UNIVERSITY OF SOUTHAMPTON

FACULTY OF MEDICINE

Human Development & Health

Association of prognostic cardiovascular biomarkers with non-alcoholic fatty liver disease and effects of high-dose n-3 fatty acids treatment

by

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BSc(Hons) MBChB MRCP

Thesis for the degree of Doctor of Philosophy

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ABSTRACT

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Non-alcoholic fatty liver disease (NAFLD) is an increasingly prevalent condition affecting up to one-third of the population worldwide, associated with increased cardiovascular (CV) mortality. Little is known about how prognostic CV biomarkers may be altered in association with changes in NAFLD severity over time. Importantly, there is currently no established pharmacological treatment option for NAFLD.

The WELCOME* trial was a randomised, double-blind placebo-controlled study testing the effects of 15-18 months of high-dose n-3 fatty acids (FA; Omacor 4g/day) versus placebo in 103 NAFLD subjects to reduce liver fat percentage (measured by magnetic resonance spectroscopy). Prespecified sub-studies also investigated whether prognostic CV biomarkers (carotid intima-media thickness (CIMT) and echocardiographic markers of left ventricular (LV) diastolic function) and insulin sensitivity were related to severity of NAFLD; and also if these biomarkers improved with n-3 FAs in relation to liver fat reduction. We also measured erythrocyte docosahexaenoic acid (DHA) enrichment as a biological measure of treatment compliance.

We found that significantly increased DHA enrichment through n-3 FA supplementation over 15-18 months resulted in a significant reduction in liver fat, as well as improving hepatic insulin sensitivity. Conversely, this did not have a beneficial effect on prognostic CV biomarkers with respect to reducing CIMT progression or improving key LV diastolic function indices. However, we also described for the first time, an independent association between percentage liver fat reduction and reduced CIMT progression in the entire cohort over the duration of study. Similarly, we found an independent association between liver fat reduction over 15-18 months and an improvement in markers of LV diastolic function across the entire cohort.

In conclusion, n-3 FAs may be a viable therapeutic option for treating liver fat and improving hepatic insulin sensitivity. Reducing liver fat in NAFLD over 15-18 months was independently associated with improvements in prognostic CV biomarkers.

*WELCOME study (Wessex evaluation of fatty liver and cardiovascular markers in NAFLD with Omacor therapy; www.clinicaltrials.gov NCT00760513)

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DECLARATION OF AUTHORSHIP

I, Lokpal Bhatia, declare that this thesis and the work presented in it are my own and has been generated by me as the result of my own original research.

Thesis title: **Association of prognostic cardiovascular biomarkers with non-alcoholic fatty liver disease and effects of high-dose n-3 fatty acids treatment**

I confirm that:

1. This work was done wholly or mainly while in candidature for a research degree at this University;
2. Where any part of this thesis has previously been submitted for a degree or any other qualification at this University or any other institution, this has been clearly stated;
3. Where I have consulted the published work of others, this is always clearly attributed;
4. Where I have quoted from the work of others, the source is always given. With the exception of such quotations, this thesis is entirely my own work;
5. I have acknowledged all main sources of help;
6. Where the thesis is based on work done by myself jointly with others, I have made clear exactly what was done by others and what I have contributed myself;
7. Parts of this work have been published as:

   - Hodson L, **Bhatia L (joint 1st author)**, Scorletti E, Smith DE, Jackson NC, Shojaee-Moradie F, Umpleby M, Calder PC, Byrne CD. Docosahexanoic acid enrichment in NAFLD is associated with improvements in hepatic metabolism and hepatic insulin sensitivity: a pilot study. *Eur J Clin Nutr.* 2017; 71(8): 973-979
   - **Bhatia L,** Scorletti E, Curzen NP, Clough GF, Calder PC, Byrne CD. Improvement in non-alcoholic fatty liver disease severity is associated with a reduction in carotid intima-media thickness progression. *Atherosclerosis*. 2016; 246: 13-20
   - Scorletti E, **Bhatia L**, McCormick K, Clough GF, Nash K, Hodson L, Moyses HE, Calder PC, Byrne CD. Effects of purified eicosapentaenoic and docosahexaenoic acids in nonalcoholic fatty liver disease: Results from the WELCOME* study. *Hepatology.* 2014; 60(4): 1211-1221
   - Scorletti E, **Bhatia L**, McCormick K, Clough GF, Nash K, Calder PC, Byrne CD. Design and rationale of the WELCOME trial: A randomised, placebo controlled study to test the efficacy of purified long chain omega-3 fatty treatment in non-alcoholic fatty liver disease. *Contemp Clin Trials.* 2014; 37(2):301-11.

- **Bhatia LS**, Scorletti E, Shojaee-Moradie F, Umpleby M, Clough GF, Curzen NP, Calder PC, Byrne CD. High-dose n-3 fatty acid treatment in non-alcoholic fatty liver disease is independently associated with reduced hepatic steatosis and improved hepatic insulin sensitivity. *Diabet Med.* 2014; 31:S53

- **Bhatia L**, Scorletti E, Shojaee-Moradie F, Umpleby M, Fletcher A, Bateman A, Curzen NP, Clough GF, Calder P, Byrne CD. Peripheral and hepatic insulin resistance are key determinants of progression of non-alcoholic fatty liver disease severity independent of body fat percentage. *Diabet Med.* 2013; 30:S60

- **Bhatia LS**, Curzen NP, Byrne CD. Nonalcoholic fatty liver disease and vascular risk. *Curr Opin Cardiol.* 2012; 27(4):420-8.

- **Bhatia LS**, Curzen NP, Calder PC, Byrne CD. Non-alcoholic fatty liver disease: a new and important cardiovascular risk factor? *Eur Heart J.* 2012; 33(10):1190-200

- **Bhatia LS**, Fletcher A, McCormick KG, Umpleby AM, Curzen NP, Peebles C, Shojaee-Moradie F, Scorletti E, Darekar A, Clough GF, Calder PC, Nash K, Byrne CD. Liver fat content in non-alcoholic fatty liver disease is related to whole body insulin resistance independent of body fat percentage. *Diabet Med.* 2012; 29:S61-62

The NIHR and Diabetes UK-funded WELCOME study was a complex trial with a large number of associated substudies and collaborations with several different local departments, as well as external research collaborations, including the University of Oxford and University of Surrey. With regards to this thesis and the WELCOME trial studies, I would like to briefly state and clarify the original work done by myself as well as work performed by others involved in the trial relating specifically to the studies I have included in this thesis. It should also be noted that all my original work was discussed throughout the project with my supervisors.

1. The idea, concept, planning and inclusion of the carotid IMT and echocardiography substudies was my work.

2. I undertook all carotid and echocardiography studies for the trial, including performing all measurements and analysis.

3. The methodology of the clamp studies was tried and tested and my training was provided by our collaborators at the University of Surrey. However, all clamp studies were performed by me throughout the trial and I also prepared and analysed all the results myself using pre-programmed computational software with supervision and guidance by our colleagues at the University of Surrey.

4. All statistical analysis and its interpretation for my three 'results' chapters were undertaken by me. For the main WELCOME trial, I undertook the analysis in collaboration with another colleague with the help of our statistician.

5. I was the main researcher-clinician 'on the ground' for the entire duration of the WELCOME study and was in charge of supervising all three research nurses, the data manager and being the first point of contact for any queries from nurses or research participants. I was also in charge of consenting all participants as well as submitting substantial amendements and data to the MHRA as necessary, including other trial administration, under the supervision of the P.I., Prof. Christopher Byrne. I was also involved in other research work relating to the trial that I have not included in my thesis.

6. Investigations that were not undertaken by me include MRI/MRS scans, DEXA scans and analysis of biochemical samples including erythrocyte fatty acid analysis and clamp isotope analysis. However, I interpreted all results provided to me.

7. I researched, prepared and performed all the writing of this thesis, of which some work had already been published (see above). However, I was the first author in all publications pertaining to the 'introduction' and 'results' chapters in my thesis.

Signed:

Dr Lokpal Bhatia

Date: 27 July 2018

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I would like to especially thank Lucinda England, for all her invaluable administrative and research governance support, as well as all the research nurses at the Southampton NIHR BRU and Wellcome Trust involved in the WELCOME trial, in particular, Gillian Wise, Bridget Clancy, Sanchia Triggs and Norma Draper, without whose help the study would not have been possible and for their friendship as well throughout the course of the study.

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I am also grateful to Helen Moyses for advice regarding statistical support, and Karen Long for data management.

I am also grateful to Pronova Biopharma and Abbott who kindly provided the Omacor and placebo supplements for the study, as well as the pharmacy team at Southampton Hospital. I am grateful to NIHR which helped with financial support for running the study.

Importantly, I would like to thank all the volunteers involved in the WELCOME study for giving up their precious time to participate in this important research, as well as making the time I spent at the research facility very enjoyable. Without their support, this project would not have been possible.

Finally, I would like to thank my family for having to put up with me taking so much time out of my career to pursue and complete this project, rather than spending it with them. Their understanding and support in helping me to complete this thesis is overwhelmingly appreciated.

Definitions and Abbreviations

A wave	peak late LV diastolic filling velocity
a’	pulsed-wave TDI-derived mitral annular late LV diastolic velocity
ACS	acute coronary syndrome
AF	atrial fibrillation
ALA	alpha-linolenic acid
ALT	alanine transaminase
AST	aspartate transaminase
AT	adipose tissue
ATP	adenosine triphosphate
AUROC	area under receiver operating characteristic curve
BMI	body mass index
BP	blood pressure
BSA	body surface area
CAC	coronary artery calcium
CAD	coronary artery disease
CFR	coronary flow reserve
CHD	coronary heart disease
CHF	congestive heart failure
ChREBP	carbohydrate responsive element-binding protein
CI	confidence interval
CIMT	carotid intima-media thickness
CK-18	cytokeratin-18
CT	computed tomography
CV	Cardiovascular
DEXA	dual energy X-ray absorptiometry
DHA	docosahexaenoic acid

DNL	de novo lipogenesis
E wave	peak early LV diastolic filling velocity
E wave DT	time interval from peak E wave along slope of LV filling to zero-velocity baseline
e’	pulsed-wave TDI-derived mitral annular early LV diastolic velocity
e’/a’	ratio of tissue doppler-derived mitral annular early to late LV diastolic velocity
E/A ratio	ratio of early to late LV diastolic filling velocities
E/e’	ratio of early LV diastolic filling velocity to tissue doppler-derived early LV relaxation velocity
EAT	epicardial adipose tissue
ECG	electrocardiogram
EGP	endogenous glucose production
EPA	eicosapentaenoic acid
F/U	follow-up
FFA	free fatty acids
FMD	flow-mediated dilatation
FRS	Framingham risk score
GGT	gamma-glutamyltransferase
HA	hyaluronic acid
HDL	high-density lipoprotein
HGP	hepatic glucose production
HOMA-IR	homeostasis model assessment of insulin resistance
HR	hazard ratio
hsCRP	high sensitivity C-reactive protein
IL-6	interleukin-6
IMT	intima-media thickness
IQR	inter-quartile range
IR	insulin resistance
ITT	intention to treat

LA	left atrial
LAVI	left atrial volume measurement indexed to body surface area
LDL	low-density lipoprotein
LV	left ventricular
MCR	metabolic clearance rate
MetS	metabolic syndrome
MI	myocardial infarction
MRI	magnetic resonance imaging
MRS	magnetic resonance spectroscopy
N-O	Newcastle Ottowa
NAFLD	Nonalcoholic fatty liver disease
NASH	non-alcoholic steatohepatitis
NEFA	non-esterified fatty acids
NF-κB	nuclear factor kappa-B
NFS	NAFLD fibrosis score
NT-proBNP	N-terminal pro-brain natriuretic peptide
OR	odds ratio
PAI-1	plasminogen activator inhibitor-1
PIIINP	procollagen type 3 N-terminal propeptide
PIS	patient information sheet
PPARα	peroxisome proliferator-activated receptor alpha
PUFA	Polyunsaturated fatty acids
RCT	randomised controlled trial
RFs	risk factors
RR	relative risk
s’	pulsed-wave TDI-derived mitral annular peak LV systolic velocity
SCD	sudden cardiac death
SD	standard deviation
SMR	standardised mortality ratio

SREBP-1c	sterol regulatory element binding protein 1c
TDI	tissue Doppler imaging
TG	triglycerides
TIMP-1	tissue inhibitor of metalloproteinase 1
TNF-α	tumour necrosis factor-alpha
TTR	tracer-to-tracee ratio
US	ultrasound
VAT	visceral adipose tissue
VLDL	very low density lipoprotein
VO_2 max	maximal oxygen consumption
VOI	volume of interest
WELCOME	Wessex Evaluation of fatty liver and cardiovascular markers in NAFLD with Omacor therapy

Chapter 1: Association of NAFLD with cardiovascular disease

1.1 Introduction

Non-alcoholic fatty liver disease (NAFLD) is an increasingly prevalent condition affecting between 10-33% of the worldwide population, depending on the population studied and diagnostic methods used.[1-3] It is the commonest cause of chronic liver disease and is defined by the presence of liver fat accumulation >5% of hepatocytes in the absence of excessive alcohol intake (<20g (2.5U) per day) or other causes of liver disease (e.g. viral, autoimmune, drug-induced, etc), although it can co-exist with any chronic liver disease state[4] (Figure 1-1).

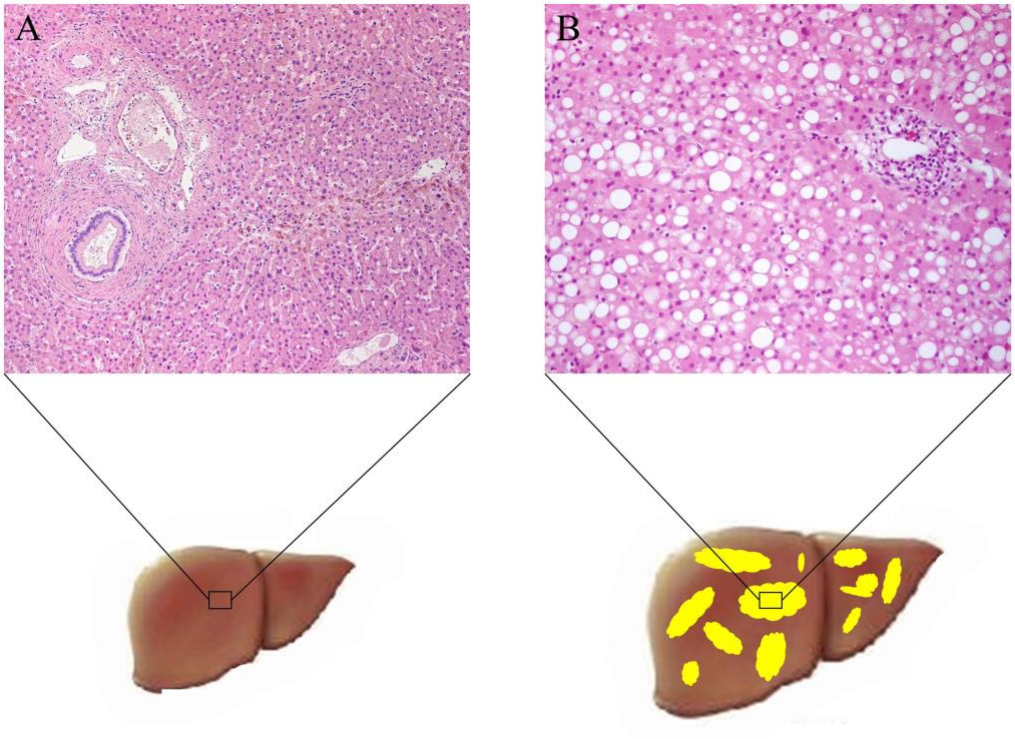

Figure 1-1 (*A*) Histological section of normal liver tissue compared with (*B*) simple steatosis, showing fat accumulation in hepatocytes.

NAFLD represents a spectrum of progressive stages of liver disease ranging from simple steatosis to non-alcoholic steatohepatitis (NASH), which includes hepatocyte inflammation, necrosis and liver fibrosis, to ultimately liver cirrhosis and a subsequent potential for hepatocellular carcinoma (Figure 1-2).[1, 5, 6]

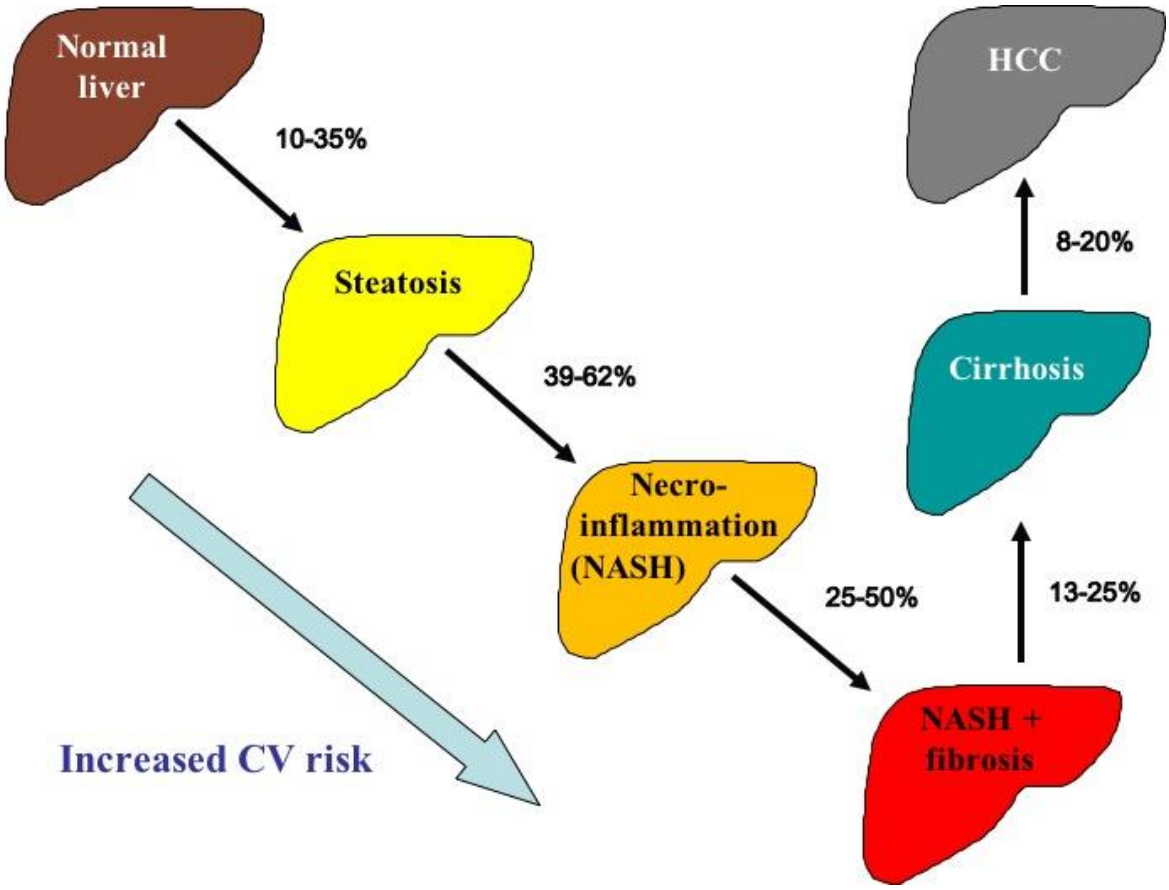

Figure 1-2 Variable progression of stages of NAFLD severity (usually over several years), with different grades of severity at each stage of simple steatosis and NASH. Each stage is reversible, apart from more severe forms of NASH and fibrosis. Cardiovascular risk increases as NAFLD severity progresses.[1, 7, 8]

NAFLD is strongly linked to insulin resistance (IR), type 2 diabetes and obesity, being prevalent in up to 95% of obese subjects and up to 70% of people with type 2 diabetes,[9] with most cases unrecognised. Given that the metabolic syndrome (MetS) is present in 30-88% of NAFLD subjects depending on which internationally recognised MetS diagnostic criteria is used, as well as severity of the disease,[2, 10] NAFLD is commonly regarded as the hepatic expression of the MetS. A recent population-based study showed that apart from obesity and diabetes, NAFLD subjects also have a high prevalence of hypertension (70%), dyslipidaemia (76%) and hypercholesterolaemia (69%).[11] Importantly, a meta-analysis of 40 cohort studies reviewing the natural history of NAFLD showed that all-cause mortality is increased in NAFLD subjects (odds ratio (OR):1.57, 95% confidence interval (CI): 1.18-2.10) compared with an age- and sex-matched reference population,[1] with higher rates of mortality in NASH compared to simple steatosis.[12] As NAFLD shares many common risk factors with CV disease, it is therefore unsurprising that CV mortality is the commonest cause of death in NAFLD subjects, followed by cancer and then liver-related mortality.[7, 8, 12, 13] With

increasing rates of obesity and type 2 diabetes worldwide, the potential future burden of NAFLD on public health-care utilisation and costs is likely to be significant.[14, 15] As such, the cardiometabolic risk conferred by NAFLD merits increased collaborative study between diabetologists, hepatologists, and especially cardiologists, given that CV disease appears to largely influence major clinical outcomes in NAFLD.[16-18]

1.2 Diagnosis of NAFLD

The accurate diagnosis and quantification of severity of NAFLD is paramount in risk stratification and management of the disease, but this remains challenging. Mildly abnormal liver enzymes, especially increased alanine transaminase (ALT) activity, are often the only biochemical marker of suspected NAFLD. However, up to 80% of NAFLD patients may have normal ALT,[2] making it an insensitive diagnostic or prognostic tool. Other common non-invasive measures such as ultrasound or computed tomography (CT) can only reliably detect NAFLD in subjects with liver fat accumulation of more than 20-30%.[19] Magnetic resonance spectroscopy (MRS) is the most sensitive and accurate method of quantifying liver fat content, but like all the other non-invasive markers, it cannot identify inflammation or fibrosis within the liver. Liver biopsy currently remains the 'gold-standard' reference for identifying, staging and monitoring NAFLD, particularly for NASH, but is highly invasive and is prone to sampling variability, especially if focal disease is present.[20] Importantly, it is not feasible to offer a routine liver biopsy to all NAFLD patients due to the small, but real risk of serious complications. It also does not represent a cost-effective and clinically practical method of ongoing monitoring of disease progression.[21]

As such, several different algorithms combining clinical, biochemical and new radiological methods have been developed over the last few years to improve non-invasive diagnosis and risk-stratification of NAFLD.[21, 22] Non-invasive imaging methods like ultrasound-based transient elastography (with controlled attenuation parameter) to detect liver 'stiffness' as a marker of fibrosis have compared well to liver biopsy scores, with area under receiver operating characteristic curve (AUROC) of 0.88,[23] although magnetic resonance elastography has shown superior diagnostic performance to transient elastography in detecting the different stages of liver fibrosis.[24]

At present, no single biomarker can accurately discriminate between simple steatosis and NASH, with several disparate biomarker algorithm models combining at least two or more variables to strengthen predictive accuracy.[22] Serum cytokeratin-18 (CK-18), a major intermediate filament protein found in hepatocytes and a marker of hepatocyte apoptosis and necrosis, appears to hold some promise in differentiating NASH from simple steatosis (AUROC 0.82, sensitivity 0.78,

specificity 0.87), albeit in combination with other non-invasive biomarkers to improve diagnostic accuracy.[1, 25, 26] Notably, a post-hoc analysis of the NASH Clinical Research Network study to validate CK-18 as a marker for NASH diagnosis showed promising results compared to histological specimens. This showed that CK-18 was a strong independent predictor of NASH with an AUROC of 0.83 (0.75,0.91) and increased levels of CK-18 were significantly related to the presence of liver fibrosis.[27] Conversely, another study only showed a modest accuracy of CK-18 for the diagnosis of NASH (66% sensitivity, 82% specificity),[28] although it is possible the differing results may have been due to the disparate CK-18 immunoassays used between studies. A recent meta-analysis of the accuracy of non-invasive biomarkers to diagnose NASH reported pooled sensitivity and specificity values for CK-18 (M30) as 0.75 and 0.77, respectively; and CK-18 (M65) as 0.71 and 0.77, respectively.[29] Currently, no single non-invasive test exists which is validated for the diagnosis of NASH.[30] Further research is required to find the 'holy grail' of one single, highly predictive non-invasive biomarker which will allow more accessible and better risk stratification and monitoring of patients according to NAFLD stage.

1.3 Epidemiology of cardiovascular disease in NAFLD

Numerous epidemiological studies have reported an increased incidence of adverse CV events in NAFLD subjects compared to the general population[7, 8, 12, 13, 16, 17, 31-38] (Table 1-1). As NAFLD is the commonest cause of abnormal liver enzymes in developed countries,[4] many epidemiological studies have employed these as biochemical surrogates of NAFLD. Several studies have shown a significant association between increased gamma-glutamyltransferase (GGT) levels and CV mortality over an average median of 12 years follow-up, even after adjusting for typical CV risk factors and body mass index (BMI).[31-33] Additionally, a meta-analysis of 10 pooled population-based cohort studies confirmed the independent association between elevated GGT (as a marker of NAFLD) and increased fatal and non-fatal CV events, even after adjustment for CV risk factors (hazard ratio=1.34, 95% CI = 1.22-1.48).[34] However, GGT is also expressed in atherosclerotic plaques and has a role in oxidative stress,[39] as well as being associated with all components of the metabolic syndrome.[40] ALT has been reported to be more closely related to liver fat content than GGT.[41] Similarly, several large population-based cohort studies have reported an independent association between elevated ALT and CV mortality after adjusting for CV risk factors.[35-37] Importantly, the correlation of raised ALT or GGT with CV disease in these studies may simply reflect their significant association with IR[42] which is itself a strong risk factor for CV disease, rather than as a marker for the presence or severity of NAFLD. In a large, Italian population-based observational study over 15 years, an independent association between fatty liver index (validated algorithm derived from serum triglyceride level, body mass index, waist circumference

and GGT) and CV mortality was found, with CV disease representing the leading cause of death (45%), followed by cancer-related (36%), and then hepatic-related mortality (7%).[43] However, IR appeared to be a key factor in driving mortality from CV causes in NAFLD in this study.

Employing ultrasound imaging as a more specific diagnostic determinant of NAFLD than liver enzymes, four large community-based prospective cohort studies also documented a significant independent association with CV events[13, 16, 17, 44] (Table 1-1). Of note, Hamaguchi *et al.* undertook a prospective analysis of 1637 healthy subjects recruited from a health check-up program, and found 19% with ultrasound evidence of NAFLD. At 5 years follow-up, 5.2% of the NAFLD group suffered an adverse CV event, compared to 1.0% of the non-NAFLD group ($p < 0.001$).[17] By multivariate analysis, the association between NAFLD and future CV events was shown to be independent of the MetS, as well as conventional cardiac risk factors. Although these studies are strongly indicative of NAFLD as a predictor of CV disease independent of diabetic status, they are limited by the lack of sensitivity of ultrasound determination of NAFLD.

Conversely, one large prospective North American cohort study found that NAFLD was associated with neither an increased risk of death from all causes nor CV disease.[45] However, there were important methodological flaws in how the authors discriminated between simple steatosis and NASH, as well as including patients with 'mild hepatic steatosis' or unwittingly 'advanced NASH' in the control group, due to the insensitivity of ultrasonography and liver enzymes in classifying cases. Furthermore, some patients may not have had the disease at baseline but could have developed it during the median 14.5-year follow-up period. This misclassification of cases and controls is likely to have nullified any excess risk of NAFLD (especially NASH) with respect to overall and CV mortality. Finally, a recent meta-analysis of six studies (n = 25837) all utilising liver ultrasound as the diagnostic modality for NAFLD, found a significantly higher risk of CV events and CV mortality in NAFLD subjects compared to controls, even after controlling for the usual cardiac risk factors.[46]

Utilising liver biopsy-proven NAFLD, smaller long-term prospective studies showed significantly higher total mortality rates compared to a matched reference population, with CV disease representing the main mode of death, outnumbering cancer and liver-related mortality.[8, 12] Of note, only subjects with NASH rather than simple steatosis had significantly reduced survival, although in one study even subjects with simple steatosis showed a trend to reduced survival ($p = 0.06$), primarily from CV-related causes over a median follow-up of 24 years.[12] However, these studies are limited by modest sample sizes and inclusion of select cohorts requiring liver biopsy for clinical reasons, which therefore necessitate cautious interpretation of the reported 'benign' nature of simple steatosis.

Table 1-1 Main epidemiological studies relating NAFLD to increased cardiovascular risk

Authors	**Study characteristics [& N-O assessment of quality*]**	**Diagnosis of NAFLD**	**Main findings**	**Risk estimates (95% CI or p-value)**	**Comments/Limitations**
Ruttmann E *et al.*[31]	Austrian population-based cohort (n=163,944), median F/U of 12 years [3,1,3]	Liver enzymes (GGT)	CV mortality increased in NAFLD, independent of traditional CV RFs, alcohol and BMI	HR: Men 1.66 (1.40-1.98), Women 1.64 (1.36-1.97)	Poor sensitivity of GGT in NAFLD
Wannamethee G *et al*.[32]	British population-based cohort (n=7613 middle-aged men), median F/U of 11.5 years [3,1,3]	Liver enzymes (GGT)	Total and CHD mortality increased in NAFLD, independent of CV RFs, alcohol and BMI	RR 1.42 (1.12-1.80)	Men-only cohort. Poor sensitivity of GGT in NAFLD
Lee DH *et al.*[33]	Finnish population-based cohort (n=28,838), median F/U 11.9 years [3,1,3]	Liver enzymes (GGT)	CHD mortality and non-fatal MI increased in NAFLD independent of CV RFs and alcohol	HR: Men 1.20 (1.10-1.31), Women 1.14 (1.03-1.27)[25]	Poor sensitivity of GGT in NAFLD
Fraser A *et al.*[34]	Meta-analysis of 10 pooled population-based cohort studies†	Liver enzymes (GGT)	CV events (fatal & non-fatal) increased in NAFLD after adjustment for CV RFs and alcohol	HR 1.34 (1.22-1.48)	Heterogeneity of studies (I^2=73%), GGT poor marker of NAFLD
Fraser A *et al.*[42]	British Women's Heart & Health Study, population-based (n=2961 older women), median F/U of 4.6 years [3,1,3]	Liver enzymes (ALT & GGT)	No independent association between NAFLD and fatal & non-fatal CV events	ALT: HR 0.94 (0.65-1.37) GGT: HR 1.17 (0.93-1.48)	Women-only cohort, ALT/GGT not sensitive markers of NAFLD, relatively short follow-up
Schindhelm RK *et al.*[35]	Hoorn Study, population – based (n=1439 middle-aged), F/U of 10 years [3,2,2]	Liver enzymes (ALT)	Fatal & non-fatal CHD increased in NAFLD, independent of CV & MetS RFs	HR 1.88 (1.21-2.92)	ALT not a sensitive marker of NAFLD
Dunn W *et al.*[36]	NHANES-III, population-based cohort (n=7574), mean F/U of 8.7 years [3,1,3]	Liver enzymes (ALT)	Total & CV mortality increased in NAFLD but only in 45-54 year age	HR 8.15 (2.00-33.20)	ALT not a sensitive marker of NAFLD

Authors	Study characteristics [& N-O assessment of quality*]	Diagnosis of NAFLD	Main findings	Risk estimates (95% CI or p-value)	Comments/Limitations
			group, independent of CV RFs		
Yun KE *et al.*[37]	Korean population-based cohort (n=37,085), median F/U of 5 years [3,1,3]	Liver enzymes (ALT)	CV or diabetes-related mortality increased in NAFLD, independent of CV RFs, alcohol, BMI & socio-economic status	RR 2.26 (1.22-4.19)	ALT not a sensitive marker of NAFLD
Targher G *et al.*[16]	Valpolicella Heart Diabetes Study, community-based diabetic cohort, free of CV disease (n=2103), mean F/U of 6.5 years [4,2,2]	Liver ultrasound	Increased fatal & non-fatal CV events in NAFLD, independent of CV RFs, diabetes control & MetS	HR 1.87 (1.20-2.60)	Exclusive diabetic cohort, liver ultrasound poor sensitivity with liver fat < 30%
Hamaguchi M *et al.*[17]	Japanese community-based healthy cohort (n=1637), mean F/U of 5.8 years [4,2,1]	Liver ultrasound	Increased adverse CV events in NAFLD, independent of CV RFs & MetS	OR 4.12 (1.58-10.75)	Largely volunteer-reported CV events, 25% lost to F/U, use of ultrasound to diagnose NAFLD
Haring R *et al.*[13]	Study of Health in Pomerania population-based German cohort (n=4160 middle-aged), median F/U of 7.3 years [3,1,3]	GGT and liver ultrasound	Increased CV mortality in men with NAFLD & raised GGT (but not women) after adjustment for cardio-metabolic RFs	HR: Men 6.22 (1.22-31.62), Women 0.98 (0.11-8.84)	Significantly older age & increased baseline CV disease in men versus women, inadequate NAFLD sample size in women → type 2 error?
El Azeem *et al*.[44]	Middle-Eastern prospective cohort (n=747), mean F/U of 3 years [4,1,2]	Liver ultrasound	NAFLD best predictor for increased adverse CV events, stroke and renal impairment.	RR 2.20 (p<0.001)	35% did not complete F/U. Use of diagnostic liver ultrasound may have missed mild NAFLD cases.
Adams LA *et al.*[7]	Community-based North American cohort (n=420), mean F/U 7.6 years [3,0,3]	Majority had liver ultrasound (liver imaging or biopsy in all subjects)	Increased total mortality (mainly CV-related or cancer) in NAFLD compared to matched reference population	SMR 1.34 (1.003-1.76)	Liver ultrasound poor sensitivity with liver fat < 30%, wide variability of length of follow-up

Authors	Study characteristics [& N-O assessment of quality*]	Diagnosis of NAFLD	Main findings	Risk estimates (95% CI or p-value)	Comments/Limitations
Ekstedt M *et al.*[8]	Swedish hospital-based consecutive biopsy cases (n=129), mean F/U of 13.7 years [3,0,3]	Liver biopsy	Increased total mortality primarily CV-related (only in NASH patients but not in simple steatosis) compared to matched reference population	RR 1.38 (p=0.006)	No diabetes screening at baseline, small sample size (due to liver biopsy as diagnostic modality)
Soderberg C *et al.*[12]	Swedish hospital-based consecutive biopsy cases (n=118), median F/U 24 years [3,0,3]	Liver biopsy	Increased total mortality in NAFLD was predominantly CV-related, compared to matched reference population	SMR 1.69 (1.24-2.25)	Small sample size (due to liver biopsy as diagnostic modality)
Schwimmer JB *et al.*[38]	Cross-sectional consecutive autopsy biopsy cases of child death (n=817) from accidental or unnatural causes [3,0,3]	Liver biopsy (autopsy)	Increased coronary & aortic atherosclerosis in NAFLD, independent of obesity	OR 1.80 (p<0.001)	Limitations with autopsy studies

NAFLD, non-alcoholic fatty liver disease; F/U, follow-up; GGT, gamma-glutamyltransferase; ALT, alanine aminotransferase; CV, cardiovascular; RFs, risk factors; BMI, body mass index; CHD, coronary heart disease; MI, myocardial infarction; MetS, metabolic syndrome; NASH, non-alcoholic steatohepatitis; CI, confidence interval; HR, hazard ratio; RR, relative risk; OR, odds ratio; SMR, standardised mortality ratio.

*N-O, Newcastle-Ottawa Scale for assessing the quality of non-randomised studies based on study selection (0-4), comparability (0-2), outcome/exposure (0-3).[47]

†Meta-analysis adhered to the Meta-analysis of observational studies in Epidemiology (MOOSE) group standards of reporting.[48]

All of these studies highlight an extremely important point with respect to all studies researching aspects of NAFLD. Given its heterogeneous disease spectrum, the slow and variable progression between NAFLD stages, as well as the increasing risk of adverse clinical outcomes with disease progression, it is imperative that studies ensure accurate classification of the presence and severity of NAFLD so as to avoid misrepresentation of the true outcomes of simple hepatic steatosis or NASH. Unfortunately this inevitably still occurs given the lack of internationally agreed collaborative standards for the diagnosis and staging of NAFLD in clinical and research settings.[4, 9]

1.4 Evidence of association of NAFLD with cardiovascular disease

1.4.1 Cardiovascular risk assessment scores in NAFLD

Given that traditional CV risk factors are commonly prevalent in NAFLD subjects, investigators have applied validated CV risk prediction scores to evaluate the risk profile of NAFLD patients, with most of these studies showing that NAFLD independently confers an increased CV risk score (Table 1-2).[49-53] One study also documented that high sensitivity C-reactive protein (hsCRP), a well-established marker of adverse CV outcome, was significantly elevated compared to the non-NAFLD group in both sexes.[50] Additionally, a strong association has been shown between histological severity of NAFLD and calculated estimates of CV risk (both QRISK2 and Framingham risk score (FRS)) independently of markers of glucose control and obesity.[54]

Although these global risk prediction studies may help to describe part of the association between NAFLD and increased CV risk, they are flawed by the inherent limitations of using risk scores based on traditional CV risk factor-derived multivariable statistical models to identify at-risk patients.[55] Furthermore, we know that some of the important determinants of NAFLD such as IR, obesity and raised triglycerides which all also increase risk of CV disease, are not generally accounted for in these risk assessment models. Indeed, the FRS is already known to underestimate the risk of CV disease in MetS,[56] which shares many features in common with NAFLD. It might therefore not be appropriate to risk-stratify patients with NAFLD solely based on current CV risk scoring systems. Further research is necessary to determine simple and cost-effective robust biomarkers (or algorithm-based scores) of NAFLD status including its direct cardiometabolic effects, before we can evaluate its added discriminant value when applied to current CV risk prediction models in cohort studies.

Table 1-2 Cardiovascular risk assessment score studies relating NAFLD to increased cardiovascular risk

Authors	**Study characteristics**	**Diagnosis of NAFLD**	**Main findings**	**Comments/ Limitations**
Ioannou GN *et al.*[49]	NHANES-III, population-based cohort (n=7526), cross-sectional analysis.	Liver enzymes (ALT)	Increased FRS in NAFLD, but non-significant after adjusting for insulin resistance & obesity	ALT not sensitive marker of NAFLD. Inclusion of subjects <30 yrs in FRS calculations.
Sung KC *et al.*[50]	South Korean community-based healthy non-obese cohort (n=30,172)	Liver ultrasound in all (24% had NAFLD). Subjects with normal ALT deemed as having simple steatosis and ↑ALT as NASH.	Increased prevalence of FRS >10% in NAFLD (NASH > simple steatosis), independent of age, BMI & smoking. Increased hsCRP in NAFLD.	↑ALT in NAFLD poor differentiator between simple steatosis & NASH. Liver ultrasound poor sensitivity with liver fat < 30%.
Gastaldelli A *et al.* [51]	RISC Study: European population-based healthy cohort aged 30-60 years (n=1307).	Fatty liver index (validated algorithm based on BMI, waist circumference, triglycerides & GGT)	Increased FRS in NAFLD independent of age & gender (subjects not diabetic or hypertensive).	Use of fatty liver index as a marker of NAFLD rather than imaging. Lack of testing for viral hepatitis as a cause of fatty liver.
Villanova N *et al.* [52]	Case-control: 52 non-diabetic NAFLD patients & 28 healthy age- & sex-matched controls.	Liver biopsy	Increased FRS & PROCAM score in NAFLD, but not adjusted for components of MetS.	Small sample size, unable to adjust for possible confounders.
Dogan S *et al*.[53]	Cross-sectional prospective study, n=155.	Liver ultrasound to diagnose NAFLD. NFS to estimate liver fibrosis.	Increased FRS in NAFLD subjects compared to controls ($p<0.05$). Positive correlation between FRS and NFS ($r=0.37$, $p<0.001$)	Use of NFS to estimate liver fibrosis rather than 'gold-standard' liver biopsy

NAFLD, non-alcoholic fatty liver disease; NHANES-III, National Health and Nutrition Examination Survey III; ALT, alanine aminotransferase; FRS, Framingham Risk Score; BMI, body mass index; hsCRP, high sensitivity C-reactive protein; RISC, Relationship between Insulin sensitivity and Cardiovascular Disease; GGT, gamma-glutamyltransferase; PROCAM, Prospective Cardiovascular Munster Heart Study; MetS, metabolic syndrome; NFS, NAFLD fibrosis score.

1.4.2 Studies evaluating coronary disease in NAFLD

Coronary artery calcium (CAC) scoring with cardiac computed tomography (CT) is a very sensitive method of demonstrating the presence and extent of coronary atherosclerosis and significantly improving CV risk prediction in asymptomatic individuals beyond traditional risk factor scoring systems.[57] Several studies demonstrate a significantly increased coronary atherosclerotic burden as well as increased risk of CAC progression in the presence of NAFLD (Table 1-3),[58-62] with one study also reporting a significant association between "vulnerable plaque" and NAFLD in patients undergoing multislice CT for clinical suspicion of coronary artery disease (CAD).[59] This finding is consistent with data showing that NAFLD patients have significantly higher plasma markers of oxidative stress and inflammation, which are in part derived from the diseased liver causing a systemic inflammatory and pro-thrombotic state.[63, 64] Furthermore, in the Study of Inherited Risk of Coronary Atherosclerosis (SIRCA) of 860 asymptomatic non-diabetic participants, investigators found that the IR index was a robust and independent predictor of CAC score even after controlling for traditional CV risk factors, MetS and CRP.[65] More recently, a meta-analysis of 16 cross-sectional studies involving over 58,000 subjects showed that NAFLD was significantly associated with a coronary artery calcium score > 100 (at least moderate risk) independent of traditional CV risk factors with a pooled odds ratio of 1.24 (95% CI 1.02-1.52, I^2=42%).[66]

A strong association between NAFLD and prevalence of significant CAD determined by coronary angiography has also been consistently reported, albeit with variable thresholds of 'significant' CAD between studies (Table 1-4).[67-71] Although these studies indicate an independent association between NAFLD and increased CAD in terms of angiographic appearance even after adjusting for traditional CV risk factors and components of the MetS, none of them evaluated the functional significance of these coronary lesions. Given that the presence of ischaemia rather than coronary anatomy dictate clinical outcome,[72, 73] the significance of these findings in association with NAFLD should not be overestimated.

However, two further studies have shown an increase in short and long-term CV adverse events as well as mortality, independently related to the presence and severity of NAFLD, in patients admitted with acute coronary syndromes (both non-ST and ST elevation myocardial infarction).[74, 75] Presence and complexity of coronary disease was also significantly higher in patients with more severe NAFLD.[74]

Table 1-3 Coronary artery calcium scoring studies relating NAFLD to increased cardiovascular risk

Authors	**Study characteristics**	**Diagnosis of NAFLD**	**Main findings**	**Comments/Limitations**
Chen CH *et al*.[58]	Cross-sectional study: consecutive self-paid health screening asymptomatic 'healthy' subjects (n=295), NAFLD in 41%.	Liver ultrasound or CT	NAFLD associated with CAC score>100 (moderate to high risk of obstructive CAD); Increased prevalence of NAFLD with higher CAC scores.	Unusually high prevalence of NAFLD in cohort. Unable to exclude possible confounders of age, gender, diabetes as small sample size.
Akabame S *et al*.[59]	Cross-sectional: consecutive referrals for coronary CT to investigate CAD (n=298), NAFLD in 20%.	Liver CT	Increased prevalence of remodelling coronary lesions/lipid core plaques (markers of potential plaque rupture) in NAFLD, independent of CV RFs.	Cohort of patients with clinical suspicion of CAD. Reduced sensitivity of liver CT in diagnosing NAFLD
Assy N *et al*.[60]	Cross-sectional case-control: 29 NAFLD subjects & 32 age-, sex- & BMI-matched controls (all free of documented or suspected CAD)	Liver CT	Increased prevalence of coronary atherosclerosis (calcified & non-calcified plaques) in NAFLD, independent of MetS components, CV RFs & CRP. Degree of NAFLD only independent predictor of increased number of coronary plaques.	Small sample size, reduced sensitivity of liver CT in diagnosing NAFLD
Moon JH *et al*.[61]	South Korean cross-sectional study (health check-up	Liver ultrasound	Mean CAC score significantly higher in NAFLD vs controls, but	Liver ultrasound poor sensitivity with liver fat < 30%. Generally low mean CAC score in

Authors	Study characteristics	Diagnosis of NAFLD	Main findings	Comments/Limitations
	referrals): 350 non-diabetic, normotensive NAFLD subjects & 400 healthy controls.		NAFLD only showed a trend towards a positive association with presence of CAC (p=0.079). Only insulin resistance (HOMA-IR) independently associated with presence of CAC.	NAFLD (20.7) vs controls (13.0), suggestive of low-risk NAFLD cohort
Sinn DH *et al*.[62]	Large South Korean retrospective cohort study (health screening), n= 4731 with NAFLD prevalence 44.1%	Liver ultrasound	Over mean F/U of 3.9 years, significant increase in annual CAC progression rates in NAFLD subjects (22% vs 17%, p<0.001), even after adjusting for all CV RFs	Retrospective study design. Reduced sensitivity of liver USS. Low-risk cohort, mean CAC score 2.0.

NAFLD, non-alcoholic fatty liver disease; CT, computed tomography; CAC, coronary artery calcium; CAD, coronary artery disease; CV, cardiovascular; RFs, risk factors; BMI, body mass index; MetS, metabolic syndrome; CRP, C-reactive protein; HOMA-IR, homeostasis model assessment of insulin resistance.

Table 1-4 Coronary angiography studies relating NAFLD to increased cardiovascular risk

Authors	**Study characteristics**	**Diagnosis of NAFLD**	**Main findings**	**Comments/Limitations**
Arslan U *et al*.[67]	Turkish cross-sectional study: consecutive patients admitted with ACS (n=92). NAFLD in 70%.	Liver ultrasound	NAFLD independent predictor of significant CAD (>50% stenosis of ≥1 major coronary artery), after adjustment for CV RFs and components of MetS.	Unusually high prevalence of NAFLD in cohort. Liver ultrasound poor sensitivity with liver fat < 30%. No functional assessment of coronary stenosis.
Acikel M *et al*.[68]	Turkish cross-sectional study: consecutive patients admitted for coronary angiography (acute & elective, n=355). NAFLD in 32%.	Liver ultrasound	NAFLD independent predictor of significant CAD (>50% stenosis of ≥1 major coronary artery) after adjustment for CV RFs. Moderate or severe NAFLD independently associated with greater extent of CAD.	Use of liver ultrasound to define NAFLD, as well as to grade severity (unreliable). No functional assessment of coronary stenosis.
Mirbagheri SA *et al*.[69]	Iranian cross-sectional study: patients admitted for elective coronary angiography (n=317). NAFLD in 54%.	Liver ultrasound	NAFLD strongest independent predictor of 'clinically relevant' CAD (>30% stenosis of ≥1 major coronary artery) after adjustment for CV RFs and components of MetS.	Liver ultrasound poor sensitivity with liver fat < 30%. Arbitrary threshold of 'clinically relevant' CAD – no functional assessment made.
Alper AT *et al*.[70]	Turkish cross-sectional study: patients with MS admitted for coronary angiography (acute & elective, n=80). NAFLD in 54%.	Liver ultrasound	NAFLD only independent predictor of severe CAD (>70% stenosis of ≥1 major coronary artery) after adjustment of CV RFs & components of MetS.	Use of liver ultrasound to define NAFLD, as well as to grade severity (unreliable). No functional assessment of coronary stenosis. Higher risk cohort (all with MetS).
Boddi M *et al*.[71]	Italian cross-sectional study: consecutive non-diabetic patients (n=95) admitted with STEMI	Liver ultrasound	Prevalence of NAFLD 87%. 'Moderate/severe' NAFLD independently associated with 3-fold risk of multi-vessel	Limitations of use of liver ultrasound to define NAFLD, as well as to grade severity. Arbitrary distinction of 'severe' vs

Authors	Study characteristics	Diagnosis of NAFLD	Main findings	Comments/Limitations
	underwent coronary angioplasty and investigation for NAFLD		disease compared to 'mild' NAFLD.	'mild' NAFLD based on ultrasound.

NAFLD, non-alcoholic fatty liver disease; ACS, acute coronary syndrome; CAD, coronary artery disease; CV, cardiovascular; RFs, risk factors; MetS, metabolic syndrome.

1.4.3 Studies evaluating carotid disease in NAFLD

Measurement of carotid intima-media thickness (CIMT) and plaque burden by ultrasound is a well-validated and widely accepted screening tool for the prediction of CV disease in asymptomatic subjects.[76, 77] Several studies link NAFLD independently with carotid disease, although a few have described a weaker association after adjusting for MetS (Table 1-5).[51, 78-84] Importantly, severity of histological features of NAFLD appear to correlate independently with increasing CIMT,[80] concordant with epidemiological data documenting NASH patients having a higher CV risk than simple steatosis. Additionally, a systematic review of seven published studies (total of 3497 subjects) reported a significant association between NAFLD and CIMT, showing an estimated increase of 13% in CIMT for NAFLD cases compared to controls. Prevalence of carotid plaque was also more frequent in NAFLD subjects.[85]

However, two subsequent studies not included in this meta-analysis did not show an association between NAFLD and increased CIMT (Table 1-5).[86, 87] Importantly, both were conducted in primarily diabetic subjects, with one study reporting a majority of their cohort on insulin treatment.[87] Given that insulin therapy is known to decrease liver fat in type 2 diabetics, possibly through reduction in glucose and free fatty acid (FFA) levels,[88] these results must be interpreted with caution. Furthermore, diabetes itself is considered a coronary-risk equivalent and so may have masked the association between NAFLD and carotid disease, especially when analysing relatively small sample sizes. Additionally, neither of these studies evaluated the presence of carotid plaque, which appears to have similar or greater predictive power for CV events than CIMT alone.[89]

A very recent meta-analysis of 13 studies involving a total of over 12,000 participants (34.5% with NAFLD) showed that NAFLD was associated with increased CIMT or carotid plaques with a pooled odds ratio 1.74 (95% CI, 1.47-2.06; $p < 0.00001$; $I^2 = 86\%$).[90] In summary, there is a large amount of epidemiological evidence suggesting an independent correlation between NAFLD and increased carotid disease, adding to the weight of evidence supporting a significant link between NALFD and increased CV risk.

Table 1-5 Main carotid studies relating NAFLD to increased cardiovascular risk

Authors	**Study characteristics**	**Diagnosis of NAFLD**	**Main findings**	**Comments/Limitations**
Volzke H *et al.*[78]	Study of Health in Pomerania population-based German cohort (n=2432), age≥45 years.	Liver ultrasound	Increased prevalence of carotid plaques in NAFLD, independent of CV RFs. Increased CIMT in NAFLD, but non-significant after CV RF & BMI adjustment.	Liver ultrasound poor sensitivity with liver fat < 30%. Representative of middle-aged/elderly population only.
Targher G *et al.*[80]	Case-control: 85 consecutive NAFLD patients & 160 age-, sex-, BMI-matched controls.	Liver biopsy	Increased CIMT & plaque prevalence in NAFLD, also correlating with histological severity; independent of CV RFs, MetS components & IR.	Cross-sectional study, small sample size (due to liver biopsy as diagnostic modality)
Targher G *et al.*[79]	Cross-sectional outpatient cohort with type 2 diabetes: 400 NAFLD & 400 age-, sex-matched controls.	Liver ultrasound	Increased prevalence of carotid plaques in NAFLD, independent of CV RFs, but non-significant after adjustment for MetS.	No CIMT assessment done. Use of liver ultrasound as diagnostic modality. Exclusive diabetic cohort.
Fracanzani A *et al.*[81]	Paired-sample case-control: 125 NAFLD & 250 age-, sex-, BMI-matched healthy controls.	Liver ultrasound	Increased CIMT and carotid plaque prevalence in NAFLD, independent of CV RFs, IR & MS components.	Liver ultrasound screen not performed in controls, only in NAFLD cases.
McKimmie *et al.*[86]	Diabetes Heart Study (cross-sectional cohort of diabetic families): 192 NAFLD & 431 unmatched	Liver computed tomography	No difference in CIMT in NAFLD vs controls after adjustment for selected CV RFs, visceral fat & C-reactive protein.	Preponderance of diabetics in study sample, unmatched controls, sample not representative of general population (family members).

Authors	Study characteristics	Diagnosis of NAFLD	Main findings	Comments/Limitations
	controls (from family members of index cases).			
Gastaldelli A *et al.*[51]	RISC Study: European population-based healthy cohort aged 30-60 years (n=1307).	Fatty liver index (validated algorithm based on BMI, waist circumference, TG & GGT)	Positive correlation between CIMT and NAFLD, independent of CV RFs, Framingham risk score & insulin sensitivity.	Limitations with use of fatty liver index as a marker of NAFLD. Lack of testing for viral hepatitis as a potential cause of fatty liver.
Petit JM *et al.*[87]	Hospital-based cross-sectional cohort, all diabetics (n=101). 60% with NAFLD.	Magnetic resonance spectroscopy of liver	No difference between CIMT in NAFLD & controls.	Older age group (mean 60 years), results not adjusted for age. Unmatched controls. Exclusively diabetic cohort with majority on insulin therapy.
Caserta CA *et al.*[82]	Italian cross-sectional population-based adolescent cohort (n=642), NAFLD in 12.5%.	Liver ultrasound	NAFLD independent marker of increased CIMT, along with obesity and systolic blood pressure.	Liver ultrasound poor sensitivity with liver fat < 30%. Exclusive adolescent cohort. Unable to exclude possible confounders of obesity and blood pressure.
Kim HC *et al.*[83]	Korean cross-sectional population-based cohort (n=1021), 50% with NAFLD.	Liver ultrasound	Increased CIMT in NAFLD vs controls independent of CV RFs and obesity, but only in subjects with MetS.	Use of liver ultrasound, unexplained high prevalence of NAFLD in 'healthy' cohort.
Kim HJ *et al*.[84]	Korean retrospective cohort study (n=819), 40.3% with NAFLD.	Liver ultrasound	Increased mean CIMT in NAFLD vs controls, independent of CV RFs, OR 1.90 (p=0.047). No significant difference in CAC scores.	Poor sensitivity of liver ultrasound, smoking status undocumented in study

1.4.4 Studies evaluating cardiac function in NAFLD

Studies in subjects with MetS have consistently shown increased left ventricular (LV) mass index and diastolic function impairment when compared to controls, which are in the main secondary to the effects of IR, obesity and hypertension on cardiac structure and function.[91, 92] When studies have focused specifically on NAFLD subjects, the findings of abnormal LV geometry and diastolic dysfunction has similarly been reported (Table 1-6).[93-98] One study also demonstrated a strong positive correlation between the degree of diastolic dysfunction and amount of liver fat, with diastolic dysfunction and IR the only independent parameters associated with NAFLD.[94]

Another study reported that echocardiographic measures of coronary flow reserve (CFR) were significantly lower in NAFLD compared to healthy controls, after adjusting for obesity, traditional CV risk factors and the presence of MetS.[99] Just under half of NAFLD patients had an impaired CFR whereas all controls had normal CFR values, and histological liver fibrosis score was the only independent predictor of impaired CFR. Although they correctly postulated that this result likely reflects impaired coronary endothelial function in the NAFLD group, they were unable to exclude the possibility of these patients having asymptomatic epicardial CAD. The consistent finding of subtle cardiac dysfunction in an asymptomatic population with NAFLD is perhaps not surprising, given that LV dysfunction and LV mass are strongly correlated with IR, as well as subsequent prognosis.[100]

Lee *et al*. recently also studied the relationship between liver fibrosis and subclinical myocardial dysfunction in asymptomatic NAFLD subjects without heart disease.[101] They reported that both hepatic fibrosis and steatosis were significantly associated with diastolic LV dysfunction, even after adjustment for traditional CV risk factors, although increased BMI attenuated the relationship with liver fat only. However, there was a strong dose-dependent association between measures of LV diastolic dysfunction and both liver fibrosis and liver fat, which again serves to highlight the graded relationship between severity of NAFLD and increased CV risk.

Table 1-6 Echocardiography studies relating NAFLD to increased cardiovascular risk

Authors	**Study characteristics**	**Diagnosis of NAFLD**	**Main findings**	**Comments/Limitations**
Goland S *et al.*[93]	Case-control: 38 non-diabetic, normotensive NAFLD patients & 25 age- & sex-matched controls.	Liver ultrasound & liver biopsy (29%)	Increased LV mass index & increased prevalence of diastolic impairment in NAFLD. Reduced E' only independent parameter associated with NAFLD on multivariate analysis.	Small sample size, no measure of insulin resistance, controls not BMI-matched (?obesity confounder), 46% of NAFLD group had MetS.
Fallo F *et al*.[94]	Case-control: Newly diagnosed untreated hypertensive patients (non-obese, non-diabetic): 48 NAFLD vs 38 controls.	Liver ultrasound	Prevalence of diastolic dysfunction increased in NAFLD, including positive correlation with degree of liver fat. Diastolic dysfunction and insulin resistance independently associated with NAFLD on multivariate analysis.	No tissue doppler indices of diastolic function employed (better load-independent measure), no stress testing to exclude subclinical ischaemia done (although unlikely in relatively young, healthy cohort).
Fotbolcu H *et al.*[95]	Case-control: 35 non-diabetic, normotensive NAFLD patients & 30 controls.	Liver ultrasound	Increased prevalence of impaired systolic & diastolic function using tissue doppler in NAFLD vs controls.	Small sample size – unable to exclude confounders. Controls not BMI or BP-matched, subclinical ischaemia not excluded.
Yilmaz Y *et al.*[99]	Case-control: 59 asymptomatic NAFLD patients & 77 age- & gender-matched healthy controls.	Liver biopsy	Reduced CFR in NAFLD, independent of obesity, CV RFs & MetS. Histological liver fibrosis score only independent predictor of impaired CFR.	No exclusion of subclinical myocardial ischaemia.

Authors	Study characteristics	Diagnosis of NAFLD	Main findings	Comments/Limitations
Jung JY *et al*.[96]	Large Korean cross-sectional 'health checkup' study (n = 20,821). NALFD in 29.7%.	Liver ultrasound	Abnormal LV relaxation and LV remodelling in NAFLD subjects compared to controls, which showed a proportionate response according to severity of liver fat. This was significant even after adjustment for CV RFs and BMI.	Young population (mean age 39.7 years) with possible selection bias. Liver ultrasound lacks sensitivity. Not all LV diastolic indices measured.
VanWagner LB *et al*.[97]	Cross-sectional analysis of CARDIA study, n = 2713. Asymptomatic subjects, no cardiac history. NAFLD in 10%.	Liver computed tomography	Significantly impaired LV diastolic function in NAFLD subjects, even after adjustment for CV RFs, BMI/VAT and physical activity.	48% cohort black, 59% women. CT liver lacks sensitivity with mild NAFLD.
Mantovani A *et al*.[98]	Cross-sectional outpatient-based study, consecutive 'healthy' type 2 diabetics, n = 222. NAFLD in 71%.	Liver ultrasound	Significantly increased LV diastolic dysfunction after adjusting for CV RFs, diabetes severity and BMI (adjusted odds ratio 3.08, p = 0.003).	Liver ultrasound lacks sensitivity. Only studied diabetic subjects, so not representative of general population.

NAFLD, non-alcoholic fatty liver disease; LV, left ventricular; E', mitral annular tissue doppler early diastolic velocity; BMI, body mass index; MetS, metabolic syndrome; BP, blood pressure; CV, cardiovascular; RFs, risk factors; CFR, coronary flow reserve. CARDIA, Coronary Artery Risk Development in Young Adults; VAT, visceral adipose tissue; CT, computed tomography.

1.4.5 Studies evaluating atrial fibrillation in NAFLD

The finding of an association of increased CV risk in NAFLD has led to further research exploring the prevalence of atrial fibrillation (AF) in the NAFLD population. This is because AF is a common cardiac disorder and is associated with increased mortality and adverse CV outcomes, including heart failure, myocardial infarction and most importantly, cerebrovascular events.[102] The incidence of AF increases with age and up to a quarter of middle-aged adults in developed countries will have the disease.[103] In fact, AF shares many risk factors with NAFLD, including hypertension, diabetes, obesity and coronary disease.

Two large community-based studies documented an association between elevated liver enzymes and increased AF prevalence.[104, 105] This was also more recently confirmed in two meta-analyses, which found that NAFLD patients had up to 2.5 times significantly more risk of developing AF than subjects without NAFLD,[106, 107] although statistical heterogeneity in the studies was high, which was a limitation of the meta-analyses. Utilising liver ultrasound to diagnose NAFLD, studies have also shown that fatty liver appears to be an independent risk factor of developing AF, in diabetic[108] and hypertensive[109] populations. Although there has also been conflicting evidence against an association between AF prevalence and NAFLD utilising computed tomography as a diagnostic modality in a subset of participants in the Framingham Heart Study,[110] the population studied here had a significantly lower incidence of AF compared to the previous positive studies, which may have led to a type 2 error.

Apart from the shared aetiological risk factors between NAFLD and AF as mentioned above, there are other postulated mechanisms for the association. NAFLD is known to be strongly associated with unfavourable changes in left ventricular mass and geometry, as well as the prevalence of diastolic dysfunction,[93-95] which increases the risk of AF.

In summary, there is good evidence to suggest that NAFLD represents a risk factor for developing AF and that the severity of NAFLD also increases the risk, especially in diabetic populations.[111]

1.4.6 Studies evaluating endothelial dysfunction and myocardial metabolism in NAFLD

Endothelial dysfunction is recognised as one of the most important and earliest detectable component in the development of atherosclerosis.[112] In both diabetic and non-diabetic cohorts, studies have shown an independent association between impaired endothelium-dependent flow-mediated dilation (FMD) and NAFLD.[52, 113, 114] In addition, lower FMD was observed in NASH compared to simple steatosis, again confirming the graded association of CV risk with severity of

NAFLD.[52, 114] A recent meta-analysis also confirmed that impaired FMD was increased in NAFLD subjects compared to controls, with an odds ratio of 3.73 (95% CI, 0.99 – 14.09).[90]

To gain further insight on the causes of subclinical cardiac dysfunction in NAFLD, the effects of hepatic steatosis on myocardial metabolism have also been examined.[115, 116] One study found a novel positive association between hepatic fat content and myocardial IR. Patients with high liver fat content not only showed significantly lower whole-body insulin sensitivity as expected, but also reduced myocardial glucose uptake and extraction rate, reduced coronary flow reserve, and increased plasma levels of inflammatory markers and vascular adhesion molecules. Only liver fat content remained significantly associated with impaired myocardial metabolism even after adjusting for IR, visceral fat mass and other important variables.[115] Another study assessed myocardial energy metabolism in NAFLD, utilising ^{31}P-MRS to determine the ratio of phosphocreatine to ATP in a young, healthy cohort.[116] The authors reported significantly impaired LV energy metabolism as well as increased epicardial fat in NAFLD compared to controls. This was despite normal LV morphological features and systolic/diastolic function in both groups, and was independent of usual CV risk factors. This suggests that in patients with hepatic steatosis, abnormalities in myocardial metabolism may precede functional and structural cardiac remodelling, leading to increased LV mass and diastolic dysfunction.

The precipitating factor for this dysfunctional cardiac phenotype appears to be the development of systemic and hepatic IR, leading to hyperinsulinaemia and increased FFA availability with associated myocardial IR. This produces inefficient energy metabolism by cardiomyocytes, switching to fat rather than glucose oxidation in physiologically demanding states, and yielding less ATP per oxygen molecule consumed. With progressive workload placing the heart under increasing strain, this potentiates myocardial dysfunction ultimately leading to myocardial adaptive remodelling and myocardial injury. The excess FFA supply also leads to cardiac lipotoxicity by causing intracellular lipid accumulation and overwhelming normal cardiomyocyte oxidative capacity, resulting in increased oxidative stress and consequent cardiac apoptosis and dysfunction.[100, 117]

1.5 Pathogenesis of cardiovascular disease in NAFLD

1.5.1 Insulin resistance and CV disease in NAFLD

Insulin resistance has been known to be strongly associated with atherosclerosis for several decades. In the 1960s, Reaven first reported the association between myocardial infarction and insulin resistance in non-diabetics.[118] He subsequently coined the term ‘Syndrome X’ for the

insulin resistance syndrome with common features that we now know as the metabolic syndrome.[119] Several epidemiological studies have also confirmed a link between increased insulin levels and CV risk.[65, 120, 121] However, given that IR is inextricably linked to each of the MetS entities and many of these study participants had some features of the MetS, it is uncertain if insulin resistance itself, is an independent risk factor for CV disease. In fact, studies have suggested that the MetS (with its implied IR) does not appear to impart CV disease risk over and above its individual components.[122, 123]

Whereas NAFLD is strongly associated with IR and increased CV risk, the majority of the previously mentioned studies suggest this increased CV risk is independent of cardiometabolic RFs. Additionally, the presence of the MetS appears to be a powerful predictor of incident diabetes, but is inferior to the Framingham Risk Score as a predictor of CV events.[124] This suggests that NAFLD imparts increased CV risk beyond its close association with IR, despite liver fat content being the best independent predictor of IR in skeletal muscle, adipose tissue and the liver.[125] Importantly, increased CV risk appears to be associated with liver fat/inflammation in a monotonic relationship, progressively increasing with more advanced stages of NAFLD.[80, 94] This parallels epidemiologic evidence showing a progressive relationship between glucose levels and CV disease extending from well below the diabetic threshold.[126] Ultimately, the development and progression of IR appears to be the key mediator in the initiation and propagation of NAFLD, primarily through adverse changes in glucose, fatty acid and lipoprotein metabolism, with both conditions subsequently driving each other in a synergistic fashion. Alterations in cellular FFA transport, possibly through hyperinsulinaemia, are involved in the pathogenesis of ectopic fat distribution by diverting the accumulation of triglyceride away from adipose tissue and toward other key metabolic organs, such as skeletal muscle and liver. This results in impaired insulin signalling in these tissues, and further exacerbates IR and the consequent cardiometabolic dysfunctional cascade.[127] These processes are also exacerbated by associated subclinical inflammation, deranged adipokines and increased ectopic fat accumulation in other organs including the heart, all ultimately contributing to increased CV risk (Figure 1-3).

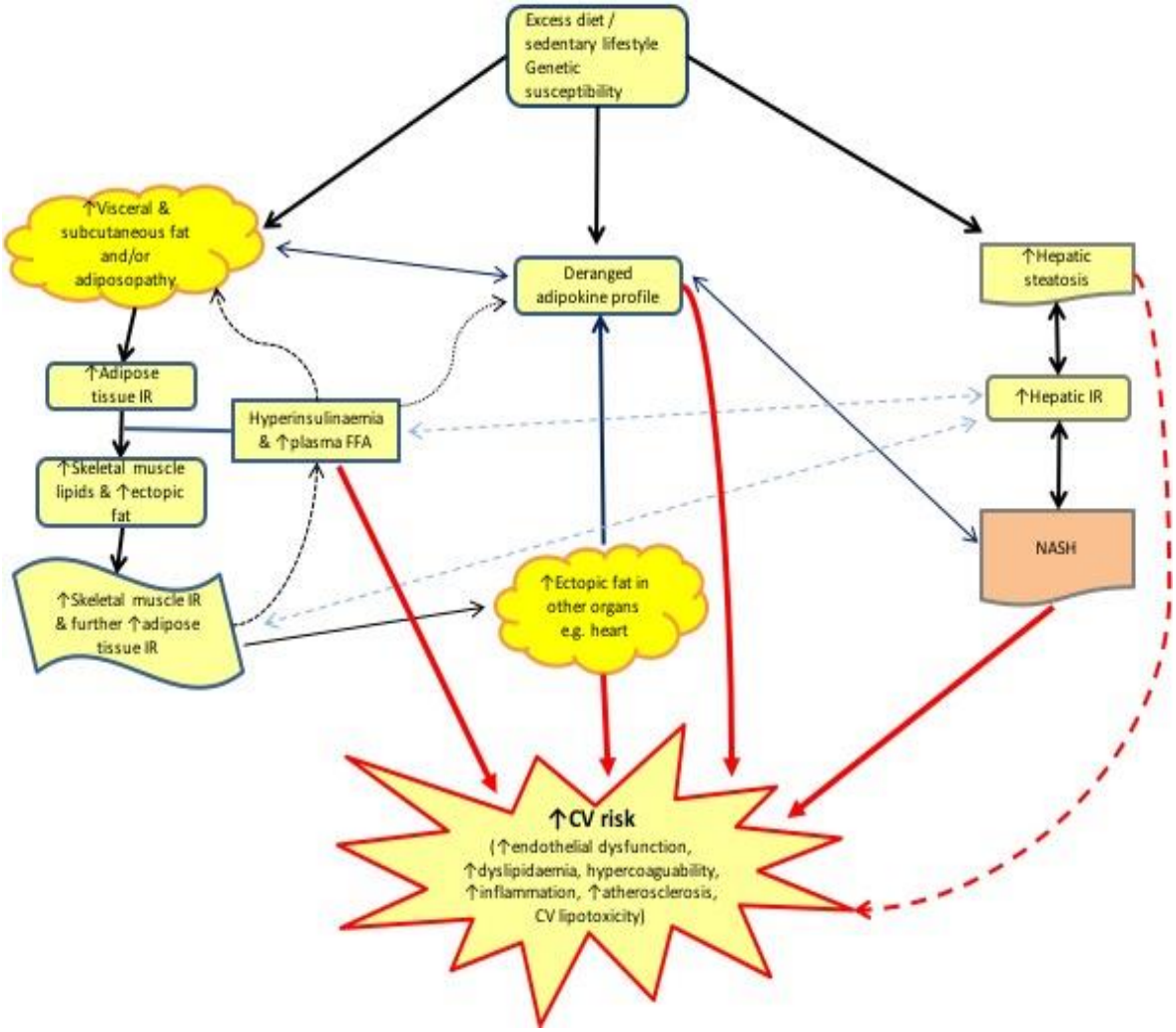

Figure 1-3 Schematic diagram of the pathophysiological processes involved in NAFLD leading to increased CV risk, highlighting the complex inter-relationships between visceral adipose tissue, adipocytokines, insulin resistance, ectopic fat accumulation and NAFLD.

1.5.2 Visceral fat

Visceral adipose tissue (VAT) appears to have a strong independent positive correlation with liver fat.[128] This is not surprising given that plasma FFA appear to be the main source of hepatic triglycerides in NAFLD, arising in part by greater lipolysis from insulin-resistant adipose tissue. This helps to explain somewhat the close association between the MetS and NAFLD, in that increased waist circumference is a mandatory criteria in the International Diabetes Federation guidelines for diagnosing MetS. Additionally, the independent link between centrally obese individuals and increased CV morbidity and mortality is well established.[129] Therefore, could VAT itself explain the increased CV risk seen with NAFLD, rather than liver fat content *per se*?

Studies show that increased VAT mass is independently associated with impaired glucose tolerance, IR and dyslipidaemia, conferring an increased risk of CV disease, irrespective of diabetic status.[130] Furthermore, the "portal hypothesis" suggests that increased VAT lipolysis secondary to IR leads to an elevated flux of FFA into the portal vein for direct transport to the liver, resulting in increased hepatic fat, which would suggest that visceral fat is an important mediator of liver fat content.[131] In fact, in the Quebec Cardiovascular Study, elevated FFA levels yielded a twofold increase in the risk of ischaemic heart disease, regardless of the presence of diabetes.[132] Additionally, high FFA concentrations in patients with angiographic CAD independently predicted CV mortality.[133] Apart from being a fat-storage organ, visceral fat is also metabolically active, secreting several adipokines, cytokines and hormones that serve to regulate inflammation, liver fat, IR and modify CV disease outcome (Table 1-7).[117, 134-146] Importantly, obesity in certain situations represents a chronic low-grade systemic inflammatory state that contributes to vasculopathy and CV risk through the release of these proinflammatory and atherogenic bioactive molecules.[147]

However, the mechanisms linking visceral fat or obesity to CV disease are strongly related to IR, which itself is robustly associated with CV risk and atherosclerosis.[148] It is therefore unclear whether VAT actually confers direct CV risk through secreted factors, or indirectly via IR-related processes, or both. Importantly, studies from patients with lipodystrophy suggest that even with little or no adipose tissue, fatty liver and IR can still develop quite markedly,[149] which undermines the portal hypothesis. Epidemiological and case-control studies also support the key role of liver fat, rather than VAT, as a marker of obesity-related metabolic dysfunction and a strong predictor of multi-organ IR, which is independent of obesity, VAT or plasma adipokine levels.[127, 150, 151] Despite these findings, adipose tissue is likely to still contribute to metabolic dysfunction as it is the specific characteristics of adipose tissue rather than the amount that is important. Accordingly, fat cell hypertrophy, macrophage infiltration of adipose tissue causing inflammation, increased adipose tissue lipolytic activity and adipose tissue hypoxia are all associated with IR.[152] It is therefore plausible that the established link between obesity and CV outcome may in fact be mediated through both ectopic fat accumulation (i.e. liver and cardiac tissue) as well as the effects of adiposopathy or 'sick fat'.[153] This occurs when adipose tissue becomes chronically inflamed and releases proinflammatory adipokines and cytokines that ultimately contribute to atherosclerosis and CV disease. Therefore NAFLD can be considered a sensitive marker of pathological dysfunction of adipose tissue, which appears to be more relevant to CV outcome than simply adipose tissue mass.

Table 1-7 Adipokines & cytokines produced or upregulated by visceral adipose tissue: associations and effects on liver, cardiovascular system, insulin resistance and adipose tissue (adapted from references[117, 134-146]

Adipocytokine	**Function**	**Liver**	**CV disease**	**Insulin resistance**	**Adipose Tissue**
Adiponectin	Insulin sensitizer Anti-inflammatory Anti-lipogenic	Correlates with ↓ NAFLD severity ↑ FA oxidation ↓ DNL (↓ SREBP-1c)	↓ in MI, CV disease ↓ foam cells Link with ↓CAC score ?↓CIMT	↓IR (muscle & liver)	↓ in obesity ↓ lipolysis - ↓FFA
TNF-α	Proinflammatory Regulates other cytokines Lipogenic Regulates cell viability	Correlates with ↑ NAFLD severity ↑ DNL (↑ SREBP-1c) ↑ CRP release from liver	↑atherosclerosis	↑IR	↑ in obesity, ↓ adiponectin levels ↑lipolysis - ↑FFA
IL-6	Proinflammatory ↑ CRP release	↑ in NAFLD	↑atherosclerosis ↑endothelial dysfunction Link with ↑CAC score	↑IR	↑ in obesity
Resistin	Proinflammatory (mainly acts on liver) Stimulates TNF-α & IL-6 actions ↓ insulin signalling	Correlates with ↑ NAFLD severity	↑ endothelial dysfunction ↑ in ACS and associated with ↓ prognosis	↑IR	↑ in obesity
Leptin	Regulates appetite & energy balance Regulates glucose homeostasis ↓ ectopic fat accumulation	↓ liver fat (↓ SREBP-1c) ? worsens NASH	↑ lipid-rich plaques ↑ platelet aggregation Link with ↑CAC score Link with CV risk	↓IR (muscle & liver)	↑ in obesity (due to leptin resistance)
Serum RBP-4	Proinflammatory	↑ in NAFLD	Link to ↑cerebral infarction	↑IR (muscle & liver)	↑ in obesity
Visfatin	?Insulin-mimetic ?Pro-inflammatory	Unknown	↑plaque destabilisation	?↓ IR	↑ in obesity

Adipocytokine	Function	Liver	CV disease	Insulin resistance	Adipose Tissue
Angiotensinogen	Precursor of angiotensin	↑ in NAFLD	↑BP, vasoconstrictor ↓NO & ↑oxygen free radicals ↑atherosclerosis	↑ in IR	↑ in obesity
Serum Amyloid A	Proinflammatory	Unknown	↑atherosclerosis ↑ in ACS & CV disease Link with CIMT Potential CV risk predictor	↑ IR	↑ in obesity, ↑ in adiposity ↑ lipolysis
CRP	Proinflammatory. Primarily produced from liver	Correlates with ↑ NAFLD severity	↑ in CVD & atherosclerosis ↑ endothelial dysfunction Useful for CV risk assessment	↑ in IR	↑ in obesity
PAI-1	Proinflammatory Prothrombotic	Correlates with ↑ NAFLD severity	↑ in MI, CV disease	↑ in IR	↑ in obesity

NAFLD, non-alcoholic fatty liver disease; FA, fatty acid; DNL, *de novo* lipogenesis; SREBP-1c, sterol regulatory element binding protein 1c; MI, myocardial infarction; CV, cardiovascular; CAC, coronary artery calcium; CIMT, carotid intima-media thickness; IR, insulin resistance; FFA, free fatty acids; TNF-α, tumour necrosis factor-alpha; CRP, C-reactive protein; IL-6, interleukin-6; ACS, acute coronary syndrome; RBP-4, retinol binding protein-4; BP, blood pressure; NO, nitric oxide; PAI-1, plasminogen activator inhibitor-1.

1.5.3 Epicardial fat

Given that NAFLD and excessive visceral abdominal fat represent abnormal ectopic fat deposition in the body, with associated VAT-secreted adipocytokines contributing to subclinical inflammation and atherosclerosis, the role of epicardial adipose tissue (EAT) which is itself a visceral fat layer has been further evaluated. Its anatomical location and proximity to the myocardium and adventitial layer of the coronary arteries, as well as sharing the same microcirculation, make it an ideal entity to exert a paracrine and vasocrine effect on the heart and its blood vessels.[154] Imaging studies have already shown that epicardial thickness or pericardial (epicardial and paracardial) fat volume correlate with the amount of VAT in both obese and non-obese subjects.[155-158] Furthermore, EAT thickness is also positively associated with left ventricular dysfunction[159] as well as the presence and severity of angiographic CAD.[157, 160, 161] Increased epicardial or pericardial fat volume measured by CT are also each independently associated with the presence of coronary artery calcium.[162, 163] Importantly, adiponectin expression was found to be significantly lower in epicardial fat isolated from patients with severe CAD compared to those without CAD,[164] and pericardial fat volume also correlates with multiple markers of inflammation and oxidative stress,[165] thus signifying potential similarities in proinflammatory adipokine function between EAT and VAT.

Iacobellis *et al.* have validated a simple echocardiographic method of quantifying EAT involving measurement over the anterior right ventricular wall in the parasternal view, showing an excellent correlation with MRI-determined values.[158] Furthermore, they have proposed EAT threshold values for cardiometabolic risk stratification,[166] having reported significant correlations of EAT with several anthropometric, CV and metabolic risk factors including IR.[167, 168] Importantly, pericardial fat volume appears to independently predict major adverse cardiac event risk in asymptomatic subjects, even after adjusting for FRS, CAC score and BMI.[169]

Studies have also shown a significant association between NAFLD and EAT. In an obese cohort, epicardial fat thickness was significantly greater in NAFLD subjects compared to controls and appeared to be the best independent correlate of hepatic steatosis.[170] Greater epicardial fat also appears to be associated with increased severity of NAFLD, in terms of simple hepatic steatosis and worsening liver fibrosis.[171, 172] In the Rotterdam study, Wolff *et al* also showed that increased liver fat was independently associated with increased epicardial fat as well as greater coronary artery calcification, after adjustment for traditional CV risk factors.[173]

Weight reduction through exercise training or a low calorie diet has been shown to decrease EAT thickness, as well as reduce VAT and increase insulin sensitivity.[174, 175] Notably, improvement in LV

diastolic function correlated better with EAT than waist circumference reduction.[174] Furthermore, increased epicardial fat has a significant negative correlation with cardiac index, and also correlates directly with intramyocardial triglyceride levels.[176] Therefore it remains unclear whether the LV dysfunction is due to lipotoxicity from excess FFA availability and subsequent oxidative stress, as well as the deleterious effects of increased LV mass, or secondary to adipokine-mediated myocardial inflammation and damage; or both.[177] However, it is likely that increased epicardial and myocardial fat both represent abnormal ectopic fat storage and may indeed be a marker of the cumulative effects of NAFLD and IR in the setting of pathological adiposity,[176, 178] with consequent associated adverse CV outcome.[179]

1.5.4 Inflammation and thrombosis

The liver is a key metabolic organ and central to the regulation of systemic inflammation. It is a generator as well as a target of various inflammatory and humoral factors (as summarised in Table 1-7), working in concert and against secreted molecules from adipose tissue, macrophages and endothelial cells in the context of CV disease initiation and progression.[136, 138, 140] Increasing severity of NAFLD likely represents worsening inflammatory and insulin-resistant states, with poorer cardiometabolic outcomes. hs-CRP, which is primarily produced by the liver and a marker of inflammation, is an independent predictor of CV events in several large studies.[180] Similarly, fibrinogen and plasminogen activator inhibitor-1 (PAI-1) also originate from hepatic tissue and are activators of the coagulation system, enhancing atherothrombosis.[146] Importantly, hepatic and plasma PAI-1 levels have been shown to correlate with the degree of hepatic steatosis as well as severity of NASH.[146, 181] Targher *et al.* also showed that biopsy-proven NASH patients had significantly higher levels of hs-CRP, fibrinogen and PAI-1 activity compared to controls. Furthermore, the severity of NASH by liver histology correlated significantly with these CV risk biomarkers after adjustment for potential confounders, including IR and visceral adiposity.[135] A similar correlation was found for serum IL-6 levels, as well as serum and hepatic TNF-α in NASH patients.[182] Additionally, these studies suggest that increased liver-secreted factors in NAFLD play an important role in the pathogenesis of systemic inflammation and atherosclerosis.

Nuclear factor kappa-B (NF-κB) is a hepatocellular transcription factor that plays a key role in intrahepatic inflammation. In rodent models, a high-fat diet results in hepatic steatosis and upregulation of NF-κB activity, which leads to hepatic production of proinflammatory cytokines IL-6, IL-1β and TNF-α, as well as activation of Kupffer cells and macrophages, possibly worsening hepatic inflammation.[183] This study also demonstrated that isolated hepatic inflammation in the absence of steatosis through selective activation of NF-κB, resulted in hepatic and skeletal muscle IR. Hepatic steatosis can also induce hepatic inflammation through lipotoxicity and endoplasmic

reticulum oxidative stress responses, as well as through mitochondrial dysfunction via increased oxidation of excess fatty acids.[184] Mitochondrial dysfunction and damage are associated with IR and atherosclerosis in several studies,[148] representing a plausible link between NAFLD and increased CV risk.

1.5.5 Dyslipidaemia

The liver plays a central role in lipid metabolism through a combination of processes including lipoprotein synthesis and export, *de novo* lipogenesis (DNL) and lipid breakdown.[185] NAFLD is characterised by an atherogenic lipid profile, consisting of high triglyceride (TG) levels, low high-density lipoprotein (HDL) cholesterol, an increase in small, dense low-density lipoprotein (LDL) particles, increased very low-density lipoprotein (VLDL) cholesterol levels and elevated apolipoprotein B100 concentration.[186, 187] This type of atherogenic dyslipidaemia is strongly linked to adverse CV outcome.[188, 189] The increased hepatic production of TG-rich VLDL provides a limited compensatory mechanism for reducing liver fat content.[190] However, this also results in abnormal HDL metabolism causing HDL reduction as well as compositional alterations. In fact, the amount of liver fat has a significant negative correlation with subfractions of HDL known to be athero-protective, which are reduced in NAFLD independently of peripheral insulin sensitivity.[191]

1.6 Treatment of NAFLD

Various therapeutic modalities for NAFLD have been postulated and trialled to date and a summary of these treatments, as well as each of its associated CV benefits and risks, are shown in table 1-8.[30, 192-202] For a more detailed overview, readers are encouraged to refer to recently published guidelines[30, 193] as well as a meta-analysis of randomised trials for the treatment of NAFLD.[203] To summarise, there is currently no established pharmacological treatment for NAFLD,[193] and lifestyle interventions such as increasing exercise, reducing dietary fat intake and encouraging weight loss are the only recommended therapeutic strategies with proven benefit.[194] From a cardiovascular perspective, lipid-lowering drugs (e.g. statins), insulin-sensitisers (e.g. thiazolidinediones, metformin) and anti-hypertensive agents have not as yet shown adequate added risk/benefit value in NAFLD over and above already established evidence-based guidelines for the individual treatment of dyslipidaemia, diabetes and hypertension. Given the increased CV risk associated with NAFLD attributed to its pro-atherogenic and pro-inflammatory states, it is perhaps surprising that statins, with their anti-atherosclerotic and pleiotropic (anti-oxidant, anti-inflammatory) effects, have thus far not shown a consistent benefit in NAFLD outcomes. One potential explanation for this could be that statins are also known to indirectly impair insulin

Table 1-8 Summary of NAFLD outcomes in different treatment/intervention trials in NAFLD and associated cardiovascular benefits/risks of intervention[30, 192-202]

Treatment	**Summary of benefit in NAFLD**	**Associated CV benefit/risk**	**Comments**
Lifestyle Intervention (e.g. weight loss, increased physical exercise, dietary intervention)	↓liver enzymes, ↓hepatic fat(MRS & US), ↑insulin sensitivity, improved or unchanged NAFLD histological staging	Improved LV function. Improved VO2max. ↓blood pressure.	NAFLD improvement only with >5-7% weight loss. Regular exercise showed improvements in NAFLD independent of body weight or visceral fat changes. Rapid weight loss can lead to ↑hepatic fat
Thiazolidinediones (e.g. pioglitazone)	↓liver enzymes, ↓hepatic fat (MRS), ↔insulin sensitivity, improved NASH histological staging	↑risk of non-fatal MI (rosiglitazone only) and CHF. Pioglitazone: ↓risk of major adverse CV events (excluding CHF) in diabetics.	Causes significant weight gain and oedema.
Metformin	↓liver enzymes, unchanged or↓hepatic fat (US), ↑insulin sensitivity, no significant change in histological staging	↓risk of MI and overall mortality in overweight diabetics.	Gastrointestinal symptoms common. Risk of lactic acidosis (rare)
Statins	↓liver enzymes, unchanged or↓hepatic fat (US), no change in histological staging.	↓↓risk of adverse CV events and death in primary and secondary prevention, regardless of lipid levels.	Generally well-tolerated with large evidence base for CV benefit. Safe in NAFLD – no need for liver enzyme monitoring.
Ezetimibe	↓liver enzymes, ↓hepatic fat (US), no significant change in histological NASH	↓risk of adverse CV events in combination with statin.	Very well tolerated, safe in NAFLD.
Fibrates	↓liver enzymes, no histological improvement observed.	↓TG, ↑HDL & ↓small, dense LDL, but overall no CV mortality benefit across all groups. ↓in CV events only seen in atherogenic dyslipidaemic patients.	Useful only in certain population groups (e.g. ↑↑TG)
N-3 polyunsaturated fatty acids (PUFAs)	↓liver enzymes, ↓hepatic fat(MRS & US), ↑insulin	↓mortality & SCD post-MI, ↓mortality & HF hospitalisations in CHF,	Generally well-tolerated, but dose important (only high

Treatment	Summary of benefit in NAFLD	Associated CV benefit/risk	Comments
	sensitivity or unchanged.	possible ↓ in AF burden, ↓triglycerides, carotid plaque stabilisation. May ↑LDL. May ↑ ventricular arrhythmias in angina patients.	dose effective for NAFLD).
Angiotensin 2 antagonists	↓liver enzymes, ↑insulin sensitivity, improved NAFLD histological staging (telmisartan only)	↓blood pressure. May improve impaired glucose tolerance.	Limited studies.
Antioxidants (Vitamin E)	↓liver enzymes, ↔insulin sensitivity, improved NAFLD & NASH histology	Overall no conclusive benefit.	Dose and duration likely to be important.
Weight-loss drugs (e.g. orlistat, rimonabant (now withdrawn))	↓liver enzymes, ↓hepatic fat (CT & US), ↑insulin sensitivity	Similar to associated weight loss benefits.	Limited studies.
Bariatric Surgery	↓liver enzymes, ↑insulin sensitivity, improved NAFLD & NASH histological staging.	Similar to associated weight loss benefits. ↓ CV mortality	Long-term benefit appears to be dependent on improvement in insulin sensitivity, rather than weight loss.
NAFLD, non-alcoholic fatty liver disease; MRS, magnetic resonance spectroscopy; US, ultrasound; LV, left ventricular; VO2 max, maximal oxygen consumption; MI, myocardial infarction; CHF, congestive heart failure; CV, cardiovascular; TG, triglyceride; HDL, high-density lipoprotein; LDL, low-density lipoprotein; SCD, sudden cardiac death; HF, heart failure; AF, atrial fibrillation; CT, computed tomography.			

sensitivity,[204] which may result in an overall net neutral effect in treating NAFLD. Other possible reasons could include inadequate trial durations to allow inflammatory changes to translate into beneficial clinical outcomes, or the enrolment of low-risk NAFLD cohorts. It is noteworthy that patients with hepatic steatosis have not been shown to be at increased risk for statin hepatotoxicity,[205] and the Liver Expert Panel stated in a report in 2006 that statins can indeed be safely used in NAFLD and NASH, without the need for routine liver enzyme monitoring.[206]

1.7 N-3 polyunsaturated fatty acids

1.7.1 Nature and actions of n-3 fatty acids

N-3 fatty acids are essential polyunsaturated fatty acids (PUFAs), also commonly known as omega-3 fatty acids. The three types of n-3 fatty acids important for normal human metabolism are α-linolenic acid (ALA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). Animals are unable to synthesize n-3 fatty acids *in vivo* and the latter two fatty acids are commonly found in marine oils, while ALA is found in plant oils.[207] ALA can be converted to EPA and DHA in the liver through desaturation and elongation (to form more carbon double bonds), but *in vivo* conversion rates are poor, especially into DHA.[208]

N-3 fatty acids influence cell function through a variety of mechanisms, primarily by its effect on altering cell membrane fatty acid compostion. These mechanisms include altering cell signalling pathways and modifying gene expression, changes in synthesis of lipid mediators and altered membrane structure and fluidity.[208] These result in several physiological actions that have potential clinical benefits, especially for the CV system. These include lipid regulation (primarily lowering of plasma triglyceride concentrations), blood pressure lowering effects, anti-inflammatory effects, improvement in endothelial function and arterial plaque stabilisation, regulation of coagulation and platelet function leading to decreased thrombosis and anti-arrhythmic effects.[209]

1.7.2 N-3 PUFAs in NAFLD

N-3 PUFAs represent a potentially viable pharmacological treatment option in NAFLD. This group of fatty acids has an excellent side-effect profile and in high dose is effective in reducing plasma triglycerides and FFA levels,[210] both increased in NAFLD and associated with increased CV risk. Animal studies have also shown n-3 PUFA to be negative regulators of hepatic lipogenesis and the inflammatory response, as well as improving insulin sensitivity.[207] This is mainly achieved through alterations in hepatic nuclear transcription factor regulation involving peroxisome proliferator-activated receptor alpha (PPARα), sterol regulatory element-binding protein-1 (SREBP-1) and carbohydrate responsive element-binding protein (ChREBP), all involved in hepatic lipid and glucose metabolism as well as inflammatory pathways.[211] Although preliminary human trials showed a beneficial effect of n-3 PUFA in treating NAFLD, they were limited by small sample sizes, lack of randomisation or placebo arms.[212-214] Following on from our research, a recent meta-analysis of twenty-two studies investigating n-3 PUFAs to treat NAFLD showed a pooled significant

reduction in liver enzymes and hepatic steatosis (assessed with MRI or MRS) in the PUFA-treated group compared to controls.[215] In subjects with NASH, n-3 PUFAs did not significantly improve histological measures of disease in a pooled analysis. However, only four studies with wide heterogeneity were able to be adequately assessed for histological outcomes and the lack of a consistent positive finding is likely to be related to several factors, including inadequate doses or short durations of treatment, poor compliance and lack of controlled histological assessments. Therefore although there is now ample evidence to suggest n-3 PUFAs may be useful in reducing hepatic steatosis and lipid levels, no robust evidence as yet exists for its use in the treatment of NASH at present.[215] Furthermore, prior to my research, no studies had yet examined the effect of reducing liver fat or NAFLD severity on proxy markers of CV risk, which was a novel experimental aspect of my research.

1.7.3 N-3 PUFAs in cardiovascular disease

Initial epidemiological evidence from the Inuit eskimo population challenged the long-held view that a high fat intake was harmful, and their significantly lower CV mortality was thought to be primarily related to the protective effects of a large dietary intake of omega-3 fatty acids contained in oily fish.[216] Subsequent observational and case-control studies from various populations further suggested that adequate dietary intake of n-3 PUFAs reduced CV mortality risk,[217] and this was confirmed in two meta-analyses in 2004 which showed a dose-response relationship between oily fish consumption and a 17-38% lower incidence of CV mortality, although the association with non-fatal MI was less robust, possibly due to inadequate doses.[218, 219]

The landmark GISSI-Prevenzione study was a large, randomised-controlled trial of post-MI subjects which showed that dietary supplementation with 1g n-3 PUFA daily resulted in a significant decrease in cardiac mortality over 3.5 years of follow up, mainly attributable to sudden cardiac death.[220, 221] This result led to various national guideline recommendations for the use of Omacor (the n-3 PUFA preparation used in the GISSI-P study) in a secondary prevention setting to reduce CV mortality.[222] Another subsequent large randomised trial in patients with hypercholesterolaemia, JELIS (Japan EPA Lipid Intervention Study), showed a significant 19% reduction in major adverse coronary events over 5 years in a primary and secondary prevention cohort who took high doses of EPA supplementation compared to controls, although most of the benefit appeared to come from the secondary prevention group in post hoc analysis.[223] The GISSI Heart Failure (GISSI-HF) study in 2008 also showed beneficial results for n-3 PUFA supplementation in heart failure patients, with a 10% reduction in CV mortality.[224] However, two further large secondary prevention post-MI trials in 2010 did not show any CV outcomes benefit

of n-3 PUFAs compared to controls.[225, 226] Importantly, there were a number of key differences between the earlier and later trials that were likely to have confounded results. Firstly, statin use in the GISSI trials was <30% of the cohort studied compared to >85% in the later trials, and other CV medications (e.g. antiplatelets, antihypertensives) were also more widely used, which could have easily nullified any beneficial effect of n-3 PUFAs in terms of CV outcomes in the later trials. Secondly, GISSI-P and JELIS were open-label trials as no placebo capsules were available at the time. Finally, doses of combined EPA/DHA were lower in the two later trials. A meta-analysis published in 2012 of twenty randomised controlled trials (RCTs) involving 68,680 subjects in either high-risk primary prevention or secondary prevention, demonstrated a significant reduction in CV mortality (possibly due to SCD), but no effect on all-cause mortality, non-fatal MI or stroke, in subjects on n-3 PUFAs compared to controls.[227]

Following this meta-analysis, further RCTs involving n-3 PUFAs were performed in the context of more up to date and widespread usage of other evidence-based beneficial cardiovascular treatments, with a number of them showing no beneficial CV or survival outcomes with n-3 PUFAs versus placebo.[228, 229] Finally, a very recent meta-analysis by the Omega-3 Treatment Trialists' Collaboration of ten trials (including the ones mentioned already) involving 77,917 subjects over a mean of 4.4 years, suggested that n-3 fatty acids compared to placebo do not significantly reduce cardiac death, non-fatal MI or stroke.[230] This finding highlights the insufficient evidence to date from RCTs regarding the optimal dosing, duration, appropriate source (dietary vs supplemental) of n-3 fatty acids and ideal therapeutic ratios of DHA to EPA formulations that may be necessary to truly improve CV outcomes in high-risk CV patients including secondary prevention.[231] In fact, there is some evidence to suggest that DHA supplementation has far greater benefit compared to EPA in terms of reducing CV risk.[232, 233] Despite the inconsistent data to date, current North American guidelines still recommend n-3 PUFAs in certain patient groups, including secondary prevention of coronary heart disease and SCD as well as in patients with heart failure and impaired LV systolic function.[234] However, the conflicting evidence certainly necessitates further RCTs to evaluate if higher doses than the recommended 1g n-3 PUFA will prove to have significant beneficial CV outcomes including in a wider range of populations such as high-risk primary prevention, diabetics, NAFLD subjects or patients with heart failure and preserved ejection fraction, all of whom are at increased CV risk.

1.8 Aims of my PhD study

Although there have been several cross-sectional and case-control studies evaluating proxy markers of CV disease in NAFLD subjects as already described, there is very limited information on how these prognostic CV biomarkers may be altered in association with changes in NAFLD severity over time.[235, 236] There is also limited information on the effects of *high-dose* n-3 fatty acid supplementation on prognostic CV biomarkers, with previous studies using only up to 2g of n-3 fatty acid supplementation.[237] This has led to inconsistent results in terms of potential CV benefits, possibly due to inadequate dosing.

The WELCOME trial (Wessex evaluation of fatty liver and cardiovascular markers in NAFLD with Omacor therapy, clinicaltrials.gov NCT00760513) was a phase IV randomised, double-blind placebo-controlled trial. The primary aim of the trial was to test whether 15-18 months of n-3 PUFAs (Omacor 4g/day) versus placebo in 103 NAFLD subjects reduced liver fat percentage and two histologically-validated algorithmically-derived biomarker scores of liver fibrosis.

Embedded in the WELCOME trial were pre-specified substudies with secondary outcome measures, which formed the main part of my PhD study and 'Results' chapters. In brief, these were to evaluate whether either prolonged n-3 PUFA supplementation in NAFLD subjects or reduction in NAFLD severity over 15-18 months:

a) improved whole-body and hepatic insulin sensitivity

b) improved markers of left ventricular diastolic function (e.g. tissue Doppler indices)

c) lead to favourable changes in carotid intima-media thickness (CIMT)

These prognostic CV biomarkers have yet to be fully investigated in association with changes in NAFLD severity over time, as well as in relation to high-dose n-3 fatty acid supplementation.

Chapter 2: Methods

2.1 Study design

The WELCOME study (**W**essex **E**valuation of fatty **L**iver and **C**ardiovascular markers in NAFLD with **OM**acor th**E**rapy; www.clinicalTrials.gov NCT00760513) was a phase IV randomised double-blind placebo-controlled trial undertaken at the Wellcome Trust Research Facility, Southampton General Hospital, which was part-funded by the NIHR and Diabetes UK. The primary aim of the trial was to test whether treatment with 15-18 months of n-3 PUFAs (pharmaceutical grade preparation Omacor 4g/day) versus placebo in 103 NAFLD subjects reduced liver fat percentage and improved two histologically-validated algorithmically-derived biomarker scores of liver fibrosis. The primary end points of the study were tested using intention-to-treat (ITT) analysis and per protocol analysis. All participants had baseline investigations prior to randomisation and then paired follow-up investigations following 15-18 months treatment. There were a number of pre-specified sub-studies embedded in the main trial, a few of which formed the mainstay of my PhD research, as stated above (Section 1.8).

2.2 Ethical approval

The WELCOME study had approval by the local research ethics committee (REC: 08/H0502/165). It also had approval from the Medicines and Health Regulatory Authority (EudraCT no: 2008-003766-26) . All participants gave written informed consent.

2.3 Research Facility

All investigations took place at the Wellcome Trust Clinical Research Facility at Southampton University Hospitals NHS Trust. This unit is dedicated to full-time research and staffed by fully trained research nurses. It is equipped with a day ward and side-rooms for various investigations to be carried out, with all necessary equipment and clinical support as required. There is also a sample laboratory on site staffed by a fully-trained laboratory scientist where blood samples can be centrifuged, separated and frozen.

2.4 Recruitment

WELCOME trial recruitment: Subjects with NAFLD were identified from Hepatology and Diabetes clinics in Southampton Hospital from the period January 2010 to August 2011. The diagnosis of

NAFLD was based on either radiological or liver biopsy criteria for NAFLD.[4] These subjects were then contacted by the research team either personally at their hospital clinic attendance or by a letter of invitation from the research team. Subjects were also identified at hospitals in Portsmouth, Poole, Bournemouth, Winchester, Basingstoke and the Isle of Wight through collaborations with clinicians there, who acted as 'post boxes' and informed them about the study using a patient information sheet (PIS) prepared by the research team. Potential volunteers were asked to get in touch with our research team if they were interested in participating using the contact telephone numbers on the PIS. However, all formal recruitment and investigations were performed at Southampton Hospital.

2.5 Subjects

2.5.1 Inclusion criteria

1. Age > 18 years

2. Either i) Histological diagnosis of non-alcoholic steatosis or steatohepatitis in keeping with the Kleiner scoring system[238] or ii) steatosis diagnosed by ultrasound, computed tomography or magnetic resonance imaging in patients who also had either diabetes and/or features of the metabolic syndrome. Liver biopsy or liver imaging was required within 3 years of recruitment to the study and on recruitment, all subjects underwent a further assessment of liver fat percentage by MRS to establish their baseline liver fat percentage at entry into the trial.

3. Alcohol consumption < 35 units/week for women, < 50 units/week for men. These thresholds were chosen because at the time of the study design, alcohol intake above these levels was considered harmful to the liver.[239] However, at recruitment, only 1 man was consuming >21 units/week of alcohol and one woman was consuming >14 units/week.

2.5.2 Exclusion criteria

1. No known aetiological factors for underlying liver disease (e.g. hepatitis A, B & C, primary biliary cirrhosis, autoimmune hepatitis, haemochromatosis or Wilson's disease). These conditions were excluded with blood tests.

2. Decompensated acute or chronic liver disease, liver cirrhosis, pregnancy or lactation, taking liver-toxic drugs or oral steroids, hypersensitivity to Omacor, soya or any of the excipients.

2.6 Study groups and randomisation

2.6.1 Treatment groups

178 potential participants were identified who met the inclusion criteria. 72 people declined for personal reasons following initial contact. 106 subjects were consented for the study at the outset. However, one person withdrew after consenting for personal reasons and two further participants withdrew after the initial baseline visit (again for personal reasons) prior to randomisation. 103 subjects were subsequently randomised 1:1 in a double-blind fashion to either omega-3 fatty acid ethyl esters 4g/day or placebo (olive oil) 4g/day for a minimum of 15 months and maximum of 18 months of treatment. As 4g/day Omacor is the highest licensed (and safe) dose used in the treatment of hypertriglyceridaemia,[240] we chose this dose to ensure maximal efficacy as our research was a proof of concept study in the treatment of NAFLD. The benefit of n-3 fatty acids in treating NAFLD was still largely uncertain at the time of initiating the study.

2.6.2 Randomisation

Participants were randomised according to standardised procedures (computerized block randomisation) in a blinded fashion by a research pharmacist at Southampton Hospital. Simple randomisation in blocks of four, either to trial medication or placebo was used.

2.6.3 Active group

51 participants were randomised to receive 4g/day of omega-3 fatty acids in the form of Omacor (Pronova Biopharma ASA, Lysaker, Norway; Abbott Laboratories, Southampton, UK). This was provided free of charge by the pharmaceutical company. Omacor 1g contains EPA 460mg and DHA 380mg as ethyl esters.

2.6.4 Placebo group

52 participants were randomised to receive 4g/day of olive oil. 1 g of olive oil contains 600mg of oleic acid and lesser amounts of linoleic, palmitic, stearic and α-linolenic acids. Both Omacor and placebo capsules were gelatine-coated and of similar appearance and taste.

2.6.5 Compliance with treatment

Compliance with the allocated medication was monitored by recording returned unused capsules at fixed intervals during the study. Adverse events were recorded. Additionally, erythrocyte EPA and DHA enrichment (between baseline and end of study) was assessed to test adherence to the intervention in the Omacor group, as well as monitor contamination with DHA/EPA in the placebo group (e.g. over-the-counter omega-3 supplements). Dietary and lifestyle changes had already been recommended to all participants as part of their routine clinical care[4] and this was continued throughout the study. There were no additional specific weight-loss programs or strict dietary restrictions placed on the participants as part of the study.

2.7 Study outcome measures

2.7.1 Primary outcomes

From the main WELCOME study, the two primary outcome measures were: 1) a decrease in liver fat percentage as measured by MRS scan and 2) an improvement in two histologically-validated algorithmically-derived biomarker scores for liver fibrosis.[241, 242] The sensitivity and specificity of these two biomarker scores (NAFLD fibrosis score and liver fibrosis score) for the diagnosis of liver fibrosis has previously been summarised.[243] The liver fibrosis score using a combination of tissue inhibitor of metalloproteinase 1 (TIMP-1), hyaluronic acid (HA) and procollagen III N-terminal propeptide (PIIINP) has excellent performance (AUROC 0.9) for the diagnosis of severe fibrosis, but performs gradually less well with reducing levels of fibrosis (AUROC 0.82 for moderate fibrosis, AUROC 0.76 for no fibrosis).[244] The NAFLD fibrosis score uses an algorithm based on age, hyperglycaemia, BMI, platelet count, albumin and AST/ALT ratio.[242] This score has good sensitivity (82%) for diagnosing no fibrosis, but poorer sensitivity for diagnosing advanced fibrosis (51%). Specificity for excluding advanced fibrosis was excellent at 98%, but only 77% specificity for excluding no fibrosis. Consequently, because these two scores provide different sensitivities and specificities according to the amount of fibrosis present, both markers were used in the study to evaluate the effects of the intervention.

2.7.2 Secondary outcomes

In addition to the primary outcomes of the WELCOME study, there were several hypothesis-generating secondary outcomes, which formed a large part of my PhD thesis work. As already described, these secondary outcome measures were to evaluate whether either prolonged n-3 PUFA supplementation in NAFLD subjects or improvement in NAFLD severity (assessment of

hepatic steatosis or NAFLD-related serum biomarkers e.g. CK18-M65) over 15-18 months: i) improved whole-body and hepatic insulin sensitivity (in a subgroup of patients who had an additional two-step hyperinsulinaemic–euglycaemic clamp study), ii) improved echocardiographic markers of left ventricular diastolic function and iii) lead to favourable changes in carotid intima-media thickness.

2.8 Sample size & power calculations

2.8.1 Primary outcomes

Based on the small amount of inconclusive published literature at the time of the initial design of the WELCOME study protocol in 2008, it was estimated that a 15% decrease in liver fat may result from n-3 fatty acid treatment. Subsequently in 2012, a systematic review and meta-analysis of omega-3 fatty acid supplementation in the treatment of NAFLD[245] suggested a Hedge's g pooled effect size of omega-3 fatty acid treatment to decrease liver fat of -0.97 (95%CI -1.35, -0.58; $p < 0.0001$). With a sigma of 0.3, a pooled effect size of -0.97 represented a -30% reduction in liver fat percentage with omega-3 fatty acid treatment. Assuming a sigma of 0.3, and an alpha of 0.05, as well as allowing for a 15% drop-out rate, the estimated sample size of 50 participants in each group provided 99% power to detect a 30% change in liver fat percentage (two-tailed test). There would be 86% power to detect the more conservative estimate of a 20% difference in liver fat with omega-3 fatty acid treatment.

The exact change in liver fibrosis score that equated to a clinically meaningful change was uncertain, but it was assumed that a 0.6-1.0 unit change in fibrosis score might be clinically significant.[241] To detect a 1.0 unit change, a total of 32 subjects would be required, with 80% power at the 5% significance level. To detect a 0.6 unit change, a total of 100 subjects would be required, with 80% power at the 5% significance level. Since we speculated that an ideal change in fibrosis score would likely be between 0.6 and 1.0, we estimated that 100 subjects should be recruited, allowing for a 15% drop-out of subjects from the study. A sample size calculation was not undertaken for the NAFLD fibrosis score.[242]

2.8.2 Secondary outcomes

For the subgroup of 24 subjects undergoing hyperinsulinaemic-euglycaemic clamp measurements, there were no prior studies of n-3 PUFA treatment on hepatic insulin sensitivity in NAFLD at the time we undertook this research, but a previous study had shown that a 6-week modest exercise intervention improved endogenous glucose production (EGP) by 30% in overweight people.[246] As

such, it was estimated that a study sample size of 12 subjects in each arm provided 86% power to detect a more modest 25% difference in hepatic insulin sensitivity between treatment groups assuming similar SDs for mean EGP.

At the time of our study, there was also no published literature on the effects n-3 PUFAs on subclinical measures of LV function (e.g. diastolic function) in NAFLD. One study testing the effects of n-3 PUFAs in people with dilated cardiomyopathy showed an approximate 10% improvement in ejection fraction.[247] Based on these data and similar SDs in our study, we would have 92% power to detect a 10% difference associated with Omacor therapy for subclinical measures of LV function, although it must be stressed that the echocardiographic outcome measures in our study were different.

At the time of our study, there was also no published literature on the effects of n-3 PUFAs in reducing carotid IMT progression in NAFLD, although a systematic review of seven cross-sectional studies with a total of 3497 subjects found a significant association between NAFLD and carotid IMT, suggesting an estimated increase of 13% in carotid IMT for NAFLD cases compared to controls.[85] Prevalence of carotid plaque was also significantly more frequent in NAFLD subjects. Given that carotid IMT represents a well-validated screening tool for predicting CV disease in asymptomatic subjects and that NAFLD confers an increased CV risk, we felt it would have been useful to investigate the effect of n-3 PUFAs on carotid IMT change in NAFLD subjects after 15-18 months treatment.

2.9 Statistical analysis

Statistical analysis was carried out with SPSS version 23 (SPSS, Inc., Chicago, IL.). The normal distribution of the data was tested by the Shapiro-Wilk and Kolmogorov-Smirnov tests. Mean values and standard deviations were calculated for continuous variables, or median and interquartile range values for non-normally distributed variables. Univariate comparisons of normally distributed data were performed with independent Student's t-tests. Mann-Whitney U or Wilcoxon signed rank tests were used for non-parametric data and Chi-squared tests for binary data. Pearson and Spearman correlations were used for normal and non-normally distributed data respectively. Log transformation was undertaken for non-normal variables where necessary. Exposure variables which showed a significant univariate association with the outcome variable, as well as key baseline variables that might confound the association between exposures and the outcome of interest, were included in the multivariable stepwise regression model. A "difference" variable, which represented the arithmetic difference between the measurement at the end of the study minus the baseline measurement, was calculated for key exposures and potential

confounders (e.g. weight change). Between-group comparisons were made using ANCOVA with the baseline value as the covariate. For the primary outcome measures, both intention-to-treat (ITT) and per protocol analysis was undertaken. For ITT analysis, complete case analysis was undertaken with exclusion of cases with missing data, regardless of protocol violators or non-compliance with treatment. Per protocol analysis included all participants who consumed > 50% of their omega-3 supplement in the time period from randomization to final visit and had a baseline mean liver fat percentage > 5%. Per-protocol analysis was adopted in two out of the three prespecified substudies (carotid IMT and echocardiography sub-studies, excluding the much smaller clamp sub-study) because the intention of these prespecified studies was hypothesis-generating and to assess efficacy and mechanisms of change rather than effectiveness. All comparisons were two-sided and a p-value of < 0.05 was considered to be statistically significant. Due to the nature of the study, there was no interim analysis of the trial.

2.10 Baseline and end of study measurements

2.10.1 Biochemical and anthropometric measurements

A full medical history and physical examination was taken at the baseline visit. Blood samples were drawn from participants after an overnight fast (>12 hours) and glucose, urea & electrolytes, HbA1c, total cholesterol and its subfractions, triglycerides, platelets, ALT, AST and GGT were measured using commercially available kits according to manufacturers' instructions, after serum was separated within 1 hour of phlebotomy. Plasma and serum samples were also frozen at -70 ^{o}C for further analysis in batches (insulin and cytokeratin-18 (CK-18) subfraction M-65 measured by M65 EpiDeath enzyme-linked immunosorbent assay (ELISA) kit (PEVIVA, Bromma, Sweden)). TIMP-1 and HA were analysed using an ELISA kit Dynex DS2 platform. PIIINP assay was performed with a UniQ radiommunoassay kit supplied by Orion Diagnostica (Product no. 68570). For the primary outcome measure, two different algorithm scores for liver fibrosis were generated as previously stated. One was the liver fibrosis score, comprising an algorithm of HA, PIIINP and TIMP-1[241], while the other was the NAFLD fibrosis score algorithm based on age, hyperglycaemia (yes/no), BMI, platelet count, albumin concentration and AST/ALT ratio.[242] Homeostasis model assessment of insulin resistance (HOMA-IR) is a validated mathematical model used to quantify insulin resistance and is based on the formula: (Fasting glucose x fasting insulin) divided by 22.5, and it correlates well with estimates of insulin resistance derived from the euglycaemic clamp.[248]

Blood pressure was measured using a Marquette Dash 3000 monitor (GE Healthcare, Little Chalfont, Bucks, UK) on the non-dominant arm in the supine position after a minimum of 60 min rest. A mean of 3 measurements 5 min apart was calculated. BMI was calculated as weight (kg)

divided by the square of height (m). Metabolic syndrome was defined using the International Diabetes Federation criteria.[249]

2.10.2 DHA and EPA enrichment of erythrocytes

We measured DHA and EPA concentrations in erythrocytes at the beginning and at the end of the study to evaluate erythrocyte enrichment during the study in all participants. Enrichment was defined as the difference between end of study and baseline measurements. We were specifically interested in measuring enrichment in participants randomised to Omacor but we were also interested in checking that no enrichment occurred in the placebo group, to evaluate if there might be contamination from over-the-counter omega-3 supplements. Measurement of omega-3 fatty acids in erythrocytes is a validated proxy for liver tissue concentrations of omega-3 fatty acids.[250, 251] Measurement of omega-3 fatty acids in erythrocytes at baseline and end of study was also used as a measure to assess compliance with study allocation to Omacor in that arm of the trial.

To quantify the magnitude of tissue enrichment with omega-3 fatty acids due to the effects of Omacor treatment, erythrocyte fatty acids were analysed by gas chromatography at both baseline and upon completion of the trial period of intervention. Thawed packed red cells (1 ml) were mixed vigorously with 5 ml chloroform:methanol (2:1 vol/vol); butylated hydroxytoluene (50 mg/L) was included in the chloroform:methanol as an antioxidant. After centrifugation the organic phase that includes the extracted total lipid was collected. This was dried down under nitrogen at 40 °C and redissolved in 0.5 ml toluene. Fatty acid methyl esters (FAMEs) were formed by incubation of the entire lipid extract with 1 ml methanol containing 2% (vol/vol) H2SO4 at 50 °C for 2 h. After cooling, samples were neutralized by addition of 1 ml of a solution of 0.25 M KHCO3 and 0.5 M K2CO3. Then FAMEs were extracted into 1 ml hexane, dried down, redissolved in a small volume (150 µl) of hexane, and separated by gas chromatography. Gas chromatography was performed on a Hewlett Packard 6890 gas chromatograph fitted with a BPX-70 column (30 m × 0.22 mm × 0.25 µm). Inlet temperature was 300 °C. Oven temperature was initially 115 °C and this was maintained for 2 min post-injection. Then the oven temperature was programmed to increase to 200 °C at the rate of 10 °C/min, to hold at 200 °C for 16 min, and then to increase to 240 °C at the rate of 60 °C/min and then to hold at 240 °C for 2 min. The total run time was 37 min. Helium was used as the carrier gas. FAMEs were detected by a flame ionization detector held at a temperature of 300 °C. The instrument was controlled by, and data were collected using, HPChemStation (Hewlett Packard). FAMEs were identified by comparison of retention times with those of authentic standards run previously. Intra-assay coefficient of

variance CVs for EPA, DPA and DHA were 3.0%, 1.0% and 2.0% respectively. Inter-assay CVs for EPA, DPA and DHA were 5.0%, 6.1% and 2.2% respectively.

2.10.3 Dual energy X-ray absorptiometry (DEXA)

DEXA scan to assess body fat percentage was undertaken on a Delphi W instrument (Hologic, Bedford, MA, USA) using a standard visual method to divide images into trunk, limb and head. Subjects were weighed and measured on the day of the assessment and total fat, regional fat and lean mass were calculated. The investigation was carried out by a qualified technician from the bone densitometry department where the machine was located.

2.10.4 Magnetic resonance imaging (MRI)

MR imaging and spectroscopy were undertaken and evaluated by a qualified MRI radiographer who was blinded to study data or treatment allocation. This was performed at the Cardiac MRI department at Southampton Hospital. MRI was used to accurately evaluate visceral fat. MRI images were acquired from five non-contiguous slices of the abdomen, extending from 5 cm below to 15 cm above L4–L5, to obtain a more accurate estimation of visceral fat than from a single slice. Axial scans were acquired with participants in the supine position. Participants were scanned on a 1.5 T MR scanner (Siemens Avanto, Syngo software release B17; Siemens AG, Munich, Germany) using a 32-channel body coil. A gradient echo 2D FLASH (fast low angle shot) sequence (TR = 111 ms, TE = 4.18 ms, flip angle = 70°, slice width = 10 mm, slice spacing = 50 mm) was used to obtain T1-weighted images. In order to accommodate the circumference of the individual being scanned within the image, the field of view was varied. The MR images were analysed using a proprietary software package (Mimics 14.0; Materialise NV, Leuven, Belgium) to identify regions of subcutaneous and visceral fat within the cross-sectional abdominal MR images. This package enabled identification of subcutaneous and visceral fat. By examining the histogram of pixel values present in the image, threshold levels could be set. Since fat pixels were the highest value pixels in the image, fat tissue could be identified from other tissue in the images. Some manual intervention was required when using this technique, as there was some variation in signal intensity across the image, which is often the case in large field-of-view MR images. Three different masks were created; one comprising the whole cross-section of the body, one containing the visceral fat region and one containing the subcutaneous fat region. It was possible to determine the number of pixels contained within each of these masks, and hence calculate the areas of subcutaneous fat and visceral fat, and compare them with the total cross-sectional area. Adipose tissue volume was converted to mass in kg using a density of 0.92 kg/l for adipose tissue.

2.10.5 Magnetic resonance spectroscopy (MRS) for liver fat percentage

Participants underwent MRS of the liver to measure the quantity of liver fat accumulated in three discrete liver zones, at baseline and follow-up. 20 × 20 × 20 mm3 spectroscopic volumes of interest (VOI) were positioned within segments 3 (inferior sub-segment of the lateral segment), 5 (inferior sub-segment of the anterior segment) and 8 (superior sub-segment of the anterior segment) of the liver, avoiding major blood vessels, intra-hepatic bile ducts, and the lateral margin of the liver. For the second visit scan, these VOI positions were copied from the first scan, to ensure consistency. A PRESS (point resolved spectroscopy) spin echo pulse sequence was used to acquire the spectroscopic data. The pulse sequence used a TR = 1500 ms, TE = 3 ms, flip angle = 90°, bandwidth = 1000 Hz, 8 averages and acquisition duration of 1024 data points, with no water suppression. The acquisition was obtained in a breath hold examination of 18 s. Spectra were post-processed using Siemens scanner software. This was a fully automated process and involved several steps, starting with filtering the data using a Hanning filter, zero-filling the data, baseline correction, phase correction and finally curve fitting was performed (with 4 iterations) to identify the water and lipid peaks. Values for the lipid and water peak integrals were produced for each VOI and recorded for each subject. The average of the 3 zones was calculated to give a mean liver fat percentage.

2.11 Carotid ultrasonography

Carotid intima-media thickness (IMT) is a well-validated screening tool for the prediction of CV disease in asymptomatic subjects.[76, 77] The carotid arteries were studied with a duplex scanner using a 7.5 MHz linear array transducer (Philips IE33, Koninklijke Philips N.V., Netherlands) with ECG monitoring. This was performed at the Cardiac Echocardiography Department at Southampton Hospital. Ultrasound parameters (dynamic range, depth range, power output and greyscale) for B-Mode carotid imaging were adjusted during image acquisition to optimize image quality. All scans were carried out according to a standardised protocol.[252] Briefly, subjects lay supine with the neck slightly rotated and a transverse scan was first performed as a screening measure and also to identify the carotid bifurcation. Longitudinal images of the near and far walls of the common, proximal portion of the internal and external carotid artery and the carotid bifurcation were examined. Multiple images of four cine-loop cycles of the carotid artery were recorded and stored digitally for subsequent off-line analysis using Philips Q-Lab version 8 software (Koninklijke Philips N.V., Netherlands). For each subject, a 10 mm plaque-free segment of IMT at the far wall of the common carotid artery immediately proximal to the carotid bulb was measured using QLAB automated software. An average of three different cardiac cycle measurements of IMT from each of the left and right common carotid arteries was calculated. The

presence of carotid plaque at the distal common carotid, carotid bulb and proximal internal carotid arteries was also recorded.[252] All measurements were performed and analysed off-line by a single trained operator blinded to subject treatment allocation. A random sample of 36 scans (baseline and end-of-study) underwent repeat analysis of CIMT values at a later date to test intraobserver reproducibility and the coefficient of variation was 2.8%, which was within the limit of 6% recommended by the American Society of Echocardiography.[252]

2.12 Echocardiography

Transthoracic echocardiography was performed at the Cardiac Echocardiography Department at Southampton Hospital using the Philips iE33 ultrasound system and 2.5 MHz transducers. Standard parasternal and apical views of the heart were acquired with 3-lead ECG monitoring with the participant in the semi-recumbent, left lateral position. Complete 2D and M-mode echo-cardiogram, conventional Doppler, and tissue Doppler imaging (TDI) were obtained for all study participants according to the American Society of Echocardiography guidelines.[253, 254] Standard 2D measurements (left ventricular (LV) end-diastolic and end-systolic dimensions (mm), septal and posterior wall thickness at end-diastole (mm)) were determined. LV ejection fraction (%) was calculated using biplane modified Simpson's method.[255] LV mass was calculated using the formula proposed by Levy *et al.*[256] and normalized for body surface area (LV mass index, g/m^2). Left atrial (LA) dimension (mm) was measured in the 2D parasternal view, and LA volume (ml/m^2) was measured using the area-length method in the apical view and normalized for body surface area. Transmitral flow velocities were obtained by pulsed-wave Doppler echocardiography, positioning a 3 mm sample volume at the level of the mitral leaflets tip in an apical four-chamber view. Mitral flow parameters, including peak velocities at early diastole (E) and late diastole (A) and E-wave deceleration time were measured and E/A ratio was calculated.

Tissue Doppler measurements of the mitral annulus LV insertion points from the apical four-chamber view were recorded. The sampling window was positioned as parallel as possible to the mitral annulus longitudinal plane of motion to ensure optimal angle of imaging. Pulsed-wave tissue Doppler imaging was performed by placing a 5 mm sample volume separately at the septal and lateral mitral annulus in the apical four-chamber view, and peak myocardial systolic, early diastolic and late diastolic velocities (s', e', and a' respectively) were measured at end-expiration with the optimal velocity scale setting at sweep speeds of 50 – 100 mm/s. All Doppler and tissue Doppler measurements reflected the average of three cardiac cycles. The ratio of mitral to myocardial early diastolic peak velocity (E/e') was calculated, after averaging the mean of the e' medial and e' lateral annulus measurements.[257] E/e' can be used to predict LV filling pressures[258]

and E/e' is a good prognostic indicator of survival in established cardiac disease,[259] as well as an independent predictor of primary CV events.[260]

All measurements were performed and analysed off-line by a single trained operator blinded to subject treatment allocation. Tissue Doppler data was excluded if the angle between the scan line and LV wall was more than 20 degrees in order to preclude angle-dependency of tissue Doppler parameters.[257] Tissue Doppler measurements were done at the peak of the upper edge of the solid Doppler curve, with scale optimised and low gain setting. A random sample of 22 scans (baseline and end-of-study) underwent repeat analysis at a later date to test intraobserver reproducibility and the coefficient of variation for tissue Doppler variables (e.g. E/e') was between 3.0% and 6.5%, which was in keeping with other published studies.[259-261]

2.13 Hepatic and peripheral insulin sensitivity

A subgroup of twenty-four subjects from the main study who were randomly allocated to the substudy underwent further detailed investigation of hepatic and peripheral insulin sensitivity. In essence, participants who had consented and enrolled into the main study were also invited to take part in the substudy prior to randomisation. The extra exclusion criteria for the substudy was i) subjects who were on insulin therapy or who had poorly controlled diabetes. Out of the first twenty-eight consecutive participants that had enrolled into the main study and who did not meet exclusion criteria, twenty-five participants gave further consent to the substudy. Those who had declined consent did so for personal reasons as taking part in the substudy necessitated extra visits to the research facility and would be time-consuming. One further participant withdrew his consent for the substudy, again due to personal time constraints (Figure 5-1).

Insulin sensitivity was measured using a two-step hyperinsulinaemic–euglycaemic clamp technique with deuterated glucose infusion in the sub-group of 24 subjects from the main study. Hepatic insulin sensitivity was determined by insulin-mediated suppression of endogenous glucose production at a low-dose insulin infusion, while peripheral insulin sensitivity was determined by whole-body glucose disposal at a high-dose insulin infusion.[262, 263]

Subjects attend the Clinical Research Facility at 08:30 hours, having abstained from vigorous exercise for 48 h and having fasted for a minimum of 12 hours prior. They were weighed on arrival. Intravenous cannulae were inserted into both antecubital fossae for blood sampling and infusion of stable isotopes, insulin and glucose. Baseline unenriched blood samples were taken and then a 170mg bolus of deuterated [6,6-$^{2}H_{2}$] glucose was administered prior to commencing a infusion of deuterium-labelled glucose. The infusion was 765mg (7.65 ml) deuterated glucose made up with 42.35 ml of normal saline and then given as a continuous infusion at 6.7 ml/h

(1.7mg/min deuterated glucose). The infusion was continued for 120 min to reach a steady-state and blood samples were taken from the contralateral arm at 100, 110, 115, and 120 mins. These were stored in ice immediately and then centrifuged at 2000G at 4°C. Flouride and heparinised blood samples were subsequently stored at -70°C for later analysis of deuterium enrichment and insulin respectively.

At 120 min, the first step of the hyperinsulinaemic-euglycaemic clamp commenced. Weight-related low-dose insulin was infused in a primed continuous fashion (0.3mU/kg/min) made up to 48mls of normal saline (and 2 mls of patient's own blood to prevent insulin adhering to the syringe) at a continuous infusion rate of 2.5 ml/h for the next 120 mins. This step was to measure insulin sensitivity of hepatic glucose production (HGP). The deuterated glucose infusion continued throughout the clamp study. At 240 min, high-dose insulin was subsequently infused at a constant rate (1.0mU/kg/min, 8.4ml infusion/hr). This second step of the clamp study was to measure insulin sensitivity of peripheral glucose uptake. Euglycaemia was maintained throughout the clamp by adjusting a 20% glucose infusion spiked with deuterated glucose (8mg [6,6-$^{2}H_{2}$] glucose per 1 g dextrose for low-dose insulin step, then 10mg [6,6-$^{2}H_{2}$] glucose per 1 g dextrose for high-dose insulin step) infused at a variable rate, according to 5 min plasma glucose measurements. These plasma glucose measurements were performed every 5 mins from 100 mins of the study using a glucose oxidase method (Yellow Springs Analyser, USA). Blood samples for glucose, isotope enrichment and insulin levels were taken at 30 min intervals until the final 30 min of each stage (210 – 240 min for low-dose, 330 – 360 min for high-dose) when samples were taken at intervals of 10 min for steady-state calculations. The M value of peripheral insulin sensitivity was defined as the glucose infusion rate (mg/kg/min) during the final 30 mins of the clamp study.[262]

The isotopic enrichment of glucose was measured by gas chromatography-mass spectrometry on a HP 5971A MSD (Agilent Technologies, Berkshire, UK) by a qualified laboratory scientist. Plasma glucose concentration and enrichment time-courses were smoothed using optimal segments analysis.[264] Tracer to tracee ratios (TTR) were calculated as the ratio of the two areas from the mass spectrometry data. A modified version of the equations formulated by Steele[265] was used to calculate total rate of appearance of glucose (Ra; μmol/kg/min), endogenous glucose production (EGP; μmol/kg/min) and rate of disappearance or uptake of glucose (Rd; μmol/kg/min) adjusted to fat-free mass. For the Steele equations, 65% was used as the effective fraction and 0.22 l/kg as the distribution volume of glucose to calculate Ra and Rd.[263, 266] EGP was calculated at steady state basally (90–120 min) and following low-dose insulin (210–240 min), corrected for fat-free mass and (since EGP is inversely related to insulin) multiplied by mean steady-state insulin (pmol/ml) at low-dose. Glucose Rd was calculated at steady state following high-dose insulin (330–360 min) and metabolic clearance rate (MCR; ml/kg/min) was calculated at basal and high-dose insulin

steady state (330–360 min) as glucose Rd/glucose. Glucose MCR and Rd were corrected for fat-free mass and (since they are directly related to insulin) divided by mean steady-state insulin (pmol/ml) at basal and high dose. Adipose tissue IR (Adipose-IR) was calculated as fasting NEFA multiplied by fasting insulin.[267] We also measured a validated index of hepatic insulin sensitivity by dividing the basal EGP rates by the basal fasting insulin concentration.[268] All measurements and calculations were done in collaboration with colleagues from the Diabetes and Metabolic Medicine Department at the University of Surrey, Guildford, UK.

Chapter 3: Carotid intima-media thickness: association with NAFLD and effects of n-3 fatty acids treatment

3.1 Preamble: Summary of primary outcomes from main WELCOME trial

It would be prudent to briefly discuss the primary outcome results from our group's main trial[269] first before reporting the secondary outcomes in this and the subsequent chapters, as changes to liver fat in the cohort were a central theme in all secondary outcomes, as was the effect of n-3 fatty acid supplementation.

To reiterate the WELCOME trial briefly (see methods chapter for more detailed information), this was a randomised, double-blind, placebo-controlled study to investigate the effects of 15-18 months treatment with 4g Omacor (DHA and EPA) versus placebo on reduction of liver fat percentage and improvement of two liver fibrosis marker scores, in 103 subjects with NAFLD.[269] Liver fat percentage was assessed by magnetic resonance spectroscopy (MRS) at baseline and end of study, and liver fibrosis was measured using two validated algorithm-based scores. Adherence to the intervention (Omacor) group and contamination in the placebo group (with DHA/EPA) was tested by measuring erythrocyte percentage DHA and EPA enrichment with gas chromatography, which is a validated proxy for liver tissue concentrations of omega-3 fatty acids.[250, 251] It was felt that Omacor treatment should produce a minimum 2% increase in erythrocyte DHA and a minimum 0.7% increase in erythrocyte EPA to produce an effect.[270, 271]

51 subjects were randomised to the Omacor group and 52 subjects to the placebo group. 8 subjects withdrew during the study and did not complete end of study measurements for various personal reasons unrelated to any study-related adverse events. Inadequate MRS data were obtained in 4 subjects, which left 91 subjects for the primary outcome analysis. From capsule counts, all subjects consumed >50% of their medications and 78% consumed >75%. No medication-related serious adverse events occurred. Interestingly, when assessing DHA and EPA enrichment of erythrocytes (relative to baseline) in intervention and placebo groups to evaluate compliance and contamination, respectively, it was found that 5 and 6 subjects in the Omacor group did not reach the prespecified threshold for EPA and DHA enrichment, respectively. In the placebo group, in which no enrichment should have occurred, 3 and 4 subjects reached the prespecified thresholds for EPA and DHA enrichment, respectively. This would suggest that these participants in the placebo group may have been taking over-the-counter fish oil supplements.

This would certainly underestimate the effect of the intervention versus placebo in the intention-to-treat (ITT) analysis.

In the Omacor group, liver fat percentage decreased from 23% (IQR 19.3%) to 16.3% (IQR 22.0%) from baseline to end of study measurement, respectively, compared to 21.7% (IQR 19.3%) to 19.7% (IQR 18.0%) in the placebo group. The difference between the two groups was not statistically significant ($p = 0.48$). In the fully adjusted model taking into account age, sex, weight change, change in CK-18 levels as well as baseline liver fat percentage, there was a 3.64% relative decrease in liver fat percentage ($\beta = -3.64$; 95% CI: -8.0, 0.8; $p = 0.1$) with Omacor treatment. However, when secondary analysis was undertaken to evaluate the effect of DHA and EPA enrichment on liver fat reduction, erythrocyte DHA enrichment was independently associated with a decrease in liver fat percentage (-1.7% for each 1% DHA enrichment; $\beta = -1.7$; 95% CI: -2.9, -0.5; $p = 0.007$ in the fully adjusted model as above). However, erythrocyte EPA enrichment was not significantly associated with liver fat reduction ($\beta = -1.0$; 95% CI: -2.7, 0.6; $p = 0.20$). When the liver fibrosis marker score outcomes were analysed, it was also noted that in the entire cohort, there were only 9 subjects with high NAFLD fibrosis score[242] and 14 with high liver fibrosis score[241] at baseline. There was no improvement in either the NAFLD or liver fibrosis scores with Omacor treatment versus placebo, or with DHA or EPA enrichment.[269]

This was the first randomised, double-blind, placebo-controlled study to test the efficacy of a high-dose DHA + EPA intervention on liver fat percentage in NAFLD and also relate changes in erythrocyte DHA + EPA enrichment to changes in liver fat percentage. Although there was no significant effect of Omacor on the primary outcomes in the ITT analyses, this study showed that in secondary analyses, erythrocyte DHA enrichment with Omacor was linearly associated with decreased liver fat percentage, and each 1% DHA enrichment was associated with a 3.3% reduction in liver fat percentage ($\beta = -3.3$; 95% CI: -4.8, -1.8; $p < 0.001$). This secondary analysis was important as there was likely poor compliance and contamination issues seen in the study with Omacor and placebo, respectively, so it was necessary to explore further the physiological relationship between DHA enrichment and liver fat percentage reduction, rather than just from an ITT analysis. This compliance/contamination issue affected 11% of the cohort and would have therefore biased the ITT analysis result toward the null, attenuating any effect of the DHA + EPA intervention on the primary outcomes. In fact, a subsequent smaller RCT in a paediatric cohort with NAFLD reported that DHA supplementation significantly reduced hepatic fat by 53% assessed by MRS, versus 23% in the placebo group ($p = 0.04$).[235] Measurement of DHA enrichment in that study showed excellent compliance in the treatment group and little contamination in the placebo group, which further supports our secondary analysis of DHA enrichment-based outcomes.

In terms of the liver fibrosis marker outcomes, it was unsurprising that the fibrosis scores were unchanged by the intervention, given the small numbers of subjects with high scores at baseline, suggesting a lower risk NAFLD cohort under investigation. A very recent meta-analysis also showed that there is currently no evidence that n-3 fatty acid treatment improves NASH or liver fibrosis.[215] The main limitation of this study was the small sample size which would have amplified the compliance/contamination issues. However, this study does suggest that substantial decreases in liver fat percentage can be achieved with high levels of erythrocyte DHA enrichment in patients with NAFLD. Further larger randomised studies are necessary to investigate these findings as high-dose n-3 fatty acids represent a potentially viable and safe pharmacological treatment option in NAFLD.

3.2 Introduction

Non-alcoholic fatty liver disease (NAFLD) is a common condition associated with obesity and type 2 diabetes, and is prevalent in up to a third of the general population.[2] Current evidence demonstrates a robust association between NAFLD and atherosclerotic disease such that, for example, cardiovascular (CV) mortality represents the main mode of death in NAFLD and is also linked to the severity of liver disease.[272] This progressive disease spectrum ranges from simple hepatic steatosis to varying grades of non-alcoholic steatohepatitis (NASH) and ultimately cirrhosis.[7] Carotid intima-media thickness (IMT) is a well-established marker of subclinical atherosclerosis and is predictive of subsequent CV events in asymptomatic subjects.[76] Measurement of carotid IMT progression is commonly used in intervention trials as a surrogate end-point for adverse clinical events, as well as evaluating efficacy of specific atherosclerotic-modifying therapies.[273] Importantly, carotid IMT offers good inter-scan as well as good inter- and intra-observer reproducibility with intra-class correlation coefficients greater than 0.90 in previous trials utilising carotid IMT progression as a primary end-point.[274] Although NAFLD is described as an independent risk factor for increased carotid IMT,[51] no studies to date have investigated whether a reduction in the degree of fatty liver disease is associated with modification of carotid IMT progression. There are currently no established pharmacological treatments for NAFLD.[193] However, in the WELCOME trial, we found a significant reduction in liver fat percentage after 18 months treatment with high-dose n-3 fatty acids i.e. DHA+EPA (Omacor/Lovaza 4 g/day) versus placebo, in subjects who showed a high level (>2%) of erythrocyte DHA enrichment.[269] Furthermore, studies from other groups have shown a positive association between high dietary intake of n-3 fatty acids and less carotid atherosclerotic plaque burden,[275] as well as a plaque-stabilizing effect on carotid atheroma and improved arterial elasticity with n-3 fatty acid treatment.[276, 277]

The aims of this prespecified substudy of the WELCOME trial[278] were: (i) to investigate the effects of high-dose n-3 fatty acids treatment over 18 months on carotid IMT progression in NAFLD subjects and (ii) to describe associations between changes in markers of NAFLD disease severity (both in terms of simple steatosis and steatohepatitis) and carotid IMT progression over that time period.

3.3 Methods

3.3.1 Subjects & Study design

This study included participants from the WELCOME study, which was a phase IV randomised, double-blind, placebo-controlled clinical trial undertaken in asymptomatic patients with NAFLD testing the effects of 15-18 months treatment with high-dose (3.6 g/day) purified ω-3 polyunsaturated fatty acids (PUFA) comprising DHA (46%) and EPA (38%) (Omacor 4 g/day; Pronova Biopharma ASA, Lysaker, Norway; Abbott Laboratories, Southampton, UK) on improving liver fat and/or fibrosis markers as its primary outcome (www.clinicaltrials.gov NCT00760513). The design and rationale for the WELCOME study have already been described in Chapter 2. Briefly, subjects were included if they had recent imaging evidence of liver fat (ultrasound, magnetic resonance imaging or computerised tomography scan) and/or histological confirmation of NAFLD as well as exclusion of all other liver conditions causing liver fat accumulation such as excess alcohol intake or evidence of cirrhosis. The study was approved by the Southampton and South West Hampshire NHS Ethics Committee and all participants gave their written informed consent.

103 participants were randomised to either Omacor 4g/day (n=51) or placebo (olive oil capsules, n=52) in a 1:1 double-blind fashion, with treatment duration of between 15-18 months. Compliance with the allocated medication was monitored by recording returned capsules at fixed intervals during the study. We also assessed erythrocyte EPA and DHA enrichment (between baseline and end of study) by gas chromatography[278] to test adherence to the intervention in the DHA+EPA group and to monitor dietary contamination with DHA and EPA in the placebo group. Dietary and lifestyle changes had already been recommended to all participants as part of their routine clinical care[4] and this was continued throughout the study. There were no additional specific weight-loss programs or strict dietary restrictions placed on the participants as part of the study.

3.3.2 Laboratory and anthropometry measurements

Blood samples were drawn after an overnight fast (>12 hours) and serum was separated within 1 hour to undergo routine biochemical assay by conventional enzymatic methods (lipids, glucose, liver transaminases). Plasma and serum samples were also frozen at -70°C for further analysis in batches (insulin and cytokeratin-18 (CK-18) subfraction M65 measured by M65 EpiDeath enzyme-linked immunosorbent assay kit (PEVIVA, Bromma, Sweden). CK-18 is a major intermediate filament protein in hepatocytes, which is released into the extracellular compartment following epithelial cell death. M65 levels not only reflect hepatocellular apoptosis and necrosis consistent with changes seen in NASH with an AUROC of 0.82,[29] but they have also been shown to correlate with fibrosis progression.[279]

Blood pressure was measured using a Marquette Dash 3000 monitor (GE Healthcare, Bucks, UK) on the non-dominant arm in the supine position after a minimum of 60 minutes rest and a mean of 3 measurements 5 minutes apart was taken. Body mass index (BMI) was calculated as weight (kg) divided by the square of height (m).

3.3.3 Liver fat assessment

Magnetic resonance spectroscopy (MRS) is a quick and safe, non-invasive tool to accurately quantify liver fat in NAFLD and correlates well with liver biopsy results. Due to its high sensitivity, it can detect small percentage differences in liver fat accumulation in NAFLD.[280] The full methodology of our MRS technique has already been described in Chapter 2 Methods section. Briefly, subjects had liver MRS scans at the start and end of the study. Three 20 x 20 x 20mm^3 spectroscopic volumes of interest (VOI) were positioned in three standard areas of the liver and the average of each VOI's lipid spectroscopic peak was used to calculate the percentage liver fat. VOIs remained constant for end of study measurements.

3.3.4 Carotid ultrasonography

The carotid arteries were studied with a duplex scanner using a 7.5 MHz linear array transducer (Philips IE33, Koninklijke Philips N.V., Netherlands) with ECG monitoring. Ultrasound parameters (dynamic range, depth range, power output and greyscale) for B-Mode carotid imaging were adjusted during image acquisition to optimize image quality. All scans were carried out according to a standardized protocol.[252] Briefly, subjects lay supine with the neck slightly rotated and a transverse scan was first performed as a screening measure and also to identify the carotid bifurcation. Longitudinal images of the near and far walls of the common, proximal portion of the internal and external carotid arteries and the carotid bifurcation were examined and multiple

images of 4 cine-loop cycles of the carotid artery were recorded and stored digitally for subsequent off-line analysis using Philips Q-Lab version 8 software (Koninklijke Philips N.V., Netherlands). For each subject, a 10 mm plaque-free segment of IMT at the far wall of the common carotid artery immediately proximal to the carotid bulb was measured at ECG-gated end-diastole using QLAB automated software. An average of three different cardiac cycle measurements of IMT from each of the left and right common carotid arteries was calculated. The presence of carotid plaque at the distal common carotid, carotid bulb and proximal internal carotid arteries was also recorded.[252] All measurements were performed and analysed off-line by a single trained operator blinded to subject treatment allocation. A random sample of 36 scans (baseline and end-of-study) underwent repeat analysis at a later date to test intraobserver reproducibility and the coefficient of variation was 2.8%, which is within the limit of 6% recommended by the American Society of Echocardiography.[252]

3.3.5 Statistical analysis

Statistical analysis was carried out with SPSS Version 23 (SPSS, Inc., Chicago, IL.). Mean values and standard deviations were calculated for continuous variables, or median and interquartile range values for non-normally distributed variables. Univariate comparisons of normally distributed data were performed with independent Student's t-tests. Mann-Whitney U or Wilcoxon signed rank tests were used for non-parametric data and Chi-squared tests for binary data. Pearson and Spearman correlations were used for normal and non-normally distributed data respectively. Log transformation was undertaken for non-normal variables where necessary. Exposure variables which showed a significant univariate association with the outcome variable, as well as key baseline variables that might confound the association between exposures and the outcome of interest were included in the multivariable stepwise regression model. A "difference" variable, which represented the arithmetic difference between the measurement at the end of the study minus the baseline measurement, was calculated for key exposures and potential confounders. All comparisons were two-sided and a p-value of < 0.05 was considered to be statistically significant.

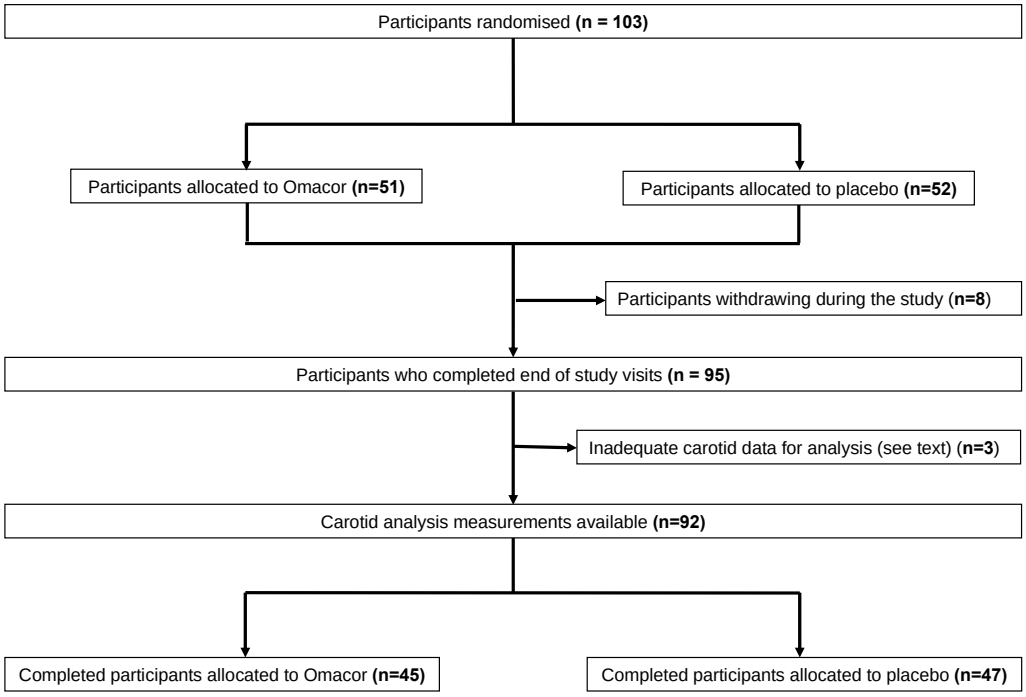

Figure 3-1 Consort diagram for the WELCOME sub-study and the numbers of subjects available for carotid analysis to test the effects of the intervention

3.4 Results

Data from 47 participants randomized to placebo and 45 randomized to DHA+EPA treatment was available for analysis out of n = 103 (Figure 3-1). Eight participants did not complete the study due to personal reasons (unrelated to study medication) comprising mainly time constraints. A further three were excluded from the carotid analysis due to inadequate carotid data (one subject had images corrupted during transfer offline) or unsuitable images for adequate analysis (one had poor imaging quality and another had untreated familial hyperlipidaemia with significant widespread carotid plaque disease). From capsule counts at 6, 12 and 18 months, we estimated that all participants consumed >50% of their study medication and 78% consumed >75%. There were no serious adverse events attributable to study drugs. All participants consumed less than 21 units of alcohol per week at randomization apart from 1 volunteer who drank 25 units/week, with no significant difference between groups (p=0.33). Alcohol consumption was also not associated with baseline liver fat (p=0.45) and did not vary significantly for each subject at the end of the study (p=0.92). The baseline characteristics of the participants according to randomization group are shown in Table 3-1.

Table 3-1 Baseline characteristics of participants in placebo and DHA+EPA groups at randomization

	Placebo (n=47)	**DHA + EPA (n=45)**	**p-value**
Age (years)	54.2 ± 9.4	48.6 ± 11.2	0.09
Male/Female	31/16	22/23	0.10
Diabetes (%)	34.0	35.6	0.88
Dietary control (%)	4.3	2.1	0.56
Oral anti-diabetic (%)	19.1	26.7	0.51
Insulin use (%)	10.6	6.7	0.48
Hypertension (%)	48.9	53.3	0.67
Smoker (%)	10.6	11.1	0.94
Ex-smoker (%)	36.2	31.1	0.59
Antihypertensive use (%)	36.2	40.0	0.71
Statin use (%)	46.8	40.0	0.51
Total cholesterol (mmol/L)	4.72 ± 1.10	5.05 ± 1.19	0.17
Low density lipoprotein cholesterol (mmol/L)	2.77 ± 0.83	2.99 ± 0.99	0.29
High density lipoprotein cholesterol (mmol/L)	1.11 ± 0.26	1.02 ± 0.27	0.12
Serum triglycerides (mmol/L)	1.4 (1.1, 2.0)	1.8 (1.4, 2.6)	0.03
Fasting glucose (mmol/L)	5.4 (5.1, 6.9)	5.5 (4.9, 6.2)	0.37
Haemoglobin A1c (% total Hb)	6.1 (5.7, 7.3)	5.9 (5.5, 6.7)	0.29
Fasting insulin (µU/ml)	11.5 (8.0, 21.1)	13.9 (7.8, 19.4)	0.90
Alanine transaminase (iu/L)	56 (40, 73)	53 (30, 72)	0.38
Aspartate aminotransferase (iu/L)	41 (31, 54)	37 (27, 49)	0.15
Weight (kg)	93.1 ± 14.6	98.7 ± 17.7	0.10
Body mass index (kg/m^2)	31.7 (28.7, 33.7)	32.8 (30.6, 37.4)	0.03
Systolic blood pressure (mmHg)	138 ± 16	138 ± 17	0.86
Diastolic blood pressure (mmHg)	85 ± 8	85 ± 12	0.89
CK-18 M65 (U/L)	605 (267, 792)	388 (245, 785)	0.28
MRS Liver fat (%)	22.8 (13.7, 32.3)	23.3 (12.7, 47.5)	0.75

	Placebo (n=47)	**DHA + EPA (n=45)**	**p-value**
Carotid IMT (mm)	0.674 ± 0.098	0.649 ± 0.095	0.21
Presence of any carotid plaque (%)	68.1	55.6	0.22
Erythrocyte EPA (20:5n3) (%)	0.95 ± 0.39	0.89 ± 0.36	0.44
Erythrocyte DHA (22:6n3) (%)	4.21 ± 1.49	3.87 ± 1.29	0.24
Data are means ± standard deviation (SD), or median (25^{th}, 75^{th} percentiles). Abbreviations: DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; CK-18 M65, cytokeratin-18 M65 subfraction; MRS, magnetic resonance spectroscopy; IMT, intima-media thickness.			

Baseline characteristics for the treatment and placebo groups were very similar, apart from the treatment group having higher baseline fasting serum triglyceride concentration (1.8 vs 1.4 mmol/L, p = 0.03), and a higher baseline BMI (32.8 vs 31.7, p = 0.03) compared to the control group. Liver fat, CK-18 and carotid IMT measurements were not significantly different between groups at baseline (Table 3-1), with mean carotid IMT of 0.674mm in the placebo group and 0.649mm in the treatment group (p=0.21). There was no significant change between baseline and end-of-study statin and anti-hypertensive use between the two groups (data not shown).

After 15-18 months of DHA+EPA (Omacor) treatment, carotid IMT in the Omacor group progressed by 0.012mm (0.005, 0.020mm) or 1.7% (0.6, 3.2%), compared to 0.015mm (0.007, 0.025mm) or 2.4% (0.9, 3.8%) in the placebo group (p=0.17). Furthermore, even in subjects with a > 2% absolute erythrocyte DHA enrichment between baseline and end of study which has been shown to be significantly associated with liver fat reduction,[269] there was no significant difference between carotid IMT percentage progression between the > 2% DHA enrichment group (1.7% (0.6, 3.3%)) and the < 2% DHA enrichment group (2.3% (0.9, 3.8%) (p=0.14). Importantly, there was also no significant difference in weight change between treatment and placebo groups over the 18 months (treatment, 0.62 ± 4.68 kg vs placebo, -0.17 ± 4.53 kg, p=0.42).

We also examined changes (difference between end and baseline measurements) in all clinically relevant variables over the 18-month study period to evaluate univariate associations between these parameters and carotid IMT progression from baseline in the whole cohort. The correlation coefficients and p-values are presented in Table 3-2.

Table 3-2 Univariate associations between percentage carotid IMT progression and changes in relevant clinical variables between start and end of study (18 months) in the entire cohort

	Correlation coefficient	**p-value**
Systolic blood pressure difference (mmHg)	-0.12	0.26
Diastolic blood pressure difference (mmHg)	-0.04	0.69
Weight difference (kg)	0.30	0.004
Serum TG difference (mmol/L)	0.27	0.01
Serum cholesterol difference (mmol/L)	0.23	0.03
LDL-cholesterol difference (mmol/L)	0.14	0.26
HDL-cholesterol difference (mmol/L)	-0.06	0.55
HOMA-IR % difference	0.22	0.08
ALT difference (iu/L)	0.11	0.28
AST difference (iu/L)	0.10	0.35
EPA % change	-0.05	0.64
DHA % change	-0.16	0.14
CK-18 M65 difference (U/L)	0.27	0.009
Liver fat difference (%)	0.49	<0.001
Abbreviations: IMT, intima-media thickness; TG, triglycerides; LDL, low-density lipoprotein; HDL, high-density lipoprotein; HOMA-IR, homeostasis model assessment of insulin resistance; ALT, Alanine transaminase; AST, Aspartate aminotransferase; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; CK-18 M65, cytokeratin-18 M65 subfraction.		

Over the 18-month period, percentage carotid IMT progression was associated with an increase in weight among subjects (r=0.30, p=0.004), and inversely associated with differences in serum triglyceride (r=0.27, p=0.01) and total cholesterol concentrations (r=0.23, p=0.03). A decrease in liver fat percentage was strongly associated with reduced carotid IMT progression over 18 months (r=0.49, p<0.001) and change in CK-18 concentration was also positively correlated with carotid IMT progression (r=0.27, p=0.009) (Figure 3-2).

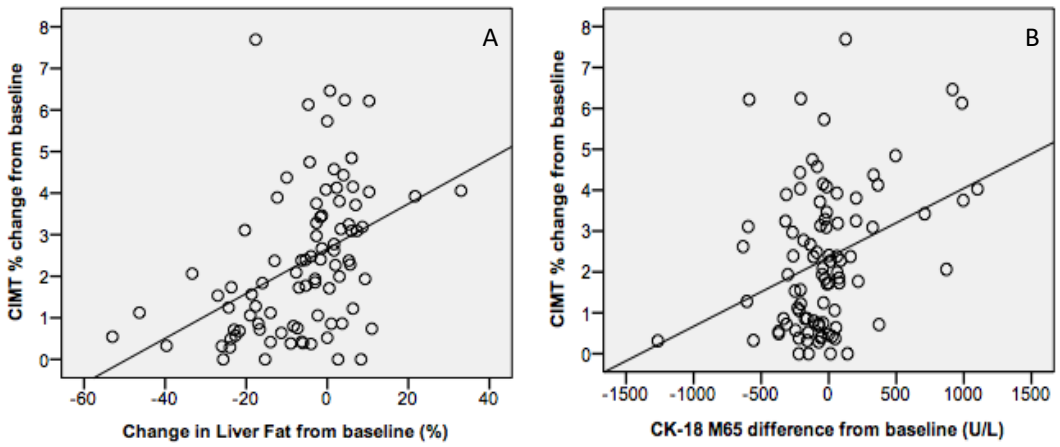

Figure 3-2 Scatter plot of relationship between carotid IMT percentage change and liver fat change (A) and CK-18 concentration change (B) between baseline and end of study in the entire cohort

When we also analysed the relationship between carotid IMT progression and the same clinical variables stratified by treatment group, we found similar associations. In the DHA+EPA group, reduced carotid IMT progression was significantly associated with liver fat decrease (r=0.52, p<0.001), CK-18 reduction (r=0.47, p=0.001), weight reduction (r=0.36, p=0.01) and decrease in cholesterol levels (r=0.34, p=0.03). In the placebo group, reduced carotid IMT progression was also significantly associated with liver fat decrease (r=0.43, p=0.004) and decrease in triglyceride levels (r=0.31, p=0.04), while decrease in weight only showed a trend toward reduced carotid IMT progression in the placebo group (p=0.06), as did reduced CK-18 levels (p=0.13).

In multivariable linear regression analyses of the entire cohort, after adjusting for age, sex, diabetes, smoking, BMI, triglyceride concentration, statin and antihypertensive usage at baseline, as well as changes in weight and cholesterol concentration between the start and end of study, the only independent predictors of reduced carotid IMT progression over time were decreased liver fat (standardized β-coefficient 0.32, p=0.005), reduced CK-18 levels (standardized β-coefficient 0.22, p=0.04), and use of antihypertensive drugs at baseline (standardized β-coefficient

-0.31, p=0.009). The overall model fit was R^2 = 0.39 (Table 3-3). Weight change was not independently associated with carotid IMT progression over 18 months in the multivariate regression model (p=0.50).

Table 3-3 Associations between key explanatory variables and percentage carotid IMT difference between start and end of study (18 months) in the entire cohort

Independent variables	**β-coefficient**	**Standardized β-coefficient**	**95% CI for β-coefficient**	**p-value**
Age	0.002	0.102	-0.003 to 0.008	0.39
Sex	0.004	0.009	-0.097 to 0.105	0.94
Diabetes	0.068	0.138	-0.036 to 0.173	0.20
Smoking	-0.002	-0.003	-0.143 to 0.139	0.98
BMI at baseline	<0.001	-0.011	-0.011 to 0.010	0.92
Statin use at baseline	0.011	0.022	-0.091 to 0.113	0.84
Antihypertensive use at baseline	-0.150	-0.305	-0.261 to -0.038	0.009
Weight difference (kg)	0.004	0.074	-0.008 to 0.016	0.50
Serum total cholesterol difference (mmol/L)	0.023	0.095	-0.027 to 0.074	0.37
Serum TG difference (mmol/L)	0.027	0.096	-0.035 to 0.088	0.39
Liver fat difference (%)	0.006	0.321	0.002 to 0.010	0.005
CK-18 M65 difference (U/L)	<0.001	0.221	<0.001 to <0.001	0.04
DHA % change	-0.006	-0.048	-0.038 to 0.026	0.70

Model fit R^2 = 0.39

Abbreviations: IMT, intima-media thickness; BMI, body mass index; TG, triglycerides; CK-18 M65, cytokeratin-18 M65 subfraction; DHA, docosahexaenoic acid

("Difference" or "change" variable represents the arithmetic difference between the measurement at the end of study minus the baseline measurement for the independent variables included in the regression model).

As already described for the WELCOME study primary outcomes,[269] liver fat percentage decreased in both the treatment and the placebo arms of the trial, between baseline and end of study. Although there was a greater reduction in liver fat percentage in the DHA+EPA group, this result was not statistically significant (Omacor, -8.1% ± 17.4% vs placebo, -4.5% ± 9.2% (p=0.23).

Consequently, relevant putative univariate predictors of liver fat reduction across the entire study cohort were evaluated and these are presented in Table 3-4.

Table 3-4 Univariate associations between percentage liver fat difference and changes in relevant putative aetiological variables between start and end of study (18 months) in the entire cohort

	Correlation coefficient	**p-value**
Systolic blood pressure difference (mmHg)	-0.20	0.86
Diastolic blood pressure difference (mmHg)	0.003	0.98
Weight difference (kg)	0.32	0.003
Serum TG difference (mmol/L)	0.21	0.06
Serum Cholesterol difference (mmol/L)	0.18	0.10
LDL-cholesterol difference (mmol/L)	0.12	0.34
HDL-cholesterol difference (mmol/L)	-0.11	0.29
HOMA-IR % difference	0.23	0.08
EPA % change	-0.09	0.41
DHA % change	-0.26	0.01
Abbreviations: TG, triglycerides; LDL, low-density lipoprotein; HDL, high-density lipoprotein; HOMA-IR, homeostasis model assessment of insulin resistance; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid.		

Percentage liver fat change over the 18 months was significantly associated with weight change (r=0.32, p=0.003) as well as DHA percentage change (r=-0.26, p=0.01), and showed a non-significant trend towards an association with change in fasting serum triglyceride concentration (r=0.21, p=0.06) and homeostasis model assessment of insulin resistance (HOMA-IR) percentage change (r=0.23, p=0.08). When we tested factors that were associated with percentage liver fat change in the entire cohort with multivariable linear regression modelling, after adjusting for age, sex, baseline percentage liver fat, change in weight and DHA percentage change, only change in weight (standardized β-coefficient 0.30, p<0.001) and DHA percentage change (standardized β-coefficient -0.19, p=0.027) were still independently associated with liver fat change. The overall model fit was R^2 = 0.48. Baseline percentage liver fat was also independently associated with liver fat change (standardized β-coefficient -0.53, p<0.001), which suggested that subjects with higher

amounts of liver fat would have a larger absolute reduction in liver fat with increasing DHA enrichment or decrease in weight.

3.5 Discussion

These results document a novel association between an improvement in NAFLD severity, both in terms of markers of simple steatosis and steatohepatitis, and attenuation of carotid IMT progression in a randomized double-blind, placebo-controlled trial.[269] Although increased n-3 fatty acid intake appears to be associated with reduced carotid atheroma burden in observational studies,[281] our study did not show that high-dose n-3 fatty acids over 18 months had a significant independent beneficial effect on carotid IMT progression in subjects with NAFLD, even in those with significant increases in erythrocyte DHA levels compared to baseline. This lack of an effect of n-3 fatty acid therapy is in contrast to the results of previous cross-sectional studies, which showed that high erythrocyte DHA (but not EPA) concentrations were associated with decreased carotid IMT and plaque burden.[233, 282] Our findings are instead, consistent with a recent randomized study by Lonn *et al*., which showed that a much lower daily dose of n-3 PUFAs (1g) given to subjects with varying degrees of insulin resistance, had no favourable effect on carotid IMT progression compared to placebo.[283] Consequently, although n-3 fatty acids have been reported to have several CV benefits including antithrombotic, anti-atherosclerotic and anti-inflammatory effects, as well as improvements in blood pressure and endothelial function,[284] randomised trials have so far failed to show consistent benefits of n-3 PUFAs over placebo in reducing CV outcomes.[209] One criticism of these studies has been that these trials tested too low doses of n-3 fatty acid treatment. However, despite our study dose of 4g/day being the highest licensed dose for n-3 fatty acid therapy, we did not find any benefit of n-3 fatty acids on carotid IMT progression compared to placebo.

A previous systematic review reported a significant association between NAFLD and carotid IMT, showing an estimated increase of 13% in carotid IMT for patients with liver fat, compared to controls without liver fat.[85] When we evaluated our entire cohort to investigate univariate associations between risk factors for CVD and carotid IMT progression over 18 months, we found that changes in weight, serum triglyceride and total cholesterol concentration all had significant positive correlations with increased carotid IMT progression. This result is perhaps not unexpected given the strong association between lipid levels and the metabolic syndrome with carotid disease.[285] However, and importantly, we also showed that a decrease in simple hepatic steatosis severity quantified by liver MRS as the gold-standard for non-invasive assessment of liver fat percentage, was independently associated with reduced carotid IMT progression over 18 months; even after adjusting for all measured confounding factors including standard CV risk

factors, weight change and relevant medication use (i.e. statins and antihypertensives), which are known to have an impact on carotid IMT progression.[286-289] Furthermore, we found that changes in CK-18 levels from baseline to the end of study were also independently associated with carotid IMT progression after adjusting for the same confounding factors as above. As CK-18 levels have been shown to correlate well with histological features of hepatic inflammation and fibrosis, including its use for monitoring disease progression in NASH,[27, 290, 291] our findings suggest that an improvement in steatohepatitis severity may also be independently associated with reduced carotid IMT progression. In a cross-sectional study, Targher et al. previously showed a progressive independent relationship between carotid IMT and increasing severity of NAFLD in 85 patients even after adjusting for classical CV risk factors and the metabolic syndrome.[80] Our prospective, randomized-controlled trial confirms as well as extends that finding, showing that increasing severity of NAFLD is independently associated with carotid IMT progression over 18 months.

Several observational and case-control studies have shown an increased incidence of adverse CV events in NAFLD subjects compared to the general population,[272] with a higher rate of CV-related mortality in NASH compared to simple hepatic steatosis.[8, 12] The aetiology of increased CV risk appears to be multifactorial, with NAFLD acting as a pathological marker of ectopic fat accumulation, insulin resistance and low-grade systemic inflammation. These factors result in multiple deranged pathophysiological processes including abnormal metabolism of glucose, fatty acids and lipoproteins, worsening subclinical inflammation, increased oxidative stress, hypercoaguability, endothelial dysfunction and progression of atherosclerosis.[285, 292] These observations appear to be consistent with our finding of an association between a reduction in markers of NAFLD severity and decreased carotid IMT progression. When we also evaluated the potential causes of improvement in liver fat using multivariable linear regression modelling, we found that percentage DHA enrichment (either through dietary contamination in the placebo arm or good compliance with n-3 fatty acids supplementation in the treatment arm) and weight loss during the trial in the entire cohort were independently associated with improvements in liver fat. Thus, we reason that both these factors contributed to improvements in liver fat during the study. Although weight loss in obesity has been shown to significantly attenuate carotid IMT progression,[293-295] we did not observe an independent association in our study after adjusting for relevant variables, including changes in NAFLD severity. Plausible explanations for this could be that our sample size was too small to detect a difference, or that weight changes were not large enough to observe a response (i.e. more than 5% body weight[295]).

There are limitations to our study. As this was a pre-specified sub-study of the WELCOME trial[278] with carotid IMT progression as a key secondary outcome of the trial, we did not undertake sample size or power calculations to determine the appropriate number of participants necessary

to test the effect of the intervention with respect to carotid IMT modification. Consequently, our study may have lacked sufficient power to prove that treatment with high dose DHA+EPA caused a decrease in carotid IMT progression. Furthermore, although we observed a significant independent association between two markers of NAFLD severity (liver fat percentage and CK-18 concentration) and carotid IMT progression, our study comprised relatively small numbers of participants, and we suggest that further larger studies are necessary to confirm these findings. Thirdly, we used CK-18 M65 subfraction as our non-invasive marker of steatohepatitis. Although it can be useful as a marker of NASH (see Section 1.2 for details), the gold-standard of quantifying liver inflammation and fibrosis would have been a liver biopsy. Due to concerns of the potential, albeit small, risk associated with serial liver biopsies, we did not consider invasive assessment of NALFD severity an appropriate component of our study methodology. Fourthly, the sensitivity of carotid IMT as a discriminatory measure is likely to vary widely among studies using different methodologies. However, we sought to minimize this by adhering to the recommended guidelines on the optimal measurement and reporting of carotid IMT studies.[252] As a comparison, an analysis of the placebo groups from several large randomized placebo-controlled trials showed that the overall weighted rate of change in mean carotid IMT was 0.0147 mm/year,[273] which is higher than our placebo group estimated change of 0.010 mm/year. However, our cohort represents a lower CV risk population compared to most previous trial data, of which the majority encompass secondary prevention CV disease cohorts and this could consequently explain the smaller annual progression rate in our study. Finally, it is possible that 18 months of high-dose PUFA therapy is inadequate to observe a significant biological effect on carotid IMT progression.

In conclusion, we have shown for the first time that an improvement in NAFLD severity over 18 months is associated with a beneficial effect on carotid IMT progression, a surrogate marker for cardiovascular outcomes.[76] However, we observed no significant effect of the n-3 PUFA intervention over 18 months on carotid IMT progression. Given that there is now increasing evidence that NAFLD portends a poorer CV outcome independent of several CV risk factors including the metabolic syndrome,[272] and that worsening grades of NAFLD also contribute to progressive cardiometabolic risk, we suggest that further larger prospective studies should be performed to confirm these findings including the evaluation of other biomarkers of increased CV risk or adverse CV outcomes in NAFLD patients. Although lifestyle changes such as weight loss, increased exercise and reducing dietary fat intake are the only universally recommended therapeutic strategies with proven benefit to reduce NAFLD severity,[4] and there are currently no established licensed pharmacological treatments for this disease, our findings of improved CIMT progression should encourage the ongoing trials of various therapeutic strategies, including active

lifestyle intervention, to reduce NAFLD severity,[195] which may then ultimately confer improved CV outcomes in the NAFLD population.

Chapter 4: Cardiac structure and function: association with NAFLD and effects of n-3 fatty acids treatment

4.1 Introduction

Non-alcoholic fatty liver disease (NAFLD) is the commonest cause of chronic liver disease and is defined by the presence of hepatic steatosis > 5% (determined either histologically or with radiological imaging) in the absence of other chronic liver disease or significant alcohol consumption.[193] The prevalence of NAFLD in the general population is estimated to be approximately 17 – 46% of adults, with differences according to the diagnostic method, age, sex and ethnicity of the population studied.[5] As the pathogenesis of the condition is closely linked to insulin resistance (IR), its prevalence parallels that of increasing rates of obesity and type 2 diabetes worldwide, with up to 95% of obese persons and 75% of diabetics likely to have NAFLD,[296] with most cases unrecognised. Given that the metabolic syndrome (MetS) is present in up to 88% of NAFLD patients, and all components of the MetS confer greater cardiovascular (CV) risk, it is unsurprising that CV mortality is the commonest cause of death in NAFLD patients, followed by cancer and then liver-related mortality.[8, 12] However, numerous studies have in fact shown that NAFLD represents an independent predictor of adverse CV events and mortality, even after adjusting for conventional cardiometabolic risk factors.[272]

NAFLD comprises several entities in its disease spectrum, with variable progression from simple hepatic steatosis to nonalcoholic steatohepatitis (NASH) which includes hepatocyte inflammation, ballooning and necrosis, to liver fibrosis, and ultimately cirrhosis and a potential for hepatocellular carcinoma.[1] CV risk appears to increase in parallel with worsening grades of NAFLD, with severe NASH and fibrosis portending the highest risk.[8, 18]

Echocardiographic evaluation with tissue Doppler imaging is a robust method of risk-stratifying asymptomatic subjects at increased CV risk, or even in the general population, independent of traditional CV risk factors.[260, 297] Different markers of diastolic dysfunction are known to be an independent predictor of all-cause mortality even in the presence of normal LV systolic function.[298] In addition, worsening of diastolic function over time appears to be independently associated with increased risk of mortality.[299, 300] Numerous cross-sectional and case-control studies on asymptomatic NAFLD subjects have reported abnormal LV geometry and diastolic dysfunction compared to controls.[93-98] There is also evidence of an independent graded relationship between severity of NAFLD and the degree of LV diastolic dysfunction, even after

adjusting for traditional CV risk factors.[94, 101] However, no studies have as yet evaluated whether changes in NAFLD severity over time are associated with changes in LV diastolic function, which would have important clinical and treatment implications for the NAFLD population.

The WELCOME study previously described a significant reduction in liver fat percentage after 18 months treatment with high-dose n-3 fatty acids (Omacor 4 g/day) versus placebo in subjects who showed a significantly increased level of erythrocyte DHA enrichment, although the effect of Omacor on liver fat reduction was non-significant in the intention-to-treat (ITT) analysis due to issues with medication compliance and placebo contamination.[269] Apart from its potential benefit in treating NAFLD, n-3 fatty acids have also shown evidence of reducing adverse CV outcomes in secondary prevention cohorts.[227] Although previous studies on the use of n-3 fatty acid supplementation in heart failure patients suggested benefit in terms of reducing mortality and improving LV systolic function, these benefits have largely only been seen in patients with at least moderately impaired LV systolic function.[224, 247, 301] Conversely, a recent meta-analysis of nine RCTs involving 800 patients taking into account more recent studies, concluded that LV ejection fraction did not significantly improve in heart failure patients receiving omega-3 PUFAs versus placebo.[302] Much of the negative data arose from patients who had only mildly impaired or relatively preserved LV systolic function, who showed no incremental benefit with standard n-3 fatty acid doses. Moreover, there is very limited data to date on the effects of high-dose n-3 fatty acid supplementation in subjects with LV diastolic dysfunction but normal LV systolic function, with previous studies using standard doses showing little or no incremental benefit in patients with nearly preserved cardiac function. The only known study so far assessing this was in an asymptomatic paediatric NAFLD cohort which showed no evidence of LV diastolic function improvement following a 6-month treatment with DHA versus placebo.[235]

The aims of this prespecified echocardiographic substudy of the WELCOME trial were: (i) to investigate the effects of high-dose n-3 fatty acids treatment over 15 - 18 months on improvement of markers of LV diastolic function in NAFLD subjects, and (ii) to describe the association between change in liver fat percentage and changes in cardiac geometry and LV diastolic function markers over the same time period.

4.2 Methods

4.2.1 Subjects and Study design

This study was part of the WELCOME trial, which has already been fully described previously in Chapter 2. Briefly, this echocardiographic study included participants from the WELCOME study,

which was a randomised, double-blind, placebo-controlled clinical trial undertaken in asymptomatic subjects with NAFLD. Subjects were included if they had recent imaging evidence of liver fat (ultrasound, magnetic resonance imaging or computerised tomography scan) and/or histological confirmation of NAFLD as well as exclusion of all other liver conditions causing liver fat accumulation such as excess alcohol intake or evidence of cirrhosis. The main trial's primary outcomes were to test the effects of 15-18 months treatment with Omacor 4g/day (high-dose purified n-3 fatty acids comprising DHA (46%) and EPA (38%); Pronova Biopharma ASA, Lysaker, Norway; Abbott Laboratories, Southampton, UK) on improving liver fat (quantified by magnetic resonance spectroscopy) and serum fibrosis markers. The WELCOME study had approval from the local ethics committee and all particpants gave their written informed consent. All participants in the WELCOME study also had echocardiographic examinations including tissue Doppler imaging. However, additional exclusion criteria were previous myocardial infarction, clinical heart failure, the presence of LV systolic impairment, significant valvular abnormalities or evidence of cardiomyopathy on the baseline echocardiogram.

103 participants were enrolled into the WELCOME study and randomised to either Omacor 4g/day (n=51) or placebo (olive oil capsules, n=52) in a 1:1 double-blind fashion, with treatment duration of between 15-18 months. Compliance with the allocated medication was monitored by recording returned capsules at fixed intervals during the study. We also assessed erythrocyte EPA and DHA enrichment (between baseline and end of study) by gas chromatography[278] to test adherence to the intervention in the DHA+EPA group and to monitor dietary contamination with DHA and EPA in the placebo group. Dietary and lifestyle changes had already been recommended to all participants as part of their routine clinical care[4] and this was continued throughout the study. There were no additional specific weight-loss programs or strict dietary restrictions placed on the participants as part of the study.

4.2.2 Laboratory and anthropometry measurements

Fasting blood samples were drawn and serum was separated within 1 hour to undergo routine biochemical assay by conventional enzymatic methods (lipids, glucose, liver transaminases). Plasma and serum samples were also frozen at -70°C for further analysis in batches (insulin and NT-proBNP). Serum N-terminal pro-brain natriuretic peptide (NT-proBNP) was measured using the commercially available MSD proBNP sandwich immunoassay (Meso Scale Diagnostics, Gaithersburg, MD, USA). NT-proBNP is produced predominantly by cardiac ventricular myocytes and is released in response to ventricular volume expansion and increased filling pressure.[303] Natriuretic peptide levels are widely used in clinical practice and CV research as a diagnostic and prognostic tool for the presence and severity of heart failure.[304, 305]

Body surface area (BSA) was measured using the Du Bois formula: BSA (m^2) = 0.007184 x Weight(kg)$^{0.425}$ x Height(cm)$^{0.725}$.[306] Blood pressure was measured using a Marquette Dash 3000 monitor (GE Healthcare, Bucks, UK) on the non-dominant arm in the supine position after a minimum of 60 minutes rest and a mean of 3 measurements 5 minutes apart was taken. Body mass index (BMI) was calculated as weight (kg) divided by the square of height (m).

4.2.3 Quantification of liver fat percentage

The full methodology of our MRS technique has already been described in Chapter 2 Methods section. To summarise briefly, MRS is a quick and safe, non-invasive tool to accurately quantify liver fat in NAFLD and correlates well with liver biopsy results. Due to its high sensitivity, it can detect small percentage differences in liver fat accumulation in NAFLD.[280] It is currently the gold-standard for the quantification of simple steatosis in NAFLD.[30] Subjects had liver MRS scans at the start and end of the study. Three 20 x 20 x 20mm^3 spectroscopic volumes of interest (VOI) were positioned in three standard areas of the liver and the average of each VOI's lipid spectroscopic peak was used to calculate the percentage liver fat. VOIs remained constant for end of study measurements.

4.2.4 Echocardiography

All participants underwent baseline and end of study transthoracic echocardiography. This was performed at the Cardiac Echocardiography Department at Southampton Hospital using the Philips iE33 ultrasound system and 2.5 MHz transducers by a single trained British Society of Echocadiography-accredited sonographer. Standard parasternal and apical views of the heart were acquired with 3-lead ECG monitoring attached with the participant in the decubitus, left lateral position. Complete 2D and M-mode echocardiogram, conventional Doppler, and tissue Doppler imaging (TDI) were obtained for all study participants according to the American Society of Echocardiography guidelines.[253, 254] No subjects had any evidence of echocardiographic exclusion criteria, e.g. significant valvular disease or significant mitral annular calcification, impaired LV systolic function, regional wall motion abnormality or evidence of cardiomyopathy. Subjects also did not have any evidence of significant atrial arrhythmias or atrial fibrillation which would make echocardiographic measurements less accurate. Standard 2D measurements (left ventricular (LV) end-diastolic and end-systolic dimensions (mm), septal and posterior wall thickness at end-diastole (mm)) were determined. LV ejection fraction (%) was calculated using biplane modified Simpson's method.[255] LV mass was calculated using the formula proposed by Levy *et al.*[256] and normalized for body surface area (LV mass index, g/m^2). Left atrial (LA) dimension (mm) was measured in the 2D parasternal view, and LA volume (ml/m^2) was measured

using the area-length method in the apical view and normalized for body surface area. Transmitral flow velocities to assess LV filling[307] were obtained by pulsed-wave Doppler echocardiography, positioning a 3 mm sample volume at the level of the mitral leaflets tip during diastole in an apical four-chamber view. Spectral gain and wall filter settings were optimised to clearly display the onset and cessation of LV inflow and obtain crisp velocity profiles. Mitral flow parameters, including peak velocities at early diastole (E) and late diastole (A) and E-wave deceleration time were measured and E/A ratio was calculated.

Tissue Doppler measurements of the mitral annulus LV insertion points from the apical four-chamber view were recorded. The sampling window was positioned as parallel as possible to the mitral annulus longitudinal plane of motion to ensure optimal angle of imaging. Pulsed-wave tissue Doppler imaging was performed by placing a 5 mm sample volume separately at the septal and lateral mitral annulus in the apical four-chamber view, and peak myocardial systolic, early diastolic and late diastolic velocities (s', e', and a' respectively) were measured at end-expiration with the optimal velocity scale setting at sweep speeds of 50 – 100 mm/s. All Doppler and tissue Doppler measurements reflected the average of three cardiac cycles. The ratio of mitral to myocardial early diastolic peak velocity (E/e') was calculated, after averaging the mean of the e' medial and e' lateral annulus measurements.[257] E/e' can be used to predict LV filling pressures[258] and E/e' is a good prognostic indicator of survival in established cardiac disease,[259] as well as an independent predictor of primary CV events.[260] Table 4-1 summarises the important echocardiographic variables used to evaluate LV diastolic function including their clinical correlates.[308]

Table 4-1 Definition of echocardiographic variables used to evaluate LV diastolic function* and its clinical correlates

Echocardiographic variable	**Definition**	**Clinical correlate**
E wave (cm/s)	Peak early LV diastolic filling velocity	Early diastolic LA-LV pressure gradient
A wave (cm/s)	Peak late LV diastolic filling velocity	Late diastolic LA-LV pressure gradient
E/A ratio	Ratio of early to late LV diastolic filling velocities	Simple classification of LV filling patterns
E-wave deceleration time (ms)	Time interval from peak E wave along slope of LV filling to zero-velocity baseline	Simple classification of LV filling patterns
e' (cm/s)	Pulsed-wave TDI-derived mitral annular early LV diastolic velocity	Related to LV relaxation and LV filling pressures
E/e' ratio	Ratio of early LV diastolic filling velocity to tissue doppler-derived early LV relaxation velocity	Important predictor of LV filling pressure
a' (cm/s)	Pulsed-wave TDI-derived mitral annular late LV diastolic velocity	Related to LV end-diastolic pressure
s' (cm/s)	Pulsed-wave TDI-derived mitral annular peak LV systolic velocity	Related to longitudinal LV systolic function, not an index of diastolic function
e'/a'	Ratio of tissue doppler-derived mitral annular early to late LV diastolic velocity	Related to LV filling pressure
LAVI (ml/m^2)	Left atrial volume measurement indexed to BSA	Can reflect cumulative effects of increased LV filling pressures over time
Abbreviations: LV, left ventricle; LA, left atrium; TDI, tissue Doppler imaging; LAVI, left atrial volume index; BSA, body surface area. *s' not an index of diastolic function		

All measurements were performed and analysed off-line by a single trained operator blinded to subject treatment allocation. Tissue Doppler data was excluded if the angle between the scan line and LV wall was more than 20 degrees in order to preclude angle-dependency of tissue Doppler parameters.[257] Tissue Doppler measurements were done at the peak of the upper edge of the solid Doppler curve, with scale optimised and low gain setting. A random sample of 22 scans (baseline and end-of-study) underwent repeat analysis at a later date to test intraobserver reproducibility and the coefficient of variation for tissue Doppler variables (e.g. E/e') was between 3.0% and 6.5%, which was in keeping with other published studies.[259-261]

4.2.5 Statistical analysis

Statistical analysis was carried out with SPSS Version 23 (SPSS, Inc., Chicago, IL.). Mean values and standard deviations were calculated for continuous variables, or median and interquartile range values for non-normally distributed variables. Univariate comparisons of normally distributed data were performed with independent Student's t-tests. Mann-Whitney U or Wilcoxon signed rank tests were used for non-parametric data and Chi-squared tests for binary data. Pearson and Spearman correlations were used for normal and non-normally distributed data respectively. Log transformation was undertaken for non-normal variables where necessary. Exposure variables which showed a significant univariate association with the outcome variable, as well as key baseline variables that might confound the association between exposures and the outcome of interest were included in the multivariable stepwise regression model. A "difference" variable, which represented the arithmetic difference between the measurement at the end of the study minus the baseline measurement, was calculated for key exposures and potential confounders. As assessment of LV diastolic function can use several different but linked variables, adjustment for multiple comparisons was not performed because of co-linearity between certain echocardiographic variables (e.g. E, e', E/e' etc) and the increased risk of type II error following adjustment. One-way ANCOVA was used to determine whether there were any statistically significant group differences on the dependent variable after adjusting for the covariate. All comparisons were two-sided and a p-value of < 0.05 was considered to be statistically significant. Coefficient of variation was determined as the absolute difference between the two sets of measurements divided by the mean of the measurements, and expressed as a percentage.

4.3 Results

Data from 48 subjects randomised to placebo and 47 randomised to DHA+EPA treatment was available for analysis out of n = 103. Eight subjects did not complete the study due to personal commitments, mostly relating to time-constraints. From capsule counts at 6, 12 and 18 months, it

was estimated that all subjects consumed > 50% of their study medication and 78% consumed > 75%. No serious adverse events occurred that were attributed to medication. Alcohol consumption was not associated with baseline liver fat percentage (p = 0.93) and did not vary significantly for each subject at the end of the study (p = 0.90). The baseline demographic and clinical characteristics of the subjects according to randomisation group are shown in table 4-2.

Table 4-2 Baseline demographic and clinical characteristics of subjects in placebo and DHA+EPA groups at randomisation

	Placebo (n = 48)	**DHA + EPA (n = 47)**	**p-value**
Age (years)	54.0 ± 9.6	48.6 ± 11.1	0.09
Male/Female	32/16	23/24	0.08
Diabetes (%)	9.0	9.0	0.90
Hypertension (%)	50.0	55.3	0.60
Systolic BP (mmHg)	137.7 ± 15.9	138.2 ± 16.7	0.90
Diastolic BP (mmHg)	85.3 ± 8.1	84.7 ± 11.8	0.80
Smoker/Ex/Non-smoker	5/18/25	5/15/27	0.84
Antihypertensive use (%)	37.5	38.3	0.94
Statin use (%)	47.9	38.3	0.34
Total chol (mmol/L)	4.8 ± 1.3	4.9 ± 1.1	0.40
LDL-chol (mmol/L)	2.7 ± 0.8	3.0 ± 0.9	0.30
HDL-chol (mmol/L)	1.1 ± 0.3	1.0 ± 0.2	0.10
Serum triglycerides (mmol/L)	1.4 (0.9)	1.8 (1.2)	0.04
Fasting glucose (mmol/L)	6.2 (2.0)	6.2 (2.8)	0.30
Haemoglobin A1c (% total Hb)	6.1 (1.6)	5.9 (1.2)	0.20
Fasting insulin (µU/ml)	11.3 (12.2)	13.6 (11.9)	0.80
Alanine transaminase (iu/L)	56 (34)	54 (43)	0.60
Aspartate aminotransferase (iu/L)	42 (19)	38 (24)	0.20
Weight (kg)	93 ± 14.4	97 ± 17	0.20
Body mass index (kg/m^2)	32.0 ± 4.3	34.3 ± 5.8	0.02
DEXA total fat mass (kg)	32.4 (8.1)	37.5 (15.1)	0.08
DEXA total lean mass (kg)	58.5 ± 10.9	58.0 ± 12.4	0.80

	Placebo (n = 48)	DHA + EPA (n = 47)	p-value
NT-proBNP (pg/ml)	72.9 (130.9)	79.9 (185.6)	1.0
CK-18 M65 (U/L)	599.5 (509.3)	388.0 (545.5)	0.32
MRS liver fat (%)	21.7 (19.3)	23.0 (36.2)	0.75
MRI visceral fat (%)	16.7 ± 4.5	15.6 ± 5.1	0.30
Erythrocyte DHA (%)	4.2 ± 1.4	3.9 ± 1.2	0.32
Erythrocyte EPA (%)	0.9 ± 0.4	0.8 ± 0.3	0.43

Data are means ± standard deviation (SD), median (IQR).

Abbreviations: DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; BP, blood pressure; LDL chol, low-density lipoprotein cholesterol; HDL, high-density lipoprotein; DEXA, dual energy X-ray absorptiometry; NT-proBNP, N-terminal pro-brain natriuretic peptide; CK-18 M65, cytokeratin-18 M65 subfraction; MRS, magnetic resonance spectroscopy; MRI, magnetic resonance imaging.

Baseline characteristics of the DHA+EPA and placebo groups were very similar, apart from the DHA+EPA group having higher baseline fasting triglycerides (1.8 vs 1.4, p = 0.04) and higher BMI (34.3 vs 32.0, p = 0.02) compared to the placebo group. Liver fat, NT-proBNP and CK-18 were not significantly different between groups at baseline.

Table 4-3 shows a comparison of baseline and end of study key echocardiographic variables in subjects randomised to DHA+EPA and placebo. Baseline variables were largely similar except for transmitral E wave velocity and transmitral E/A ratio which were lower in the placebo group compared to the Omacor group (E wave: Omacor, 71.9 cm/s vs placebo, 64.3 cm/s ($p < 0.01$) and E/A ratio: Omacor, 1.03 vs placebo, 0.91 ($p = 0.01$)). This finding is likely a result of the slightly older cohort in the placebo group compared to the Omacor group (placebo, 54.0 yrs vs Omacor, 48.6 yrs ($p = 0.09$)), although this result did not reach statistical significance. Age is a key determinant in reducing the mitral E velocity and E/A ratio among normal, healthy populations.[309] The baseline mean E/e' ratio was also lower in the placebo group compared to the Omacor group, although this finding just failed to reach significance (placebo, 8.11 vs Omacor, 8.91 ($p = 0.05$)). This difference may have been due to the significantly higher BMI noted in the Omacor group at baseline compared to placebo (Omacor, 34.3 kg/m^2 vs placebo, 32.0 kg/m^2 ($p = 0.02$)), as obesity and overweight independently predict LV diastolic dysfunction, specifically in relation to the E/e' ratio.[310]

Table 4-4 shows the changes between baseline and end of study measurements, stratified by randomisation group, for the main anthropometric and biochemical variables in the study. In the Omacor group, there was a significant reduction in median liver fat percentage at the end of study

compared to baseline (22.7% vs 16.3%, p = 0.01). However, it was interesting to note that there was also a lesser, but nonetheless significant reduction in liver fat percentage in the placebo group as well (21.7% to 19.7%, p < 0.01). As mentioned already in Chapter 3 when describing the WELCOME main trial results, there was likely to have been contamination in the placebo group with some subjects increasing their oily fish intake or taking over-the-counter omega-3 supplements. This can also be seen from the significant increases in erythrocyte DHA enrichment in both groups over the 18 months, albeit to a lesser extent in the placebo group (Omacor, 3.93% vs 6.97%; p < 0.001 and placebo, 4.21% vs 4.87%; p < 0.01). As expected in the Omacor group, fasting triglycerides decreased significantly over the 18 months (1.8 mmol/l vs 1.6 mmol/l, p = 0.02), and HDL-cholesterol increased from 1.03 mmol/l to 1.14 mmol/l (p < 0.001). There was also a small but significant reduction in both systolic and diastolic BP in the Omacor group between baseline and end of study (Table 4-4), which is not an unexpected finding as a consequence of high-dose n-3 fatty acid treatment.[209]

Table 4-3 Comparison of baseline and end of study echocardiographic variables in subjects randomised to DHA +EPA or placebo treatment over 15-18 months

	Placebo (n = 48)			DHA+EPA (n = 47)			p-value for baseline group comparison	p-value for adjusted changes between groups (Adjusted for baseline value ANCOVA)
	Baseline	End of study	Within group p-value	Baseline	End of study	Within group p-value		
Left atrial volume index (ml/m2)	24.0 ± 4.0	24.0 ± 4.1	0.94	23.5 ± 4.8	23.5 ± 4.7	0.99	0.59	0.92
LV septal diameter (cm)	1.12 ± 0.19	1.12 ± 0.19	0.82	1.08 ± 0.15	1.08 ± 0.13	0.87	0.27	0.89
LV end-systolic diameter (cm)	2.84 ± 0.43	2.86 ± 0.42	0.20	2.76 ± 0.44	2.81 ± 0.45	<0.01	0.38	0.17
LV end-diastolic diameter (cm)	4.58 ± 0.43	4.58 ± 0.41	0.70	4.52 ± 0.44	4.53 ± 0.43	0.36	0.51	0.77
LV mass index (g/m2)	90.3 ± 21.5	90.5 ± 20.5	0.75	83.3 ± 13.8	83.2 ± 13.6	0.88	0.06	0.38
LV ejection fraction (%)	65.3 ± 5.0	65.3 ± 5.1	0.89	68.9 ± 6.1	68.5 ± 6.1	0.02	<0.01	0.21
Transmitral E wave velocity (cm/s)	64.3 ± 11.7	58.7 ± 10.5	0.001	71.9 ± 14.8	66.3 ± 12.9	0.001	<0.01	0.08
Transmitral E wave deceleration time (ms)	260 ± 39	272 ± 36	0.10	256 ± 34	257 ± 37	0.70	0.55	0.11
Transmitral E/A ratio	0.91 ± 0.17	0.86 ± 0.18	0.04	1.03 ± 0.27	1.00 ± 0.28	0.27	0.01	0.23
Mean tissue doppler e' velocity (cm/s)	8.16 ± 1.87	8.00 ± 1.50	0.53	8.34 ± 1.91	8.30 ± 2.18	0.83	0.65	0.53
Mean tissue doppler a' velocity (cm/s)	9.67 ± 1.57	9.62 ± 1.51	0.83	9.40 ± 1.66	9.08 ± 1.93	0.15	0.42	0.21
Mean tissue doppler s' velocity (cm/s)	9.08 ± 1.75	8.96 ± 1.47	0.65	8.99 ± 1.37	9.16 ± 1.58	0.37	0.79	0.38
Mean e'/a' ratio	0.87 ± 0.26	0.86 ± 0.23	0.57	0.91 ± 0.27	0.97 ± 0.41	0.10	0.42	0.10
Mean E/e' ratio	8.11 ± 1.68	7.72 ± 1.63	0.02	8.91 ± 2.21	8.75 ± 2.89	0.53	0.05	0.37
Data are means ± standard deviation (SD)								

Table 4-4 Comparison of baseline and end of study main anthropometric and biochemical variables randomised to DHA+EPA or placebo

	Placebo (n = 48)			DHA+EPA (n = 47)		
	Baseline	**End of study**	**p-value**	**Baseline**	**End of study**	**p-value**
BMI (kg/m$^{2)}$	31.7 ± 4.3	31.6 ± 4.3	0.75	34.3 ± 5.9	34.5 ± 5.5	0.31
Weight (kg)	93.1 ± 14.4	92.9 ± 14.5	0.77	97.7 ± 17.9	98.3 ± 16.6	0.39
Systolic BP (mmHg)	137.8 ± 15.9	134.0 ± 12.1	0.14	138.1 ± 16.9	133.4 ± 13.4	<0.01
Diastolic BP (mmHg)	85.2 ± 8.1	83.1 ± 7.8	0.08	84.7 ± 12.0	81.1 ± 10.3	<0.01
Total chol (mmol/l)	4.83 ± 1.33	4.67 ± 0.96	0.29	5.09 ± 1.16	4.89 ± 1.12	0.17
HDL-chol (mmol/l)	1.12 ± 0.27	1.12 ± 0.26	0.91	1.03 ± 0.27	1.14 ± 0.31	<0.001
LDL-chol (mmol/l)	2.72 ± 0.79	2.64 ± 0.84	0.38	3.05 ± 0.96	2.82 ± 0.84	0.13
TG (mmol/l)	1.5 (1.0)	1.7 (1.3)	0.05	1.8 (1.2)	1.6 (1.5)	0.02
Fasting glucose (mmol/l)	5.4 (1.8)	5.5 (2.8)	0.07	5.4 (1.3)	5.4 (2.0)	0.77
HbA1c (% total Hb)	6.1 (1.6)	6.2 (1.6)	0.15	5.9 (1.2)	5.9 (1.4)	0.71
NT-proBNP (pg/ml)	72.5 (131.3)	106.5 (138.8)	0.02	80.0 (192.0)	120.0 (146.0)	0.50
MRS liver fat	21.7 (18.7)	19.7 (18.0)	<0.01	22.7 (35.5)	16.3 (22.0)	0.01
MRI visceral fat (%)	16.8 ± 4.6	17.3 ± 5.1	0.36	15.8 ± 5.3	16.0 ± 4.7	0.68
Erythrocyte EPA (%)	0.96 ± 0.40	1.03 ± 0.26	0.18	0.89 ± 0.36	2.91 ± 1.43	<0.001
Erythrocyte DHA (%)	4.21 ± 1.47	4.87 ± 1.07	<0.01	3.93 ± 1.29	6.97 ± 1.35	<0.001

Data are means ± standard deviation (SD), median (IQR).

Abbreviations: BMI, body mass index; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; BP, blood pressure; LDL-chol, low-density lipoprotein cholesterol; HDL-chol, high-density lipoprotein cholesterol; TG, serum triglycerides; HbA1c, glycosylated haemoglobin; NT-proBNP, N-terminal pro-brain natriuretic peptide; MRS, magnetic resonance spectroscopy; MRI, magnetic resonance imaging.

At the end of the 18 month study, after adjustment for pre-intervention E/e' ratios, there did not appear to be a statistically significant difference in post-intervention E/e' ratios between the Omacor and placebo groups ($F(1, 92) = 0.81$, $p = 0.37$, partial $\eta^2 = 0.009$) (Table 4-3). No significant difference was also seen in any of the other markers of LV diastolic function when comparing the Omacor to placebo groups after controlling for the relevant pre-intervention echocardiographic baseline variable between groups (table 4-3). Given the issues with lack of compliance in the Omacor group and some contamination in the placebo group (refer to Chapter 3, Introduction), this would have certainly underestimated the effect of the DHA+EPA intervention versus placebo in the ITT analysis. Therefore, a further analysis was performed to compare the effects of an absolute > 2% increase in erythrocyte DHA enrichment from baseline versus < 2% absolute enrichment, which had been associated with a significant reduction in liver fat percentage in the main WELCOME trial (see Chapter 3), on changes in LV diastolic function in this study. It was found that 54 subjects had < 2% erythrocyte DHA enrichment versus 41 subjects with > 2% absolute DHA enrichment in the study. After adjustment for pre-intervention E/e' ratios, there was also no statistically significant difference in post-intervention E/e' ratios between the > 2% DHA enrichment group and the < 2% DHA enrichment groups ($F(1, 92) = 0.05$, $p = 0.82$, partial $\eta^2 = 0.001$). No significant difference was also seen in any of the other markers of LV diastolic function when comparing the > 2% and < 2% DHA enrichment groups after controlling for the relevant pre-intervention echocardiographic baseline variable between groups.

As part of our prespecified aims in this study, we also wanted to investigate the association between change in liver fat percentage and changes in cardiac geometry and LV diastolic function in the entire cohort over the 18-month study period. It was relevant to evaluate the entire cohort, as liver fat percentage had reduced significantly compared to baseline values, in both the treatment and placebo groups. As a post-hoc analysis, we therefore stratified the entire cohort into two groups; Group 1 was characterised by any liver fat increase or no change, between baseline and end of study (n = 36), and Group 2 was characterised by any liver fat reduction between baseline and end of study (n = 55). Of note, only two subjects had no change in liver fat percentage at the end of the study, with one subject having been randomised to Omacor, and the other to placebo. Table 4-5 shows the baseline demographic and clinical characteristics of the cohort stratified in this way.

Table 4-5 Baseline characteristics of subjects divided by liver fat reduction or increase/no change between baseline and end of study (15-18 months)

	Liver fat increase/no change Group 1 (n=36)	**Liver fat reduction Group 2 (n=55)**	**p-value**
Age (years)	53.0 ± 10.6	50.7 ± 10.7	0.31
Male/Female	17/19	36/19	0.09
Diabetes (%)	36.1	30.9	0.61
Oral anti-diabetic (%)	36.1	29.1	0.48
Insulin use (%)	11.1	5.5	0.32
Hypertension (%)	52.8	52.7	1.0
Smoker/Ex/Non-smoker	5/12/19	5/17/33	0.71
Antihypertensive use (%)	38.9	34.5	0.67
Statin use (%)	52.8	36.4	0.12
Total cholesterol (mmol/L)	4.83 ± 0.88	5.08 ± 1.45	0.32
LDL-cholesterol (mmol/L)	2.88 ± 0.82	2.94 ± 0.95	0.80
HDL-cholesterol (mmol/L)	1.10 ± 0.26	1.06 ± 0.29	0.43
Triglycerides (mmol/L)	1.5 (0.7)	1.9 (1.3)	0.12
Fasting glucose (mmol/L)	5.4 (2.0)	5.4 (1.3)	0.62
Haemoglobin A1c (% total Hb)	6.0 (1.2)	6.1 (1.6)	0.83
Fasting insulin (μU/ml)	8.9 (13.5)	13.7 (10.2)	0.17
Alanine transaminase (iu/L)	44 (45)	57 (34)	0.11
Weight (kg)	92.1 ± 19.0	97.2 ± 14.7	0.17
Body mass index (kg/m^2)	32.0 ± 5.8	33.6 ± 4.9	0.18
Systolic blood pressure (mmHg)	138 ± 19	139 ± 14	0.88
Diastolic blood pressure (mmHg)	84 ± 9	86 ± 11	0.46
MRS Liver fat (%)	16.7 (11.5)	31.5 (32.5)	<0.001
NT-proBNP (pg/ml)	78.6 (125.6)	68.9 (185.1)	0.86
Omacor treatment (%)	52.8	49.1	0.73
Erythrocyte DHA (22:6n3) (%)	4.28 ± 1.23	3.98 ± 1.51	0.29

Data are means ± standard deviation (SD), median (interquartile range), or number (percentage). Abbreviations: HDL, high density lipoprotein; LDL, low density lipoprotein; DHA, docosahexaenoic acid; MRS, magnetic resonance spectroscopy; NT-proBNP, N-terminal pro-brain natriuretic peptide.

All baseline demographic and clinical variables, apart from baseline liver fat percentage, were very similar with no significant differences between groups. It was interesting to note that median liver fat percentage in the group that had any liver fat reduction at the end of the study was significantly higher at baseline (31.5%), compared to the group with any liver fat increase/no change at the end of the study (16.7%) ($p < 0.001$). This might suggest that factors involved in reducing liver fat across the entire cohort over the 18 months, including the intervention, were more likely to succeed in the presence of more severe grades of hepatic steatosis at baseline. Baseline erythrocyte DHA enrichment was similar between both groups also (Table 4-5).

Table 4-6 shows the baseline group comparisons as well as the changes in echocardiographic variables between the end of study liver fat increase/no change and liver fat reduction groups. Of note is that the baseline mean E/e' ratio was significantly higher in Group 2 (liver fat increase/no change) compared to Group 1 (liver fat reduction) (8.85 vs 8.00; $p < 0.05$). Higher LV E/e' ratios are consistent with worsening diastolic function, although there are certain threshold ranges that are highly specific for diagnosing diastolic dysfunction in clinical practice.[308] This finding of increased baseline E/e' in Group 2 may have been as a result of subjects in Group 2 having significantly higher liver fat percentage compared to Group 1 (table 4-5), which would be consistent with previous studies showing a direct association between fatty liver and diastolic dysfunction (see table 1-6). Importantly, in a multivariable linear regression analysis of the entire cohort with baseline E/e' as the dependent variable, after adjusting for key baseline clinical variables that could impact on LV diastolic function (table 4-7), the only independent predictors of worsening E/e' were increased liver fat (standardized β-coefficient 0.23, $p = 0.01$) and increased left atrial volume index (standardized β-coefficient 0.65, $p < 0.001$). The regression model fit was an R^2 of 0.48 with an adjusted R^2 of 0.41. Left atrial volume is already known to be strongly associated with LV diastolic impairment, and is a good measure of the chronicity of increased LV filling pressures.[308] Increased left atrial volume is also an important factor in the development and maintenance of AF,[311] and this finding is in keeping with current evidence suggestive of NAFLD representing a risk factor for the development of AF.[106, 107]

Table 4-6 Changes in echocardiographic variables in subjects with liver fat increase/no change or liver fat reduction at the end of study (after 15-18 months) and baseline group comparisons

	Liver fat increase / no change Group 1 (n=36)			**Liver fat reduction Group 2 (n=55)**			**p-value for baseline group comparison**	**p-value for adjusted changes between groups (Adjusted for baseline value ANCOVA)**
	Baseline	**End of study**	**Within group p-value**	**Baseline**	**End of study**	**Within group p-value**		
Left atrial volume index (ml/m2)	23.3 ± 4.3	23.5 ± 4.3	0.1	24.0 ± 4.7	23.9 ± 4.7	0.29	0.45	0.42
LV septal diameter (cm)	1.08 ± 0.13	1.08 ± 0.13	0.48	1.13 ± 0.19	1.12 ± 0.18	0.41	0.14	0.53
LV end-systolic diameter (cm)	2.81 ± 0.42	2.83 ± 0.42	0.10	2.78 ± 0.45	2.81 ± 0.45	0.02	0.79	0.72
LV end-diastolic diameter (cm)	4.52 ± 0.36	4.55 ± 0.36	0.06	4.55 ± 0.49	4.55 ± 0.47	0.97	0.74	0.26
LV mass index (g/m2)	86.3 ± 15.1	85.8 ± 14.6	0.42	88.4 ± 20.4	88.7 ± 19.6	0.55	0.58	0.23
LV ejection fraction (%)	66.1 ± 5.6	66.1 ± 5.7	1.0	67.6 ± 6.0	67.4 ± 6.0	0.09	0.26	0.30
E wave velocity (cm/s)	66.6 ± 15.1	64.5 ± 13.0	0.20	68.8 ± 12.9	61.3 ± 11.4	<0.001	0.48	0.02
E wave deceleration time (ms)	259 ± 44	274 ± 39	0.03	260 ± 32	260 ± 34	0.88	0.91	0.03
Transmitral E/A ratio	0.92 ± 0.21	0.92 ± 0.22	0.91	0.99 ± 0.24	0.93 ± 0.27	0.03	0.15	0.22
Mean tissue doppler e' velocity (cm/s)	8.46 ± 1.68	7.99 ± 1.62	0.04	8.06 ± 1.99	8.24 ± 2.02	0.36	0.30	0.06
Mean tissue doppler a' velocity (cm/s)	9.68 ± 1.56	9.37 ± 1.73	0.19	9.54 ± 1.65	9.35 ± 1.74	0.42	0.69	0.84
Mean tissue doppler s' velocity (cm/s)	9.00 ± 1.42	9.01 ± 1.42	0.97	9.11 ± 1.69	9.20 ± 1.55	0.71	0.74	0.63
Mean e'/a' ratio	0.90 ± 0.27	0.90 ± 0.36	0.89	0.87 ± 0.26	0.92 ± 0.32	0.11	0.56	0.23
Mean E/e' ratio	8.00 ± 1.90	8.66 ± 2.72	<0.01	8.85 ± 2.04	8.02 ± 2.20	<0.001	<0.05	<0.001
Data are means ± standard deviation (SD)								

Table 4-7 Multivariate linear regression analysis of associations between baseline key explanatory variables and baseline E/e' as the dependent outcome

Independent variables <u>at baseline</u>	**β-coefficient**	**Standardized β-coefficient**	**95% CI for β-coefficient**	**p-value**
Age	-0.007	-0.038	-0.049 to 0.034	0.72
Sex	0.373	0.091	-0.532 to 1.278	0.41
Liver fat (%)	0.024	0.233	0.005 to 0.043	0.01
LV mass index (g/m^2)	-0.012	-0.106	-0.033 to 0.010	0.29
Use of anti-hypertensives	-0.176	-0.041	-1.067 to 0.716	0.70
Diabetes presence	0.183	0.042	-0.608 to 0.974	0.65
Left atrial volume (indexed) (ml/m^2)	0.287	0.645	0.204 to 0.371	<0.01
Weight (kg)	-0.013	-0.101	-0.040 to 0.013	0.31
Use of statins	0.707	0.169	-0.092 to 1.505	0.08
MRI visceral fat (%)	0.006	0.014	-0.077 to 0.089	0.89
Model fit R^2 = 0.48 and adjusted R^2 = 0.41 Abbreviations: CI, confidence interval; MRI, magnetic resonance imaging				

When evaluating changes in measures of LV diastolic dysfunction in both groups between baseline and end of study, there were several significant findings suggestive of changes in liver fat being directly associated with changes in LV diastolic dysfunction (table 4-6). In the 'liver fat increase/no change' group, the transmitral E wave velocity increased while the mean e' velocity decreased significantly from baseline to end of study (E wave: 259 ms to 275 ms, p = 0.03 and e': 8.46 cm/s to 7.99 cm/s, p = 0.04, respectively). Both these changes were in keeping with worsening of diastolic function markers. Furthermore, there was a significant increase in the E/e' ratio between baseline and end of study in this group (8.00 to 8.66, p < 0.01) which is again reflective of possible deterioration of LV diastolic function.[308] Similarly, when evaluating the 'liver fat reduction' group, there were significant reductions in transmitral E wave velocity and E/A ratio

between baseline and end of study (E wave: 68.8 cm/s to 61.3 cm/s, $p < 0.001$ and E/A ratio: 0.99 to 0.93, $p = 0.03$, respectively). These changes were in keeping with improvement in LV diastolic function markers. More importantly, there was a significant decrease in the E/e' ratio between baseline and end of study in this group (8.85 to 8.02, $p < 0.001$), consistent with a possible improvement in diastolic function.[257] Finally, after adjustment for pre-intervention E/e' ratio, transmitral E wave and E wave deceleration time, there was a statistically significant difference in each of these post-intervention key variables associated with LV diastolic dysfunction, between Group 1 and Group 2 (E/e': $F(1, 88) = 29.53$, $p < 0.001$, partial $\eta^2 = 0.251$ E wave: $F(1, 88) = 5.43$, $p = 0.02$, partial $\eta^2 = 0.058$; E deceleration: $F(1, 77) = 4.72$, $p = 0.03$, partial $\eta^2 = 0.058$, respectively). In summary, these results suggest that an increase in liver fat is associated with a deterioration in several markers of LV diastolic function, whilst a reduction in liver fat is conversely associated with an improvement in several markers of LV diastolic function.

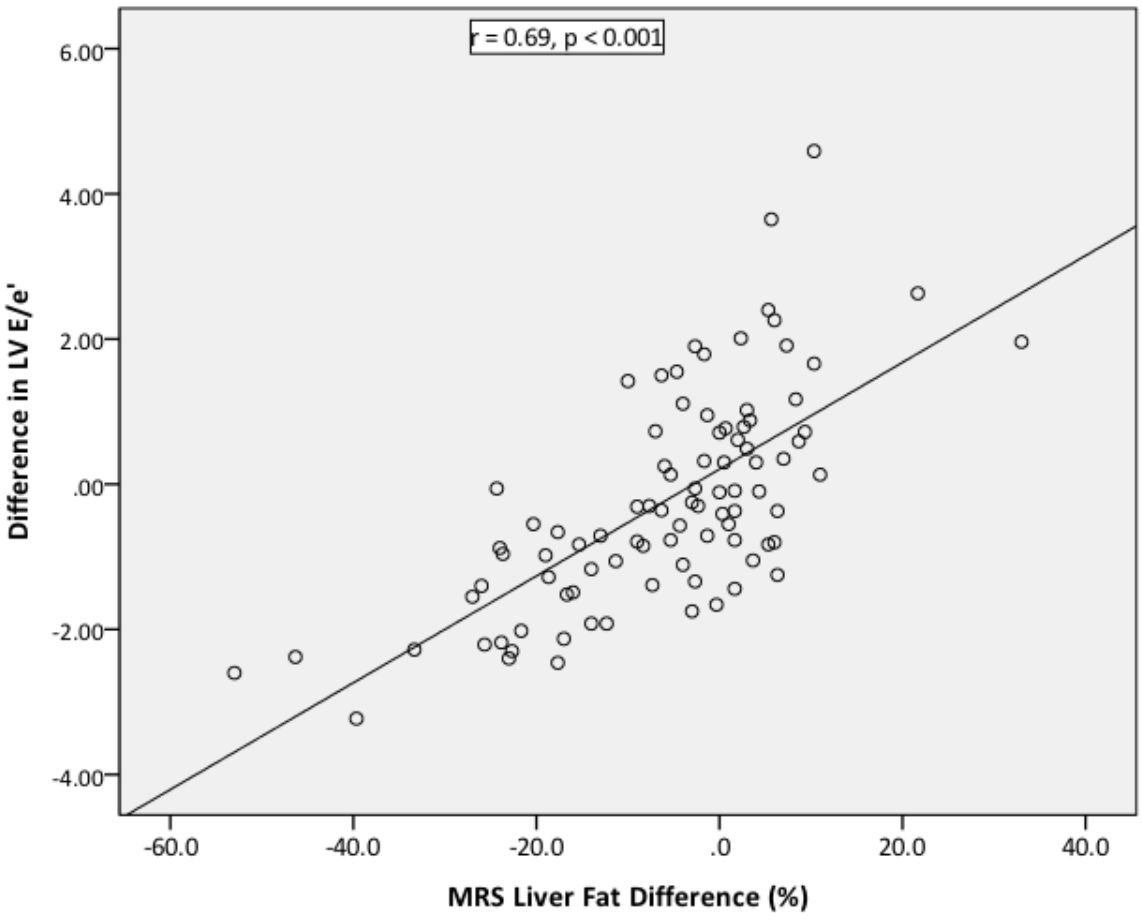

Figure 4-1 Scatter plot of relationship between percentage liver fat difference and E/e' difference between baseline and end of study measurements in the entire cohort ($r = 0.69$, $p < 0.001$)

Across the entire cohort, the change in E/e' between baseline and end of study (E/e' difference) was significantly positively correlated with the change in liver fat percentage (liver fat difference) over the same time period ($r = 0.69$, $p < 0.001$) (Figure 4-1). Furthermore, multivariable linear regression analysis of the entire cohort with E/e' difference as the dependent variable, after adjusting for key clinical variables that could impact on LV diastolic function including controlling for baseline E/e' (table 4-8), the only independent predictors of worsening E/e' across the whole cohort were increased percentage liver fat (standardized β-coefficient 0.58, $p < 0.001$) and increased left atrial volume index (standardized β-coefficient 0.29, $p < 0.001$). The regression model fit was an R^2 of 0.60 with an adjusted R^2 of 0.57.

Table 4-8 Multivariate linear regression analysis of associations between key explanatory variables and difference in E/e' between baseline and end of study in the entire cohort

Independent variables	**β-coefficient**	**Standardized β-coefficient**	**95% CI for β-coefficient**	**p-value**
Age	-0.007	-0.051	-0.029 to 0.015	0.51
Sex	0.433	0.145	-0.012 to 0.878	0.06
Diabetes presence	0.341	0.109	-0.115 to 0.797	0.14
Use of anti-hypertensives	0.032	0.010	-0.444 to 0.508	0.89
Baseline E/e' ratio	0.032	0.044	-0.076 to 0.140	0.56
Liver fat difference (%)	0.062	0.580	0.045 to 0.079	<0.001
Weight difference (kg)	0.005	0.015	-0.046 to 0.057	0.84
Difference in left atrial volume (indexed) (ml/m^2)	0.209	0.294	0.099 to 0.319	<0.001
Model fit $R^2 = 0.60$ and adjusted $R^2 = 0.57$ Abbreviations: CI, confidence interval				

The regression model was also tested in a stepwise fashion with several other possible explanatory baseline and 'difference' variables (to take into account changes in these variables over the 15-18 months) including difference in triglyceride levels, HbA1c difference, HOMA-IR difference, cholesterol difference, DHA and EPA enrichment change, left ventricular mass index

change, MRI visceral fat percentage difference, use of statins at baseline, blood pressure difference and smoking status, but none of these variables improved the model in terms of R and R^2, suggesting they were not helpful in explaining the variance of E/e' in this model.

4.4 Discussion

This is the first study to document a novel direct association between changes in liver fat percentage and changes in LV diastolic function markers in a NAFLD cohort over 15-18 months during a randomised double-blind, placebo-controlled trial. However, although previous studies have shown a beneficial effect of n-3 fatty acids in reducing adverse CV outcomes,[221, 223] as well as specifically in heart failure patients with LV systolic impairment,[224, 247] we did not find that high-dose n-3 fatty acids treatment improved markers of LV diastolic function compared to placebo in our study. This finding was consistent even when we analysed subjects with significant erythrocyte DHA enrichment (>2% absolute increase from baseline), given that >2% DHA enrichment has been shown to be associated with a significant reduction in liver fat percentage in the WELCOME trial,[269] as well as evidence showing that reduced DHA levels correlate significantly with reduced LV function and increased LV wall stress.[312] This finding of a lack of benefit of n-3 fatty acids in improving echocardiographic markers of LV diastolic function is consistent with a randomised, placebo-controlled study by Pacifico *et al*, which showed that a 6-month treatment with 250mg DHA supplementation in a paediatric overweight NAFLD cohort, did not significantly alter markers of LV diastolic function despite significantly reducing liver fat in the DHA group. All of the subjects in our study had normal LV systolic function and were asymptomatic, thereby representing a low-risk cohort, which could explain why we did not see any beneficial effect of n-3 fatty acid supplementation over 15-18 months on subclinical measures of cardiac function, even at the highest licensed dose of 4g/day.

There have been a number of cross-sectional and case-control studies highlighting the independent association between the presence of NAFLD and LV diastolic dysfunction, even after controlling for traditional CV risk factors and overweight/obesity in an asymptomatic population (see table 1-6). The degree of diastolic function has also been shown to correlate independently with the amount of liver fat, although the estimation of liver fat using ultrasound in these studies was a limitation in terms of its diagnostic sensitivity.[94, 96] Our results also show a graded direct independent relationship between baseline liver fat percentage and markers of LV diastolic dysfunction, although our use of MRS to quantify liver fat percentage represents the gold-standard diagnostic modality. This significant association was independent of CV risk factors,

weight and visceral fat. Graner *et al*. also showed an independent stepwise association between MRS-quantified hepatic steatosis and LV diastolic function in NAFLD, but this was not independent of visceral adipose tissue, which itself correlated with degree of diastolic dysfunction.[172] Given that visceral adipose tissue has a strong independent correlation with liver fat,[128] and that both entities are driven primarily by insulin resistance, it is likely that both factors are inter-dependent and act synergistically in a bidirectional manner.[313]

Our novel finding of an independent, direct association between changes in liver fat and changes in markers of LV diastolic function over 15-18 months extends previous cross-sectional and case-controlled data on the link between NAFLD and LV diastolic dysfunction (Table 1-6). We showed that several markers of LV diastolic function were significantly related to both increases and reductions in liver fat percentage over time. In the assessment of LV diastolic function, mitral E/e' ratio has been shown to have good prognostic value in established cardiac disease,[259] as well as being a powerful predictor of primary cardiac events in an asymptomatic population.[260] Large randomised studies have also used E/e' as a surrogate marker of diastolic function, as it is easy to measure, reproducible and relatively pre-load independent.[260, 314] Using E/e', our results showed that changes in liver fat over the study period across the entire NAFLD cohort were independently associated with changes in LV diastolic function. This was even after adjusting for CV risk factors, as well as changes in putative aetiological variables (e.g. weight changes, lipid level changes, blood pressure differences and visceral fat changes) from baseline to end of study, that might have affected the change in diastolic function.

Cassidy *et al*. showed that in an exercise intervention program in patients with type 2 diabetes, liver fat was significantly reduced and MRI-assessed cardiac structure (LV wall mass) and function (early diastolic filling rates) improved, both related to the exercise program.[236] However, the authors did not show any direct relationship between liver fat reduction and diastolic function improvement in that study. It is likely the study was underpowered to detect this association given that baseline mean liver fat percentage in the cohort was low at 7%, including the small sample size of 28 subjects. A study on the effects of weight loss in obesity through lifestyle modifications over 6 months also showed improvements in ventricular function from baseline, being independently associated with weight reduction and reduced insulin resistance, although no evaluation of liver fat was performed in that study.[315] Insulin resistance was also assessed in our study through HOMA-IR measurements and it did not show any association with diastolic function changes, but this was only evaluated in the non-diabetics so was likely underpowered to detect any potential relationship.

Our finding of an independent association between diastolic function change and liver fat change is important as it may encourage wider and more thorough cardiac screening of asymptomatic NAFLD patients. Given that isolated LV diastolic dysfunction is an independent predictor of all-cause mortality and adverse cardiac outcomes,[260, 300] better screening and identification of diastolic dysfunction in NAFLD patients may improve risk-stratification.[30] Importantly, regardless of the therapeutic method to reduce liver fat (e.g. exercise, reduced calorie intake, weight loss or potential new drugs), our results suggest for the first time that reducing liver fat may also improve LV diastolic function. Given that improvement in diastolic function is associated with improved long-term survival and vice-versa,[299, 300] our novel results should encourage further research into discovering established treatments for this increasingly prevalent condition with its associated increased CV risks. Putting our results into context, the ASCOT substudy showed that one unit rise in the E/e' ratio was associated with a 17% increment in the risk of a cardiac event over four years (HR 1.17, CI 1.05-1.29; p = 0.003).[260] Using our results from Table 4-8, that would translate into a 16% increase in liver fat conferring a similar risk.

There are limitations to this study. As this was a pre-specified sub-study of the WELCOME trial[278] with evaluation of LV diastolic function as a key secondary outcome of the trial, we did not undertake sample size or power calculations to determine the appropriate number of participants necessary to test the effect of the intervention with respect to changes in markers of LV diastolic function. Consequently, our study may have lacked sufficient power to prove that treatment with high-dose n-3 fatty acids caused an improvement in markers of LV diastolic function. Secondly, although MRS is the gold-standard for quantification of liver fat, we were not able to qualify severity of NAFLD in terms of steatohepatitis and fibrosis, which would only have been evaluated accurately with liver biopsy. We may have therefore missed potential associations between more severe grades of NAFLD and LV diastolic function changes. Thirdly, although E/e' has powerful predictive value in terms of prognostication, its diagnostic accuracy to reliably estimate LV filling pressures as a sole marker in subjects with normal LV systolic function is still debatable.[316] There is also poor specificity for E/e' ratios between 8-13 ('grey zone') and only values > 14 have high specificity for increased LV filling pressures in clinical practice.[308] The majority of the subjects in our study were in the E/e' 'grey zone'. However, our results using E/e' ratios are still very valid as a prognostic measure, given the findings of the ASCOT substudy as described above.[260] We also described similar changes in other markers of LV diastolic function, which in fact adds to the strength of our findings, given that individual tissue Doppler indices can independently predict adverse CV events in the long-term.[297] Additionally, the transition from normal diastolic function (in terms of E/e') to heart failure with preserved ejection fraction i.e. significant diastolic dysfunction, appears to have a progressive element, with E/e' ratio gradually increasing in some

cases prior to reaching the threshold for diastolic dysfunction diagnosis, which suggests its use as a continuous variable for prognostication may have some merit.[317]

In conclusion, we have shown for the first time a direct, independent association between changes in liver fat percentage and changes in markers of LV diastolic function in an asymptomatic NAFLD cohort over 15-18 months. However, we observed no significant effect of high-dose n-3 fatty acid supplementation on improving LV diastolic function markers over the same time period. Given that the most common cause of death in NAFLD is related to cardiovascular events independent of other cardiometabolic risk factors, as well as the high prevalence of this disease in the general population, there is an urgent need to ensure wider CV screening e.g. identification of LV diastolic dysfunction, to better risk-stratify NAFLD patients to try and reduce CV risk.[193] As this was a proof-of-concept study, further larger studies are needed to more thoroughly investigate the potential role of high-dose n-3 fatty acid supplementation in the treatment of liver fat as well as LV diastolic dysfunction, including evaluation of LV diastolic function markers in the management and prognostication of CV risk in NAFLD.

Chapter 5: Peripheral and hepatic insulin sensitivity in NAFLD and effects of n-3 fatty acids treatment

5.1 Introduction

Non-alcoholic fatty liver disease (NAFLD) is the commonest cause of chronic liver disease and is defined by the presence of liver fat accumulation of more than 5% of hepatocytes in the absence of excessive alcohol intake or other secondary causes of liver disease.[30] It encompasses a spectrum of conditions ranging from hepatic steatosis through to cirrhosis. Insulin resistance (IR) is a key determinant as well as a consequence of NAFLD.[318] Insulin resistance is defined as the reduced ability of insulin to exert its biological effects on target tissues, namely skeletal muscle, liver and adipose tissue. It is the reciprocal of insulin sensitivity. Impaired glucose metabolism is the main consequence of insulin resistance. As a result, there is a decrease in glucose uptake by skeletal muscle, reduced inhibition of endogenous glucose production (EGP) by the liver and increased lipolysis in adipose tissue.[319]

Although NAFLD shares common links with obesity, type 2 diabetes and the metabolic syndrome, mainly through underlying IR, liver fat content appears to be the best independent predictor of peripheral (whole body) and hepatic insulin resistance.[125] There appears to be limited and conflicting data on the effects of reducing hepatic fat and its consequences on changes in insulin resistance, with small studies utilizing HOMA-IR as a surrogate marker of IR showing significant improvement in IR,[213, 320] whilst other studies suggest neutral or negative results.[321, 322] However, reproducibility of HOMA-IR is poor with coefficients of variation (CV) of ~30%, although CVs improve when used in larger cohort studies, suggesting that HOMA-IR should be used to estimate IR in epidemiological cohorts with repeated mesaurements over long time periods rather than in smaller studies. The hyperinsulinaemic-euglycaemic clamp has been referred to as the 'gold standard' method for measuring insulin sensitivity.[262] It also has the advantage of evaluating peripheral as well as hepatic insulin sensitivity using a two-step technique,[323] but is laborious and time-consuming so is best suited for more detailed phenotypical characterisaton of smaller sample sizes.

High-dose n-3 fatty acids (eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA)) are a licensed treatment to reduce plasma triglyceride (TG) concentrations.[324] The WELCOME study previously described a significant reduction in liver fat percentage after 18 months treatment with high-dose n-3 fatty acids (Omacor 4 g/day) versus placebo in subjects who showed a significantly

increased (>2%) level of erythrocyte DHA enrichment, although the effect of Omacor on liver fat reduction was non-significant in the intention-to-treat (ITT) analysis due to issues with medication compliance and placebo contamination.[269] At the time of this study, the data on the efficacy of n-3 fatty acids in reducing hepatic steatosis was inconsistent and limited.[245] However, a very recent meta-analysis of the effectiveness of n-3 fatty acids in NAFLD (which included the WELCOME trial) showed a pooled clinically meaningful reduction in liver fat content of 5.2%.[215]

This study was a pre-specified sub-study of the WELCOME trial already mentioned.[269] The aim of this pilot sub-study was to test if a pre-specified increase (>2%) in erythrocyte enrichment of DHA was associated with an improvement in hepatic and peripheral insulin sensitivity. As described previously, it was felt in the main trial that Omacor treatment should produce a minimum 2% increase in erythrocyte DHA and a minimum 0.7% increase in erythrocyte EPA to produce a biological effect.[270, 271] As this sub-study was hypothesis-generating, it was deemed necessary to only analyse subjects who had significant versus non-significant DHA enrichment rather than on an intention-to-treat analysis, given the lack of compliance with intervention and contamination with placebo issues in the main trial we encountered, as previously mentioned.

5.2 Methods

5.2.1 Subjects and study design

Twenty-four individuals recruited from the main WELCOME trial were randomly allocated to the sub-study (n=12 randomised to EPA+DHA, 4 g/day and n=12 randomised to placebo (olive oil, 4 g/day) (Figure 5-1). The duration of intervention was 15-18 months and inclusion and exclusion criteria have been described previously.[278] For the main trial patients were randomised according to standardised procedures (computerised block randomisation in blocks of four), either to trial medication or placebo. This randomisation strategy was maintained for the sub-study. The study was approved by the Southampton and South West Hampshire Local Research Ethics Committee (REC 08/H0502/165). All subjects gave written informed consent for both the main trial and the sub-study.

Three participants withdrew from the sub-study before completing all tests. Four patients with diabetes were not included in the analysis as their anti-diabetic regimens increased between baseline and end-of-study clamp tests, which would have influenced change in insulin sensitivity measurements. Similarly, one participant who had lost > 10 kg in weight over the course of the trial was also excluded (Figure 5-1).

We compared participants who showed an absolute increase in erythrocyte DHA enrichment of >2% between baseline and end of the study, with participants showing little change in erythrocyte DHA enrichment (DHA <2%). In the DHA >2% group, eight participants had been randomised to EPA+DHA intervention and one participant had been randomised to placebo; the latter had a 4.2% increase in erythrocyte DHA enrichment between baseline and end of study. All seven subjects in the DHA <2% group had been randomised to the placebo (Figure 5-1).

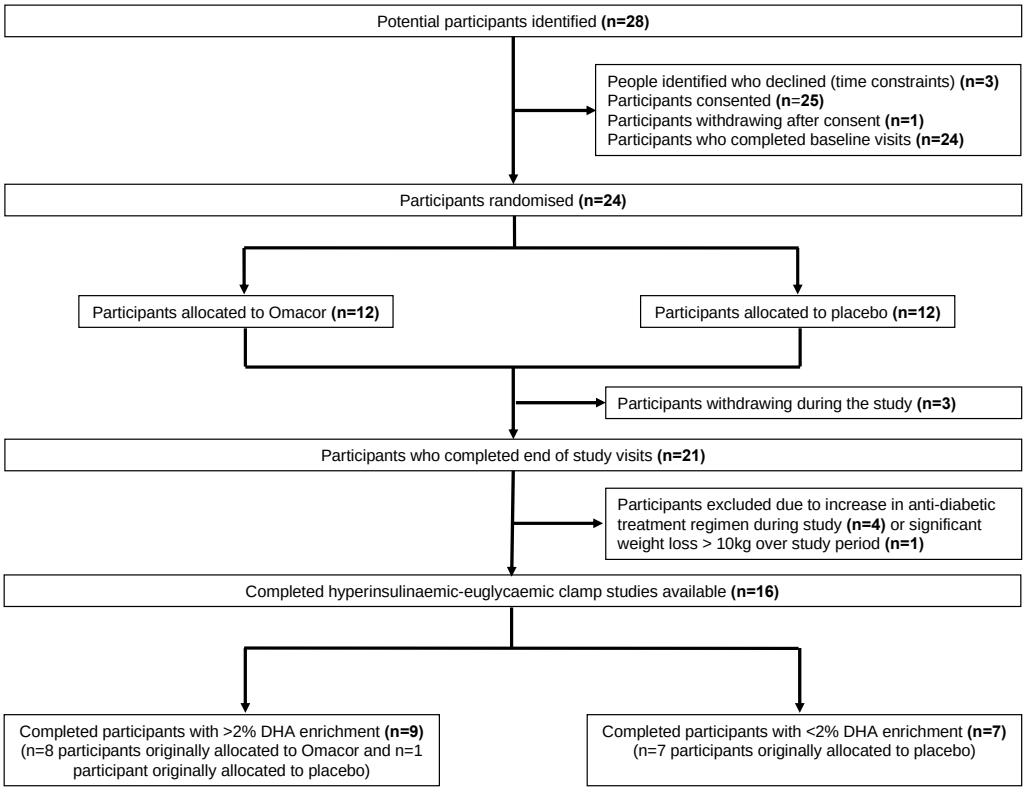

Figure 5-1 Consort diagram showing recruitment for the WELCOME sub-study and numbers of participants available for clamp studies in each group

5.2.2 Laboratory and anthropometry measurements

See Chapter 2 Methods section for detailed procedure. Briefly, blood samples were taken after an overnight fast (12 h) and serum separated within 1 hour to undergo routine biochemical assay. Blood pressure was measured using a Marquette Dash 3000 monitor (GE Healthcare, Bucks, UK), body composition by dual-energy X-ray absorptiometry (DEXA) and liver fat content measured at baseline and end of the study by magnetic resonance spectroscopy (MRS).

To determine specific fatty acid composition erythrocyte ghosts were prepared, membranes isolated, total lipids isolated, fatty acid methyl esters (FAMEs) prepared and fatty acid compositions

determined by gas chromatography (GC), as described previously. Measurement of erythrocyte percentage DHA and EPA enrichment with GC is a validated proxy for liver tissue concentrations of n-3 fatty acids.[250, 251]

5.2.3 Hyperinsulinaemic-euglycaemic clamp

The full methodology of this procedure has already been described in Chapter 2's Methods section (page 54). As mentioned, the hyperinsulinaemic-euglycaemic clamp is considered the 'gold standard' method for the measurement of insulin sensitivity under constant insulin-stimulated conditions.[262] Glucose is 'clamped' at a predetermined level (e.g. 5 mmol/l) by titrating a variable-rate infusion of glucose against a fixed-rate insulin infusion (priming dose followed by maintenance dose). Insulin infusion rates are determined according to BSA or body weight.[325] In normal individuals, administration of insulin stimulates glucose disposal, of which about 80% is taken up by skeletal muscle, which results in the plasma glucose to fall. This is prevented by adjusting the variable glucose infusion rate, which is computed on the basis of bedside blood glucose concentrations measured at 5-minute intervals throughout the clamp study. Our mean baseline basal steady-state fasting glucose was 5.6 mmol/l and did not change significantly at the end of the study at 5.4 mmol/l. 'Clamp' levels were done at around 0.5 mmol/l below basal steady-state glucose concentrations.[325]

During the first step of the clamp, insulin is infused at a 'low-dose' (0.3 mU/kg/min) to enable assessment of hepatic insulin sensitivity, compared to the second step where insulin is infused at a 'high-dose' (1.0 mU/kg/min) to assess whole-body insulin sensitivity with suppression of endogenous glucose production (EGP).[326] During the first step, EGP can be quantified from the dilution of exogenous-labelled (deuterated) glucose by endogenous glucose release. The decrease of endogenous glucose release between basal and clamp conditons i.e. suppression of EGP can then be used as a measure of hepatic insulin sensitivity. During the second step with high-dose insulin, the rate of glucose infusion at steady state required to maintain euglycaemia is expressed as glucose infusion rate (GIR, ml/h) or as whole-body glucose metabolism (M-value, mg/kg/min) and this reflects peripheral insulin sensitivity.[262] M-values should be corrected for fat-free mass (FFM) to account for gender-related differences in fat mass and obesity.[327] In our clamp studies, we used clamps of 120 mins for each stage to reach steady-state, whereas other groups suggest 180 mins. However, M-value measurements have been shown to be reproducible with both time periods.[328]

The hyperinsulinaemic-euglycaemic clamp has an intra-individual CV of ~10%.[328] However, despite being the 'gold standard', it does have limitations. It is very labour-intensive, time-consuming and

requires some training. It also potentially puts the research participant at risk of hypokalaemia as hyperinsulinaemia causes potassium to shift intracellularly, although this can be countered if necessary with a slow intravenous potassium chloride infusion.

5.2.4 Sample size and statistical analysis

The sample size for the main WELCOME study was powered to detect a change in the primary outcome, namely liver fat content, as already described in Chapter 2. The sub-study reported here was run as a hypothesis-generating pilot study. Statistical analysis was carried out with SPSS Version 23 (SPSS, Inc., Chicago, IL.). Mean values and standard error of the means (s.e.m) were calculated for continuous variables, or median and interquartile range values for non-normally distributed variables. Pearson and Spearman correlations were used for normal and non-normally distributed data respectively. All data sets were tested for normality according to the Shapiro-Wilk test. Log transformation was undertaken for non-normal variables where necessary. Baseline results were compared with end of study results using paired t-tests for normally distributed data and Wilcoxon singed rank test for non-parametric data. Comparisons between the two groups groups were undertaken using independent t-test or Mann Whitney U tests for non-parametric data. Statistical significance was set at $p < 0.05$.

5.3 Results

5.3.1 Baseline results analysis

Although only sixteen participants were fully analysed for this sub-study in terms of comparing baseline with end of study data, we also analysed the twenty-four participants who completed just the baseline clamp study so as to describe baseline associations in our sub-study (Figure 5-1). This baseline data will be presented separately now.

Baseline characteristics of the twenty-four participants not according to randomisation (i.e. entire sub-study cohort) are shown in Table 5-1. Age of the cohort was was 50.6 ± 11.9 years, with 71% men and 71% with pre-enrolment hypertension. 75% met the criteria for the MetS and six participants were diabetic. Median body mass index was 32.2 kg/m^2 (6.9 kg/m^2) with a mean body fat percentage of 36.1 ± 7.2%. Median percentage liver fat was 25.7% (19.6%). Mean M-value was 3.3 mg/kg/min. In non-obese individuals, the estimated threshold for diagnosing whole-body insulin resistance is at values below 4.7 mg/kg/min,[325] suggesting that the vast majority of our cohort had peripheral insulin resistance (only 4 out of 24 subjects had M-values > 4.7 mg/kg/min), with the caveat that all of our cohort was overweight/obese.

Table 5-1 Baseline demographic, clinical characteristics and clamp measurements of all sub-study participants not stratified by randomisation group (i.e. entire sub-study cohort)

Variable	**Baseline values (n = 24)**
Age (years)	50.5 ± 2.4
Men	17 (71%)
Hypertension	17 (71%)
Diabetes	6 (25%)
Systolic BP (mmHg)	140 ± 4
Diastolic BP (mmHg)	86 ± 2
Body mass index (kg/m^2)	32.2 (6.9)
Metabolic syndrome (IDF criteria)	18 (75%)
Alanine transaminase (ALT) (IU/L)	65 (48)
Fasting glucose (mmol/l)	5.6 ± 0.2
Haemoglobin A1c (% total Hb)	6.1 ± 0.1
Total cholesterol (mmol/l)	4.9 ± 0.2
LDL-cholesterol (mmol/l)	2.6 ± 0.1
HDL-cholesterol (mmol/l)	1.0 ± 0.1
Serum triglycerides (mmol/l)	1.8 (1.5)
Liver fat %	25.7 (19.6)
Body fat % (DEXA)	36.1 ± 1.5
Basal endogenous glucose production (Ra; μmol/min/kg FFM)	14.3 ± 0.44
Low-dose insulin EGP (μmol/min/kg FFM)	7.7 ± 0.49
High-dose insulin glucose disposal (Rd; μmol/min/kg FFM)	32.9 ± 2.1
Hepatic insulin sensitivity index (μmol/min/kg FFM; mU/l) x 10^2	0.55 (0.45)
M-value (mg/kg/min)	3.3 ± 0.3
Adipose-IR x 10^{-2}	84.2 ± 11.2
Abbreviations: BP, blood pressure; LDL, low-density lipoprotein; HDL, high-density lipoprotein; DEXA, dual energy X-ray absorptiometry; EGP, endogenous glucose production; M-value, glucose infusion rate (high-dose insulin); IR, insulin resistance Data presented as mean ± s.e.m, median (IQR) or total number (%)	

In the entire cohort at baseline, M-value (peripheral insulin sensitivity) was strongly inversely correlated with liver fat percentage (r = -0.54, p < 0.01) (Figure 5-2) and hepatic insulin sensitivity index was also inversely correlated with liver fat percentage (r = -0.44, p = 0.04) (Figure 5-3).

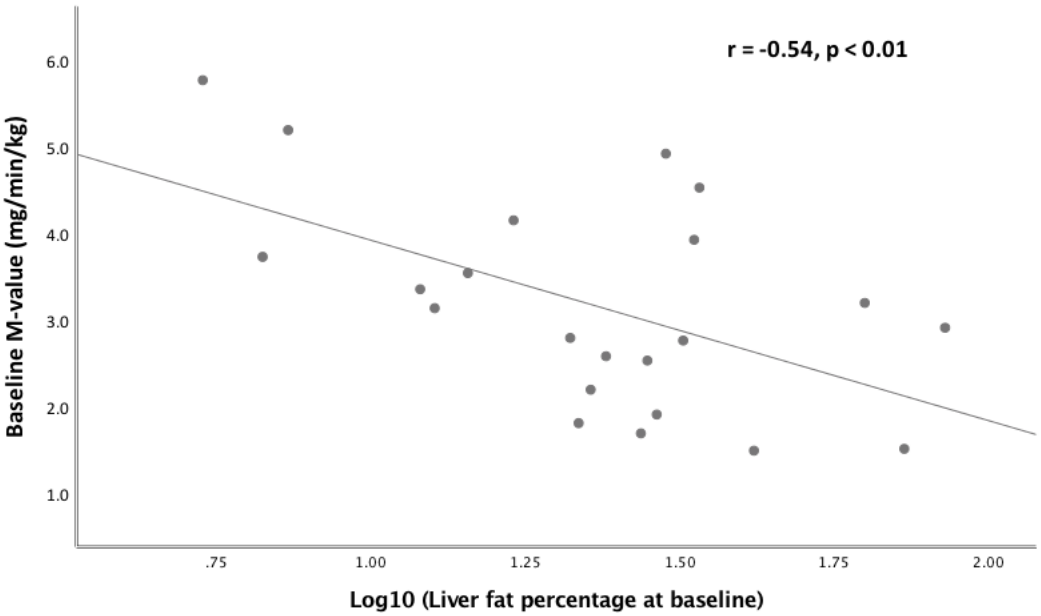

Figure 5-2 Scatter plot of relationship between liver fat percentage (Log10) and M-value in the entire cohort at baseline

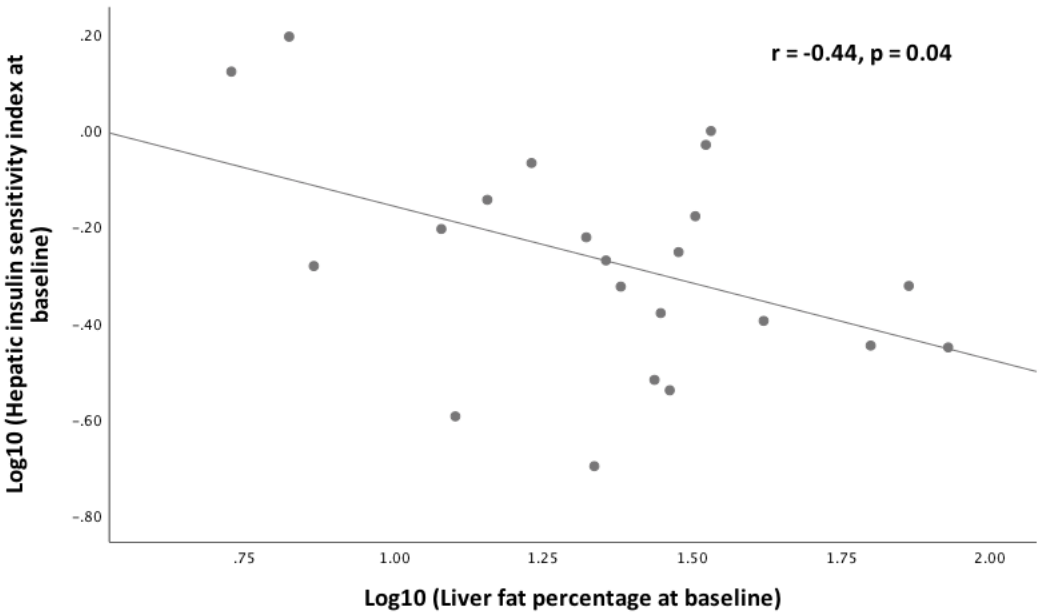

Figure 5-3 Scatter plot of relationship between liver fat percentage (Log10) and hepatic insulin sensitivity index in the entire cohort at baseline

5.3.2 Baseline and end of study analysis

Baseline and end of study characteristics of participants with a change in erythrocyte DHA enrichment of >2 % (n = 9) or <2% (n = 7) are shown in Table 5-2. One participant randomised to the placebo group had a significant increase in erythrocyte EPA and DHA enrichment (0.8% and 4.2%, respectively) between baseline and end of study measurements. This increase is most likely due to the participant consuming more oily fish or over-the-counter fish oil capsules during the course of the trial. As previously described regarding the WELCOME trial, there were a few cases of placebo contamination and lack of compliance with the intervention.[269] There was no significant change in body mass index or body fat percentage between baseline and end of study in either group (Table 5-2). Baseline liver fat percentage ranged from 5.5% to 85% and although liver fat decreased to a greater extent in the DHA > 2% compared with the DHA < 2% group, this difference did not reach statistical significance.

We measured erythrocyte FA composition as a surrogate marker for tissue, specifically liver, FA enrichment.[250, 329] The DHA > 2% group had a significant ($p < 0.001$) change in the erythrocyte enrichment of EPA (by > 300%) and DHA (by 92%) between baseline and end of study measurements, but there was no change in the DHA < 2% group (Table 5-2).

There was no difference between baseline and end of study measurements in the fasting plasma concentrations of glucose, insulin, non-esterified fatty acid, total cholesterol, LDL- or HDL-cholesterol (Table 5-2). Fasting plasma TG concentrations were significantly decreased by 0.6 mmol/L in the DHA > 2% group ($p < 0.001$), whilst concentrations remained unchanged in the DHA < 2% group (Table 5-2).

Whole-body insulin sensitivity (M-value) and peripheral glucose disposal (Rd) during the high-dose insulin stage did not change in either the DHA > 2% or DHA < 2% group between baseline and end of study (Table 5-3). Hepatic insulin sensitivity significantly increased in the DHA > 2% group over the course of the study ($p < 0.01$), with no change being observed in the DHA < 2% group (Table 5-3). We investigated whether a change in liver fat percentage was associated with changes in insulin sensitivity. In exploratory analyses, we stratified the cohort into two equal groups by the median change in liver fat percentage during the trial. Group 1 represented a “high” reduction in liver fat (range -3% to -53%), whilst Group 2 represented minimal change or increase in liver fat (range -1.3% to +33%). When we analysed the difference in percentage suppression of endogenous glucose production at the low-dose insulin step between the groups (as another measure of hepatic insulin sensitivity), percentage suppression was significantly better in Group 1 vs Group 2 (13.7% vs -3.8% (95% C.I. 1.4, 33.5, $p < 0.05$)). However, the difference in percentage increase of glucose disposal (measure of whole-body insulin sensitivity) was not significantly

Table 5-2 Comparison between baseline and end of study participant demographics and clinical characteristics stratified by change in erythrocyte DHA enrichment (> 2% or < 2%)

	DHA > 2% (n = 9)		**DHA < 2% (n = 7)**	
Variables	**Baseline**	**End of study**	**Baseline**	**End of study**
Group (treatment/placebo)	8 / 1		0 / 7	
Sex (M/F)	5 / 4		6 / 1	
Age (years)	45.7 ± 4.4		56.7 ± 2.5	
Weight (kg)	94.9 ± 5.4	95.4 ± 5.6	98.3 ± 1.6	95.9 ± 2.6
BMI (kg/m^2)	33.3 ± 1.2	33.4 ± 0.9	32.8 ± 1.3	31.8 ± 0.8
Waist circumference (cm)	110.8 ± 2.9	110.9 ± 2.9	111.8 ± 1.6	109.6 ± 1.2
DEXA % body fat	40.0 ± 2.1	39.8 ± 2.2	33.8 ± 3.3	34.8 ± 2.6
MRS liver fat (%)	34.4 ± 8.5	25.3 ± 6.1	18.9 ± 5.4	15.9 ± 12.3
MRI visceral mass (kg)	3.36 ± 0.43	3.53 ± 0.32	3.79 ± 0.34	3.41 ± 0.19
MAP (mmHg)	102.7 ± 3.6	99.4 ± 3.4	104.2 ± 4.8	102.9 ± 4.2
HbA1c (% total Hb)	5.8 ± 0.1	5.8 ± 0.2	6.0 ± 0.2	5.9 ± 0.3
Erythrocyte EPA (%)	0.82 ± 0.13	3.44 ± 0.47[b]	1.00 ± 0.10	0.90 ± 0.08
Erythrocyte DHA (%)	3.68 ± 0.60	7.08 ± 0.47[b]	4.62 ± 0.40	4.81 ± 0.26
Fasting glucose (mmol/L)	5.6 ± 0.2	5.9 ± 0.3	5.6 ± 0.2	5.5 ± 0.2
Fasting insulin (mU/L)	35 ± 6	33 ± 8	40 ± 11	21 ± 3
Total cholesterol (mmol/L)	4.5 ± 0.3	4.7 ± 0.4	5.1 ± 0.6	4.6 ± 0.3
HDL-cholesterol (mmol/L)	1.2 (0.7)	1.3 (0.5)	1.0 (0.3)	1.1 (0.1)
LDL-cholesterol (mmol/L)	2.6 ± 0.2	3.0 ± 0.3	2.5 ± 0.3	2.5 ± 0.4
TG (mmol/L)	2.1 ± 0.3	1.5 ±0.2[a]	2.3 ± 0.4	2.6 ± 0.5
NEFA (µmol/L)	550 ± 55	570 ± 74	653 ± 79	663 ± 80

Data presented as mean ± SEM or median (IQR)

Abbreviations: BMI, body mass index; DEXA, dual energy X-ray absorptiometry; MRS, magnetic resonance spectroscopy; MRI, magnetic resonance imaging; MAP, mean arterial pressure; HbA1c, glycated haemoglobin A1c; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; NEFA, non-esterified fatty acids

[a]$p < 0.01$; [b]$p < 0.001$ between baseline and end of study measurements within the respective groups

different (Group 1, 26.7% vs Group 2, 8.7% (95% C.I. -56.2, 92.3, p = 0.61)).

Table 5-3 Comparison between baseline and end of study markers of hepatic and peripheral insulin sensitivity in non-diabetic participants stratified by change in erythrocyte DHA enrichment (> 2% or < 2%)

	DHA > 2% (n = 9)		**DHA < 2% (n = 7)**	
Variables	**Baseline**	**End**	**Baseline**	**End**
Basal endogenous glucose production (Ra; µmol/min/kg FFM)	15.2 ± 0.8	14.4 ± 0.7	13.4 ± 0.7	14.0 ± 1.0
Low-dose insulin EGP (µmol/min/kg FFM)	8.7 ± 0.9	7.8 ± 0.7	7.1 ± 0.5	6.7 ± 1.0
High-dose insulin total body glucose disposal (Rd; µmol/min/kg FFM)	35.0 ± 3.1	34.3 ± 4.2	30.4 ± 3.5	35.9 ± 5.5
High-dose insulin total body glucose clearance (MCR; ml/min/kg FFM)	7.17 ± 0.84	6.79 ± 0.75	6.12 ± 0.73	7.26 ± 1.16
M-value (mg/kg/min)	3.22 ± 0.33	3.21 ± 0.34	3.23 ± 0.61	3.77 ± 0.73
Hepatic insulin sensitivity index (µmol/min/kg FFM) $(mU/L)^{-1} \times 10^2$	0.54 (0.46)	0.63 (0.47)[a]	0.52 (0.37)	0.55 (0.96)
Adipose-IR x 10^{-2}	75.5 ± 11.0	109.0 ± 38.9	110.0±27.6	67.9 ± 10.1

Data presented as mean ± SEM or median (IQR)

Abbreviations: Ra, Rate of appearance of glucose; Rd, rate of glucose disposal; EGP, endogenous glucose production; FFM, fat-free mass; MCR, metabolic clearance rate; M-value, glucose infusion rate (high-dose insulin); IR, insulin resistance

[a]$p < 0.01$ between baseline and end of study measurements within the respective groups

5.4 Discussion

We report here data demonstrating that individuals with NAFLD, who have an increase in erythrocyte DHA enrichment of > 2% (as a marker of tissue enrichment) through treatment with high-dose n-3 fatty acids for 15-18 months, showed favourable changes in hepatic, but not whole-body insulin sensitivity. We also confirm previous findings[125] of a significant inverse association between liver fat percentage and both peripheral as well as hepatic insulin sensitivity. Further analysis of our data revealed that a reduction in liver fat was also significantly associated with

improved hepatic, but not peripheral insulin sensitivity. Given that increased liver fat is associated with defects in insulin-mediated suppression of glucose production,[330] our study extends this observation by showing a significant improvement in hepatic insulin sensitivity over 18 months in association with reduced liver fat.

Our findings appear to be consistent with data reported by Petersen *et al*., who showed that moderate weight loss in obese, diabetic subjects through dietary intervention resulted in a significant reduction of liver fat to normal levels as well as an improvement in hepatic insulin resistance, but not whole-body IR.[331] Our study extends this finding also to a non-diabetic cohort. However, more recently, Cuthbertson *et al.* showed that supervised moderate-intensity exercise in non-diabetics reduced liver fat and improved peripheral IR but did not alter hepatic IR, despite significant liver fat reduction and weight loss.[332] This finding is surprising, given that the exercise group in that study had a 48% reduction in liver fat compared to the 26% reduction in the > 2% DHA enriched group in our study. Previous studies have already shown that hepatic insulin sensitivity is directly related to liver fat content, independent of age, sex, percent body fat, BMI and visceral fat.[127, 318, 333, 334] Although we only had a non-significant 26% reduction in liver fat in subjects who had high DHA enrichment, yet still showed significant improved hepatic insulin sensitivity, this may be explained by the pleiotropic effects of high-dose n-3 fatty acids on reducing intrahepatic inflammation. N-3 FAs are known to modulate transcription factors like nuclear factor-κB, which is known to precipitate hepatic inflammation, increasing local and circulating interleukin-6 and resulting in hepatic insulin resistance.[183] Hepatic tumour necrosis factor-α levels have also been shown to decrease with n-3 FA supplementation in a model of IR and NAFLD in rodents, augmenting PPARα expression and ameliorating fatty liver and the degree of liver damage.[335] Other postulated anti-inflammatory benefits of n-3 fatty acids include its effect on reducing reactive oxygen species, increasing expression of adiponectin (a potent insulin-sensitising adipokine produced in adipose tissue) and down-regulating expression of pro-inflammatory cytokines, chemokines and adhesion molecules,[207, 211, 213] all of which may promote hepatic insulin sensitivity.

Apart from its postulated anti-inflammatory effects, supplementation with n-3 fatty acids has been reported to notably decrease liver fat in some[269, 320] but not all[336] studies. In the present study as mentioned, participants who increased their erythrocyte DHA enrichment by > 2% had, on average a non-significant 26% decrease in liver fat content. In the main WELCOME study[269] we noted some individuals benefitted markedly from treatment with 4 g/day DHA+EPA whilst others derived no benefit; a result which could not be explained through lack of adherence to DHA+EPA treatment. The results of the present sub-study provide some insight to plausible mechanisms to explain why some individuals fail to derive a benefit from n-3 FA treatment in NAFLD, as we show

here that there are marked differences in hepatic insulin sensitivity according to whether DHA enrichment was < 2% or > 2%. Furthermore, we have also shown in a separate WELCOME sub-study that certain genotypes (e.g. PNPLA3 variant) can significantly influence DHA enrichment and liver fat percentage change despite high-dose n-3 FA supplementation, resulting in a blunted response to treatment.[337]

It is important to consider the strengths and limitations of our study. Although the sample size is small in this proof of concept study, we have undertaken a randomised double-blind, placebo-controlled trial testing the effects of the high-dose omega-3 FA intervention over a minimum period of 15 months. We have also used the gold standard test for evaluating hepatic and peripheral insulin sensitivity. Our sub-study was also affected by placebo contamination, which also affected a small proportion of the participants in the main WELCOME trial, and this may have attenuated the results. However, we used erthyrocyte DHA enrichment as a marker of 'compliance', which ensured we stratified the groups accurately to investigate our hypothesis. We also used 120-min steady state clamp stages, rather than 180-min which could have allowed us to be more assured of reaching a steady-state with the longer duration, although we were conscious of not wanting to inconvenience and tire the participants given the two-stage clamp study. However, there is also data to support good reproducibility of 120-min compared to 180-min clamp stages.[328]

In conclusion, this study confirms previous data reporting a significant inverse association between liver fat percentage and hepatic and whole-body insulin sensitivity. Importantly, it also shows that non-diabetic individuals with NAFLD treated with high-dose n-3 fatty acids with an associated significant increase in erythrocyte DHA enrichment (> 2 %), are conferred a significant improvement in hepatic insulin sensitivity despite a non-significant reduction in liver fat percentage. This suggests that the benefits of high-dose n-3 fatty acids in treating NAFLD go beyond liver fat reduction and may also include pleiotropic anti-inflammatory properties to improve insulin sensitivity. However, further research with larger trials are needed to explore these potentially interesting findings which have important therapeutic implications in the management of NAFLD.

Chapter 6: Discussion

In this research project, we have investigated several prognostic cardiovascular biomarkers, including carotid intima-media thickness, indices of left ventricular diastolic function and markers of insulin resistance, in association with NAFLD and the effects of high-dose n-3 polyunsaturated fatty acids treatment. This was all performed in the context of a randomised, double-blind, placebo-controlled trial (the WELCOME trial)[269] testing the effects of 15-18 months treatment with high-dose n-3 fatty acids (Omacor 4g/day) versus placebo in 103 asymptomatic subjects with NAFLD, on the reduction of percentage liver fat as its primary outcome. The importance of this research relates to the fact that NAFLD is an increasingly prevalent condition affecting up to 33% of the general population in varying degrees, and is strongly linked to insulin resistance, obesity and type 2 diabetes.[1] Importantly, NAFLD is not a benign condition and all-cause mortality appears to be increased in NAFLD subjects compared with an age and sex-matched reference population (OR = 1.57, 95% CI 1-18 – 2.10). Ample data suggest that cardiovascular mortality is the commonest cause of death in NAFLD,[7, 8, 12] and a recent meta-analysis reported an increased risk of CV mortality in NAFLD versus non-NAFLD groups (RR: 2.09; 95% CI: 1.46 – 2.98, $p < 0.001$).[46] Research into identifying potential prognostic CV biomarkers and the effect of therapeutic agents in NAFLD remains an unmet need, which is why we embarked on these studies.

In our main primary outcomes trial, although we did not report a significant effect of Omacor over 15-18 months on reducing liver fat percentage compared to placebo, we did show in secondary analysis that subjects who had a significant increase in erythrocyte DHA enrichment (a marker of tissue enrichment), in fact obtained a significant reduction in liver fat compared to subjects with little or no DHA enrichment. This was an important analysis as our WELCOME study suffered with a combined 11% 'biologically-tested' treatment deviation with likely poor compliance in the intervention group and equally, contamination in the placebo group with subjects likely taking increased over-the-counter (OTC) fish oil supplements. This is an important issue relating to such randomised-controlled trials and in fact was highlighted as a precautionary aspect in a recent systematic review and meta-analysis of interventional trials with omega-3 fatty acids in NAFLD.[215] The authors suggest that it is imperative that biological measures of compliance are assessed to ensure participants in the active intervention group consume the n-3 PUFA supplements and those in the control group do not increase their intakes of n-3 PUFAs (e.g. from marine sources or OTC supplements). With respect to our sub-studies, we had ensured that they were evaluated using the DHA enrichment analysis in addition to the Omacor ITT analysis. The other common strength in all of our studies is the use of the 'gold-standard' MRS as a diagnostic modality for assessing liver fat content.[30]

In the carotid IMT sub-study, we reported a novel independent association between improvement in NAFLD severity and reduced progression of carotid IMT over 18 months, after adjustment for traditional cardiometabolic risk factors. This finding was consistent for liver fat percentage as well as CK-18, a surrogate marker of NASH. These are very promising findings in terms of not only adding to the mounting evidence confirming increased CV risk with NAFLD, but more importantly, suggesting that CV risk in NAFLD can be modifiable by reducing NAFLD severity. As CIMT is known to be an independent predictor of future adverse CV events in an asymptomatic population,[76] our findings suggest that by reducing liver fat content in NAFLD subjects, we could potentially reduce their CV risk independently of other traditional cardiac risk factors. However, we must acknowledge the limitations of using CK-18 as a surrogate marker of NASH, as despite it being the most extensively studied non-invasive NASH biomarker with a pooled AUROC of 0.82,[29] it still has inconsistencies in terms of type of assay or subfraction used, so results from our study relating to NASH (rather than simple hepatic steatosis) must be interpreted with some caution.

In this study, we could not show any benefit of high-dose n-3 PUFA supplementation or significant DHA enrichment on CIMT outcomes compared to placebo. It is likely our study was underpowered to detect a potentially significant difference over this time period. Additionally, our cohort appeared to be at a lower CV risk than most of the previous populations studied using CIMT, given that our cohort's placebo group mean CIMT progression was up to 47% less per year compared to pooled results of placebo groups from previous studies, mostly representing secondary prevention cohorts.[273] To date, there has been a lack of suitably-sized RCTs that have evaluated this issue using the highest licensed doses of n-3 PUFA and this is certainly an area that merits further investigation.

In keeping with the CIMT study, we did not find any beneficial effect of high-dose n-3 PUFAs in improving several markers of LV diastolic function compared to placebo after 18 months. This finding was consistent even when we analysed the groups stratified according to significantly increased DHA enrichment or not. Current American Heart Association guidelines still recommend n-3 PUFAs for patients with heart failure and reduced LV ejection fraction but not for primary prevention, primarily due to the scarcity of RCTs evaluating the use of n-3 PUFAs in this setting.[234] Our negative result may again be related to our small cohort size as well as the low-risk subjects enrolled in the trial. Under 10% of subjects had 'severe' LV diastolic dysfunction if using the E/e' diagnostic thresholds.[308] To ensure suitable study power to potentially detect a significant improvement in LV diastolic markers with n-3 PUFAs, future studies should enrol a higher-risk cohort e.g. heart failure patients with preserved LV ejection fraction (HFpEF) in the first instance, all of whom will have significant LV diastolic dysfunction.[234]

Our study also showed an independent positive association between baseline percentage liver fat and baseline E/e', which adds to the current evidence suggesting that increased severity of fatty liver is linked to worse CV risk.[18] Importantly, our study extended that observation by showing for the first time a direct, independent association between percentage liver fat change and changes in markers of LV diastolic function over 18 months. This finding was independent of several putative aetiological variables that may have affected the association, including traditional CV risk factors, differences in lipid levels, change in weight, insulin resistance and percentage visceral fat difference, over the course of the study. Given that impaired LV diastolic function (with normal systolic function) is a potent, independent predictor of mortality and adverse CV outcomes,[260, 300] this finding suggests for the first time that reducing liver fat may also improve LV diastolic function over time, thereby decreasing the medium to long-term risk of adverse CV outcomes. However, given that this study was hypothesis-generating, further studies are required to confirm these findings in larger cohorts or extend these observations to higher-risk subjects e.g. patients with documented NASH who are at higher CV risk[272] and may be easier and more cost-effective to target in the clinical setting.

Given the strong link between insulin resistance and CV risk,[65, 120, 121] we also evaluated insulin resistance in a smaller sub-study using a two-step hyperinsulinaemic-euglycaemic clamp study, which is the gold-standard for assessing hepatic and whole-body insulin sensitivity. From baseline associations, we reported a significant relationship between liver fat percentage and both hepatic and peripheral insulin resistance, which is consistent with previous data showing that liver fat is the best independent predictor of insulin resistance.[125] We also found that hepatic, but not whole-body insulin sensitivity, was significantly improved in subjects with significantly increased (> 2%) erythrocyte DHA enrichment as a result of n-3 PUFA supplementation, despite a non-significant reduction in liver fat percentage over 18 months. We postulated that this beneficial effect on hepatic insulin sensitivity was likely, in part due to the pleiotropic anti-inflammatory effects of n-3 PUFAs.[211] Inflammation and insulin resistance can act synergistically to drive one another, through the effects of oxidative stress, increased production of proinflammatory cytokines from inflamed adipose tissue and the liver, resulting in a further increase in hepatic and peripheral insulin resistance, and ultimately causing endothelial dysfunction, hypercoagubility and CV disease[144] (Figure 1-3). This would suggest that high-dose n-3 fatty acid supplementation has a role in reducing liver fat as well as inflammation, and potentially could be used to treat more severe forms of NAFLD, such as steatohepatitis and liver fibrosis. However, a recent meta-analysis suggested no benefit in four studies evaluating n-3 PUFA treatment in NASH, using serial liver biopsies.[215] However, it is difficult to draw definitive conclusions regarding this as all of the studies were relatively small, compliance was poorly assessed and importantly, the DHA dose was

potentially subtherapeutic at < 800mg/day. Further research into the potential benefits of high-dose n-3 PUFA supplementation in treating NASH patients are desperately needed, given the higher CV and liver-associated risks of this group compared to simple steatosis.[8, 12]

To summarise and collate the findings from our research, we have found that significantly increased DHA enrichment through n-3 fatty acid supplementation over 15-18 months caused a significant reduction in liver fat, as well as improving hepatic insulin sensitivity. Conversely, this did not have a beneficial effect on prognostic CV biomarkers such as reducing carotid IMT progression or improving key LV diastolic function indices. However, we also found for the first time, an independent association between percentage liver fat reduction and reduced carotid IMT progression in the entire cohort over the duration of the study. Similarly, we described an independent association between liver fat reduction over 15-18 months and a reduction in markers of LV diastolic function across the entire cohort.

In conclusion, NAFLD is a marker of pathological ectopic fat accumulation combined with a low-grade chronic inflammatory state affecting adipose tissue and characterised almost universally by IR. This results in several deleterious pathophysiological processes including abnormal glucose, fatty acid and lipoprotein metabolism, increased oxidative stress, deranged adipokine profile, worsening subclinical inflammation, hypercoaguability, endothelial dysfunction and progression of atherosclerosis, ultimately leading to a dysfunctional cardiometabolic phenotype with potentially unfavourable CV outcome.[272] There is convincing evidence that worsening grades of NAFLD contribute to progressive cardiometabolic risk, such that NASH represents a marker as well as a mediator of increased CV risk more than simple steatosis. The majority of the studies discussed in this thesis, including our own research, point to an independent link between NAFLD and increased CV risk or adverse CV outcome. However, there is considerable heterogeneity in many of these studies in terms of outcomes measured as well as confounding variables not adequately adjusted for, but most importantly, in the method of NAFLD diagnosis and quantification of severity of NAFLD. This appears to be of paramount importance due to the disparate pathophysiological and metabolic consequences of the various stages of simple steatosis and NASH, both strongly linked to hepatic and peripheral IR.[318]

Although future studies should quantify liver fat using MR spectroscopy as a gold-standard, as we have performed in our research, there remains an issue over how to obtain reproducible non-invasive measures of hepatic necroinflammation and fibrosis to document NASH improvement, especially in randomised studies. Importantly, as steatohepatitis becomes more advanced, there is often a reduction in liver fat due to replacement of fat-laden hepatocytes with necrosed and fibrotic tissue, rendering liver fat measurements as a marker of NAFLD severity inaccurate.[1] It is

therefore imperative that future therapeutic trials in NAFLD also aim to include measurements of a range of validated cardiac, metabolic and inflammatory biomarkers linked to clinical outcome, to serve as alternative objective measures of the change in NAFLD status and its associated cardiometabolic phenotype. This may also allow better risk prediction when adjusting for the effect of conventional risk factors in determining the true CV risk of NAFLD.

Importantly, current research evaluating easily accessible novel biomarkers or combined clinical and biochemical algorithms to accurately grade the severity of NAFLD tend to focus too narrowly on liver–based outcomes, ignoring the detrimental cardiometabolic effects which are often the main cause of adverse clinical events. As we have shown with our research, the use of CV biomarkers such as CIMT and LV diastolic function markers have a role in prognostication in NAFLD. Furthermore, the cardiometabolic consequences of NAFLD are remarkably heterogeneous in terms of its interplay with visceral adiposity, IR and subclinical inflammation, and given that up to a third of the general population are estimated to have this condition,[2] a targeted strategy for pharmacological intervention would be challenging without outcome-based risk stratification. Therefore further research in this area is urgently needed to establish robust methods of predicting increased CV risk, as well as identifying novel treatments to improve the adverse CV outcome currently associated with NAFLD.

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