SD or median (IQR). Significance testing with independent student t-test, unless otherwise stated. †Kruskal-Wallis test, ‡Chi-squared test
Medication use is defined as any medication, regardless of dose.

Table 12.3a: Factors from univariate analysis associated with ALT resolution, with associations expressed as the likelihood (odds ratio) of resolution with increasing units of each variable.

Variables		**OR (95% CI)**	**p =**
Age		1.01 (0.98 – 1.04)	0.548
Male gender		2.83 (1.52 – 5.29)	0.001
Smoking status	Non-smoker	Ref	
	Ex-smoker	5.41 (0.69 – 42.64)	0.109
	Current smoker	1.15 (0.63 – 2.11)	0.657
Hypertension		0.86 (0.41 – 1.82)	0.696
Antihypertensives		0.86 (0.49 – 1.50)	0.601
Type II diabetes		1.10 (0.58 – 2.05)	0.777
Diabetic medication (OHG and insulin)		0.88 (0.48 – 1.61)	0.68
Insulin use		0.74 (0.30 – 1.82)	0.505
Hypercholesterolaemia		0.99 (0.50 – 1.97)	0.976
Cholesterol lowering medication		0.70 (0.39 – 1.27)	0.245
Waist circumference		0.94 (0.92 – 0.95)	<0.001
Systolic BP		1.00 (0.99 – 1.01)	0.552
Diastolic BP		1.01 (1.00 – 1.02)	0.201
TBWL	<5%	Ref	
	5-10%	2.57 (1.86 – 3.53)	<0.001
	10-15%	3.64 (2.58 – 5.12)	<0.001
	>15%	6.36 (4.25 – 9.53)	<0.001
BMI group	<30	Ref	
	30-35	0.35 (0.17 – 0.73)	0.005
	35-40	0.13 (0.06 – 0.28)	<0.001
	40-45	0.09 (0.04 – 0.21)	<0.001
	>45	0.06 (0.02 – 0.17)	<0.001
GGT		0.92 (0.90 – 0.94)	<0.001
AST		0.77 (0.74 – 0.80)	<0.001
ALP		0.98 (0.96 – 0.99)	<0.001
Total cholesterol		0.99 (0.95 – 1.02)	0.467
Triglycerides		0.42 (0.31 – 0.58)	<0.001
HDL cholesterol		0.99 (0.97 – 1.02)	0.602
LDL cholesterol		0.98 (0.93 – 1.03)	0.455
hsCRP		0.98 (0.96 – 1.00)	0.101
Insulin		0.74 (0.30 – 1.82)	0.505
BSL		0.95 (0.84 – 1.07)	0.365
Steatosis	<33%	Ref	
	33-66%	0.52 (0.26 – 1.42)	0.083
	>66%	0.24 (0.12 – 0.50)	<0.001
Inflammation	None	Ref	
	<1 foci/lobule	0.61 (0.26 – 1.42)	0.255
	1-2 foci/lobule	0.42 (0.16 – 1.12)	0.082
	3-4 foci/lobule	-	-
Ballooning	None	Ref	
	Few	0.34 (0.12 – 0.97)	0.044
NAS category	NAS 0-2	Ref	
	NAS 3-4	0.43 (0.21 – 0.84)	0.014
	NAS ≥ 5	0.24 (0.11 – 0.55)	0.001
Fibrosis	F0	Ref	
	F1	1.13 (0.55 – 2.32)	0.735
	F2	1.79 (0.76 – 4.20)	0.183
	F3 and F4	-	-

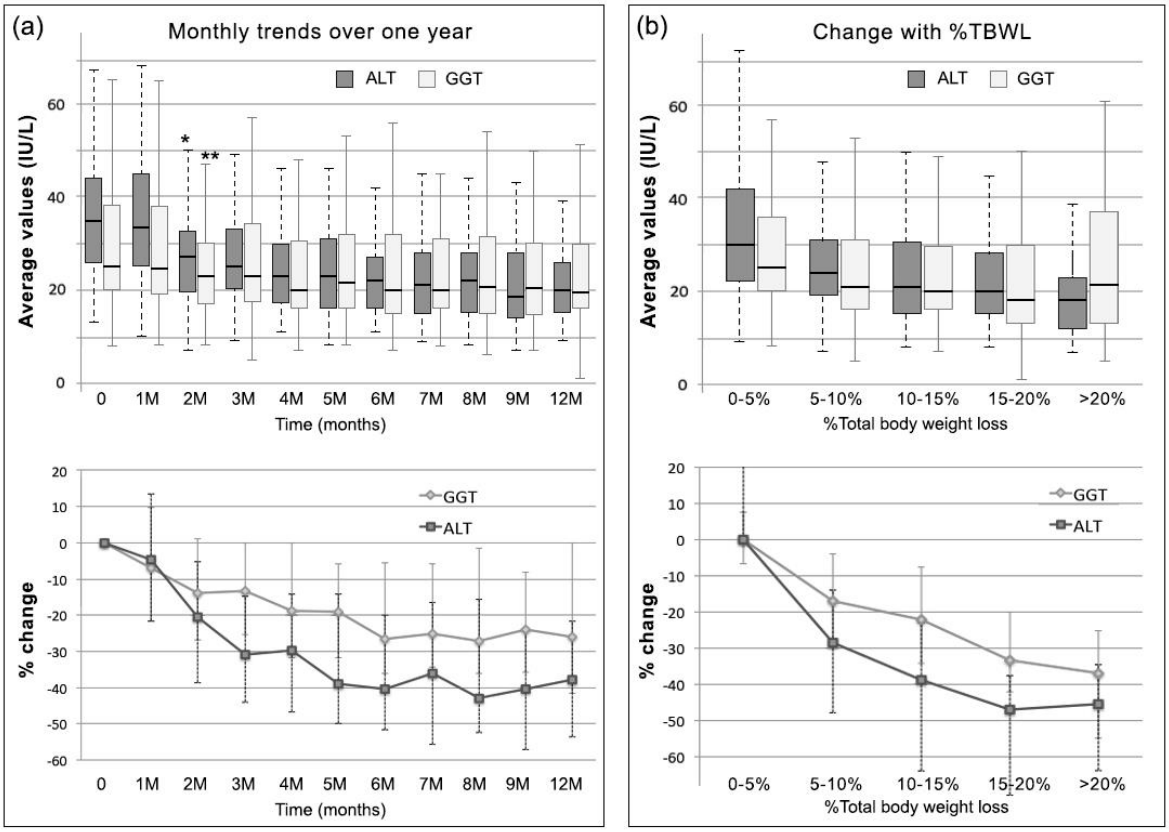
OHG – oral hypoglycaemics; BP – blood pressure; TBWL – total body weight loss; BMI – body mass index; GGT – gamma glutamyltransferase; AST – aspartate aminotransferase; ALP – alanine phosphatase; HDL – high density lipoprotein; LDL – low density lipoprotein; hsCRP – highly sensitive C-reactive protein; BSL – blood sugar level; NAS – NASH activity score.

Table 12.3b: Factors from multivariate analysis associated with ALT resolution, with associations expressed as the likelihood (odds ratio) of resolution with increasing units of each variable.

Variables		**OR (95% CI)**	**p =**
Male gender		30.40 (13.29 – 69.54)	<0.001
TBWL	<5%	Ref	
	5-10%	1.65 (0.93 – 2.93)	0.089
	10-15%	2.49 (1.31 – 4.73)	0.005
	>15%	3.56 (1.71 – 7.43)	0.001
GGT		0.96 (0.94 – 0.98)	<0.001
AST		0.73 (0.69 – 0.78)	<0.001
Triglycerides		0.59 (0.38 – 0.92)	0.021
Total NAS		0.28 (0.14 – 0.57)	<0.001

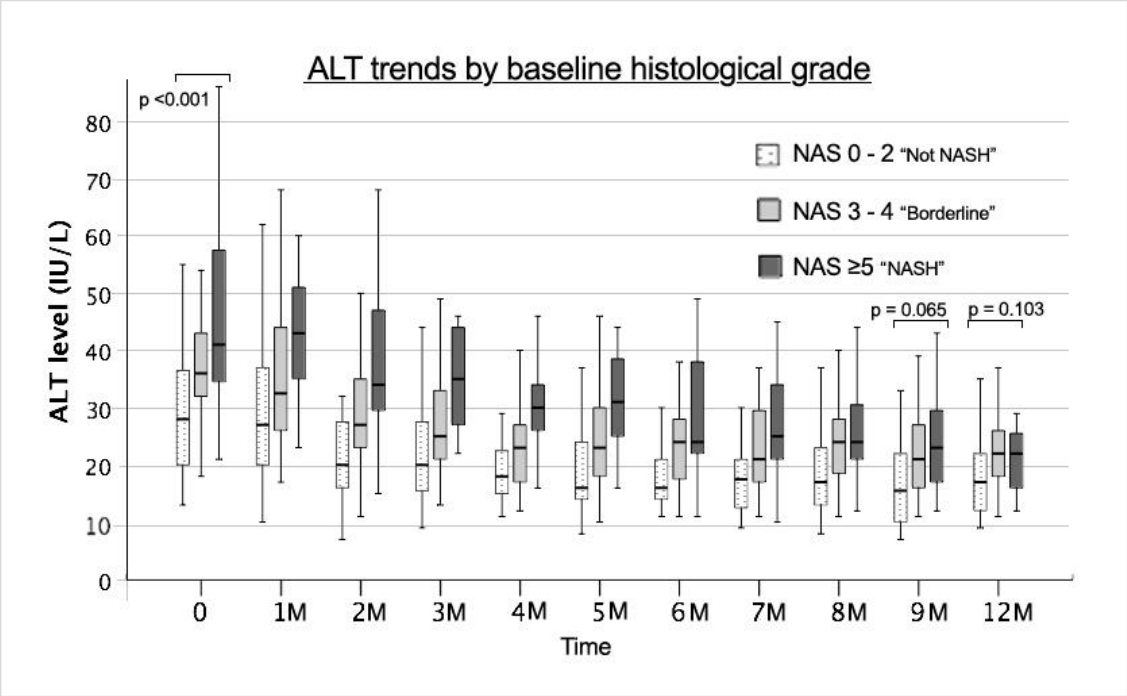
TBWL – total body weight loss; GGT – gamma glutamyltransferase; AST – aspartate aminotransferase; NAS – NASH activity score.

*Figure 12.1: (a) ALT and GGT trends in all patients in the 12 months after bariatric surgery, showing rapid falls in levels with statistically significantly decreased ALT and GGT levels at 2 months (*GGT 25 vs 22.5, p=0.001, and **ALT 35 vs 27, p<0.001). (b) ALT and GGT trends with total body weight loss (%TBWL).*



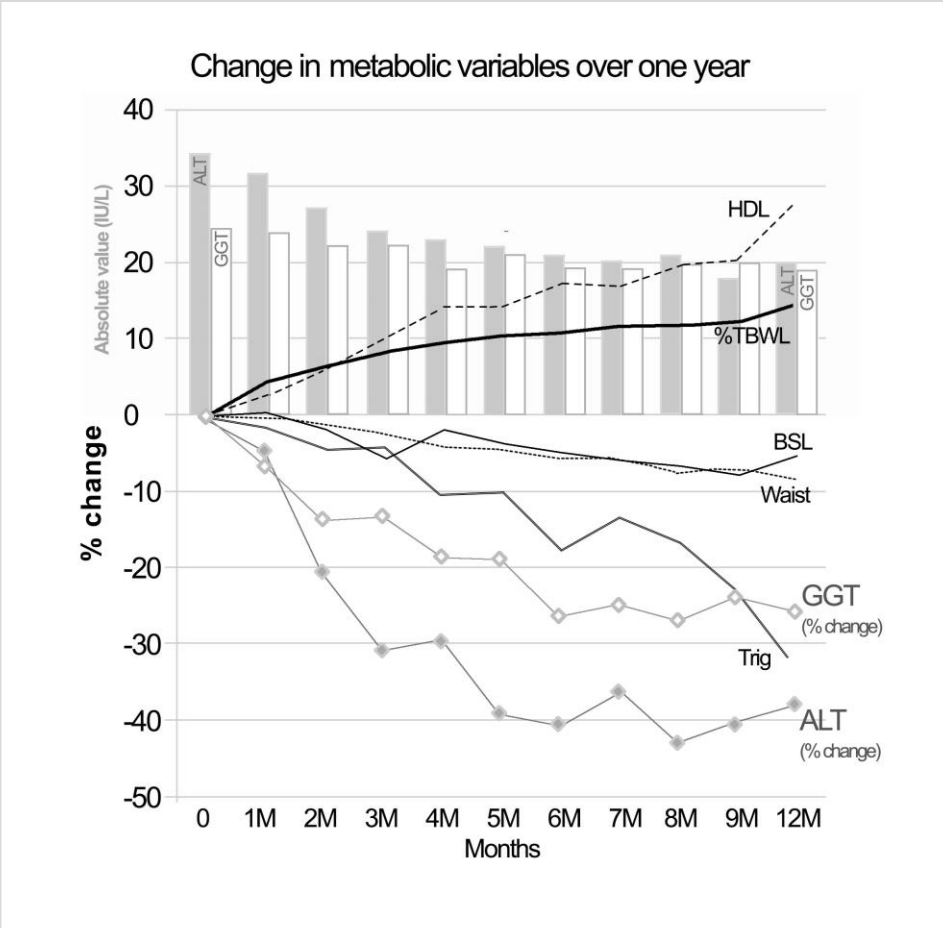
Boxplots represent median and interquartile range (IQR), with range represented as whiskers. Line graph represents median and error bars represent IQR. Significance testing for difference in GGT and ALT from baseline values, with paired Wilcoxon signed-rank test.

Figure 12.2: ALT trends divided into NAFLD activity score (NAS) categories of "unlikely NASH" (NAS 0-2), "borderline" (NAS 3-4), or "diagnostic for NASH" (NAS≥5), showing resolution across all ranges of pathology.



Boxplots represent median with whiskers representing interquartile range (IQR). Significance testing with Kruskal-Wallis test.

Figure 12.3: Twelve-month trends for ALT and GGT (absolute and % change values, in grey) and metabolic variables (% change values, in black), showing rapid change in ALT over the first 6 months, with steady progressive improvement of all other variables over the study period.



HDL – high density lipoprotein; %TBWL – percentage total body weight loss; BSL – blood sugar level; Trig – triglycerides; GGT – gamma glutamyl transferase; ALT – alanine aminotransferase.

12.5 Discussion

We have described the rate of NAFLD resolution in obese patients with weight loss by conducting a prospective observational study with serial measurements of ALT and GGT following bariatric surgery. We found a high baseline incidence of NAFLD in obese patients with metabolic syndrome, with good correlation of aminotransferases, especially ALT, with NAS histological grade. Considerable improvements were observed after a full twelve months (ALT 35 vs 20, $p<0.001$), with a mean weight loss of 15.0 ± 7.5% TBWL.

The key finding in this study was rapid and substantial improvement in aminotransferases, with modest weight loss. By performing detailed and repeated measures, we were able to observe that improvements in aminotransferases were closely related to weight loss. These changes occurred early in the weight loss curve, with significantly increased odds of ALT normalisation (ALT ≤30 IU/L for males and ≤19 IU/L for females) starting at 10% TBWL. We also demonstrated that increasing weight loss results in progressive improvement and increasing odds of normalisation in ALT.

Notably, absolute BMI is not significantly related to ALT normalisation in multivariate analysis. This suggests that dynamic weight loss plays a greater role in improvement, rather than actual weight. Thus, a morbidly obese patient may not have achieved a traditional "ideal" body weight, yet may still have significant metabolic benefits from modest weight loss.

Previous studies have looked at the benefits of modest weight loss in other comorbidities, particularly diabetes and insulin resistance. The Look AHEAD study has shown metabolic and cardiovascular improvements with just 5-10% body weight loss (577), and this is further emphasised by other studies reviews on the benefits of modest weight loss (166). Although a total body weight loss of 10% would traditionally be deemed a "failure", our study and previous evidence suggests that bariatric patients already achieve substantial metabolic benefits at this stage.

Whilst achieving 10-15% weight reduction is a realistic target, it is still beyond what is commonly observed or sustained long-term in most studies of diet and lifestyle intervention (578). Our findings have major implications for physicians treating NAFLD in the obese, who may be contemplating bariatric surgery or further measures to achieve weight loss.

However, our data showed that if this weight loss can be achieved, undertaking more aggressive therapies to achieve greater weight loss may have fewer additional benefits for NAFLD.

Changes in ALT appeared to precede improvement in metabolic markers. We observe a rapid drop in ALT over six months to a near normal, whereas other metabolic factors improved more gradually over twelve months. The mechanism behind improvements in ALT preceding improvements in metabolic variables is unclear. There are a few possibilities for this. The first is that liver abnormalities may more easily recover after weight loss than other obesity-related disorders. Indeed, previous studies have demonstrated rapid changes in the liver size, reflective of steatosis, after just two weeks of very low calorie diets (VLCD) (180). Hence, the ALT changes observed may reflect a measure of reduction in hepatic fat content. On the other hand, earlier resolution of aminotransferases also raises the possibility that underlying liver health may play a role in resolution of metabolic disease, as has been suggested by some groups (579). What we have observed in our study could be a combination of both theories, as it is well know that both NAFLD and metabolic syndrome are inter-related entities (544). Further investigation is required to determine the complex mechanisms involved.

The major strengths of this study are the detailed and repeated clinical and biochemical measurements every month for one year, in a well-defined cohort, allowing for close examination of liver health over the timeframe of significant weight change after bariatric surgery. Whilst previous studies have shown improvement in NAFLD with dramatic weight loss after years (447, 569, 570, 573, 574), the weight loss threshold required to induce improvement and resolution have never been studied in detail. This study design has enabled identification of the precise time and weight loss achieved when resolution occurs.

Secondly, the use of LAGB allows for weight loss purely through reduction in caloric intake. It thereby eliminates any hormonal effects or other biases from other more major bariatric procedures (580), but reliably induces more substantial and sustained weight loss than conservative measures (349, 581). This model would more accurately reflect the effects of progressive weight loss seen with conservative measures, which is the most accessible form of weight loss. It can hence help inform the changes in NAFLD that occur with these non-surgical methods. However, future research endeavours should investigate the effects of other

bariatric procedures, such as Roux-en-Y gastric bypass and sleeve gastrectomy, that alter the hormonal milieu. These procedures are known to improve metabolic health (572), and may have an additive effect on NAFLD improvements.

In this study, we have used modified values for ALT upper limit of normal (19 IU/L for females, and 30 IU/L for males) (229). The widely applied values for upper limit of normal of ALT (approximately 40 IU/L) were established in the 1980s, primarily for screening for viral hepatitis A and B. However, these 'normal' ranges were calculated without exclusion of patients with non-alcoholic fatty liver disease and hepatitis C virus (HCV). This results in a high prevalence of patients with these liver conditions, who have an ALT within this accepted normal range. Subsequently, many studies have challenged the definition of 'normal' ALT to more accurately reflect the populations with NAFLD and HCV, with lower thresholds, as used in this study, improving the sensitivity and negative predictive value of ALT for these conditions (279, 576, 582).

This study has some limitations that warrant discussion. Due to the risks and impracticalities involved in taking repeated liver biopsies, we have used ALT and GGT as a surrogate for NAFLD activity. The use of routine liver function tests as a proxy for NAFLD activity is contentious, with previous studies emphasizing its relative weakness in comparison to liver biopsy (13). For diagnostic purposes, the LABS consortium found that ALT does not reliably exclude significant disease, and that strong consideration should be given for routine liver biopsy during bariatric surgery and medical follow-up.

However, detailed study of NAFLD using liver biopsy is not practical due to the inherent risks, costs and logistical burden (227). Repeated aminotransferase measures, although imperfect, may be a practical proxy in approximating the rate of improvement and, perhaps, indicating substantial improvement in NAFLD. Serum ALT has been considered a reasonable marker for hepatocellular injury and inflammation in NAFLD after exclusion of other causes, with modestly good ability in predicting NASH (AUROC 0.60-0.81 (276, 277, 582). In addition, previous weight loss studies have demonstrated correlation between substantial improvements in serum ALT and GGT levels and histological improvements (447, 575, 581). Serum aminotransferases have often used in lieu of repeated liver biopsies for monitoring changes in NAFLD following intervention (276).

Regardless, the limitations of any non-invasive measure in diagnosing NAFLD remain (502, 503, 583). To verify the findings of this study, a future option would be to undertake a biopsy after 10-15% TBWL to demonstrate improvement.

In conclusion, there was rapid amelioration of NAFLD after bariatric surgery, as reflected by normalisation of ALT. These improvements were closely related to weight loss and various metabolic factors. A modest weight loss of approximately 10-15% total body weight is an appropriate and achievable target to attain substantial improvements in ALT levels.

Future endeavours should focus on better defining patterns of improvement in NAFLD in other surgical cohorts and validating this weight loss target with histological samples.

13 Weight loss after laparoscopic adjustable gastric band and resolution of the metabolic syndrome and its components

13.1 Abstract

INTRODUCTION: Substantial weight loss after bariatric surgery has considerably metabolic benefits. Yet some studies have shown improvements in obesity-related metabolic comorbidities with more modest weight loss. By closely monitoring patients, we aimed to determine weight loss goals based on the overall resolution of metabolic syndrome and the resolution of its components following bariatric surgery.

METHODS: We performed a prospective observational cohort study of obese participants with metabolic syndrome (ATPIII Criteria) who underwent laparoscopic adjustable gastric banding. Participants were assessed for all criteria of the metabolic syndrome for the first nine months, then three-monthly until 24 months.

RESULTS: We recruited 107 patients. Baseline BMI was 42.4±6.2kg, age was 48.2±10.7years, and there were 56 (63%) women. Resolution of the metabolic syndrome occurred in 60 of 89 participants (67%) at 12 months and 60 of 75 participants (80%) at 24 months. The mean weight loss when metabolic syndrome resolved was 11±8% total body weight loss (TBWL). Median weight loss at which prevalence of disease is halved is 7.0% TBWL (17.5% EWL) for hypertriglyceridaemia; 11% TBWL (26.1-28% EWL) for HDL cholesterol and hyperglycaemia; 20% TBWL (59.5% EWL) for hypertension; 29% TBWL (73.3% EWL) for waist circumference. A linear relationship between weight loss and resolution of metabolic syndrome was observed with an increased probability of resolution with more substantial weight loss.

CONCLUSIONS: In obese participants, a weight loss target of 10-15% TBWL (25-30% EWL) is a reasonable initial goal for metabolic benefits. Further metabolic improvement could be expected with additional weight loss.

13.2 Introduction

Morbid obesity and metabolic dysfunction are closely linked (54, 584). An essential element in the management of metabolic risk factors, especially in the setting of obesity, is weight loss (55, 191, 585). Whilst there is irrefutable evidence that weight loss is a powerful means of tackling obesity-related metabolic disorders (163, 165, 166, 426, 586-588), it is still unclear exactly how much weight loss is required to produce meaningful improvements. This is a crucial question, as it can assist in appropriate goal setting (589), as well as selection of weight loss strategy (190).

The metabolic syndrome refers to a cluster of inter-related metabolic risk factors that predict progression to type II diabetes and atherosclerotic cardiovascular disease (93, 585, 590). All definitions of metabolic syndrome share a common focus on elevated glucose, triglyceride, blood pressure levels and low HDL cholesterol (see **Table 3.8** in **Section 3.1.4.2 - Dyslipidaemia**). Although the utility and interpretation of the metabolic syndrome as a distinct diagnostic entity is controversial (89), it serves as a useful overall measure of metabolic derangement associated with obesity (591).

Weight loss targets based on meaningful metabolic benefits are currently poorly defined. The traditional benchmark for 'ideal' body mass index has been set at 25 kg/m^2. However, in the obese, this is rarely achieved with any weight loss method (191). Bariatric surgery is a proven method of substantial and sustained weight loss, in the range of 50-70% excess weight loss (EWL) (191). With this magnitude of weight loss, consistent and significant metabolic benefits have been seen, including remission of diabetes in up to 79% of patients (163), improved cholesterol profiles (586) and resolution of hypertension (426, 587). However, evidence from weight loss studies using lifestyle interventions suggests that significant metabolic improvement can occur with modest weight loss, starting from 5-10% EWL (165, 166, 588).

Previous studies have not measured the progressive metabolic effects of incremental weight loss at different time points. If we were able to observe these changes as they occur, this would greatly improve our understanding of the response of metabolic disease to weight loss. Importantly, this would assist in defining evidence-based targets for weight loss programs (589). Furthermore, it could aid in the choice of weight loss method and inform decisions regarding progression to pharmaceutical or surgical weight loss therapies.

We hypothesised that resolution of the metabolic syndrome and its components occur before maximal weight loss is achieved in obese patients undergoing bariatric surgery. To test this hypothesis, we prospectively followed obese patients with metabolic syndrome who underwent laparoscopic adjustable gastric band (LAGB) surgery and repeatedly measured the changes in parameters that define the metabolic syndrome. By using the LAGB as a model of weight loss, we were able to observe steady and significant weight loss over two years without effects on gastric emptying or the hormonal milieu that accompany other bariatric procedures (195).

The primary aim of this study was to define the relationship between weight loss and resolution of the metabolic syndrome in the setting of obesity. We also aimed to investigate the relative sequence of resolution of its component metabolic risk factors. The secondary aims were to assess reduction in cardiovascular risk with progressive weight loss, and to identify factors associated with resolution of metabolic syndrome with weight loss.

13.3 Methods

All participants provided informed written consent to participate. Ethical approval was obtained from the Avenue Ethics Committee (reference no. 099). The trial was registered with the Australian Clinical Trials Register (ACTRN12610000049077).

We undertook a two-year prospective observational study of obese patients with the metabolic syndrome who underwent LAGB placement between April 2009 and March 2010 by one of six surgeons affiliated with the Centre for Bariatric Surgery in Melbourne. The STROBE statement checklist was used to guide reporting of this study.

13.3.1 **Inclusion and exclusion criteria**

Inclusion criteria in the study included: (1) age ≥18 years, (2) BMI >30kg/m^2, (3) undergoing a primary LAGB procedure, and (4) metabolic syndrome as defined by the Adult Treatment Panel III (ATPIII) (see **Table 3.9** in **Section 3.1.5 – Metabolic syndrome**) (55). The ATPIII criteria were chosen to define metabolic syndrome because of their broad acceptability, as well as the lack of glucose tolerance or clamp testing, making it a practical assessment that is easily translatable into clinical practice. Patients were excluded if they were (1) pregnant, or (2) unwilling or unsuitable for bariatric surgery.

13.3.2 **Baseline and follow-up measurements**

Baseline weight was defined as the weight on the day the decision was made to proceed with bariatric surgery. Blood tests included a full blood examination, electrolytes, urea and creatinine, liver function tests, fasting blood sugar level (BSL), fasting insulin, total cholesterol, HDL cholesterol, LDL cholesterol, triglycerides (TG), thyroid function tests, and highly sensitive CRP (hsCRP). Blood pressure (BP) was taken using a bariatric blood pressure cuff and waist circumference (WC) was measured at the midpoint between the lowest point of the ribs and highest point of the iliac crests. Medications and smoking status were recorded on a study pro forma. All measures were collected prior to surgery, on the day of surgery, monthly for nine months then three-monthly until 24 months. These measurements were performed in the Centre for Bariatric Surgery clinic by dedicated research nurses, in accordance with the study protocol. Blood tests were performed by a single pathology service (Melbourne Pathology, Melbourne).

Weight loss was expressed as percentage total body weight loss (%TBWL) and percentage excess weight loss (%EWL). Excess weight was defined as the weight carried above the 'ideal' body weight corresponding to a BMI of 25 kg/m^2.

13.3.2.1 LAGB follow-up protocol

Routine LAGB post-operative follow-up at the Centre for Bariatric Surgery involves a post-operative review at four weeks, where an assessment of satiety and restriction is made, and the band is adjusted accordingly. Patients are then seen every 2-4 weeks until they reach a point where satiety and weight loss is maintained without adverse symptoms such as reflux and dysphagia (the "green zone") (592). Once this is achieved, patients are generally seen every 2-3 months for a year. At these appointments, appropriate eating styles and behaviours are reinforced, nutritional advice provided, and patient queries are addressed. However, if satiety is not satisfactory, additional appointments and adjustments occur whenever necessary. We aim to maintain follow-up indefinitely, seeing patients at least once every 6-12 months. This ensures fluid within the band can be monitored, healthy behaviours can be encouraged, and adverse symptoms can be investigated and managed in a timely manner.

13.3.2.2 Cardiovascular risk measurements

Ten-year cardiovascular risk was calculated for participants aged over 30 years old without a known history of cardiovascular disease, using an algorithm based on the new Framingham Risk Equation (FRE) (94). Input variables were age, gender, systolic blood pressure, use of antihypertensive medication, total cholesterol, HDL cholesterol, smoking status and diabetes status. High sensitivity CRP (hsCRP) was also used to stratify cardiovascular risk. Values ≥10 mg/L were excluded under the assumption that they reflected acute inflammatory processes unrelated to cardiovascular disease.

13.3.3 **Outcomes**

The primary outcome was the resolution of the metabolic syndrome and each of its component, as defined by the ATPIII criteria (55). Secondary outcomes included change in cardiovascular risk, and clinical and biochemical factors associated with metabolic syndrome resolution.

13.3.4 **Statistical analysis**

The weight loss required for half the population to remit the metabolic syndrome ("median resolution") was determined by Kaplan-Meier survival curve analysis, with %TBWL rather than time used as the longitudinal measure.

Continuous variables were expressed as mean ± standard deviation (SD) for parametric data, and median and interquartile range (IQR) for nonparametric data. Differences between parametric datasets were determined using Student t-test and one-way ANOVA with post-hoc Bonferroni test, and non-parametric data were compared using Mann Whitney U-tests, Wilcoxon signed-rank tests and Kruskal-Wallis tests. Categorical data were compared using Pearson's chi-squared or Fisher's exact tests.

A Generalised Estimating Equation (GEE) model was used to identify clinical and biochemical factors associated with metabolic syndrome resolution. The model incorporated a binomial distribution to account for binary outcome, a logit link function and an exchangeable correlation structure.

Data were stored on a secure Access™ database (Microsoft, Seattle, USA). Statistical analysis was performed using Stata V13.0 (Stata Corp, College Station, Tx, USA). A p-value ≤ 0.05 was considered statistically significant.

13.4 Results

Two hundred and thirty-two patients consented to being formally screened for metabolic syndrome at the Centre for Bariatric Surgery (Melbourne, Australia). Of these patients, 171 were diagnosed with metabolic syndrome according to the ATPIII criteria (73.7% prevalence).

One hundred and seven participants consented to involvement in the study. Eighty-nine participants followed through with data to at least one year (83.2%). The reasons for withdrawal within the first year were pregnancy (n=5), medical problems (n=2), and geographical relocation (n=3), and eight were lost to follow-up. At two years, a further 14 participants withdrew due to explant (n=3), pregnancy (n=3), and medical problems (n=1), and seven were lost to follow-up. Complete study data were available for 75 participants at two years (70.1% retention). Weight data were available for 89 participants at one year and 79 participants at two years.

13.4.1 Participant characteristics and weight loss

The baseline characteristics and changes at 24 months are shown in **Table 13.1**. On the day of the first visit (baseline), the mean BMI was 42.4±6.2 kg/m^2, mean age was 48.2±10.7 years, and 63% (n=56) were women.

13.4.1.1 Pre-operative weight loss

Fifty-nine participants (66%) undertook a pre-operative weight loss program. These participants achieved a mean TBWL 3.7±3.1% (EWL 9.0±7.8%) by the day of surgery, which was significantly greater than the weight loss observed in participants who did not undertake a formal weight loss program (TBWL 0.3±2.1% and EWL 0.6±5.6%, p <0.001).

13.4.1.2 Changes over 24 months

At 24 months, the mean TBWL was 18.4±7.6% (EWL 48.7±23.4%) (**Figure 13.1**), corresponding to a mean BMI of 34.7±6.4 kg/m^2.

All components of the metabolic syndrome improved significantly: triglycerides (1.98±1.07 vs 1.39±0.58, p<0.001), HDL cholesterol (1.17±0.29 vs 1.48±0.34, p<0.001), fasting blood

sugar (6.0 (5.3-7.9) vs 5.3 (5.1-5.9), p<0.001), systolic blood pressure (143.7±21.3 vs 136.7±13.9, p<0.001), and waist circumference (123.1±22.8 vs 106.8±15.1, p<0.001). Correspondingly, there were a substantially lower proportion of participants reaching ATPIII diagnostic criteria for each of these variables by 24 months (**Table 13.1**).

13.4.2 **Resolution of metabolic syndrome**

Complete resolution of the metabolic syndrome occurred in 60 of 89 participants at 12 months (67%) and 60 of 75 participants at 24 months (80%). This corresponded to a total of 69 of the 107 participants initially recruited with confirmed resolution of the metabolic syndrome over the two-year follow-up period (64.5%). The mean weight loss at the time that metabolic syndrome first resolved was 10.9±7.7% TBWL, or 28.4±21.0% EWL.

13.4.2.1 Weight loss for resolution of metabolic syndrome

With progressive weight loss, the proportion of participants with metabolic syndrome decreased. The prevalence of metabolic syndrome was over 80% with <5% TBWL, decreasing to below 43% when ≥20% TBWL had been achieved (**Figure 13.2a**). The prevalence of metabolic syndrome also correlated with weight, with over 70% prevalence when BMI exceeded 35 kg/m^2, and dropping to 36% and 10% with a BMI of 25-30kg/m^2 and <25kg/m^2 respectively (**Figure 13.2b**).

To determine the point of significant change in metabolic syndrome, a generalised estimating equation (GEE) analysis of weight loss and BMI categories was conducted (**Table 13.2**). This showed that compared to <2.5% TBWL, a TBWL of 10-12.5% was significantly associated with double the odds of metabolic syndrome resolving (OR 2.09, p=0.025). The odds of resolution increased with increasing weight loss, with an odds ratio of 14.10 (95% CI: 6.52-30.47) if >25% TBWL was achieved. Compared to the reference BMI of 25 kg/m^2, a BMI ≥30 kg/m^2 was associated with significantly reduced odds of metabolic syndrome resolution (OR 0.08, 95% CI: 0.01-0.64, p=0.017).

13.4.2.2 Weight loss for resolution of components of the ATP III criteria

Using a survival analysis, the weight loss at which the prevalence of the metabolic syndrome is halved was 12.8% (11.3-14.4%) TBWL (or 33.1% (23.0-43.2%) EWL) (**Figure 13.3**).

When survival analysis was applied to individual components of the metabolic syndrome, early factors to resolve were hypertriglyceridaemia (median resolution at 7.0% (4.7-9.3%) TBWL (or 17.5% (12.0-23.0%) EWL)), HDL cholesterol (11.1% (8.5- 13.8%) TBWL (or 28.0% (22.5-33.5%) EWL)) and hyperglycaemia (11.2% (6.7-15.7%) TBWL (or 26.1% (8.1-44.0%) EWL)). Nearly 20% TBWL was required for median resolution of hypertension (19.8% (16.8-22.8%) TBWL or 59.5% (36.8-81.8%) EWL). Waist circumference was the last component to resolve, requiring a TBWL of 28.7% (24.9-32.5%) (or 73.3% (66.3-80.3%) EWL) for half the population to attain normal measures.

13.4.2.3 Factors associated with resolution of metabolic syndrome

Using a univariate generalised estimating equation (GEE) analysis of baseline and related variables, metabolic syndrome resolution was not associated with baseline characteristics, but was significantly associated with fasting insulin and C-peptide concentration (**Table 13.2**).

13.4.3 Change in cardiovascular risk scores

The median hsCRP at baseline decreased from 4.7 (2.7-9.6) mg/L to 2.0 (0.8-4.2) mg/L by 24 months ($p<0.001$). **Figure 13.4a** shows the drop in median hsCRP with weight loss, from 3.65 mg/L with 0-5% TBWL, to 1.6 mg/L when >25% TBWL had been achieved.

Figure 13.4b shows the hsCRP risk stratification of the population, with progressive improvement with weight loss.

The new Framingham Risk Equation (FRE) could be applied to 74 participants at baseline. For the other fifteen participants, it could not be calculated due to young age (n=10), or previous cardiovascular disease (n=5). Ten-year absolute cardiovascular disease risk at baseline was 17.0% (7.2-33.5%). A statistically significant decrease in FRE risk occurred when 5-10% TBWL was achieved (17.0% vs 15.8%, $p<0.001$), with continuing improvement until 20% TBWL (FRE 9.0% (3.6-16.6%)) (**Figure 13.4c**).

Table 13.1: Participant baseline and 24 month characteristics

Variable	Baseline	24 months	p-value
Female gender	56 (62.9%)	-	-
Age	48.2 ±10.7	-	-
Weight	119.0 ±20.3	97.2 ±20.5	<0.001
Height	1.67 ± 0.09	-	-
BMI	42.4 ±6.2	34.7 ±6.4	<0.001
Excess weight	48.7 ±17.4	27.5 ±17.3	<0.001
EWL	-	48.7 ± 23.4%	-
TBWL	-	18.4 ± 7.6%	-
Non-smoker	49 (55.1%)	-	-
Ex-smoker	3 (3.4%)	-	-
Current smoker	37 (41.6%)	-	-
Baseline cholesterol	5.13 ±1.11	5.10 ±1.09	0.647
Baseline HDL	1.17 ±0.29	1.48 ±0.34	<0.001
HDL <1.0mmol/L (ATP III)	21 (23.6%)	3 (4.0%)	0.001
Baseline triglyceride	1.98 ±1.07	1.39 ±0.58	<0.001
Triglyceride >1.7mmol/L (ATP III)	46 (51.7%)	15 (20.0%)	<0.001
Baseline waist circumference (WC)	123.1 ±22.8	106.8 ±15.1	<0.001
WC >102cm/>88cm (ATP III)	89 (100%)	58 (80.6%)	-
Baseline blood sugar level (BSL)	6.0 (5.3 – 7.9)	5.3 (5.1 – 5.9)	<0.001†
BSL > 5.6mmol/L (ATP III)	54 (60.7%)	26 (34.2%)	<0.001
Baseline systolic BP	143.7 ±21.3	136.7 ±13.9	<0.001
Baseline diastolic BP	88.5 ±14.5	81.4 ±14.8	<0.001
BP > 135/80mmHg (ATP III)	78 (87.6%)	37 (63.8%)	0.012
Baseline insulin	10.9 (7.0 – 17.3)	10.3 (7.6 – 16.6)	0.750†
hsCRP	4.7 (2.7, 9.6)	2.0 (0.8 – 4.2)	<0.001†
C-peptide	0.94 ±0.45	1.04 ±0.51	0.287
Use of antihypertensive medication	47 (52.8%)	45 (50.6%)	0.500
Use of oral hypoglycemics	28 (31.5%)	26 (29.2%)	0.500
Use of insulin	10 (11.2%)	9 (10.1%)	1.000
Use of cholesterol lowering medication	33 (37.1%)	31 (34.8%)	0.500

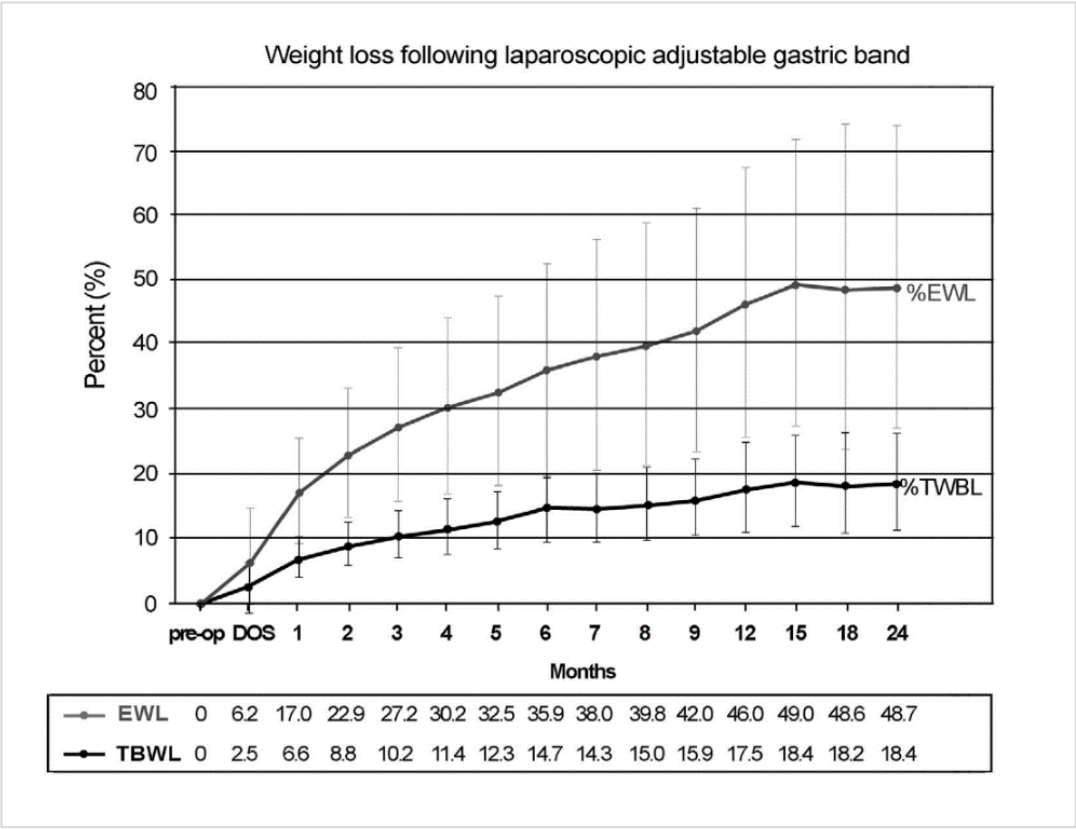
Values represented as mean±SD, or median (Q1, Q3), or number (percentage). Significance testing with Student t-test for continuous variables or McNemar test for dichotomous paired variables, unless otherwise stated.
†Wilcoxon signed-rank test.
BMI – body mass index; TBWL – total body weight loss; ATP III – Adult treatment panel III; WC – waist circumference; BSL – blood sugar level; BP – blood pressure; hsCRP – high sensitivity C-reactive protein; ns – not significant.

Table 13.2: Factors related to resolution of the metabolic syndrome.

Variables	Odds ratio (95% CI)	p-value
Total body weight loss (TBWL)	1.10 (1.08 - 1.12)	<0.001
TBWL category		
0-2.5%	ref	
2.5 - 5.0%	1.34 (0.64 - 2.82)	0.445
5.0 - 7.5%	1.65 (0.84- 3.25)	0.146
7.5 - 10.0%	1.58 (0.81 – 3.0)	0.178
10.0 - 12.5%	2.09 (1.10 – 4.00)	0.025
12.5 - 15.0%	2.55 (1.33 – 4.89)	0.005
15.0 - 17.5%	3.34 (1.69 – 6.61)	0.001
17.5 - 20.0%	3.67 (1.85 – 7.26)	<0.001
20.0 - 22.5%	7.41 (3.56 – 15.44)	<0.001
22.5 - 25.0%	6.46 (2.93 – 14.26)	<0.001
>25.0%	14.10 (6.52 – 30.47)	<0.001
Body mass index (BMI)	0.97 (0.92 – 1.02)	0.185
BMI category		
<25 kg/m^2	ref	
25-30 kg/m^2	0.23 (0.03 – 1.77)	0.159
30-35 kg/m^2	0.08 (0.01 – 0.64)	0.017
35-40 kg/m^2	0.03 (0.00 – 0.24)	0.001
40-45 kg/m^2	0.02 (0.00 – 0.16)	<0.001
45-50 kg/m^2	0.02 (0.00 – 0.13)	<0.001
>50 kg/m^2	0.03 (0.00 – 0.30)	0.003
Male gender	0.97 (0.54 - 1.74)	0.917
Age	0.99 (0.86 - 1.02)	0.443
Smoking		
Non-smoker	ref	
Ex-smoker	3.07 (0.63 - 14.94)	0.164
Smoker	0.72 (0.41 - 1.29)	0.269
Insulin	0.89 (0.86 - 0.91)	<0.001
hsCRP	0.98 (0.96 - 1.00)	0.096
C-peptide	0.27 (0.19 - 0.39)	<0.001

Values presented as odds ratio (95% confidence interval).
TBWL – total body weight loss; BMI – body mass index; hsCRP – high sensitive C-reactive protein

Figure 13.1: Weight loss following laparoscopic adjustable gastric banding over the 24 month trial period.



EWL – excess weight loss; TBWL – total body weight loss; DOS – day of surgery

Figure 13.2: Percentage population with resolution of the metabolic syndrome with (a) total body weight loss (TBWL) category and (b) body mass index (BMI) category

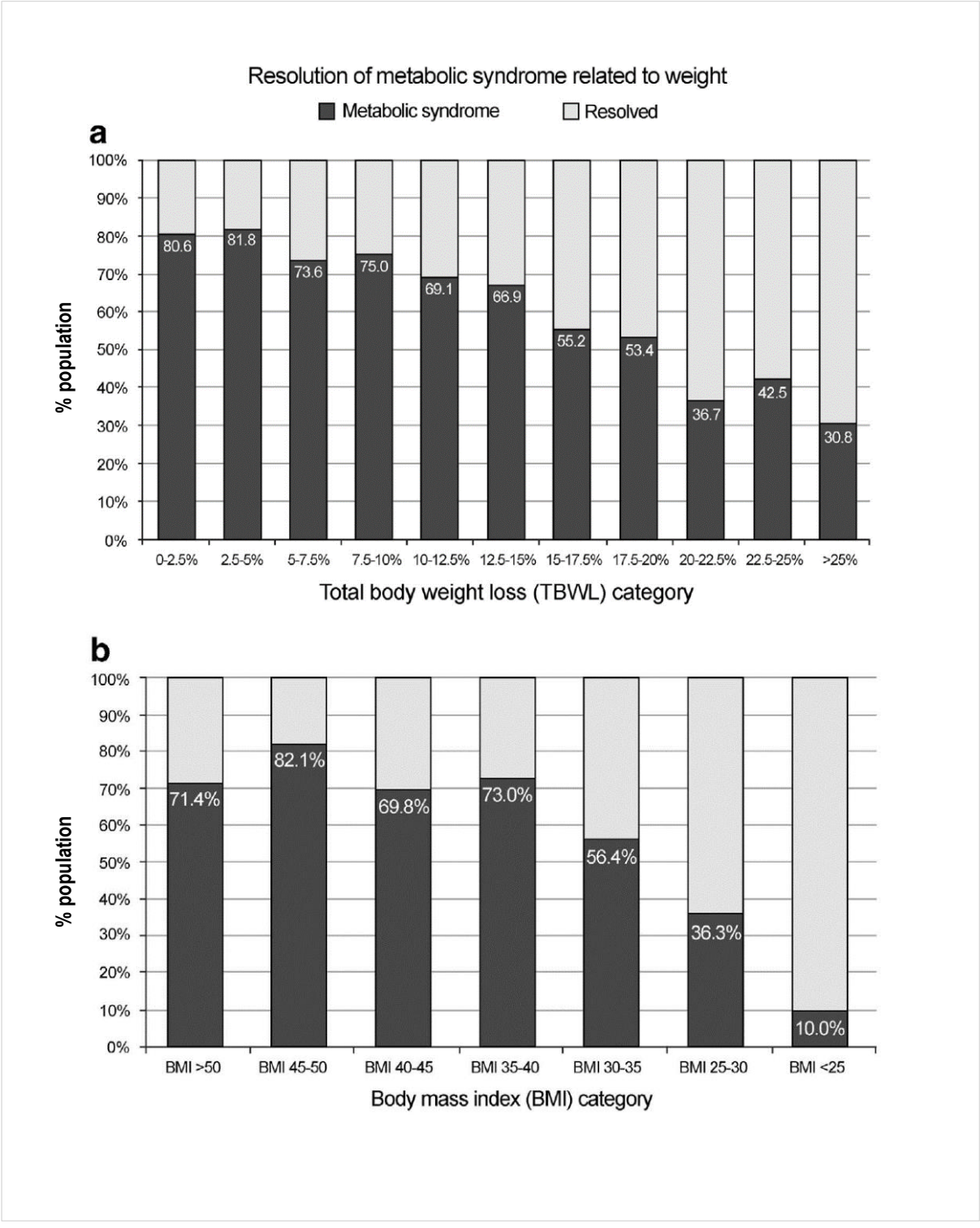


Figure 13.3: Progressive resolution of the metabolic syndrome (as per ATP III criteria) and resolution of each component of the ATP III criteria (triglyceride level, HDL level, glycose, BP and waist circumference).

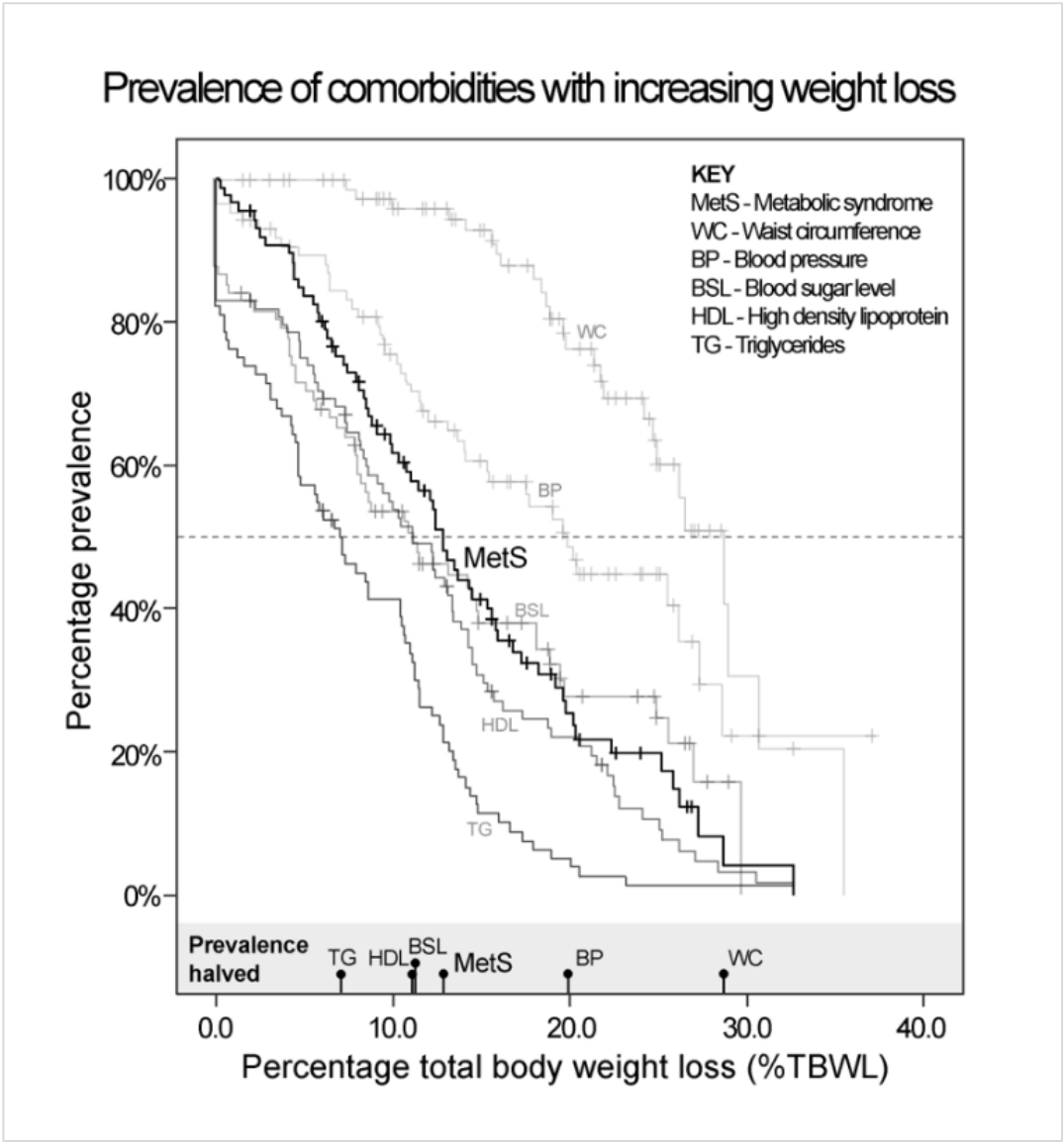
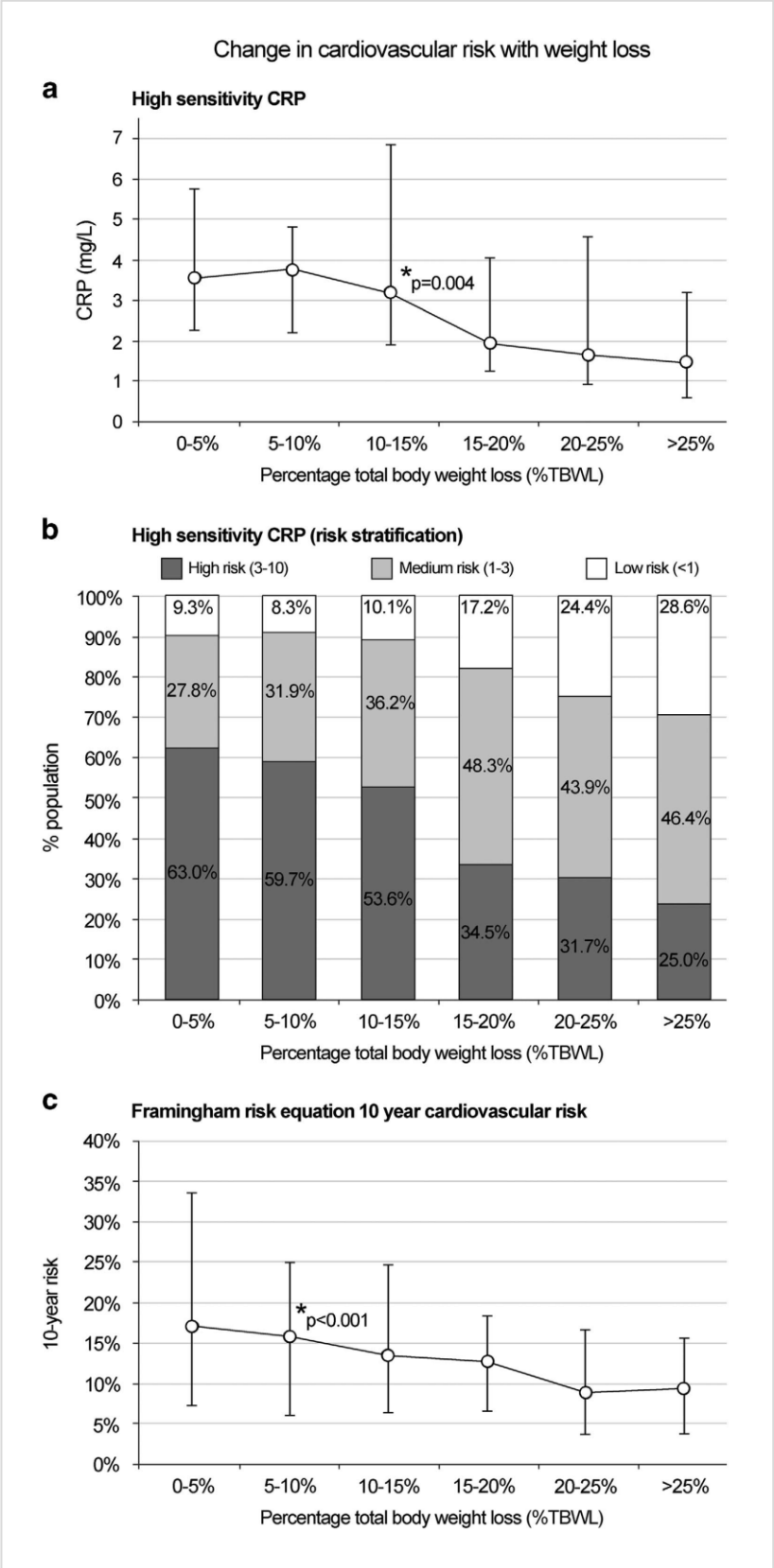


*Figure 13.4: Changes in measures of cardiovascular risk with weight loss (%TBWL). (a) Median CRP levels (with interquartile range) and (b) changes in hsCRP, showing the percentage of population stratified into risks. (c) Changes in median 10-year absolute cardiovascular risk according to the Framingham Risk Equation for participants at risk. *Statistically significant reduction from baseline (p<0.05).*



13.5 Discussion

We have prospectively measured the effect of incremental weight loss on the metabolic syndrome and its components in obese patients, in an attempt to identify weight loss targets for meaningful regression or improvement of the metabolic syndrome. The key finding of this study was the linear relationship of weight loss to resolution of the metabolic syndrome, with significantly increased odds of resolution with 10-12.5% TBWL and improved probability of resolution with decreasing BMI.

Components of the metabolic syndrome resolved at different stages of weight loss. Biochemical markers were the first to normalise, with only 7.0% TBWL required for prevalence of hypertriglyceridaemia to halve. This was closely followed by HDL (at 11.1% TBWL) and hyperglycaemia (at 11.2% TBWL). These parameters had the lowest percentage prevalence by 24 months follow-up, and likely contributed most to the resolution of metabolic syndrome by ATPIII criteria. Both blood pressure and waist circumference required more significant weight loss to resolve, at 19.8% and 28.7% TBWL respectively. The mechanisms underlying these differences are unclear. Early changes in hepatic metabolism and skeletal muscle function, as observed in other studies (18), may alter the metabolism of lipids and glucose to expedite these biochemical changes. Early alterations in the inflammatory milieu with weight loss, as reflected by change in hsCRP, may also contribute to biochemical improvements.

Baseline insulin and C-peptide concentrations were significantly associated with resolution of the metabolic syndrome. This is perhaps not surprising given that insulin resistance is central to all definitions of the metabolic syndrome. This finding is also consistent with reports of change in cardiovascular risk that identified fasting insulin concentration as the only predictor of decreased cardiovascular risk following bariatric surgery (426).

Predictors of cardiovascular risk substantially improved with 10-15% TBWL, with minimal further improvement in risk reduction observed after 20% TBWL was achieved. These data complement the changes observed in the metabolic syndrome at this weight loss threshold. Other studies have shown that bariatric surgery nearly halves overall cardiovascular risk, assessed by the Framingham Risk Equation (593). However, this should be interpreted with caution, as cardiovascular risk equations are not validated in the weight loss setting and reductions may not necessarily deliver fewer cardiovascular events, as was reported in the

LOOK-AHEAD study (577). However, reductions in metabolic risk factors with weight loss, together with other evidence showing direct beneficial effects on cardiovascular function and coronary vascular integrity (594), provide compelling evidence for the positive effect on future cardiovascular health.

Overall, these data suggest that 10-12.5% TBWL (25-30% EWL) is significantly associated with metabolic benefit in morbidly obese patients. This represents only a fraction of the weight loss required to reach a 'healthy' BMI of 25 kg/m^2 and could be both feasible and sustainable with conservative programs, especially those that include pharmacotherapies (595, 596), Appropriate and achievable goal setting is a vital step of successful weight loss programs (589), and we suggest that these data be used as the basis for such goals. However, we have also observed a linear improvement in metabolic syndrome with further weight loss. Hence, if there is no improvement with more modest degrees of weight loss, further weight loss should be actively sought, with possible consideration of pharmacotherapy or bariatric surgery.

These data challenge how we define both success and failure after bariatric surgical procedures. Traditionally failure following bariatric surgery has been defined as <50% EWL. This definition was based on work from the 1980s where statistical quartiles appear to have been arbitrarily chosen (597) and fell in to common usage with little validation of their appropriateness (598). However, definitions of success and failure should also incorporate measures of metabolic improvement, in addition to simple weight loss targets. We have previously used EWL<25% (approximately 10-15% TBWL) to define failure after bariatric surgery (599) on the basis of our clinical observation that little metabolic benefit was achieved below this level. These current data validate this alternate definition.

The metabolic syndrome as a distinct diagnostic entity is controversial (89). It was first conceptualised as a common cluster of metabolic risk factors, linked to cardiovascular disease (600). The major limitations have been extensively discussed and its value debated (89). Such issues include the lack of consensus definition, a lack of a unifying pathogenic hypothesis, and the ambiguity in prediction of cardiovascular disease and type II diabetes. Detractors also question the dichotomisation of continuous variables that may undervalue the magnitude of risk, especially in those who do not quite reach criteria definition (89). However, proponents have suggested a multiplicative model of the metabolic syndrome, with over two times the risk of cardiovascular disease (CVD), myocardial infarction and stroke in

those with the metabolic syndrome compared to those without (91). Additionally, there is unaccounted additional risk after treatment of component metabolic risk factors, suggesting that the metabolic syndrome as a sum is probably greater than its parts (92).

Regardless of the controversy, the metabolic syndrome clearly identifies individuals at higher risk of diabetes and cardiovascular disease (93, 590). It is easily applicable in the clinical setting for primary care physicians and those not subspecialised in the field, and can trigger interventions and treatment strategies to combat cardiovascular disease.

Our study differs substantially from previous studies. Firstly, we prospectively undertook repeated and detailed measures of metabolic syndrome, in a cohort of patients who progressively lost a substantial amount of weight. This allowed us to examine the effects of various levels of weight loss and provided insight into the steps behind its progressive resolution. Our study design helped answer the key question about optimal weight loss goals for significant metabolic outcome.

Secondly, our cohort of patients focussed on the more severely obese with metabolic disease, and did not include those in the overweight category. It confirmed the benefits of modest weight loss in this population. Despite our cohort remaining within the obese category after bariatric surgery and not reaching ‘ideal’ weight, early weight loss achievements were shown to induce meaningful metabolic improvements.

Finally, in contrast to other surgical cohorts, weight loss following LABG is not dependent on changing gut anatomy nor the hormonal milieu. Rather, weight loss is achieved by induction of satiety, leading to caloric restriction (195). Weight loss following LAGB, therefore, closely mimics the mechanism of conservative weight loss programs and as such, the benefits seen in this study should better model expected outcomes with lifestyle intervention.

There are several study limitations that warrant discussion. This was a relatively short-term study, spanning only two years post-operatively. Although we know that weight loss after bariatric surgery is durable, we would expect the metabolic syndrome to partially recur over time, given the associations of age with glucose, blood pressure and lipid measures. Secondly, we have not measured other lifestyle factors that influence health outcomes, such

as exercise or specific dietary changes. These changes often accompany bariatric surgery, and are known to influence metabolic disease. Thirdly, this study examined only those who met the criteria for metabolic syndrome. The prevalence of the metabolic syndrome in obese populations is reported between 24-78%, with only 2-28% having no metabolic risk factors (601). The prevalence within our centre was nearly 75%, which may reflect the BMI range and bariatric surgical population. Despite this, the exclusion of obese participants without criteria definition of metabolic syndrome may limit the applicability of these results to the general obese population.

The use of survival analysis in this study allowed us to study the relative resolution of metabolic syndrome components. One limitation in this analysis and the dataset is the inability to capture the exact weight at time of resolution, as patients are assessed at intervals. Interpretation of the weight loss required for resolution needs to be interpreted with caution. However, our frequent review of patients, especially during their time of maximal weight loss, will hopefully have assisted in mitigating this effect.

Lastly, a potential source of bias will occur with loss to follow-up. By 24 months, 15 participants were lost to follow-up and 17 had withdrawn due to various reasons. These include significant medical issues, relocation or explant, which meant that patients were unable or unwilling to continue with the intense follow-up of the study. Patients who became pregnant were withdrawn, as the biochemical and physical changes of pregnancy will naturally affect measurement of the metabolic syndrome. Regardless of cause for withdrawal, these losses may affect the data, as those who are not followed up may have poor weight loss and more severe comorbidities. In a worse-case scenario, the rate of resolution would have been 56.1% (n=60) and 64.5% (n=69) at one and two years, and in the best-case scenario, resolution: 72.9% (n=78) and 86.0% (n=92).

In conclusion, this study validates weight loss as a central treatment for obese patients with the metabolic syndrome. A weight loss target of 10-12.5% TBWL (25-30% EWL) is a reasonable initial goal for morbidly obese people, as it is associated with significant odds of improvement in the metabolic syndrome. If improvements are not seen with this weight loss, further weight loss should be encouraged as we observed additional metabolic health benefits

with increasing weight loss. Future studies should seek to validate these findings and confirm the long-term cardiovascular impact of remitting metabolic syndrome in the morbidly obese.

14 Detailed description of change in serum cholesterol profile with incremental weight loss after restrictive bariatric surgery

14.1 Abstract

INTRODUCTION: Dyslipidaemia affects up to 75% of morbidly obese individuals, and is a key driver of cardiovascular disease. Weight loss is an established strategy to improve metabolic risk, including dyslipidaemia. We aimed to determine weight loss goals for resolution of serum lipid abnormalities, by measuring improvements during progressive weight loss in obese individuals.

METHODS: We performed a prospective cohort study of obese individuals with the metabolic syndrome undergoing adjustable gastric banding. Lipid levels were monitored monthly for nine months, then three monthly until 24 months.

RESULTS: There were 101 participants included, age 47.4±10.9 years with body mass index 42.6±5.9kg/m^2. At 24 months, total body weight loss (TBWL) was 18.3±7.9%. This was associated with significant improvements in high density lipoprotein (HDL) (1.18 vs 1.47, $p<0.001$), triglyceride (2.0 vs 1.4, $p<0.001$) and total cholesterol to HDL ratio (TC:HDL) (4.6 vs 3.6, $p<0.001$). Over this time, progressive and linear improvements in HDL, triglycerides and TC:HDL were seen with incremental weight loss (observed at 2.5% TBWL intervals). Significant improvements occurred after a threshold weight loss of 7.5-12.5% TBWL was achieved, with odds ratio (OR) 1.48-2.50 for normalisation. These odds improved significantly with increasing weight loss (OR 18.2-30.4 with >25% TBWL). Despite significant weight loss, there was no significant change in low density lipoprotein (LDL).

CONCLUSION: Significant improvements in triglycerides, HDL and TC:HDL occur after 7.5-12.5% TBWL, with ongoing benefit after greater weight loss. LDL needs to be addressed independently, as this was not observed to respond to weight loss alone.

14.2 Introduction

Dyslipidaemia is strongly associated with obesity, affecting up to 75% of morbidly obese individuals undergoing bariatric surgery (602). It is instrumental in the development of cardiovascular disease (603), through the promotion of atherosclerosis, vascular inflammation and thrombosis (603, 604). Cardiovascular disease burden is now the principal causes of death in obesity, and significantly reduces life expectancy (54). Consequently, effective and timely management of dyslipidaemia is key to reducing cardiovascular risk and mortality in the obese (25, 605).

Weight loss is a proven method to improve the metabolic syndrome and obesity-associated risks (164, 165). Bariatric surgery produces effective sustained long-term weight loss in morbidly obese individuals, in the range of 15-30% total body weight loss (TBWL) (191, 424). Several studies have also shown that it leads to marked changes in lipid levels, with near complete resolution of cholesterol abnormalities, such as high density lipoprotein (HDL), triglyceride and total cholesterol levels (168, 602, 606). However, many of these studies included patients undergoing significant intestinal diversionary procedures, which have independent effects on serum lipid concentration through alterations in gastrointestinal hormonal milieu (216) or bile acid diversion (607). By contrast, lifestyle changes and restrictive bariatric surgery, such as the laparoscopic adjustable gastric band (LAGB) (195), induces weight loss through caloric restriction.

Whilst it is established that substantial weight loss has significant effects on resolution of dyslipidaemia (168, 602, 606), the relationship of progressive weight loss to improvement in component circulating lipids has not been clearly defined. Evidence suggests that lipid metabolism improves with as little as 5-10% TBWL (18, 557). Understanding the rate and magnitude of improvement in different lipid parameters would increase our understanding of the role of weight loss in management of dyslipidaemia and cardiovascular risk. Importantly, it would assist in setting evidence-based targets for weight loss aimed at managing lipid abnormalities. It could also inform decisions to continue or trial cessation of lipid lowering medication in those who achieve weight loss.

We hypothesised that improvements in lipid levels begin to occur with early weight loss in the morbidly obese undergoing laparoscopic adjustable gastric band (LAGB) surgery, but that different weight loss targets are required to resolve different lipid parameters. To test this

hypothesis, we closely observed the changes in lipid parameters in obese individuals after LAGB surgery over two years to obtain a detailed description of lipid profile changes with weight loss.

The primary aim of this study was to examine the improvement in lipid levels with incremental weight loss in obese individuals after LAGB surgery. By so doing, we aimed to define the weight loss thresholds required to produce significant benefit to lipid profile, particularly triglyceride levels, high-density lipoprotein (HDL) levels, total cholesterol to HDL ratio (TC: HDL), and low-density lipoprotein (LDL) levels, and investigate the impact of more substantial weight loss. The secondary aim was to identify factors associated with improvement in lipid profile.

14.3 Methods

All participants provided informed written consent to participate. Ethics approval was obtained from the Avenue Ethics Committee (reference no. 099). The trial was registered with the Australian Clinical Trials Register (ACTRN12610000049077).

14.3.1.1 Patients

We undertook a two-year prospective observational study of consecutive eligible obese patients with the metabolic syndrome who underwent LAGB placement between April 2009 and March 2010 by one of six surgeons affiliated with the Centre for Bariatric Surgery in Melbourne, Australia.

Criteria for inclusion in the study included: (1) age ≥18 years, (2) BMI >30kg/m^2, (3) undergoing a primary LAGB procedure, and (4) metabolic syndrome as defined by the Adult Treatment Panel (ATP) III. The decision to proceed with LAGB was based on a clinical assessment by the individual surgeons, and in discussion with the patient. Due consideration was given to all patients with BMI≥30kg/m^2, and based on individual obesity-related risk (193, 608). Patients were approached for participation in this study once LAGB surgery was planned.

14.3.2 Outcomes

14.3.2.1 Baseline and weight data

Patients underwent a complete medical history and examination within two weeks of surgery. This included an assessment of previous diagnosis of dyslipidaemia and use of cholesterol lowering medication. Comorbid illnesses and medications were recorded on a standardised questionnaire. Body mass index (BMI) was categorised using the World Health Organization classification, with a BMI>30kg/m^2 defined as obesity (21). Fasting blood tests were taken on the same day as the surgery.

14.3.2.2 Follow-up

Patients were reviewed every month for nine months, then three monthly until 18 months, and then at 24 months.

Data collected at each visit included: weight in kilograms (for calculation of BMI and total body weight loss); fasting blood tests, including a full cholesterol assessment and measures of insulin resistance and metabolic health (performed at a single pathology service (Melbourne Pathology, Melbourne, Australia)); medical comorbidities; and medication use.

Specific cholesterol levels of interest were: triglyceride (TG) (reference range <1.7 mmol/L) (55); high density lipoprotein (HDL) (reference range >1.0 for males, >1.3 for females); total cholesterol : HDL ratio (TC:HDL) (reference range <4.5); and low density lipoprotein (LDL) (reference range <2.6) (25).

14.3.3 **Statistical analysis**

Data were analysed in subgroups according to (1) gender and (2) baseline abnormal lipid level. Changes in lipid variables were analysed against weight loss to determine the effects of weight loss on serum lipid levels. The weight and weight loss required for complete resolution of abnormal lipid levels according to reference levels (see **Table 3.8** in **Section 3.1.4.2 - Dyslipidaemia**) was noted and analysed.

Continuous variables were expressed as mean ± standard deviation (SD) for parametric data, and median and interquartile range (IQR) for nonparametric data. Normality was assessed by Shapiro-Wilks test. Student t-test and one-way ANOVA with post-hoc Bonferroni test was used for parametric data, and Mann Whitney U-test, Wilcoxon signed-rank test and Kruskal-Wallis test for non-parametric data. Categorical variables were expressed as numbers (with percentages). Pearson's chi-squared or Fisher's exact test were used for independent categorical variables. McNemar's test was used for dependent categorical variables. A p-value ≤ 0.05 was considered statistically significant.

A Generalised Estimating Equation (GEE) model was used to identify clinical and biochemical factors associated with normalisation of lipid levels. The model incorporated a binomial distribution to account for binary outcome, a logit link function and an exchangeable correlation structure. For the multivariate model, starting from the most significant variable identified in the univariate analysis, we included the next most significant variable and used the likelihood ratio test to see if there was a significant improvement to the fit of the model. This was performed sequentially for all variables that were significant in the univariate analysis. Data analysis was performed in Stata V14 (Stata Corp, College Station, Tx, USA) and level of significance set at 5%.

14.4 Results

14.4.1 **Patients**

One hundred and one obese patients undergoing bariatric surgery were included (**Table 14.1**). The average age was 47.4±10.9 years, and average weight was 119±19.2kg corresponding to an average body mass index (BMI) of 42.6±5.9 kg/$m^{2.}$ There were 34 men (33.7%).

There were high rates of comorbidities, with 81 (80.2%) having hypertension, 28 (27.7%) with impaired fasting glucose and 37 (36.6%) having diabetes. Seventy-four (73.3%) patients had a diagnosis of dyslipidaemia, and 36 (35.6%) were on lipid lowering medications.

By 24 months, there were 80 patients with adequate data. Reasons for withdrawal included pregnancy (n=8), LAGB explant (n=7) and inadequate follow up (<50% of study requirements) (n=6). The average BMI at 24 months decreased to 34.7±6.4 kg/m^2 ($p<0.001$), corresponding to a percentage total body weight loss (%TBWL) of 18.3±7.9%. Four patients were taken off their lipid lowering medications by the end of the trial (35.6% on medication at baseline vs 33.7% at 24 months, p=0.80). There were no other patients who had their lipid lowering medication regime altered.

14.4.2 **Changes in lipid profile**

14.4.2.1 Baseline and 24 month outcomes

Baseline and 24 month biochemical results are seen in **Table 14.1**. At baseline, 41 (41.6%) patients had elevated triglyceride levels, 77 (76.3%) had decreased HDL levels, 46 (45.6%) had an abnormal TC:HDL ratio, and 63 (62.4%) had elevated LDL levels. By 24 months, there were significant improvements in triglyceride levels (2.0 vs 1.4 mmol/L, $p<0.001$), HDL (1.18 vs 1.47, $p<0.001$), and TC:HDL ratio (4.6 vs 3.6, $p<0.001$). Correspondingly, there was a substantial reduction in proportion of individuals with abnormal triglyceride level (41.6% vs 7.5%, $p<0.001$), HDL level (76.3% vs 16.3%, $p<0.001$) and TC:HDL ratio (45.6% vs 16.3%, $p<0.001$). These reductions were seen in both male and female cohorts.

There was no significant change in measured LDL cholesterol (3.1 vs 3.0, p=0.739), nor proportion of patients with abnormal LDL levels (62.4% vs 61.3%, p=0.661)

Measures of insulin resistance also showed significant reductions (blood glucose 6.9 vs 5.7 mmol/L, p<0.001; insulin 164.3 vs 87.2 pmol/L, p<0.001; homeostatic model assessment index-2 of insulin resistance (HOMA2-IR) 3.2 vs 1.7, p<0.001).

14.4.2.2 Trends in lipid profile with weight loss

Figure 14.1 and **Figure 14.2** show the progressive changes in lipid variables for both male and female cohorts with incremental weight loss. Percentage reduction from baseline is seen in **Table 14.2** (with average values in **Supplementary Table 14.1**), divided into male and female cohorts, and those with and without dyslipidaemia at baseline.

Triglyceride levels

There were progressive reductions in triglyceride levels, with early reductions occurring with early weight loss (**Figure 14.1** and **Figure 14.2**). Normal triglyceride levels were achieved after 5-7.5% (males) and 7.5-10% TBWL (females) in those with abnormal baseline levels. By 25% TBWL, there was an average reduction of 52.2% for males and 35.9% for females (**Table 14.2**).

With generalised estimating equation (GEE) analysis, odds of normalisation of triglycerides were significantly related to both weight loss (in %TBWL) and body weight (BMI) (**Table 14.3**). For men, significant odds of normalisation were achieved at 10-12.5% TBWL (OR 2.22, p=0.021), with increasing odds up to 7.05 (p=0.007) after 20% TBWL. Significant odds of normalisation of 1.64 (p=0.012) were seen at 7.5-10% TBWL for females, increasing to 18.15 (p<0.001) at ≥25% TBWL.

Percentage TBWL was significantly associated with triglyceride resolution in multivariate GEE analysis for both males and females (OR 1.10, p<0.001 and OR 1.07, p<0.001) (**Table 14.4**).

High density lipoprotein (HDL) levels

HDL levels incrementally increased with weight loss, and reached average normal values by 7.5-10% and 12.5-15% TBWL for males and females respectively (**Figure 14.1-14.2**). When 25% TBWL was achieved, HDL increased by an average of 37.9% (males) and 26.2% (females). More marked changes were seen in those with abnormal levels at baseline (66.7% (males) and 36.1% (females)) (**Table 14.2**).

Significant odds of resolution occurred at 10-12.5% TBWL for both males (OR 1.86, p=0.034) and females (OR 1.67, p=0.032). These odds increased as increasing weight loss was achieved, up to OR 13.07-14.25 at 22.5-25% TBWL (**Table 14.3**). In multivariate analysis, TBWL was significantly associated with normalisation of HDL (OR 1.06 and OR 1.09 for males and females) (**Table 14.4**).

Total cholesterol to HDL ratio (TC: HDL)

Total cholesterol to HDL ratio (TC: HDL) showed incremental improvement, up to 38.1% (males) and 22.8% (females) after >25% TBWL was achieved (**Table 14.2**).

There was significant improvement at 7.5-10% TBWL (OR 2.50 and OR 1.45 for males and females). This increased to an odds ratio of 30.4 and 20.0 after >25% TBWL (**Table 14.3**). In multivariate analysis, odds of improvement increased with increasing %TBWL (**Table 14.4**).

Low density lipoprotein (LDL)

There were no significant clear trends in LDL levels with total body weight loss, for both female and male cohorts (**Figure 14.1**, **Figure 14.2** and **Table 14.2**). For females, increasing BMI was related to LDL abnormalities, however this was not evident in multivariate analysis (**Table 14.4**). Percentage TBWL was weakly associated with LDL normalisation for females after multivariate analysis, but not for males.

14.4.2.3 Factors associated with lipid level normalisation

In a multivariate analysis, total body weight loss was globally associated with normalisation of triglyceride, HDL and TC:HDL in males and females. Measures of insulin resistance and metabolic health, such as HbA1c and HOMA2-IR, were similarly associated with resolution of some lipid parameters. Other variables significantly associated with lipid level normalisation are shown in **Table 14.4**.

Table 14.1: Baseline and 24 month variables for all patients, and separated into male and female cohorts.

Variable	All patients			Male			Female		
	Baseline	24 months	p-value	Baseline	24 months	p-value	Baseline	24 months	p-value
n=	101	80		34	29		67	51	
Age	47.4±10.9			50.1±9.2			46.0±11.5		
Male gender	34 (33.7%)								
Weight (kg)	119.0±19.2	96.9±20.3	<0.001	114.2±16.8	90.7±19.0	<0.001	128.4±20.4	108.3±18.0	<0.001
Ideal weight (kg)	70.0±7.5			77.3±6.5			66.2±4.6		
Excess weight (kg)	49.1±16.2			51.1±16.6			48.0±16.0		
BMI (kg/m^2)	42.6±5.9	34.7±6.4	<0.001	43.1±6.2	34.7±7.0	<0.001	41.4±4.9	34.7±5.1	<0.001
%EWL	0	48.5±23.9%		0	51.2±25.5		0	43.5±20.0	
%TBWL	0	18.3±7.9%		0	19.3±8.0		0	16.5±7.3	
LIPID LEVELS									
Triglycerides (TG)	2.0±1.0	1.4±0.6	<0.001	1.9±0.8	1.4±0.5	0.013	2.1±1.4	1.4±0.8	<0.001
Abnormal TG (>1.7)	41 (41.6%)	6 (7.5%)	<0.001	14 (41.1%)	1 (3.4%)	<0.001	27 (41.3%)	5 (9.8%)	<0.001
High density lipoprotein (HDL)	1.18±0.28	1.47±0.33	<0.001	1.26±0.26	1.56±0.35	<0.001	1.02±0.26	1.31±0.22	<0.001
Abnormal HDL (<1.3, <1)	77 (76.3%)	13 (16.3%)	<0.001	24 (80.6%)	2 (6.9%)	<0.001	53 (79.1%)	11 (21.6%)	<0.001
Total to HDL cholesterol (TC:HDL)	4.6±1.2	3.6±1.0	<0.001	4.36±1.13	3.54±1.01	0.001	4.93±1.39	3.75±1.11	<0.001
Abnormal TC:HDL(>4.5)	46 (45.6%)	13 (16.3%)	<0.001	19 (55.9%)	7 (24.1%)	<0.001	27 (40.3%)	6 (11.8%)	<0.001
Total cholesterol (TC)	5.1±1.1	5.1±1.1	0.905	5.3±1.1	5.3±1.1	0.867	4.8±1.1	4.8±1.0	0.983
Abnormal TC (>5.5)	39 (38.6%)	21 (26.3%)	<0.001	9 (26.4%)	3 (10.3%)	0.031	30 (44.8%)	18 (35.3%)	<0.001
Low density lipoprotein (LDL)	3.1±1.0	3.0±1.0	0.739	3.2±1.0	3.1±1.1	0.774	2.9±0.9	2.8±1.0	0.873
Abnormal LDL (>2.6)	63 (62.4%)	49 (61.3%)	0.661	17 (50.0%)	15 (51.7%)	0.636	46 (68.7%)	34 (66.7%)	0.802
Non-HDL	3.97±1.06	3.65±1.10	0.056	4.06±1.09	3.76±1.12	0.209	3.79±0.98	3.46±1.05	0.153
TG:HDL ratio	1.9±1.6	1.0±0.5	<0.001	1.6±0.9	0.95±0.44	0.004	2.43±2.45	1.09±0.69	<0.001
OTHER BIOCHEMICAL MARKERS									
Fasting glucose (mmol/L)	6.9±2.4	5.7±1.2	<0.001	6.5±2.3	5.3±0.6	0.023	7.7±2.6	6.4±1.7	<0.001
Fasting insulin (pmol/L)	164.3±116.7	87.2±48.6	<0.001	150.8±97.0	84.8±46.8	0.001	190.6±145.6	91.4±52.4	<0.001
HOMA2 IR	3.2±2.1	1.7±0.9	<0.001	2.9±1.8	1.6±0.9	<0.001	3.8±2.6	1.8±1.0	<0.001
HbA1c	6.7±1.6	5.8±0.7	<0.001	6.4±1.5	5.7±0.6	<0.001	7.3±1.6	6.1±0.8	<0.001
C-peptide	1.24±0.55	1.04±0.52	0.020	1.17±0.51	0.99±0.48	0.147	1.37±0.60	1.13±0.59	0.057
hsCRP	7.4±5.8	3.3±4.1	<0.001	8.6±5.8	5.7±0.6	0.008	5.16±5.22	2.18±2.18	<0.001

BMI – Body mass index; %EWL – percentage excess weight loss; %TBWL – percentage total body weight loss; TBWL – total body weight loss; HDL – high density lipoprotein; Trig – triglycerides; TC:HDL – total cholesterol to HDL ratio; HbA1c – haemoglobin A1c; BSL – blood sugar level; HOMA2-IR – homeostatic model assessment index 2 insulin resistance; hsCRP – high sensitivity C-reactive protein.

Table 14.2: Percentage change from baseline of triglycerides, high density lipoprotein (HDL), total cholesterol to HDL ratio (TC:HDL) and low density lipoprotein (LDL) with percentage total body weight loss in 2.5% intervals in males and females. Patients further subgrouped into those with normal lipid levels at baseline, and those with abnormal lipid levels.

% change	**Triglyceride**			**High density lipoprotein (HDL)**			**Total cholesterol to HDL (TC:HDL)**			**Low density lipoprotein (LDL**		
	All patients	**Normal at start**	**Abnormal at start**	**All patients**	**Normal at start**	**Abnormal at start**	**All patients**	**Normal at start**	**Abnormal at start**	**All patients**	**Normal at start**	**Abnormal at start**
Males												
n=	n=34	n=20	n=14	n=34	n=10	n=24	n=34	n=15	n=19	n=34	n=14	n=20
%TBWL												
0-2.5%	ref	ref	ref	ref	ref	ref	ref	ref	ref	ref	ref	ref
2.5-5.0%	2.4%	11.6%	-27.1%	-4.9%	-7.6%	2.5%	8.6%	0.8%	4.6%	7.8%	0	-2.8%
5.0-7.5%	-22.7%	-4.1%	-35.8%	8.7%	4.2%	12.3%	-11.2%	-7.8%	-7.4%	-3.0%	5.2%	-12.7%
7.5-10.0%	-25.6%	-12.2%	-37.1%	13.6%	10.9%	30.9%	-19.1%	-8.6%	-17.0%	-11.6%	1.6%	-23.2%
10.0-12.5%	-19.8%	-5.4%	-37.1%	5.8%	0.8%	25.9%	-6.5%	-10.7%	-12.1%	0	12.4%	-15.9%
12.5-15.0%	-36.7%	-24.5%	-49.8%	10.7%	2.5%	28.4%	-13.1%	-11.8%	-22.2%	2.2%	13.5%	-13.6%
15.0-17.5%	-36.7%	-29.9%	-49.8%	16.5%	8.4%	32.1%	-17.6%	-23.1%	-24.8%	-0.7%	5.7%	-14.2%
17.5-20.0%	-38.2%	-20.4%	-52.3%	15.5%	1.7%	43.2%	-13.8%	-24.4%	-18.3%	4.9%	2.1%	-8.5%
20.0-22.5%	-43.5%	-26.5%	-57.6%	13.6%	4.2%	25.9%	-11.4%	-16.6%	-20.6%	11.9%	24.4%	-8.5%
22.5-25.0%	-50.7%	-41.5%	-55.5%	29.1%	24.4%	46.9%	-26.0%	-20.1%	-32.8%	1.9%	11.9%	-7.9%
>25.0%	-52.2%	-34.0%	-68.5%	37.9%	24.4%	66.7%	-38.1%	-23.6%	-47.3%	-19.4%	3.1%	-11.3%
Females												
n=	n=67	n=40	n=27	n=67	n=14	n=53	n=67	n=40	n=27	n=67	n=51	n=16
%TBWL												
0-2.5%	ref	ref	ref	ref	ref	ref	ref	ref	ref	ref	ref	ref
2.5-5.0%	-9.4%	-7.5%	-15.6%	-1.6%	-4.8%	2.7%	-1.6%	-0.3%	-10.3%	-4.2%	24.8%	-10.1%
5.0-7.5%	3.3%	-2.7%	-0.8%	-4.1%	-4.1%	1.8%	2.4%	3.2%	-7.0%	-5.8%	20.1%	-6.9%
7.5-10.0%	-6.1%	-4.1%	-12.3%	3.3%	-3.4%	7.2%	-2.8%	-5.1%	-7.0%	-4.5%	16.4%	-15.3%
10.0-12.5%	-8.3%	3.4%	-22.5%	10.7%	4.8%	3.6%	-6.1%	-5.9%	-13.0%	-1.0%	-3.7%	0
12.5-15.0%	-11.0%	-0.7%	-25.0%	13.1%	6.2%	10.8%	-5.4%	-7.3%	-11.7%	2.9%	-2.8%	3.7%
15.0-17.5%	-21.0%	-12.3%	-28.7%	22.1%	13.0%	18.9%	-15.1%	-8.1%	-30.5%	-2.6%	3.3%	-6.1%
17.5-20.0%	-20.4%	-13.0%	-30.7%	21.3%	15.8%	17.1%	-14.6%	-11.3%	-24.4%	-3.5%	-2.8%	-4.0%
20.0-22.5%	-22.7%	-15.8%	-33.2%	17.2%	18.5%	16.2%	-11.3%	-6.5%	-23.6%	0.6%	6.4%	-0.6%
22.5-25.0%	-30.9%	-26.7%	-25.8%	28.7%	28.8%	26.1%	-17.6%	-21.2%	-20.8%	0	12.1%	-0.9%
>25.0%	-35.9%	-30.1%	-37.7%	26.2%	12.3%	36.0%	-22.8%	-17.5%	-35.2%	-8.1%	5.1%	-12.1%

%TBWL – percentage total body weight loss; HDL – high density lipoprotein, TC:HDL – total cholesterol to HDL ratio. Values expressed as mean±standard deviation. Independent Student t-test for comparison between normal and abnormal values.

Table 14.3: Univariate analysis of variables associated with resolution of high density lipoprotein (HDL), triglyceride level , total cholesterol to HDL ratio (TC:HDL) and low density lipoprotein (LDL), in males and females.

	Male								**Female**							
	Trig		**HDL**		**TC:HDL**		**LDL**		**Trig**		**HDL**		**TC:HDL**		**LDL**	
Variables	Odd ratio	p-value	Odd ratio	p-value	Odds ratio	p-value	Odds ratio	p-value	Odds ratio	p-value	Odds ratio	p-value	Odds ratio	p-value	Odds ratio	p-value
Age	1.038	0.236	1.107	**0.002**	1.109	**0.007**	1.001	0.963	1.031	**0.035**	1.064	**<0.001**	1.073	**<0.001**	1.034	0.050
Baseline weight	1.027	0.112	1.004	0.743	1.015	0.321	1.011	0.485	1.007	0.407	0.974	**0.011**	0.998	0.850	1.015	0.227
Weight	0.963	**<0.001**	0.947	**<0.001**	0.960	**<0.001**	1.064	0.157	0.966	**<0.001**	0.927	**<0.001**	0.962	**<0.001**	1.030	0.417
BMI	0.862	**<0.001**	0.809	**<0.001**	0.856	**<0.001**	1.024	0.256	0.883	**<0.001**	0.833	**<0.001**	0.884	**<0.001**	0.985	0.316
<30 kg/m2	ref		ref		ref		ref		ref		ref		ref		ref	
30-35 kg/m2	0.160	0.100	0.091	0.044	0.617	0.248	0.589	0.051	0.294	**0.016**	0.203	**<0.001**	0.538	**0.037**	0.371	**0.048**
35-40 kg/m2	0.100	**0.046**	0.045	**0.012**	0.342	**0.014**	0.833	0.521	0.113	**<0.001**	0.071	**<0.001**	0.240	**<0.001**	0.356	**0.043**
40-50 kg/m2	0.069	**0.026**	0.017	**0.001**	0.190	**0.001**	0.777	0.446	0.076	**<0.001**	0.032	**<0.001**	0.149	**<0.001**	0.256	**0.009**
>50 kg/m2	0.024	**0.017**	0.027	**0.024**	-	-	1.741	0.528	0.074	**<0.001**	0.043	**<0.001**	0.120	**<0.001**	0.331	**0.039**
%TBWL	1.097	<0.001	1.112	<0.001	1.080	<0.001	0.989	0.239	1.084	**<0.001**	1.014	**<0.001**	1.071	**<0.001**	1.013	0.074
<2.5%	ref		ref		ref		ref		ref		ref		ref		ref	
2.5-5%	1.140	0.765	0.531	0.121	0.823	0.596	1.271	0.453	1.300	0.284	1.073	0.808	0.956	0.836	1.362	0.215
5-7.5%	0.934	0.961	0.935	0.832	1.224	0.483	0.901	0.680	1.404	0.099	1.112	0.673	1.249	0.230	1.631	0.023
7.5-10%	1.892	0.070	1.657	0.095	2.496	**0.001**	1.010	0.968	1.638	**0.012**	1.493	0.083	1.484	**0.025**	1.463	0.060
10-12.5%	2.222	**0.021**	1.855	**0.034**	2.854	**<0.001**	1.015	0.950	2.367	**<0.001**	1.668	**0.032**	2.035	**<0.001**	1.334	0.173
12.5-15%	3.894	**0.003**	3.879	**<0.001**	3.135	**<0.001**	0.878	0.606	3.099	**<0.001**	3.269	**<0.001**	2.142	**<0.001**	1.216	0.348
15-17.5%	2.852	**0.032**	5.607	**<0.001**	3.292	**0.001**	0.741	0.308	3.331	**<0.001**	3.363	**<0.001**	2.305	**<0.001**	1.443	0.103
17.5-20%	5.670	**0.004**	3.281	**0.003**	2.759	**0.004**	0.916	0.756	3.547	**<0.001**	4.217	**<0.001**	2.487	**<0.001**	1.202	0.416
20-22.5%	7.045	**0.007**	5.150	**0.001**	3.515	**0.002**	0.659	0.183	6.612	**<0.001**	4.351	**<0.001**	2.681	**<0.001**	1.435	0.192
22.5-25%	-		14.252	**0.005**	7.991	**0.001**	0.738	0.430	8.353	**<0.001**	13.068	**<0.001**	7.173	**<0.001**	1.241	0.484
>25%	-		-		-		1.017	0.961	18.15	**<0.001**	30.396	**<0.001**	20.029	**<0.001**	1.927	0.011
Impaired fasting glucose	1.270	0.738	2.572	0.111	1.181	0.789	0.373	0.114	2.579	**0.026**	1.653	0.179	1.483	0.283	1.364	0.478
Type II diabetes mellitus	0.630	0.457	0.862	0.758	1.899	0.247	3.691	**0.027**	0.593	0.108	0.827	0.610	1.842	0.080	2.116	0.087
Dyslipidaemia	0.182	0.303	1.016	0.983	1.760	0.484	2.050	0.444	0.490	0.088	1.074	0.842	0.831	0.616	0.663	0.331
Medication: Insulin	1.549	0.638	0.534	0.317	1.561	0.580	2.194	0.338	0.866	0.766	0.277	**0.035**	2.088	0.187	5.579	**0.012**
Medication: Lipid lowering	1.101	0.865	0.961	0.931	2.772	**0.037**	3.819	0.014	0.952	0.877	0.797	0.527	2.275	**0.013**	10.30	**<0.001**
Albumin	0.966	0.497	1.101	**0.026**	0.993	0.860	0.971	0.346	1.005	0.866	1.140	**<0.001**	1.024	0.316	0.897	**<0.001**
HbA1c	0.602	**0.002**	0.502	**<0.001**	0.607	**0.001**	1.187	0.172	0.642	**<0.001**	0.621	**0.003**	0.751	**0.003**	1.155	0.203
Fasting glucose	0.843	**0.014**	0.900	0.102	0.836	**0.003**	0.946	0.273	0.831	**<0.001**	0.757	**<0.001**	0.858	**<0.001**	0.948	0.342
Fasting insulin	0.972	**0.007**	0.990	0.330	0.972	**0.002**	0.979	**0.006**	0.969	**<0.001**	0.965	**<0.001**	0.976	**0.002**	1.007	0.321
HOMA2-IR	0.760	**0.001**	0.927	0.328	0.759	**<0.001**	0.866	**0.016**	0.748	**<0.001**	0.749	**<0.001**	0.814	**0.001**	1.031	0.571
C-peptide	0.484	**0.007**	0.801	0.375	0.507	**0.003**	0.565	**0.004**	0.434	**<0.001**	0.434	**<0.001**	0.488	**<0.001**	1.082	0.657
Uric Acid	0.011	**0.029**	0.112	0.207	0.005	**0.002**	0.118	0.117	0.032	**0.002**	0.003	**<0.001**	0.002	**<0.001**	0.012	**0.001**
hsCRP	1.011	0.654	1.005	0.805	0.972	0.083	1.007	0.631	0.994	0.492	0.966	**0.001**	0.965	**0.001**	0.992	0.292

HDL – high density lipoprotein; Trig – triglycerides; TC:HDL – total cholesterol to HDL ratio; BMI – Body mass index; %TBWL – percentage total body weight loss; HbA1c – haemoglobin A1c; BSL – blood sugar level; HOMA2-IR – homeostatic model assessment index 2 insulin resistance; hsCRP – high sensitivity C-reactive protein.

Table 14.4: Multivariate analysis of variables associated with resolution of high density lipoprotein (HDL), triglyceride level and total cholesterol to HDL ratio (TC:HDL), in males and females.

	Males			Females		
	Variable	**OR**	**p-value**	**Variable**	**OR**	**p-value**
Triglycerides	%TBWL	1.097	<0.001	%TBWL	1.067	<0.001
				HOMA2-IR	0.824	0.006
HDL	%TBWL	1.061	0.004	%TBWL	1.088	<0.001
	Age	1.100	0.011	Uric acid	0.008	<0.001
	HbA1c	0.625	0.013	HOMA2-IR	0.721	0.001
				Albumin	1.141	0.002
				Age	1.042	0.007
TC:HDL	%TBWL	1.077	<0.001	%TBWL	1.060	<0.001
	Age	1.074	0.027	Age	1.054	0.002
	Uric acid	0.020	0.030	Uric acid	0.027	0.013
LDL	Cholesterol medication	4.062	0.012	Cholesterol medication	11.12	<0.001
	HOMA2-IR	0.840	0.014	%TBWL	1.019	0.035
				Albumin	0.878	0.002

OR – odds ratio; HDL – high density lipoprotein; %TBWL – percentage total body weight loss; HbA1c – haemoglobin A1c; HOMA2-IR – homeostatic model assessment index 2 insulin resistance; TC:HDL – total cholesterol to HDL ratio.

*Figure 14.1: Changes in lipid levels per percentage total body weight loss (%TBWL) interval and body mass index (BMI) category, divided into those with dyslipidaemia at baseline, and those without (males). Shaded area indicates abnormal levels. *significant odds of resolution from baseline, based on generalised estimating equation (GEE) analysis.*

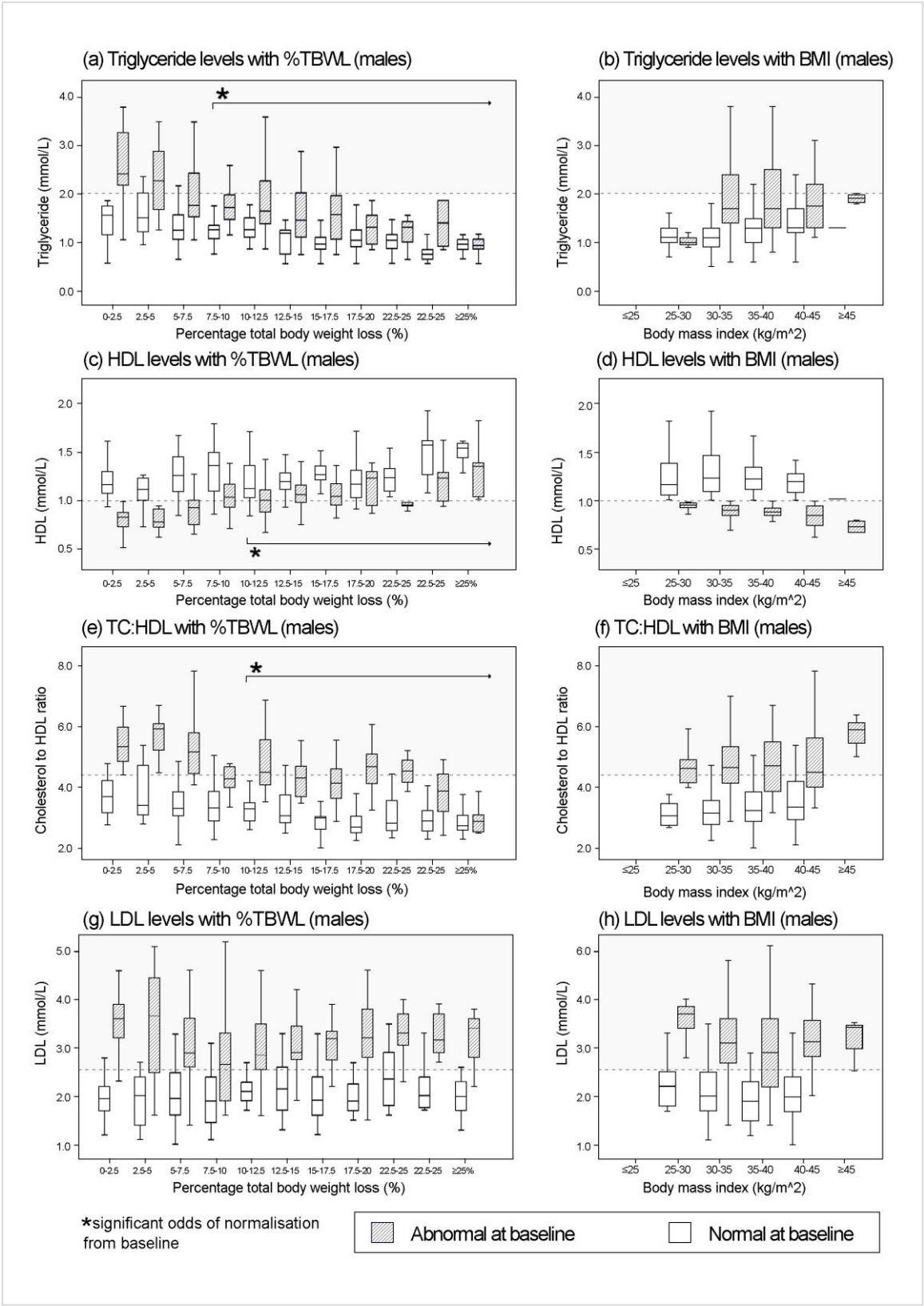
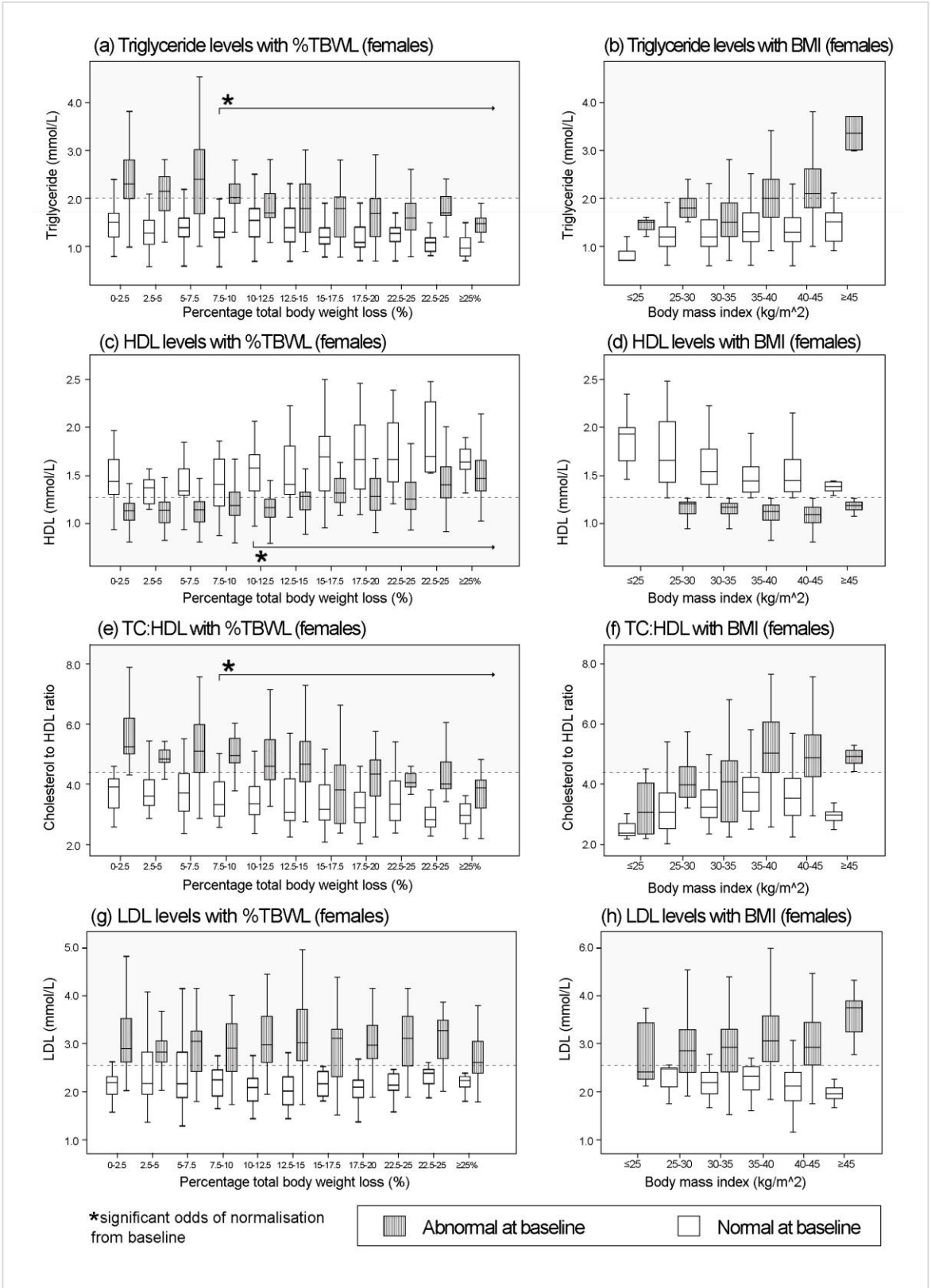


*Figure 14.2: Changes in lipid levels per percentage total body weight loss (%TBWL) interval and body mass index (BMI) category, divided into those with dyslipidaemia at baseline, and those without (females). Shaded area indicates abnormal levels. *significant odds of resolution from baseline, based on generalised estimating equation (GEE) analysis.*



14.5 Discussion

In this study, we have closely detailed progressive changes in serum lipid components with incremental weight loss, in order to define weight loss goals for meaningful improvements. We found that progressive weight loss after laparoscopic adjustable gastric banding (LAGB) steadily improved triglyceride levels, HDL and TC:HDL ratio, but did not have significant effects on LDL levels. A threshold total body weight loss of 7.5-12.5% conferred significant benefits for triglyceride levels, HDL levels and TC:HDL. This threshold represents a lower target than what is usually defined as 'success' after bariatric surgery, and is a far more achievable goal for non-operative weight loss methods. It is consistent with mechanisms previously investigated, which show significant changes in triglyceride levels and modulation of biological lipid pathways after 5% TBWL (18). We found that further progressive improvements occurred with increasing weight loss, with substantially increased chances of achieving a normal cholesterol profile after >25% TBWL (OR 18.2-30.4).

In this cohort, there was little change in measured LDL levels, despite marked weight loss up to 25% TBWL and significant concurrent improvement in insulin resistance and other lipid parameters. Low density lipoprotein (LDL) is an important measure for cardiovascular risk, and remains the recommended primary target of therapy due to its atherogenic tendencies and substantial link with adverse cardiovascular events (25, 609). The failure to achieve reduction in LDL brings into question the therapeutic effects of caloric restriction weight loss alone on LDL levels. This lack of LDL response has been demonstrated by other studies using lifestyle changes or purely restrictive bariatric surgery, which have shown reductions in triglycerides and HDL, but less effect on LDL cholesterol (437, 610-612). However, other bariatric procedures utilizing intestinal diversion, such as Roux-en-Y gastric bypass, has shown efficacy in decreasing total LDL levels at equivalent weight loss thresholds (426, 435, 606). This suggests that more invasive procedures affect LDL cholesterol via mechanisms independent of weight loss alone.

Another potential explanation for failure of LDL response may be due to the inability of standard laboratory measures to reflect beneficial changes in LDL profile. Small dense LDL (sdLDL) subtype is known to have greater atherogenic potential (613), and may be more closely associated with coronary heart disease endpoints (614-616). Krauss *et al* has previously shown that the small dense LDL fraction and LDL peak diameter decreased with weight loss and dietary change (617), showing that when the *quality* of LDL particles are

measured, significant changes can be observed. Zambon *et al* similarly showed improvements in the atherogenic LDL profile with weight loss after LAGB (618). Furthermore, studies have suggested that sdLDL levels are inversely related to other LDL subfractions (619), which may explain the somewhat stable nature of overall LDL levels, despite theoretical benefits. Further study would be required to interrogate the exact nature of change in sdLDL with weight loss.

We found that changes in these lipid levels were independently associated with measures of insulin resistance, including fasting glucose, insulin, and HOMA2-IR. It is well established that insulin resistance causes alterations in lipid metabolism leading to elevated triglycerides, reduced HDL cholesterol and increased low density lipoprotein (LDL) cholesterol (620). This is often referred to as 'atherogenic dyslipidaemia' due to the associated risk of cardiovascular disease (621). Improved insulin sensitivity in adipose tissue, muscle and liver are known to occur with early weight loss, in the vicinity of 5-10% (18). This early restoration of insulin sensitivity has a plethora of effects on lipid metabolism, including decreased adipose tissue lipolysis, hepatic lipoprotein synthesis and export, and improved lipid clearance (621), ultimately resulting in reversal of mechanisms driving dyslipidaemia.

In this study, we focused on four important clinical lipid measures – triglyceride levels, high density lipoprotein (HDL) cholesterol, total cholesterol to HDL ratio (TC:HDL), and low density lipoprotein (LDL) cholesterol. All are currently used widely for an overall assessment of cardiovascular risk (622). LDL is the most extensively studied lipoprotein. Despite some controversies regarding its measurement and interpretation, it has been strongly associated with risk of cardiovascular disease (623). Low levels of HDL cholesterol have been linked to increased coronary events, due to its cardioprotective effects, role in reverse cholesterol transport, effect on endothelial vascular cells and antioxidant characteristics (624). Triglycerides contribute to the metabolic pathway of atherogenic lipoproteins, reflects insulin resistance and is an important biomarker of cardiovascular disease (CVD) risk (622). Finally, TC:HDL ratio is a reliable and easy-to-calculate marker of atherogenic particle burden including LDL particle concentration (625, 626). Importantly, improvements in all these lipid parameters are associated with improved metabolic and cardiovascular health, and are often used to monitor treatment and risk reduction (609, 627, 628).

Overall, these data suggest that 7.5-12.5% TBWL is the threshold weight loss for meaningful benefits in HDL, triglyceride levels and TC:HDL in patients with obesity. As further weight

is lost, this beneficial effect on lipid profile significantly increases in a dose-dependent manner. However, LDL levels shows little to no improvement with pure caloric restriction weight loss. Further work is required to elicit the mechanisms and implications of these findings. Healthcare providers should be wary of ceasing lipid lowering medications, even in the setting of significant weight loss, as LDL levels appear to remain elevated.

This study differs significantly from previous studies. Firstly, by taking multiple repeated lipid levels over two years following gastric banding, we have been able to closely monitor the effects of incremental and significant weight loss on lipid parameters. This has given us substantially greater detail regarding the relationship of dyslipidaemia to weight loss and importantly, has allowed us to define weight loss targets for meaningful lipid improvement.

Secondly, the use of LAGB as the mechanism of weight loss in this study allows observation of changes in metabolic and lipid parameters without significant changes in gut anatomy or gastrointestinal hormones, which can affect lipid absorption and metabolism (629). The LAGB achieves weight loss by inducing satiety and reducing caloric intake (195), thereby modelling the effects of weight loss achieved through lifestyle change. Study of weight loss through caloric reduction is pertinent, as this is the most accessible, and often only, means of weight loss for many individuals with obesity.

There are some limitations that warrant discussion. Firstly, cholesterol-lowering medication was used by a third of the study cohort, with cessation of treatment in four participants over the study period. Decisions to cease medication were based on participants' treating clinicians, independent of study protocol. Low rates of medication cessation, despite good responses in lipid variables, may be due to guideline recommendations for their use in cardiovascular risk reduction, particularly in those with other cardiometabolic risk factors (630). The number of participants was too low to perform a meaningful subgroup analysis for this medication use or change. However, the effect of cessation of cholesterol-lowering medication will likely increase or plateau lipid levels in those individuals and hence, at worst, there will be an underestimation of weight loss effect on lipid levels. Additionally, our data was not significantly altered when these four subjects were removed from the analysis.

Secondly, changes in lifestyle choices, such as exercise and dietary changes, have not been quantified in this cohort. As this was not the primary focus of this study, we have not

measure alterations in lifestyle and behaviour. However, these changes often accompany bariatric surgery, and multiple studies have shown beneficial effects of lifestyle interventions on insulin sensitivity as well as lipid profile (18, 166, 631). A consideration for future bariatric surgical studies is the incorporation of measures of lifestyle change, to identify factors that independently or synergistically result in benefit.

Thirdly, the relatively small study size may affect the ability to draw robust conclusions. This is particularly as we have necessarily divided the cohort into gender, and baseline dyslipidaemia subgroups. However, we have been able to perform multiple repeated outcomes measures on this cohort to conduct an in-depth and detailed analysis of changes with weight loss. Additionally, we were still able to find significant differences despite our relatively low numbers.

Finally, a potential source of bias could occur due to loss to follow-up and withdrawals, due to patients being unable or unwilling to continue with intense follow-up requirements, particularly after LAGB explant. Additionally, patients who became pregnant were withdrawn, due to inherent physiological changes in biochemical markers. Regardless of cause for withdrawal, these losses may introduce bias to the data. This is partially mitigated by our focus on weight loss, rather than time, as the primary scale for analysis of lipid change.

In conclusion, this study has demonstrated the meaningful benefit of incremental weight loss on important lipid measures in obesity. Steady and significant improvements in triglycerides, HDL and TC:HDL were seen after achieving a moderate weight loss target of 7.5-12.5% TBWL, and was closely associated with improvement in insulin resistance measures. This magnitude of weight loss should be the initial therapeutic target in patients with dyslipidaemia. Furthermore, in those with persistent dyslipidaemia, additional weight loss is likely to result in further improvements in lipid profile. Notably, LDL levels were not substantially improved with weight loss after LAGB. Further study should investigate mechanisms behind persistence of LDL abnormalities after caloric restriction weight loss, and the change in atherogenic LDL profile.

Research Theme 4

Developing an understanding of pathophysiological drivers of NAFLD in obesity

Study 10

Nonalcoholic fatty liver disease (NAFLD) is defined by the abnormal accumulation of fat within the liver (3). Ectopic fat deposition and lipotoxicity has been theorised to contribute to the pathogenesis and progression of NAFLD. Numerous animal and experimental models show that specific lipid species in NAFLD have a substantial role in inflammation (20). Describing the relationship of lipid species with NAFLD in larger human studies is needed to verify these findings.

Establishing the role of lipids in NAFLD is important for several reasons. If shown to be a central player in disease progression, therapeutics may be developed to decrease levels of target lipid species, and thereby ameliorate accumulation of pathogenic lipids. Secondly, lipid accumulation in the liver may be represented in blood, and thus establishing the relationship of serum lipids to liver disease may assist in development of much needed biomarkers of NAFLD. This is particularly important, as there are substantial weaknesses in current diagnostic tests, demonstrating an ongoing need for exploration of novel techniques targeted at obese individuals.

This last chapter aimed to explore the role of lipids in the pathogenesis of NAFLD, and its potential as a blood biomarker.

15 Lipidomic analysis of nonalcoholic fatty liver disease in obesity: Alterations in liver lipid profile and parallel serum changes with progressive disease

15.1 Abstract

BACKGROUND: Energy excess in states of obesity can lead to ectopic lipid accumulation in the liver, resulting in nonalcoholic fatty liver disease (NAFLD). Lipid accumulation can disrupt normal lipid pathways and homeostasis. Certain lipid subtypes are known to be pathogenic, and their accumulation are strongly linked with progressive metabolic and liver disease.

AIMS: To characterise the liver, adipose and plasma lipid profile of progressive NAFLD in obesity. To evaluate the utility of plasma lipids as specific identifiers of liver disease in the setting of obesity.

METHODS: We recruited 181 obese patients undergoing bariatric surgery. Blood, liver, visceral (VAT) and subcutaneous adipose tissue (SAT) biopsies were obtained. Liver histology was examined to determine NAFLD severity. Lipidomic analysis was performed on all specimens. Correlation between liver, VAT, SAT and serum lipidome and liver disease was performed.

RESULTS: Increasing hepatic steatosis was associated with substantial changes in liver lipid composition. Significant increases were seen in sphingolipids, including ceramide (2.08-3.18% increase in lipid per unit steatosis, $p<0.001$), dihydroceramide (1.31-2.01%, $p<0.001$), hexosyl-ceramide and GM3 ganglioside species. There were significant alterations in multiple phospholipids. Plasma lipidome, particularly dihydroceramide (1.17-1.90%, $p<0.025$) and ceramide species (0.73-0.98%, $p<0.05$), was significantly related to hepatic steatosis. Notably, these plasma lipid species showed strong correlations with liver lipid species (dihydroceramide, r 0.508-0.663, $p<0.001$). VAT and SAT lipid levels were unrelated to changes in liver, and did not correlate to plasma levels. We did not find any lipid species significantly related to presence of NASH, independent of steatosis severity.

CONCLUSIONS: Worsening steatosis in NAFLD is associated with a range of changes in the liver lipidome, including increases in liver ceramide and dihydroceramide species. This is paralleled by similar lipidomic changes in plasma. These changes are unique to liver and plasma, and are not significantly influenced by adipose tissue in the setting of obesity. These findings indicate the potential for plasma lipidome to be used as a non-invasive marker of nonalcoholic fatty liver disease.

15.2 Introduction

Nonalcoholic fatty liver disease (NAFLD) is endemic in morbid obesity, affecting 84-96% of obese individuals (278). It is characterised by hepatocyte accumulation of triglycerides, and can progress to nonalcoholic steatohepatitis (NASH) and fibrosis, which respectively affects 25-55% and 34-47% of those with obesity (8). The significance of NAFLD is its potential to progress to liver cirrhosis, hepatocellular carcinoma (HCC) and end stage liver failure (632). Given the already large prevalence of NAFLD, increasing rates of obesity and associated metabolic disease, the clinical burden of NAFLD is projected to become enormous (232).

Hepatic steatosis occurs due to an imbalance between fatty acid uptake and production, and fatty acid oxidation and lipid export. Free fatty acids (FFAs) are primarily derived from dietary lipid sources and tissue adipose lipolysis (354), which is exacerbated by insulin resistance. *De novo* hepatic lipogenesis, upregulated by hyperinsulinaemic insulin-resistant states, further contributes to fatty acid production and is most prominent in the liver.(6) Whilst the majority of free fatty acids are stored in triglycerides (TG) within lipid droplets, an imbalance between production and disposal of fatty acids or upregulation of specific lipid biosynthetic pathways can result in the accumulation of a wide variety of lipid species that can impact cell functions (633).

Lipotoxicity is the term used to describe cellular dysfunction and injury mediated by the accumulation of excess free fatty acids and lipid intermediates in non-adipose tissues (634). Emerging evidence points to lipotoxicity as a significant pathogenic mechanism in NAFLD. Several human studies have shown substantial perturbations in the lipid profile (lipidome) of liver with increasing steatosis (386), including variations in glycerophospholipids (390), sphingolipids (389, 391), and fatty acids (388, 635). Several of these lipid intermediates have been implicated in the development of NASH (390, 391, 395). Most prominently, sphingolipids, and particularly ceramide species, have been linked to NAFLD and metabolic disease in both human and animal studies (636).

Obesity is characterised by an overwhelming excess in adiposity, however, the relationship between the adipose tissue and liver lipidome has not been well described. It has previously been established that visceral adipose tissue, particularly in morbid obesity, is associated with severity of NAFLD via inflammatory and immune cell changes (493, 637). On the other hand, subcutaneous adipose tissue contributes ~70% of the free fatty acids in the circulation

(638), indicating that rather than visceral adipose tissue, subcutaneous adipose tissue might instead impact the liver lipidome in obesity by supplying excess FFA substrate. Moreover, recent studies in transgenic mice overexpressing acid ceramidase in liver or adipose tissue, provide evidence that sphingolipids are shunted between the liver and adipose tissue (639), indicating that the accumulation of lipid species, such as ceramides, are regulated by complex intracellular mechanisms and previously unappreciated inter-organ cross-talk. A detailed lipidomic examination of adipose tissue stored in various anatomical locations could provide insight into their impact on liver disease in humans, and their effect on the plasma lipidome.

Lipidomic analysis of unique changes associated with NAFLD could also address current difficulties in diagnosis. Liver biopsy remains the most reliable approach for identification of NAFLD, NASH and fibrosis (3). The plasma lipidome may provide insight into the liver lipidomic changes and non-invasively enable stratification of liver disease. However, the relationship between liver, adipose tissue and plasma has not been well described in humans. This avenue of investigation might be especially important in obesity where, due to differences in clinical, biochemical, and physical characteristics, there are ongoing challenges in developing non-invasive tests (311, 503).

The overarching aim of this study was to characterise the lipid profile associated with increasing severity of NAFLD and the presence of NASH. We specifically aimed to explore the changes in the liver, visceral adipose tissue (VAT), subcutaneous adipose tissue (SAT) and plasma lipidomes, to identify lipids that may be associated with liver disease in each depot. Using this information, we explored the relationships between the plasma lipid profile and the liver and adipose tissue lipidomes in NAFLD to investigate the utility of specific plasma lipid species as an indication of liver disease.

15.3 Methods

All participants provided informed consent to participate in this study. Ethics approval was obtained from Alfred (no. 195/15), Avenue (no. 190) and Cabrini (no. 09-31-08-15) Human Research Ethics Committee. This study was registered with the Australian Clinical Trials Register (ACTRN12615000875505).

15.3.1 **Patients**

We prospectively enrolled consecutive eligible severely and morbidly obese patients undergoing bariatric surgery in three metropolitan hospitals in Melbourne, Australia, between July 2015 and November 2016.

Inclusion criteria included: (1) age ≥18 years, (2) BMI≥35 kg/m^2, (3) alanine aminotransferase (ALT) or aspartate aminotransferase (AST) >0.5 times the upper limit normal (ULN) (equivalent to ALT 19IU/L for women and 21IU/L for men(229)), or gamma-glutamyl transferase (GGT)>ULN. Patients were excluded if they had evidence of other liver disease, including viral hepatitis, medication-related, autoimmune, familial/genetic causes or a history of excessive alcohol use, as defined by the American Association for the Study of Liver Diseases (3).

15.3.2 **Outcomes**

15.3.2.1 Clinical and biochemical data

Patients underwent a complete medical history and physical examination on the day of operation. Metabolic comorbidities were noted, including the presence of hypertension (>140/90mmHg or on antihypertensive medication), diabetes (previously diagnosed by oral glucose tolerance testing, or on antidiabetic medication) and hypercholesterolaemia (fasting total cholesterol ≥4.0mmol/L, high density lipoprotein (HDL) <1.0mmol/L, low density lipoprotein (LDL) ≥2.0mmol/L, triglyceride ≥2.0mmol/L, or on lipid lowering medication) (25).

Fasting venous blood samples were taken prior to induction of anaesthesia, in patients fasted for 8-12 hours. Blood was mixed in a 10ml K_2EDTA tube, spun for 10 minutes at 4000 rpm (~2000 RCF), with plasma collected and stored at -80ºC before analysis. Plasma was

assessed for liver function tests (alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma glutamyltransferase (GGT) and alkaline phosphatase (ALP)), cholesterol profile, glucose, insulin, glycosylated haemoglobin (HbA1c), C-peptide, full blood examination, electrolytes and screening blood tests for liver disease.

Full details on biochemical analysis are found in **Appendix 9: Supplementary Materials**.

15.3.2.2 Bariatric surgery and intraoperative biopsy

Intraoperative wedge liver biopsies, at least 1cm in depth, were taken. A section was frozen in dry ice and stored at -80ºC for lipidomic analysis, and a section formalin fixed for histological assessment. A single pathologist graded the biopsies in a blinded manner, according to the NAFLD activity score (NAS) (481) and Kleiner classification of liver fibrosis.(240) As the NAS is a heterogenous classification of various degrees of steatosis and inflammation, this classification was used for documentation, but final definition of NASH was provided by the pathologist (481).

Histological outcomes used for analysis

Steatosis severity by image analysis: Image analysis with Fiji ImageJ (Madison, WI, USA) (482) was used to objectively quantify liver steatosis. The area of lipid vacuolisation was measured across five representative images from each patient specimen and averaged. This was represented as a continuous variable, being percentage area of liver affected by steatosis.

Pathologist-defined NAFLD: Patients were partitioned into the following groups based on pathologist defined features of steatosis, inflammation, ballooning and fibrosis: "*Normal"* - no significant steatosis or abnormality; "*Nonalcoholic fatty liver (NAFL)"* - any degree of steatosis, without inflammation or with minor inflammation only (one point of inflammation/ballooning); "*Nonalcoholic steatohepatitis (NASH)"* - defined as NASH according to the NAS grading system, presence of significant inflammation (≥2 points of inflammation/ ballooning), or presence of fibrosis.

Visceral adipose tissue (VAT) and abdominal subcutaneous adipose tissue (SAT) were collected during the operation, frozen in dry ice and stored at -80ºC for subsequent lipidomic analysis.

15.3.3 **Lipidomic analyses**

Full details of tissue and lipidomic analysis of samples are found in the **Appendix 9: Supplementary Materials**.

Liver, VAT and SAT samples were homogenised in ice-cold phosphate buffered saline (PBS). Lipids were extracted from tissue and plasma samples using a single phase chloroform/methanol extraction process, incorporating an internal standard mix, as previously described (640).

Lipid analysis was performed by liquid chromatography, electrospray ionization-tandem mass spectrometry using an Agilent 1200 liquid chromatography system combined with an Applied Biosystems API 4000 Q/TRAP mass spectrometer with a turbo-ionspray source (350^{o}C and Analyst 1.5 data system, as previously described (640). Lipid species of the following classes were measured: dihydroceramide (dhCer), Ceramide (Cer), monohexosylceramide (Hex1Cer, MHC), dihexosylceramide (Hex2Cer, DHC), trihexosylceramide (Hex3Cer, THC), GM3 ganglioside (GM3), sphingomyelin (SM), phosphatidylcholine (PC), alkylphosphatidylcholine (PC-O), alkenylphosphatidylcholine (plasmalogen, PC-P), lysophosphatidylcholine (LPC), lysoalkylphosphatidylcholine (LPC-O), lysoalkenylphosphatidylcholine (LPC-P), phosphatidylethanolamine (PE), alkylphosphatidylethanolamine (PE-O), alkenylphosphatidylethanolamine (plasmalogen, PEP), lysophosphatidylethanolamine (LPE), phosphatidylinositol (PI), phosphatidylserine (PS), phosphatidylglycerol (PG), cholesterol ester (CE), free cholesterol (COH), diacylglycerol (DG) and triacylglycerol (TG). Lipid concentrations were calculated by relating the peak area of each species to the peak area of the corresponding stable isotope or non-physiological internal standard. The lack of availability of suitable stable isotope internal standards for every individual species requires the use of representative standards for each lipid class and precludes the creation of calibration curves for each lipid species. This consideration should be taken into account when interpreting the data. Whilst the comparison of lipid species between individuals will provide good estimates of differences in lipid abundance (i.e., high assay precision), exact quantification and subsequent distribution of lipids within a class should be recognized as approximations only (640).

15.3.4 **Statistical analysis**

Continuous variables were expressed as mean ± standard deviation (SD) for parametric data, and median and interquartile range (IQR) for nonparametric data. Normality was assessed by Shapiro-Wilks test. Independent student t-test was used for parametric data, and Mann Whitney U-test for non-parametric data. Categorical variables were expressed as numbers (with percentages). Pearson's chi-squared or Fisher's exact test were used for independent categorical variables. A p-value ≤ 0.05 was considered statistically significant.

Logarithmic transformation of lipidomic data was conducted prior to analysis. Linear and logistic regression analyses were performed on categorical and continuous outcomes, respectively. Covariates used included age, gender, body mass index (BMI) and homeostatic model of assessment 2 insulin resistance (HOMA2-IR). Results are presented as IQR odds ratios (OR) with 95% confidence intervals (CI) for logistic regression, and beta coefficients with 95% CI for linear regressions. P-values are corrected for multiple comparisons (Benjamini-Hochberg method), with $p<0.05$ considered as significant.

Correlations of lipids between depots were conducted, using Pearson correlation coefficient to determine significant correlation.

Data analysis was performed in SPSS v23 (SPSS Inc., Chicago, IL, USA) , MATLAB (The MathWorks Inc., Natick, MA, USA), and Prism 7 (GraphPad Software Inc., La Jolla, CA, USA).

15.4 Results

15.4.1 **Patients**

We recruited 181 severely and morbidly obese bariatric surgical patients (**Table 15.1**). The average age was 45±12 years and average BMI was 45.1±8.3 kg/m^2 with a female predominance (75.7%). There was a high prevalence of dyslipidaemia (n=160, 88.4%), hypertension (n=82, 45.6%) and type 2 diabetes mellitus (n=40, 22.2%).

Liver histology revealed that 128 participants (71.7%) had some degree of NAFLD. Using our groupings for lipidomic analysis, 53 participants had *normal* liver (29.3%), 68 had *non-NASH NAFL* (37.6%) and 60 participants had *NASH* (33.1%). **Supplementary Table 15.1** shows a comparison of these groups.

15.4.2 **Liver lipidomic profile**

15.4.2.1 Steatosis

Increasing steatosis was associated with significant changes in the liver lipidome. **Figure 15.1a** summarises the changes in all measured lipids with increasing liver steatosis. Liver steatosis has been measured objectively by image analysis, as percentage area of the histological slide with lipid vacuolisation. Significant changes in total lipid classes and subclasses measured are shown in **Table 15.2**.

As expected, significantly increased triacylglycerol (TG) and diacylglycerol (DG) levels were seen globally for all TG species (3.9-12.7% increase in lipid, and 7.8% increase in total TG per unit increase in steatosis, $p<0.001$), and almost all DG species (5.11% increase in total DG, $p<0.001$). Increases in cholesterol and variable changes in cholesterol esters were seen with increasing steatosis (**Supplementary Table 15.2a**).

Worsening steatosis was associated with increases in most sphingolipids (**Figure 15.1a** and **Supplementary Table 15.2b**). Total dihydroceramides (total dhCer, 2.53% increase per unit steatosis, $p<0.001$), total ceramides (total Cer, 1.23%, $p<0.001$), and total trihexosylceramide (total THC, 1.09%, $p=0.015$) species were increased significantly. Increases were seen in the majority of sphingolipid species, with all six dhCer, five of six Cer, two of six DHC, three of five THC and four of six GM3 ganglioside (GM3) species. Increases in total dhCer and

Cer(d18:0/22:0 are demonstrated in **Figure 15.1b**, showing statistically significant increases in ceramide species from minimal steatosis to significant steatosis.

Phospholipid levels changed variably with liver steatosis (**Figure 15.1a** and **Supplementary Table 15.2c**). Significant decreases were seen in the majority of phosphatidylcholines (PC) (-1.00% to -2.26% decrease per unit increase in steatosis), phosphatidylethanolamine (PE) and phosphatidylinositol (PI) species with increasing steatosis. Conversely, significant increases were seen in other phospholipid species, including lysophosphatidylcholines (LPC), lysoalkylphosphatidylcholine (LPC(O)), PE, lysophosphatidylethanolamine (LPE), PI, lysophosphatidylinositol (LPI) and phosphatidylglycerol (PG) species. In particular, PI and LPI were some of the top species with the most significant and substantial changes, ranging from 1.23% to 3.08% increases with every unit increase in percentage steatosis (**Figure 15.1b**).

Overall, these data show substantial and systematic change in the liver lipidome with increasing hepatic steatosis severity.

15.4.2.2 Nonalcoholic steatohepatitis

We divided the study cohort according to the presence of steatosis alone (non-NASH NAFLD or "NAFL") and those with signs of inflammation or fibrosis ("NASH"). Histological NASH compared to normal was associated with substantial changes in lipid species (**Figure 15.2a**). These changes were similar to those seen with increases in steatosis severity, as well as to those changes seen between normal and NAFL (**Supplementary Table 15.3**). The most significant and substantial differences were seen in the sphingolipid and ceramide species (**Figure 15.2b**).

To identify changes uniquely associated with inflammation, we compared NAFL to NASH. This comparison showed that there were no significant differences in lipid levels between NAFL and NASH (i.e. no inflammation vs inflammation) (**Figure 15.2a** – left panel).

15.4.3 **Correlation of the adipose tissue lipidome with NAFLD**

15.4.3.1 Associations of visceral and subcutaneous adipose tissue lipidome with steatosis

Significant differences were seen in phospholipid species in visceral adipose tissue (VAT), with increasing liver steatosis (**Figure 15.3**). Significant increases were seen in PC(P), and a single PE(P) species. The remaining lipids were significantly decreased, including three PC, one LPC, five PE, one PI and two LPI species. Notably, the changes in VAT lipid species were markedly different compared to the liver lipidome, with no similarities in any of significantly altered lipids.

There were no significant associations between subcutaneous adipose tissue (SAT) lipids and liver steatosis (**Supplementary Figure 15.2b**).

15.4.3.2 Associations of VAT and SAT with inflammation

There were no significant differences in the VAT or SAT lipidome with histologically defined NASH compared to normal (data not shown).

Overall, the adipose tissue lipidome was not reflective of liver disease or the liver lipidomic profile.

15.4.4 **Plasma lipidomic profile**

15.4.4.1 Steatosis

Distinct changes in the plasma lipidome were observed with increasing liver steatosis severity. Many plasma sphingolipids and phospholipids mirrored the increases observed in the liver lipidome with increasing liver steatosis (**Figure 15.1a** and **Figure 15.1c**). This was evident for total dhCer, Cer, and LPI (**Table 15.2**). Several dhCer, Cer, PE, PI, and LPI species also showed concurrent significant changes in plasma levels compared to liver levels (**Supplementary Table 15.4**). In particular, 4 of 6 dhCer and 3 of 6 Cer showed significant increases in the serum as well as the liver lipidome with increasing hepatic steatosis severity. Similar to the liver lipidome, significant increases in DG (1.81% increase in total TG with unit increase in steatosis, $p<0.001$) and TG (1.30% increase in total TG, $p=0.001$) were seen, potentially reflective of dyslipidaemic state.

15.4.4.2 Nonalcoholic steatohepatitis

There were several changes in the plasma lipidome with the diagnosis of histological NASH compared to normal liver. These included significantly altered total dhCer (32.5% increase with NASH compared to normal, p=0.010), total LPI (21.3%, p=0.016), total PC(O) (-10.2%, p=0.032), total DG (44.9%, p=0.010), and total TG (29.4%, p=0.011). Importantly, increases in the plasma total dihydroceramides and several dihydroceramide species (Cer(d18:0/18:0), Cer(d18:0/22:0) and Cer(d18:0/24:1)) are seen, reflecting changes similar to the liver (**Figure 15.2c** and **Supplementary Table 15.5**).

No differences have been found between the plasma lipidome of those with NAFL and those with NASH, including DG and TG species, indicating that there are no lipids independently associated with histological inflammation (data not shown).

Collectively, these data show that the changes in the plasma lipidome occur with increasing liver steatosis severity, and many of these changes reflect changes that occur in the liver lipidome. No changes have been seen in the plasma lipidome with NASH, independent of steatosis.

15.4.5 **Correlation of plasma and tissue lipidome**

When correlating the plasma lipidome to each of the tissue lipidomes, the main finding was the significant and substantial correlation of plasma lipid levels with liver lipid levels (**Figure 15.4a and 15.4b**, **Supplementary Figure 15.1**). . There were 44 lipids in the plasma that showed strong (r >0.5) and significant (p<0.05) correlation to liver lipid levels. A further 77 showed moderately strong (r 0.3-0.5) correlation. In particular, total dhCer (r 0.360, p<0.001), dhCer (Cer(18:0/18:0) r 0.663, p<0.001) and phospholipid species (PC(32:1) r 0.854, p<0.001; PE(16:0/16:1) r 0.733, p<0.001; PI(16:0/16:0) r 0.808, p<0.001) showed substantial and significant correlation between liver and plasma (**Figure 15.4a and 15.4b**). Importantly, these serum lipid species were also significantly related to severity of histological liver steatosis (**Table 15.3**). dhCer and PI species showed substantial correlation between plasma and liver, as well as disease severity (r: 0.508-0.870) (**Figure 15.3** and **Supplementary Figure 15.1**).

This is in contrast to both the SAT and VAT lipidome, where there were few significant or substantial correlations (**Figure 15.4a** and **Supplementary Figure 15.1**). In the VAT

lipidome, there were four lipid species levels strongly (r >0.5) related to the serum lipid levels (SM(d18:0/14:0), SM(d18:2/14:0, PE(P-20:0/22:6), DG(16:0_22:6)). With these SM and PE species, the liver lipid levels were more strongly correlated to serum lipid levels than the VAT lipid levels (r 0.593 vs 0.527, r 0.714 vs 0.670, and r 0.583 vs 0.516, respectively). In the SAT lipidome, there was only one lipid that correlated strongly with the serum lipid levels (SM(d18:2/14:0)), with weaker correlation than liver lipids (r 0.714 vs 0.613).

15.4.6 **Utility of serum lipids as biomarkers of NAFLD**

As serum lipid levels are significantly related to both liver steatosis severity as well as liver lipid levels, we explored their potential utility as biomarkers of NAFLD.

For detection of steatosis, total dhCer, Cer(d18:0/22:0) and PI(16:0/16:0) have fair AUROC values of 0.670 (0.590-0.750, p<0.001), 0.680 (0.501-0.760, p<0.001) and 0.712 (0.636-0.788, p<0.001). An optimal threshold of 937pmol/ml for Cer(d18:0/22:0) has a sensitivity of 85.7% and specificity of 50.5%.

For detection of NASH, total dhCer and Cer(d18:0/24:1) have an AUROC of 0.630 (0.546-0.715, p=0.005) and 0.634 (0.549-0.720, p=0.004). An optimal threshold of 1663pmol/ml has a sensitivity of 60.7% and specificity of 66.1% for detection of NASH.

Table 15.1: Characteristics of cohort

Variable		n=181
Clinical variables		
Age		45 ± 12
Gender (males)		44 (24.3%)
Body mass index (BMI), kg/m2		45.1 ± 8.3
Weight		126.4 ± 28.6
Type 2 diabetes mellitus (T2DM)		40 (22.2%)
Dyslipidaemia		160 (88.4%)
Hypertension		82 (45.6%)
Biochemical variables		
Alanine aminotransferase (ALT), IU/L		33 (24-52)
Aspartate aminotransferase (AST), IU/L		27 (22-35)
Gamma glutamyl transferase (GGT), IU/L		32 (20-42)
Alkaline phosphatase (ALP), IU/L		72 ± 21
Bilirubin, mmol/L		10 ± 5
Total cholesterol (TC), mmol/L		4.1 ± 1.0
High density lipoprotein (HDL), mmol/L		1.0 ± 0.3
Low density lipoprotein (LDL), mmol/L		2.4 ± 0.8
Triglyceride levels (TG), mmol/L		1.5 ± 0.7
Blood sugar level (BSL), mmol/L		5.8 ± 1.9
HbA1c, %		5.7 (5.4-6.1)
Insulin, mU/L		7.1 (4.3-12.2)
C-peptide, pmol/L		773 (572-1081)
Histological variables		
Steatosis (image analysis), %area		4.87 (0.63-14.13)
Steatosis (histology)	S0 (<5%)	53 (29.3%)
	S1 (5-33%)	52 (28.7%)
	S2 (34-66%)	61 (33.7%)
	S3 (≥67%)	15 (8.3%)
Inflammation	0	107 (59.1%)
	1	67 (37.0%)
	2	7 (3.9%)
Ballooning	0	114 (63.0%)
	1	51 (28.2%)
	2	16 (8.8%)
NAFLD activity score (NAS) grade	Not NASH (≤2)	112 (61.9%)
	Equivocal (3-4)	47 (26.0%)
	NASH (≥5)	22 (12.2%)
Fibrosis	F0	138 (76.2%)
	F1	37 (20.4%)
	F2	3 (1.7%)
	F3	3 (1.7%)
	F4	-
Histological classification for analysis	Normal liver	53 (29.3%)
	Steatosis/non-NASH	68 (37.6%)
	Inflammation/NASH	60 (33.1%)

Data presented in mean ± standard deviation, median (interquartile range), or number (percentage %).

Table 15.2: Changes in total lipid species in liver and serum with increasing liver steatosis

Lipid class/subclass	Liver lipidome		Serum lipidome	
	Percentage change (95% CI) per unit increase in liver steatosis	**p-value**	**Percentage change (95% CI) per unit increase in liver steatosis**	**p-value**
Total dhCer	**2.53 (1.72 - 3.34)**	**<0.001**	**1.32 (0.62, 2.02)**	**0.002**
Total Cer	**1.23 (0.68 - 1.79)**	**<0.001**	**0.66 (0.15, 1.18)**	**0.049**
Total MHC	-0.09 (-0.64 - 0.47)	0.858	0.24 (-0.38, 0.87)	0.505
Total DHC	0.94 (-0.1 - 1.99)	0.122	-0.31 (-0.84, 0.22)	0.343
Total THC	**1.09 (0.34 - 1.86)**	**0.015**	-0.26 (-0.77, 0.26)	0.438
Total GM3	0.53 (-0.01 - 1.06)	0.108	0.3 (-0.18, 0.78)	0.335
Total SM	-0.26 (-0.61 - 0.09)	0.222	-0.07 (-0.43, 0.3)	0.726
Total PC(O)	0.34 (-0.14 - 0.82)	0.233	-0.36 (-0.72, -0.01)	0.098
Total PC(P)	-0.36 (-0.91 - 0.2)	0.278	-0.42 (-0.83, 0)	0.098
Total LPC	1.04 (-0.07 - 2.17)	0.115	0.18 (-0.24, 0.59)	0.477
Total LPC(O)	1.17 (0.1 - 2.25)	0.071	-0.51 (-0.97, -0.04)	0.089
Total LPC(P)	1.28 (-0.05 - 2.62)	0.109	-0.53 (-1.02, -0.04)	0.089
Total PE	-0.03 (-0.29 - 0.23)	0.889	0.86 (0.05, 1.68)	0.089
Total PE(O)	0 (-0.62 - 0.63)	0.995	-0.2 (-0.83, 0.43)	0.555
Total PE(P)	0 (-0.55 - 0.55)	0.995	-0.41 (-1.28, 0.46)	0.438
Total LPE	**1.64 (0.53 - 2.76)**	**0.013**	0.19 (-0.34, 0.73)	0.523
Total LPE(P)	1.75 (0.21 - 3.3)	0.068	-0.8 (-2, 0.41)	0.307
Total PI	**0.44 (0.16 - 0.72)**	**0.01**	0.33 (-0.1, 0.76)	0.235
Total LPI	**1.43 (0.57 - 2.29)**	**0.006**	**0.89 (0.35, 1.43)**	**0.008**
Total PG	0.71 (0.06 - 1.36)	0.071	**1.3 (0.54, 2.05)**	**0.006**
Total PS	0.49 (-0.34 - 1.34)	0.311	-1.53 (-3.69, 0.67)	0.287
Total CE	0.24 (-0.63 - 1.12)	0.707	-0.4 (-0.77, -0.03)	0.089
Total COH	**1.31 (0.64 - 1.97)**	**0.001**	0.5 (0.05, 0.95)	0.089
Total DG	**5.11 (3.87 - 6.37)**	**<0.001**	**1.81 (1.02, 2.61)**	**<0.001**
Total TG	**7.77 (6.59 - 8.96)**	**<0.001**	**1.30 (0.68, 1.93)**	**0.001**

Table 15.3: Key lipids with significant relationships to liver steatosis, and substantial correlation between serum and liver lipidomes.

Lipid	**Liver lipidome correlated to steatosis**		**Serum lipidome correlated to steatosis**		**Liver vs Serum lipids (Correlation)**	
	Percent change with steatosis	***p-value***	***Percent change with steatosis***	***p-value***	***Pearson's correlation***	***p-value***
Total dhCer	2.5%	<0.001	1.3%	0.002	0.360	<0.001
Cer(d18:0/18:0)	3.2%	<0.001	1.9%	0.003	**0.663**	<0.001
Cer(d18:0/22:0)	2.8%	<0.001	1.5%	0.001	**0.533**	<0.001
Cer(d18:0/24:0)	2.1%	<0.001	1.2%	0.021	**0.508**	<0.001
Cer(d18:0/24:1)	2.6%	<0.001	1.4%	0.002	**0.618**	<0.001
Cer(d18:1/16:0)	1.8%	<0.001	0.9%	0.035	0.393	<0.001
Cer(d18:1/20:0)	1.6%	<0.001	1.0%	0.011	0.365	<0.001
Cer(d18:1/24:0)	1.3%	<0.001	0.7%	0.021	0.175	0.043
GM3(d18:1/20:0)	2.1%	0.002	0.9%	0.012	0.463	<0.001
PE(16:0_16:1)	1.5%	0.012	2.1%	0.005	**0.733**	<0.001
PI(16:0/16:0)	2.9%	<0.001	3.2%	<0.001	**0.808**	<0.001
PI(16:0_16:1)	2.5%	<0.001	3.2%	<0.001	**0.870**	<0.001
PI(34:0)	1.6%	0.038	2.0%	0.003	**0.756**	<0.001
PI(34:1)	1.6%	0.001	2.0%	<0.001	**0.688**	<0.001
PI(18:0_22:5) (n3)	2.3%	<0.001	1.3%	<0.001	**0.550**	<0.001
DG(14:0_18:1) (a)	6.3%	<0.001	3.1%	<0.001	**0.519**	<0.001
DG(14:0_18:1) (b)	8.7%	<0.001	2.9%	0.001	0.485	<0.001
DG(14:0_18:2) (a)	5.0%	<0.001	2.2%	0.02	0.363	<0.001
DG(14:0_18:2) (b)	7.2%	<0.001	2.6%	0.005	0.323	<0.001
DG(16:0_16:0) (b)	9.6%	<0.001	6.0%	<0.001	0.321	<0.001
DG(16:1_18:1) (b)	6.5%	<0.001	2.4%	<0.001	0.327	<0.001
TG(14:0_18:0_18:1)	8.9%	<0.001	3.9%	<0.001	0.499	<0.001
TG(14:1_16:1_18:0)	8.9%	<0.001	2.8%	0.001	0.337	<0.001
TG(15:0_16:0_18:1)	9.0%	<0.001	2.1%	<0.001	0.324	<0.001
TG(16:0_16:0_16:0)	8.8%	<0.001	4.8%	<0.001	0.446	<0.001
TG(16:0_16:0_18:0)	10.1%	<0.001	4.1%	<0.001	0.419	<0.001
TG(16:1_16:1_16:1)	10.0%	<0.001	2.3%	0.011	0.370	<0.001
TG(16:1_16:1_18:0)	8.2%	<0.001	2.2%	0.005	0.394	<0.001
TG(16:0_16:1_17:0)	9.2%	<0.001	3.1%	<0.001	0.384	<0.001
TG(14:0_17:0_18:1)	8.4%	<0.001	2.3%	0.001	0.350	<0.001
TG(14:0_16:0_18:1)	8.4%	<0.001	2.6%	0.001	0.322	<0.001

***Bold** shows lipids that have substantial Pearson correlation (r > 0.5).*

Figure 15.1a: Representation of all measured lipids (~450 lipids) in liver lipidome (left) and serum lipidome (right), showing changes with increasing liver steatosis severity. Represented as percentage change in lipid species level associated with increase in percentage area of liver steatosis.

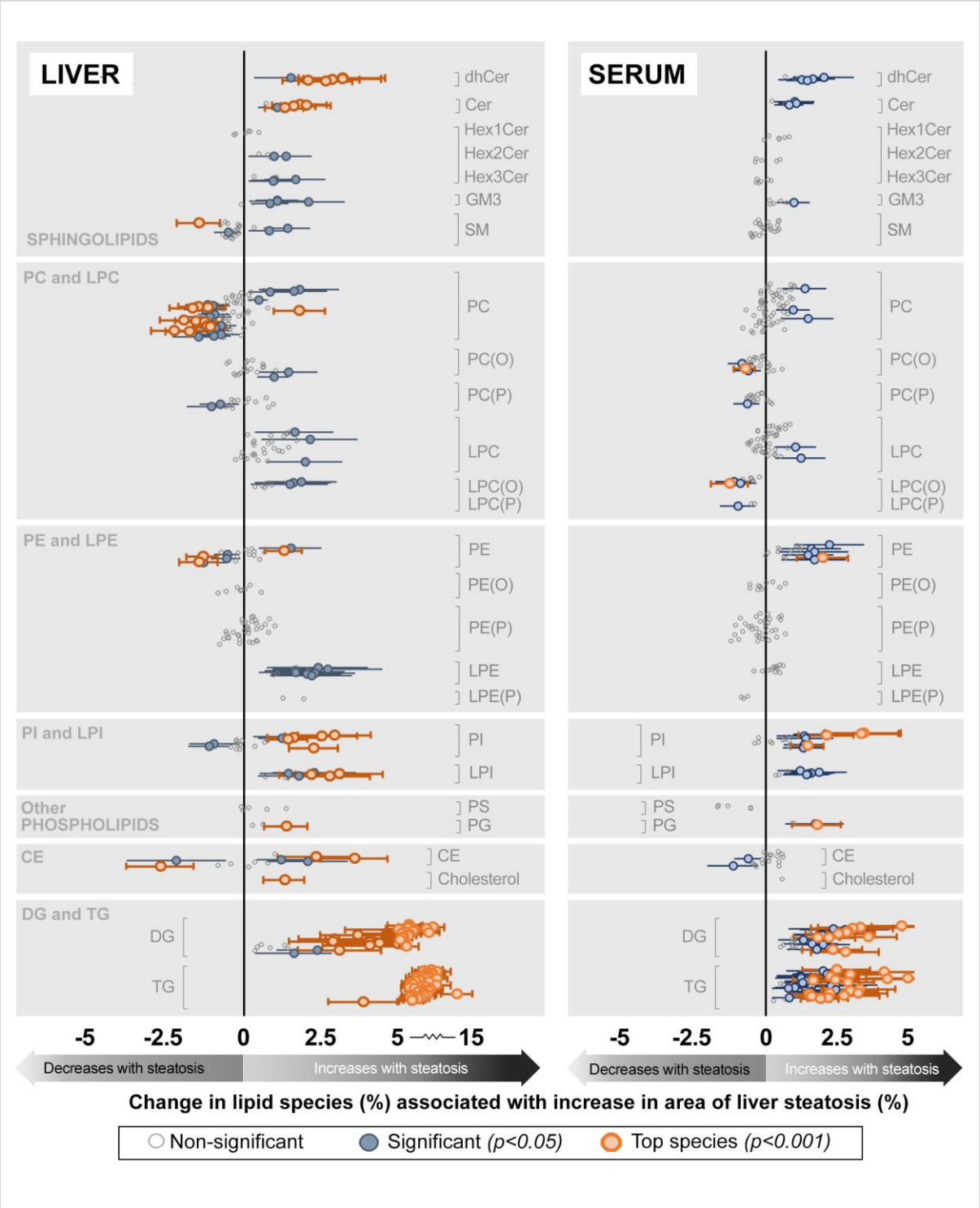


Figure 15.1b: Examples of specific liver lipid subclass and species (total dhCer, Cer(d18:0/22:0), PI(16:0/16:0) and PI(16:0_16:1) that are significantly increased with increasing liver steatosis (<5% vs 5-15% vs >15% area of histological steatosis). Significant increases represented with horizontal bars.

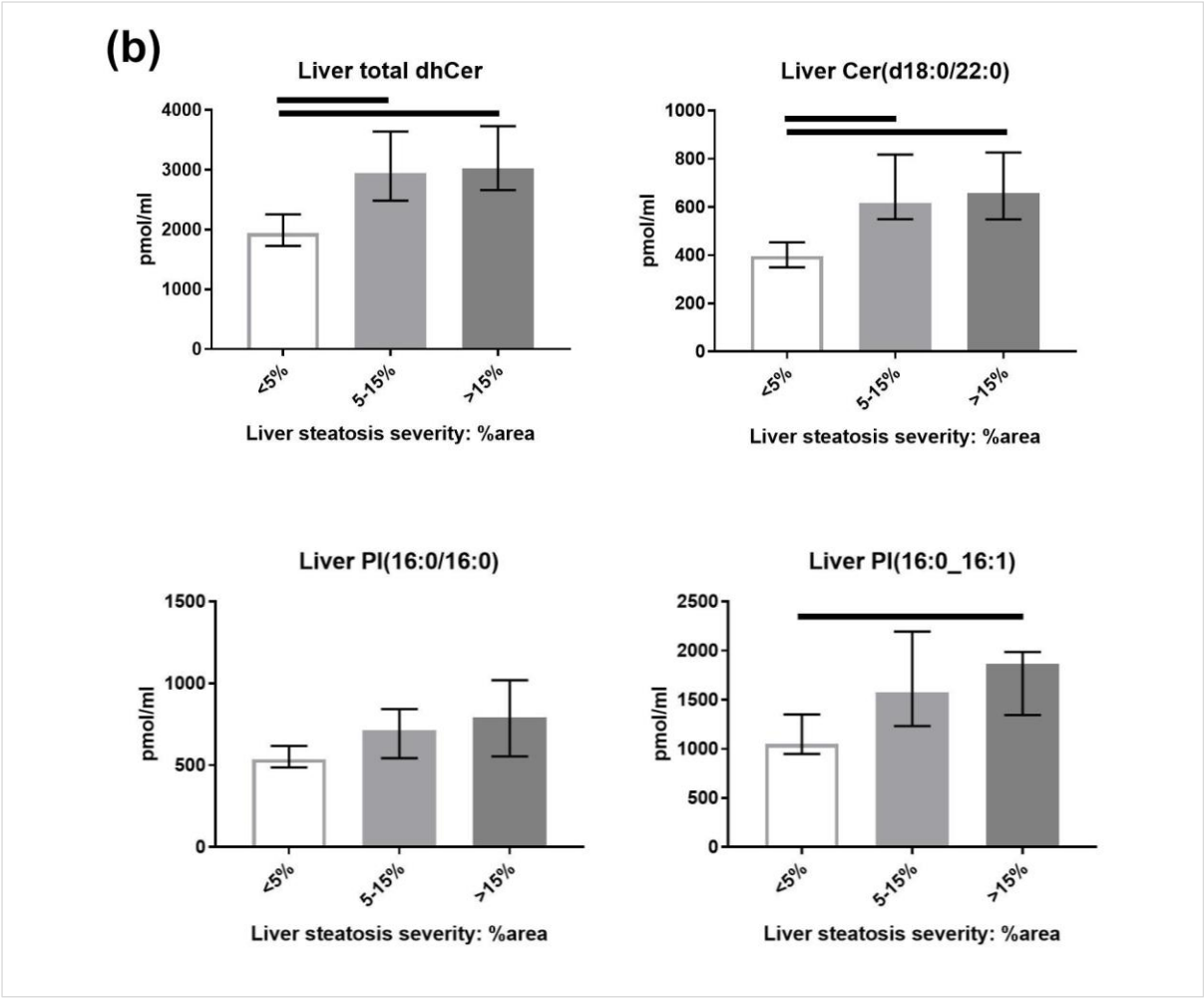


Figure 15.1c: Examples of specific lipid subclass and species measured in serum, showing significantly increased levels with increasing liver steatosis. Significant increases represented with horizontal bars.

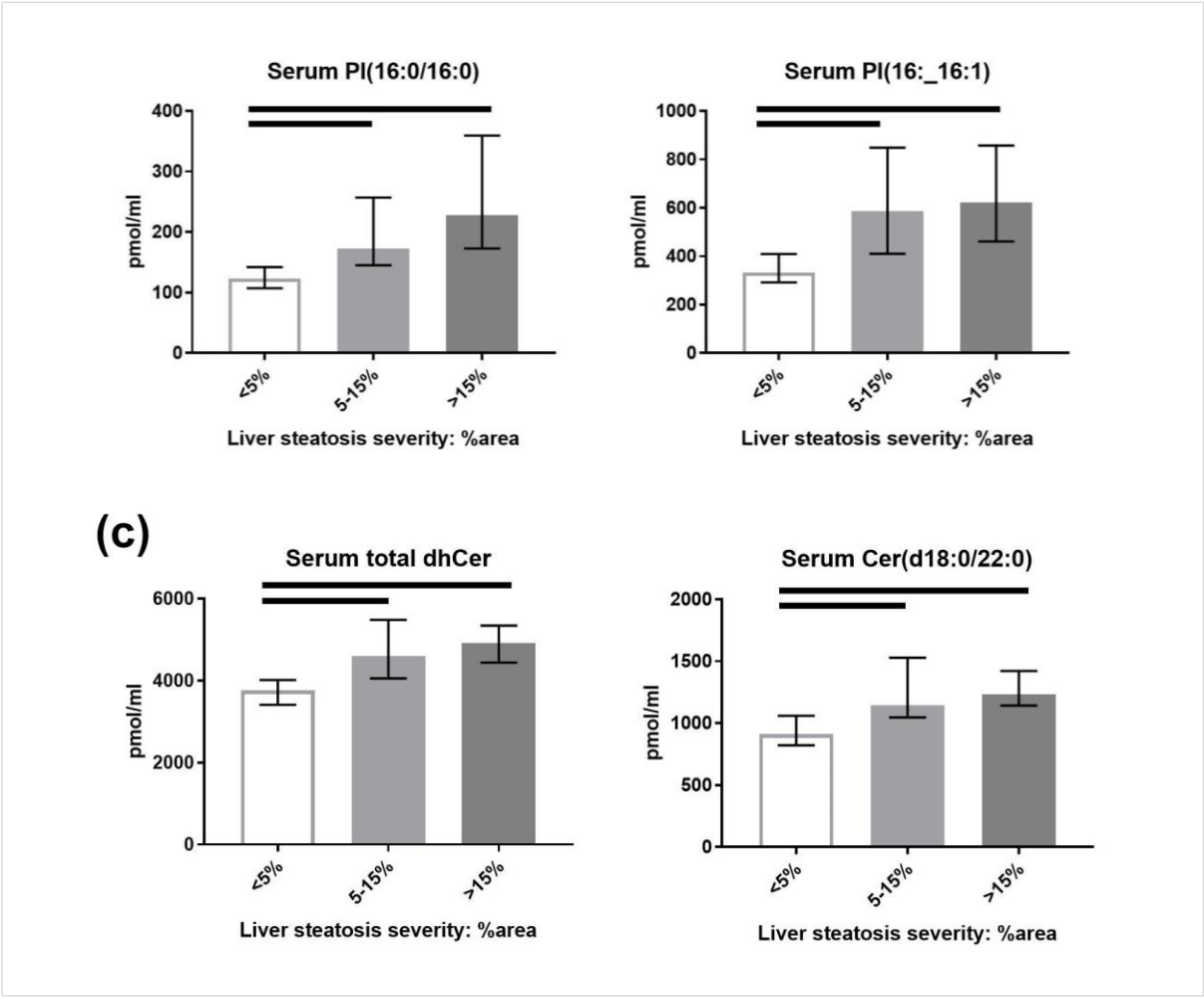


*Figure 15.2a: Representation of all measured lipids (~450 lipids) in liver lipidome, showing differences in normal liver vs NAFL (being steatosis without significant inflammation) (**left**), normal vs NASH (presence of significant inflammation) (**middle**) and NAFL vs NASH (**right**). Represented as percentage change in lipid species level associated with change from one state to another.*

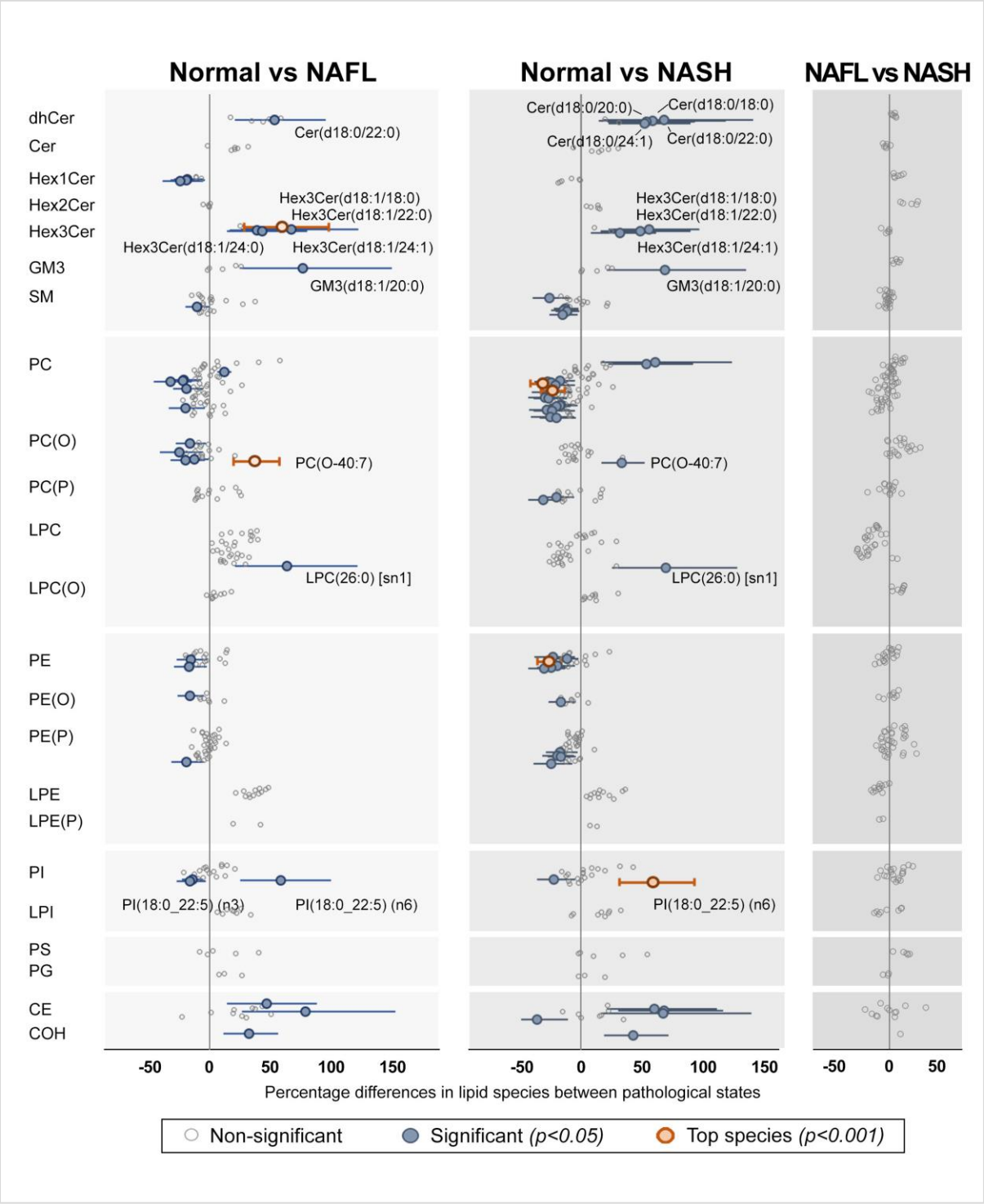


Figure 15.2b: Examples of specific liver lipid subclass and species (total dhCer, Cer(d18:0/18:0), Cer(d18:0/20:0), Cer(d18:0/22:0),Cer(d18:0/24:1), and PI(16:0_16:1) that are significantly increased with changes from normal to NAFL to NASH. Significant increases represented with horizontal bars.

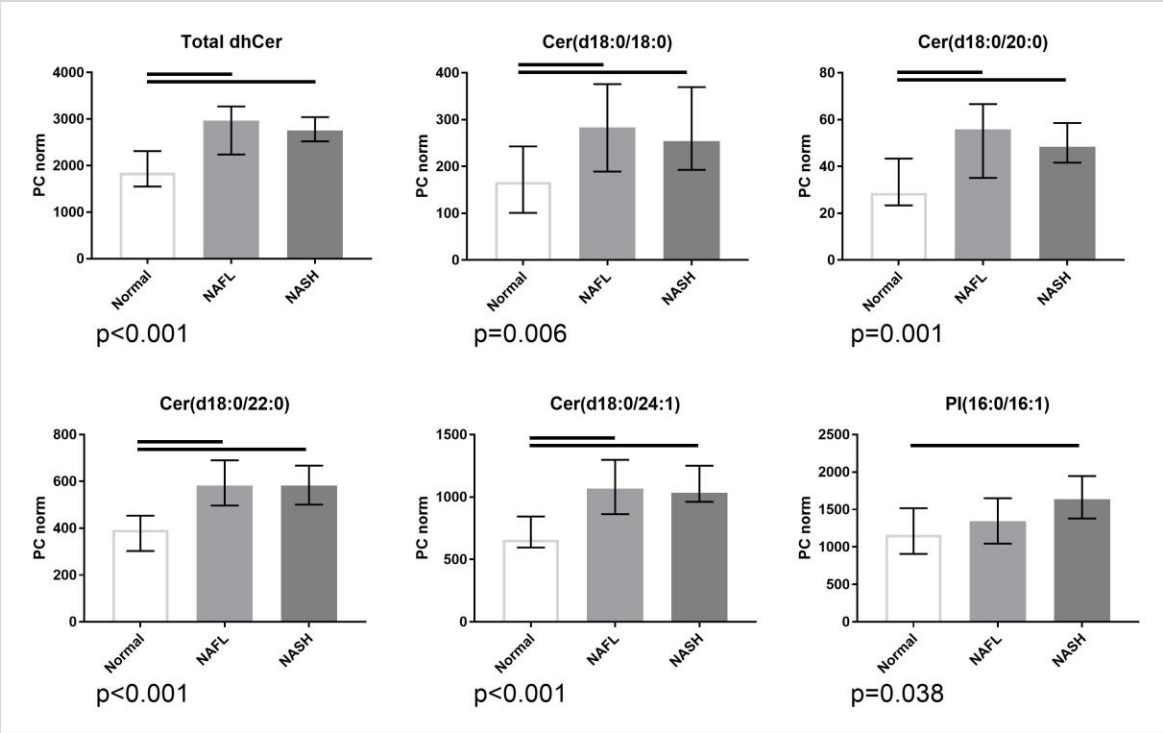


Figure 15.2c: Examples of specific lipid subclass and species measured in serum, showing significantly increased levels with changes from normal to NAFL to NASH. Significant increases represented with horizontal bars.

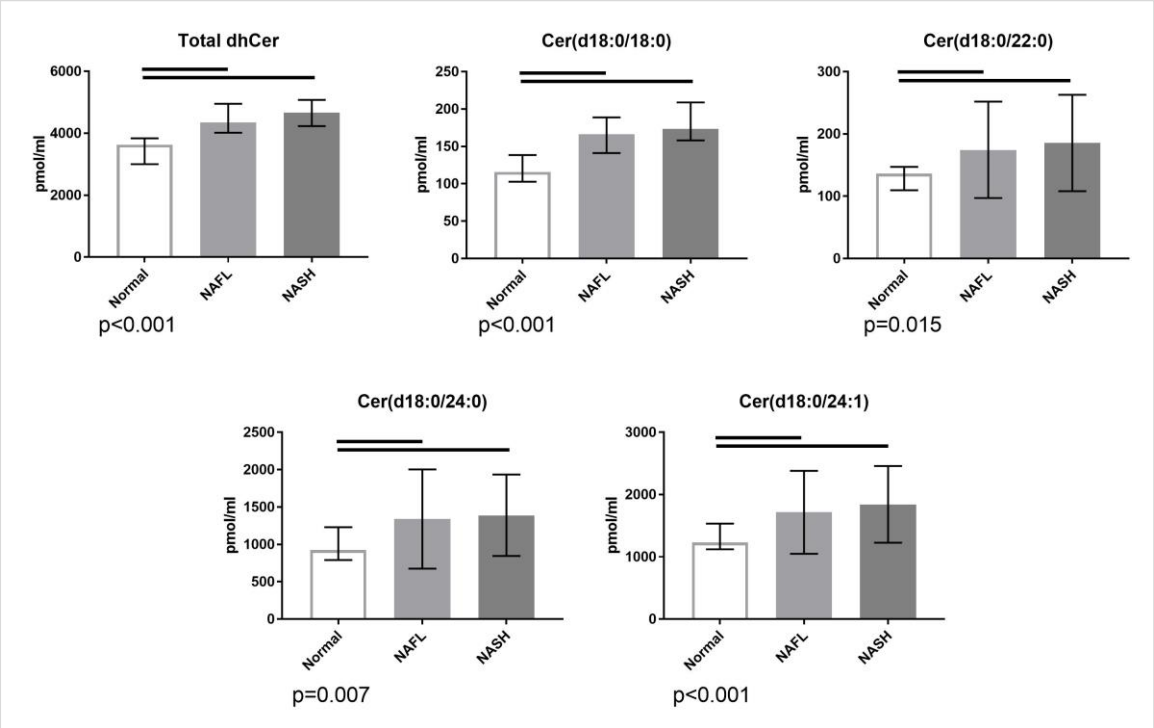


Figure 15.3: (left) Representation of all measured lipids (~450 lipids) in visceral adipose tissue (VAT) lipidome, showing changes with increasing liver steatosis severity. Represented as percentage change in lipid species level associated with increase in percentage area of liver steatosis. (right) Representation of all measured lipids (~450 lipids) in abdominal subcutaneous adipose tissue (SAT) lipidome. This shows no significant changes in any lipid species in abdominal SAT with severity of liver steatosis.

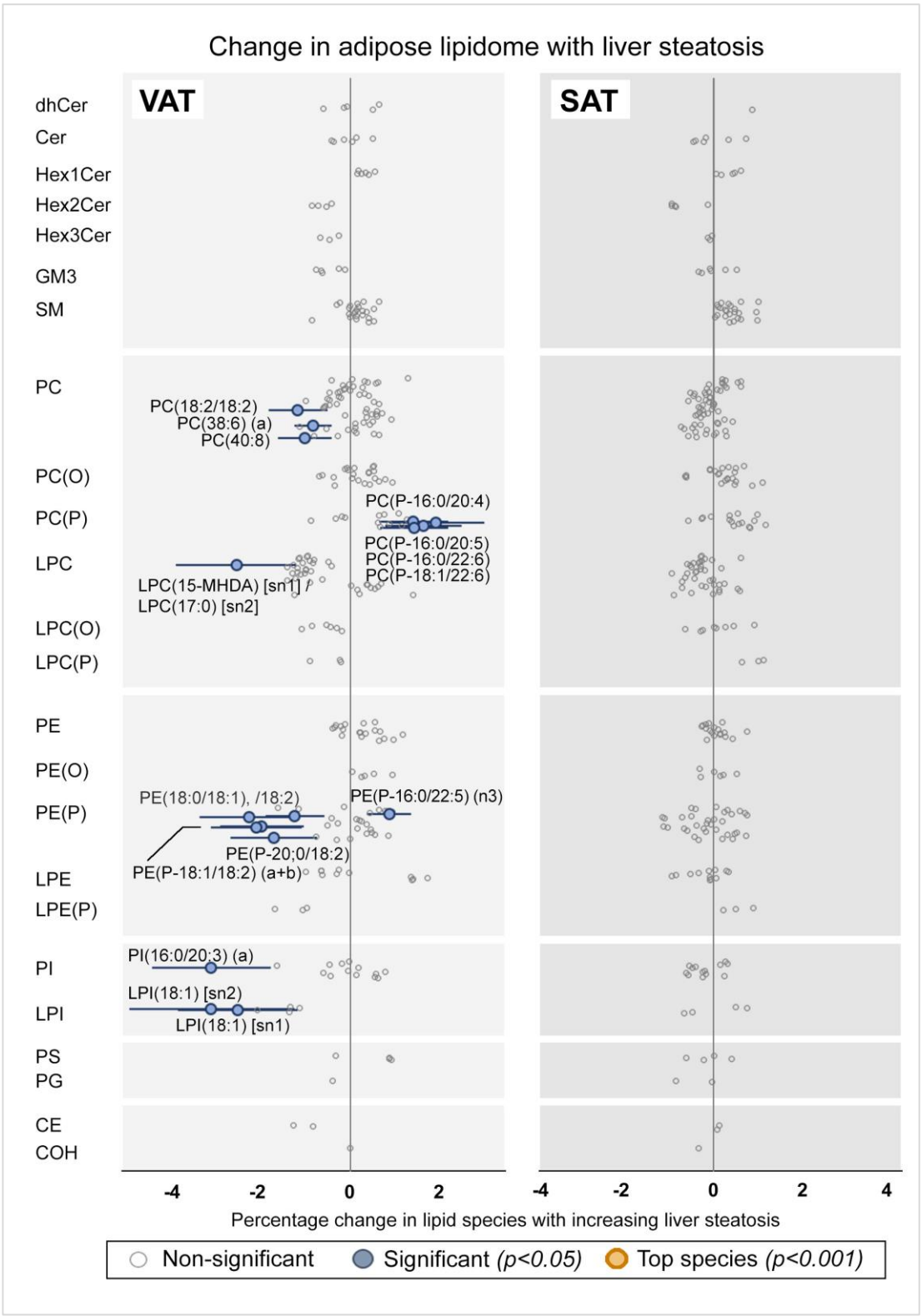


Figure 15.4a: Correlation of serum lipid levels with (a) liver, (b) visceral adipose tissue (VAT) and (c) subcutaneous adipose tissue (SAT). This shows the lipid species Cer(18:0/18:0), PE(16:0/16:1) and PI(16:0/16:0), demonstrating significant correlation between serum and liver, but minimal correlation of serum to VAT and SAT.

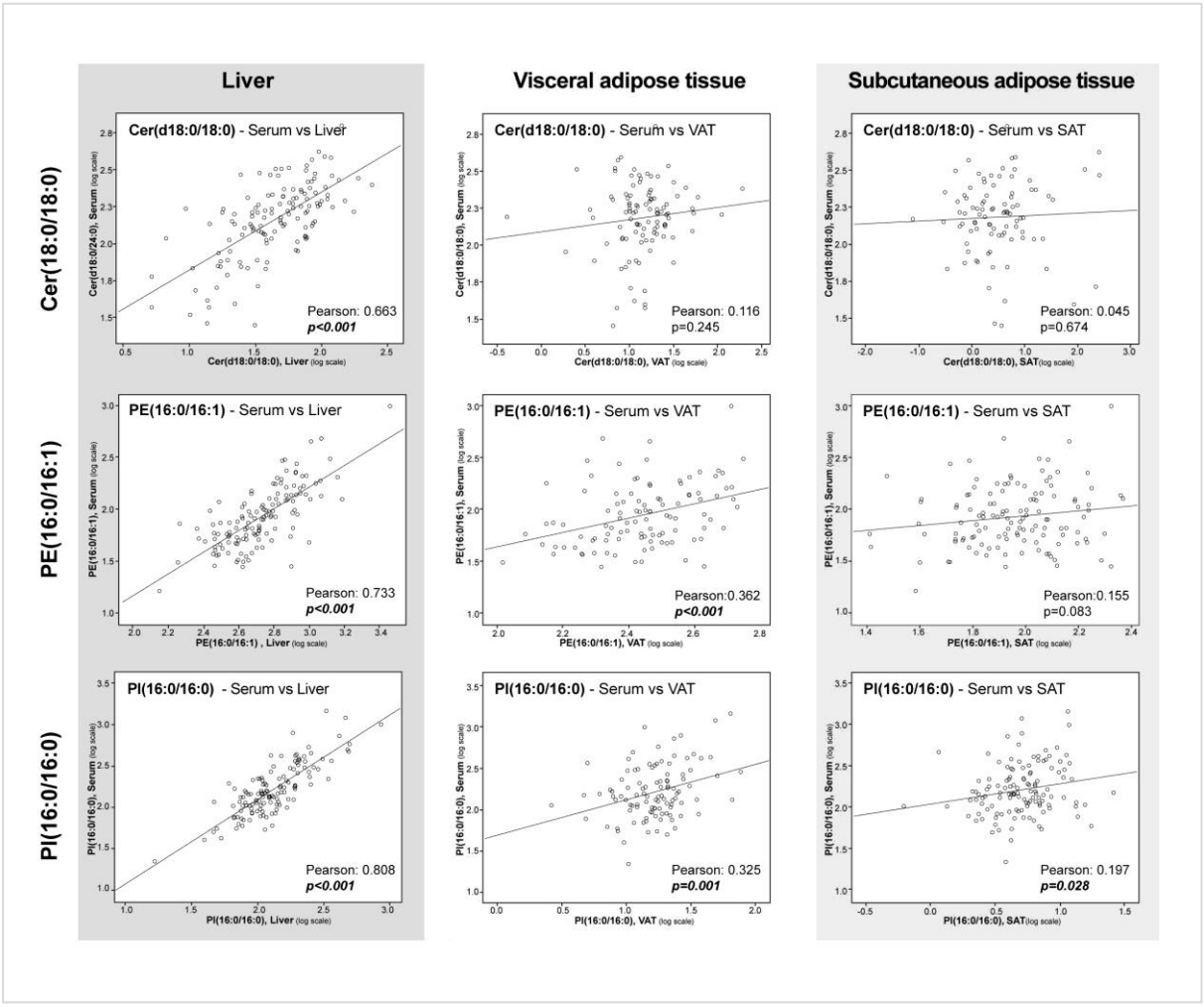
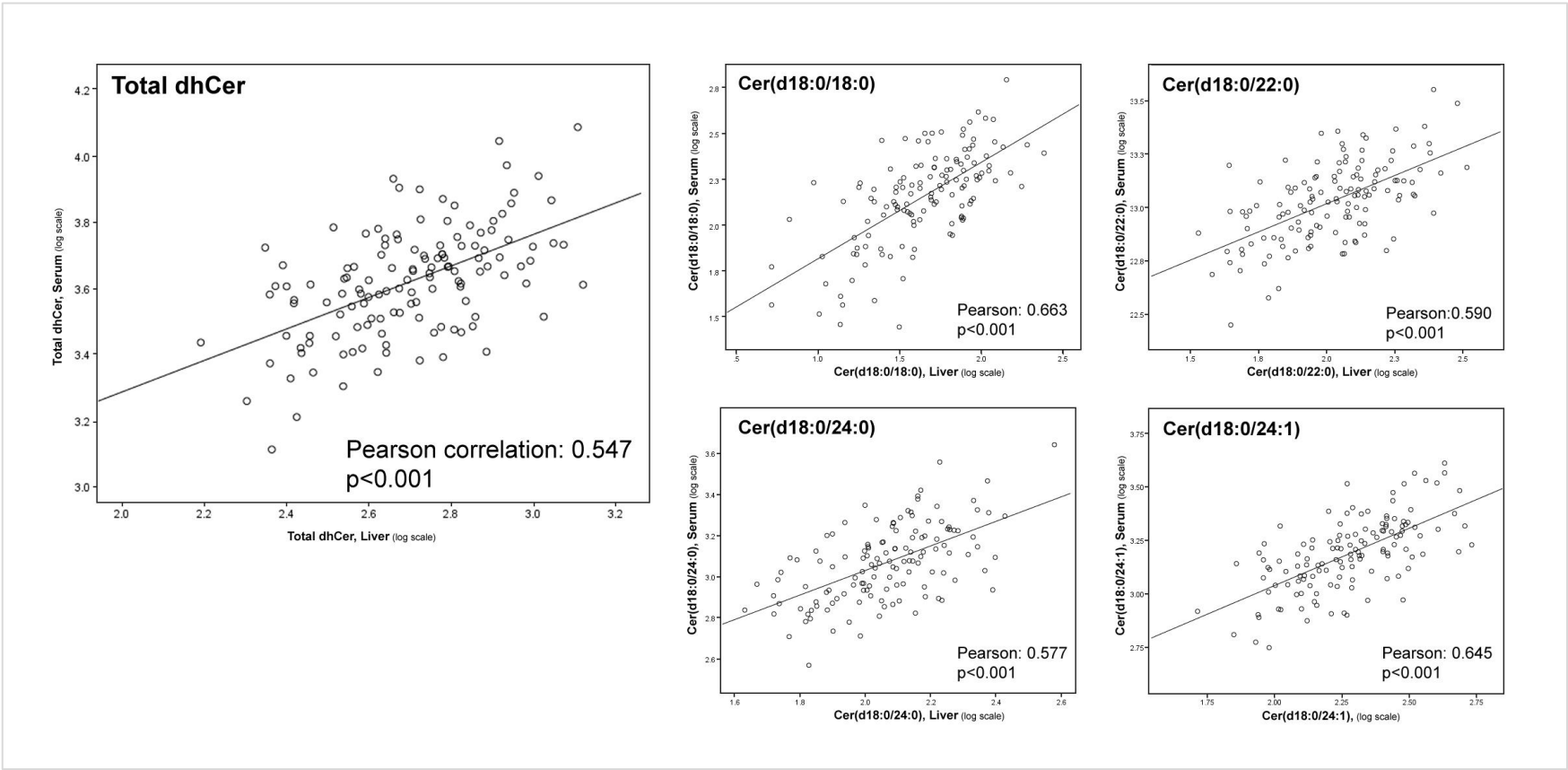


Figure 15.4b: Correlation between liver (x-axis) and serum (y-axis) lipid levels dihydroceramides (dhCer), showing total dhCer (left) and dhCer species (right). This shows significant and substantial correlation between serum and liver levels of these lipids.



15.5 Discussion

The defining feature of NAFLD is the excess accumulation of lipids within hepatocytes. Through the simultaneous assessment of patient-matched liver, adipose and plasma samples, this study provides an in-depth assessment of the lipid profile associated with NAFLD.

We found a range of significant changes in the liver lipidome with progressive liver steatosis. Most notably, multiple sphingolipids were increased in liver in association with increasing steatosis. Ceramide and dihydroceramide species were significantly elevated, suggesting increase in the *de novo* synthesis of ceramides. Numerous complex sphingolipids, such as GM3 gangliosides, dihexosylceramide (DHC) and trihexosylceramide (THC) species, were also elevated. There were significant but variable changes in phospholipids, with phosphatidylcholine (PC) and phosphatidylethanolamine (PE) species showed variable change. Notably, lyso- species (LPC, LPE and LPI) showed substantial and significant increases. This profile is in keeping with other studies showing alterations in ceramides (391, 641), lysophosphatidylcholine (LPC) (412, 642). phosphatidylcholine (PC) and phosphatidylethanolamine (PE) (386, 411) in animal and human NAFLD.

Ceramide species have been linked with the development of nonalcoholic fatty liver disease (391) and insulin resistance (643, 644). Experimental models using pharmacological inhibition of ceramide synthase or transgenic manipulation of ceramide synthase (CerS) in mice have reported a close association between diet-induced NAFLD and hepatic ceramide accumulation (403-405, 645). Specifically, the accumulation of C16:0 and C18:0 ceramide levels are more prominently associated with NAFLD in mice fed high sugar/high fat NAFLD-inducing diets and are often accompanied by lower C24:0 and C24:1 species (646). We also report the strongest association between C16:0 and C18:0 ceramide and NAFLD progression, but interestingly, C20:0 and C24 ceramides were also increased. In light of the increase in all dihydroceramide species in NAFLD (i.e. markers of *de novo* ceramide synthesis), the present data indicate that ceramide content is increased by greater fatty acid substrate delivery and concomitant upregulation of various ceramide synthase (CerS) isoforms that catalyse the conversion of dihydroceramide to specific ceramide species by acylation with a range of fatty acyl-CoA substrates. To the best of our knowledge, the expression of CerS isoforms is not described in human NAFLD. It is also likely that decreased activity of certain sphingomyelinases contributed to the increase in ceramide, as indicated by reduced content of several sphingomyelin species in NAFLD.

There remains limited data that document the role of ceramides in NAFLD progression in humans. To date, only three studies have provided sphingolipid data from liver biopsies of NAFLD patients. Gordon *et al* reported no difference in hepatic ceramides when comparing individuals with no pathology and those with steatosis (390), while data from a small clinical trial showed 50% and 33% increases in total ceramides with NASH and steatosis respectively, as well as correlation of hepatic dihydroceramides species (16:0. 22:0 and 24:1) with NASH (647). In support of this latter report, Luukkonen *et al* (391) reported increases in most ceramide species in obese NAFLD patients with insulin resistance and a greater prevalence of NASH compared with NAFLD patients with relatively low liver fat and a relatively healthy metabolic profile. Our data in a larger, heterogenous patient cohort contributes to the understanding of ceramide and other sphingolipid species changes with NAFLD in humans, and provides evidence of its association with progressive liver disease.

Ceramides are strongly associated with a wide range of deleterious metabolic and cardiovascular effects that are often evident with NAFLD (385, 636), including impaired insulin sensitivity, pancreatic β cell dysfunction, and the development of type 2 diabetes (648, 649). Ceramides have also been linked with atherosclerosis, vascular disease, hypertension, and can contribute independently to cardiac dysfunction. Studies in mice have shown that down-regulating ceramide production ameliorates these effects, and the current data add to the evidence base supporting ceramide-reducing approaches as a promising target for potential therapy in NAFLD patients (636).

A second major finding of this work was that no specific lipid was specifically related to NASH, independent of steatosis or NAFL. The progressive changes in hepatic lipid composition with NAFLD severity was previously reported in mice fed a high-fat, high-cholesterol diet (650). Comparison of lipidomes in mice fed for 16 and 52 weeks, representing NAFL and NASH, revealed remarkable lipidomic remodelling highlighted by reduced cholesterol ester, PC, and PE levels and changes in the levels of many sphingolipids including increased ceramide C16:0 and C24:1 (and decreased C24:0), sphingosine and globotrioseacylceramides, which were not assessed in the present study. Unfortunately, the capacity to identify specific lipids and lipid pathways that were related to disease progression were compromised by differences in the animals age, which was not factored into the analyses. Inflammation and lipid signalling are interrelated bidirectional modulators of cell function. While it is well known that activation of various inflammatory signalling pathways

can impact lipid metabolism in the liver (651), the present results indicate that the inflammation accompanying NASH is not sufficient to modulate lipid species in the presence of pre-existing NAFL. Naturally, these conclusions do not account for the spatial-temporal changes in lipids or inflammation and are a snapshot in time. In addition, several factors limit the broad applicability of these observations. Firstly, the pathophysiological mechanisms that drive steatohepatitis and fibrosis are complex (355), and the effects of lipid species on development of inflammation may not be easily demonstrated by correlation analysis. Secondly, severe NASH was not well represented in this cohort, as demonstrated by raw inflammation and ballooning scores. This may dampen the power to find significant differences in our cohort. Finally, despite corrections for baseline characteristics, heterogeneity that exists within human cohorts make it more difficult to observe differences in lipid species that have been found in experimental models of NASH. Overall, further study of the lipidome is required, to determine the contribution of lipids to inflammation and NASH pathogenesis in humans.

The lipidome of visceral (VAT) or subcutaneous adipose tissue (SAT) depots did not show any convincing association with NAFLD. These findings support those from a study by Anjani *et al* (395), examining the lipid efflux from VAT and SAT in 46 women, which similarly showed very few associations with NASH. Hence, whilst adipose tissue, particularly VAT, may have strong links to NAFLD via inflammatory and immune cell changes (493, 637, 652, 653), our findings demonstrate that the adipose lipidome is unlikely to be a direct contributor to the development of liver disease.

In contrast, the circulating plasma lipidome showed significant changes in association with NAFLD. These included total ceramides and dihydroceramides, ceramide and dihydroceramide species, and several phosphatidylinositol (PI) and lysophosphatidylinositol (LPI) species. Importantly, many of these lipids that increased in plasma were the same lipid species elevated in liver with worsening NAFLD. Whilst previous studies were not able to demonstrate these association in circulating plasma lipidome with NAFLD (395), a possible explanation for these findings are the greater patient population in this present study, and the ability to now examine a wider range of lipid species. This study suggests that the circulating lipidome demonstrates significant changes with worsening liver disease, and furthermore, these changes reflect the lipidomic changes shown within the liver. This also reflects

previous studies demonstrating that the vast majority of plasma ceramides are contained with lipoproteins derived from the liver (649).

A direct and significant association of the plasma and liver lipidome, but not the adipose tissue lipidome, is observed. There were 44 moderately to strongly related lipids (Pearson correlation >0.5), including many sphingolipids and phospholipid species. In particular, strong correlations were seen between liver and plasma dihydroceramide levels, with Pearson correlation of 0.547-0.663 ($p<0.001$). These lipid species were also associated with states of steatosis and NASH in both the liver and plasma lipidome. In contrast, despite significant obesity and excess adiposity in this cohort, the circulating lipid profile showed little appreciable relationship to either the visceral or subcutaneous adipose tissue lipidome. This suggests a clear and reflective contribution of the liver to the plasma lipidome. Hence, not only is the plasma lipidome significantly associated with liver disease, and mirrors lipidomic changes seen in the liver, but there is a direct relationship between the liver and plasma lipidomes. This suggests that the plasma lipidome reflects pathological changes in liver lipidome, even in patients with significant obesity, and may be used as a non-invasive means of assessing the liver lipidome.

Our studies differ from previous studies in several ways. Firstly, it is one of the largest studies into the lipidomic profile of NAFLD in humans. Secondly, we performed a global assessment of lipid profiles in liver, VAT, SAT and plasma. This has allowed not only an analysis of associations of each depot with nonalcoholic fatty liver disease, but also an examination of the associations between each depot. Secondly, we have recruited over 180 well-characterised severely and morbidly obese participants for this study. Having this number of participants has provided additional power to find statistical differences. Finally, in this study, we have analysed over 440 lipid species, to gain an even finer understanding of lipid alterations in NAFLD. With the ever-growing field of metabolomics, advances will likely further improve our ability to define the lipidome in the future.

There are some drawbacks that warrant discussion. Firstly, this is a cross-sectional examination of tissue specimens, and therefore only demonstrates correlation between lipids and disease states. However, this study provides a detailed description of the lipid profile, and corroborates findings from experimental studies, showing alterations in lipid profile with disease severity. Secondly, fibrosis was not well represented in this cohort, and therefore no meaningful analysis could be performed. Finally, there was substantial heterogeneity in

medication used within this cohort for treatment of metabolic disease. Twenty percent of patients used an anti-diabetic medication, 15% used a lipid lower medication and 40% used an antihypertensive. These may alter lipid metabolism, however heterogeneity in medications used and combination therapies made subgroup analysis problematic. Further study should investigate the effects of medications on hepatic lipid profile.

In conclusion, substantial alterations in liver lipidome occur with worsening nonalcoholic fatty liver disease (NAFLD). Dihydroceramide and ceramide species, in particular, are significantly increased in the liver lipidome with increasing steatosis and NASH. Parallel increases of specific ceramide and dihydroceramide species are seen in the plasma lipidome. Furthermore, strong associations between the liver and plasma lipidome are seen. This is not seen between either adipose tissue depot (visceral or subcutaneous) and plasma. These findings indicate the potential for the plasma lipidome to be used as a non-invasive marker of nonalcoholic fatty liver disease.

16 Concluding remarks

Nonalcoholic fatty liver disease (NAFLD) and obesity are closely interrelated, yet research on NAFLD specifically within obese cohorts is lacking. The overall goal of this thesis was to address some of these knowledge deficiencies, by focusing on the intersection of NAFLD, severe obesity and bariatric surgery. These studies have been performed using different research methodologies covering several themes, where significant opportunities existed for improving diagnostic tools, treatment pathways and pathophysiological understanding.

The premise for this series of studies stemmed firstly from the clinical observation that NAFLD was an endemic disease, increasing in prevalence and severity, particularly in the bariatric surgical cohort. Despite this, few guidelines existed that aid bariatric surgeons and physicians with NAFLD management, reflecting a poor evidence base in this domain. It was also noted that bariatric surgery remained under-utilised in the management of NAFLD. This is in contrast to other obesity-related metabolic diseases, where clear pathways for management exist and the role of bariatric surgery is better defined.

The basis for these research methodologies was the recognition that the bariatric surgical cohort provided an ideal medium for research into NAFLD. Firstly, the patient population was well-characterised and regularly reviewed, which created an excellent foundation for patient selection and follow-up. There was a high prevalence of NAFLD within this population, owing to greater degrees of obesity and higher rates of metabolic disease. Bariatric surgical patients were often at least morbidly obese, with increased prevalence of super obesity, making them an ideal population for studying more severe forms of obesity. Finally, bariatric surgery allowed relatively safe access to tissues, including liver, adipose tissue and blood. With abundant pathophysiological data from animal models, bariatric surgery provided an opportune platform for translation of these findings into human disease.

Collectively, these studies have contributed to our understanding of various clinical and pathophysiological aspects of NAFLD in the context of severe obesity, as well as the impact of bariatric surgery and weight loss on disease resolution. Key outcomes from this thesis can be categorised into three broad areas:

- **Clinical applicability**: Specific results within Research Theme 1, 2 and 3 have readily translatable clinical application, via improved diagnostic tools in obesity and a

greater understanding of weight loss as a tool for primary treatment of NAFLD. These data will better inform clinicians who specifically manage obesity, by providing more accurate context and population-based evidence.

- **Pathophysiology**: An improved understanding of the contribution of lipotoxicity to NAFLD was provided in Research Theme 4, with the capacity to analyse other pathophysiological mechanisms via stored data and tissue collected as part of this overall endeavour.
- **Collaboration and future direction**: Work within Research Theme 1 and 4 illustrated the advantages of interfacing clinical and laboratory work, and have established the foundation for current and future collaborative research endeavours that are already achieving success. It is hoped that those studies will lead to further shifts in the understanding, diagnosis, management and treatment of NAFLD.

16.1 Major findings and implications

The major findings of this thesis are discussed below.

Research Theme 1: Current scope of the problem

A prospective study of NAFLD recruiting consecutive eligible bariatric surgical patients showed that NAFLD was common (74.1%), however more severe NASH (12.0%) and steatofibrosis (5.1%) was far less abundant than previously described. Due to the study design and setting within a bariatric population, this may better represent the prevalence in general obese populations. These findings are concordant with other bariatric surgical studies, where lower NASH and steatofibrosis prevalence have been observe. These prevalence data can help us to understand the realistic risk of NASH/steatofibrosis within the bariatric patient population.

Both increasing obesity as well as metabolic abnormalities independently increase the odds of NASH/steatofibrosis by over threefold. A combination of both super obesity (>50 kg/m^2) and metabolic abnormality markedly increased these odds to 9.71 for NASH/steatofibrosis. This presents a simple means by which to stratify patients into risk categories within bariatric populations. It can also prompt investigations for NAFLD and guide pre-operative counselling.

Contrary to previous evidence, we did not find any relationship of adipose tissue or systemic inflammation with NAFLD status. The reason for this is uncertain, however it could reflect the complex nature of obesity-related inflammation in the setting of severe and morbid obesity.

Research Theme 2: Challenges of diagnosing NAFLD in obesity

A systematic literature review assessing diagnostic accuracy of 11 common non-invasive tests for NAFLD-related fibrosis revealed that complex scores (such as ELF) and imaging tests (such as MRE and TE) have the best diagnostic accuracy in obesity. However, several drawbacks currently exist. Complex scores were poorly validated in obese cohorts, and feasibility issues hindered the widespread use of imaging tests. Furthermore, there were only 12 existing studies that examined these tests in exclusively obese cohorts. This review demonstrated the current limitations that exist in diagnosis tests for obese individuals, and highlighted the need for further validation and development in this area.

When we validated simple NAFLD fibrosis risk scores in an obese cohort, we found that they were not optimally developed in this population. Factors such as excessive weighting on BMI and high aminotransferase thresholds skewed the risk scores. However, a simple modification of the Forn index, by lowering thresholds, improved diagnostic accuracy in the setting of obesity. Strategies such as these may increase the utility of these simple tests in obese populations.

Imaging tests for NAFLD in the context of obesity had the best diagnostic accuracy. Magnetic resonance spectroscopy (MRS) had excellent accuracy for steatosis, with AUROC 0.852, sensitivity 81.3% and specificity of 87.5%. However, due to low feasibility (65.3%), these figures decreased significantly after intention-to-diagnose analysis (50% sensitivity and 60.9% specificity). Transient elastography (TE) and controlled attenuation parameter (CAP) had a reasonable balance of good diagnostic accuracy and feasibility. Combining the Forn index with TE, and ALT with CAP yielded reasonable overall accuracy for detecting fibrosis and steatosis. This simple algorithm could be a practical clinical approach in the setting of obesity.

Identification of liver abnormalities in bariatric surgical patients intraoperatively is common. A simple assessment of liver colour, size and surface texture assisted in stratifying patients who likely have liver pathology and would most benefit from an intraoperative liver biopsy.

This structured approach had a better accuracy than an 'overall impression'. A score of zero (completely normal appearing liver) was a relative contraindication to liver biopsy.

Research Theme 3: Impact of weight loss on NAFLD and related metabolic diseases

In the Metabolic Syndrome Study, we took monthly repeated measures of metabolic parameters over two years. By doing this, we were able to analyse the effects of incremental weight loss on disease resolution. This was in significant contrast to previous literature, which report results after substantial weight loss. In these studies, we were able to identify weight loss targets for meaningful improvements in disease, changes with modest weight loss, and effects of increasing weight loss.

These studies consistently showed that meaningful change was observed after 10% TBWL in the setting of obesity. There were significant odds of normalisation of aminotransferase levels after this weight loss threshold. Substantial improvements in metabolic syndrome and lipid parameters also occurred at 10-12.5% TBWL. We saw that early weight loss resulted in almost immediate improvement in biochemical markers, with rapidly decreasing levels of ALT, HDL, glucose and triglyceride levels post-operatively. This early improvement supports the notion of early changes in metabolic function prior to significant weight loss. Further weight loss resulted in greater improvements in all NAFLD and metabolic parameters.

These data challenge our definition of weight loss success and failure, showing that meaningful metabolic improvements occurred early, without having to achieve radical weight loss targets. Future investigation into these early changes would help us understand the mechanisms behind benefits of weight loss, and could potentially assist in developing therapies to augment response.

Research Theme 4: Developing an understanding of pathophysiological drivers of NAFLD in obesity

Advanced lipidomic techniques were able to map out the characteristic changes in hepatic lipidomic profile that occurred with advancing NAFLD. Significant changes could be seen, including increased sphingolipids, ceramides, triacylglycerol, and changes in glycerophospholipids. Similar hepatic lipid profiles were seen with NASH, however there were no unique patterns distinguishing simple steatosis from NASH. Sphingolipids, particularly ceramides, have now been implicated in other metabolic disease processes, such

as insulin resistance and cardiovascular disease. These data contribute to this body of evidence, and help to further unveil the role of ceramides in NAFLD pathogenesis.

Comparison of plasma and liver lipidome showed strong correlation of ceramide, dihydroceramide, phosphatidylinositol and phosphatidylethanolamine species. No substantial correlation was seen between adipose tissue lipid species and plasma lipids. Plasma ceramides also directly reflected NAFLD severity. This demonstrated the potential utility of the plasma lipidome as a biomarker of NAFLD.

In summary, the series of studies within this thesis have demonstrated the unique characteristics of NAFLD in obesity, and have contributed to our understanding of clinical and pathological aspects of this disease. This is vital, as the burden of both obesity and NAFLD increases. Ultimately, these studies can better inform primary care physicians, hepatologists and bariatric clinicians, by providing specific tools and knowledge for management of NAFLD in the growing obese population.

16.2 Limitations

Limitations in this work can be divided into those specific to individual research themes, and general limitations to the overall thesis.

A key general limitation was related to the intrinsic design of this PhD as a broad-based undertaking. The scope of this thesis was also limited by practical considerations around the duration of tenure and time investment in patient recruitment and data collection. Whilst this prevented more in-depth interrogation of collected data and tissues, these studies were able to address key clinical issues, covering a broad range of areas around the intersection of NAFLD, obesity and bariatric surgery. As such, this thesis has addressed its primary aims. Furthermore, it has created the framework for future exploration of the clinical and laboratory data already collected.

Specific constraints relating to findings in the prospective NAFLD studies warrant discussion. Firstly, prevalence of NASH and steatofibrosis was low in this cohort. This was unexpected, given the previous literature on epidemiology, as well as the significant rates of morbid obesity within this cohort, and accompanying metabolic disease. Whilst this has been an interesting finding in itself, it has diminished the power within many subsequent studies.

One key consideration in the bariatric surgical cohort is the use of very low-calorie diet (VLCD) pre-operatively. The use of VLCD is known to reduce liver volume, and therefore fat content, with the aim of improving surgical access and perioperative risk. The effects on inflammation and fibrosis is not known, but short term VLCD is less likely to have a significant impact on these. Therefore, the rates of NASH and steatofibrosis were likely to be similar regardless of VLCD use. Furthermore, VLCD was used inconsistently amongst surgeons, for differing time periods and with varying compliance, making meaningful subgroup analysis difficult.

The *Metabolic Syndrome* study follow-up spanned two years, to include the period of greatest weight loss after bariatric surgery. Although we know that weight loss after bariatric surgery is durable, metabolic disease, cholesterol abnormalities and insulin resistance tend to recur over time, given their associations with age. Greater follow-up would determine whether the observed changes are durable in the long term, or whether recurrence occurs over time.

Another source of heterogeneity in both study cohorts was the use of medications, particularly for diabetes, dyslipidaemia and hypertension. There was a 26-37% prevalence of diabetes, with a variety of oral hypoglycaemic agents and insulin used. Medication records were documented, however due to the substantial variation and the nature of the study aims, no specific sub-analysis was performed based on medication use.

More generally, the focus on obese (and frequently morbidly or super obese) individuals limits the application of this data to the obese, and not overweight or normal weight individuals. Undoubtedly, normal weight individuals also suffer from NAFLD and metabolic disease, although this group is relatively small. Other implications of not recruiting normal weight participants include the lack of control subjects for studies comparing inflammatory or lipidomic change. Further studies including normal weight individuals with and without NAFLD could better elucidate obesity-related change from NAFLD-related change.

Finally, integration of new knowledge and pathways take time to fully develop and requires validation. Currently, many of the findings of these studies remain preliminary. Their clinical utility will remain uncertain and somewhat limited, until external evaluation and validation can be performed.

16.3 Future directions

A range of future research directions have been established from this thesis. The projects within this PhD have established the framework for in-depth investigations around the themes of obesity, NAFLD and metabolic disease.

At this stage, several collaborative research efforts have been completed, whilst others are underway or being planned as intermediate and longer-term studies. The key to the success of these projects has been the formation of strong collaborations between clinicians and basic scientists, as well as the exploitation of bariatric surgery as a platform for basic science research. Such future projects include the following:

- In direct continuation from the final study in this thesis, future research is aimed at validating the findings of plasma lipidomic markers of liver steatosis in a larger cohort of patients. Recruitment of more patients with severe NASH could yield greater power in finding specific lipids associated with inflammation and fibrosis. Subsequent experimental models could potentially elucidate the exact mechanisms that result in lipidomic changes linked with NASH progression. These data provide a springboard for hypothesis testing to determine which lipid pathways could be involved in disease progression. Ultimately, this could result in potential therapeutics that could block lipotoxicity and potentially ameliorate disease.
- Investigating novel serum biomarkers of NAFLD through interrogation of whole liver secretome (NAFLD Secretome Study). This study is currently underway, having currently recruited over 40 participants. It applies techniques established in animal models to human tissue, whereby fresh liver tissue is collected and the whole protein secretory profile is analysed. Secreted proteins will be investigated as a novel non-invasive biomarker of these diseases. Once identified, target proteins will be validated within a separate bariatric population. Achievement of significant competitive funding of this project is testament to its perceived value and feasibility (NHMRC project grant APP1162511, 2018).
- Mechanisms behind NASH development via T-cell protein tyrosine phosphatase (TCPTP). This study has demonstrated two separate pathways towards NASH and HCC development in mice mediated by TCPTP. Laboratory findings were validated within a human population. It has recently been published in Cell (Grohmann et al,

Obesity drives STAT-1-dependent NASH and STAT-3-dependent HCC, *Cell* (2018), 175:1-18. https://doi.org/10.1016/j.cell.2018.09.053).

- Characterisation of pre-adipocyte characteristics of fat depots, pre-adipocyte differentiation into mature adipocytes and their associations with metabolic and liver disease. This study showed that pre-adipocytes can differentiate into three types of mature adipocytes, each with different metabolic profiles. This research has been submitted as a revised manuscript to Cell Metabolism (Raajendiran *et al*, Identification of metabolically distinct adipocyte progenitor cells in human adipose tissues).
- Evaluation of pre-clinical models of NASH-dependent HCC. This research validated different animal models of NASH against the molecular characteristics human disease to elucidate the most reliable model for HCC-targeted therapies. This study has recently been accepted in Cell Metabolism (Febbraio et al, Preclinical models for studying NASH-driven HCC: how useful are they?, *Cell Metabolism* (2018)).

Further research endeavours in the area of NAFLD, obesity and metabolic disease could be aided by two major undertakings:

1. **Formal collaborative effort**: The development of focused collaborative groups involving basic scientists, hepatologists and bariatric clinicians. This would allow us to link key clinical questions in NAFLD with feasible methods and scientific breakthroughs, translating benchtop metabolic research into clinical practice.
2. **Metabolic biobank**: The development of a comprehensive Metabolic Biobank, based on bariatric surgical population, to collect detailed patient data coupled with tissues for basic research. This would be a valuable resource for future research into NAFLD and obesity-related disorders.

This collaborative approach to studying NAFLD in the bariatric surgical population has opened multiple opportunities in an area of research need. Ultimately, this framework can accelerate major advances in knowledge, and address current deficiencies in our understanding of NAFLD, obesity and metabolic disease.

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18 Appendices

18.1 Appendix 1: Supplementary materials - Effect of body mass index, metabolic health and adipose tissue inflammation on the severity of nonalcoholic fatty liver disease in bariatric surgical patients: A prospective study

18.1.1 Supplementary Methods

18.1.1.1 Collection of intraoperative biopsies

All tissue specimens were collected by the operating surgeon. A wedge biopsy, at least 1cm in depth, was taken from the left lobe of liver. All half of the wedge liver biopsy was formalin fixed (10% buffered formalin) and paraffin embedded for subsequent histopathological assessment. The remaining section of liver was frozen in dry ice for storage at -80^{o}C.

Approximately 5-10mL of omental visceral adipose tissue (VAT) and abdominal subcutaneous adipose tissue (SAT) were collected. These were divided, with half undergoing formalin fixation (10% buffered formalin) and paraffin embedded, and half frozen in dry ice then stored at -80^{o}C.

18.1.1.2 Histology

Paraffin embedded liver samples were sectioned and stained with hematoxylin and eosin (H&E), and Masson's trichrome. Liver biopsies were assessed by a single histopathologist, blinded to clinical information, according to the NASH CRN Scoring System (481) and fibrosis stage as described by Kleiner (240).

18.1.1.3 Image analysis of tissue

Adipose tissue was sectioned and H&E stained. Image analysis was used to objectively quantify adipose tissue cell size (Fiji, ImageJ, Madison, WI, USA) (482).

18.1.1.4 Liver and adipose tissue mRNA expression

mRNA extraction

Liver and adipose tissue was thawed on ice, then homogenised for 2 minutes at 50/second in 1mL of TRI Reagent (Sigma-Aldrich, St Louis, USA). 200µl chloroform was vigorously mixed to the homogenate for 15 seconds and incubated for three minutes at room temperature. This was centrifuged at 12,000G at 4^{o}C for 15 minutes. The aqueous top phase was transferred and stored in separate fresh tubes. 500µl isopropanol was mixed in, and then this was incubated at room temperature for 10 minutes. Samples were centrifuged at 12,000G at 4^{o}C for 10 minutes. The supernatant was discarded leaving the RNA pellet. 1mL of 100% ethanol was added, and the sample vortexed to dislodge the pellet. The sample was then centrifuged at 10,000G at 4^{o}C for 15 minutes. The supernatant was discarded and the samples left to air dry at room temperature. The pellet was suspended in 30µl of RNAase free water.

Samples were analysed on a GmbH nanophotometer (Implen, Munich, Germany). The concentration of samples was determined by absorbance at 260nm. RNA purity was assessed by the ratio of absorbance at 260nm/280nm, and excess ethanol was detected by 260nm/230nm absorbance ratio. DNAase was added to eliminate DNA contamination.

Reverse Transcription to cDNA

1000µg mRNA was aliquoted from the samples. Nuclease free water was added to a total volume of 15µl. Reverse transcription of mRNA to cDNA was performed using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories, California USA). 4µL of iScript Reaction Mixture, and 1µL of iScript Reverse Transcriptase was added to each tube. A T100 Thermal Cycler (Bio-Rad Laboratories, California, USA) was used to heat the samples. Samples were heated at 25^{o}C for 5 minutes for priming, then for 20minutes at 46^{o}C to facilitate reverse transcription and at 95^{o}C for 1 minute to terminate the reaction.

Quantitative real time polymerase chain reaction (qRT-PCR)

mRNA expression of interleukin 6 (IL-6), interleukin 1β (IL-1β) and C-C motif chemokine ligand 2 (CCL-2) was determined by qRT-PCR reactions. Initially a series of primers were tested for their efficiency at dilutions of 0.2, 0.1, 0.02, 0.01, once primers were determined to be efficient the samples were run. Samples were run in triplicate on a 384 well plate. Firstly, a master mix was made; containing 5µl of Sybr Green 2x (Qiagen, Hilden, Germany) 0.5µL

of Forward Primer (20mM) and 0.5μL of Reverse Primer (20mM). This was added in each well to 4μL of cDNA to a total of 10μL per well. The plate was centrifuged at 1000 rpm, for 1 minute at room temperature. Samples were placed in a thermal cycler, (CFX384 Touch™ Real-Time PCR Detection System) (Bio-Rad Laboratories, California, USA) to run the reaction. Samples were heated to 94^{o}C for 2 minutes for initial denaturation, followed by 40 cycles at 94^{o}C for 15 seconds for further denaturing, and at 60^{o}C for 1 minute for annealing, extension and for reading of the fluorescence. A housekeeping gene, HRPT was used to control for differences in cDNA loading. A critical threshold (C_T) method was used to calculate the relevant quantities of each transcript.

18.1.1.5 Serum cytokine levels

Concentrations of the cytokines IL-1β, IL-6, IL-10 and TNF-α in serum was determined by enzyme-linked immunosorbent assays (ELISA), using the Human IL-6, Human IL-1β, Human IL-10 and Human TNF-α ELISA kits (Invitrogen, Maryland, USA).

Samples and standards were run in triplicates. Serum and standards (100μL each) were added to wells on a 96-well plate, and incubated for 2 hours at room temperature. Samples were thoroughly aspirated and well washed 4 times. 100μL of biotinylated anti-IL-1β, anti-IL-6, anti-IL-10 or TNF-α antibody solution (Invitrogen, Maryland, USA) was added to each well and incubated for 1 hour at room temperature. Samples were again thoroughly aspirated and well washed 4 times. 100μL of Streptavidin-HRP Working Solution (Invitrogen, Maryland, USA) was added to each well and incubated at room temperature for 30 minutes. Samples were once more thoroughly aspirated and well washed 4 times. Finally, 100μL of Stabilised Chromogen (Invitrogen, Maryland, USA) and incubated for 30 minutes at room temperature in a dark room before Stop Solution (Invitrogen, Maryland, USA) was used to cease the reaction. Absorbance of each well was read at 450nm on a spectrophotometer (Implen, Munich, Germany).

Standard curves were constructed with a minimum acceptable coefficient of determination (r^2) of 0.91. Sample absorbance of duplicates was averaged and plotted on standard curves to determine cytokine concentration in pg/ml.

18.1.2 **Supplementary Tables**

Supplementary Table 6.1: Definitions of metabolically healthy obesity.

Group	Metabolic health definition
NCEP ATP III (55)	Less than three of: - SBP >130mmHg and/or DBP >85mmHg - Triglycerides ≥1.70 mmol/L - HDL <1.03 mmol/L (males) or <1.29 mmol/L (females) - FBG ≥5.6 mmol/L
Aguilar-Salinas, 2008 (654)	All of: - Systolic blood pressure (SBP) <140mmHg and diastolic blood pressure (DBP) <90mmHg, or no treatment - High density lipoprotein (HDL) ≥1.04 - Fasting blood glucose (FBG) <7.00 and no treatment
Brochu (655)	Percent body fat ≥35% Insulin sensitivity by hyperinsulinaemic-euglycaemic (HE) clamp (>8.0 mg/min x kg of lean body mass)
Karelis, 2005 (656)	BMI >27 Ratio of glucose disposal (M) by HE clamp test to fat free mass (FFM). - Metabolically healthy obese (MHO) were those in the upper quartile (M /FFM ≥12.62) - Metabolically abnormal obese (MAO) were those in the lower quartile (M/FFM ≤9.29)
Karelis, 2004 (657)	Four or more of: - Triglycerides ≤1.70 - HDL ≥1.30 and no treatment - Low density lipoprotein (LDL) ≤2.60 and no treatment - Total cholesterol ≥5.20 - Homeostatic model of assessment (HOMA) ≤1.95
Blüher (658)	All of: - FBG <7.0 mmol/L and glycosylated haemoglobin (HbA1c) <6.0% - SBP <140mmHg and DBP <85mmHg - Leucocyte count <800 Gpt/l - C-reactive protein (CRP) <5.0mg/dl No clinical evidence of cardiovascular or peripheral vascular disease
Wildman, 2008 (659)	Less than two of: - SBP ≥130mmHg or DBP ≥85mmHg or treatment - Triglycerides ≥1.70 - HDL <1.04 (males) or <1.30 (females) or treatment - FBG ≥5.55 or treatment - HOMA >90th percentile - CRP >90th percentile
Meigs, 2006 (660) (Metabolic syndrome variables)	Less than three of: - SBP ≥130 or DPB ≥85 or treatment - Triglycerides ≥1.70 - HDL <1.04 (males) or <1.30 (females) or treatment - FBG ≥5.55 or treatment - Waist circumference >102cm (males) or >88cm (females)
Meigs, 2006 (660) (Homeostatic model only)	HOMA <75th percentile among non-diabetic subjects

SBP – systolic blood pressure; DBP – diastolic blood pressure; MHO – metabolically healthy obese; MAO – metabolically abnormal obese; HE test - hyperinsulinaemic-euglycaemic clamp test; M – glucose disposal measured by HE test; FFM – fat free mass; BSL – blood sugar level; FBG – fasting blood glucose; HDL – high density lipoprotein; LDL – low density lipoprotein; HbA1c – glycosylated haemoglobin; HOMA – homeostatic model of assessment; CRP – C-reactive protein

Supplementary Table 6.2: Distribution of liver pathology in body mass index and metabolic health status categories

Histological variable		All patients	BMI <40	BMI 40-50	BMI >50	p-value	MHO	Borderline	MAO	p-value
n=		216	58 (26.9%)	106 (49.1%)	52 (24.1%)		18 (8.3%)	73 (33.8%)	125 (57.9%)	
Steatosis	S0 (<5% steatosis)	60 (27.7%)	21 (36.2%)	29 (27.3%)	10 (19.2%)	0.334	5 (27.7%)	23 (31.5%)	32 (25.6%)	0.692
	S1 (5-33% steatosis)	63 (29.1%)	18 (31%)	30 (28.3%)	15 (28.8%)		7 (38.8%)	22 (30.1%)	34 (27.2%)	
	S2 (34-66% steatosis)	69 (31.9%)	14 (24.1%)	37 (34.9%)	18 (34.6%)		4 (22.2%)	23 (31.5%)	42 (33.6%)	
	S3 (67-100% steatosis)	24 (11.1%)	5 (8.6%)	10 (9.4%)	9 (17.3%)		2 (11.1%)	5 (6.8%)	17 (13.6%)	
Inflammation	0 (no lobular inflammation)	121 (56%)	35 (60.3%)	65 (61.3%)	21 (40.3%)	0.082	10 (55.5%)	46 (63%)	65 (52%)	0.684
	1 (<2 foci per x200 field)	84 (38.8%)	22 (37.9%)	35 (33%)	27 (51.9%)		7 (38.8%)	24 (32.8%)	53 (42.4%)	
	2 (2-4 foci per x200 field)	11 (5%)	1 (1.7%)	6 (5.6%)	4 (7.6%)		1 (5.5%)	3 (4.1%)	7 (5.6%)	
	3 (>4 foci per x200 field)	-	-	-	-		-	-	-	
Ballooning	0 (no ballooning)	134 (62%)	38 (65.5%)	69 (65%)	27 (51.9%)	0.412	13 (72.2%)	48 (65.7%)	73 (58.4%)	0.336
	1 (few ballooned cells)	63 (29.1%)	17 (29.3%)	27 (25.4%)	19 (36.5%)		4 (22.2%)	22 (30.1%)	37 (29.6%)	
	2 (many ballooned cells)	19 (8.7%)	3 (5.1%)	10 (9.4%)	6 (11.5%)		1 (5.5%)	3 (4.1%)	15 (12%)	
Fibrosis	F0	150 (69.4%)	45 (77.5%)	77 (72.6%)	28 (53.8%)	0.215	12 (66.6%)	58 (79.4%)	80 (64%)	0.273
	F1	55 (25.4%)	12 (20.6%)	24 (22.6%)	19 (36.5%)		5 (27.7%)	14 (19.1%)	36 (28.8%)	
	F2	4 (1.8%)	0 (0%)	2 (1.8%)	2 (3.8%)		1 (5.5%)	1 (1.3%)	2 (1.6%)	
	F3	4 (1.8%)	1 (1.7%)	1 (0.9%)	2 (3.8%)		0 (0%)	0 (0%)	4 (3.2%)	
	F4	3 (1.3%)	0 (0%)	2 (1.8%)	1 (1.9%)		0 (0%)	0 (0%)	3 (2.4%)	
CRN NAS classification (481)	Not NASH	128 (59.5%)	38 (65.5%)	66 (62.2%)	24 (47%)	0.069	11 (61.1%)	48 (65.7%)	69 (55.6%)	0.088
	Equivocal	56 (26%)	16 (27.5%)	26 (24.5%)	14 (27.4%)		6 (33.3%)	20 (27.3%)	30 (24.1%)	
	Diagnostic for NASH	31 (14.4%)	4 (6.8%)	14 (13.2%)	13 (25.4%)		1 (5.5%)	5 (6.8%)	25 (20.1%)	
Histological groups for analysis	Normal	56 (25.9%)	20 (34.4%)	28 (26.4%)	8 (15.3%)	0.108	4 (22.2%)	23 (31.5%)	29 (23.2%)	0.066
	Non-NASH NAFLD	123 (56.9%)	32 (55.1%)	62 (58.4%)	29 (55.7%)		13 (72.2%)	44 (60.2%)	66 (52.8%)	
	NASH	26 (12%)	5 (8.6%)	11 (10.3%)	10 (19.2%)		0 (0%)	5 (6.8%)	21 (16.8%)	
	Steatofibrosis	11 (5%)	1 (1.7%)	5 (4.7%)	5 (9.6%)		1 (5.5%)	1 (1.3%)	9 (7.2%)	
BMI category	BMI <40	58 (26.9%)					8 (44.4%)	13 (17.8%)	36 (29.0%)	0.078
	BMI 40-50	106 (49.1%)					7 (38.9%)	44 (60.3%)	55 (44.4%)	
	BMI >50	52 (24.1%)					3 (16.7%)	16 (21.9%)	33 (26.6%)	
Metabolic health status	MHO	18 (8.3%)	8 (14.0%)	7 (6.6%)	3 (5.8%)	0.078				
	Borderline	73 (33.8%)	13 (22.8%)	44 (41.5%)	16 (30.8%)					
	MAO	125 (57.9%)	36 (63.2%)	55 (51.9%)	22 (63.5%)					

Data shown as number (percentage). Chi-square test used for assessment of significance

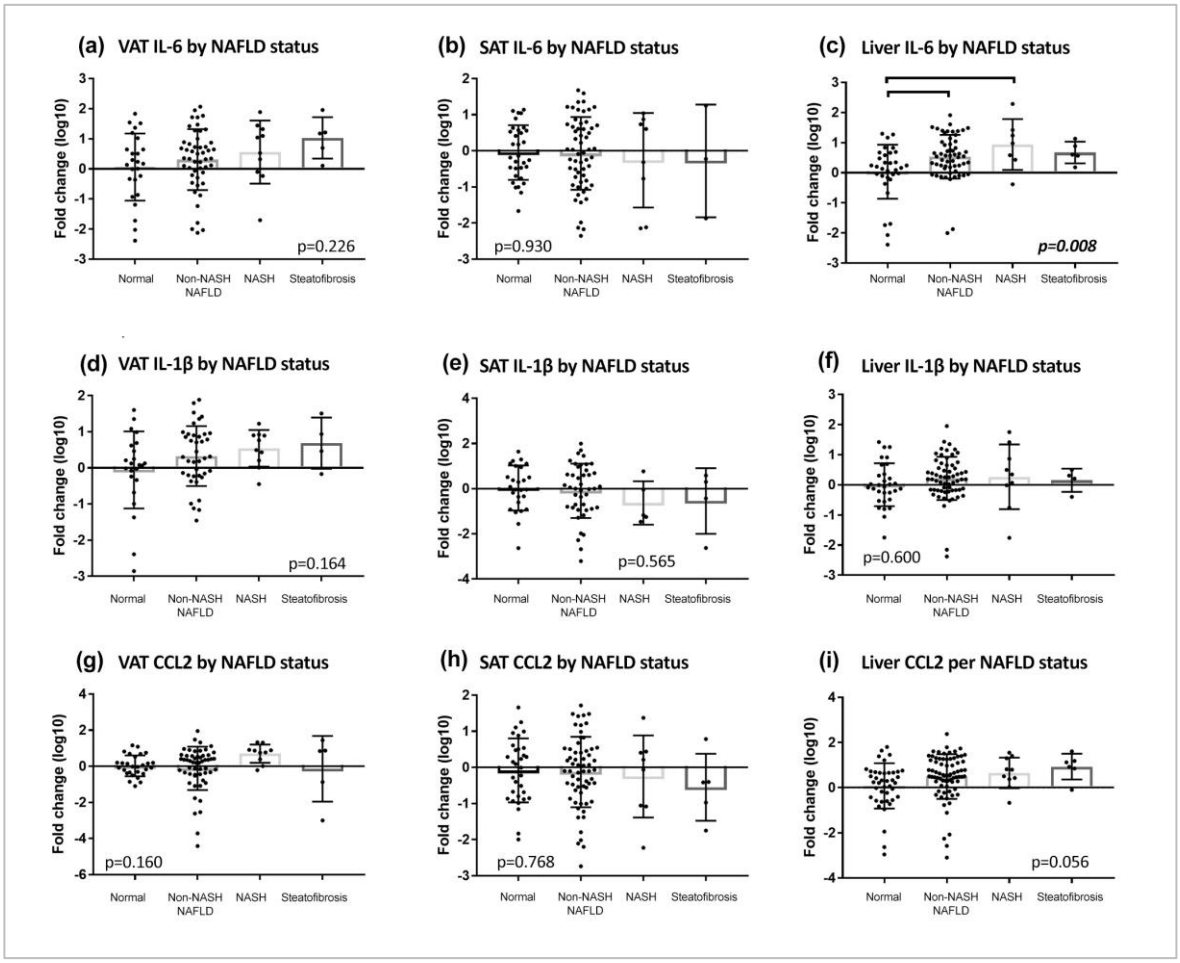
Supplementary Table 6.3: Univariate and multivariate analysis of clinical factors associated with histological NASH, fibrosis and steatofibrosis.

Histological diagnosis	Variable	Beta (SE)	p-value
Multivariate analysis			
NASH/steatofibrosis	Body mass index	0.063 (0.020)	**0.002**
	Metabolic health status	1.436 (0.458)	**0.002**
Any fibrosis	Body mass index	0.013 (0.002)	**<0.001**
	Gender	0.223 (0.074)	**0.003**
Steatofibrosis	Body mass index	0.004 (0.002)	**0.019**
	Gender	0.088 (0.036)	**0.017**
Univariate analysis			
NASH/steatofibrosis	Metabolic health status	1.103 (0.388)	**0.004**
	Body mass index	0.062 (0.019)	**0.001**
	Age	0.004 (0.015)	0.805
	Gender	0.632 (0.388)	0.103
	Smoking	0.069 (0.256)	0.789
	OSA	0.307 (0.382)	0.422
Any fibrosis	Metabolic health status	0.347 (0.242)	0.151
	Body mass index	0.056 (0.017)	**0.001**
	Age	0.000 (0.012)	0.999
	Gender	0.869 (0.329)	**0.008**
	Smoking	0.139 (0.211)	0.510
	OSA	0.650 (0.314)	**0.039**
Steatofibrosis	Metabolic health status	0.730 (0.612)	0.233
	Body mass index	0.053 (0.027)	0.052
	Age	0.046 (0.028)	0.102
	Gender	1.800 (0.649)	**0.006**
	Smoking	0.143 (0.438)	0.745
	OSA	0.724 (0.625)	0.247

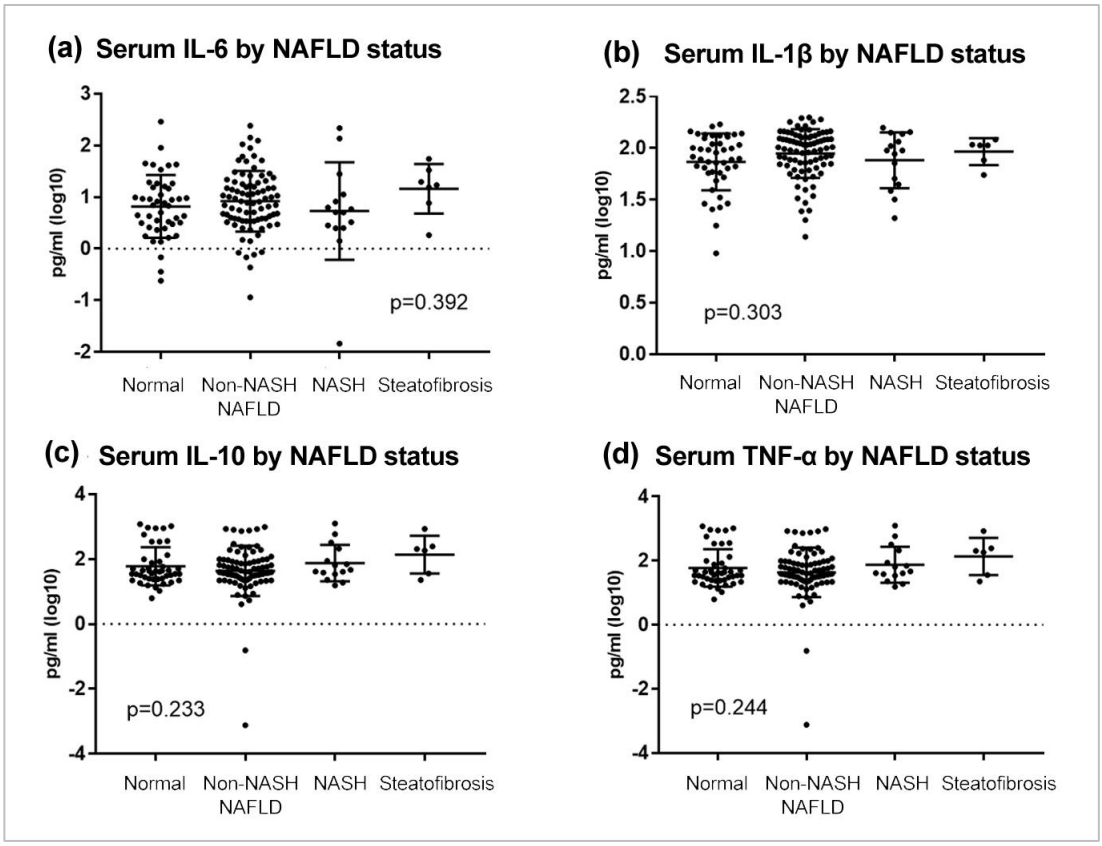
NASH – nonalcoholic steatohepatitis; SE – standard error.
Variables entered: Metabolic health status (Metabolically healthy obesity (MHO)/ borderline MHO/ metabolically abnormal obesity (MAO)), body mass index, age, gender, smoking status, obstructive sleep apnoea status.

18.1.3 **Supplementary Figures**

Supplementary Figure 6.1: Changes in visceral adipose tissue (VAT), subcutaneous adipose tissue (SAT) and liver inflammatory markers with nonalcoholic fatty liver disease (NAFLD) category. (a-c) interleukin-6 (IL-6), (d-f) interleukin 1β (IL-1β) and (g-i) C-C motif chemokine ligand 2 (CCL2)



Supplementary Figure 6.2: Comparison of blood cytokines (interleukin-6 (IL-6), interleukin-1β (IL-1β), interleukin-10 (IL-10) and tumour necrosis factor alpha (TNF-α)) between nonalcoholic fatty liver disease categories, showing no differences in circulating cytokines.



18.2 Appendix 2: Supplementary materials - Systematic review and meta-analysis: Non-invasive detection of nonalcoholic fatty liver disease related fibrosis in the obese

18.2.1 Supplementary Tables

Supplementary Table 7.1: Study specific QUADAS-2 criteria

Domain 1: Patient selection

A. Risk of Bias		
Describe method of patient selection	What was the setting they were recruited from? Consecutive, random, other. Inclusion and exclusion criteria? Healthy people selected?	
Was a consecutive or random sample of patients enrolled?	Yes	If stated in the text.
	No	If other methods for enrolment stated.
	Unclear	If these details are not stated.
Was a case-control design avoided?	Yes	If healthy controls or other control group (e.g. HCV, ASH) was used as a comparison.
	No	If no comparison group recruited.
	Unclear	If unable to determine whether a control group was recruited.
Did the study avoid inappropriate exclusions?	Yes	If appropriate exclusions, e.g. other causes of liver disease (e.g. HCV, ASH, haemochromatosis).
	No	If patients excluded for no stated reason, or for reasons not stated in the exclusion criteria.
	Unclear	If exclusions not stated, or patients not included in final analysis for no stated reason.
Could the selection of patients have introduced bias?	Low	If all of the above is Yes.
	High	If any of the above is No.
	Unclear	If any of the above is unclear.
B. Concerns regarding applicability		
Describe included patients	Tested prior or initial test? How did they present to the setting? Why were the index tests performed? (research, diagnosis, confirmation)	
Is there concern that the included patients do not match the review question? (see above)	Low	Matches the review question – Obese patients. Diagnosing NAFLD.
	High	From non-obese population.
	Unclear	Adequate description of recruitment not available.

Domain 2: Index test(s)

A. Risk of Bias		
Describe the index test	How was it conducted? How was it interpreted? (elastography techniques – Same person performing test? Expert? Blinded?) (Patented blood tests – Same lab? Same company?)	
Were the index test results interpreted without knowledge of the results of the reference standard?	Yes	If blinding of any tests that require interpretation/subjective assessment (e.g. Fibroscan) was done.
	No	If no blinding done.
	N/A	If objective (e.g. blood test, or calculated score with defined algorithm)
	Unclear	If not stated.
If a threshold was used, was it pre-specified?	Yes	If standard or pre-specified threshold are used.
	No	If threshold developed within study to be optimal, and not validated within study.
	N/A	If study developed the score, developed the threshold, and subsequently validated this.
	Unclear	If not stated.
Could the conduct or interpretation of the index test have introduced bias?	Low	If Yes to questions.
	High	If No to any of questions.
	Unclear	If any of above are not stated.
B. Concerns regarding applicability		
Is there concern that the index test, its conduct or interpretation differ from the review question?	Low/High/Unclear High, if not one of the index texts specified by review, or alteration to test.	

Domain 3: Reference standard

A. Risk of Bias		
Describe the reference standard and how it was conducted and interpreted.	Liver biopsy (as per inclusion criteria). Intraoperative or percutaneous? Quality assessment performed? How many pathologists? Specialist liver pathologist? Blinded?	
Were the reference standard results interpreted without knowledge of the results of the index test?	Yes	If blinding was done.
	No	If blinding was not done.
	Unclear	If not stated.
Could the reference standard, its conduct or its interpretation have introduced bias.	Low	Blinded pathologist, specialist liver pathologist, and quality of liver biopsies stated.
	High	Not blinded. Non-specialist or not experienced pathologist. No quality assessment of liver biopsy.

	Unclear	Not stated if blinded, not stated if quality assessed or liver pathologist used.
B. Concerns regarding applicability		
Is there concern that the target condition as defined by the reference standard does not match the review question?		Not applicable (all chosen with liver biopsy)

Domain 4: Flow and timing

A. Risk of Bias		
Describe any patients who did not receive either the index or reference standard (from flow diagram).		
Describe the time interval and any interventions between index test and reference standard.		Within 6 months.
Was there an appropriate interval between index test and reference standard?	Yes	If liver biopsy and test performed within 6 months.
	No	If performed beyond 6 months.
	Unclear	If not stated.
Did all patients receive the reference standard?	Yes	If everyone had a liver biopsy.
	No	If not everyone got a liver biopsy (e.g. control group, or did not reach threshold for liver biopsy).
	Unclear	If liver biopsy status not stated (not mentioned whether it was performed for all patients, or just a proportion of patients)
Were all patients included in the analysis?	Yes	All patients included in the analysis.
	No	Some patients initially recruited and included were left out (e.g. did not get liver biopsy)
	Unclear	Does not specify who was included in the analysis.
Could the patient flow have introduced bias?	Low	If yes to all above.
	High	If no to any above.
	Unclear	(If yes or unclear to the above questions).

Supplementary Table 7.2a: Summary of number of studies and participants used to assess each test for each fibrosis level in all studies.

	Any fibrosis (F1-4)	Significant fibrosis (F2-4)	Advanced fibrosis (F3-4)	Cirrhosis (F4)
NFS	3 studies n=577 (101-331)	6 studies n=1337 (52-452)	22 studies* n=5462 (52–827)	1 study n=88
BARD	3 studies n=504 (101-258)	4 studies n=1108 (101-452)	17 studies* n=4025 (56–827)	1 study n=242
FIB4	2 studies n=246 (101-145)	4 studies n=1108 (101-452)	13 studies* n=2953 (56–541)	1 study n=242
Fibrometer	1 study n=452	2 studies n=540 (88-452)	4 studies n=827 (88-452)	1 study n=88
Fibrotest	1 study n=288	4 studies n=1249 (242-452)	3 studies n=961 (242-452)	1 study n=242
ELF	1 study n=192	2 studies n=233 (41-192)	2 studies n=248 (56–192)	
Hepascore		2 studies n=694 (242-452)	2 studies n=694 (242-452)	1 study n=242
TE		9 studies n=1249 (41–452)	7 studies n=1145 (52–452)	3 studies n=454 (75–291)
ARFI	2 studies n=153 (28-125)	3 studies n=457 (41-291)	3 studies n=588 (125-291)	2 studies n=416 (125-291)
SWE		1 study n=232	1 study n=232	1 study n=232
MRE	2 studies n=242 (117-124)	2 studies n=242 (117-125)	4 studies* n=486 (102-142)	2 studies n=242 (117-125)

**includes study with some overlapping patients, but not included in meta-analysis*

Supplementary Table 7.2b: Summary of number of studies and participants used to assess each test for each fibrosis level in Obese-only studies.

	Any fibrosis (F1-4)	Significant fibrosis (F2-4)	Advanced fibrosis (F3-4)	Cirrhosis (F4)
NFS	2 studies n=432 (101-331)	3 studies n=745 (101-331)	6 studies n=1216 (88-331)	
BARD	2 studies n=359 (101-258)	2 studies n=414 (101-313)	3 studies n=672 (101-313)	
FIB4	1 study n=101	2 studies n=414 (101-313)	2 studies n=414 (101-313)	
Fibrotest	1 study n=288	1 study n=288		
ELF		1 study n=41		
TE		3 studies n=216 (75–100)	2 studies n=175 (75-100)	1 study n=75
ARFI	1 study n=28	1 study n=41		

Supplementary Table 7.3a: Meta-analysis with pooled positive likelihood ratio (LR+), negative likelihood ratio (LR-) and diagnostic odds ratio (DOR) for detection of each level of fibrosis

	Studies (n_{total}=)	SROC	LR+ Pooled	Hetero-geneity	LR- Pooled	Hetero-geneity	DOR Pooled	Hetero-geneity
F1-4								
NFS (low threshold)	2 (432)		1.484 (1.257-1.753)	Chi-sq=0.63 p=0.427 I^1=0%	0.481 (0.368-0.629)	Chi-sq=0.29 p=0.590 I^1=0%	6.932 (3.835-12.532)	Chi-sq=0.81, p=0.368 I^2=0.0%
NFS (high threshold)	2 (476)		2.598 (0.970-6.954)	Chi-sq=6.74 p=0.009 I^1=85.2%	0.389 (0.078-1.933)	Chi-sq=20.65 p=0 I^1=95.2%	3.100 (2.033-4.725)	Chi-sq=0.42, p=0.518 I^2=0.0%
FIB4	2 (246)		2.502 (0.199-31.502)	Chi-sq=9.72 p=0.002 I^1=89.7%	0.670 (0.253-1.775)	Chi-sq=44.70 p=0 I^1=97.8%	3.751 (0.137-102.77)	Chi-sq=13.17, p<0.001 I^2=92.4%
BARD	3 (504)	0.6068 ±0.1143 Q* 0.5805 ±0.0871	1.393 (1.246-1.558)	Chi-sq=1.61 p=0.448 I^1=0%	0.545 (0.352-0.844	Chi-sq=2.82 p=0.244 I^1=29.2%	2.796 (1.440-5.430)	Chi-sq=2.62, p=0.270 I^2=23.6%
MRE	2 (242)		5.530 (2.912-10.501)	Chi-sq=0.15 p=0.699 I^1=0%	0.537 (0.405-0.714)	Chi-sq=2.44 p=0.118 I^1=59.0%	10.513 (4.909-22.513)	Chi-sq=0.48, p=0.490 I^2=0.0%
F2-4								
NFS (low threshold)	3 (471)	0.7680 ±0.0346 Q* 0.7084 ±0.0291	2.655 (1.010-6.979)	Chi-sq=15.33 p=0 I^1=87.0%	0.478 (0.372-0.614)	Chi-sq=1.61 p=0.448 I^1=0%	5.179 (2.907-9.226)	Chi-sq=2.39, p=0.303 I^2=16.2%
NFS (high threshold)	2 (432)		1.901 (0.436-8.290)	Chi-sq=3.94 p=0.047 I^1=74.6%	0.829 (0.489-1.405)	Chi-sq=20.35 p=0 I^1=95.1%	2.263 (0.333-15.366)	Chi-sq=5.16, p=0.023 I^2=80.6%
BARD	2 (343)		1.301 (1.003-1.687)	Chi-sq=1.51 p=0.220 I^1=33.6%	0.783 (0.640-0.956	Chi-sq=0.21 p=0.650 I^1=0%	1.871 (1.156-3.030)	Chi-sq=0.01, p=0.938 I^2=0.0%
FIB4	2 (343)		4.478 (2.816-7.121)	Chi-sq=0.25 p=0.621 I^1=0%	0.701 (0.267-1.840)	Chi-sq=59.60 p=0 I^1=98.3%	8.565 (4.592-15.973)	Chi-sq=0.02, p=0.901 I^2=0.0%
Fibrotest (low threshold)	2 (509)	0.8265 ±0.0292 Q* 0.7595 ±0.0265	2.798 (1.999-3.917)	Chi-sq=4.88 p=0.087 I^1=59.0%	0.392 (0.224-0.685)	Chi-sq=7.75 p=0.021 I^1=74.2%	7.225 (3.019-17.294)	Chi-sq=7.24, p=0.027 I^2=72.4%
Fibrotest (high threshold)	2 (555)	0.7292 ±0.4068 Q* 0.6764 ±0.3305	8.489 (3.022-23.851)	Chi-sq=0.14 p=0.935 I^1=0%	0.905 (0.835-0.980)	Chi-sq=2.48 p=0.289 I^1=19.4%	9.822 (3.283-29.386)	Chi-sq=0.08, p=0.962 I^2=0.0%
ELF	2 (231)		3.661 (2.529-5.300)	Chi-sq=0.51 p=0.473 I^1=0%	0.368 (0.259-0.522)	Chi-sq=0.04 p=0.846 I^1=0%	9.706 (5.042-18.682)	Chi-sq=0.15, p=0.704 I^2=0.0%
TE	8 (709)	0.8509 ±0.0171 Q* 0.7820 ±0.0162	4.108 (2.402-7.026)	Chi-sq=49.70 p=0 I^1=85.9%	0.302 (0.221-0.411)	Chi-sq=17.57 p=0.014 I^1=60.2%	13.898 (8.441-22.883)	Chi-sq=11.65, p=0.113 I^2=39.9%
ARFI	3 (398)	0.8366 ±0.0239 Q* 0.7687 ±0.0221	2.589 (1.302-5.151)	Chi-sq=24.52 p=0 I^1=87.8%	0.318 (0.164-0.617)	Chi-sq=14.17 p=0.003 I^1=78.8%	10.406 (6.491-16.682)	Chi-sq=2.82, p=0.421 I^2=0.0%
MRE	2 (242)		9.892 (4.863-20.120)	Chi-sq=1.36 p=0.244 I^1=26.4%	0.379 (0.274-0.524)	Chi-sq=0.21 p=0.644 I^1=0%	26.417 (11.187-62.382)	Chi-sq=1.16, p=0.281 I^2=14.0%

SROC–summary receiver operator characteristic curve; LR+ - positive likelihood ratio; LR- - negative likelihood ratio; DOR–diagnostic odds ratio; NFS–NAFLD fibrosis score, TE–transient elastography, ELF–enhanced liver fibrosis score; MRE–magnetic resonance elastography.

Supplementary Table 7.3b: Meta-analysis with pooled positive likelihood ratio (LR+), negative likelihood ratio (LR-) and diagnostic odds ratio (DOR) for detection of each level of fibrosis

	Studies (n_{total}=)	SROC	LR+ Pooled	Hetero-geneity	LR- Pooled	Hetero-geneity	DOR Pooled	Hetero-geneity
F3-4								
NFS (low threshold)	16 (2528)	0.7947 ±0.0198 Q* 0.7313 ±0.0172	2.296 (1.903-2.769)	Chi-sq=157.19 p=0 I^1=88.5%	0.353 (0.290-0.429)	Chi-sq=29.19 p=0.046 I^1=38.3%	7.469 (5.471-10.195)	Chi-sq=33.01, p=0.017 I^2=45.5%
NFS (high threshold)	12 (2038)	0.8129 ±0.0531 Q* 0.7472 ±0.0472	7.258 (3.978-13.244)	Chi-sq=111.87 p=0 I^1=87.5%	0.607 (0.497-0.742)	Chi-sq=102.41 p=0 I^1=86.3%	14.415 (7.810-26.605)	Chi-sq=48.52, p<0.001 I^2=71.1%
BARD	12 (2589)	0.7253 ±0.0157 Q* 0.6732 ±0.0127	1.775 (1.585-1.989)	Chi-sq=33.50 p=0.001 I^1=61.2%	0.486 (0.383-0.617)	Chi-sq=37.62 p=0 I^1=65.4%	4.348 (3.523–5.365)	Chi-sq=13.02, p=0.446 I^2=0.2%
FIB4 (low threshold)	9 (2057)	0.8313 ±0.0147 Q* 0.7638 ±0.0134	2.964 (2.434-3.609)	Chi-sq=38.23 p=0 I^1=73.8%	0.330 (0.284-0.383)	Chi-sq=4.40 p=0.927 I^1=0%	10.165 (7.332-14.093)	Chi-sq=15.96, p=0.101 I^2=37.3%
FIB4 (high threshold)	6 (1247)	0.7696 ±0.0515 Q* 0.7098 ±0.0434	9.402 (4.185-21.120)	Chi-sq=36.57 p=0 I^1=80.9%	0.699 (0.622-0.786)	Chi-sq=12.03 p=0.100 I^1=41.8%	13.252 (8.769-20.025)	Chi-sq=6.90, p=0.439 I^2=0.0%
Fibrometer	4 (827)	0.7740 ±0.0923 Q* 0.7135 ±0.0782	2.278 (1.355-3.830)	Chi-sq=20.42 p=0 I^1=85.3%	0.471 (0.233-0.949)	Chi-sq=38.80 p=0 I^1=92.3%	4.893 (1.705-14.041)	Chi-sq=23.34, p<0.001 I^2=87.1%
Hepascore	2 (694)		3.592 (2.150-6.001)	Chi-sq=5.70 p=0.017 I^1=82.5%	0.374 (0.259-0.540)	Chi-sq=2.13 p=0.145 I^1=53.0%	9.832 (4.067-23.770)	Chi-sq=4.38, p=0.036 I^2=77.2%
Fibrotest (low threshold)	2 (719)	0.3587 ±0.2443 Q* 0.3931 ±0.1884	2.567 (1.700-3.874)	Chi-sq=15.44 p=0 I^1=87.0%	0.228 (0.101-0.511)	Chi-sq=3.54 p=0.170 I^1=43.5%	12.022 (3.562-40.575)	Chi-sq=5.28, p=0.071 I^2=62.1%
Fibrotest (high threshold)	2 (509)	0.8468 ±0.0615 Q* 0.7782 ±0.0578	6.688 (4.321-10.351)	Chi-sq=1.54 p=0.464 I^1=0%	0.643 (0.439-0.942)	Chi-sq=10.39 p=0.006 I^1=80.7%	14.025 (7.530-26.122)	Chi-sq=0.35, p=0.839 I^2=0.0%
ELF	2 (248)	0.9620 ±0.0396 Q* 0.9074 ±0.0588	10.244 (5.335-19.668)	Chi-sq=2.76 p=0.252 I^1=27.5%	0.179 (0.102-0.315)	Chi-sq=2.13 p=0.345 I^1=6.0%	84.860 (20.229–355.99)	Chi-sq=3.75, p=0.153 I^2=46.7%
TE	6 (1002)	0.8591 ±0.0167 Q* 0.7898 ±0.0160	3.557 (2.649-4.777)	Chi-sq=28.50 p=0 I^1=78.9%	0.175 (0.082-0.371)	Chi-sq=41.65 p=0 I^1=85.6%	16.647 (10.142-27.324)	Chi-sq=8.89, p=0.180 I^2=32.5%
ARFI	3 (496)	0.9017 ±0.0351 Q* 0.8330 ±0.378	4.355 (2.547-7.447)	Chi-sq=23.68 p=0 I^1=87.3%	0.173 (0.059-0.505)	Chi-sq=27.40 p=0 I^1=89.0	24.254 (10.518-55.930)	Chi-sq=7.97, p=0.0.47 I^2=62.4%
MRE	3 (384)	0.9651 ±0.0146 Q* 0.9121 ±0.0224	10.172 (7.241-14.291)	Chi-sq=1.46 p=0.691 I^1=0%	0.153 (0.094-0.249)	Chi-sq=1.93 p=0.587 I^1=0%	72.025 (35.623-145.62)	Chi-sq=2.09, p=0.553 I^2=0.0%
F4								
TE	3 (386)	0.8906 ±0.0240 Q* 0.8213 ±0.0250	4.724 (2.542-8.779)	Chi-sq=26.05 p=0 I^1=88.5%	0.190 (0.068-0.533)	Chi-sq=7.11 p=0.068 I^1=57.8%	20.513 (10.685-39.379)	Chi-sq=1.93, p=0.588 I^2=0.0%
ARFI	2 (361)	0.8781 ±0.0567 Q* 0.8085 ±0.0570	4.743 (2.231-10.084)	Chi-sq=14.21 p=0.001 I^1=85.9%	0.301 (0.087-1.040)	Chi-sq=14.69 p=0.001 I^1=86.4%	14.614 (5.216-40.945)	Chi-sq=4.95, p=0.0.84 I^2=59.6%
MRE	2 (242)		9.923 (6.061-16.245)	Chi-sq=0.04 p=0.849 I^1=0%	0.264 (0.114-0.615)	Chi-sq=0.97 p=0.324 I^1=0%	41.258 (11.783-144.47)	Chi-sq=0.64, p=0.422 I^2=0.0%

SROC–summary receiver operator characteristic curve; LR+ - positive likelihood ratio; LR- - negative likelihood ratio; DOR–diagnostic odds ratio; NFS–NAFLD fibrosis score, TE–transient elastography, ELF–enhanced liver fibrosis score; MRE–magnetic resonance elastography.

Supplementary Table 7.4: Transient elastography (TE) success and failure rates as reported by studies.

Study	Average BMI	Probe used	Failure rates	Unreliable	Comments
Aykut 2014	30.2	M, XL	-	-	Not reported
Boursier 2016	31.1±5.8	M	n=83 (14.1%)	n=47 (8.0%)	
Cassinotto 2016	32.1±6	M	n=42 (14.4%)	n=26 (8.9%)	
Dincses 2015	30.8±5.4	M, XL	-	-	Not reported
Ergelen 2015	30.6±5.4	M, XL	n=1 (1.1%)	-	M probe used routinely, with XL probe used for obese patients.
Ergelen 2016	30.4	M	n=1 (1.6%)	-	
Karlas 2015	46.8 (32.1-57.1)	XL	n=1 (2.4%)	n=20 (49%)	
Myers 2012	30 (29-38)	M, XL	16% (M probe) 1.1% (XL probe) 4.9% (XL probe and BMI≥40)	35% (M probe) 7% (XL probe)	Failure rate related to BMI category, but mitigated by using XL probe. Elasticity consistently lower with XL probe compared to M probe.*
Naveau 2014	42.3	M, XL	n=15 (11.0%)	n=11 (8.1%)	

**Results of XL probe used for meta-analysis; BMI – Body mass index.*
Values expressed as mean±standard deviation or median (interquartile range).

18.2.2 **Supplementary Text**

18.2.2.1 Diagnostic accuracy of biomarkers

Diagnostic accuracy of all tests assessed by the included studies are shown in **Supplementary Figures 7.2-7.5**.

NAFLD fibrosis score (NFS)

A description of for detection ≥F3 fibrosis is found in the main manuscript.

Three studies examined the accuracy of NAFLD fibrosis score for detection of ≥F1 fibrosis. The AUROC ranged from 0.615-0.740. With a low threshold, the pooled sensitivity was 48.9% (43.2-54.6%), specificity 81.6% (748-87.2%) and DOR 6.93 (3.84-12.5) (**Table 7.3**). High threshold yielded pooled sensitivity 76.6% (71.2-81.5%), specificity 48.7% (40.6-56.9%) and DOR 3.10 (2.03-4.73).

Six studies assessed accuracy in detecting ≥F2, with AUROC ranging from 0.593-0.734. For low threshold, the SROC was 0.768 ±0.035, with pooled sensitivity of 72.9% (65.2-79.7%), specificity 50.9% (45.3-56.6%) and DOR of 5.18 (2.91-9.23). For high threshold, the sensitivity was 35.1% (26.4-44.6%), specificity 88.1% (84.0-91.4%) and DOR 2.26 (0.33-15.37).

There was only one study that examined the detection of F4 fibrosis, with AUROC 0.678, sensitivity of 66.7% and specificity of 72.2%.

BARD

Seventeen studies assessed the accuracy of the BARD index in detecting ≥F2, ≥F3 and F4 fibrosis. Sixteen studies assessed the accuracy for ≥F3, with AUROC 0.601-0.816, sensitivity 33-100% and specificity 26-90%. The pooled estimates of sensitivity, specificity and DOR were 73.2% (69.7-76.5%), 57.8% (55.6-59.9%) and 4.3 (3.5-5.4) (**Table 7.3**). The area under the SROC was 0.725 (SE 0.016) with Q* 0.673 (**Figure 7.3**).

Four studies assessed ≥F2, with AUROC 0.503-0.698, pooled sensitivity 50.8% (41.6-60.1%) and pooled specificity 57.4% (50.6-64.0%). For ≥F1, the SROC was 0.507 (SE 0.114), with pooled sensitivity 73.6% (66.8-79.6%) and specificity 36.2% (30.9-41.8%). Only one study assessed accuracy for F4, with AUROC 0.746, sensitivity 52% and specificity 84%.

Fibrosis-4 (FIB4)

Fourteen studies assessed ≥F3 disease, with AUROC 0.744–0.866. The sensitivity using the low cut-off value (FIB4 <1.24-1.54) was 71–87%. Using the high cut-off value (FIB4 >2.67-3.25), the specificity was 78-100%. For studies using dual cut-off values, the indeterminate fraction varied from 5.7% to 37.8%, with an average of 28.9% of the cohort with scores between high and low thresholds. The pooled estimates of sensitivity, specificity and DOR using a high cut-off were 36.0% (30.7-41.7%), 95.0% (93.6-96.2%) and 13.3 (8.8-20.0). Using a low cut-off, these were 76.6% (72.9-80.0%), 72.6% (70.4-74.8%) and 10.2 (7.3-14.1) (**Table 7.3**). The area under the SROC was 0.770 (SE 0.052) with Q* 0.710 and 0.831 (SE 0.015) with Q* 0.7638 for high and low thresholds respectively (**Figure 7.3**).

For ≥F2, the AUROC ranged from 0.640-0.743. Pooled sensitivity and specificity were 45.0% (35.9-54.3%) and 91.9% (87.5-95.1%) respectively, reported by two studies using variable thresholds. For ≥F1 and F4, AUROC was 0.605-0.821 and 0.860.

Enhanced liver fibrosis score (ELF)

The results for ≥F3 fibrosis are described in the main manuscript.

One study examined the accuracy of ELF for detection for ≥F1 fibrosis. The AUROC was reported as 0.76, with sensitivity of 61% and specificity of 80%. Two studies assessed ≥F2 fibrosis, with only one reporting an AUROC of 0.820. The pooled sensitivity was 70.9% (59.6-80.6%), with pooled specificity of 81.6 (74.5-87.4%) and DOR 9.71 (5.04-18.7).

There were no studies looking at the accuracy of ELF for detection of cirrhosis.

Fibrometer

For Fibrometer, the AUROC for ≥F3 disease in the obese population was assessed by four studies at 0.706-0.862. The SROC was 0.774 (SE 0.092), with pooled sensitivity of 67.6% (62.1-72.8%), pooled specificity of 69.1% (64.9-73.1%) and DOR of 4.9 (1.7-14.0).

For ≥F1, ≥F2 and F4, the AUROC was 0.801, 0.622-0.764 and 0.745, assessed by one or two studies.

Fibrotest

There were four studies assessing diagnostic accuracy of Fibrotest. For ≥F3, three studies reported an AUROC of 0.736–0.920. With low cut-off (0.30–0.316), the sensitivity and

specificity were reported at 81-92% and 57-71%. The sensitivity and specificity for high cut-off (0.47-0.7) was 25-60% and 90-98%. The indeterminate fraction varied from 31.2-35.4%. The pooled sensitivity, specificity and DOR for low and high thresholds were 83.2% (77.4-88.0%), 63.0% (58.7-67.2%) and 12.0 (3.6-40.6), and 46.1% (35.4-57.0%), 94.3% (91.6-96.3%) and 14.0 (7.5-26.1) (**Table 7.3**). The area under the SROC for studies that used a high threshold was 0.846 (SE 0.062) with Q* 0.778 (**Figure 7.3**).

For ≥F2, the AUROC ranged from 0.707-0.860 in four studies. The sensitivity and specificity were 5-83% and 73-100% using a variety of threshold values. For those with dual thresholds, the indeterminate fraction varied from 30.7-34.9%. The pooled sensitivity, specificity and DOR for low and high thresholds were 66.7% (59.0-73.7%), 75.1% (70.1-79.6%) and 7.2 (3.0-17.3), and 13.2% (7.0-21.9%), 98.9% (97.5-99.6%) and 9.8 (3.3-29.4) (**Table 7.3**). The area under the SROC for studies using low and high thresholds were 0.827 (SE 0.029) with Q* 0.759 and 0.729 (SE 0.407) with Q* 0.676.

Hepascore

There were only two studies assessing the Hepascore for NAFLD in an obese population. The AUROC for detecting ≥F2, ≥F3 and F4 was 0.729-0.752, 0.778-0.814 and 0.907. Using optimal thresholds based on Youden cut-offs, the sensitivity and specificities were 51% and 88% for ≥F2 (threshold 0.44), 67-75% and 76-84% for ≥F3 (threshold 0.32-0.37) and 87% and 89% for F4 (threshold 0.70). The pooled sensitivity, specificity and DOR for detection of ≥F3 was 69.3% (62.9-75.3%), 79.3% (75.4-82.9%) and 9.8 (4.1-23.8).

Transient elastography (TE)

The results for the diagnostic accuracy of TE for ≥F3 fibrosis are described in the main manuscript.

Nine studies examined the diagnostic accuracy of TE for ≥F2 fibrosis. The AUROC ranged from 0.831-0.938. The SROC was 0.851 ±0.017, with pooled sensitivity of 75.8% (71.8-79.5%), specificity of 77.3 (73.0-81.3%) and DOR 13.9 (8.44-2.88).

Detection of F4 was examined in 3 studies, with excellent AUROC, ranging from 0.870-0.950. The SROC was 0.891 ±0.024. Pooled sensitivity was 81.8% (72.2-89.2%), specificity 78.3% (74.5-81.8%) and DOR 20.5 (10.7-39.4).

No studies attempted to assess diagnostic accuracy of TE for ≥F1 fibrosis in the setting of obesity.

Acoustic radiation force imaging (ARFI)

There were five studies assessing ARFI at all four fibrosis levels. The AUROC were good to excellent for ≥ F2, ≥F3 and F4, at 0.770-0.8484, 0.840-0.900 and 0.770-0.848. This correlated with good to excellent SROC of 0.8366 (SE 0.024), 0.912 (SE 0.035) and 0.878 (SE 0.057) respectively. Variable thresholds were used to assess diagnostic accuracy at each of these levels, with generally good to excellent sensitivity (**Supplementary Figures 7.2-7.5**). Pooled sensitivity, specificity and DOR for these three levels of fibrosis are seen in **Table 7.3**. Valid and reliable results were highly dependent on BMI, with decreasing accuracy as BMI increased.

Shearwave elastography (SWE)

One study assessed the diagnostic accuracy of SWE in this review (16), measuring accuracy for ≥F2, ≥F3 and F4 at optimum sensitivity and specificity (90%). The AUROC was 0.860, 0.890 and 0.880 for each level of fibrosis respectively. Based on an optimal sensitivity of 90%, specificity for ≥F2, ≥F3 and F4 was 50%, 71% and 72% with variable cut-off values of 6.3, 8.3 and 10.5.

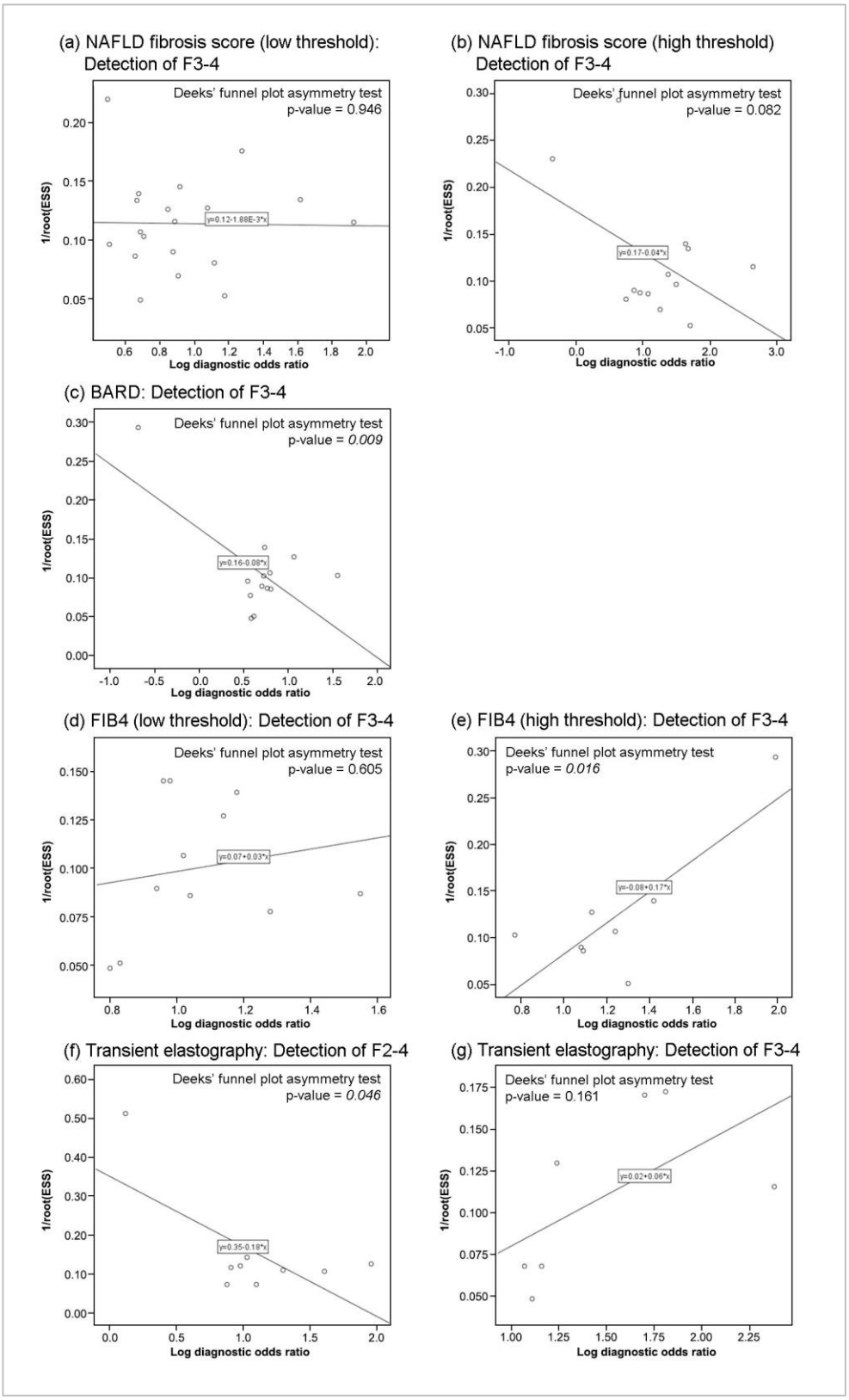
Magnetic resonance elastography (MRE)

The description of the diagnostic accuracy for MRE for ≥F3 fibrosis is in the main manuscript.

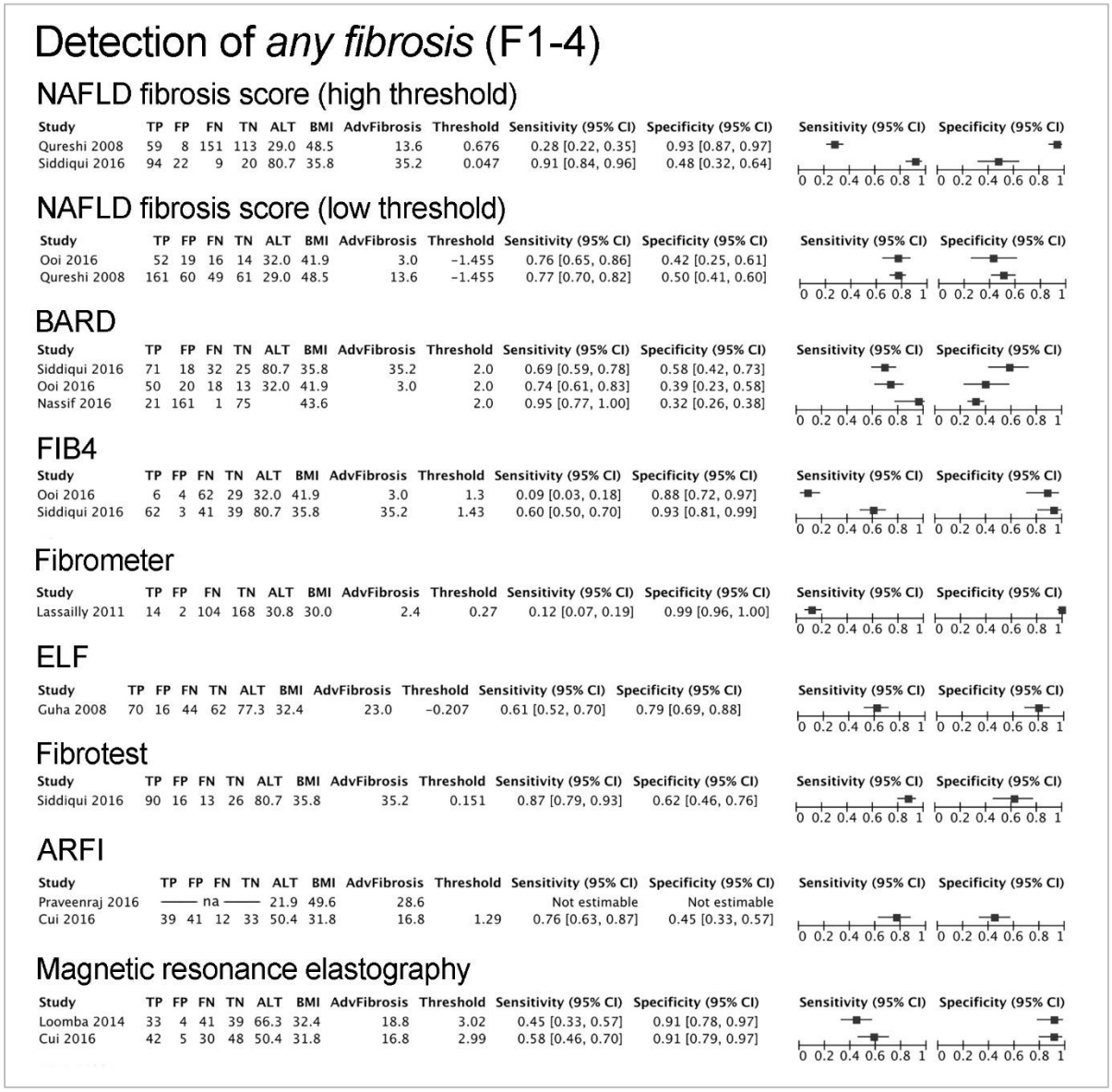
Two studies examined the diagnostic accuracy of MRE for ≥F1, ≥F2 and F4 fibrosis. Pooled sensitivity and specificity for ≥F1 was 51.4% (43.0-59.7%) and 90.6% (83.0-95.6%), and for ≥F2 was 64.7% (52.2-75.9%) and 93.7 (89.0-96.8%). For F4 fibrosis, the pooled sensitivity was 78.9% (54.4-93.9%), specificity was 91.9% (87.5-95.1%) and diagnostic OR was 41.3 (11.8-114.5).

18.2.3 **Supplementary Figures**

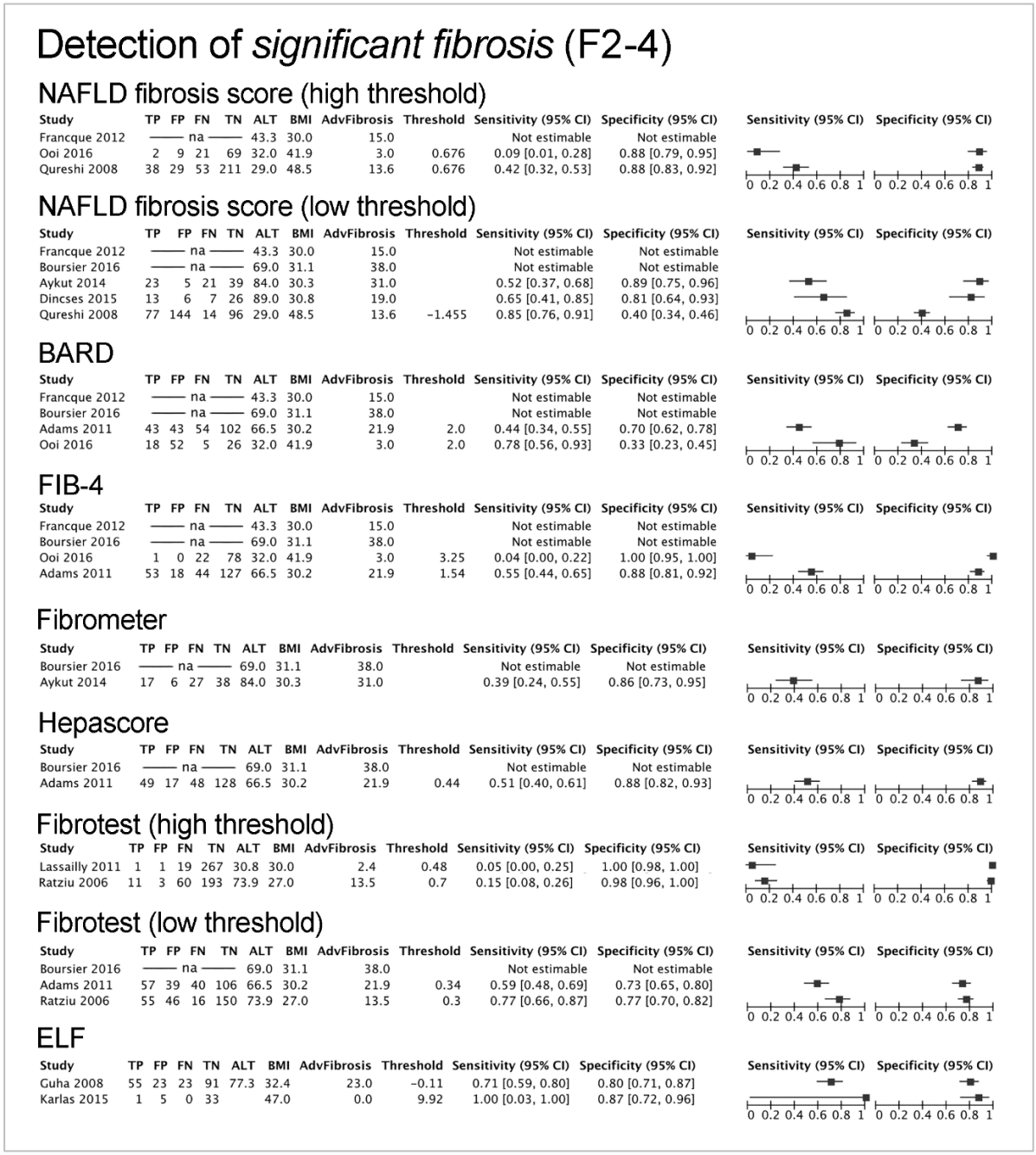
Supplementary Figure 7.1: Deeks' funnel plot analysis for evidence of publication bias for tests with ≥7 studies available for meta-analysis.



Supplementary Figure 7.2: Diagnostic accuracy of tests for detection of any fibrosis (F1-4)



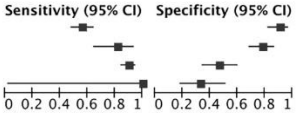
Supplementary Figure 7.3: Diagnostic accuracy of tests for detection of significant fibrosis (F2-4)



Detection of *significant fibrosis* (F2-4) *(continued)*

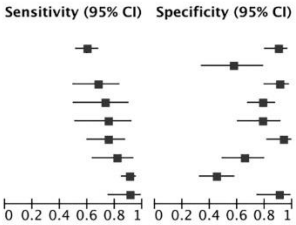
ARFI

Study	TP	FP	FN	TN	ALT	BMI	AdvFibrosis	Threshold	Sensitivity (95% CI)	Specificity (95% CI)
Cassinotto 2016	90	7	70	69	71.2	32.1	43.3	1.32	0.56 [0.48, 0.64]	0.91 [0.82, 0.96]
Cui 2016	27	20	6	72	50.4	31.8	16.8	1.34	0.82 [0.65, 0.93]	0.78 [0.68, 0.86]
Cassinotto 2016	144	35	16	31	71.2	32.1	43.3	0.95	0.90 [0.84, 0.94]	0.47 [0.35, 0.60]
Karlas 2015	1	24	0	12		47.0		1.35	1.00 [0.03, 1.00]	0.33 [0.19, 0.51]



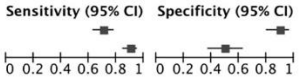
Transient elastography

Study	TP	FP	FN	TN	ALT	BMI	AdvFibrosis	Threshold	Sensitivity (95% CI)	Specificity (95% CI)
Boursier 2016	na				69.0	31.1	38.0		Not estimable	Not estimable
Cassinotto 2016	93	7	63	60	71.2	32.1	43.3	9.8	0.60 [0.51, 0.67]	0.90 [0.80, 0.96]
Karlas 2015	0	9	0	12		47.0	0.0	7.6	Not estimable	0.57 [0.34, 0.78]
Ergelen 2015	23	5	11	48	77.8	30.6	21.8	9.6	0.68 [0.49, 0.83]	0.91 [0.79, 0.97]
Naveau 2014	16	17	6	61	38.0	42.3	9.0	7.6	0.73 [0.50, 0.89]	0.78 [0.67, 0.87]
Dincses 2015	15	7	5	25	89.0	30.8	19.0	7.9	0.75 [0.51, 0.91]	0.78 [0.60, 0.91]
Aykut 2014	33	3	11	41	84.0	30.3	31.0	7.9	0.75 [0.60, 0.87]	0.93 [0.81, 0.99]
Myers 2012	26	15	6	28	55.0	30.0	29.3	6.4	0.81 [0.64, 0.93]	0.65 [0.49, 0.79]
Cassinotto 2016	141	37	15	30	71.2	32.1	43.3	6.2	0.90 [0.85, 0.95]	0.45 [0.33, 0.57]
Ergelen 2016	29	3	3	28		30.4		9.8	0.91 [0.75, 0.98]	0.90 [0.74, 0.98]



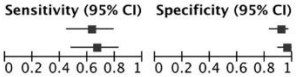
Shearwave elastography

Study	TP	FP	FN	TN	ALT	BMI	AdvFibrosis	Threshold	Sensitivity (95% CI)	Specificity (95% CI)
Cassinotto 2016	116	7	48	61	71.2	32.1	43.3	8.7	0.71 [0.63, 0.78]	0.90 [0.80, 0.96]
Cassinotto 2016	148	34	16	34	71.2	32.1	43.3	6.3	0.90 [0.85, 0.94]	0.50 [0.38, 0.62]

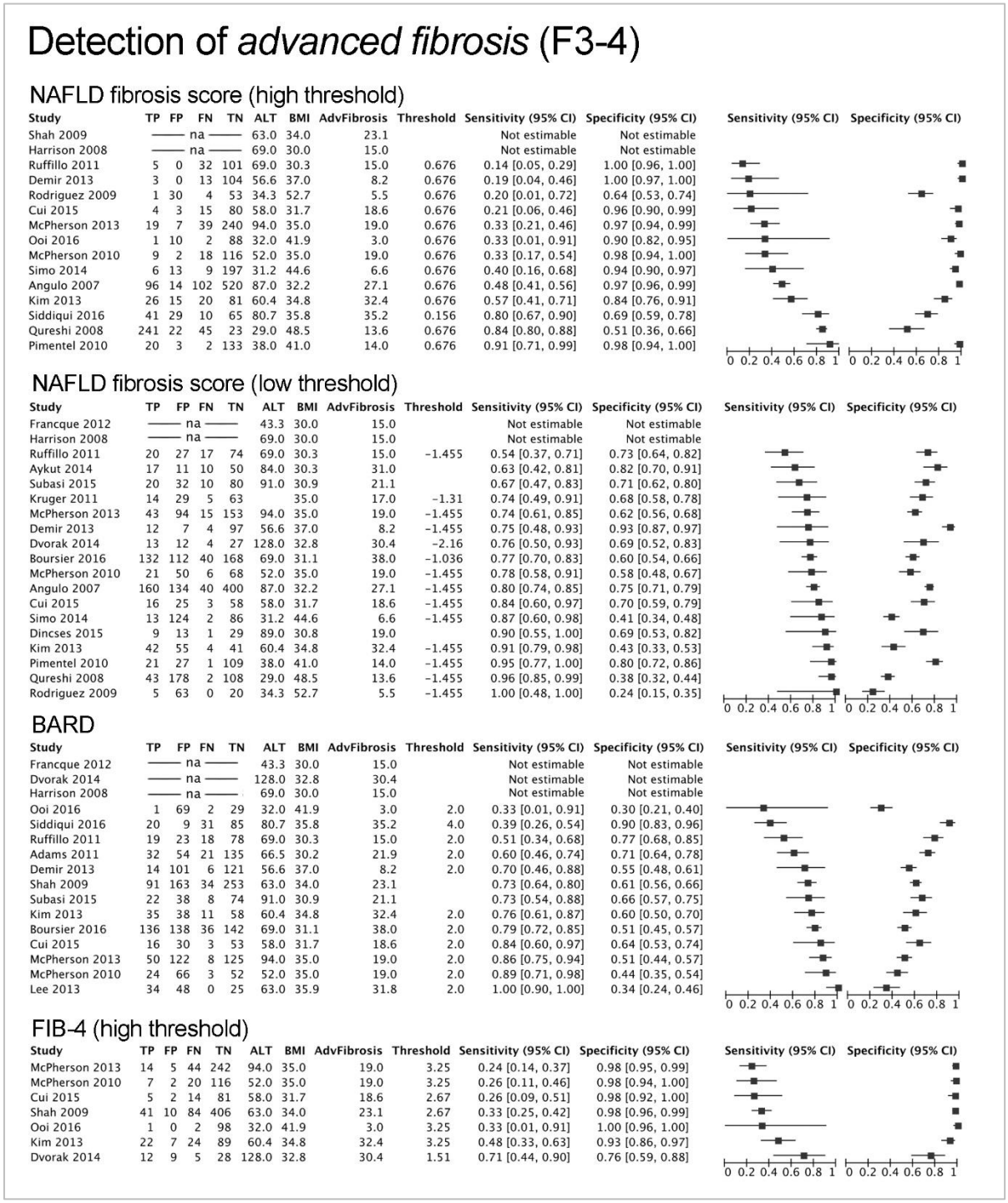


Magnetic resonance elastography

Study	TP	FP	FN	TN	ALT	BMI	AdvFibrosis	Threshold	Sensitivity (95% CI)	Specificity (95% CI)
Loomba 2014	22	7	13	75	66.3	32.4	18.8	3.58	0.63 [0.45, 0.79]	0.91 [0.83, 0.96]
Cui 2016	22	4	11	88	50.4	31.8	16.8	3.62	0.67 [0.48, 0.82]	0.96 [0.89, 0.99]



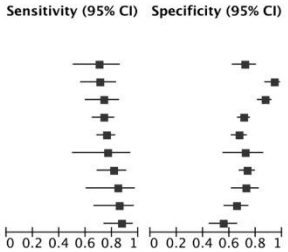
Supplementary Figure 7.4: Diagnostic accuracy of tests for detection of advanced fibrosis (F3-4)



Detection of *advanced fibrosis* (F3-4) (continued)

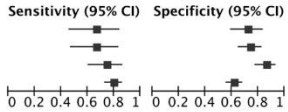
FIB-4 (low threshold)

Study	TP	FP	FN	TN	ALT	BMI	AdvFibrosis	Threshold	Sensitivity (95% CI)	Specificity (95% CI)
Francque 2012	na				43.3	30.0	15.0		Not estimable	Not estimable
Demir 2013	na				56.6	37.0	8.2		Not estimable	Not estimable
Subasi 2015	21	32	9	80	91.0	30.9	21.1		0.70 [0.51, 0.85]	0.71 [0.62, 0.80]
Siddiqui 2016	36	6	15	88	80.7	35.8	35.2	1.961	0.71 [0.56, 0.83]	0.94 [0.87, 0.98]
Adams 2011	39	25	14	164	66.5	30.2	21.9	1.54	0.74 [0.60, 0.85]	0.87 [0.81, 0.91]
Shah 2009	92	122	33	294	63.0	34.0	23.1	1.3	0.74 [0.65, 0.81]	0.71 [0.66, 0.75]
Boursier 2016	130	92	42	188	69.0	31.1	38.0	1.515	0.76 [0.68, 0.82]	0.67 [0.61, 0.73]
Dvorak 2014	13	11	4	28	128.0	32.8	30.4	1.24	0.76 [0.50, 0.93]	0.72 [0.55, 0.85]
McPherson 2013	47	66	11	181	94.0	35.0	19.0	1.3	0.81 [0.69, 0.90]	0.73 [0.67, 0.79]
Cui 2015	16	23	3	60	58.0	31.7	18.6	1.3	0.84 [0.60, 0.97]	0.72 [0.61, 0.82]
McPherson 2010	23	41	4	77	52.0	35.0	19.0	1.3	0.85 [0.66, 0.96]	0.65 [0.56, 0.74]
Kim 2013	40	43	6	53	60.4	34.8	32.4	1.3	0.87 [0.74, 0.95]	0.55 [0.45, 0.65]



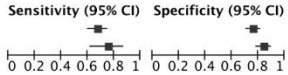
Fibrometer

Study	TP	FP	FN	TN	ALT	BMI	AdvFibrosis	Threshold	Sensitivity (95% CI)	Specificity (95% CI)
Aykut 2014	18	17	9	44	84.0	30.3	31.0		0.67 [0.46, 0.83]	0.72 [0.59, 0.83]
Subasi 2015	20	29	10	83	91.0	30.9	21.1		0.67 [0.47, 0.83]	0.74 [0.65, 0.82]
Siddiqui 2016	38	13	13	81	80.7	35.8	35.2	0.589	0.75 [0.60, 0.86]	0.86 [0.78, 0.92]
Boursier 2016	137	107	35	173	69.0	31.1	38.0	0.311	0.80 [0.73, 0.85]	0.62 [0.56, 0.68]



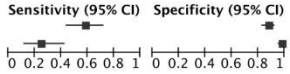
Hepascore

Study	TP	FP	FN	TN	ALT	BMI	AdvFibrosis	Threshold	Sensitivity (95% CI)	Specificity (95% CI)
Boursier 2016	116	67	56	213	69.0	31.1	38.0	0.322	0.67 [0.60, 0.74]	0.76 [0.71, 0.81]
Adams 2011	40	30	13	159	66.5	30.2	21.9	0.37	0.75 [0.62, 0.86]	0.84 [0.78, 0.89]



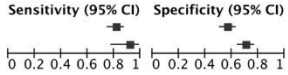
Fibrotest (high threshold)

Study	TP	FP	FN	TN	ALT	BMI	AdvFibrosis	Threshold	Sensitivity (95% CI)	Specificity (95% CI)
Adams 2011	32	19	21	170	66.5	30.2	21.9	0.47	0.60 [0.46, 0.74]	0.90 [0.85, 0.94]
Ratziu 2006	9	5	27	226	73.9	27.0	13.5	0.7	0.25 [0.12, 0.42]	0.98 [0.95, 0.99]



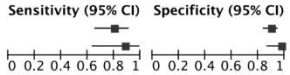
Fibrotest (low threshold)

Study	TP	FP	FN	TN	ALT	BMI	AdvFibrosis	Threshold	Sensitivity (95% CI)	Specificity (95% CI)
Boursier 2016	140	121	32	159	69.0	31.1	38.0	0.316	0.81 [0.75, 0.87]	0.57 [0.51, 0.63]
Ratziu 2006	33	68	3	163	73.9	27.0	13.5	0.3	0.92 [0.78, 0.98]	0.71 [0.64, 0.76]



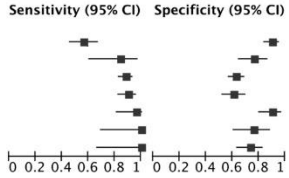
ELF

Study	TP	FP	FN	TN	ALT	BMI	AdvFibrosis	Threshold	Sensitivity (95% CI)	Specificity (95% CI)
Guha 2008	36	15	9	132	77.3	32.4	23.0	0.358	0.80 [0.65, 0.90]	0.90 [0.84, 0.94]
Dvorak 2014	15	1	2	38	128.0	32.8	30.4	-3.37	0.88 [0.64, 0.99]	0.97 [0.87, 1.00]



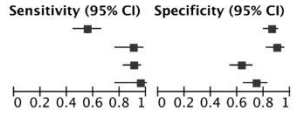
Transient elastography

Study	TP	FP	FN	TN	ALT	BMI	AdvFibrosis	Threshold	Sensitivity (95% CI)	Specificity (95% CI)
Myers 2012	na				55.0	30.0	29.3		Not estimable	Not estimable
Cassinotto 2016	52	13	40	118	71.2	32.1	43.3	12.5	0.57 [0.46, 0.67]	0.90 [0.84, 0.95]
Ergelen 2015	16	16	3	52	77.8	30.6	21.8	9.0	0.84 [0.60, 0.97]	0.76 [0.65, 0.86]
Boursier 2016	152	104	20	176	69.0	31.1	38.0	8.7	0.88 [0.83, 0.93]	0.63 [0.57, 0.69]
Cassinotto 2016	83	51	9	80	71.2	32.1	43.3	8.2	0.90 [0.82, 0.95]	0.61 [0.52, 0.69]
Aykut 2014	26	6	1	55	84.0	30.3	31.0	7.9	0.96 [0.81, 1.00]	0.90 [0.80, 0.96]
Dincses 2015	10	10	0	32	89.0	30.8	19.0	7.9	1.00 [0.69, 1.00]	0.76 [0.61, 0.88]
Naveau 2014	9	24	0	67	38.0	42.3	9.0	7.6	1.00 [0.66, 1.00]	0.74 [0.63, 0.82]



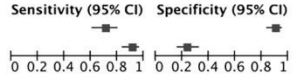
Acoustic radiation force imaging

Study	TP	FP	FN	TN	ALT	BMI	AdvFibrosis	Threshold	Sensitivity (95% CI)	Specificity (95% CI)
Cassinotto 2016	55	15	38	128	71.2	32.1	43.3	1.53	0.59 [0.48, 0.69]	0.90 [0.83, 0.94]
Palmeri 2011	36	10	4	86		33.2	29.6	4.24	0.90 [0.76, 0.97]	0.90 [0.82, 0.95]
Cassinotto 2016	84	53	9	90	71.2	32.1	43.3	1.15	0.90 [0.82, 0.95]	0.63 [0.54, 0.71]
Cui 2016	20	27	1	77	50.4	31.8	16.8	1.34	0.95 [0.76, 1.00]	0.74 [0.65, 0.82]



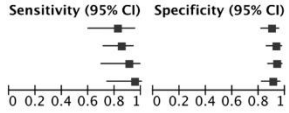
Shearwave elastography

Study	TP	FP	FN	TN	ALT	BMI	AdvFibrosis	Threshold	Sensitivity (95% CI)	Specificity (95% CI)
Cassinotto 2016	71	13	29	119	71.2	32.1	43.3	8.3	0.71 [0.61, 0.80]	0.90 [0.84, 0.95]
Cassinotto 2016	91	93	9	29	71.2	32.1	43.3	10.7	0.91 [0.84, 0.96]	0.24 [0.17, 0.32]

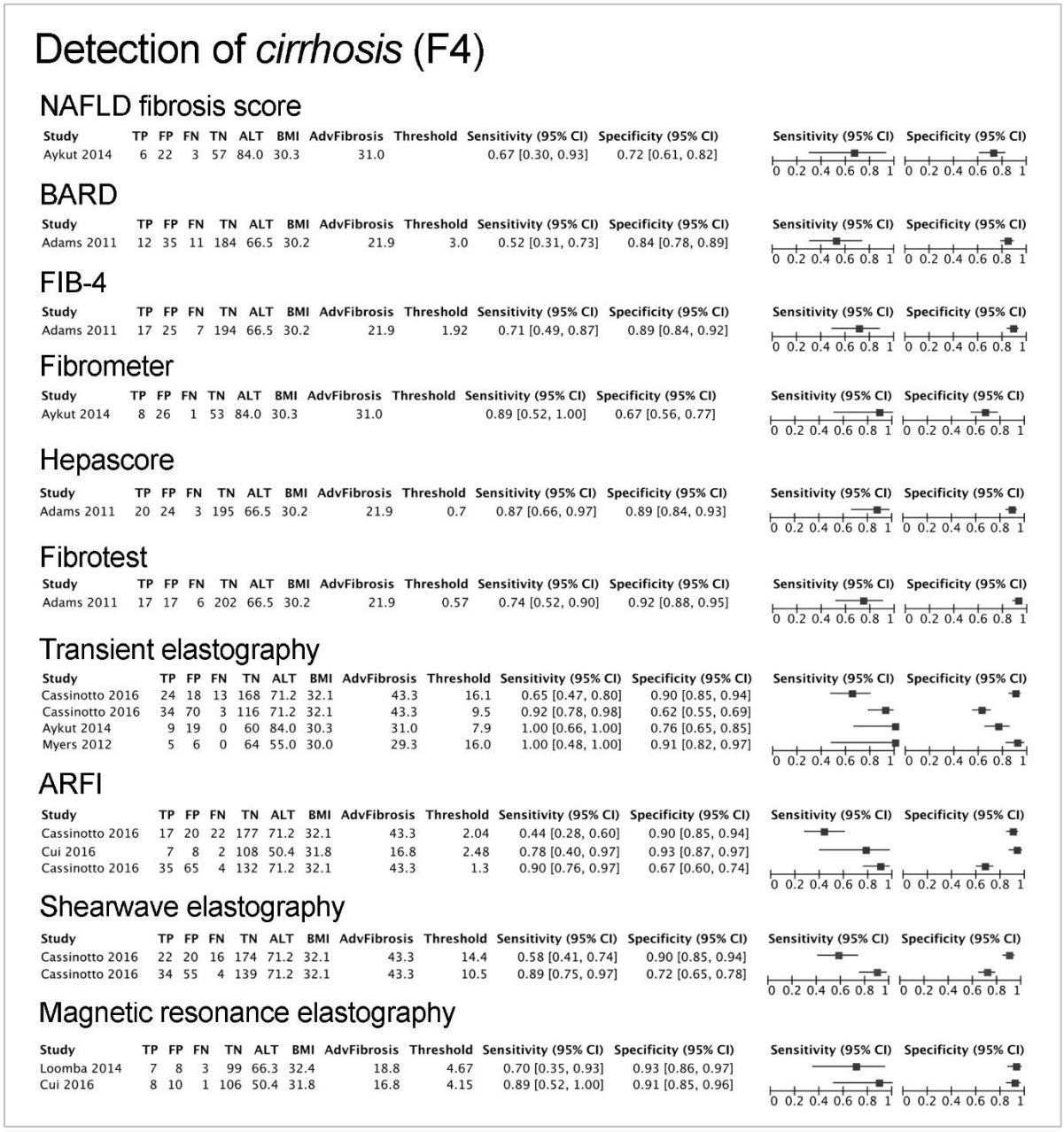


Magnetic resonance elastography

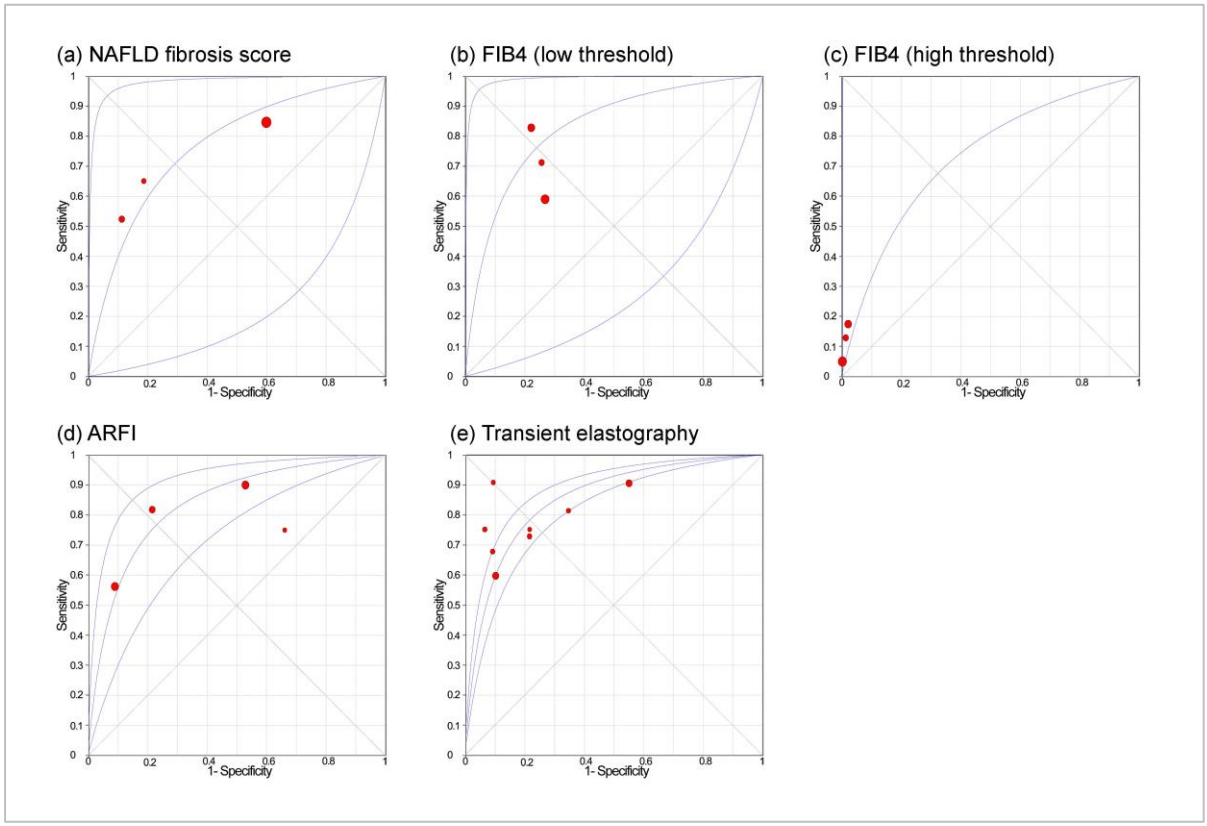
Study	TP	FP	FN	TN	ALT	BMI	AdvFibrosis	Threshold	Sensitivity (95% CI)	Specificity (95% CI)
Loomba 2014	18	10	4	85	66.3	32.4	18.8	3.64	0.82 [0.60, 0.95]	0.89 [0.81, 0.95]
Kim 2013	39	7	7	89	60.4	34.8	32.4	4.15	0.85 [0.71, 0.94]	0.93 [0.86, 0.97]
Cui 2016	19	7	2	97	50.4	31.8	16.8	3.62	0.90 [0.70, 0.99]	0.93 [0.87, 0.97]
Cui 2015	18	8	1	75	58.0	31.7	18.6	3.64	0.95 [0.74, 1.00]	0.90 [0.82, 0.96]



Supplementary Figure 7.5: Diagnostic accuracy of tests for detection of cirrhosis (F4)



Supplementary Figure 7.6: Summary receiver operator curves for detection of F2-4 fibrosis with serum panels and elastography techniques



18.3 Appendix 3: Supplementary materials - Modified thresholds for fibrosis risk scores in nonalcoholic fatty liver disease are necessary in the obese

18.3.1 **Supplementary Tables**

*Supplementary Table 8.1: Training and validation cohorts compared. ^Fisher exact test *Mann Whitney U test*

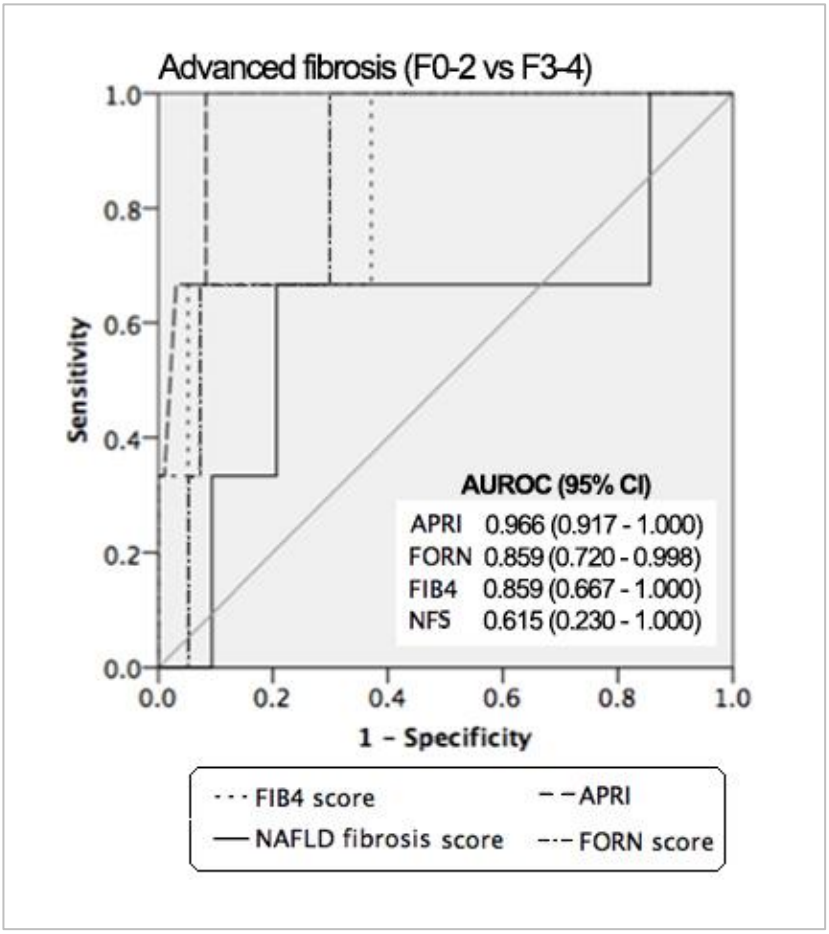
	All patients	**Training cohort**	**Validation cohort**	**p =**
n =	**154**	**101**	**53**	
Demographics				
Age	47 (35 – 54)	49 (38 – 54)	43 (32 – 52)	**0.047***
Male	50 (32.5%)	23 (33.7%)	16 (30.2%)	0.662
BMI	43.3 (39.3 – 47.8)	41.9 (39.1 – 46.5)	46.6 (40.1 – 52.6)	**0.007***
Weight	121.7 (106.8 -135.9)	118.8 (106.4 – 132.4)	129.2 (112.0 – 139.4)	**0.022***
Comorbidities				
Type II diabetes	48 (31.2%)	35 (34.7%)	13 (24.5%)	0.197
IGT	34 (22.1%)	30 (29.7%)	4 (7.5%)	**0.002**^
Hypertension	104 (67.5%)	80 (79.2%)	24 (45.3%)	**<0.001**
High chol	84 (54.9%)	74 (73.3%)	10 (19.2%)	**<0.001**
Fibrosis grade				
F0	77 (50.0%)	33 (32.7%)	34 (64.2%)	**0.006**
F1	57 (37.0%)	45 (44.6%)	12 (22.6%)	
F2	26 (16.9%)	20 (19.8%)	6 (11.3%)	
F3	1 (0.6%)	1 (1.0%)	0	
F4	3 (1.9%)	2 (2.0%)	1 (1.9%)	
Fibrosis scores				
APRI	0.25 (0.19 – 0.35)	0.24 (0.18 – 0.30)	0.29 (0.20 – 0.41)	**0.026***
Forn	3.84 ± 1.66	3.63 ± 1.64	4.25 ± 1.65	**0.027**
FIB4	0.79 (0.60 – 1.10)	0.79 (0.60 – 1.01)	0.79 (0.52 – 1.26)	0.765*
NFS	-0.502 ± 1.410	-0.742 ± 1.356	-0.049 ± 1.410	**0.003**
BARD	102 (66.2%)	71 (70.3%)	31 (58.5%)	0.141

Supplementary Table 8.2: Sensitivity, specificity, PPV and NPV for scores using standard high cut-off points (left) for classification of advanced fibrosis (F3-4). Diagnostic accuracy of modified scores (right) calculated from threshold that gives the highest Youden index.

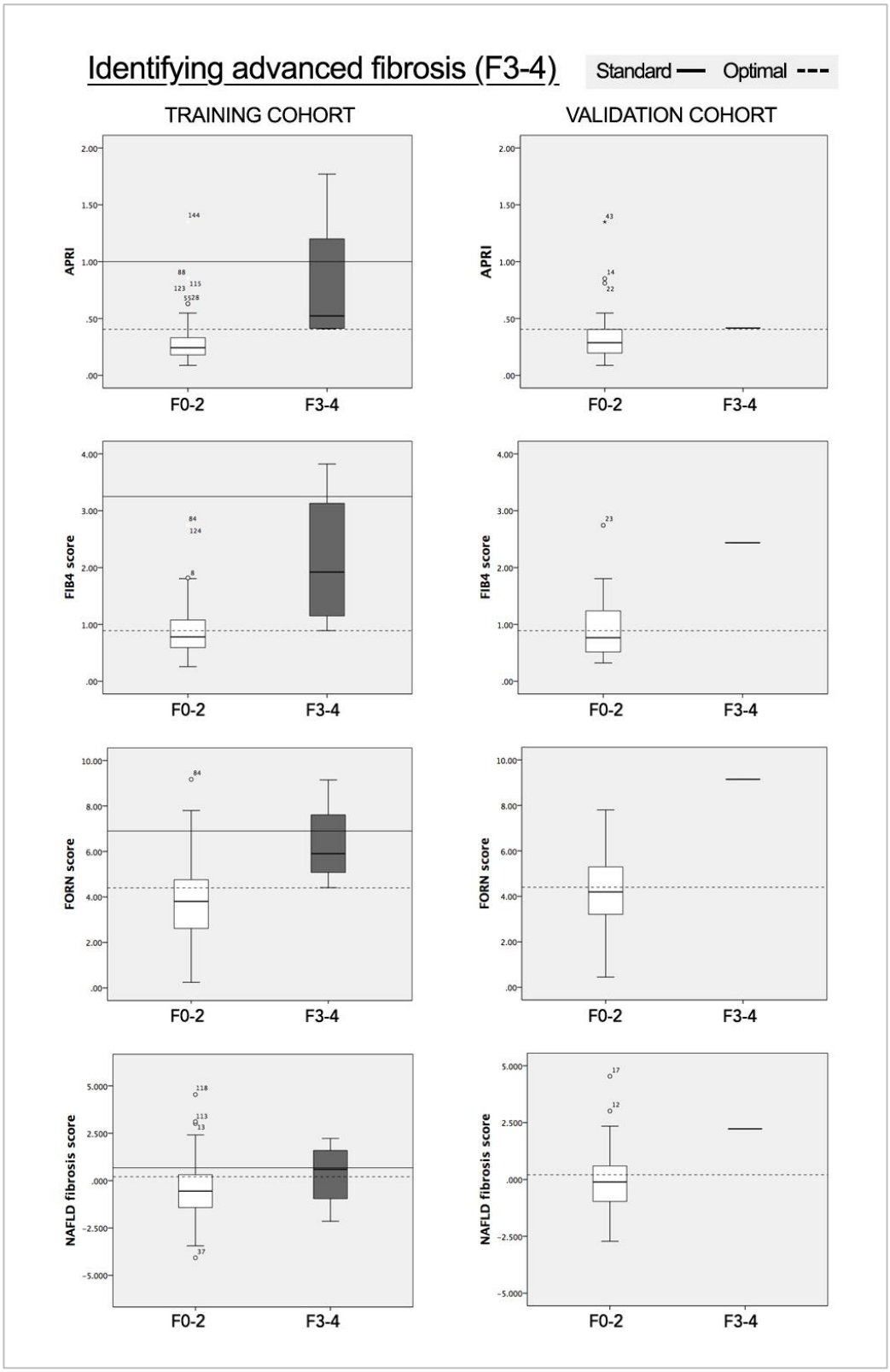
Diagnostic accuracy of scores		**Training cohort**								**Validation cohort**			
		Standard cut-off				Modified cut-off				Modified cut-off			
		APRI	**Forn**	**FIB4**	**NFS**	**APRI**	**Forn**	**FIB4**	**NFS**	**APRI**	**Forn**	**FIB4**	**NFS**
Advanced fibrosis	*Cut-off*	*>0.7*	*>6.9*	*>3.25*	*>0.676*	*>0.405*	*>4.4*	*>0.89*	*>0.206*	*>0.405*	*>4.4*	*>0.89*	*>0.206*
	Sensitivity	33.3%	0%	33.3%	33.3%	100%	100%	100%	66.7%	100%	100%	100%	100%
	Specificity	99.0%	97.9%	100%	89.8%	91.8%	70.4%	62.2%	79.6%	75%	59.6%	57.7%	63.5%
	PPV	50.0%	0%	0%	9.1%	27.3%	9.4%	7.5%	9.1%	7.1%	4.6%	4.4%	5.0%
	NPV	98.0%	96.9%	98.0%	97.8%	100%	100%	100%	98.7%	100%	100%	100%	100%

18.3.2 **Supplementary Figures**

Supplementary Figure 8.1: AUROC for scores for identification of advanced fibrosis (F3-4).



Supplementary Figure 8.2: Boxplots for training and validation cohorts divided into F0-2 vs F3-4 fibrosis. Standard thresholds (solid line) and optimised thresholds (dotted line) shown.



18.4 Appendix 4: Supplementary materials - Evaluating feasibility and accuracy of non-invasive tests for nonalcoholic fatty liver disease in obese patients

18.4.1 Supplementary Tables

Supplementary Table 9.1: Non-invasive tests for NAFLD

Score	Calculation	Thresholds	Fibrosis grade
TESTS FOR FIBROSIS			
AST to platelet ratio index (APRI) (661)	(**AST** [U/L]/upper limit normal)/**platelet** count [10^9/L] x 100	≥0.7: High-risk	F2-F4
		≥0.98: High-risk	F3-F4
		≥1.0: High-risk	F4
FIB-4 (315)	(**Age** [years] x **AST** [U/L]) / (**platelet** [10^9/L] x $\sqrt{}$ **ALT** [U/L])	<1.3: Low risk 1.3 - 2.67: Indeterminate ≥2.67: High risk	F3-F4
Forn index (518)	7.811 – 3.131 x $\log_e$(**platelet** [10^9/L]) + 0.781 x $\log_e$(**GGT** [U/L]) + 3.467 x $\log_e$(**age** [years]) – 0.014 x **cholesterol** [mg/dl]	<4.2: Low risk 4.2 – 6.9: Indeterminate >6.9: High-risk	F3-F4
NAFLD fibrosis score (NFS) (310)	-1.675 + 0.037 x **age** [years] + 0.094 x **BMI** + 1.13 x **diabetes status** + 0.99 x **AST/ALT** – 0.013 x **platelet** [10^9/L] – 0.66 x **albumin** [g/dl]	< -1.455: Low risk -1.455–0.676: Indeterminate ≥ 0.676: High-risk	F3-F4
BARD score (525)	**BMI** ≥ 28 = 1 **AST/ALT ratio** ≥ 0.8 = 2 **Diabetes status** = 1	≥2: High-risk	F3-F4
Fibroscan	Based in principles of elastography		
TESTS FOR STEATOSIS			
Fatty Liver Index (FLI) (283)	$e^{(0.953 \times \log e (\textbf{triglycerides}) + 0.139 \times \textbf{BMI} + 0.718 \times \log e (\textbf{GGT}) + 0.053 \times \textbf{waist circumference} - 15.745)} / (1+e^{0.953 \times \log e (\textbf{triglycerides}) + 0.139 \times \textbf{BMI} + 0.718 \times \log e (\textbf{GGT}) + 0.053 \times \textbf{waist circumference} - 15.745)} \times 100$	<30: Low risk 30-60: Indeterminate ≥60: High risk	
NAFLD liver fat score (NLFS) (285)	-2.87 + 1.18 x **metabolic syndrome** (yes=1, no=0) + 0.45 x **T2DM** (yes=2, no=0) + 0.15 x **insulin** [mU/L] + 0.04 x **AST** [U/L] – 0.94 **AST/ALT**	>-0.640: High risk	
Lipid accumulation product (LAP) (286)	Men: (**WC** [cm] - 65) x (**triglycerides** [mmol/L]) Women: (**WC** [cm] – 58) x (**triglycerides** [mmol/L])	Continuous marker of liver steatosis.	
Hepatic steatosis index (HSI) (288)	8 x (**ALT/AST ratio**) + **BMI** + 2 x **gender** (male=0, female=1) + 2 x **diabetes** (yes=1, no=0).	<30.0: Low risk 30-36: Indeterminate ≥36: High risk	
Controlled attenuation parameter		283 dB/m(662)	

Supplementary Table 9.2: Steatosis scores and measures, averages according to thirds.

	All patients	<5%	5-33%	34-66%	67-100%	p-value
n =	182	31	68	58	25	
ALT	44.0±39.4	22.2±8.2*	36.4±22.9^	50.3±47.5	66.4±58.3*^	0.001
AST	34.0±29.8	21.1±6.0*	28.1±15.1^	38.0±33.4	51.2±50.9*^	0.002
FLI	95.7 (86.2-99.0)	91.2 (82.4-99.2)	95.5 (87.2-98.6)	97.8 (92.4-99.2)	96.4 (90.0-99.2)	0.109
Low	1 (0.6%)	1 (3.2%)	0	0	0	0.151
Med	7 (3.9%)	3 (9.7%)	3 (4.6%)	1 (1.8%)	0	
Hi	170 (95.5%)	27 (87.1%)	62 (95.4%)	56 (98.2%)	25 (100%)	
NAFLD liver fat score	0.21 (-1.17-1.61)	-0.84 (-1.71-0.76)	-0.21 (-1.33-0.82)	1.02 (-0.62-1.82)	1.62 (0.35-4.79)	<0.001
Low	58 (34.9%)	18 (62.1%)	25 (41.7%)	13 (24.5%)	2 (8.3%)	<0.001
Hi	108 (65.1%)	11 (37.9%)	35 (58.3%)	40 (75.5%)	22 (91.7%)	
LAP	86 (54.6-124.2)	62.4 (49.5-109.5)	83.3 (54.4-109.2)	96.6 (71.4-132.6)	100.8 (67.6-162.4)	0.011
Hep Steatosis index	52.5 (48.4-58.1)	52.5 (47.4-58.5)	52.4 (47.6-58.6)	52.8 (49.0-57.5)	52.5 (48.5-58.1)	0.992
^{1}H-MRS % fat	6.2 (3.0-11.4)	1.5 (0.3-2.7)	3.8 (2.7-6.1)	8.6 (4.0-14.3)	13.8 (8.1-16.6)	0.003
CAP	313 (279-362)	262 (233-305)	298 (277-332)	359 (295-400)	323 (279-362)	0.010

*Values expressed as mean ± standard deviation or median (interquartile range). One-way ANOVA and post-hoc Bonferroni performed unless otherwise indicated. *Significant difference with S0, ^Significant difference with S1, ≠Significant difference with S2. +Kruskal-Wallis test performed for asymmetrical data.*

Supplementary Table 9.3: Diagnostic accuracy of tests and variables for detecting steatosis

	Any steatosis (S1-3)		Significant steatosis (S2-3)		Advanced steatosis (S3)	
	AUROC	**p-value**	**AUROC**	**p-value**	**AUROC**	**p-value**
ALT	0.686 (0.586-0.785)	**0.001**	0.699 (0.623-0.776)	**<0.001**	0.717 (0.618-0.816)	**0.001**
AST	0.696 (0.597-0.794)	**0.001**	0.703 (0.627-0.779)	**<0.001**	0.718 (0.622-0.813)	**<0.001**
Fatty liver index	0.595 (0.473-0.718	0.096	0.596 (0.512-0.679)	**0.028**	0.536 (0.415-0.656)	0.568
NAFLD liver fat score	0.644 (0.529-0.758)	0.015	0.687 (0.606-0.769)	**<0.001**	0.726 (0.551-0.900)	**0.013**
Liver accumulation product index	0.633 (0.517-0.748)	0.020	0.631 (0.550-0.713)	**0.003**	0.599 (0.467-0.731)	0.112
Hepatic steatosis index	0.491 (0.376-0.606)	0.878	0.510 (0.424-0.596)	0.818	0.501 (0.375-0.626)	0.992
Computed attenuation parameter	**0.749 (0.556-0.942)**	**0.012**	0.688 (0.563-0.812)	**0.007**	0.540 (0.378-0.702)	0.676
MR spectroscopy	0.908 (0.756-1.000)	0.056	**0.852 (0.705-0.998)**	**0.001**	**0.849 (0.715-0.982)**	**0.005**

AUROC – Area under the receiver operator characteristic curve; ALT – alanine aminotransferase; AST – aspartate aminotransferase; NAFLD liver fat score; MR spectroscopy – magnetic resonance spectroscopy.

Supplementary Table 9.4: Sensitivity and specificity for various thresholds of steatosis scores, controlled attenuation parameter (CAP) and magnetic resonance spectroscopy (^{1}H-MRS).

	Test	Threshold	Sens	Spec	PPV	NPV	LR+	LR-	CC
S1-3	*ALT*	*17/21*	91.3	29.0	86.2	40.9	1.287	0.299	80.7
	AST	*17/21*	90.5	25.8	85.4	36.4	1.220	0.367	79.3
	FLI	*30**	100	3.2	83.1	100	1.033	0	83.1
		*60**	97.3	12.9	84.1	50	1.117	0.211	82.6
		75	92.5	16.1	84	31.1	1.103	0.464	79.2
		99	24.5	74.2	81.8	17.2	0.949	1.018	33.1
	NLFS	*-2.047*	90.5	17.2	83.8	27.8	1.094	0.550	77.7
		*-0.640**	70.8	62.1	89.8	31.0	1.867	0.470	69.3
		1.817	22.6	86.2	88.6	19.1	1.641	0.898	33.7
	LAP	*50.5*	85.8	32.3	85.8	32.3	1.267	0.440	76.5
		142	14.2	87.1	84.0	17.5	1.100	0.985	26.5
	*HSI^*	*45*	87.6	9.7	81.9	14.3	0.970	1.283	73.9
		67	6.9	90.3	76.9	17.2	0.713	1.031	21.6
	CAP	*270*	89.8	60	93	50	2.246	0.169	85.5
		355	32.2	90	95	18.4	3.22	0.753	40.6
	^{1}H-MRS	*2.95*	80	100	100	25	-	0.200	81.3
S2-3	*ALT*	*17/21*	95.1	18.0	48.4	81.8	1.159	0.274	52.5
	AST	*17/21*	95.1	18.6	49.7	81.8	1.168	0.263	53.6
	FLI	*30**	100	1	45.8	100	1.010	0	46.1
		*60**	98.8	7.2	47.1	87.5	1.064	0.171	48.9
		80	87.7	16.5	46.7	61.5	1.050	0.748	48.9
		99	30.9	80.4	56.8	58.2	1.576	0.860	57.9
	NLFS	*-2.047*	92.1	13.3	47.3	66.7	1.063	0.592	49.4
		*-0.640**	80.3	47.8	56.5	74.1	1.537	0.413	62.7
		1.817	31.6	87.8	68.6	60.3	2.584	0.779	62.0
	LAP	*50.5*	87.7	21.4	48	67.7	1.116	0.576	51.4
		142	21.0	91.8	68.0	58.4	2.571	0.860	59.8
	*HSI^*	*45*	86.4	10.5	45.2	47.6	0.966	1.290	45.5
		67	7.4	92.6	46.2	54.0	1.005	1.000	53.4
	CAP	*285*	84.8	47.2	59.6	77.3	1.608	0.321	65.2
		355	42.4	83.3	70	61.2	2.545	0.691	63.8
	^{1}H-MRS	*6.6*	81.3	87.5	86.7	82.4	6.500	0.214	84.4
S3	*ALT*	*17/21*	85.7	14.1	15.2	84.6	0.998	1.013	25.0
	AST	*17/21*	100	14.3	15.9	100	1.167	0	26.3
	FLI	*30**	100	0.7	14.1	100	1.007	0	14.6
		*60**	100	5.2	14.7	100	1.055	0	18.5
		92	72	35.9	15.5	88.7	1.124	0.779	41.0
		99	32.0	76.5	18.2	87.3	1.360	0.889	70.2
	NLFS	*-2.047*	94.4	15.5	12.0	95.8	1.118	0.357	24.1
		*-0.640**	91.7	39.4	20.4	96.6	1.514	0.211	47.0
		1.817	89.3	28.6	82.4	41.7	1.250	0.374	76.5
	LAP	*50.5*	84	17.5	14.2	87.1	1.019	0.913	26.8
		142	32.0	89.0	32.0	89.0	2.899	0.764	81.0
	*HSI^*	*45*	84	11.3	13.5	81.0	0.947	1.421	21.6
		67	8	92.7	15.4	85.9	1.098	0.992	80.7
	CAP	*310*	54.5	50.0	17.1	85.3	1.091	0.909	61.3
		400	9.1	82.8	9.1	82.8	0.527	1.098	71.0

**Standard thresholds. ^Standard threshold yielded no negative results. Sens – sensitivity; Spec – specificity; PPV – positive predictive value; NPV – negative predictive value; LR+ - positive likelihood ratio; LR- - negative likelihood ratio; CC – correctly classified; ALT – alanine aminotransferase; AST – aspartate aminotransferase; FLI – fatty liver index; NLFS – NAFLD liver fat score; LAP – lipid accumulation parameter; HSI – hepatic steatosis index; CAP – controlled attenuation parameter; MRS – magnetic resonance spectroscopy.*

Supplementary Table 9.5: Multivariate linear regression analysis actors significantly influencing CAP readings. Entered variables – steatosis grade, inflammation grade, ballooning grade, fibrosis grade, age, body mass index, gender.

Model Summary[g]

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate
1	.515[a]	.266	.181	53.8641
2	.513[b]	.263	.191	53.5322
3	.508[c]	.258	.199	53.2630
4	.499[d]	.249	.202	53.1822
5	.486[e]	.237	.201	53.1989
6	.457[f]	.209	.185	53.7532

ANOVA[a]

Model		Sum of Squares	df	Mean Square	F	Sig.
1	Regression	64004.905	7	9143.558	3.151	.007[b]
	Residual	176981.646	61	2901.338		
	Total	240986.551	68			
2	Regression	63313.213	6	10552.202	3.682	.003[c]
	Residual	177673.338	62	2865.699		
	Total	240986.551	68			
3	Regression	62259.127	5	12451.825	4.389	.002[d]
	Residual	178727.424	63	2836.943		
	Total	240986.551	68			
4	Regression	59972.597	4	14993.149	5.301	.001[e]
	Residual	181013.954	64	2828.343		
	Total	240986.551	68			
5	Regression	57028.641	3	19009.547	6.717	.001[f]
	Residual	183957.910	65	2830.122		
	Total	240986.551	68			
6	Regression	50285.383	2	25142.691	8.702	.000[g]
	Residual	190701.168	66	2889.412		
	Total	240986.551	68			

Model		Unstandardized Coefficients B	Unstandardized Coefficients Std. Error	Standardized Coefficients Beta	t	Sig.
1	(Constant)	178.148	55.729		3.197	.002
	Steatosis	10.113	8.788	.159	1.151	.254
	Fibrosis	5.364	9.000	.071	.596	.553
	Inflam	11.728	12.949	.115	.906	.369
	Ballooning	14.907	12.552	.163	1.188	.240
	Age	.311	.637	.064	.488	.627
	Gender	9.471	15.652	.075	.605	.547
	Baseline BMI	2.008	.885	.272	2.269	.027
2	(Constant)	197.534	38.865		5.083	.000
	Steatosis	10.755	8.636	.169	1.245	.218
	Fibrosis	5.424	8.944	.072	.606	.546
	Inflam	9.820	12.270	.096	.800	.427
	Ballooning	14.393	12.431	.157	1.158	.251
	Gender	12.327	14.427	.097	.854	.396
	Baseline BMI	1.877	.838	.254	2.239	.029
3	(Constant)	197.864	38.666		5.117	.000
	Steatosis	11.496	8.506	.180	1.352	.181
	Inflam	10.854	12.090	.106	.898	.373
	Ballooning	14.947	12.335	.163	1.212	.230
	Gender	14.221	14.015	.112	1.015	.314
	Baseline BMI	1.858	.833	.251	2.229	.029
4	(Constant)	198.148	38.606		5.133	.000
	Steatosis	13.422	8.218	.210	1.633	.107
	Ballooning	16.318	12.222	.178	1.335	.187
	Gender	14.276	13.993	.113	1.020	.311
	Baseline BMI	1.906	.830	.258	2.295	.025
5	(Constant)	203.893	38.205		5.337	.000
	Steatosis	13.073	8.214	.205	1.592	.116
	Ballooning	18.563	12.026	.203	1.544	.128
	Baseline BMI	1.871	.830	.253	2.254	.028
6	(Constant)	189.858	37.494		5.064	.000
	Steatosis	19.673	7.086	.308	2.776	.007
	Baseline BMI	2.133	.821	.289	2.598	.012

Supplementary Table 9.6: Multivariate linear regression analysis actors significantly influencing ^{1}H-MRS readings. Entered variables – steatosis grade, inflammation grade, ballooning grade, fibrosis grade, age, body mass index, gender.

Model Summary[f]

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate
1	.732[a]	.535	.400	5.5748
2	.732[b]	.535	.424	5.4633
3	.731[c]	.535	.445	5.3609
4	.715[d]	.511	.439	5.3923
5	.705[e]	.497	.443	5.3690

ANOVA[a]

Model		Sum of Squares	df	Mean Square	F	Sig.
1	Regression	859.554	7	122.793	3.951	.005[b]
	Residual	745.872	24	31.078		
	Total	1605.426	31			
2	Regression	859.248	6	143.208	4.798	.002[c]
	Residual	746.178	25	29.847		
	Total	1605.426	31			
3	Regression	858.196	5	171.639	5.972	.001[d]
	Residual	747.230	26	28.740		
	Total	1605.426	31			
4	Regression	820.340	4	205.085	7.053	.001[e]
	Residual	785.086	27	29.077		
	Total	1605.426	31			
5	Regression	798.284	3	266.095	9.231	.000[f]
	Residual	807.141	28	28.826		
	Total	1605.426	31			

Coefficients[a]

Model		Unstandardized Coefficients B	Unstandardized Coefficients Std. Error	Standardized Coefficients Beta	t	Sig.
1	(Constant)	-12.289	11.088		-1.108	.279
	Steatosis	3.120	1.443	.391	2.162	.041
	Fibrosis	.463	2.951	.030	.157	.877
	Inflam	-2.665	2.357	-.210	-1.131	.269
	Ballooning	4.796	2.317	.378	2.070	.049
	Age	.107	.103	.180	1.042	.308
	Gender	.317	3.193	.016	.099	.922
	Baseline BMI	.219	.193	.195	1.132	.269
2	(Constant)	-12.104	10.712		-1.130	.269
	Steatosis	3.090	1.384	.387	2.233	.035
	Fibrosis	.529	2.818	.035	.188	.853
	Inflam	-2.611	2.248	-.206	-1.162	.256
	Ballooning	4.779	2.264	.377	2.111	.045
	Age	.110	.099	.184	1.110	.277
	Baseline BMI	.214	.183	.190	1.171	.253
3	(Constant)	-12.597	10.190		-1.236	.227
	Steatosis	3.169	1.293	.397	2.452	.021
	Inflam	-2.460	2.060	-.194	-1.194	.243
	Ballooning	4.867	2.173	.384	2.239	.034
	Age	.111	.097	.186	1.148	.262
	Baseline BMI	.222	.174	.198	1.274	.214
4	(Constant)	-3.756	6.710		-.560	.580
	Steatosis	3.232	1.299	.405	2.488	.019
	Inflam	-3.454	1.880	-.272	-1.837	.077
	Ballooning	5.035	2.181	.397	2.308	.029
	Baseline BMI	.139	.159	.124	.871	.391
5	(Constant)	1.769	2.177		.813	.423
	Steatosis	3.505	1.255	.439	2.792	.009
	Inflam	-3.447	1.872	-.272	-1.841	.076
	Ballooning	5.142	2.168	.405	2.371	.025

Supplementary Table 9.7: Diagnostic accuracy of tests and variables for detecting fibrosis

	Any fibrosis (F1-4)		**Significant fibrosis (F2-4)**		**Advanced fibrosis (F3-4)**	
	AUROC	**p-value**	**AUROC**	**p-value**	**AUROC**	**p-value**
DEMOGRAPHIC PREDICTORS						
Weight	0.729 (0.605-0.853)	**0.001**	0.638 (0.346-0.931)	0.352	0.500 (0.210-0.790)	1.000
BMI	0.663 (0.536-0.790)	**0.020**	0.406 (0.173-0.638)	0.526	0.282 (0.073-0.491)	0.201
Waist circumference	0.632 (0.488-0.777)	0.060	0.696 (0.573-0.818)	0.188	0.663 (0.551-0.774)	0.340
AST	**0.841 (0.747-0.934)**	**<0.001**	0.710 (0.497-0.923)	0.158	0.494 (0.202-0.785)	0.970
ALT	**0.794 (0.679-0.910)**	**<0.001**	0.634 (0.344-0.924)	0.367	0.690 (0.464-0.916)	0.266
Cholesterol	0.485 (0.353-0.616)	0.832	0.328 (0.000-0.665)	0.247	0.103 (0.000-0.248)	0.020
HDL	0.622 (0.483-0.761)	0.084	0.514 (0.278-0.749)	0.927	0.387 (0.083-0.691)	0.507
Triglycerides	0.545 (0.404-0.687)	0.529	0.633 (0.456-0.809)	0.372	0.659 (0.409-0.908)	0.352
TC:HDL ratio	0.591 (0.442-0.741)	0.206	0.337 (0.063-0.611)	0.273	0.073 (0.000-0.148)	0.012
LIVER FIBROSIS TESTS						
TE	**0.762 (0.616-0.909)**	**0.003**	**0.903 (0.787-1.000)**	**0.007**	**0.910 (0.759-1.000)**	**0.017**
APRI	**0.859 (0.781-0.938)**	**<0.001**	**0.792 (0.624-0.960)**	**0.049**	**0.859 (0.759-0.959)**	**0.036**
NFS	0.659 (0.536-0.781)	**0.024**	**0.772 (0.565-0.979)**	**0.042**	**0.932 (0.877-0.988)**	**0.011**
BARD	0.524 (0.380-0.668)	0.732	**0.823 (0.662-0.984)**	**0.030**	**0.840 (0.651-1.000)**	**0.047**
FIB4	0.546 (0.404-0.687)	**0.041**	**0.790 (0.494-1.000)**	**0.030**	**0.957 (0.911-1.000)**	**0.008**
Forn	0.642 (0.503-0.781)	0.512	**0.800 (0.571-1.000)**	**0.025**	**0.970 (0.928-1.000)**	**0.006**

AUROC – Area under the receiver operator characteristic curve; TE – transient elastography; APRI – AST to platelet ratio index; NFS – NAFLD fibrosis score; FIB-4 – Fibrosis-4 score; AST – aspartate aminotransferase; ALT – alanine aminotransferase; BMI – body mass index; HDL – high density lipoprotein; TC:HDL ratio – total cholesterol to HDL ratio

Supplementary Table 9.8: Sensitivity and specificity of fibrosis tests for levels of fibrosis

	Test	Threshold	Sens	Spec	PPV	NPV	LR+	LR-	CC
F1-4	*TE*	*>5.25kPa^*	92.9	42.3	30.2	95.7	1.61	0.17	53.0
		*>7kPa**	78.6	59.6	34.4	91.2	1.95	0.36	63.6
		>5.25kPa^ (ITD)	65.0	35.5	24.5	75.9	1.01	0.99	42.7
		>7kPa (ITD)*	55.0	50.0	26.2	77.5	1.10	0.90	51.2
	APRI	*>0.7*	19	94.2	50.0	79.1	3.262	0.860	76.5
	BARD	*≥2*	69	35.8	25.2	78.7	1.076	0.864	43.8
	NFS	*Low: >-1.455*	90.2	21.1	26.1	87.5	1.143	0.463	37.4
		High: >0.676	29.3	78.9	30.0	78.4	1.390	0.896	67.2
	FIB4	*Low: >1.3*	38.1	82.8	41.0	81.0	2.219	0.747	72.2
		High: >2.67	2.4	97.8	25.0	76.2	1.063	0.999	75.0
	Forn	*Low: >4.2*	57.1	49.3	25.8	78.8	1.126	0.870	51.1
		High: >6.9	7.1	97.1	42.9	77.2	2.429	0.957	75.8
F2-4	*TE*	*>7.0kPa^*	100	54.8	12.5	100	2.21	0	57.6
		*>9.0kPa**	100	74.2	20	100	3.88	0	75.8
		>7.0kPa^ (ITD)	100	43.6	8.3	100	1.773	0	46.3
		>9.0kPa (ITD)*	100	59.0	11.1	100	2.44	0	61.0
	APRI	*>0.7*	14.3	92.4	7.1	96.3	1.868	0.928	89.3
	BARD	*≥2*	100	36.1	6.1	100	1.565	0	38.6
	NFS	*Low: >-1.455*	100	19.2	4.9	100	1.237	0	22.4
		High: >0.676	57.1	78.4	10	97.8	2.651	0.546	77.6
	FIB4	*Low: >1.3*	57.1	79.3	10.3	97.8	2.759	0.541	78.4
		High: >2.67	0	97.6	0	95.9	0	1.024	93.8
	Forn	*Low: >4.2*	71.4	48.5	5.4	97.6	1.388	0.589	49.4
		High: >6.9	42.9	97.7	42.9	97.7	18.321	0.585	95.5
F3-4	*TE*	*>10.3kPa^*	66.7	81.0	14.3	98.1	3.50	0.41	80.3
		*>12.85kPa**	66.7	90.5	25.0	98.3	7.00	0.37	89.4
		>10.3kPa^ (ITD)	66.7	64.6	6.7	98.1	1.88	0.52	64.6
		>12.85kPa (ITD)*	66.7	72.2	8.3	98.3	2.39	0.46	72.0
	APRI	*>0.98*	0	91.9	0	97.5	0	1.088	89.8
	BARD	*≥2*	100	35.5	3.5	100	1.550	0	36.9
	NFS	*Low: >-1.455*	100	18.8	2.8	100	1.232	0	20.7
		High: >0.676	100	78.8	10	100	4.722	0	79.3
	FIB4	*Low: >1.3*	75.0	79.1	7.7	99.3	3.583	0.316	79.0
		High: >2.67	0	97.7	0	97.7	0	1.024	95.5
	Forn	*Low: >4.2*	100	48.9	4.3	100	1.955	0	50.0
		High: >6.9	75.0	97.7	42.9	99.4	32.625	0.256	97.2

*APRI – AST to platelet ratio index; NFS – NAFLD fibrosis score; FIB-4 – Fibrosis-4 score; TE – transient elastography; PPV – positive predictive value; NPV – negative predictive value; LR+ – Positive likelihood ratio; LR- – negative likelihood ration; CC – Correctly classified. ^Previously cited threshold value. *Based on Youden index. ITD – intention to diagnose analysis*

Supplementary Table 9.9: Multivariate model of factors potentially affecting transient elastography liver stiffness measure (kPa)

Coefficients[a]

Model		Unstandardized Coefficients B	Unstandardized Coefficients Std. Error	Standardized Coefficients Beta	t	Sig.	95.0% Confidence Interval for B Lower Bound	95.0% Confidence Interval for B Upper Bound
1	(Constant)	-1.955	3.070		-.637	.527	-8.087	4.178
	Fibrosis_0_CM	3.996	.593	.550	6.737	.000	2.811	5.181
	SkinToCaps_0 (mm)	.345	.068	.482	5.048	.000	.208	.482
	Baseline BMI	-.090	.129	-.132	-.695	.489	-.347	.168
	Baseline weight	.029	.040	.152	.713	.479	-.052	.110
	Gender (0=female, 1=male)	.213	1.420	.018	.150	.881	-2.624	3.051
	NAS_0_CM	-.126	.426	-.039	-.295	.769	-.976	.725
	Waist circumference	-.002	.033	-.007	-.060	.952	-.067	.063
	Steatosis%histology	-.015	.028	-.069	-.556	.580	-.071	.040
2	(Constant)	-2.062	2.477		-.833	.408	-7.008	2.884
	Fibrosis_0_CM	3.992	.585	.550	6.829	.000	2.825	5.160
	SkinToCaps_0 (mm)	.344	.066	.481	5.207	.000	.212	.476
	Baseline BMI	-.092	.124	-.135	-.738	.463	-.340	.156
	Baseline weight	.028	.040	.150	.716	.476	-.051	.108
	Gender (0=female, 1=male)	.184	1.323	.015	.139	.890	-2.459	2.827
	NAS_0_CM	-.121	.414	-.037	-.291	.772	-.948	.707
	Steatosis%histology	-.015	.027	-.069	-.557	.579	-.070	.039
3	(Constant)	-1.996	2.413		-.827	.411	-6.814	2.821
	Fibrosis_0_CM	3.999	.578	.550	6.915	.000	2.844	5.153
	SkinToCaps_0 (mm)	.344	.066	.481	5.250	.000	.213	.475
	Baseline BMI	-.103	.094	-.151	-1.096	.277	-.290	.085
	Baseline weight	.032	.028	.171	1.141	.258	-.024	.089
	NAS_0_CM	-.124	.411	-.038	-.301	.764	-.944	.696
	Steatosis%histology	-.015	.027	-.068	-.558	.579	-.069	.039
4	(Constant)	-1.907	2.378		-.802	.425	-6.653	2.840
	Fibrosis_0_CM	3.972	.568	.547	6.996	.000	2.839	5.106
	SkinToCaps_0 (mm)	.342	.065	.477	5.292	.000	.213	.471
	Baseline BMI	-.102	.093	-.150	-1.098	.276	-.288	.084
	Baseline weight	.032	.028	.167	1.129	.263	-.024	.088
	Steatosis%histology	-.021	.017	-.097	-1.244	.218	-.056	.013
5	(Constant)	-3.432	1.933		-1.775	.080	-7.290	.426
	Fibrosis_0_CM	4.119	.553	.567	7.451	.000	3.016	5.222
	SkinToCaps_0 (mm)	.343	.065	.480	5.313	.000	.214	.472
	Baseline weight	.007	.017	.039	.429	.670	-.027	.042
	Steatosis%histology	-.024	.017	-.110	-1.429	.158	-.058	.010
6	(Constant)	-3.018	1.665		-1.813	.074	-6.339	.303
	Fibrosis_0_CM	4.134	.548	.569	7.539	.000	3.040	5.228
	SkinToCaps_0 (mm)	.359	.053	.502	6.786	.000	.254	.465
	Steatosis%histology	-.023	.017	-.105	-1.388	.170	-.056	.010
7	(Constant)	-3.392	1.654		-2.051	.044	-6.690	-.094
	Fibrosis_0_CM	3.947	.535	.543	7.378	.000	2.880	5.014
	SkinToCaps_0 (mm)	.348	.053	.487	6.611	.000	.243	.454

a. Dependent Variable: FibroScLSM_0

Model Summary[h]

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate
1	.808[a]	.652	.609	3.4886
2	.808[b]	.652	.615	3.4617
3	.807[c]	.652	.620	3.4359
4	.807[d]	.652	.626	3.4125
5	.803[e]	.645	.624	3.4177
6	.803[f]	.644	.629	3.3974
7	.796[g]	.634	.624	3.4198

a. Predictors: (Constant), Steatosis%histology, Gender (0=female, 1=male), Baseline BMI, Fibrosis_0_CM, SkinToCaps_0 (mm), Waist circumference, NAS_0_CM, Baseline weight
b. Predictors: (Constant), Steatosis%histology, Gender (0=female, 1=male), Baseline BMI, Fibrosis_0_CM, SkinToCaps_0 (mm), NAS_0_CM, Baseline weight
c. Predictors: (Constant), Steatosis%histology, Baseline BMI, Fibrosis_0_CM, SkinToCaps_0 (mm), NAS_0_CM, Baseline weight
d. Predictors: (Constant), Steatosis%histology, Baseline BMI, Fibrosis_0_CM, SkinToCaps_0 (mm), Baseline weight
e. Predictors: (Constant), Steatosis%histology, Fibrosis_0_CM, SkinToCaps_0 (mm), Baseline weight
f. Predictors: (Constant), Steatosis%histology, Fibrosis_0_CM, SkinToCaps_0 (mm)
g. Predictors: (Constant), Fibrosis_0_CM, SkinToCaps_0 (mm)

Supplementary Table 9.10: Diagnostic accuracy of controlled attenuation parameter (CAP) in combination with ALT, with intention to diagnose (ITD) analysis.

Combination of tests	Both tests positive^		Both tests negative^		Biopsies saved	Indetermi-nate	Correctly classified
	Sensitivity	PPV	Specificity	NPV			
S1-3 (threshold CAP ≥270 dB/m, ^{1}H-MRS ≥2.95%)							
CAP alone	89.8%	93.0%	60.0%	50.0%	12 (17.4%)	-	59 (85.5%)
CAP alone (ITD)	77.9%	89.8%	50.0%	28.6%	12 (15.0%)	-	59 (73.8%)
^{1}H-MRS alone	80.0%	100%	100%	25%	8 (25.0%)	-	26 (81.3%)
^{1}H-MRS alone (ITD)	53.3%	92.3%	3.3%	8.7%	8 (16.3%)	-	26 (53.1%)
CAP + ALT	80.6%	93.1%	8.3%	50.0%	2 (2.5%)	19 (23.8%)	55 (69.6%)
^{1}H-MRS + ALT	77.8%	94.6%	50.0%	33.3%	6 (12.2%)	6 (12.2%)	37 (75.5%)
S2-3 (threshold CAP≥285 dB/m, ^{1}H-MRS ≥6.6%)							
CAP alone	84.8%	59.6%	47.2%	77.3%	22 (31.9%)	-	45 (65.2%)
CAP alone (ITD)	75.7%	51.9%	39.5%	65.4%	22 (27.5%)	-	45 (56.3%)
^{1}H-MRS alone	81.3%	86.7%	87.5%	82.4%	17 (53.1%)	-	27 (84.4%)
^{1}H-MRS alone (ITD)	50.0%	59.1%	60.9%	51.9%	17 (34.7%)	-	27 (55.1%)
CAP + ALT	77.8%	56.0%	9.3%	100%	4 (5.0%)	25 (31.3%)	32 (40.5%)
^{1}H-MRS + ALT	84.6%	78.6%	26.1%	100%	6 (12.2%)	15 (30.6%)	28 (57.1%)
S3 (threshold CAP≥310 dB/m, ^{1}H-MRS ≥9.7%)							
CAP alone	54.5%	17.1%	50.0%	85.3%	34 (49.3%)	-	35 (50.7%)
CAP alone (ITD)	50.0%	13.3%	42.6%	82.9%	34 (42.5%)	-	35 (43.8%)
^{1}H-MRS alone	71.4%	55.6%	84.0%	91.3%	23 (71.9%)	-	26 (81.3%)
^{1}H-MRS alone (ITD)	55.6%	20.8%	52.5%	84.0%	23 (46.9%)	-	26 (53.1%)
CAP + ALT	14.3%	9.1%	15.4%	100%	6 (7.5%)	7 (8.8%)	7 (15.2%)
^{1}H-MRS + ALT	77.8%	30.4%	17.5%	100%	29 (59.2%)	19 (38.8%)	14 (28.6%)

TE–transient elastography; APRI–AST to platelet ratio index; NFS–NAFLD fibrosis score; FIB4–Fibrosis-4 score; PPV–positive predictive value; NPV–negative predictive value; (n=) in brackets
^Both negative or both positive, or serum result in the absence of successful TE reading.

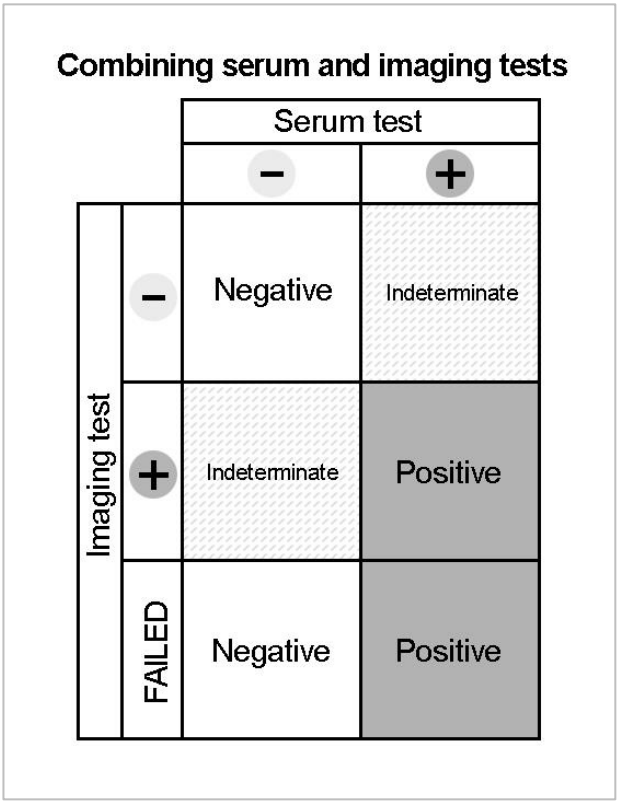
Supplementary Table 9.11: Diagnostic accuracy of transient elastography (TE) in combination with tests, with intention to diagnose (ITD) analysis.

Combination of tests*	Both tests positive^		Both tests negative^		Biopsies saved	Indeterminate	Correctly classified
	Sensitivity	PPV	Specificity	NPV			
F1-4 (TE threshold ≥7kPa)							
TE alone (ITD)	55.0%	26.2%	50.0%	77.5%	41.5% (34)	19.5% (16)	51.2% (42)
TE+APRI	20.0%	66.7%	65.6%	85.1%	58.0% (47)	34.6% (28)	42.0% (44)
TE+BARD	50.0%	32.3%	26.2%	72.7%	27.2% (22)	34.6% (28)	32.1% (26)
TE+NFS (low)	80.0%	35.6%	14.5%	100%	11.0% (9)	34.1% (28)	30.5% (25)
TE+FIB4 (low)	35.0%	41.2%	57.4%	81.4%	53.1% (43)	25.9% (21)	51.9% (42)
TE+Forn (low)	45.0%	36.0%	31.1%	76.0%	30.9% (25)	38.3% (31)	34.6% (28)
F2-4 (TE threshold ≥9kPa)							
TE alone (ITD)	100%	11.1%	59.0%	100%	56.1% (46)	19.5% (16)	60.9% (50)
TE+APRI	0	0	77.9%	100%	74.1% (60)	24.7% (20)	74.1% (60)
TE+BARD	100%	18.2%	32.5%	100%	30.9% (25)	42.0% (34)	35.8% (29)
TE+NFS (high)	75%	21.4%	61.5%	100%	58.5% (48)	24.4% (20)	62.2% (51)
TE+FIB4 (low)	75%	27.3%	63.6%	100%	60.5% (49)	25.9% (21)	64.2% (52)
TE+Forn (high)	75%	60%	75.5%	100%	72.8% (59)	21.0% (17)	76.5% (62)
F3-4 (TE threshold ≥12.85kPa)							
TE alone (ITD)	66.7%	8.3%	72.2%	98.3%	70.7% (58)	19.5% (16)	72.0% (59)
TE+APRI	0	-	89.7%	98.6%	67.9% (71)	12.3% (10)	86.4% (70)
TE+BARD	66.7%	14.3%	37.2%	100%	28.4% (29)	46.9% (38)	38.3% (31)
TE+NFS (high)	66.7%	25.0%	68.4%	100%	65.9% (54)	24.4% (20)	68.3% (56)
TE+FIB4 (low)	66.7%	33.3%	71.8%	100%	69.1% (56)	23.5% (19)	71.6% (58)
TE+Forn (high)	66.7%	66.7%	88.5%	100%	85.2% (69)	11.1% (9)	87.7% (71)

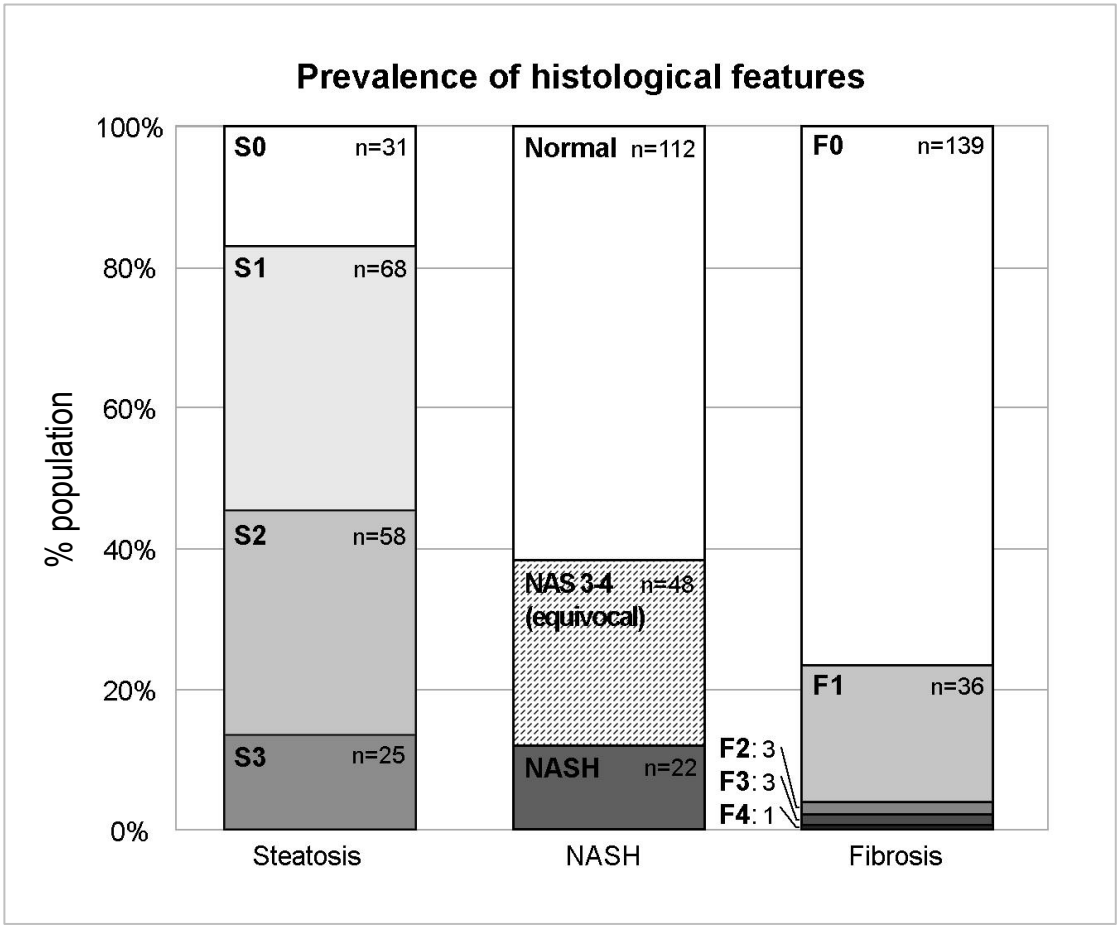
TE–transient elastography; APRI–AST to platelet ratio index; NFS–NAFLD fibrosis score; FIB4–Fibrosis-4 score; PPV–positive predictive value; NPV–negative predictive value; (n=) in brackets
**All analyses performed with intention to diagnose analysis. ^Both negative or both positive, or serum result in the absence of successful TE reading.*

18.4.2 **Supplementary Figures**

Supplementary Figure 9.1: Combining serum and imaging tests to improve practical diagnostic accuracy.

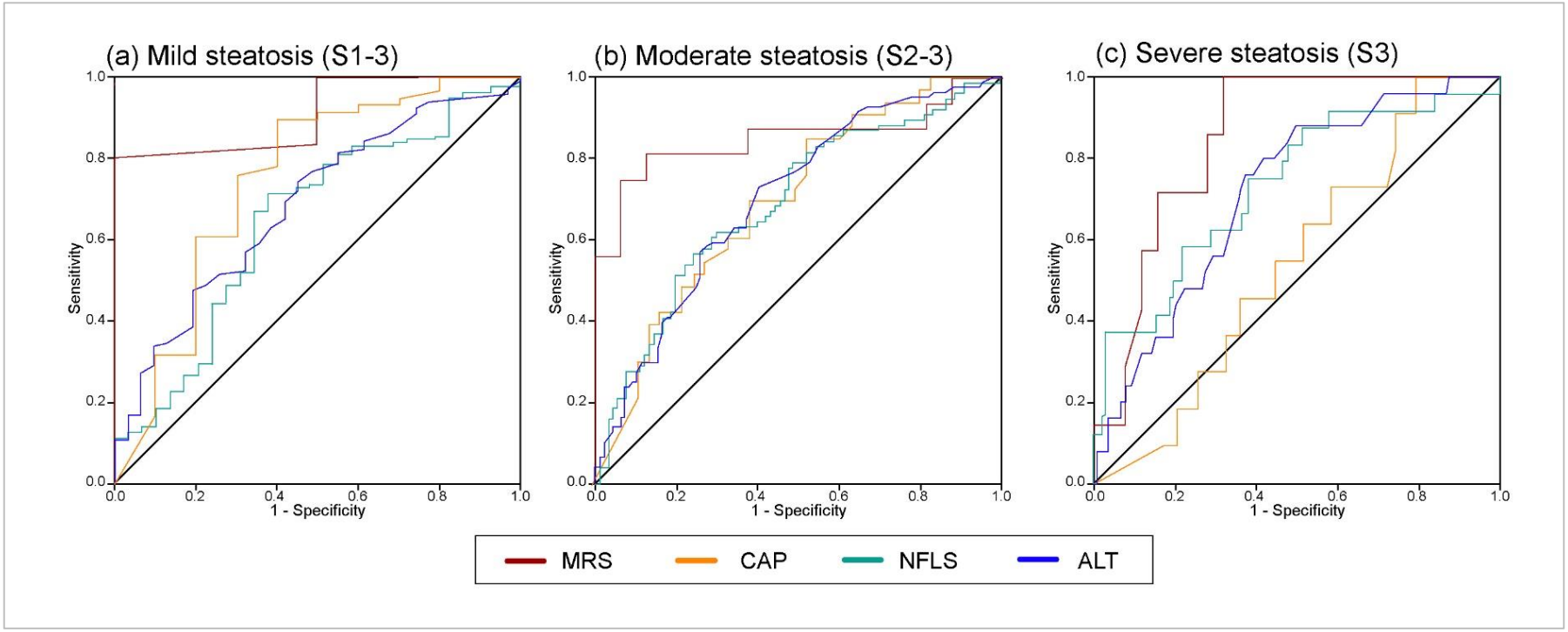


Supplementary Figure 9.2: Prevalence of histological disease features in this study cohort (steatosis, NASH and fibrosis)

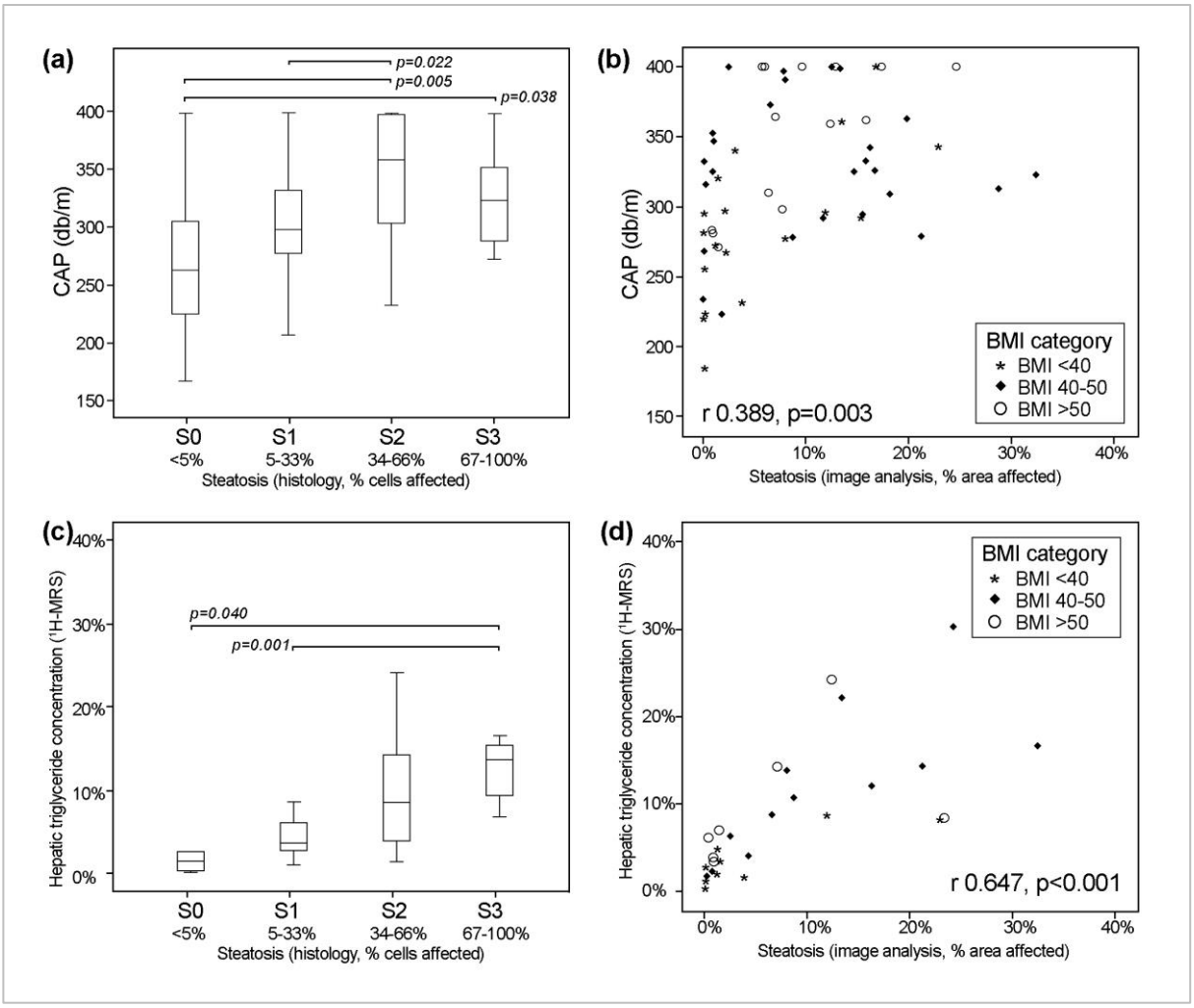


NAS – NAFLD activity score; NASH – nonalcoholic steatohepatitis

Supplementary Figure 9.3: Area under receiver operator characteristic (AUROC) curves for identification of mild steatosis (S1-3), significant steatosis (S2-3) and severe steatosis (S3) with ALT, NAFLD fatty liver score (NFLS), controlled attenuated parameter (CAP) and magnetic resonance spectroscopy (^{1}H-MRS).

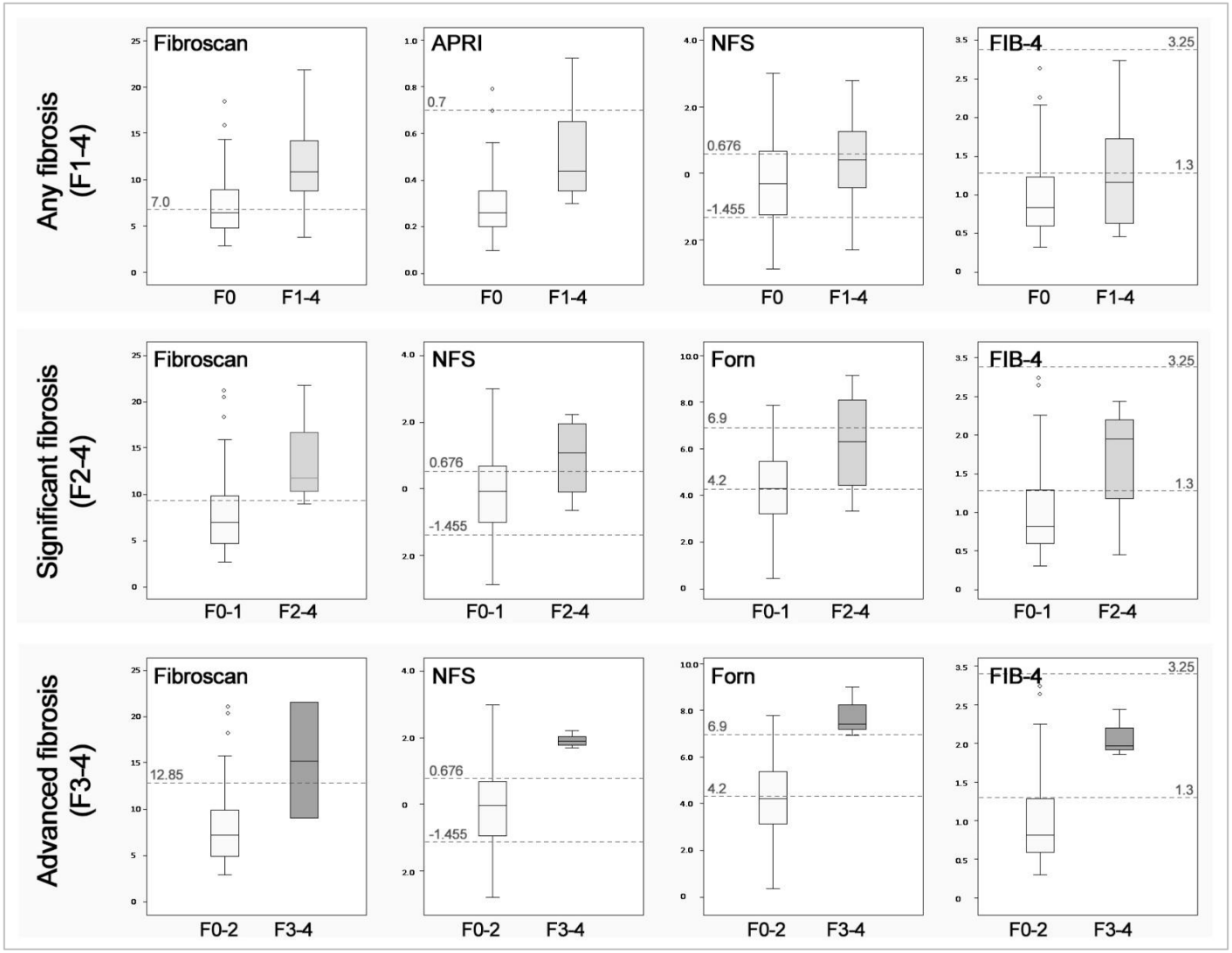


Supplementary Figure 9.4: (a) Bar graph of CAP readings per steatosis group; (b) Scatter plot of CAP readings per steatosis group as quantified by image analysis, with patients shown in BMI category; (c) Bar graph of ^{1}H-MRS readings per steatosis group; (d) Scatter plot of ^{1}H-MRS readings per steatosis group as quantified by image analysis.



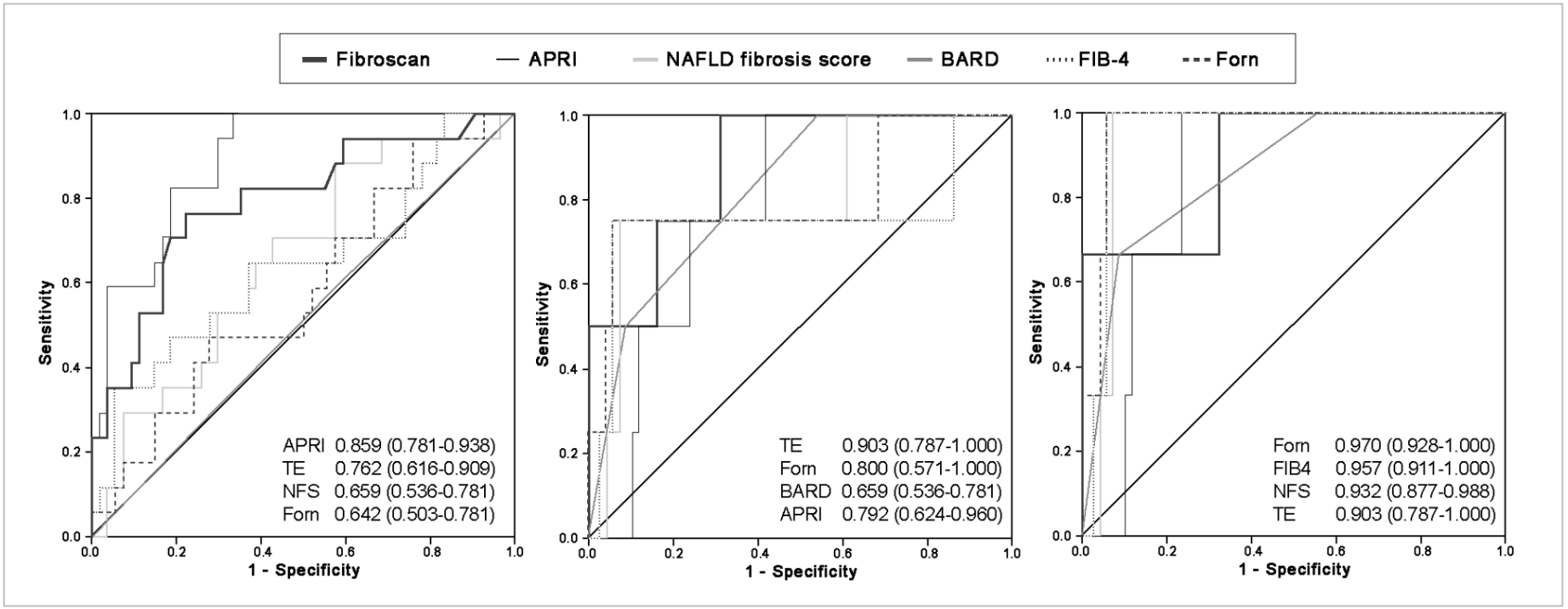
CAP – controlled attenuation parameter; BMI – body mass index; ^{1}H MRS – magnetic resonance spectroscopy

Supplementary Figure 9.5: Bar graph showing average score for each fibrosis level, for tests with best diagnostic accuracy.



APRI – AST to platelet ratio index; NFS – NAFLD fibrosis score; FIB-4 – fibrosis-4 score

Supplementary Figure 9.6: Area under receiver operator characteristic (AUROC) curves for identification of any fibrosis (F1-4), significant fibrosis (F2-4) and advanced fibrosis (F3-4).

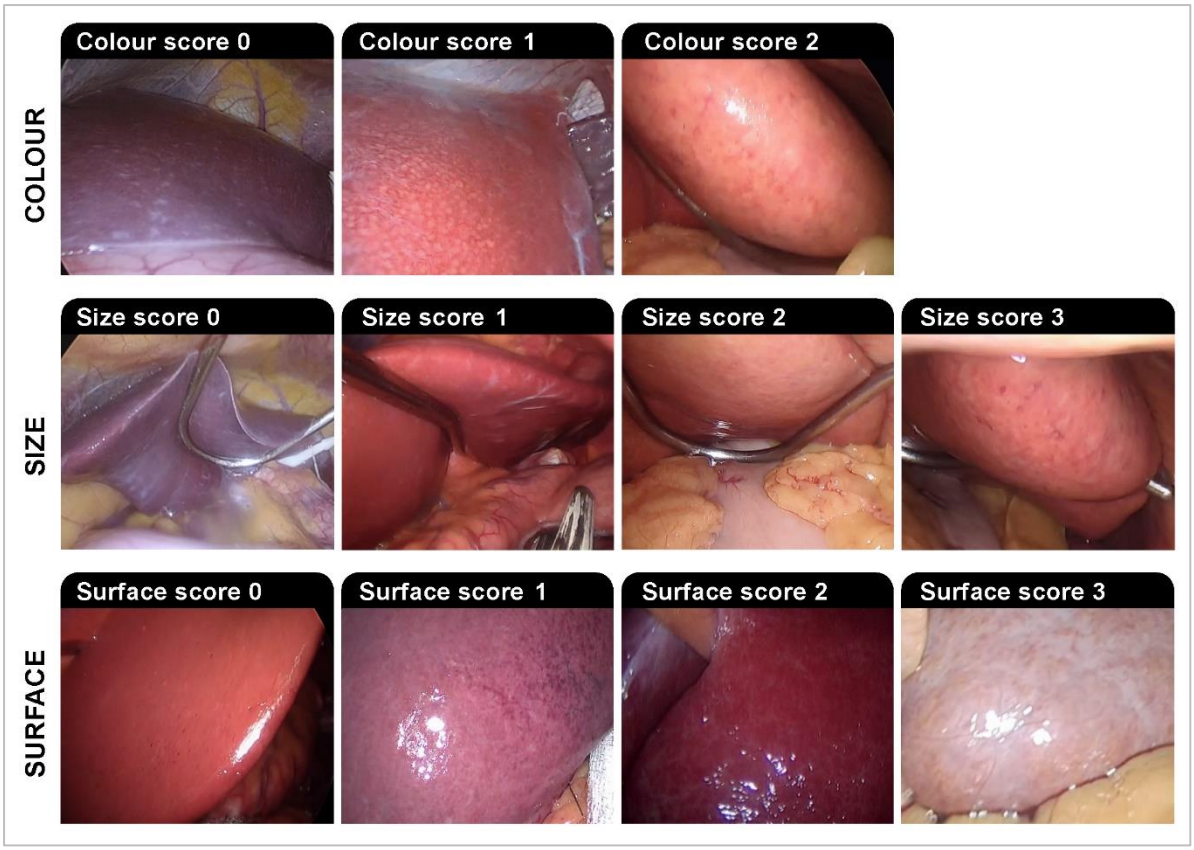


APRI – AST to platelet ratio index; NFS – NAFLD fibrosis score; BARD – BARD score; FIB-4 – fibrosis-4 score; TE – transient elastography.

18.5 Appendix 5: Supplementary materials - Visual liver score to stratify nonalcoholic steatohepatitis risk and determine selective intraoperative liver biopsy in obesity

18.5.1 **Supplementary Figures**

Supplementary Figure 10.1: Examples of associated scoring for each component of the intraoperative visual liver scoring (VLS) system.



18.5.2 **Supplementary Tables**

Supplementary Table 10.1: Baseline characteristics of total cohort, and patients with NASH.

Variable		All patients	Not NASH (NAS<5)	NASH (NAS≥5)	p-value
n=		152	132 (86.8%)	20 (13.2%)	
Age		44.9 ±11.8	45.2±12.0	43.4±10.4	0.526
Male gender		36 (23.7%)	29 (22.0%)	7 (35.0%)	0.201
Weight		126.3 ±27.0	124.4±24.6	139.1±38.1	0.110
BMI		**45.0±8.2**	**44.5±7.5**	**48.5±11.2**	**0.041**
Impaired fasting glucose		10 (6.6%)	7 (5.3%)	3 (15.8%)	0.086
T2DM		34 (22.5%)	28 (21.2%)	6 (31.6%)	0.312
Oral hypoglycaemic use		30 (21.0%)	24 (19.2%)	6 (33.3%)	0.169
Insulin use		10 (7.0%)	8 (6.4%)	2 (11.8%)	0.417
Hypertension		70 (46.4%)	59 (44.7%)	11 (57.9%)	0.281
Antihypertensive use		62 (43.7%)	53 (42.7%)	9 (50.0%)	0.562
High cholesterol		30 (19.9%)	24 (18.2%)	6 (31.6%)	0.171
Cholesterol lowering medication use		22 (15.7%)	19 (15.3%)	3 (18.8%)	0.723
Biochemistry					
Urea		4.8 ±1.8	4.8±1.9	5.0±1.3	0.543
Creatinine		70.8±21.8	70.6±22.6	72.6±14.2	0.718
Albumin		36.0±4.0	35.9±4.1	36.8±3.6	0.368
Bilirubin		8 (6-12)	8 (6-12)	9 (8-13)	0.306*
ALT		**46.2±42.7**	**43.4±41.9**	**67.2±44.1**	**0.026**
AST		35.4±32.5	33.6±32.0	48.4±34.3	0.070
GGT		**32 (20-42)**	**31 (19-41)**	**42 (31-57)**	**0.003***
ALP		74.3±21.0	73.6±19.6	78.9±29.7	0.315
Total cholesterol		4.4±3.9	4.4±4.2	4.5±0.7	0.963
HDL		1.11±1.68	1.1±1.8	0.9±0.2	0.520
LDL		2.5±0.9	2.4±0.9	2.7±0.6	0.235
Triglycerides		**1.5±0.7**	**1.4±0.7**	**2.0±0.8**	**0.003**
Total cholesterol: HDL ratio		4.74±4.00	4.66±4.24	5.33±1.24	0.503
Iron		12.7±4.7	12.5±4.6	14.3±5.6	0.128
Ferritin		129.1±147.8	125.0±147.1	158.2±153.8	0.374
Vitamin D		**63.6±25.1**	**65.4±25.0**	**50.9±22.8**	**0.026**
Fasting glucose		5.4 (4.8-6.4)	5.4 (4.8-6.1)	5.9 (5.1-7.2)	0.132*
HbA1c		5.7 (5.4-6.1)	5.7 (5.4-6.1)	5.9 (5.4-7.9)	0.325*
C-peptide		**763.5 (572-1080)**	**730 (529.5-987.5)**	**1070 (731-1780)**	**0.002***
Insulin		**6.9 (4.2-12.1)**	**6.3 (4.1-10.7)**	**14.6 (8.3-30.2)**	**0.001***
HOMA2: Insulin resistance		**1.3±1.3**	**1.2±1.1**	**2.4±1.8**	**0.011**
Haemoglobin		132.5±12.7	132.0±12.4	136.6±14.6	0.152
Platelet count		241.0±58.2	239.4±59.3	253.4±48.6	0.355
INR		1.1±0.1	1.1±0.1	1.1±0.1	0.849
Histology					
Total NAS	0-2 (not NASH)	98 (64.5%)	-	-	
	3-4 (equivocal)	34 (22.4%)	-	-	
	≥5 (NASH)	20 (13.2%)	-	-	
Steatosis	S0 (<5%)	45 (29.6%)	45 (34.1%)	0 (0.0%)	<0.001
	S1 (5-33%)	46 (30.3%)	45 (34.1%)	1 (5.0%)	
	S2 (34-66%)	49 (32.2%)	40 (30.3%)	9 (45.0%)	
	S3 (≥67%)	12 (7.9%)	2 (1.5%)	10 (50.0%)	
Inflammation	Grade 0	91 (59.8%)	91 (68.9%)	0 (0.0%)	<0.001
	Grade 1	55 (36.2%)	39 (29.5%)	16 (80.0%)	
	Grade 2	6 (4.0%)	2 (1.5%)	4 (20.0%)	
	Grade 3	0	0	0	
Ballooning	Grade 0	99 (65.1%)	98 (74.2%)	1 (5.0%)	<0.001
	Grade 1	39 (25.7%)	30 (22.7%)	9 (45.0%)	
	Grade 2	14 (9.2%)	4 (3.0%)	10 (50.0%)	
Fibrosis	F0	116 (76.3%)	112 (84.8%)	4 (20.0%)	<0.001
	F1	30 (19.7%)	17 (12.9%)	13 (65.0%)	
	F2	3 (2.0%)	1 (0.8%)	2 (10.0%)	
	F3	2 (1.3%)	2 (1.5%)	0 (0.0%)	
	F4	1 (0.7%)	0 (0.0%)	1 (5.0%)	

*Expressed as mean±standard deviation and number (percentage). Student t-test used for continuous variables, and chi-squared test used for categorical variables unless otherwise stated. *Mann Whitney U-test. ^Fisher exact test.*

Supplementary Table 10.2: Characterisation of the study population by visual liver score (VLS).

Variable			All patients	Any steatosis (S1-3)			Severe steatosis (S3)			Any fibrosis (F1-4)		
				S0	S1-3	p-value	S0-2	S3	p-value	F0	F1-4	p-value
n =			152							116 (76.3%)	36 (23.7%)	
VLS: Colour			0.61 ±0.77	0.22±0.47	0.77±0.82	<0.001	0.52±0.72	1.54±0.78	<0.001	0.48±0.72	1.00±0.83	<0.001
	Score	0	85 (61.2%)	36 (80.0%)	51 (47.7%)	<0.001	2 (15.4%)	2 (15.4%)	<0.001	75 (64.7%)	12 (33.3%)	0.002
		1	36 (25.9%)	8 (17.8%)	30 (28.0%)		2 (15.4%)	2 (15.4%)		26 (22.4%)	12 (33.3%)	
		2	18 (12.9%)	1 (2.2%)	26 (24.3%)		9 (69.2%)	9 (69.2%)		15 (12.9%)	12 (33.3%)	
VLS: Size			0.65±0.72	0.36±0.53	0.95±0.88	<0.001	2.08±0.95	2.08±0.95	<0.001	0.62±0.72	1.28±1.00	0.001
	Score	0	67 (48.2%)	30 (66.7%)	38 (35.5%)	0.001	1 (7.7%)	1 (7.7%)	<0.001	59 (50.9%)	9 (25.0%)	<0.001
		1	54 (38.8%)	14 (31.1%)	42 (39.3%)		2 (15.4%)	2 (15.4%)		43 (37.1%)	13 (36.1%)	
		2	17 (12.2%)	1 (2.2%)	21 (19.6%)		5 (38.5%)	5 (38.5%)		13 (11.2%)	9 (25.0%)	
		3	1 (0.7%)	-	6 (5.6%)		5 (38.5%)	5 (38.5%)		1 (0.9%)	5 (13.9%)	
VLS: Surface			0.09±0.35	0.04±0.21	0.14±0.46	0.082	0.38±0.77	0.38±0.77	0.190	0.04±0.20	0.33±0.72	0.022
	Score	0	129 (92.8%)	43 (95.6%)	96 (89.7%)	0.610	10 (76.9%)	10 (76.9%)	<0.001	111 (95.7%)	28 (77.8%)	0.002
		1	9 (6.5%)	2 (4.4%)	8 (7.5%)		1 (7.7%)	1 (7.7%)		5 (4.3%)	5 (13.9%)	
		2	-		2 (1.9%)		2 (15.4%)	2 (15.4%)		0	2 (5.6%)	
		3	1 (0.7%)		1 (0.9%)		-	-		0	1 (2.8%)	
Total VLS score			1.49 ±1.62	0.62±0.94	1.86±1.71	<0.001	1.26±1.39	4.00±1.83	<0.001	1.15±1.37	2.61±1.87	<0.001
Completely normal (VLS score of 0)			77 (55.4%)	36 (80.0%)	43 (40.2%)	<0.001	2 (15.4%)	2 (15.4%)	0.008^	52 (44.8%)	6 (16.7%)	<0.001
VLS grade	0-1		89 (58.6%)	38 (84.4%)	51 (47.7%)		87 (62.6%)	2 (15.4%)		79 (68.1%)	10 (27.8%)	
	2-3		39 (25.7%)	6 (13.3%)	33 (30.8%)		38 (27.3%)	1 (7.7%)		25 (21.6%)	14 (38.9%)	
	≥4		24 (15.8%)	1 (2.2%)	23 (21.5%)		14 (10.1%)	10 (76.9%)		12 (10.3%)	12 (33.3%)	
Overall impression			0.66±0.81	0.24±0.53	0.96±0.88	<0.001	1.69±0.75	1.69±0.75	<0.001	0.58±0.78	1.31±0.86	<0.001
	Normal		77 (55.4%)	36 (80.0%)	43 (40.2%)	<0.001	2 (15.4%)	2 (15.4%)	<0.001	70 (60.3%)	9 (25.0%)	<0.001
	Equivocal		32 (23.0%)	7 (15.6%)	25 (23.4%)		-	-		25 (21.6%)	7 (19.4%)	
	Abnormal		30 (21.6%)	2 (4.4%)	39 (36.4%)		11 (84.6%)	11 (84.6%)		21 (18.1%)	20 (55.6%)	
Biopsy?	No		125 (89.9%)	43 (95.6%)	88 (82.2%)	0.038^	6 (46.2%)	6 (46.2%)	<0.001	105 (90.5%)	26 (72.2%)	0.005
	Yes		14 (10.1%)	2 (4.4%)	19 (17.8%)		7 (53.8%)	7 (53.8%)		11 (9.5%)	10 (27.8%)	

^*Fisher exact test*

Supplementary Table 10.3a: Area under the receiver operator characteristic curve for visual identification of NASH, any fibrosis and significant fibrosis.

	Significant steatosis (S2-3)		**NASH (NAS≥5)**		**Significant fibrosis (F2-4)**	
Visual assessment	**AUROC**	**p-value**	**AUROC**	**p-value**	**AUROC**	**p-value**
VLS colour score	0.733 (0.649-0.817)	<0.001	0.709 (0.579-0.838)	0.003	0.637 (0.407-0.867)	0.256
VLS size score	0.726 (0.642-0.811)	<0.001	0.728 (0.593-0.864)	0.001	0.716 (0.498-0.934)	0.074
VLS surface score	0.539 (0.445-0.634)	0.410	0.546 (0.400-0.692)	0.519	0.818 (0.583-1.000)	0.008
Total VLS score	0.767 (0.687-0.847)	<0.001	0.746 (0.616-0.876)	0.001	0.841 (0.716-0.966)	0.005
Overall visual impression score	0.759 (0.678-0.841)	<0.001	0.746 (0.620-0.872)	0.001	0.797 (0.682-0.911)	0.014

Supplementary Table 10.3b: Area under the receiver operator characteristic curve for visual identification of any steatosis, severe steatosis and any fibrosis.

	Any steatosis (S1-3)		**Severe steatosis (S3)**		**Any fibrosis (F1-4)**	
Visual assessment	**AUROC**	**p-value**	**AUROC**	**p-value**	**AUROC**	**p-value**
VLS colour score	0.680 (0.594-0.766)	<0.001	**0.798 (0.654-0.942)**	0.001	0.665 (0.560-0.770)	0.003
VLS size score	0.691 (0.605-0.777)	<0.001	**0.853 (0.723-0.983)**	<0.001	0.677 (0.571-0.784)	0.001
VLS surface score	0.530 (0.431-0.628)	0.562	0.549 (0.368-0.731)	0.570	0.580 (0.464-0.695)	0.154
Total VLS score	**0.719 (0.636-0.801)**	<0.001	**0.855 (0.719-0.991)**	<0.001	**0.726 (0.627-0.825)**	<0.001
Overall visual impression score	**0.722 (0.640-0.804)**	<0.001	**0.790 (0.645-0.934)**	0.001	**0.714 (0.612-0.815)**	<0.001

Supplementary Table 10.4a: Interobserver variability in VLS, VLS components, and overall score – Overall level of agreement among multiple observers.

Parameter	Kappa	z-score	p-value
Colour component (VLS)	0.46	9.42	<0.001
Size component (VLS)	0.42	8.70	<0.001
Surface component (VLS)	0.45	9.32	<0.001
Total VLS (≥2)	0.53	10.98	<0.001
Overall impression (normal vs unsure/abnormal)	0.38	7.85	<0.001
Overall impression (normal/unsure vs abnormal)	0.38	7.87	<0.001

Supplementary Table 10.4b: Interobserver variability (kappa) in VLS components, total VLS, and overall impression score – Comparison in pairs.

Colour component (VLS)

	S2	S3	S4	S5
S1	**0.653**	**0.532**	**0.433**	**0.385**
S2		**0.455**	0.132	**0.531**
S3			**0.648**	**0.473**
S4				**0.409**

Size component (VLS)

	S2	S3	S4	S5
S1	**0.586**	**0.208**	**0.262**	**0.215**
S2		**0.262**	0.210	0.203
S3			**0.219**	**0.186**
S4				0.119

Surface component (VLS)

	S2	S3	S4	S5
S1	**0.468**	**0.776**	**0.258**	**0.613**
S2		**0.412**	**0.313**	**0.330**
S3			0.150	**0.588**
S4				**0.298**

Total VLS (≥2)

	S2	S3	S4	S5
S1	**0.897**	**0.607**	**0.527**	**0.559**
S2		**0.634**	**0.529**	**0.659**
S3			**0.385**	**0.733**
S4				**0.515**

Overall impression (Normal/Unsure vs Abnormal)

	S2	S3	S4	S5
S1	**0.588**	**0.664**	**0.280**	**0.475**
S2		**0.692**	0.222	**0.583**
S3			**0.371**	**0.672**
S4				**0.348**

Overall impression (Normal vs Unsure/Abnormal)

	S2	S3	S4	S5
S1	0.220	**0.322**	**0.343**	**0.358**
S2		**1.000**	**0.380**	**0.875**
S3			**0.371**	**0.672**
S4				**0.348**

S1-5 indicates individual surgeons. Bold indicates statistically significant. Shaded boxes are kappa≥0.500.

18.6 Appendix 6: Supplementary materials - Evaluating the histological variability of nonalcoholic fatty liver disease in obesity

18.6.1 **Supplementary Tables**

Supplementary Table 11.1a: Direct comparison of fibrosis scores between biopsy sites.

		Right core biopsy				
		F0	**F1**	**F2**	**F3**	**F4**
Left core biopsy	**F0**	44	7	3	0	0
	F1	3	9	1	0	0
	F2	1	2	0	0	0
	F3	0	1	1	3	0
	F4	0	0	0	1	0

		Left wedge biopsy				
		F0	**F1**	**F2**	**F3**	**F4**
Left core biopsy	**F0**	49	12	2	0	0
	F1	7	5	1	1	0
	F2	2	1	1	0	0
	F3	0	2	1	0	1
	F4	0	0	0	1	0

		Pathologist 2 (Overall)				
		F0	**F1**	**F2**	**F3**	**F4**
Pathologist 1 (Wedge)	**F0**	51	6	1	0	0
	F1	14	6	0	0	0
	F2	3	1	1	0	0
	F3	0	1	0	1	0
	F4	0	0	0	1	0

Supplementary Table 11.1b: Direct comparison of presence of significant fibrosis between biopsy sites.

		Right core biopsy	
		F0-1	**F2-4**
Left core biopsy	**F0-1**	63	4
	F2-4	4	5

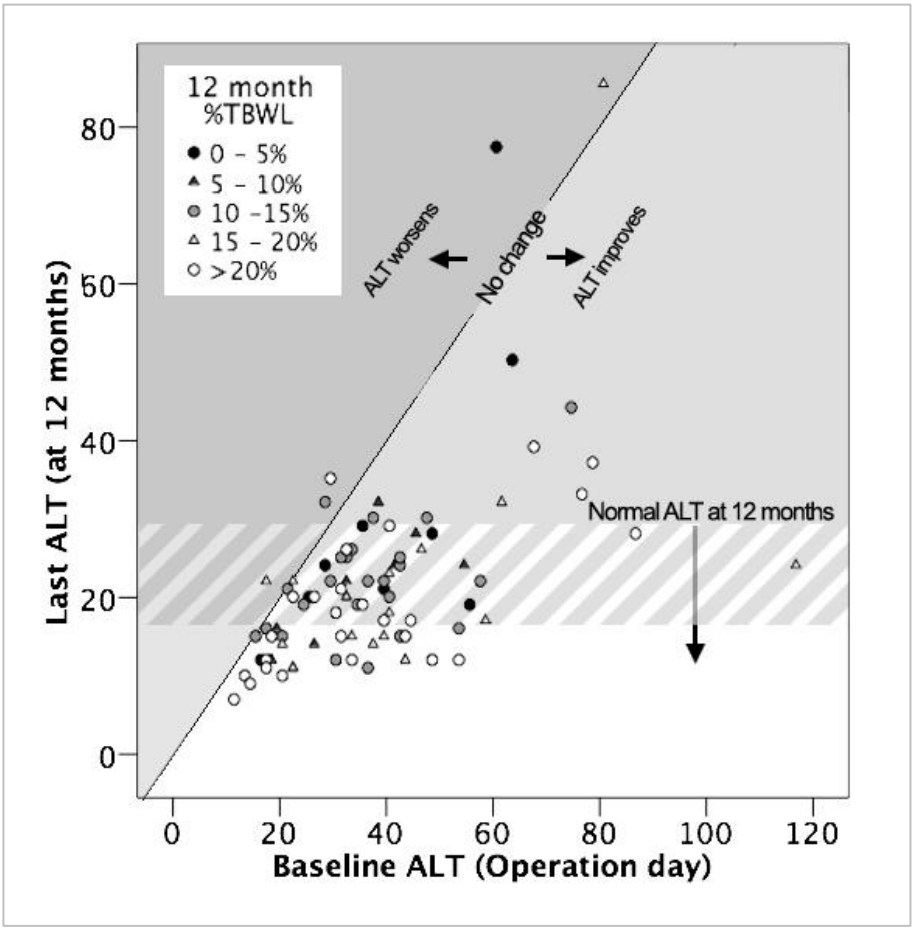
		Left wedge biopsy	
		F0-1	**F2-4**
Left core biopsy	**F0-1**	73	4
	F2-4	5	4

		Pathologist 2 (Overall)	
		F0-1	**F2-4**
Pathologist 1 (Wedge)	**F0-1**	77	1
	F2-4	5	3

18.7 Appendix 7: Supplementary materials - Effects of bariatric surgery on liver function tests in patients with nonalcoholic fatty liver disease

18.7.1 **Supplementary Figure**

Supplementary Figure 12.1: Individual patient ALT at baseline and 12 months after bariatric surgery. Twelve-month weight loss achieved depicted by key. This shows most patients with improved ALT after 12 months of weight loss, with a trend for lower 12 month ALT for those with greater weight loss. Over half the patients had a normal ALT at 12 months.



ALT – alanine aminotransferase; TBWL – total body weight loss.

18.8 Appendix 8: Supplementary materials - Detailed description of change in serum cholesterol profile with incremental weight loss after restrictive bariatric surgery

18.8.1 **Supplementary Table**

Supplementary Table 14.1: Changes in triglycerides, high density lipoprotein (HDL), and total cholesterol to HDL ratio (TC:HDL) with weight loss in males and females.

%TBWL category	Triglycerides				High density lipoprotein (HDL)				Total to HDL ratio (TC:HDL)				Low density lipoprotein (LDL)			
	All patients	Normal at baseline	Abnormal at baseline	p-value	All patients	Normal at baseline	Abnormal at baseline	p-value	All patients	Normal at baseline	Abnormal at baseline	p-value	All patients	Normal at baseline	Abnormal at baseline	p-value
Males																
0-2.5%	2.07±1.35	1.47±0.35	3.21±1.78	0.001	1.03±0.24	1.19±0.17	0.81±0.13	<0.001	4.65±1.36	3.73±0.74	5.64±1.17	<0.001	2.68±0.97	1.93±0.42	3.53±0.64	<0.001
2.5-5.0%	2.12±0.75	1.64±0.51	2.34±0.74	0.024	0.98±0.21	1.10±0.15	0.83±0.18	<0.001	5.05±1.43	3.76±0.93	5.90±0.99	<0.001	2.89±1.21	1.93±0.57	3.43±1.15	<0.001
5.0-7.5%	1.60±0.62	1.41±0.45	2.06±0.73	0.005	1.12±0.27	1.24±0.23	0.91±0.16	<0.001	4.13±1.12	3.44±0.64	5.22±0.95	<0.001	2.60±0.87	2.03±0.60	3.08±0.77	<0.001
7.5-10.0%	1.54±0.72	1.29±0.39	2.02±0.94	0.002	1.17±0.24	1.32±0.23	1.06±0.19	<0.001	3.76±1.06	3.41±0.70	4.68±1.32	0.001	2.37±0.80	1.96±0.58	2.71±0.81	<0.001
10.0-12.5%	1.66±0.70	1.39±0.35	2.02±0.87	0.001	1.09±0.24	1.20±0.26	1.02±0.20	0.002	4.35±1.28	3.33±0.57	4.96±1.20	<0.001	2.68±0.74	2.17±0.53	2.97±0.68	<0.001
12.5-15.0%	1.31±0.48	1.11±0.28	1.61±0.55	0.001	1.14+0.21	1.22±0.20	1.04±0.18	0.003	4.04±0.94	3.29±0.69	4.39±0.83	<0.001	2.74±0.71	2.19±0.61	3.05±0.56	<0.001
15.0-17.5%	1.31±0.59	1.03±0.28	1.62±0.71	0.010	1.20±0.22	1.29±0.21	1.07±0.16	0.003	3.83±1.03	2.87±0.46	4.24±0.92	<0.001	2.66±0.86	2.04±0.61	3.03±0.78	0.001
17.5-20.0%	1.28±0.58	1.17±0.40	1.53±0.83	0.097	1.19±0.23	1.21±0.24	1.16±0.20	0.521	4.01±1.10	2.82±0.50	4.61±0.78	<0.001	2.81±0.86	1.97±0.40	3.23±0.70	<0.001
20.0-22.5%	1.17±0.40	1.08±0.33	1.36±0.50	0.098	1.17±0.19	1.24±0.16	1.02±0.17	0.002	4.12±0.86	3.11±0.78	4.48±0.55	<0.001	2.99±0.78	2.40±0.67	3.23±0.70	0.008
22.5-25.0%	1.02±0.42	0.86±0.23	1.43±0.55	0.130	1.33±0.30	1.48±0.29	1.19±0.24	0.068	3.44±0.83	2.98±0.63	3.79±0.83	0.072	2.63±0.75	2.16±0.55	3.25±0.46	0.002
>25.0%	0.99±0.21	0.97±0.15	1.01±0.29	0.730	1.42±0.27	1.48±0.15	1.35±0.36	0.314	2.88±0.46	2.85±0.44	2.97±0.56	0.631	2.16±0.61	1.98±0.37	3.13±0.83	0.134
Females																
0-2.5%	1.81±0.72	1.46±0.39	2.44±0.75	<0.001	1.22±0.24	1.46±0.26	1.11±0.13	<0.001	4.25±1.11	3.72±0.56	5.54±0.91	<0.001	3.10±0.96	2.14±0.55	3.46±0.82	<0.001
2.5-5.0%	1.64±0.52	1.35±0.32	2.06±0.47	<0.001	1.20±0.24	1.39±0.26	1.14±0.19	0.001	4.18±0.88	3.71±0.63	4.97±0.65	<0.001	2.97±0.86	2.67±1.16	3.11±0.66	<0.001
5.0-7.5%	1.87±0.84	1.42±0.36	2.42±0.93	<0.001	1.17±0.22	1.40±0.29	1.13±0.18	0.005	4.35±1.17	3.84±0.93	5.15±1.06	<0.001	2.92±1.03	2.57±1.08	3.22±0.87	0.003
7.5-10.0%	1.70±0.59	1.40±0.42	2.14±0.54	<0.001	1.26±0.26	1.41±0.29	1.19±0.22	0.001	4.13±1.13	3.53±0.69	5.15±1.02	<0.001	2.96±0.98	2.49±1.11	3.20±0.83	0.001
10.0-12.5%	1.66±0.49	1.51±0.42	1.89±0.51	<0.001	1.35±0.31	1.53±0.31	1.15±0.16	<0.001	3.99±0.18	3.50±0.76	4.82±1.04	<0.001	3.07±1.02	2.06±0.50	3.46±0.89	<0.001
12.5-15.0%	1.61±0.53	1.45±0.41	1.83±0.60	0.001	1.38±0.31	1.55±0.35	1.23±0.17	<0.001	4.02±1.25	3.45±0.84	4.89±1.26	<0.001	3.19±1.16	2.08±0.70	3.59±1.02	<0.001
15.0-17.5%	1.43±0.48	1.28±0.35	1.74±0.56	0.001	1.49±0.35	1.65±0.41	1.32±0.18	<0.001	3.61±1.06	3.42±0.85	3.85±1.25	0.107	3.02±1.06	2.21±0.36	3.25±1.08	<0.001
17.5-20.0%	1.44±0.50	1.27±0.42	1.69±0.51	<0.001	1.48±0.37	1.69±0.40	1.30±0.21	<0.001	3.63±1.05	3.30±0.76	4.19±1.22	<0.001	2.99±0.98	2.08±0.45	3.32±0.91	<0.001
20.0-22.5%	1.40±0.42	1.23±0.27	1.63±0.47	0.003	1.43±0.35	1.73±0.37	1.29±0.23	0.002	3.77±0.99	3.48±0.87	4.23±1.01	0.023	3.12±0.95	2.28±0.55	3.44±0.87	<0.001
22.5-25.0%	1.25±0.47	1.07±0.32	1.81±0.41	<0.001	1.57±0.39	1.88±0.38	1.40±0.28	0.004	3.50±0.98	2.93±0.47	4.39±0.90	<0.001	3.10±0.79	2.40±0.31	3.43±0.74	<0.001
>25.0%	1.16±0.35	1.02±0.21	1.52±0.38	<0.001	1.54±0.26	1.64±0.17	1.51±0.27	0.121	3.28±0.75	3.07±0.68	3.59±0.77	0.011	2.85±0.87	2.25±0.46	3.04±0.89	<0.001

TBWL – total body weight loss; HDL – high density lipoprotein, TC:HDL – total cholesterol to HDL ratio. Values expressed as mean±standard deviation. Independent Student t-test for comparison between normal and abnormal values

18.9 Appendix 9: Supplementary materials - Lipidomic analysis of nonalcoholic fatty liver disease (NAFLD) in morbid obesity: Alterations in liver lipid profile and parallel serum changes with progressive disease

18.9.1 **Supplementary Tables**

Supplementary Tables

Supplementary Table 15.1: Characteristics of cohort at baseline and comparison between histological groups.

Variable	**All patients n=181**	***Normal* liver* n=53**	***NAFL* liver* n=68**	***NASH* liver* n=60**	***p-value***
Clinical variables					
Age	45 ± 12	45 ± 14	45 ± 11	44 ± 12	0.882
Gender (males)	44 (24:3%)	10 (18.9%)	14 (20.6%)	20 (33.3%)	0.134
Body mass index (BMI), kg/m2	45.1 ± 8.3	44.1 ± 9.1	45.0 ± 6.6	46.0 ± 9.3	0.469
Weight, kg	126.4 ± 28.6	122.6 ± 31	125.1 ± 22.5	131.3 ± 32.2	0.247
Type 2 diabetes mellitus (T2DM)	40 (22.2%)	8 (15.1%)	15 (22.1%)	17 (28.8%)	0.218
Any antidiabetic medication	37 (20.4%)	7 (13.2%)	14 (20.6%)	16 (26.7%)	0.208
Dyslipidaemia	160 (88.4%)	44 (83.0%)	62 (91.2%)	54 (90.0%)	0.340
Any lipid lowering medication	28 (15.5%)	12 (22.6%)	8 (11.8%)	8 (13.3%)	0.222
Hypertension	82 (45.6%)	24 (45.3%)	27 (39.7%)	31 (52.5%)	0.350
Any antihypertensive medication	75 (41.4%)	22 (41.5%)	26 (38.2%)	28 (46.7%)	0.626
Biochemical variables					
Alanine aminotransferase (ALT), IU/L	33 (24-52)	25 (17-35)	31 (26-49)	47 (33-66)	<0.001
Aspartate aminotransferase (AST), IU/L	27 (22-35)	22 (17-28)	27 (23-33)	32 (26-50)	<0.001
Gamma glutamyl transferase (GGT), IU/L	32 (20-42)	23 (18-37)	34 (20-41)	37 (27-49)	0.001
Alkaline phosphatase (ALP), IU/L	72 ± 21	73 ± 21	70 ± 20	73 ± 22	0.593
Bilirubin, mmol/L	10 ± 5	10 ± 6	10 ± 5	10 ± 5	0.734
Total cholesterol (TC), mmol/L	4.1 ± 1.0	4.0 ± 1.1	4.0 ± 1.0	4.2 ± 0.8	0.581
High density lipoprotein (HDL), mmol/L	1.0 ± 0.3	1.1 ± 0.3^	1.0 ± 0.2	0.9 ± 0.2^	0.004
Low density lipoprotein (LDL), mmol/L	2.4 ± 0.8	2.4 ± 0.9	2.3 ± 0.8	2.5 ± 0.8	0.730
Triglyceride levels (TG), mmol/L	1.5 ± 0.7	1.2 ± 0.5^	1.5 ± 0.7	1.7 ± 0.8^	0.001
Blood sugar level (BSL), mmol/L	5.8 ± 1.9	5.4 ± 1.1	5.8 ± 2.2	6.1 ± 2.1	0.143
HbA1c, %	5.7 (5.4-6.1)	5.6 (5.3-5.9)	5.7 (5.4-6.1)	5.9 (5.5-6.9)	0.014
Insulin, mU/L	7.1 (4.3-12.2)	5.3 (3.6-10.9)	6.8 (4.5-11.8)	8.2 (5.6-15)	0.023
C-peptide, pmol/L	773 (572-1081)	644 (478-893)	771 (588-1093)	923 (674-1294)	0.002

**Groups for lipidomics analysis. Statistical significance tested by independent student t-test and one-way ANOVA for continuous data, and Chi-square test for categorical data, unless otherwise specified. ^Significantly different on post-hoc analysis. #Kruskal-Wallis test*

Supplementary Table 15.2a: Liver cholesterol and cholesterol esters significantly associated with increasing steatosis in liver. (Full list of lipids in Supplementary Spreadsheet)

Significant decrease associated with steatosis			Significant increase associated with steatosis		
Lipid	**Percent change (95% CI)**	**p-value**	**Lipid**	**Percent change (95% CI)**	**p-value**
CE(18:2)	-2.19 (-3.79, -0.57)	0.025	CE(16:0)	1.01 (0.03, 2)	0.099
CE(22:5) (n6)	-2.71 (-3.8, -1.61)	<0.001	CE(17:0)	2.31 (1.11, 3.53)	0.001
			CE(18:0)	3.58 (2.51, 4.65)	<0.001
			CE(18:1)	1.21 (0.4, 2.02)	0.011
			CE(18:3)	2.06 (0.75, 3.38)	0.007
			COH	1.31 (0.64, 1.97)	0.001

Supplementary Table 15.2b: Liver sphingolipid species significantly correlated to severity of steatosis in liver. Full list of changes in all lipids shown in Supplementary Spreadsheet.

Significant decrease associated with steatosis			Significant increase associated with steatosis		
Lipid	**Percent change (95% CI)**	**p-value**	**Lipid**	**Percent change (95% CI)**	**p-value**
			Dihydroceramide		
			Cer(d18:0/16:0)	1.50 (0.33, 2.69)	0.034
			Cer(d18:0/18:0)	3.17 (1.77, 4.59)	<0.001
			Cer(d18:0/20:0)	3.18 (1.92, 4.44)	<0.001
			Cer(d18:0/22:0)	2.84 (1.95, 3.75)	<0.001
			Cer(d18:0/24:0)	2.08 (1.27, 2.89)	<0.001
			Cer(d18:0/24:1)	2.64 (1.77, 3.52)	<0.001
			Ceramide		
			Cer(d18:1/16:0)	1.80 (0.92, 2.68)	<0.001
			Cer(d18:1/18:0)	2.01 (1.2, 2.82)	<0.001
			Cer(d18:1/20:0)	1.61 (0.91, 2.31)	<0.001
			Cer(d18:1/22:0)	1.08 (0.47, 1.69)	0.002
			Cer(d18:1/24:0)	1.31 (0.68, 1.94)	<0.001
			Dihexosylceramide		
			Hex2Cer(d18:1/22:0)	0.96 (0.16, 1.76)	0.047
			Hex2Cer(d18:1/24:0)	1.35 (0.49, 2.21)	0.008
			Trihexosylceramide		
			Hex3Cer(d18:1/18:0)	1.04 (0.09, 2)	0.075
			Hex3Cer(d18:1/22:0)	1.65 (0.67, 2.65)	0.004
			Hex3Cer(d18:1/24:0)	0.97 (0.23, 1.73)	0.030
			Hex3Cer(d18:1/24:1)	0.93 (0.15, 1.71)	0.049
			GM3 ganglioside		
			GM3(d18:1/16:0)	1.08 (0.39, 1.77)	0.008
			GM3(d18:1/18:0)	1.07 (0.19, 1.95)	0.046
			GM3(d18:1/20:0)	2.09 (0.92, 3.27)	0.002
			GM3(d18:1/24:0)	0.84 (0.25, 1.44)	0.018
Sphingomyelin			***Sphingomyelin***		
SM(37:2)	-1.46 (-2.15, -0.77)	<0.001	SM(d18:0/16:0)	1.4 (0.65, 2.14)	0.001
SM(d18:1/20:0)/ SM(d16:1/22:0)	-0.53 (-0.96, -0.09)	0.047	SM(d18:1/17:0)/ SM(d17:1/18:0)	0.79 (0.16, 1.44)	0.041
SM(d18:2/18:0)	-0.81 (-1.52, -0.09)	0.065			
SM(d18:2/20:0)	-0.58 (-1.08, -0.09)	0.053			

CI – confidence interval;

Supplementary Table 15.2c: Liver phospholipids significantly associated with increasing steatosis in liver. (Full list of lipids in Supplementary Spreadsheet)

Significant decrease associated with steatosis			**Significant increase associated with steatosis**		
Lipid	**Percent change (95% CI)**	**p-value**	**Lipid**	**Percent change (95% CI)**	**p-value**
PC(15-MHDA_18:1)	-1.18 (-1.88, -0.48)	0.004	PC(28:0)	1.78 (0.5, 3.08)	0.020
PC(17:0_18:1)	-1.00 (-1.55, -0.44)	0.002	PC(31:0) (a)	1.61 (0.55, 2.69)	0.011
PC(15-MHDA_18:2)	-1.48 (-2.1, -0.85)	**<0.001**	PC(31:0) (b)	0.83 (0.16, 1.5)	0.041
PC(17:0_18:2)	-1.18 (-1.78, -0.57)	0.001	PC(16:0_18:2)	0.47 (0.17, 0.77)	0.009
PC(17:1_18:2)	-1.67 (-2.39, -0.94)	**<0.001**	PC(36:0)	1.79 (0.96, 2.63)	**<0.001**
PC(15:0_20:4)	-1.27 (-1.93, -0.61)	0.001			
PC(18:1_18:2)	-0.96 (-1.48, -0.43)	0.002	PC(O-36:5)	1.43 (0.5, 2.38)	0.009
PC(16:1_20:4)	-1.11 (-1.82, -0.39)	0.009	PC(O-40:7)	0.95 (0.45, 1.46)	0.001
PC(15-MHDA_20:4)	-1.96 (-2.71, -1.2)	**<0.001**			
PC(17:0_20:4)	-1.57 (-2.22, -0.9)	**<0.001**	LPC(16:0) [sn2]	1.63 (0.37, 2.91)	0.032
PC(15:0_22:6)	-1.28 (-1.85, -0.7)	**<0.001**	LPC(18:0) [sn2]	2.13 (0.59, 3.7)	0.021
PC(18:0_20:4)	-0.75 (-1.27, -0.23)	0.016	LPC(26:0) [sn1]	1.97 (0.75, 3.19)	0.006
PC(38:5) (a)	-1.2 (-1.59, -0.81)	**<0.001**			
PC(38:5) (b)	-1.11 (-1.64, -0.58)	**<0.001**	LPC(O-16:0)	1.85 (0.71, 3)	0.006
PC(38:6) (a)	-0.93 (-1.44, -0.41)	0.002	LPC(O-18:0)	1.59 (0.36, 2.84)	0.032
PC(16:1_22:6)	-1.08 (-1.7, -0.46)	0.003	LPC(O-20:0)	1.47 (0.24, 2.72)	0.05
PC(38:7)(c)	-1.06 (-1.77, -0.34)	0.013			
PC(39:5)(a)	-1.59 (-2.39, -0.79)	0.001	PE(16:0_16:1)	1.51 (0.5, 2.53)	0.012
PC(39:5)(b)	-1.77 (-2.38, -1.16)	**<0.001**	PE(18:0_18:1)	1.28 (0.69, 1.87)	**<0.001**
PC(15-MHDA_22:6)	-2.26 (-3, -1.52)	**<0.001**			
PC(17:0_22:6)	-1.77 (-2.53, -1)	**<0.001**	LPE(16:0) [sn2]	2.38 (0.77, 4.02)	0.013
PC(18:0_22:6)	-0.76 (-1.37, -0.13)	0.046	LPE(18:0) [sn2]	2.7 (0.93, 4.49)	0.01
PC(18:1_22:6) (a)	-0.98 (-1.56, -0.41)	0.004	LPE(18:0) [sn1]	2.03 (0.32, 3.77)	0.05
PC(18:1_22:6) (b)	-1.47 (-2.3, -0.64)	0.003	LPE(18:1) [sn2]	1.60 (0.68, 2.53)	0.003
			LPE(18:1) [sn1]	2.05 (0.85, 3.26)	0.003
PC(P-16:0/20:4)	-0.78 (-1.42, -0.14)	0.045	LPE(18:2) [sn2]	1.66 (0.5, 2.83)	0.016
PC(P-38:5) (a)	-1.07 (-1.83, -0.3)	0.02	LPE(18:2) [sn1]	2.29 (0.98, 3.62)	0.003
			LPE(20:4) [sn1]	2.02 (0.88, 3.18)	0.002
PE(16:0_20:4)	-0.55 (-0.96, -0.14)	0.025	LPE(22:6) [sn1]	2.17 (0.85, 3.51)	0.005
PE(38:5) (a)	-1.34 (-1.84, -0.84)	**<0.001**			
PE(16:0_22:6)	-0.58 (-1.06, -0.1)	0.049	PI(16:0/16:0)	2.92 (1.75, 4.11)	**<0.001**
PE(18:1_22:6) (a)	-1.46 (-2.08, -0.83)	**<0.001**	PI(16:0_16:1)	2.51 (1.34, 3.68)	**<0.001**
PE(18:1_22:6) (b)	-1.32 (-2.05, -0.58)	0.002	PI(34:0)	1.59 (0.33, 2.86)	0.038
			PI(34:1)	1.59 (0.77, 2.41)	0.001
PI(18:0_20:3) (b)	-0.98 (-1.77, -0.18)	0.045	PI(18:0_18:1)	1.23 (0.48, 1.99)	0.005
PI (38:5) (b)	-1.13 (-1.79, -0.47)	0.004	PI(36:2) (a+b)	1.42 (0.79, 2.06)	**<0.001**
			PI(18:0_22:5) (n3)	2.25 (1.47, 3.04)	**<0.001**
			LPI(18:0) [sn2]	2.27 (0.93, 3.63)	0.004
			LPI(18:0) [sn1]	1.43 (0.53, 2.34)	0.007
			LPI(18:1) [sn2]	3.08 (1.68, 4.49)	**<0.001**
			LPI(18:1) [sn1]	2.17 (1.16, 3.19)	**<0.001**
			LPI(18:2) [sn2]	1.76 (0.48, 3.05)	0.021
			LPI(18:2) [sn1]	2.76 (1.45, 4.09)	**<0.001**
			PG(36:2)	1.36 (0.65, 2.07)	0.001

Supplementary Table 15.3: Comparison of significant changes in liver lipid profile between normal vs nonalcoholic steatohepatitis (NASH), and normal vs non-NASH nonalcoholic fatty liver (NAFL)

Change from Normal	**Non-NASH NAFL**		**NASH**	
Lipid species	***Percent change in lipid***	***p-value***	***Percent change in lipid***	***p-value***
Cer(d18:0/18:0)	54.0	0.195	65.2	0.034
Cer(d18:0/20:0)	44.8	0.195	55.4	0.038
Cer(d18:0/22:0)	48.7	0.019	50.7	0.006
Cer(d18:0/24:1)	39.7	0.068	49.7	0.004
Hex1Cer(d18:1/22:0)	-21.9	0.019	-17.3	0.116
Hex1Cer(d18:1/24:0)	-22.6	0.038	-19.1	0.104
Hex1Cer(d18:1/24:1)	-27.3	0.037	-20.3	0.071
Hex3Cer(d18:1/18:0)	54.8	0.003	52.7	0.005
Hex3Cer(d18:1/22:0)	62.5	0.015	45.6	0.020
Hex3Cer(d18:1/24:0)	34.3	0.016	22.7	0.066
Hex3Cer(d18:1/24:1)	38.9	0.040	29.6	0.049
GM3(d18:1/20:0)	71.8	0.024	65.5	0.021
SM(37:2)	-18.0	0.079	-27.6	0.019
SM(d18:1/20:0)/SM(d16:1/22:0)	-14.2	0.041	-14.8	0.040
SM(d18:1/24:0)	-9.3	0.251	-16.2	0.044
SM(d18:1/24:1)	-8.2	0.306	-13.8	0.038
SM(d18:2/20:0)	-10.2	0.112	-17.3	0.036
PC(28:0)	53.4	0.068	58.2	0.035
PC(31:0) (a)	36.1	0.153	50.5	0.005
PC(16:0_18:1)	8.0	0.038	7.5	0.067
PC(17:0_18:1)	-15.1	0.126	-19.5	0.024
PC(15-MHDA_18:2)	-24.8	0.016	-29.2	0.001
PC(17:0_18:2)	-25.8	0.009	-26.0	0.003
PC(17:1_18:2)	-35.5	0.006	-33.0	<0.001
PC(15:0_20:4)	-11.9	0.304	-23.0	0.035
PC(18:1_18:2)	-22.2	0.019	-25.6	<0.001
PC(18:2_18:2)	-20.4	0.235	-27.4	0.030
PC(15-MHDA_20:4)	-15.3	0.295	-31.7	0.005
PC(17:0_20:4)	-16.8	0.153	-28.5	0.005
PC(38:5) (a)	-10.9	0.185	-18.2	0.009
PC(38:5) (b)	-16.2	0.103	-19.3	0.044
PC(38:6) (a)	-6.9	0.549	-21.9	0.003
PC(39:5)(a)	-28.6	0.027	-27.1	0.036
PC(39:5)(b)	-24.0	0.010	-28.2	0.005
PC(15-MHDA_22:6)	-23.5	0.040	-29.6	0.021
PC(17:0_22:6)	-23.2	0.058	-26.0	0.040
PC(18:1_22:6) (b)	-17.2	0.118	-27.2	0.042
PC(40:8)	-3.0	0.812	-22.1	0.040
PC(O-32:1)	-20.0	0.030	-9.3	0.483
PC(O-18:0/18:2)	-28.6	0.046	-14.8	0.475
PC(O-40:5)	-16.4	0.045	-12.4	0.178
PC(O-18:0/22:6)	-23.0	0.035	-19.7	0.099
PC(O-40:7)	32.9	0.002	30.5	0.002
PC(P-16:0/20:4)	-13.1	0.203	-22.0	0.025
PC(P-38:5) (a)	-13.0	0.314	-32.6	0.002
LPC(26:0) [sn1]	58.8	0.028	66.1	0.008
PE(18:1_18:2)	-23.0	0.112	-25.1	0.042
PE(16:0_20:4)	-8.0	0.304	-13.7	0.035
PE(38:5) (a)	-19.1	0.045	-28.3	<0.001
PE(18:0_22:5) (n3)	-14.2	0.153	-21.3	0.028
PE(18:1_22:6) (a)	-17.5	0.140	-26.6	0.004
PE(18:1_22:6) (b)	-20.5	0.046	-31.8	0.006
PE(O-16:0/20:4)	-19.8	0.019	-18.4	0.167
PE(O-38:6) (b)	-4.2	0.633	-18.3	0.022
PE(P-18:1/20:4) (a)	-4.3	0.681	-18.9	0.040
PE(P-18:1/22:4)	-13.3	0.211	-21.6	0.020
PE(P-18:1/22:5) (a)	-6.8	0.526	-18.6	0.021
PE(P-20:1/22:6)	-22.8	0.028	-26.7	0.025
PI (38:5) (b)	-8.8	0.376	-24.0	0.035
PI(40:4)	-17.8	0.005	-11.6	0.118
PI(18:0_22:5) (n3)	53.6	0.008	56.4	<0.001
PI(18:0_22:5) (n6)	-19.4	0.029	-3.6	0.791
CE(16:0)	42.1	0.043	19.6	0.303
CE(17:0)	39.5	0.155	56.8	0.013
CE(18:0)	31.6	0.258	65.0	0.002
CE(18:3)	73.8	0.022	64.1	0.036
CE(22:5) (n6)	-25.5	0.187	-37.4	0.031
COH	27.9	0.039	40.1	0.004

Grey indicates not significant

Supplementary Table 15.4: Lipid species in serum significantly correlated to liver steatosis severity

Significant decrease associated with steatosis			Significant increase associated with steatosis		
Lipid	**Beta-coefficient**	**p-value**	**Lipid**	**Beta-coefficient**	**p-value**
			Dihydroceramide		
			*Cer(d18:0/18:0)	1.90 (0.86, 2.94)	0.003
			*Cer(d18:0/22:0)	1.53 (0.76, 2.31)	0.001
			*Cer(d18:0/24:0)	1.17 (0.38, 1.96)	0.021
			*Cer(d18:0/24:1)	1.35 (0.65, 2.07)	0.002
			Ceramide		
			*Cer(d18:1/16:0)	0.93 (0.25, 1.6)	0.035
			*Cer(d18:1/20:0)	0.98 (0.37, 1.6)	0.011
			*Cer(d18:1/24:0)	0.73 (0.24, 1.23)	0.021
			GM3 ganglioside		
			*GM3(d18:1/20:0)	0.9 (0.33, 1.46)	0.012
Phosphatidylcholine, alkylphosphatidylcholine and alkenylphosphatidyl					
PC(O-18:1/18:1)	-0.88 (-1.32, -0.43)	0.001	PC(32:1)	1.27 (0.52, 2.03)	0.007
PC(O-38:5)	-0.76 (-1.12, -0.4)	0.001	PC(16:0_20:3) (a)	0.87 (0.29, 1.45)	0.02
PC(O-40:5)	-0.65 (-1.09, -0.22)	0.021	PC(18:0_20:3)	1.36 (0.48, 2.24)	0.015
PC(P-20:0/20:4)	-0.69 (-1.13, -0.25)	0.013			
Lysophosphatidylcholine, lysoalkylphosphatidylcholine and lysoalkenylphosphatidylcholine					
LPC(O-22:1)	-1.13 (-1.74, -0.52)	0.003	LPC(20:3) [sn2]	0.95 (0.24, 1.67)	0.041
LPC(O-24:1)	-0.92 (-1.47, -0.38)	0.007	*LPC(26:0) [sn1]	1.12 (0.27, 1.99)	0.047
LPC(O-24:2)	-1.28 (-1.89, -0.66)	0.001			
*LPC(P-20:0)	-0.99 (-1.6, -0.38)	0.011			
			Phosphatidylethanolamine		
			*PE(16:0_16:1)	2.09 (0.91, 3.3)	0.005
			PE(18:0_18:2)	1.49 (0.49, 2.51)	0.021
			PE(18:0_20:3)	1.58 (0.41, 2.77)	0.039
			*PE(16:0_22:6)	1.38 (0.52, 2.26)	0.012
			PE(18:0_22:6)	1.87 (1.01, 2.75)	0
			*PE(18:1_22:6) (b)	1.58 (0.49, 2.69)	0.025
			Phosphatidylinositol		
			*PI(16:0/16:0)	3.24 (1.97, 4.53)	0
			*PI(16:0_16:1)	3.19 (1.94, 4.45)	0
			*PI(34:0)	1.96 (0.89, 3.04)	0.003
			*PI(34:1)	2 (1.07, 2.94)	0
			*PI(18:0_18:1)	1.25 (0.33, 2.18)	0.039
			PI(16:0/20:3) (a)	1.28 (0.33, 2.25)	0.039
			PI(38:6)	1.25 (0.57, 1.94)	0.004
			*PI(18:0_22:5) (n3)	1.35 (0.79, 1.91)	0
			PI(18:0_22:6)	1.21 (0.54, 1.89)	0.004
			Lysophosphatidylinositol		
			*LPI(18:0) [sn2]	1.12 (0.35, 1.89)	0.024
			*LPI(18:1) [sn2]	1.76 (0.82, 2.71)	0.003
			*LPI(18:1) [sn1]	1.47 (0.63, 2.32)	0.005
			*LPI(18:2) [sn2]	1.36 (0.57, 2.17)	0.006
			*LPI(18:2) [sn1]	1.32 (0.55, 2.1)	0.007
			Phosphatidyglycerol		
			PG(36:1)	1.62 (0.63, 2.61)	0.01
			*PG(36:2)	1.68 (0.86, 2.51)	0.001
			Cholesterol esters		
			*CE(18:2)	-0.65 (-1.10, -1.20)	0.026
			CE(22:6)	-1.17 (-2.03, -0.31)	0.039

** indicates lipids also significantly altered in same direction in liver lipidome with increasing steatosis*

Supplementary Table 15.5: Lipid species in serum significantly correlated to nonalcoholic steatohepatitis (NASH) in liver (not corrected for steatosis severity)

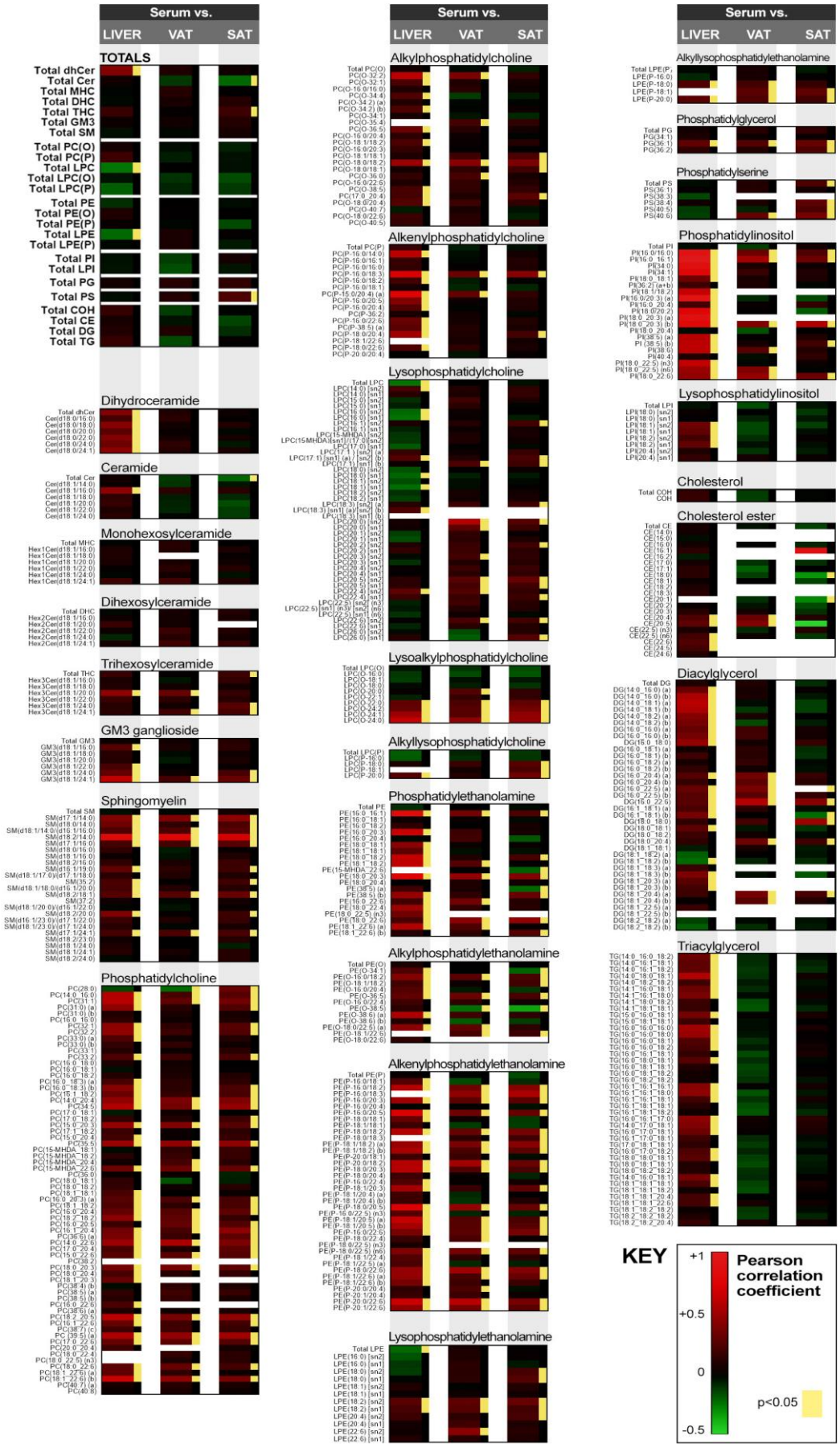
Significant decrease associated with NASH			Significant increase associated with NASH		
Lipid	**Percent change**	**p-value**	**Lipid**	**Percent change**	**p-value**
			Dihydroceramide		
			*Cer(d18:0/18:0)	55.06 (18.96, 102.12)	0.025
			*Cer(d18:0/22:0)	36.56 (14.89, 62.31)	0.013
			*Cer(d18:0/24:1)	34.72 (14.7, 58.24)	0.012
			Monohexosylceramide		
			Hex1Cer(d18:1/18:0)	24.85 (7.22, 45.39)	0.047
Alkylphosphatidylcholine					
PC(O-18:1/18:1)	-17.26 (-25.12, -8.57)	0.011			
PC(O-18:1/18:2)	-17.73 (-27.67, -6.42)	0.037			
PC(O-18:0/20:4)	-17.46 (-26.74, -7.01)	0.027			
PC(O-38:5)	-14.1 (-21.85, -5.57)	0.027			
			Phosphatidylinositol		
			*PI(18:0_22:5) (n3)	25.77 (10.83, 42.73)	0.013
			Lysophosphatidylinositol		
			LPI(18:2) [sn2]	32.78 (10.4, 59.69)	0.037
			Phosphatidylglycerol		
			PG(36:2)	40.6 (12.48, 75.75)	0.037

**indicates lipids also significant altered in the same direction in liver lipidome with the diagnosis of NASH.*

18.9.2 **Supplementary Figures**

Supplementary Figure 15.3: Heatmap showing correlation of serum lipids with liver, visceral adipose tissue (VAT) and subcutaneous adipose tissue (SAT) lipids respectively. Yellow indicates a significant correlation ($p<0.05$), with red being a positive correlation. This shows significantly greater numbers of liver lipids positively and significantly correlated with serum lipids.

(Figure 15.3 on next page)


Serum vs.
LIVER
VAT
SAT
TOTALS
Total dhCer
Total Cer
Total MHC
Total DHC
Total THC
Total GM3
Total SM
Total PC(O)
Total PC(P)
Total LPC
Total LPC(O)
Total LPC(P)
Total PE
Total PE(O)
Total PE(P)
Total LPE
Total LPE(P)
Total PI
Total LPI
Total PG
Total PS
Total COH
Total CE
Total DG
Total TG
Dihydroceramide
Ceramide
Monohexosylceramide
Dihexosylceramide
Trihexosylceramide
GM3 ganglioside
Sphingomyelin
Phosphatidylcholine
Alkylphosphatidylcholine
Alkenylphosphatidylcholine
Lysophosphatidylcholine
Lysoalkylphosphatidylcholine
Alkyllysophosphatidylcholine
Phosphatidylethanolamine
Alkylphosphatidylethanolamine
Alkenylphosphatidylethanolamine
Lysophosphatidylethanolamine
Alkyllysophosphatidylethanolamine
Phosphatidylglycerol
Phosphatidylserine
Phosphatidylinositol
Lysophosphatidylinositol
Cholesterol
Cholesterol ester
Diacylglycerol
Triacylglycerol
KEY
+1
+0.5
0
-0.5
Pearson correlation coefficient
p<0.05

18.9.3 **Supplementary Section 15.1**

18.9.3.1 Lipidomic analysis of samples

Samples were homogenized in ice-cold Phosphate Buffered Saline (PBS, pH 7.47) solution, using an electric homogeniser (Polytron), assayed for total protein content in duplicate using bicinchoninic acid (BCA) protein assay, diluted to a final stock concentration of 5mg protein/ml with ice-cold PBS (pH 7.47), and stored at -80^{o}C.

Lipids were extracted from plasma and tissue homogenates with a single phase chloroform/methanol ($CHCl_3$/MeOH (2:1), 20 times sample volume) extraction method, after the addition of 10µL internal standard mixture (containing 25 internal standards).

Lipid analysis was performed by liquid chromatography, electrospray ionization-tandem mass spectrometry using an Agilent 1200 liquid chromatography system combined with an Applied Biosystems API 4000 Q/TRAP mass spectrometer with a turbo-ionspray source (350^{o}C and Analyst 1.5 data system), as previously described.

Liquid chromatography was performed on a Zorbax C18, 1.8µm, 50×2.1 mm column (Agilent Technologies). Solvents A and B consisted of tetrahydrofuran:methanol:water in the ratio (30:20:50) and (75:20:5) respectively, both containing 10mM ammonium formate. Columns were heated to 50oC and the auto-sampler regulated to 25oC. Diacylglycerol (DG) and triacylglycerol (TG) species (1µL injection) were separated using an isocratic flow (100µL/min) of 85% B over 6 min. All other lipid species (5µL injection) were separated under gradient conditions (300µL/min) 0% B to 100% B over 8.0 min, 2.5 min at 100% B, a return to 0% B over 0.5 min, then 10.5 min at 0% B prior to the next injection. Allowing for 0.5 min injection time, this equated to 14 min between injections. Lipid species of the following classes were measured: dhCer, Cer, monohexosylceramide (Hex1Cer, MHC), dihexosylceramide (Hex2Cer, DHC), trihexosylceramide (Hex3Cer, THC), GM3 ganglioside (GM3), sphingomyelin (SM), phosphatidylcholine (PC), alkylphosphatidylcholine (PC-O), alkenylphosphatidylcholine (plasmalogen, PC-P), lysophosphatidylcholine (LPC), lysoalkylphosphatidylcholine (LPC-O), lysoalkenylphosphatidylcholine (LPC-P), phosphatidylethanolamine (PE), alkylphosphatidylethanolamine (PE-O), alkenylphosphatidylethanolamine (plasmalogen, PEP), lysophosphatidylethanolamine (LPE),

phosphatidylinositol (PI), phosphatidylserine (PS), phosphatidylglycerol (PG), cholesterol ester (CE), free cholesterol (COH), diacylglycerol (DG) and triacylglycerol (TG). Lipid concentrations were calculated by relating the peak area of each species to the peak area of the corresponding stable isotope or non-physiological internal standard.

The lipidomic analysis used in this study represents semi-quantitative measurements of over 450 lipid species. The lack of availability of suitable stable isotope internal standards for every individual species requires the use of representative standards for each lipid class and precludes the creation of calibration curves for each lipid species. Thus, care must be taken in the interpretation of the data. Whilst the comparison of lipid species between individuals will provide good estimates of differences in lipid abundance (i.e., high assay precision), exact quantification and subsequent distribution of lipids within a class should be recognized as approximations only (640).