# Personalised Nutrition in Non-Alcoholic Fatty Liver Disease: Feasibility of a Nutrigenetic Therapeutic Approach

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# Abstract

Background: This multi-phased project investigates diet lifestyle care for patients with non-alcoholic fatty liver disease (NAFLD) and explores the feasibility of a genotype-driven randomised controlled trial (RCT) investigating the differential response to a Mediterranean diet (MD) intervention of patients according to genotype for the rs738409 (I148M) variant of *PNPLA3.*

Methods: Clinicians completed an e-survey to assess current practice and perceived barriers to the effective delivery of lifestyle interventions. In a meta-analysis, data from randomised and clinical controlled trials describing the effects of MD and calorie-restricted interventions (CRI) in NAFLD were synthesised. A randomised, crossover feasibility trial was undertaken. Participants were randomised to Diet 1 (MD) or Diet 2 (control i.e., habitual diet) for 4-weeks, separated by a 4-weeks washout period. The primary outcome was the feasibility, acceptability and effectiveness of the protocol. Secondary outcomes included assessment of liver fibrosis biomarkers and the influence of *PNPLA3* genotype.

Results: ***A cross-sectional survey*** revealed that provision of diet lifestyle care differs across centres and professional roles, and deviates from standard of care guidance. ***A meta-analysis*** found that dietary interventions (MD and CRI) improved NAFLD surrogate markers in as little as two weeks and improvements were sustained for up to two years. There was a dose-response relationship between degree of calorie restriction and beneficial effects on liver function and weight loss. The MD may be an effective diet therapy. ***Experimental data*** established the feasibility of a genotype-driven RCT and the effectiveness of a MD intervention, which rapidly improved cardiovascular risk (CVR) with evidence of early benefits on hepatic fibrosis. Carriers of the I148M variant appear to benefit less in terms of CVR factors when prescribed a MD intervention.

Conclusion: There is considerable variability in diet lifestyle care for patients with NAFLD. The effectiveness of calorie restriction and diet modification, observed in the meta-analysis, suggests this strategy should remain the cornerstone of NAFLD management. The findings of the feasibility study lay the foundation for a future definitive RCT, by informing trial design and optimising the dietary treatments, instruments and procedures.

# Acknowledgements

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A special thanks to collaborators Aberystwyth University, particularly Dr Thomas Wilson who generously provided knowledge and expertise, and members associated with the EU IMI2-funded Liver Investigation: Testing Marker Utility in Steatohepatitis (LITMUS) biomarkers consortium for their expert support and input. Additionally, this endeavour would not have been possible without the generous contributions from all the participants, thank you.

Finally, I would like to express my special appreciation and thanks to my family. Thank you, John for your unparalleled support and helping me every step of the way.

# Declaration

I declare that this thesis is an original report of my research, has been written by me and has not been submitted for any other degree or professional qualification. The experimental work is almost entirely my own work; the collaborative contributions have been indicated clearly and acknowledged. Due references have been provided on all supporting literatures and resources.

Parts of this work have been published, and my contribution and those of the co-authors to this work have been explicitly indicated. I confirm that appropriate credit has been given within this thesis where reference has been made to the work of others.

# Co-author Contributions

### Chapter 2: Defining Diet Lifestyle Care for patients with NAFLD

Study 1: The Effectiveness and Acceptability of Mediterranean Diet and Calorie Restriction in NAFLD: A Systematic Review and Meta-Analysis.

| | |
|---|---|
| **Study conception and design** | Laura Haigh (LH), Professor Quentin M Anstee (QMA), and Professor John C Mathers (JCM). Linda Errington assisted the formulation of search strategies. |
| **Acquisition of data** | LH conducted the database searches. LH, Colette Kirk (CK), Jennifer Gallacher and JCM assessed article eligibility and risk of bias. LH and CK performed data extraction. |
| **Analysis and interpretation of data** | LH conducted meta-analysis and meta-regression; Khalil El Gendy assisted with meta-analysis and meta-regression. |
| **Drafting of chapter** | LH. |
| **Critical revision** | QMA and JCM. |

### Chapter 2: Defining Diet Lifestyle Care for patients with NAFLD

Study 2: Diet Lifestyle Management of NAFLD: A Cross-sectional Survey of Clinicians.

| | |
|---|---|
| **Study conception and design** | LH, QMA, JCM and Professor Stuart McPherson (SMc). Professor Shira Zelber-Sagi (SZS) assisted with refinement of the e-survey. |
| **Acquisition of data** | LH.<br>The e-survey was circulated on the British Society of Gastroenterology website and emailed directly to clinical groups of (SZS) and the European Union IMI2-funded Liver Investigation: Testing Marker Utility in Steatohepatitis (LITMUS) biomarkers consortium. |
| **Analysis and interpretation of data** | LH. |
| **Drafting of chapter** | LH. |
| **Critical revision** | QMA and JCM. |

### Chapter 3: Nutrigenetic Therapeutic Approach for Patients with NAFLD: Study Protocol for a Randomised Controlled Feasibility Trial

| | |
|---|---|
| **Study conception and design** | LH, QMA, JCM and SMc. |
| **Acquisition of data** | Not applicable. |
| **Analysis and interpretation of data** | Not applicable. |
| **Drafting of chapter** | LH. |
| **Critical revision** | QMA and JCM. |

## Chapter 4: Randomised Controlled Feasibility Trial: Feasibility and Acceptability of The Study Protocol

| | |
|---|---|
| **Study conception and design** | LH, QMA, JCM and SMc. |
| **Acquisition of data** | Elsbeth Henderson (EH) and Lorna Brownlee (LB) assisted the collection of patient-reported outcome and open-ended response data. |
| **Analysis and interpretation of data** | LH.<br>LH and CK performed the thematic analyses. QMA and JCM were consulted to refine the analytical framework. |
| **Drafting of chapter** | LH. |
| **Critical revision** | QMA and JCM. |

## Chapter 5: Randomised Controlled Feasibility Trial: The Effectiveness of The Study Protocol

| | |
|---|---|
| **Study conception and design** | LH, QMA, JCM and SMc. |
| **Acquisition of data** | LH, EH and LB conducted physical measures. EH and LB assisted the collection of dietary intake measures and accelerometers. |
| **Analysis and interpretation of data** | LH.<br>Accelerometer data processing was conducted by Dr Sarah J Charman and Dr Alasdair P Blain. Aberystwyth University (Dr Thomas Wilson) performed the quantification of urinary dietary biomarkers, advanced statistical analyses and production of associated figures. Dr Olivier Govaere supported the creation of radar plots. |
| **Drafting of chapter** | LH. |
| **Critical revision** | QMA and JCM. |

## Chapter 6: Randomised Controlled Feasibility Trial: An Assessment of Preliminary Exploratory Data

| | |
|---|---|
| **Study conception and design** | LH, QMA, JCM and SMc. |
| **Acquisition of data** | EH and LB conducted physical measures. Genotyping was conducted under the mentorship of Dr Olivier Govaere. |
| **Analysis and interpretation of data** | LH.<br>Aberystwyth University (Dr Thomas Wilson) performed the quantification of urinary dietary biomarkers, advanced statistical analyses and production of associated figures. Dr Olivier Govaere supported the analysis and interpretation of genotyping data and creation of radar plots. Nordic Bioscience A/S, Denmark (LITMUS partners) measured PRO-C3 (N-terminal propeptide of type III collagen), PRO-C4, PRO-C5, CTX-III (crosslinked type III collagen). Dr Jeremy Palmer measured growth differentiation factor 15 (GDF15). |
| **Drafting of chapter** | LH. |
| **Critical revision** | QMA and JCM. |

# Publications and Presentations Associated with this Thesis

## Poster Presentations

- The European Association of Liver Disease (EASL) International Liver Congress (ILC): A MEDITERRANEAN DIET INTERVENTION has BENEFICIAL EFFECTS on BIOMARKERS of CARDIOVASCULAR RISK and HEPATIC FIBROSIS in NON-ALCOHOLIC FATTY LIVER DISEASE (NAFLD) (June 2022)
- EASL, The Digital ILC: Diet lifestyle management of non-alcoholic fatty liver disease (NAFLD): a cross-sectional survey of clinicians (August 2020)
- EASL, The Digital ILC: The effectiveness of Mediterranean diet versus calorie restriction in non-alcoholic fatty liver disease (NAFLD): a systematic review with clinical implications (August 2020)
- British Society of Gastroenterology: Lifestyle management of non-alcoholic fatty liver disease (June 2019)

## Publications

- Haigh L, Kirk C, El Gendy K, et al. The effectiveness and acceptability of Mediterranean diet and calorie restriction in non-alcoholic fatty liver disease (NAFLD): A systematic review and meta-analysis. Clin Nutr. 2022 Sep;41(9):1913-1931.
- Haigh L, McPherson S, Mathers JC, et al. Nutrigenetics-based intervention approach for adults with non-alcoholic fatty liver disease (NAFLD): study protocol for a randomised controlled feasibility trial. BMJ Open 2021;11: e045922.
- Haigh L, Bremner S, Houghton D, et al. Barriers and Facilitators to Mediterranean Diet Adoption by Patients with Nonalcoholic Fatty Liver Disease in Northern Europe. Clin Gastroenterol Hepatol. 2019 Jun;17(7):1364-1371: e3.

# Impact of COVID-19 Statement

This statement summarises the challenges of COVID-19 on the planned research and the mitigating actions taken. My contribution to the COVID-19 response entailed additional monthly clinics to help support patient care during this period. However, the impact of COVID-19 primarily related to work package 3: A Nutrigenetics-based Intervention Approach for Patients with NAFLD (randomised controlled feasibility trial). The study opened for recruitment 13th January 2020 and was paused in line with the mandated National Institute for Health and Care Research (NIHR) announcement (19th March 2020) and in response to Government advice.

This disruption was further exacerbated by closure of the university research facilities and suspension of non-COVID-19 clinical trials and experimental medicine studies within the host NHS organisation. Although, the study reopened for recruitment 21st July 2020 the loss of several months meant that recruitment was behind schedule. There was also a cumulative effect as a consequence of COVID-19 on the experimental work being undertaken (e.g., storage and transfer of samples, access to laboratories and research facilities, and delayed acquisition of required consumables).

An action plan was developed in discussion with my supervisors, as a realistic and achievable research strategy to mitigate the unavoidable disruption and to maximise benefit from the project. This plan was made to complete the research as effectively as possible, in consideration of the significant delay due to the stalled clinical trial, and the closure/ restrictions at research facilities and laboratories.

Changes included timely protocol amendments to allow both telephone and physical visits and to support a longer wash-out period. Whilst this reflected a change to the definition of the end of study, it did not impact on the safety of participants, study costings or the integrity of data collection. Recruitment targets were met and only one participant left the study for a non-related health issue. The study included an open-response questionnaire to capture perceptions of participation. This created an opportunity to explore how COVID-19 impacted upon participants diet and lifestyle patterns. These findings and insights may help shape our understanding and improve future trial design and dietary interventions.

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# Abbreviations

| | |
|---|---|
| $^{1}$H-MRS | Proton magnetic resonance spectroscopy |
| $^{1}$H- NMR | Proton nuclear magnetic resonance |
| AGE | Advanced glycation end-product |
| ALP | Alkaline phosphatase |
| ALT | Alanine aminotransferase |
| ARFI | Acoustic radiation force impulse |
| AST | Aspartate aminotransferase |
| AUROC | Area under the receiver operating curve |
| BASL | British Association for the Study of Liver Disease |
| BCT | Behaviour change technique |
| BIA | Bioelectrical impedance analysis |
| BMI | Body mass index |
| BP | Blood pressure |
| BSG | British Society of Gastroenterology |
| CAP | Controlled attenuation parameter |
| CCT | Clinical controlled trial |
| CD | Control diet |
| CENTRAL | Cochrane Central Register of Controlled Trials |
| CI | Confidence interval |
| CKD | Chronic kidney disease |
| CLD | Chronic liver disease |
| CLDQ | Chronic liver disease questionnaire |
| CLDQ-NASH | Chronic liver disease questionnaire for non-alcoholic fatty liver disease and non-alcoholic steatohepatitis |
| Cm | Centimetres |
| CMA | Comprehensive meta-analysis software |
| CONSORT | Consolidated Standards of Reporting Trials |
| COVID-19 | Coronavirus |
| CRI | Calorie-restricted intervention |
| CRP | C-reactive protein |
| CT | Computed tomography |
| CTX-III | Crosslinked type III collagen |
| CVD | Cardiovascular disease |
| CVR | Cardiovascular risk |
| DHA | n-3 docosahexaenoic acid |
| DN | Dietitians/nutritionists |
| DNA | Deoxyribonucleic acid |
| DNL | De novo lipogenesis |
| DPP-4 | Dipeptidyl peptidase-4 |
| EASL | European Association for the Study of the Liver |
| E-DII | Empirical dietary inflammatory index |
| EDTA | Ethylenediaminetetra-acetic acid |
| ELF | Enhanced liver fibrosis test |
| ELISA | Enzyme-linked immunosorbent assay |
| EQ-5D | Euroqol five dimension scale |
| ER | Endoplasmic reticulum |
| EVOO | Extra-virgin olive oil |
| FBC | Full blood count |
| FFA | Free fatty acids |
| FFM | Fat free mass |

| | |
|---|---|
| FFQ | Food frequency questionnaire |
| FIB-4 | Fibrosis-4 index |
| FIE-HRMS | Flow infusion-high resolution fingerprinting |
| FLI | Fatty Liver Index |
| GCP | Good clinical practice |
| GDF15 | Growth differentiation factor 15 |
| GEE | Generalized estimating equations |
| GGT | Gamma-glutamyltransferase |
| GWAS | Genome-wide association studies |
| HbA1c | Glycated haemoglobin |
| HCC | Hepatocellular carcinoma |
| HDL | High-density lipoprotein |
| HIV | Human immunodeficiency virus infection |
| HOMA-IR | Homeostatic Model Assessment of Insulin Resistance |
| HRQL | Health related Quality of Life |
| HSC | Hepatic stellate cell |
| HSD17B13 | hydroxysteroid 17-beta dehydrogenase 13 |
| IR | Insulin resistance |
| IV | Inverse variance |
| Kg | Kilograms |
| LCD | Low carbohydrate diet |
| LDL | Low-density lipoprotein |
| LFD | Low fat diet |
| LFT | Liver function test |
| LITMUS | Liver Investigation: Testing Marker Utility in Steatohepatitis |
| LOD | Limit of detection |
| LOQ | Limit of quantification |
| LPA | Light physical activity |
| LSM | Liver stiffness measure |
| MAF | Minor allele frequency |
| MAFLD | Metabolic associated fatty liver disease |
| MAP | Mean arterial pressure |
| MEDAS | Mediterranean diet assessment score |
| MD | Mediterranean diet |
| MDT | Multidisciplinary team |
| MetS | Metabolic syndrome |
| MGB | Minor groove binders |
| MPA | Moderate physical activity |
| MRI | Magnetic resonance imaging |
| MRM | Multiple reaction monitoring |
| MR-PDFF | Magnetic resonance proton derived fat fraction |
| MUFA | Monounsaturated fatty acid |
| n-3 | omega-3 |
| n-6 | omega-6 |
| NAFL | Non-alcoholic fatty liver |
| NAFLD | Non-alcoholic fatty liver disease |
| NAS | NAFLD activity score |
| NASH | Non-alcoholic steatohepatitis |
| NASH-CRN | NASH-Clinical Research Network |
| NFS | NAFLD fibrosis score |
| NHS | National Health Service |
| NIHR | National Institute for Health and Care Research |

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|---|---|
| NIT | Non-invasive test |
| NMES | Non-milk extrinsic sugar |
| NuTH | The Newcastle upon Tyne Hospitals NHS Foundation Trust |
| OSA | Obstructive sleep apnoea |
| PA | Physical activity |
| PC-LDA | Principal component linear discriminant analysis |
| PCOS | Polycystic ovarian syndrome |
| PCR | Polymerase chain reaction |
| PNPLA3 | Patatin-like phospholipase domain containing 3 |
| PPAR | Peroxisome proliferator-activated receptor |
| PPIE | Patient and public involvement and engagement |
| PRISMA | Preferred Reporting Items for Systematic Reviews and Meta-Analyses |
| PRO | Patient reported outcome |
| PRO-C3 | N-terminal propeptide of type III collagen |
| PROSPERO | Prospective Register of Systematic Reviews |
| PUFA | Polyunsaturated fatty acid |
| QICC | Corrected quasi likelihood under independence model criterion |
| QoL | Quality of life |
| RCT | Randomised controlled trial |
| REC | Research Ethics Service |
| RF | Random forest |
| RoB 2 | Risk of bias for RCTs 2.0 |
| ROBINS-I | Risk of bias in non-randomised studies of interventions |
| SAF | FLIP steatosis, activity, and fibrosis |
| SC | Standard care |
| SD | Standard deviation |
| SFA | Saturated fatty acid |
| SGH | Senior gastroenterologists/ hepatologists |
| SGLT2 | Sodium-glucose cotransporter-2 |
| SMD | Standardised mean difference |
| SNP | Single nucleotide polymorphism |
| SREBP | Sterol regulatory element binding protein |
| SRM | Selected reaction monitoring |
| SSB | Sugar-sweetened beverage |
| ST | Specialist trainees |
| SWE | Shear wave elastography |
| T2DM | Type 2 diabetes mellitus |
| TC | Total cholesterol |
| TE | Transient elastography |
| TM6SF2 | Transmembrane 6 superfamily member 2 |
| TG | Triglyceride |
| TMAO | Trimethylamine-n-oxide |
| Tw | Eigenvalues |
| UHPLC | Ultra High-Performance Liquid Chromatography |
| UK | United Kingdom |
| USA | United States of America |
| USnd | Ultrasound |
| VCTE | Vibration-controlled transient elastography |
| vLCD | very low-calorie diet |
| VLDL | Very low-density lipoprotein |
| VPA | Vigorous physical activity |
| WHR | Waist-to-hip ratio |

# Chapter 1

# Introduction and Background

## 1.1 Definition of NAFLD and its Contribution to Chronic Liver Disease

Non-alcoholic fatty liver disease (NAFLD) is the most common cause of chronic liver disease (CLD) worldwide [1], an urgent public health problem that is driving healthcare resource utilisation [2-4]. NAFLD is a spectrum that spans simple steatosis (non-alcoholic fatty liver, NAFL); through steatosis plus lobular inflammation with, hepatocyte ballooning (non-alcoholic steatohepatitis, NASH) and progressive fibrosis; ultimately to cirrhosis and potentially hepatocellular carcinoma (HCC) [5]. NAFLD is defined as ≥5% hepatic steatosis without evidence of secondary causes or excessive alcohol consumption (<20 g/day for females and <30 g/day for males) [6,7]. NAFLD is regarded as two pathologically distinct subtypes, NAFL and NASH [8].

NAFLD is a disease with different rates of dynamic progression and regression between stages, and different clinical manifestations among individuals (Figure 1.1) [9]. Only a minority develop advanced liver disease [9-12]. Identifying at-risk patients is challenging, but crucial to determine potential therapeutic interventions that can mitigate disease burden [13]. Multiple factors such as environmental, clinical, genetic and epigenetics drive disease progression [14].

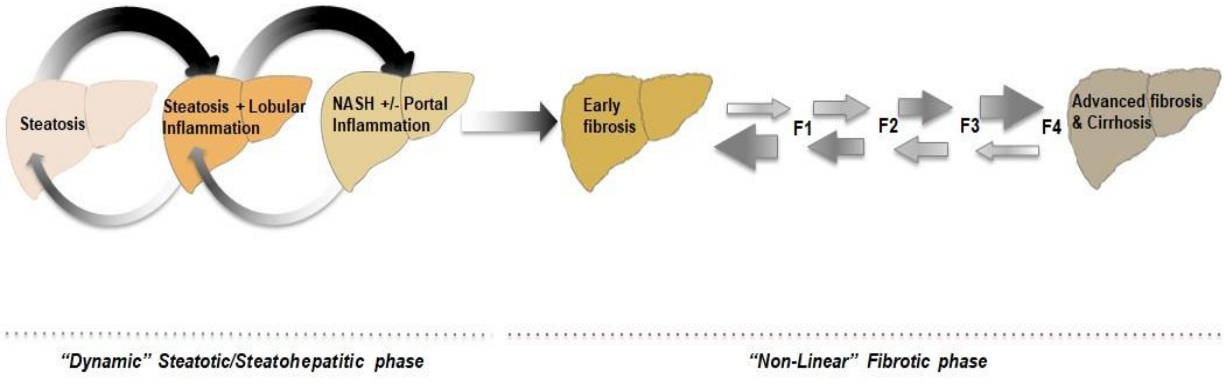



**Figure 1.1 Natural history of NAFLD adapted from reference [15] with permission of the author.** NASH is the biological driver of disease progression and fibrogenesis. The strongest predictor of liver-related mortality is advanced fibrosis stage (F3-F4).

Patients with NAFL are considered to have a relatively benign liver prognosis [16] although up to 40% will develop progressive NASH with an increased trend for advanced liver disease and liver-related mortality [11]. Recent research indicated that NAFL could be an evolving rather than benign phenotype [17]. Indeed, patients with NAFL and fibrosis stage 0 (F0) have an annual fibrosis progression rate of 0.07, corresponding to one stage per 14 years [12].

The strongest predictor of liver-related mortality is advanced fibrosis stage (F3-F4) [18]. The estimated rates of NASH progression to advanced fibrosis/cirrhosis are 10-25% over 8-14 years [19]. Currently, the most clinically relevant target is the presence of advanced fibrosis/cirrhosis [20]. The risk of developing clinically significant portal hypertension in patients with NASH cirrhosis is 52% at ten years [21]. The median survival of patients with NASH cirrhosis once decompensated is two years [22]. NASH attributed liver-related mortality is projected to increase by 178% by the end of the decade [23,24]. The growing number of patients with NAFLD, and potentially with progressive NASH, has large-scale clinical, economic and social implications [25]. NAFLD is the most rapidly increasing indication for liver transplantation both for end-stage liver disease and HCC [25,26].

Patients with NAFLD have higher cardiovascular disease (CVD) related morbidity and mortality, which is more common than liver-related mortality [5,27]. NAFLD contributes to cardiovascular disease (CVD) through a complex and interconnected relationship involving multiple mechanisms such as insulin resistance, hypertension, dyslipidaemia, proinflammatory factors and oxidative stress [28,29]. NAFLD can contribute to a prothrombotic state [29]. The presence of advanced fibrosis stage (F3-F4) further increases the risk of CVD [29].

### 1.2 Obesity and the Epidemiology of NAFLD

The prevalence of obesity (body mass index $\geq$30 kg/m$^2$ white populations; $\geq$27.5 kg/m$^2$ non-white minority ethnic populations) has nearly tripled worldwide since 1975, reaching epidemic levels [30]. In Europe, one third of children and almost two thirds of adults are overweight (body mass index 25-29.9 kg/m$^2$ white populations; 23 kg/m2-27.4 kg/m$^2$ non-white minority ethnic populations) or obese [31]. This upsurge has resulted in an increasing prevalence of obesity-related systemic diseases, including NAFLD [32]. The rising burden of NAFLD parallels the rise in prevalence of obesity [17,33,34]. Obesity increases NAFLD risk through multiple interactions of hormonal [35], metabolic and inflammatory factors, and involves adipose tissue expansion with the loss of capacity to store excess energy, insulin resistance, lipolysis and hepatic fat accumulation [17].

The prevalence of NAFLD is currently estimated to be 25%-33% of the global population [2,36]. The highest prevalence rates are in the Middle East (32%) and

South America (30%) [2], followed by Asia (27%), United States and Europe (24%) with the lowest rates in Africa (13%) [2]. NAFLD prevalence increases with increasing age [37], and is highest in males aged 40-65 years [36,38,39].

NAFLD prevalence and incidence has increased consistently over the past 20 years [2,25,40-43]. Recent meta-analytic data showed that prevalence rates increased from 26% in 2005 to 38% in 2016 [39], which also suggests previous reported prevalence rates were underestimates i.e., 15% in 2005 to 25% in 2010 [2,25]. NAFLD is highly prevalent in certain sub-populations including obese individuals and those with Type 2 diabetes (T2DM) (60-95% and 54-56% respectively) [44-48]. The prevalence of NAFLD is also increasing among non-obese individuals [49]. The prevalence of NAFL is higher among the Hispanic population, and lowest in Black populations [50-52]. A modest growth in future NAFLD cases of up to 30% across China, France, Germany, Italy, Japan, Spain, UK and USA have been projected by 2030 [53]. Nevertheless, NAFLD modelling certainty is impacted by the paucity of general population studies that accurately assess hepatic steatosis [53]. There are also insufficient population-based data on NAFLD incidence but meta-analytic data estimated the global incidence of NAFLD at 47 per 1000 person-years [36]. Further findings, indicate NAFLD incidence is significantly higher among males than females, with rates of 71 and 30 cases per 1000 person-years, respectively [36]. However, sex differences in NAFLD remain poorly understood [54]. NAFLD prevalence and incidence are forecasted to increase, driven by the persistence of obesity [24].

Similarly, the global prevalence of NASH has increased, but rates are lower than for NAFLD [25]. Current estimates suggest NASH affects between 2-6% of the general population, and up to 40% of patients with biopsy-proven NAFLD [2,46,55]. However, as the definitive diagnosis of NASH requires an invasive liver biopsy, these rates may be underestimated [6]. The prevalence of NASH increases among individuals with the metabolic syndrome (MetS) or T2DM partly due to the role of insulin resistance in the pathogenesis of NAFLD; and is higher in the Hispanic population and males [2,46,55]. The higher prevalence of NASH in the Hispanic population, can be attributed to a higher frequency of MetS and T2DM, as well the involvement of genetic factors [56,57]. The observed sex-based differences in NASH prevalence, can be influenced by biological sex differences such as genetics and hormones, as well as sociocultural background including dietary patterns [54]. The incidence of NASH-associated HCC is

estimated to be between 1-3% and can develop in the absence of cirrhosis [43,58-60]. Since HCC is the fourth-leading global cause of cancer-related mortality [61], these data underscore the growing disease burden [43].

### 1.3 The Aetiology of, and Risk factors, for NAFLD

NAFLD is strongly associated with MetS [5] and develops frequently in the presence of obesity/ abdominal adiposity (60-95%) [44,47,48], hyperlipidaemia (69%) [2], T2DM (54-56%) [45,46], and hypertension (39%) [2]. Body mass index (BMI) and waist circumference, as a measure of visceral adiposity, are positively related to risk of NAFLD [62]. Irrespective of baseline BMI status, weight gain of 3-5kg, is predictive of NAFLD development [63].

Emerging disease associations include polycystic ovarian syndrome (PCOS), obstructive sleep apnoea (OSA), thyroid disorders and psoriasis [25]. Demographic risk factors comprise age, sex, and ethnicity, with increased risk found in Hispanic and Asian populations, in males and in older adults [25,64]. In the Asian population, fat accumulation can occur at a lower BMI threshold [65]. However, more data from population-based studies in Africa are needed to elucidate the disease profile in this region [66]. Limited available evidence suggests that the rising rates of obesity and T2DM (T2DM risk occurs at a lower BMI threshold than in non-African populations) [67], as well as human immunodeficiency virus infection (HIV) burden may be contributing factors [66,68].

Genetic and epigenetic predisposition are associated with disease susceptibility. Recent advances in NAFLD-related genetics provides robust evidence of associations between variations in genes and disease trajectory [69]. Several genetic variations have been identified, including the well-established non-synonymous single nucleotide polymorphisms (SNPs) in the genes patatin-like phospholipase domain containing 3 *(PNPLA3)* rs738409 (C>G I148M) and transmembrane 6 superfamily member 2 *(TM6SF2)* rs58542926 (C>T E167K), as well as the more recently described non-coding genetic variants in hydroxysteroid 17-beta dehydrogenase 13 *(HSD17B13)* such as rs9992651 (G>A) or rs13118664 (A>T) [70]. The *PNPLA3* and *TM6SF2* variants have been associated with an increased risk of NAFLD development, and the HSD17B13 variants confer reduced risk [70-72].

Disease progression is more likely in individuals with obesity or T2DM, i.e., the 'high-risk groups' [44]. Advanced liver disease is associated with increasing number of metabolic co-morbidities [73], and T2DM the most significant risk factor for the development of HCC [43]. Limited data suggests slight differences in the natural history of male/female NASH [74], with males exhibiting more advanced disease [75,76]. Increasing age, is an important risk factor for advanced fibrosis (fibrosis stage F3-F4), reflecting either disease duration and/or a cumulative effect of metabolic co-morbidities [77,78]. Limited evidence suggests that compared to non-Asian individuals, Asians may exhibit higher grades of ballooning and more lobular inflammation [50].

Extrahepatic complications of NAFLD include increased risk of hypertension, chronic kidney disease (CKD), MetS, impaired glucose regulation, T2DM and malignancy [64]. NAFLD is an independent risk factor for development of CVD [5]. Previous research reported that NAFLD was associated with a nearly two-fold increased risk of CKD [79,80], MetS and T2DM [81]. Meta-analytic data from observational cohort studies suggests that NAFLD is associated with long-term risk for extrahepatic cancers such as gastrointestinal, breast and gynaecological [82]. Table 1.1. summarises both established and emerging disease associations, and extra-hepatic complications.

| **Extra-hepatic complications** | **NAFLD prevalence in established disease associations** | **Emerging disease associations** |
|---|---|---|
| CVD | MetS (42%) [2] | PCOS |
| CKD | Obesity (60-95%) [44,47,48] | OSA |
| Malignancy | T2DM (54-56%) [45,46] | Thyroid disorders |
| | Hyperlipidaemia (69%) [2] | Psoriasis |
| | Hypertension (39%) [2] | |

**Table 1.1 Extra-hepatic complications and disease associations of NAFLD.** CVD, cardiovascular disease; CKD, chronic kidney disease; MetS, metabolic syndrome; T2DM, Type 2 diabetes; PCOS, polycystic ovarian syndrome; OSA, obstructive sleep apnoea.

## 1.4 Diagnosis, Staging and Grading of NAFLD

A diagnosis of NAFLD is a diagnosis of exclusion based primarily on establishing increased hepatic fat content with the exclusion of excess alcohol and other causes of liver disease [18]. Increased hepatic fat content may be detected histologically or by imaging techniques like ultrasound (USnd), computed tomography (CT) or magnetic resonance imaging (MRI), in the presence of metabolic risk factors.

Elevated liver enzymes alone should not be relied upon to diagnose patients [83]. Magnetic resonance proton derived fat fraction (MR-PDFF) is the most accurate imaging to assess steatosis, but it is expensive and not readily available in clinical practice [18]. USnd is the most widely-used imaging tool with relatively high diagnostic accuracy for >30% of steatosis (85% sensitivity and 94% specificity) but has limited precision for mild steatosis [84]. As a more recent modality, controlled attenuation parameter (CAP) by transient elastography (TE) has shown clinical utility (cutoff of 248 dB/m yields 69% sensitivity and 82% specificity) [85,86], but lacks precision for mild steatosis, and is hindered by operator dependency and measurement error in patients with high BMIs [87,88]. Among biomarker panels, the Fatty Liver Index (FLI) is a simple algorithm using routine clinical data that has been proposed as a useful tool to detect patients at increased risk of steatosis [89]. The FLI has evident strengths due to its simplicity, but it is not designed to reflect dynamic changes in NAFLD status over time when serial measurements are taken. Meta-analytic findings suggest that while it may have value in risk stratification, its performance in diagnosing or excluding NAFLD is limited [90]. Moreover, FLI has been found to be inaccurate at distinguishing between mild and moderate-to-severe steatosis [91].

Once NAFLD is diagnosed, grading disease activity and staging fibrosis progression are critical to determine prognosis. Liver biopsy is the reference standard to differentiate NAFL from NASH [20]. However, this procedure is costly, invasive and associated with poor patient acceptability and risks to health, making it impractical for general screening and monitoring purposes [20,92]. Sampling errors and intra- and inter-observer variability diminishes its precision [20,93,94]. Liver biopsy has other limitations, which includes inaccurate assessment of fibrosis burden via the use of two semi-quantitative scoring systems for histological staging [20]. Both scoring systems, NASH-Clinical Research Network (NASH-CRN) and FLIP steatosis (S), activity (A), and fibrosis (F) (SAF) aims to assess steatosis, lobular inflammation, ballooning and fibrosis [95,96]. The future adoption of objective artificial intelligence-based strategies could potentially improve the assessment of hepatocyte injury and diagnose NASH [94].

These limitations have driven the pursuit of non-invasive tests (NITs) for accurate fibrosis staging and risk stratification. In the current era, some of these endeavours

have already positively impacted clinical practice [20]. The Fibrosis-4 index (FIB-4) [97], the NAFLD Fibrosis Score (NFS) [98], which combine aspartate aminotransferase (AST)/ alanine aminotransferase (ALT) and platelet count alongside other routine components [20] and the enhanced liver fibrosis (ELF) test [99] that focuses on matrix turnover are the most widely adopted serum makers [20]. NFS and FIB-4 are first-line tests that effectively rule out advanced fibrosis if patients have a low score (using age-appropriate cut-offs), whereas indeterminate or high scores require second-line investigation [18]. Based on recent data, FIB-4 and NFS have comparable prognostic performance to histology as significant predictors of clinical outcomes such as event-free survival and adverse events at 5 years (time dependent area under the receiver operating curve (tAUROC) of 0.74; 0.70; and 0·72)) respectively [100].

Second line investigation or triage might include biomarker panels such as the ELF test, and elastography procedures to confirm or exclude advanced fibrosis [18]. The ELF test demonstrates high sensitivity (i.e., >0.90), but yields limited specificity and positive predictive value (i.e., >0.80) at low cut-offs [101]. Thus, the ELF test exhibits a more limited performance in low prevalence clinical settings, in terms of its low positive predictive value [101]. MR- or USnd- based elastography are emerging techniques, found to effectively measure liver stiffness as a surrogate for hepatic fibrosis, and vibration-controlled transient elastography (VCTE) is the most adopted [20]. Although, each method has inherent strengths and limitations including confounding factors, measurement failures and operator dependency [20]. VCTE has been found to have prognostic performance (29.4% sensitivity and 92.0% specificity) that is comparable to histologically assessed liver fibrosis (33.3% sensitivity and 90.5% specificity) in predicting clinical outcomes after 5 years [100]. Moreover, in a comparative analysis, when compared with FIB-4, VCTE demonstrated acceptable accuracy for the detection of advanced fibrosis (AUROC of 0.73 versus 0·83) [102].

Given the imperfect performance of existing biomarkers, intense research efforts for more effective NITs continues apace. Emergent evidence has shown promising advances in biomarker panel development. A prospective study of 449 patients with biopsy-proven NAFLD found that plasma levels of PRO-C3 (N-terminal propeptide of type III collagen), a biomarker of active fibrogenesis, correlated with severity of NASH and fibrosis stage [103]. Across eight reviewed studies, PRO-C3, detected advanced fibrosis (area under the receiver operating curve (AUROC) of 0.79)) in 2058 patients

with NAFLD [104]. Preliminary data indicates, ADAPT [105], a combined PRO-C3, age, T2DM and platelet count algorithm, performs slightly better than either PRO-C3 alone and FIB-4 for the detection of advanced fibrosis [103]. Consistently, a large comparative diagnostic accuracy study reported that the ADAPT score, detected advanced fibrosis with acceptable accuracy (AUROC of 0·85). In the same study the SomaSignal test, outperformed all other markers with an AUROC of 0·90 [102]. However, more extensive study is needed to improve the accurate detection of NASH and clinically significant fibrosis ($\geq$F2) [101]. In the future, precision medicine-based biomarkers offer an opportunity to build on these advances to maximise clinical impacts for this patient population [20].

### 1.5 NAFLD Pathogenesis: Lifestyle and Genes

An extensive discussion of the pathogenesis of NAFLD is beyond the scope of this thesis. NAFLD is a multifaceted disease and the underlying mechanisms are not fully elucidated [106]. There is substantial inter-individual variation in NAFLD susceptibility, progression and outcome, due partly to gene-environment interactions [5,26]. A group of experts have recently challenged the NAFLD nomenclature, with an increased focus on the importance of predisposing metabolic risk factors in the complex and heterogeneous disease trajectory [107]. Hence, metabolic associated fatty liver disease (MAFLD) has been suggested as a more suitable overarching term [107]. This proposed change, which involves serious conceptual changes, has generated much debate and led to calls for further consensus discussions [108,109].

The development of NAFL is associated with obesity, visceral adiposity, insulin resistance (IR) and chronic hyperinsulinemia [110,111], encouraging delivery of lipogenic substrates to the liver [112,113]. Hepatic fat accumulation is exacerbated by hypercaloric diets, high sugar intake and sedentary lifestyles [111,114], and influenced by genetic polymorphisms [115]. Hepatic steatosis results from increased triglyceride (TG) production using fatty acids from dietary intake and/or released from adipose tissue [116,117]. Increased free fatty acids (FFAs) flux to the liver alongside increased hepatic de novo lipogenesis (DNL), leads to substrate overload [111] and major abnormalities of hepatic lipid metabolism [111,118] (Figure 1.2). FFAs, free cholesterol, as well as other lipid metabolites have been identified as toxic species [119].
The NAFL to NASH transition is incompletely characterised, although multiple parallel insults, and many pathogenic factors have been hypothesised [119,120] (Figure 1.2).

Hepatocyte lipotoxicity, the harmful effects of fat accumulation, induces inflammatory cytokine release [111] and triggers various molecular pathways of cell stress, such as mitochondrial dysfunction [120,121]. Upregulated proteins in multiple pathways lead to mitochondrial dysfunction and apoptosis [120]. Increased hepatic oxidative stress which drives inflammation arises from numerous hepatocyte-damaging factors and may also act as an upstream mechanism to activate endoplasmic reticulum (ER) stress [111,121]. Reduced ER efficiency and ER stress triggers the unfolded protein response, which can lead to apoptosis [120]. Numerous immune cell-mediated inflammatory responses are involved in disease progression [122]. NASH has been associated with alteration of the intestinal barrier, and microbiota composition and metabolome [9,111,119]. Increased retinol metabolism and changes in bile acid metabolism are potential pathways involved in the process of fibrosis [123]. Hepatic stellate cell (HSC) activation triggers fibrogenesis [111]. Accordingly, advanced NASH is associated histological evidence of hepatic inflammation, injury and fibrosis [111].

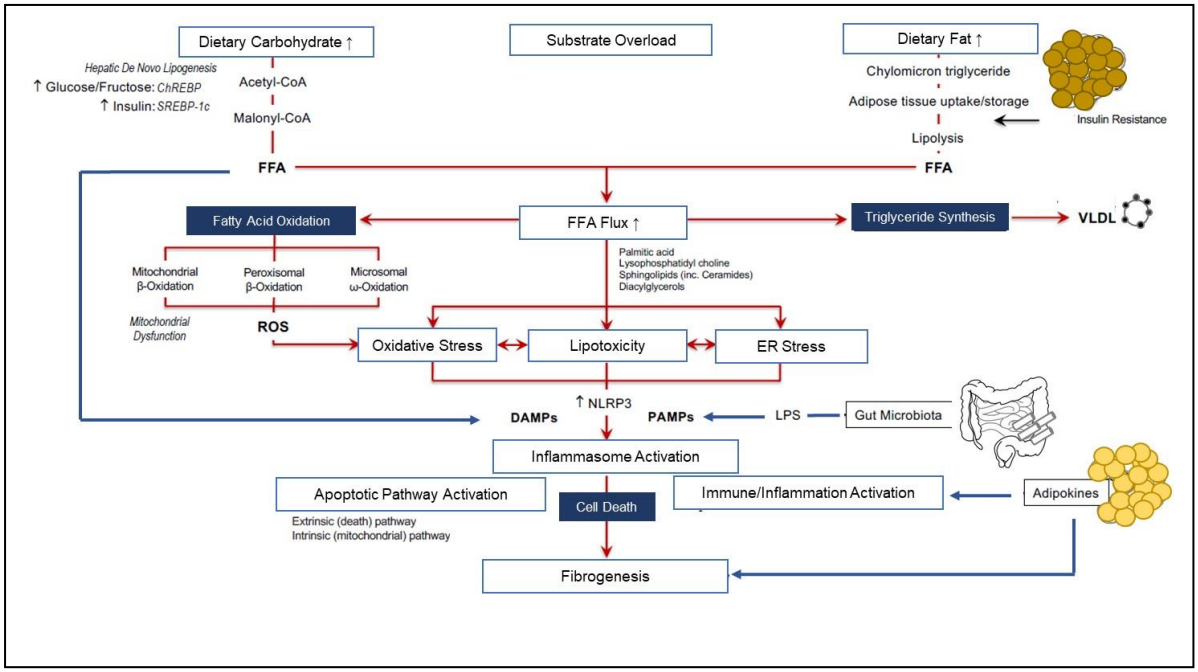



**Figure 1.2 NAFLD pathogenesis adapted with permission of the author (Professor Quentin Anstee).** This overview focuses on NAFLD progression pathophysiology. CoA, coenzyme A; ChREBP, carbohydrate-responsive element-binding protein polycystic ovarian syndrome; SREBP-1c, sterol regulatory element binding protein; VLDL, very low-density lipoprotein; ROS, reactive oxygen species; DAMPs, damage-associated molecular patterns; PAMPs, pathogen-associated molecular patterns; NLRP3, NOD-, LRR- and pyrin domain-containing protein 3; LPS, lipopolysaccharide.

### *1.5.1 Lifestyle*

A triple-hit behavioural phenotype of excess weight and/or weight gain, the western dietary pattern, and a sedentary lifestyle has been linked with disease pathogenesis [13,124]. Western diets are hypercaloric and abundant in NAFLD-promoting components; excessive refined and processed foods, alcohol, salt, red meats, sugar-sweetened beverages (SSBs) and snacks [125,126]; with inadequate amounts of fruits, vegetables, wholegrains, legumes (and therefore fibre and antioxidants), fish and low-fat dairy products [127]. Advanced glycation end-products (AGEs) levels elevated in western diets, have pathogenic significance, contributing to hepatic injury, inflammation, and fibrosis [128-131]. NAFLD patients typically consume less polyunsaturated fatty acids (PUFAs) [132,133], and more saturated FAs (SFAs) and cholesterol, and have a higher omega-6/omega-3 (n-6/n-3) PUFA ratio [134,135].

Dietary fats and carbohydrates prevent or contribute to NAFLD development [136-139]. Excess intake of trans FAs [140,141], SFAs [142-144] and n-6 PUFA [145] encourages pathogenesis. Data derived from animal research suggests that trans FAs modulate Kupffer cell function, up-regulate lipogenic gene expression and increase steatosis [146]. SFAs increase SFAs within the liver such as palmitate (16:0) [117], and also promote ceramide synthesis [147,148]. Ceramides impact molecular pathways involved in IR, mitochondrial dysfunction and inflammation [149]. Additionally, excess intake of SFAs has been associated with increased oxidative stress and impaired glutathione metabolism, both of which have been implicated in disease progression [141]. In comparison with PUFAs, excess intake of SFAs increase steatosis (via increased DNL and adipose tissue lipolysis) [141-143] and visceral adiposity [143,144]. SFAs have been shown to increase hypothalamic inflammation [139], and preliminary data from animal research suggests SFAs induce ER stress and hepatocyte injury [150].

PUFAs have been shown to suppress the expression of Sterol Regulatory Element Binding Protein-1c (SREBP-1c), which is involved in the regulation of lipogenic enzyme genes [151], leading to a decrease in DNL [152]. N-3 PUFAs have favourable effects on steatosis [153,154] and insulin sensitivity [155]. N-3 PUFAs have anti-inflammatory properties, and modulate hepatic lipid metabolism, reducing DNL [139]. However, studies investigating the effects of n-3 treatment across the NAFLD spectrum have reported conflicting findings and varying effectiveness [13]. In contrast, higher dietary intakes of n-6 PUFA may result in an increased uptake of n-6

within the liver and promote the accumulation of oxidized linoleic acid metabolites (OXLAMs) [156]. OXLAMS have been shown to promote pathogenesis in mice through NOD-like receptor protein 3 (NLRP3) inflammasome activation, and mechanisms involved in mitochondrial dysfunction and hepatocyte cell death [157]. A high monounsaturated FA (MUFA) intake has been shown to reduce steatosis in patients with T2DM [158]. High MUFA intake increases FA oxidation, through activated peroxisome proliferator-activated receptors (PPARs) activity and reduces lipogenesis through diminished SREBP activity [158].

Previous research has demonstrated that higher carbohydrate intakes are associated with increased risk of fibrosis severity [159]. Excess consumption of monosaccharides i.e., glucose and fructose or of amino acids leads to a conversion of these substrates into SFAs (e.g., palmitate (16:0)) via DNL [160-162]. Research has clarified the role that added sugars, and more prominently SSBs have in NAFLD pathogenesis [13]. The intake of fructose typically found in SSBs induces IR, stimulates DNL as a preferential substrate and increases steatosis [141,163,164]. Epidemiological studies show an association between consumption of SSBs and fibrosis severity in patients with NAFLD [162,165]. Seven or more cups of SSBs per week are associated with a 53% increased risk of NAFLD, in a dose-response relationship [166]. On the other hand, low glycaemic index foods and fibre (e.g., wholegrains, legumes, vegetables) [167] are considered beneficial in this patient population [141]. Recent findings from a cross-sectional study revealed an association between higher dietary fibre intake and lower odds of NAFLD [168]. In an RCT, improvements in three markers of liver fibrosis were observed with a higher insoluble fibre intake (≥ 7.5 grams/day), while fruit fibre consumption (≥8.8 grams/day) improved liver enzymes [169]. High fibre consumption can improve insulin sensitivity, stimulate anorexigenic hormones that suppress appetite, and has been associated with promoting weight reduction [170-173]. Fibre-rich foods have potential to improve inflammation and liver injury by modifying gut microbiota, such as increasing the beneficial bacteria Bifidobacterium [171].

Conflicting evidence suggests that choline and methionine deficiency may contribute to the pathogenesis of NAFLD [174-176]. Choline and Methionine may have a role in very low-density lipoprotein (VLDL) synthesis and hepatic β-oxidation [175]. Additionally, in animal models isoflavones have been shown to suppress DNL and

activate hepatic β-oxidation [177]. Currently, there is a scarcity of information about the association between dietary factors and HCC [13,178].

### *1.5.2 Genes*

Recent advances in NAFLD-related genetics provides robust evidence of associations between gene variants and disease trajectory [69]. Genome-wide association studies (GWAS) have identified variants associated with distinct hepatic abnormalities such as steatosis, inflammation, and fibrosis, and variants that are associated with the full disease spectrum spanning steatosis to cirrhosis [179].

A GWAS performed in 2008 among a multiethnic population provided initial evidence of the association between the *PNPLA3* rs738409 SNP and NAFLD [180]. In this study, the frequency of the *PNPLA3* variant was highest in Hispanics (49%), with lower frequencies in European Americans (23%) and African Americans (17%) [180]. Data indicates that the minor allele frequency in the general population for *PNPLA3* rs738409 ranges from 12% in African, 23% in European, 25% in South Asian, 35% in East Asian to 48% in American populations [181]. Since then, numerous GWAS have robustly shown that the *PNPLA3* rs738409 SNP is the most important genetic risk factor for NAFLD [182]; and its association with the entire spectrum of the disease has been confirmed in various populations [70,115,183-186]. The *PNPLA3* rs738409 SNP is a common modifier of disease outcome, with its impact amplified by adiposity [70,183,184,187,188]. This additive effect poses considerable concern, as patients with NAFLD tend to consume excess calories as a consequence of poor-quality diets and sedentary lifestyles [13,185,189]. Studies suggest that carriage of the *PNPLA3* polymorphism not only confers increased risk of inflammation and fibrosis, but also for HCC [183,190].

The PNPLA3 protein has lipase activity and regulates lipid droplets in hepatocytes and hepatic stellate cells [185,191] and several studies have contributed to understanding of its function in hepatic lipid handling [192]. The *PNPLA3* rs738409 SNP is a 'loss-of-function' variant resistant to ubiquitylation and degradation [193,194]. There is greater *PNPLA3* rs738409 SNP abundance on lipid droplets; leading to sequestration of CGI-58 and impairing ATGL-catalysed lipolysis and lipophagy; which results in increased lipid droplet accumulation [194]. The *PNPLA3* rs738409 SNP has the potential to influence treatment responsiveness [182,195]. Thus, among the

identified NAFLD-related genes, *PNPLA3* has the most potential as a therapeutic target for NAFLD [183-185].

A recent GWAS conducted on a large histologically defined cohort confirmed the well-established signal in *TM6SF2* [70]. Evolving evidence suggests *TM6SF2* rs58543926 SNP reduces VLDL-TG secretion from the liver, leading to hepatic TG accumulation [196]. The *TM6SF2* rs58543926 SNP has been implicated in NASH and associated with increased risk of fibrosis progression [72,197]. Paradoxically, carriers of the variant have improved lipid profile and reduced risks of atherosclerosis and myocardial infarction, indicating a cardioprotective role [72,198-200]. The minor allele frequency in the general population for *TM6SF2* rs58543926 ranges from 2% in African, 6% in American, 7% in European, 9% in East Asian to 11% in South Asian populations [201]. The rs58543926 SNP in *TM6SF2* has been identified as a promising therapeutic target in NAFLD due to its role in lipid metabolism, and its potential to ameliorate cardiovascular comorbidity [202].

Genetic associations with glucokinase regulatory protein (*GCKR*) [70], membrane-bound O-acyltransferase domain-containing 7 (*MBOAT7*) [179] and the full disease spectrum have been reported. Although, the underpinning mechanisms are unclear, the *GCKR* gene variant, specifically the rs1260326 (C>T) SNP has been associated with steatosis [203], fibrosis [70] and liver function [204]. The rs641738 (C>T) SNP in the *MBOAT7* gene has been implicated in the NAFLD pathogenesis [179], and independently associated with fibrosis development through an imbalance in phosphatidylinositol species [205]. However, the findings from a large GWAS performed on a histologically defined cohort indicate that *MBOAT7* is not a risk loci for NAFLD [70].

On the other hand, there are genetic associations that have only been identified with distinct features of NAFLD [179]. Notably, the loci associated with steatosis include pygopus family PHD finger 1 (*PYGO1*) [70], apolipoprotein E (*APOE*) and protein phosphatase 1 regulatory subunit 3B *(PPP1R3B)* [203,206-208]. Furthermore, leptin receptor (*LEPR*) has been associated with histological characterised NASH [70]. HSD17B13 is a lipid droplet-associated protein, but its function, as well as the mechanisms by which its variants reduce NAFLD risk, are still ambiguous [209].

## 1.6 NAFLD Management: Primary and Secondary Care

The clinical management of individuals with NAFLD in primary and secondary care is described in Figure 1.3. Optimum NAFLD management requires early identification, liver specific treatment and concomitant management of MetS comorbidities [6,17]. Nonetheless, the need for accurate non-invasive diagnosis, staging and grading, and approved pharmacological treatment remain unmet [210,211].

Patients stratified as low risk of advanced fibrosis based on NITs (e.g., FIB-4 score or NFS) can be routinely managed in the community. These patients should receive advice on lifestyle and cardiovascular risk reduction, with risk of fibrosis re-assessed in three years [83]. Patients stratified as indeterminate risk require further discriminatory tests such as TE or ELF, performed either in primary care or secondary care. High risk patients are referred to secondary care for further investigations [83].

Secondary care involves a confirmation of diagnosis and fibrosis stage, with liver biopsy a consideration. Selected patients may be offered NASH drug therapies with trial evidence (i.e., pioglitazone [212] or liraglutide [213] for patients with T2DM; vitamin E [214] in selected nondiabetic patients) and access to relevant clinical trials [83]. Multidisciplinary management of advanced NAFLD is recommended [6,83,215] targeting:

- The obesogenic lifestyle (weight loss, dietary change, increasing physical activity levels, metabolic surgery).
- The MetS to reduce CVD risk.
- The liver disease to ameliorate hepatic steatosis and prevent progression to fibrosis/cirrhosis.
- Minimising downstream complications such as end-stage liver disease or HCC.

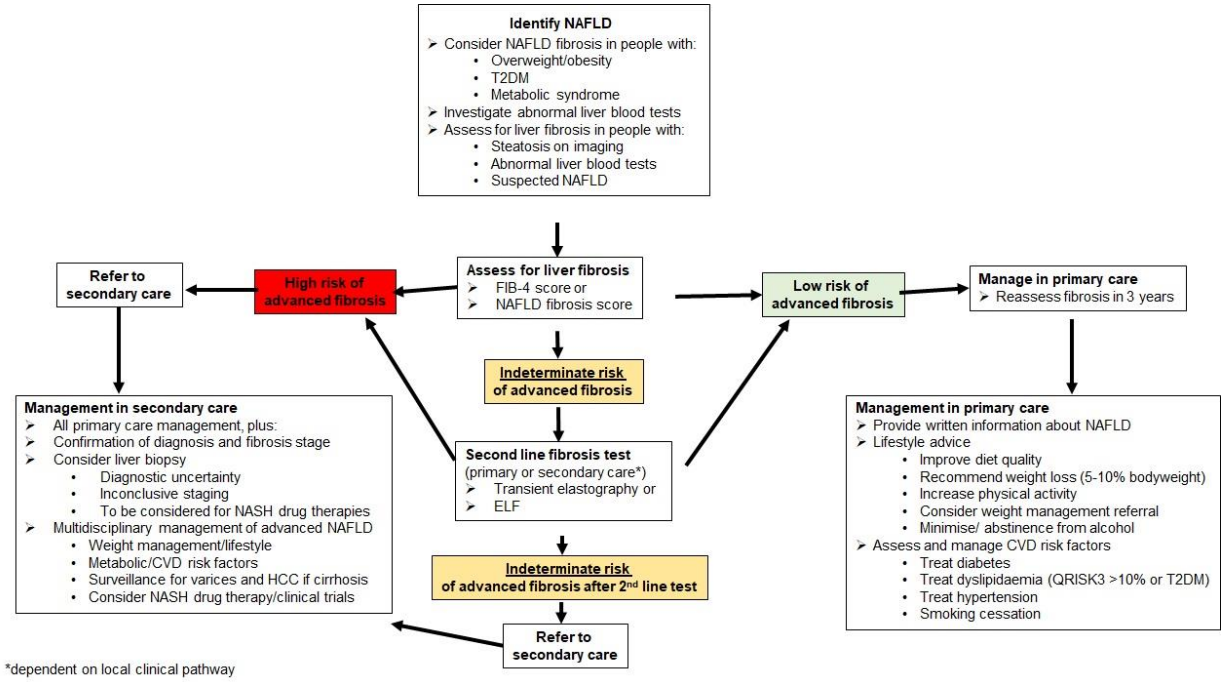



**Figure 1.3 Clinicalmanagement of individuals with NAFLD in primary and secondary care adapted from reference [83] with permission of the author.** This overview focuses on the provision of lifestyle advice and cardiovasular risk reduciton.

## 1.7 NAFLD Management: Lifestyle Interventions

There is an urgent need to identify potential therapeutic interventions that can prevent NAFLD progression and induce regression [13]. Despite some promising advances, presently there are no regulatory approved pharmacological therapies for NAFLD [216,217]. Therefore, lifestyle interventions that encourage calorie (energy) restriction to induce weight loss and disease regression are the mainstay of treatment [6,44,218]. Research has shown that calorie restriction, is effective with a dose-response relationship between weight loss and effects on NAFLD activity [6,219,220].

### *1.7.1 Calorie Restriction and Weight Loss*

There is consistent evidence that adherence to a range of calorie-restricted interventions improve measures of NAFLD activity [13,221]. Targeting weight loss is also effective for CVD and T2DM risk reduction in patients with NAFLD [13].

In a small randomised controlled trial (RCT), calorie-restriction (1000-1200 calories/day for weight <90.7kg or 1200-1500 calories/day for weight >90.7kg)

induced a weight loss of ≥7%, improved waist circumference, and NAFLD markers i.e., ALT, steatosis, and histological NAFLD Activity Score (NAS) [220]. Meta-analytic data from eight RCTs found that ≥5% weight loss improved steatosis, but ≥7% weight loss was needed to improve NAS [222]. Across a number of reviewed studies, there is consistent, evidence that weight loss of 5% in NAFLD and 7-10% in NASH is beneficial [223]. Differential responses to weight loss have been reported, i.e., participants with higher baseline BMI experience smaller improvements in liver makers [224]. Potential explanations for this observation include differences in genetics [225]. Furthermore, there is preliminary evidence indicating an association between higher BMIs, more advanced disease, and metabolic damage, ultimately impacting weight loss responsiveness [224,226].

There is encouraging data on fibrosis regression using a calorie-restricted intervention (CRI) that included 293 NASH patients with paired biopsies over 52 weeks (before and after intervention) [219]. Magnitude of weight loss was independently associated with improved NASH-related features [219]. Weight loss of 10% induced a reduction in NAS; 90% of patients had NASH resolution, and 45% of patients had fibrosis regression. Limited data from a small cohort study of 45 NASH patients, undergoing serial liver biopsies, showed that patients with ≥10% weight loss exhibited higher rates of fibrosis regression (63% vs. 9%) [227]. European clinical guidelines are congruent with these findings and recommend 7-10% weight loss [6].

However, despite the convincing evidence that weight loss is advantageous, patients frequently find it difficult to attain/sustain targets [17,228]. Around 10% of patients achieve 10% weight loss [219], less than half attain ≥7% weight loss [222], and less than 30% attain 5% weight loss [219,222]. Weight loss maintenance is also complex and weight cycling episodes are common [229]. A very low-calorie diet (vLCD) has been proposed as a more viable treatment strategy to achieve weight targets, beyond the current lifestyle approaches [230]. A small feasibility study showed that at 9 months around one third of patients attained ≥10% weight loss, 51% achieved ≥7% weight loss, and 68% achieved ≥5% weight loss, with associated improvements in liver enzymes and liver stiffness and cardiometabolic parameters [230]. However, there are few data on the adverse effects of these type of diets, and of their long-term outcomes in patients with NAFLD. Hence, 500-1000 calorie deficit per day remains the advised calorie restriction in lifestyle interventions [6].

### *1.7.2 Physical Activity and Exercise*

Physical activity and exercise have favourable effects on liver enzymes and hepatic steatosis reduction, independent of weight loss [231-233]. Preliminary data suggests that exercise may attenuate ER stress [234], as well as promoting changes in hepatic metabolism, such as decreased substrate delivery, decreased lipid anabolic processes and increased mitochondrial lipid oxidation [235]. A small RCT found that different types (dose and intensity) of aerobic exercise regimens induced comparable effects on steatosis reduction [236]. Furthermore, the results from 24 reviewed studies suggests that the activity type, whether its aerobic, resistance (strength), high intensity or a blended approach, appears to be less relevant [231]. However, in comparison with weight loss, the effects of exercise are modest (steatosis relative reduction 80% vs. 20-30%, respectively) [221]. Nonetheless, physical activity/exercise as an adjunct to dietary approaches is encouraged due to its strong cardiovascular benefits and positive effects on body composition [13]. Patients with NAFLD are recommended to perform aerobic exercise 150-200 minutes per week and resistance exercise on 2-3 days per week [6].

### *1.7.3 Pharmacological and Surgical Strategies*

Pharmacological and surgical strategies may be suitable for selected NAFLD patients [6]. Orlistat is an anti-obesity medication that improves biochemical, metabolic and anthropometric indicators [237] but its effect on fibrosis regression is inconsistent and weight regain is common [17]. Metabolic surgery can be cost-effective, regardless of fibrosis stage [238]. Meta-analytic data found that surgical approaches resulted in resolution of steatosis, ballooning and fibrosis in 66%, 50% and 40% of morbidly obese NAFLD patients, (body mass index $\geq$40 kg/m$^2$ white populations; $\geq$37.5 kg/m$^2$ non-white minority ethnic populations), respectively [239]. Nonetheless, progressive weight regain has been observed, after certain types of surgical interventions [240]. Furthermore, the potential for deleterious effects on hepatic histology following drastic weight loss post-surgery must be considered [17].

### *1.7.4 Acceptability and Characteristics of Effective Interventions*

To date, it remains unclear which is the most effective and achievable lifestyle intervention for patients with NAFLD [13]. in addition, the most advantageous lifestyle-based strategies to support sustained weight loss have not been elucidated [13].

There are challenges involved in the design of therapeutic interventions that aim to optimise patient efforts at lifestyle changes and maximise benefits on liver function [241,242].

#### *1.7.4.1 Feasibility, Acceptability and Adherence*

A successful intervention must be acceptable to both deliverers/patients and elicit strong patient adherence [13,241,243]. Currently, there is insufficient data on the factors that influence acceptability and adherence to, and completion of, lifestyle interventions. Recent guidance recommends that feasibility and acceptability measures and patient and public involvement and engagement (PPIE) are integrated into intervention design and evaluation [241,242,244]. The completion of feasibility studies with defined progression criteria can inform the refinement of interventions, by generating information relating to [242]: study design (recruitment, retention, data collection burden, outcome and analysis) and lifestyle intervention design (optimum components and delivery mechanisms, acceptability, adherence and implementation fidelity).

Feasibility studies should include methods that assess the impact of interventions on patient-related outcomes (PROs) such as health-related quality of life (HRQoL). Multiple generic and disease specific PRO instruments have been tested with patients across the NAFLD spectrum [245]. A recent systematic review of studies reporting PROs, found that NASH was associated with substantial HRQoL burden, that increased with disease severity [246]. Disease progression was also associated with physical and mental QoL burden [246]. NASH appears to impact work capacity, performing daily tasks and relationships [246]. Furthermore, data from these 23 reviewed studies indicated that multi-morbidity negatively impacted QoL [246]. An earlier prospective analysis identified substantial symptom burden among histologically defined NAFLD patients, using the chronic liver disease questionnaire (CLDQ) [247]. Further findings suggest that hepatic inflammation was associated with lower HRQL [247]. However, there remains limited data on the impact of lifestyle interventions on HRQoL in this patient population.

#### *1.7.4.2 Behavioural Strategies*

Definitive data are needed on which specific behaviour change strategies and intervention characteristics enhance intervention effectiveness in patients with

NAFLD [248,249]. However, there is evidence to support the use of behavioural approaches targeting diet and lifestyle modification in managing other metabolic diseases [249]. Specific behaviour change techniques (BCTs) [250], and intervention characteristics have been found to increase intervention effectiveness among adults at risk of/ or with T2DM [251,252]. These include social support [251]; action planning; instruction/demonstration on how to perform a behaviour and behavioural practice [252]; goal setting and self-monitoring [251]. The following intervention characteristics were identified with more effective interventions; greater frequency and intensity of contacts [251,252]; and exercise physiologist and/ or dietitian input [252]. This is in line with the findings of a meta-analysis of dietary interventions in older adults, which suggests that the BCTs of barrier identification, problem solving, and social support/social change may be useful to increase intervention effectiveness [250,253].

### 1.8 Diet Therapies

There is evidence that targeting improved dietary patterns and diet composition changes are important in achieving beneficial hepatic effects, i.e., calorie restriction alone may not be the optimum treatment for NAFLD [6,13,221,254]. Moreover, given that recommended weight loss targets are difficult to achieve and to sustain, better evidence of the efficacy of different diet composition modifications could be a basis for developing personalised dietary care. However, more evidence is needed on what dietary patterns are achievable by most patients and which promote greatest adherence.

The deleterious effects of excess sugars, refined carbohydrates and SFA on hepatic lipid metabolism have been well-elucidated [117]. The exclusion of NAFLD-promoting components such as excessive refined, processed foods and high fructose options is effective and standard of care [6,125]. Limited data also supports the substitution of SFAs with PUFAs or MUFAs within isocaloric diets [142,255]. Alcohol has a synergistic effect with other facets of the metabolic syndrome and increases the risk for severe liver disease [256]. Therefore, there is potentially no safe amount of alcohol to drink for patients with NAFLD, and it should be completely avoided in patients with NASH-cirrhosis [6,256].

### *1.8.1 Low Carbohydrate and Low Fat Diets*

Low carbohydrate (LCD) and low fat diets (LFD) are well established diet therapies in obesity treatment. The results of several RCTs demonstrate that there is no clear superiority of LFDs over other diet treatments for obesity [257]. Meta-analytic data suggests LCDs induce greater improvements in weight, TG and high-density lipoprotein (HDL) than LFDs, but less favourable changes in low-density lipoprotein (LDL) and total cholesterol (TC) levels [258]. However, both diets have been found to reduce the prevalence of MetS in obese adults [259].

There is limited evidence of the effects of these diet types in NAFLD. In a six month RCT, both a hypocaloric LCD (<90 g carbohydrates) and hypocaloric LFD (<20% fat of total energy intake) induced comparable reductions in ALT, steatosis and weight [260]. Adherence to either diet types for three months improved liver enzymes and IR, with similar weight reductions in a small RCT [261]. When combined with exercise, both diets have been found to improve liver enzymes [262,263] and NAS [263], irrespective of weight loss [262,263]. When compared with a calorie restricted diet, an LCD produced comparable reductions in liver enzymes and weight [264]. Two small meta-analyses found an equivalence of these diets on liver enzymes and steatosis improvements [222,265].

There is a paucity of comparative trials with sufficient sample sizes, using accurate NITs that have evaluated low-carbohydrate and low-fat diets in NAFLD [248]. Further clarification from experimental trials is needed to determine the precise carbohydrate restriction associated with optimum benefits, as different studies have used carbohydrate intakes ranging from reduced to low and ketogenic, with varying fat and protein ratios [266]. Studies of ketogenic diets have produced promising findings, which need to be verified in larger long-term studies [266]. More evidence of the impact of dietary interventions with different types of fat and carbohydrate on liver function is needed [266].

### *1.8.2 Mediterranean Diet*

The effects of dietary patterns on NAFLD activity have been studied extensively in the past decade, including increasing attention on Mediterranean diet (MD) characteristics and its potential effects on NAFLD.

The traditional MD reflects the habitual model of healthy eating that originates from regions surrounding the Mediterranean Sea during the 1960s [267,268]. The MD is characterised by a high quantity of plant-based foods, unrefined cereals, fruit and vegetables, olive oil, and nuts; eating white meat, fish and legumes in moderation; restricting red and processed meats, and sweets; and drinking red wine moderately [267]. Although, the exact dietary pattern varies to some extent between regions.

MD guidance has been depicted as a pyramidal visual display and has evolved in line with epidemiological evidence. An iteration of the MD pyramid with brief guidelines were produced in 2011, which is adaptable to the different nutritional and socio-economic contexts of Mediterranean regions [269,270]. Recommendations on what foods should be consumed and how often are outlined [269]. In addition, family meals, cooking from scratch and adequate rest are encouraged [269]. The most recent MD pyramid (2020) has extended towards a focus on sustainability, depicting the environmental impact of the foods outlined (Figure 1.4) [271]. Compared with previous versions, it stresses the importance of reduced red meat and bovine dairy products, and increased legumes and plant-based foods [271].

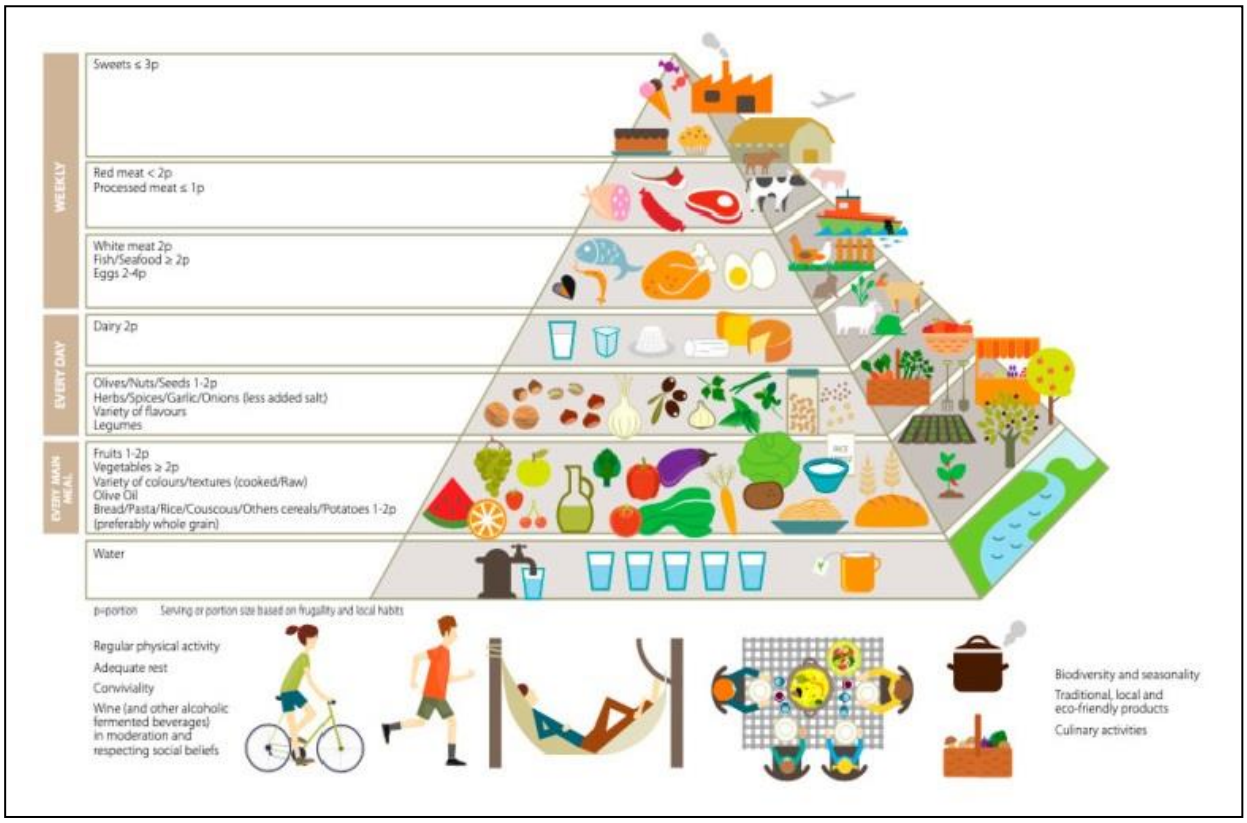



**Figure 1.4 The modern MD pyramid [271]. Creative Commons Attribution (CC BY 4.0) licence.** The new environmental dimension enhances food intake recommendations to address health and ecological issues.

The beneficial properties of a MD have been demonstrated in a broad range of diseases in numerous studies [272-275]. In addition, the MD may influence cellular and molecular hallmarks of ageing, with positive impact on longevity and age-related disease risk [276]. Protective effects of MD on T2DM and MetS features, which are strongly associated with NAFLD, have been reported [277,278]. Furthermore, individuals randomised to a MD intervention had reduced CVD [279].

Recent research has shown that greater adherence to the MD is effective for NAFLD prevention and management [6,92]. MD interventions have been shown to reduce hepatic steatosis, improve liver biochemistry and cardio-metabolic dysfunction, with or without weight loss, and is the most recommended dietary pattern in NAFLD [6,280-285]. A 6 month dietary intervention based on the MD produced significant improvements in HDL, TC/ HDL, LDL/HDL and TG/HDL [282]. There are numerous possible mechanisms through which the MD and its components can induce favourable effects independent of weight loss. The MD is abundant in phenolic compounds, which have been implicated in improved insulin sensitivity and reduced DNL [286]. A lower intake of SFAs is beneficial, as SFAs increase IR and steatosis

[144,287]. Additionally, increased dietary fibre can alter gut microbiota, which in turn can influence the gut-liver axis [286]. However, the mechanisms through which MD exerts its NAFLD-specific effects remain poorly understood [92]. Definitive data are needed on whether a MD results in greater clinical benefits in NAFLD than other dietary interventions.

### 1.9 Nutrigenetics

Differences in gene sequence can alter the activity of encoded proteins and affect the response of individuals to dietary components [288]. The most researched gene-diet interactions in clinical studies involve *PNPLA3* variants [182]. *PNPLA3* expression is regulated by modifications in dietary components and energy balance shifts [159]. Emergent research has shown nutritional regulation of *PNPLA3* [192,288] so that patients with NAFLD carrying the *PNPLA3* risk allele might benefit more from weight loss but less from omega-3 supplementation [288-291]. Evidence from a RCT, that investigated a hypocaloric intervention, found that improvements in liver fat and anthropometric indicators were greater among individuals homozygous for the rs738409 G allele, as opposed to those carrying the rs738409 C allele [291]. In vivo and in vitro data suggests that specific unsaturated FAs (oleic, linoleic, EPA and DHA) inhibit lipogenesis and *PNPLA3* expression, which is considered beneficial in this patient population [292]. However, in an RCT, carriers homozygous for the rs738409 G allele in the *PNPLA3* gene increased liver fat compared with carriers of the rs738409 C allele after omega-3 supplementation [289]. Further clarification is needed to determine the role of *PNPLA3* variants in influencing responsiveness to omega-3 PUFA.

Diet lifestyle modification is more effective in decreasing liver steatosis in *PNPLA3 I148M* carriers than in non-carriers [291,293]. Liver fat content is influenced by the interaction between *PNPLA3* variants and high carbohydrate intake, specifically sugar, or a high n-6/n-3 PUFA ratio [294,295]. Moreover, emergent evidence indicates that a higher carbohydrate intake is associated with increased risk of fibrosis severity in patients carrying the *PNPLA3* risk allele [159]. A hypocaloric low-carbohydrate diet induced greater hepatic fat reduction in carriers homozygous for the rs738409 G allele in the *PNPLA3* gene compared with carriers of the rs738409 C allele, irrespective of weight loss [296]. Thus, this interaction could be important in the disease pathogenesis and progression [159]. Carbohydrate intake stimulates *PNPLA3*

expression in hepatocytes via pathways involving carbohydrate response element protein (ChREBP) and SREBP-1c to induce DNL [297,298].

A gene-diet interaction study found a protective effect from carriage of the *PNPLA3* risk allele, with reduced risk of significant fibrosis when higher intakes of total choline, methionine and total isoflavones were consumed [159]. Although, further experimental data are needed to corroborate these associations. The role of *PNPLA3* variants in influencing responsiveness to different diet therapies remains poorly understood. The impact of other NAFLD-related gene variants such as in *TM6SF2, HSD17B13* in response to diet therapy also warrants further exploration [159] .

### 1.10 Metabolomics

The assessment of habitual dietary intake is often inadequate, with considerable reliance on self-report instruments, with well-defined risks of misreporting, errors and bias [299]. Measuring diet intakes via food frequency questionnaires (FFQs), diet record diaries or recalls increases participant burden, and often requires subsequent analyses by researchers/clinicians to calculate nutrient intakes [299]. These issues impede efforts to elucidate associations between specific dietary components/ patterns with clinical phenotypes [299,300]. However, some of these challenges can be overcome using urine-based biomarker approaches, that objectively measure food-derived metabolites [299]. The application of metabolomics has identified numerous dietary intake biomarkers and may be a promising tool to provide information about the metabolic response to diet [301-303]. There are two main approaches in metabolomics: non-targeted and targeted [304]. Non-targeted approaches are comprehensive and conducted in an unbiased manner to detect a wide range of metabolites, with the potential to discover novel biomarkers and generate hypotheses [304]. In contrast, targeted approaches involve the analysis of a predetermined panel of metabolites relevant to a study's hypothesis, supporting precise quantification [304,305]. By incorporating both approaches into study design, there is potential to achieve unbiased metabolite profiling, and monitor a specified list of known metabolites [305].

Metabolomics are a rapidly developing field, with increasing recognition that a combination of self-reported data and the quantification of urinary dietary biomarkers, may reduce measurement error in diet intake assessment [306]. Metabolomics studies

have demonstrated the feasibility of various urine sampling methods, in different settings to assess an individual's diet [299,301]. Urine is a minimally-invasive biofluid, in which a large volume of metabolic by-products can be obtained [301,307]. Nevertheless, validation of these urine-based biomarker approaches in a range of study designs are needed to determine cost-effectiveness, practicability, and utility in practice [299,301].

## 1.11 Summary of Hypotheses, Aims and Objectives

In light of the compelling evidence that the *PNPLA3* rs738409 SNP is the most important genetic risk factor for NAFLD [182]. Furthermore, that gene-diet interactions involving the *PNPLA3* variant could be important in disease pathogenesis and progression [159]. This project hypothesises that carriage of the *PNPLA3* variant influences MD responsiveness in NAFLD. Evaluating individual genes is important to attain a comprehensive understanding of the mechanisms that underlie their effects. This might lead to a clearer interpretation of the results, by potentially attributing effects to the gene being studied. To prepare for a definitive RCT, that will address the hypothesis, the current research project is split into three work packages.

Chapter 2 combines work package one and two which aimed to provide the evidence base on which to develop diet lifestyle care for patients with NAFLD. This chapter showcases the synergy between evidence-based theories and alignment with real-world clinical practice and contributes to a more comprehensive understanding of the topic. The combined findings from these two studies, as well as other research evidence, and patient and public feedback, directly informed the next stage of the project i.e., the design of a randomised controlled feasibility trial described in Chapter 3.

### *1.11.1 Work Package 1: The Effectiveness and Acceptability of Mediterranean Diet and Calorie Restriction in NAFLD: A Systematic Review and Meta-Analysis*

Chapter 2 reports the findings of a systematic review and meta-analysis, which synthesised data from 26 dietary intervention studies describing the effects of calorie restriction and MD interventions in NAFLD.

The objectives of this study were:

- To synthesise data from randomised and clinical controlled trials (RCTs/ CCTs), investigating the effects of Mediterranean diet (MD) and calorie-restricted interventions (CRI), on markers of liver function and weight loss and on the acceptability of the intervention in patients with NAFLD.

### *1.11.2 Work Package 2: Diet Lifestyle Management of NAFLD: A Cross-sectional Survey of Clinicians*

Chapter 2 presents data from a cross-sectional survey of clinicians on current practice and perceived barriers to the effective delivery of diet lifestyle interventions. The objectives of this study were:

- To evaluate the current status of diet lifestyle care for patients with NAFLD.

### *1.11.3 Work Package 3: A Nutrigenetics-based Intervention Approach for Patients with NAFLD*

Chapter 3 describes the protocol for a randomised controlled feasibility trial that was undertaken to determine the feasibility of a nutrigenetic therapeutic approach for patients with NAFLD. The aim of the randomised controlled feasibility trial was to determine whether it is feasible to conduct a RCT to investigate the impact of *PNPLA3* carriage on responsiveness to MD and NAFLD severity, and to provide preliminary data to inform the development of a definitive RCT.

The primary objective was to determine whether the protocol for a future definitive RCT is feasible, acceptable and effective:

- To determine the feasibility of recruitment and retention.
- To evaluate adherence to, and completion of, the study procedures.
- To evaluate implementation fidelity and how practicable it is to deliver the protocol in a clinical setting.
- To determine the acceptability of the diets, instruments and procedures.
- To evaluate adherence to, and completion of, the MD intervention.
- To undertake preliminary exploration of changes in key clinical and lifestyle variables.

Secondary objectives included the assessment of liver fibrosis biomarkers and the influence of *PNPLA3* genotype in response to the dietary intervention.

- To undertake preliminary exploration of changes in biomarkers of liver fibrosis.
- To undertake preliminary exploration of the influence of *PNPLA3* genotype on metabolic endpoints.

Chapters 4 to 6 presents data about the feasibility, acceptability and effectiveness of the study protocol, as well as preliminary data on liver fibrosis biomarkers and the influence of *PNPLA3* genotype.

# Chapter 2

# Defining Diet Lifestyle Care for patients with NAFLD

## 2.1 Introduction

Non-alcoholic fatty liver disease (NAFLD) is a growing health threat, associated with substantial morbidity and mortality [4]. A major challenge is practice variation in patient management of NAFLD, despite the availability of standard of care guidance [308]. Patients with NAFLD frequently find it difficult to access lifestyle interventions or achieve recommended targets [4,17,309,310]. For example, the need for optimum strategies to promote sustained weight loss remains unmet and many patients and clinicians are uncertain about which dietary approaches should be adopted [243,311].

Priorities for service improvement have been highlighted [309], including clinicians actively addressing both hepatic and extra-hepatic manifestations of the metabolic syndrome (MetS), and improved access to lifestyle interventions [309]. However, in spite of the impetus from landmark clinical guidelines in 2016 to improve care delivery [6,64], there remains a dissonance between recommended practice and actual service provision [311,312]. Regional disparities have been documented between general hepatology and a specialist NAFLD clinic in the North East of England [313]. Non-specialist clinics were less likely to induce recommended weight loss and to provide access to structured lifestyle programmes [6,219,313]. Access to lifestyle interventions is also problematic at a European level; with less than 25% of countries providing this standard of care [4].

The current evidence, from a limited number of studies, reveals significant differences in patient management and deviations from reference guidelines. However, little is known about the specific weight loss or dietary strategies that are used by clinicians in practice [313]. There is an urgent need to understand the scope and components of diet lifestyle care delivered, and the degree to which clinical practice varies between and within countries. Although calorie-restricted interventions and the Mediterranean diet interventions are recommended [6], and have been studied extensively in the past decade. Chapter 1 has highlighted the uncertainty about which dietary approaches are most beneficial and promote greatest adherence. These knowledge gaps limit the delivery of effective diet and lifestyle interventions in routine clinical practice.

## 2.2 Study Aims and Objectives

This chapter combines two studies which aimed to provide the evidence base on which to develop diet lifestyle care for patients with NAFLD.

Study 1: Effectiveness and Acceptability of Mediterranean Diet and Calorie Restriction in NAFLD: A Systematic Review and Meta-Analysis.

Study 2: Diet Lifestyle Management of NAFLD: A Cross-sectional Survey of Clinicians.

The objectives of two studies described in this chapter were:

- To synthesise data from randomised and clinical controlled trials (RCTs/ CCTs), investigating the effects of Mediterranean diet (MD) and calorie-restricted interventions (CRI), on markers of liver function and weight loss and on the acceptability of the intervention in patients with NAFLD.
- To evaluate the current status of diet lifestyle care for patients with NAFLD.

## Study 1: The Effectiveness and Acceptability of Mediterranean Diet and Calorie Restriction in NAFLD: A Systematic Review and Meta-Analysis.

## 2.3 Materials and Methods

### *2.3.1 Study Design*

This systematic review [314] was conducted according to Cochrane, the Centre for Reviews and Dissemination, and Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines [315-317] and the protocol was registered with PROSPERO, the International Prospective Register of Systematic Reviews (Registration number CRD42019118537).

### *2.3.2 Eligibility Criteria*

Only full reports of dietary interventions on markers of NAFLD, in adults (>18 years) were considered. Studies were selected according to the following criteria: a) adult participants (>18 years old) with NAFLD; b) RCTs and CCTs; c) MD interventions (that attempted to increase overall MD adherence, or which altered intake of its constituent food components), and CRI; d) NAFLD markers (i.e., hepatic steatosis, non-alcoholic steatohepatitis (NASH), hepatic fibrosis, histological biomarkers, alanine aminotransferase (ALT) and aspartate aminotransferase (AST)).

The following were exclusion criteria: a) laboratory-based feeding trials; b) studies designed to test effects of specific micronutrients or dietary supplements; c) animal studies; d) in vitro studies; e) other study designs; f) studies testing specific exercise protocols. Studies testing dietary additives or other putative therapies were included only if MD effects were reported independently. Primary outcomes included markers of NAFLD (i.e., steatosis, NASH, fibrosis, histological biomarkers, ALT and AST). Secondary outcomes included body weight, waist circumference, cardiometabolic parameters, quality of life measures and intervention acceptability (i.e., attrition rates, where these data were the only measure of acceptability reported). Diet intake modification included changes in consumption of one or more dietary components and/or improved diet adherence at follow-up, assessed by diet assessment scores, quantitative analysis of self-reported food intake data (e.g., diet record diary) and biomarkers of dietary intake.

### *2.3.3 Search Strategy*

The following five electronic databases were searched systematically (October 2021): MEDLINE, Embase, Scopus, Web of Science, and Cochrane Central Register of Controlled Trials (CENTRAL) in the Cochrane Library to identify eligible full text studies. A medical librarian (Linda Errington) assisted with the formulation of search strategies. The review of articles was restricted to those published in English with year of publication restricted to 2010-2021. The effects of dietary patterns on NAFLD activity have been studied extensively in the past decade. An initial scoping search identified this as the period in which the relevant studies appeared. Reference lists of previously published related reviews were scanned to identify other studies eligible for inclusion. The search strategy involved combining words from the following concepts: NAFLD, controlled trials, MD and weight loss. Prior to searching, an extensive exercise to identify relevant terms was undertaken. The search terms were translated into a search strategy using a combination of index terms and keywords and refined iteratively. Highly sensitive search filters for identifying RCTs were used in MEDLINE and Embase [315]. The final list of search terms for all databases is provided (Appendix A).

### *2.3.4 Article Selection and Data Extraction*

Two reviewers (Laura Haigh (first reviewer) and Colette Kirk or Jennifer Gallacher (second reviewers)) independently assessed potentially relevant articles for eligibility.

The decision to include or exclude studies was based initially on study title and abstract. Full text articles were then retrieved and evaluated to decide on final inclusion (with reasons for exclusion recorded). Where articles noted the existence of other study reports or published protocols, these were obtained to extract any potentially missing data. Data were extracted from the included studies using a standardised pre-piloted form (Appendix B). Discrepancies between reviewers on article eligibility and data extraction were resolved through discussion with a third reviewer (John C Mathers) using a consensus approach. Study authors were contacted for additional data when required (Appendix C). Extracted information included: study design (country, recruitment methods, analysis type and methods, completion rates, intervention and comparator details), participant characteristics (population and setting, inclusion/exclusion criteria, baseline characteristics), and outcome assessment. These data were uploaded into Microsoft® Excel 2019 and used to compile a narrative synthesis of the results that is reported below using descriptive statistics (e.g., percentages) and summary tables.

### *2.3.5 Assessment of Quality*

Two reviewers (Laura Haigh and Colette Kirk or Jennifer Gallacher) independently assessed study quality using the Cochrane Collaboration's tools: risk of bias for RCTs (RoB 2) [318] and risk of bias in non-randomised studies of interventions (ROBINS-I tool) [319]. The data were then integrated into Microsoft® Excel and Access databases. Disagreements were discussed and resolved with a third reviewer (John C Mathers). An overall summary score was calculated across domains using the defined criteria for each of the tools.

### *2.3.6 Meta-analysis and Meta-regression*

Meta-analysis was conducted using the Review Manager software (version 5.4, the Cochrane Collaboration, 2020), inputting mean and standard deviation (SD) data for post-intervention values. Data synthesis was undertaken, including the calculation of effects sizes with 95% confidence intervals (CI), using a random-effects model with inverse variance weighting. Results of body weight, ALT, AST and Fatty Liver Index (FLI) were summarised as difference in means. However, due to the different techniques used to assess steatosis (e.g., histologic examination and magnetic resonance imaging (MRI)) and liver stiffness (transient elastography (TE) Fibroscan™; ultrasound (USnd) acoustic radiation force impulse (ARFI), shear wave

elastography (SWE) and Multiwave)), effects on these outcomes were summarised as standardised mean difference. Heterogeneity between studies was assessed by the $\chi^2$ statistic (expressed as P value) and $I^2$ statistic (expressed as percentage) and significance was set at $p \leq 0.05$. For graphical presentation of the outcomes, forest plots were generated with studies arranged in order of effect size. Publication bias was assessed with funnel plots and by Egger's regression test.

Data not provided in the main text or tables of the original publications were extracted from the figures. Data from trials that reported their results as medians were converted to mean and SD using the formula described by Hozo *et al*., [320]. For trials with two intervention arms and a single control group, the total number of participants in each of the intervention arms was compared against half of the number of participants in the control group. A conservative method was adopted for crossover trials, with the mean and SD inputted separately for the intervention and control arms [321]. To explore the robustness of the findings, a sensitivity analyses was conducted on the main meta-analysis models by i) excluding crossover trials and ii) excluding trials that were judged as having serious, critical or high risk of bias [318,319]. A subgroup analysis was completed to explore the implications of different types of interventions; i) CRI, ii) MD interventions and iii) MD components interventions. Meta-regression analyses were used to determine whether baseline participant and study characteristics such as underlying NAFLD category, age, body mass index (BMI) and intervention duration, physical activity advice presence, sample size and attrition influenced the effect of dietary interventions on markers of liver function. Comprehensive meta-analysis (CMA) software version 3 (Biostat Inc.) was utilised to perform meta-regression analysis. The random effects model was employed to address heterogeneity across studies and account for within and between study variability.

### 2.4 Results

4041 publications were identified; from these 26 trials with a total of 3037 participants met the inclusion criteria. The summary reasons for exclusion of full text studies comprised ineligible population or intervention, insufficient detail on outcomes, and incorrect outcome measures, or analysis type (Appendix D). Finally, all but five papers [322-326] were included in the meta-analysis. Results of the screening process are described in Figure 2.1.

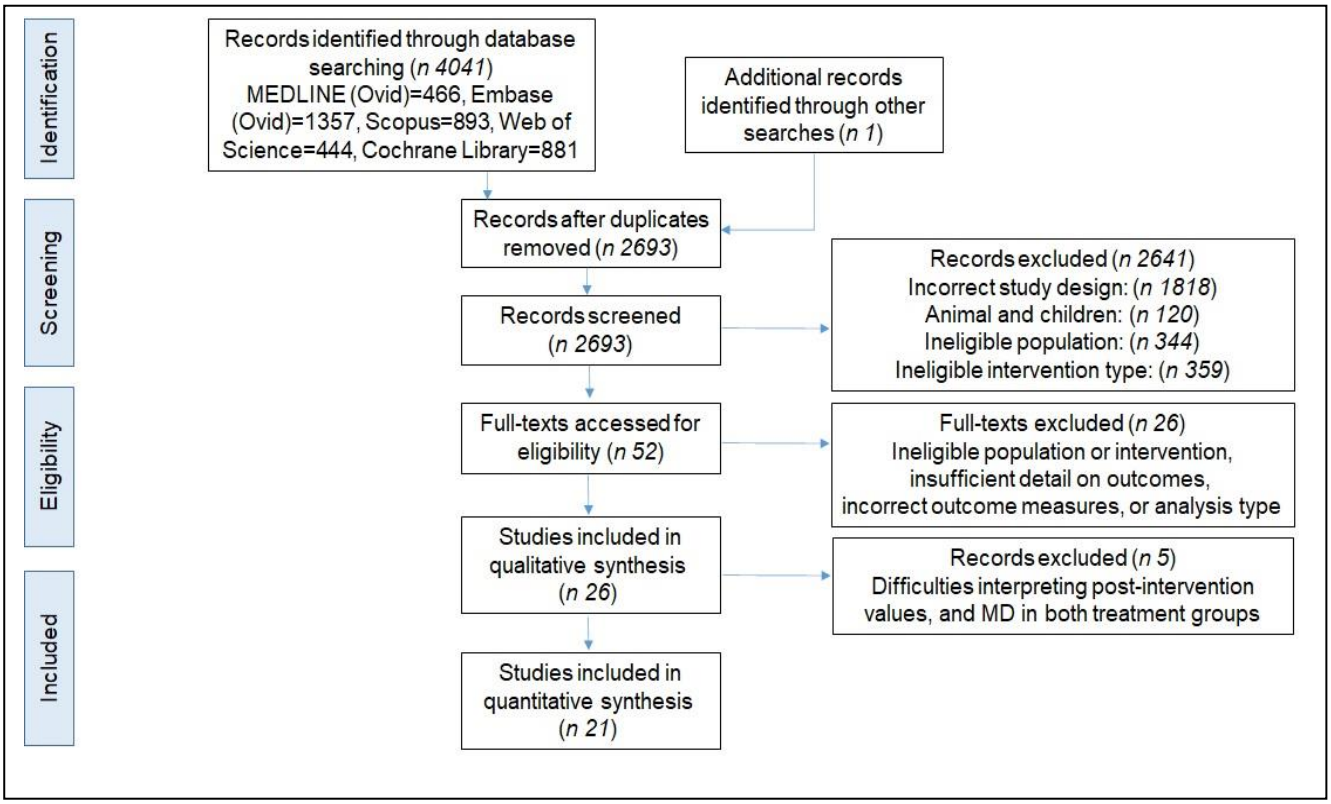



**Figure 2.1 Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) flow diagram.**

### *2.4.1 Study Characteristics*

The study characteristics are presented in Appendix E. These included 54.7% male in the mixed-sex studies, with two studies including males only [327,328]. The mean (SD) age of participants was 48.3 (5.9) years and mean (SD) BMI 31.0 (2.3) kg/m$^2$ at baseline. The mean trial follow-up duration was 32.2 weeks (range 2 to 104 weeks). Study populations comprised NAFLD [270,280,285,322,326-342]; NASH [220,343]; individuals with abdominal obesity/dyslipidaemia [323,324] and those at risk of Type 2 diabetes (T2DM) [325]. The trials were conducted in lower-middle income (*n* 5) [333,335-337,342], upper-middle income (*n* 5) [327,328,339,341,343] and high-income countries (*n* 16) [220,270,280,285,322-326,329-332,334,338,340].

#### *2.4.1.1 Eligible studies design and outcome measures*

The study designs comprised twenty-three RCTs (88.5%) [220,270,280,285,323-331,333-339,341-343] and three CCTs (11.5%) [322,332,340]. There were two crossover trials (8%) [280,332] and the remaining trials used a parallel group design [220,270,285,322-331,333-343]. A broad range of reported outcomes and assessment methods were used. Nearly all studies assessed biochemical markers ALT (*n 25,* 96%) [220,270,280,285,322,323,325-343] and AST (*n 21, 81*%) [220,323,325-343]. Studies investigated steatosis using FLI (*n 6,* 23%) [322,327-331],

imaging techniques (*n 20*, 77%) [280,285,323-331,333-341] and histology (*n 1*, 4%) [220]. Fibrosis was assessed by liver stiffness measures (LSM), using non-invasive techniques (*n 8,* 31%) [270,285,326,330,331,334,338,341] and scoring systems, NAFLD Fibrosis Score (NFS) (*n 3*, 11.5%) [270,322,327], Fibrosis-4 Index (FIB-4); (*n 1*, 4%) [322] and HepaScore (*n 1*, 4%) [285].

#### *2.4.1.2 Intervention and comparison details*

The included trials applied a diverse range of dietary interventions and comparator (control) treatments. Dietary interventions were delivered at various contact intervals, mainly face-to-face in individual consultations using nutrition counselling, and personalised approaches. Eight trials (31%) investigated multiple intervention arms (additional study arms from four trials were eligible for inclusion) [270,323,325,326]. Calorie reductions were applied in twenty studies (77%) [220,270,322,323,325-328,330-332,334,336-343], general physical activity was advised in fourteen studies (54%) [220,270,322-325,327,328,330,331,333,334,337,338] but only five studies (19%) reported specific behaviour change strategies [220,270,322,333,338]. The most common comparator (control) treatment was 'standard care', which was used in nineteen studies (73%) [220,270,322,323,325-327,329-331,333-336,338,339,341-343], followed by low-fat, high-carbohydrate diets (LFD) (*n 5*, 19%) [280,285,324,328,332]. The remaining studies applied a distinct dietary treatment or an individual dietary change component [337,340]. Seven studies (27%) provided participants with specific food items [220,280,285,323,324,339,340].

Across the twenty-six trials, nine investigated CRI (35%) [220,326,327,338-343], thirteen investigated MD interventions (50%) [270,280,285,322-324,328-334] and four investigated MD components (15%) [325,335-337] (Table 2.1). The most common interventions in the calorie-restricted trials involved broader, healthy dietary guidance (*n 5*, 19%) [220,327,338,342,343] or specific dietary treatments (*n 4*, 15%) [326,339-341]. The studies investigating MD interventions were mostly in Mediterranean regions/climates, and based on traditional Cretan diets, from descriptive food data and analysis of actual foods consumed [280,285,329], with low glycaemic index foods [329]; the MD pyramid, which adapts to different nutritional and socio-economic contexts of Mediterranean regions [269,270]; and national dietary guidelines [270,330]. Two trials adopted low-carbohydrate MD (<40g/day for two months, then $\leq$70-80g/day) supplemented with polyphenol-rich products [323,324], while a higher meal frequency (7 meals/day) was a key intervention feature of two trials [331,334]. The studies investigating MD

components increased intakes of wholegrains [325] to at least half of cereal servings each day [335]; reduced red meat consumption (substituting it with turkey, fish, or chicken) [325]; and allocated either 20g or 20% of the total fat (30% of total energy intake) to olive oil each day [336,337].

| | **Calorie-restricted interventions (*n 9*)** | **MD interventions (*n 13*)** | **MD component Interventions (*n 4*)** |
|---|---|---|---|
| Total participants | 777 | 1854 | 406 |
| Participants | 86 (18-280) | 143 (12-716) | 102 (50-178) |
| Male (%) (mixed-sex studies) | 48.6% | 63.9% | 39.1% |
| Age (years) | 50.8 | 48.0 | 43.6 |
| BMI (kg/m$^2$) | 30.2 | 31.7 | 30.8 |
| Follow-up total duration (weeks) | 32.0 (2-104) | 37.5 (8-104) | 15.5 (12-26) |
| Disease: | | | |
| NAFLD | 7 (78%) | 11 (85%) | 3 (75%) |
| NASH | 2 (22%) | 0 (0%) | 0 (0%) |
| Central obesity/ dyslipidaemia | 0 (0%) | 2 (15%) | 0 (0%) |
| Risk of T2DM | 0 (0%) | 0 (0%) | 1 (25%) |
| Intervention features: | | | |
| Calorie restriction | 9 (100%) | 8 (62%) | 3 (75%) |
| Physical activity advice | 3 (33%) | 9 (69%) | 2 (50%) |
| Behaviour change | 2 (22%) | 3 (23%) | 0 (0%) |
| Diet food items | 3 (33%) | 4 (31%) | 0 (0%) |

**Table 2.1 Characteristics of the included studies by intervention type.** Data presented as mean (range) and n (%). MD, Mediterranean diet; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; BMI, body mass index; T2DM, Type 2 diabetes.

### *2.4.2 Assessment of Risk of Bias and Publication Bias*

The risk of bias was assessed for all outcomes included in the review (Appendix F). I have presented below the risk of bias assessment for ALT as the most common primary outcome (Figures 2.2-2.4).


**Figure 2.2 Risk of bias judgements of the included randomised controlled trials studies for alanine aminotransferase (ALT) based on reference [318].** * Study not included in meta-analyses. USA, United States of America.


**Figure 2.3 Risk of bias judgements of the included randomised controlled trials, crossover studies for alanine aminotransferase (ALT) based on reference [318].**

| ROBINS-I | Confounding | Selection of participants into the study | Classifications of interventions | Deviations from intended interventions | Missing data | Measurement of outcomes | Selection of the reported result | Risk of bias |
|---|---|---|---|---|---|---|---|---|
| Mazzotti 2018, Italy* | | | | | | | | Moderate |
| Biolato 2019, Italy | | | | | | | | Serious |
| Browning 2011, USA | | | | | | | | Moderate |

**Figure 2.4 Risk of bias judgements of the included clinical controlled studies for alanine aminotransferase (ALT) based on reference [319].** * Study not included in meta-analyses. Green, low risk; yellow, moderate risk; orange, serious risk; USA, United States of America.

The overall risk of bias is summarised in (Appendix G). The majority of RCTs were judged overall as some concerns [270,280,323,324,327,329,334,337,339,341] or high risk of bias [220,285,325,328,331,333,335,336,342,343], with two RCTs judged at low risk of bias [326,338]. There was a high risk of bias in the domain of deviations from intended interventions for four RCTs [220,285,342,343] and six RCTs were judged at high risk of bias due to missing outcome data [325,328,331,333,335,336]. The CCTs were judged overall as moderate [322,340] or serious risk of bias [332]. There was either moderate [322,340] or serious risk of bias [332] due to confounding, moderate risk of bias due to missing data [322,332] and the selection of participants [322]. Investigation of potential publication bias was performed by producing funnel plots (Appendix H). Visual inspection of the funnel plots did not show evidence of publication bias for the outcomes described. Eggers's regression test confirmed the likely absence of publication bias in meta-analyses, but the power of the test was poor for three of the outcomes (FLI, LSM and steatosis) for which there were <10 trials.

### *2.4.3 Effects of Interventions on Primary and Secondary Outcomes*

The effects of dietary interventions on primary and secondary outcomes are described in the (Appendix I). The following sections consider CRI, MD interventions and MD component interventions separately.

#### *2.4.3.1 Calorie-restricted interventions (CRI)*

When compared with standard care (SC), CRI improved ALT [327,338,341], AST [343], FLI [327], and steatosis as measured by imaging (USnd/ proton magnetic resonance spectroscopy ($^1$H-MRS)) [326,327,338,339,341] and LSM [338,341]. These were associated with greater reductions in waist circumference [338] and body weight (5.8% (CRI) vs. 0.3% (SC)) [220,338,341,342]. CRI-induced body weight loss of ≥7% reduced waist circumference, and tended to improve ALT, steatosis, and histological NAFLD Activity Score (NAS) [220]. CRI improved Homeostatic Model Assessment of Insulin Resistance (HOMA-IR); fasting insulin [342,343]; as well as low-density lipoprotein (LDL) [338] and triglycerides (TG) [327]. CRI resulted in higher protein consumption [342] and, as planned, a fibre-enriched CRI increased fibre intakes [339]. Both a calorie-restricted diet and a carbohydrate-restricted diet improved AST and steatosis (proton nuclear magnetic resonance ($^1$H- NMR)), but the carbohydrate-restricted diet reduced steatosis to a greater extent (12.0% vs. 5.0%), despite similar weight loss (~4.5%) [340]. Randomisation to a carbohydrate-restricted diet resulted in higher protein and fat, and lower carbohydrate intakes [340].

#### *2.4.3.2 MD interventions*

When compared with standard care, MD improved ALT [270,323,333]; AST [323,333]; FLI [330]; steatosis assessed by imaging ($^1$H-MRS/MRI/USnd) [323,329,333,334]; LSM [270,330]; waist circumference [323,330], and resulted in larger body weight loss of 5.4% vs. 1.0% [270,323,330]. MD produced bigger reductions in HOMA-IR, fasting insulin, total cholesterol (TC) and TG [330]. MD adherence was higher in MD groups [270,331,334] with increased consumption of fibre [270], fruit [334] and nuts [323]. MD reduced calorie [331] carbohydrate [323,331,334], and sodium intake [334] alongside increased meal frequency, protein and polyunsaturated fat (PUFA) intake [331]. MD increased total plasma polyphenol and serum folate concentrations [323].

In comparison with LFD, MD induced either a comparable or greater improvement in ALT [285,328,332], AST [328], FLI [328] and steatosis as assessed by imaging ($^1$H-

MRS/MRI) [280,285,324]. MD improved, high-density lipoprotein (HDL), TG [324,328] and waist circumference [324,332]. Both MD and LFD reduced body weight to similar degrees, except for one trial [332] in which weight loss was greater with MD (5.8% (MD) vs. 0.7% (LFD)) [332]. Participants on MD had higher levels of oleic acid, n-3 docosahexaenoic acid (DHA), monounsaturated (MUFA)/ saturated fat (SFA) ratio and total MUFAs; and lower concentrations of palmitic acid and total SFAs in serum phospholipids [328]. MD resulted in higher intakes of fat, MUFA [285] and nuts [324] with lower intakes of carbohydrate [285,324], sugars, sodium [285], trans-fat and dietary cholesterol [324]. Both a web-based MD intervention and its group-based MD comparator improved ALT, FLI and FIB-4, but the web-based intervention normalised ALT more frequently, and produced bigger reductions in FLI [322].

#### *2.4.3.3 MD component interventions*

In comparison with standard care, increased consumption of wholegrains (≥½ of cereal servings/day) improved ALT, AST, fatty liver grades (USnd) and blood pressure (BP) [335]. Increased wholegrain consumption reduced calorie intake [335] and increased fibre intake [325], while a reduced red meat intervention lowered iron intakes and serum ferritin concentration [325]. Reduction in ferritin concentration correlated with improvement in liver fat content [325]. Supplemental olive oil (20g or 20% of total fat/day) led to higher MUFA [336,337] and omega-3 intakes [337] and lower PUFA intakes [336,337], and produced greater improvements in ALT, AST [336] and fatty liver grades (USnd) [337].

### *2.4.4 Attrition*

Three trials (11.5%) reported zero attrition [280,330,340]. For the remaining trials, attrition ranged from 2% to 45%, with a mean of 14%. Four trials (15%) reported attrition over 20% [322,325,331,339]. Where reported, reasons for dropouts included health problems or pregnancy [323,325,326,333,337-339,342], unwillingness to continue [323,324,327,333,334,337,339], non-adherence [285,326,327,332,337], scheduling conflicts/ travel [337-339,341], personal reasons [285,323,324,326,339], dissatisfaction with either the allocated diet group, or diet phase [324,325,332], adoption of a non-intervention diet [333], alcohol excess [285], side effects [325], self-isolation due to COVID-19 [326] and death [327]. Studies investigating CRI reported the lowest attrition (mean 11%, range 2% to 30%), followed by MD interventions (mean 16%, range 6% to 45%), then MD components (mean 19%, range 14% to 26%).

### *2.4.5 Meta-analysis and Meta-regression of Effects of Dietary Intervention on NAFLD Markers*

Twenty-one trials [220,270,280,285,327-343] were included in the meta-analysis.

#### *2.4.5.1 Primary outcomes*

*2.4.5.1.1 Effects of dietary interventions on ALT and AST*

Meta-analysis showed that dietary interventions reduced post-intervention ALT (*n* 1295 participants) [220,270,280,285,327,328,330-343] ($I^2$: 67%, p <0.001) and AST concentrations (*n* 1152 participants) [220,327,328,330-340,342,343] ($I^2$: 75%, p = 0.004) significantly, but there was substantial heterogeneity between the included trials. CRI (*n* 667 participants) [220,327,338-343] and MD interventions (*n* 418 participants) [270,280,285,328,330-334] had favourable effects on post-intervention ALT levels ($I^2$: 0%, p <0.001), and ($I^2$: 81%, p = 0.02), respectively. There was no significant effect for MD component interventions (*n* 210 participants) [335-337] ($I^2$: 74%, p = 0.09) (Figures 2.5 and 2.6). Meta-regression analyses found no significant moderating effects for underlying NAFLD category, age, BMI, intervention duration, physical activity advice presence, sample size and attrition.

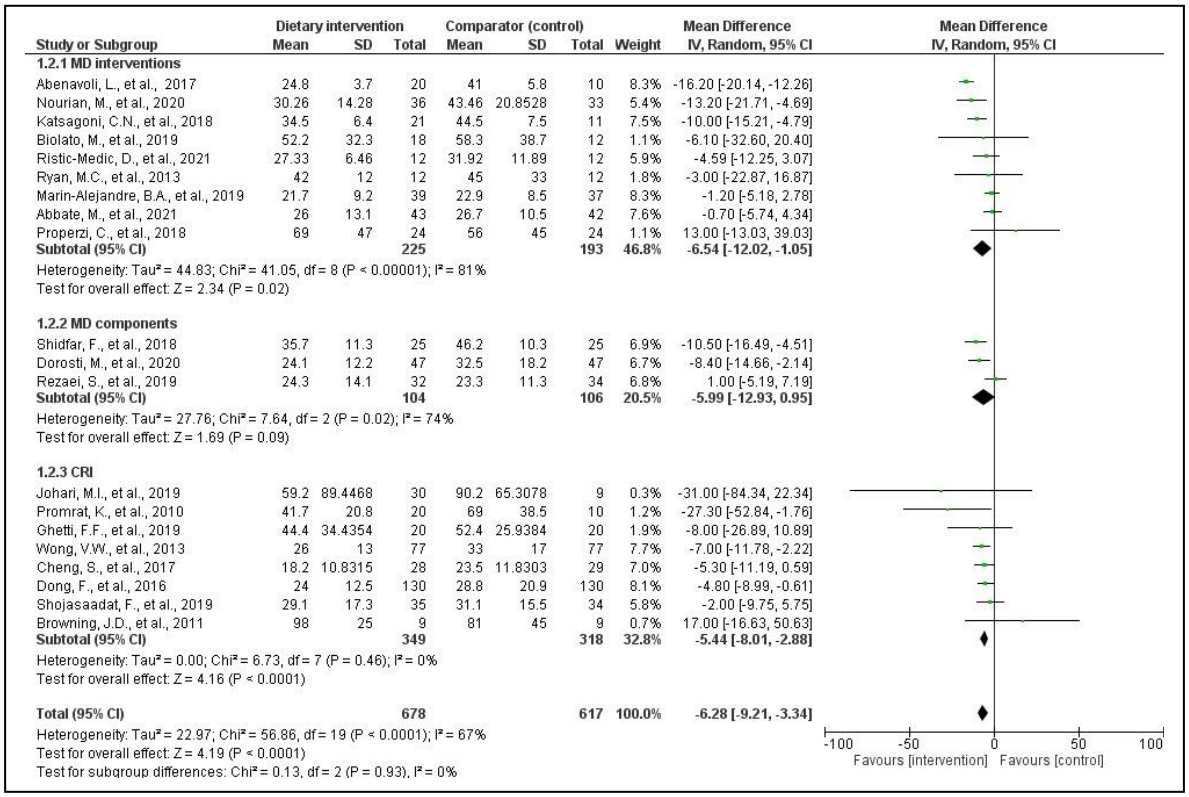



**Figure 2.5 Effects of dietary interventions on alanine aminotransferase (ALT) .** SD, standard deviation; IV, inverse variance; CI, confidence interval; MD, Mediterranean diet; CRI, calorie-restricted interventions.

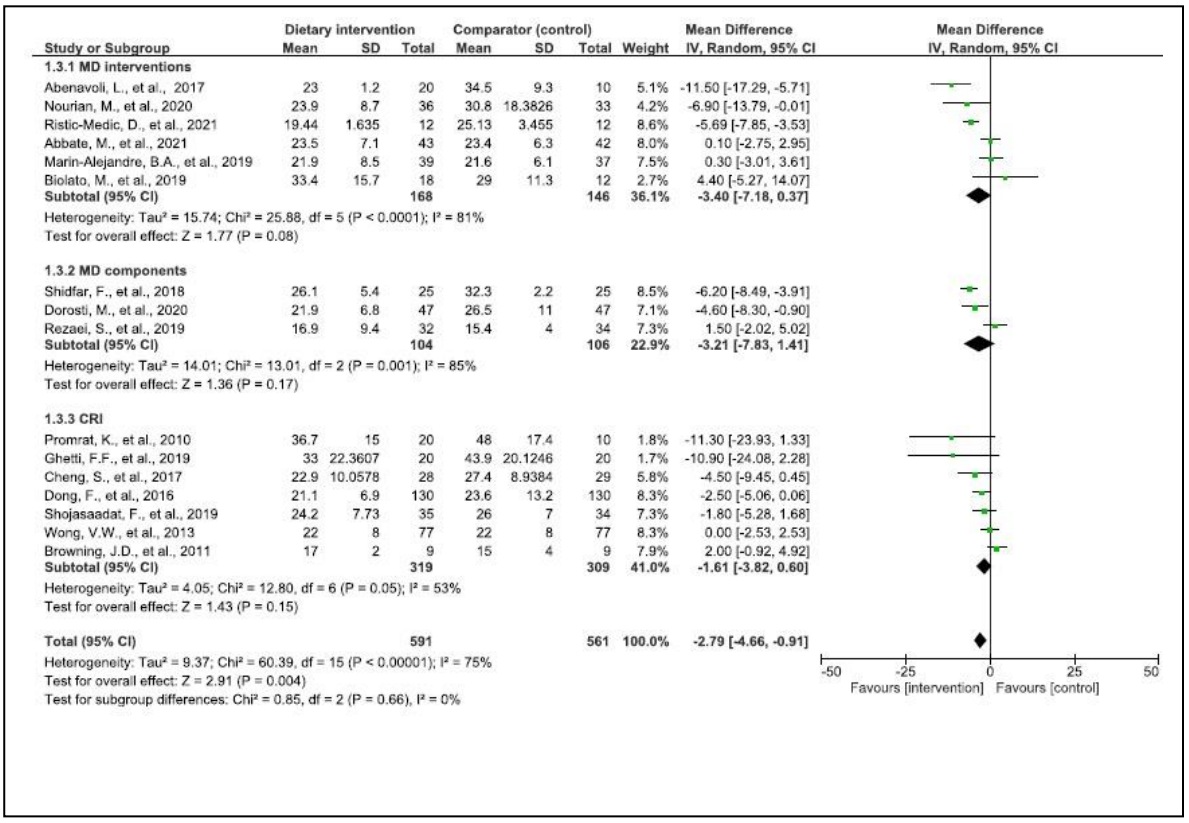



**Figure 2.6 Effects of dietary interventions on aspartate aminotransferase (AST).**
SD, standard deviation; IV, inverse variance; CI, confidence interval; MD, Mediterranean diet; CRI, calorie-restricted interventions.

### *2.4.5.1.2 Effects of dietary interventions on FLI and Hepatic Steatosis (by imaging and histology)*

Dietary interventions (*n* 488 participants) [327-331] had favourable effects on post-intervention FLI values ($I^2$: 80%, p <0.001), with substantial heterogeneity between the included trials. There were significant effects of MD interventions (*n* 228 participants) [328-331] on post-intervention FLI values ($I^2$: 71%, p <0.001) (Figure 2.7). Meta-analysis revealed a statistically significant effect of intervention (*n* 490 participants) [220,280,285,331,334,338-340] (p = 0.02) on post-intervention steatosis, but with substantial heterogeneity ($I^2$: 66%). Analysis by intervention type showed significant improvements in post-intervention steatosis following CRI (*n* 257 participants) [220,338-340] ($I^2$: 0%, p <0.001), but no effect of MD (*n* 233 participants) [280,285,331,334] ($I^2$: 76%, p = 0.70). There were statistically significant subgroup differences ($I^2$: 73.9%, p = 0.05) (Figure 2.8). Meta-regression analyses found no significant moderating effects

for underlying NAFLD category, age, BMI, intervention duration, physical activity advice presence, sample size and attrition.

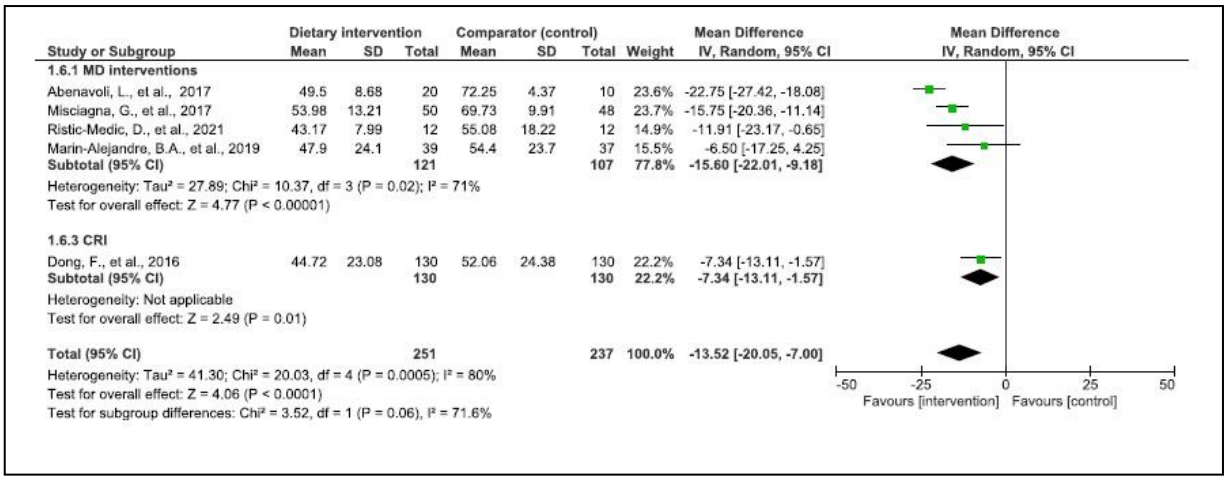



**Figure 2.7 Effects of dietary interventions on Fatty Liver Index (FLI).** SD, standard deviation; IV, inverse variance; CI, confidence interval; MD, Mediterranean diet; CRI, calorie-restricted interventions.

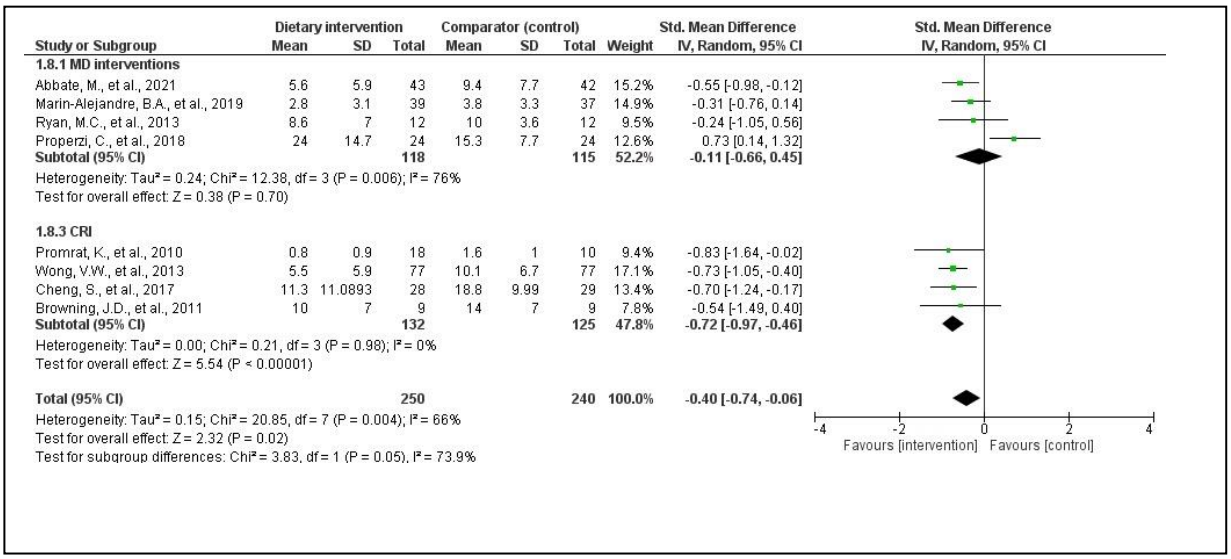



**Figure 2.8 Effects of dietary interventions on hepatic steatosis.** SD, standard deviation; IV, inverse variance; CI, confidence interval; MD, Mediterranean diet; CRI, calorie-restricted interventions.

*2.4.5.1.3 Effects of dietary interventions on Liver Stiffness*

Dietary interventions (*n* 464 participants) [270,285,330,331,334,338,341] lowered post-intervention liver stiffness, a surrogate for liver fibrosis, ($I^2$: 81%, p = 0.01) but there was evidence of substantial heterogeneity. CRI (*n* 193 participants) [338,341] and MD interventions (*n* 271 participants) [270,285,330,331,334] had favourable effects on post-intervention liver stiffness ($I^2$: 0%, p = 0.009, and $I^2$: 87%, p = 0.05, respectively) (Figure 2.9). Meta-regression analyses revealed that diet-induced improvement in

liver stiffness decreased as participant age increased (p <0.001) (Figure 2.10). There were no significant moderating effects of underlying NAFLD category, BMI, intervention duration, physical activity advice presence, sample size and attrition.

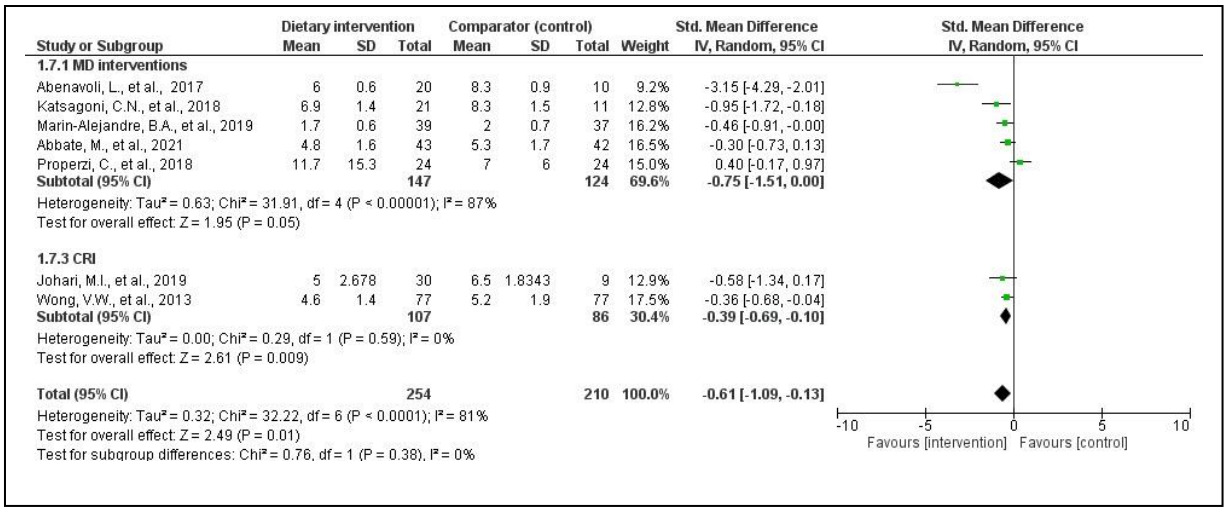



**Figure 2.9 Effects of dietary interventions on liver stiffness measurement (LSM).** SD, standard deviation; IV, inverse variance; CI, confidence interval; MD**,** Mediterranean diet; CRI, calorie-restricted interventions.

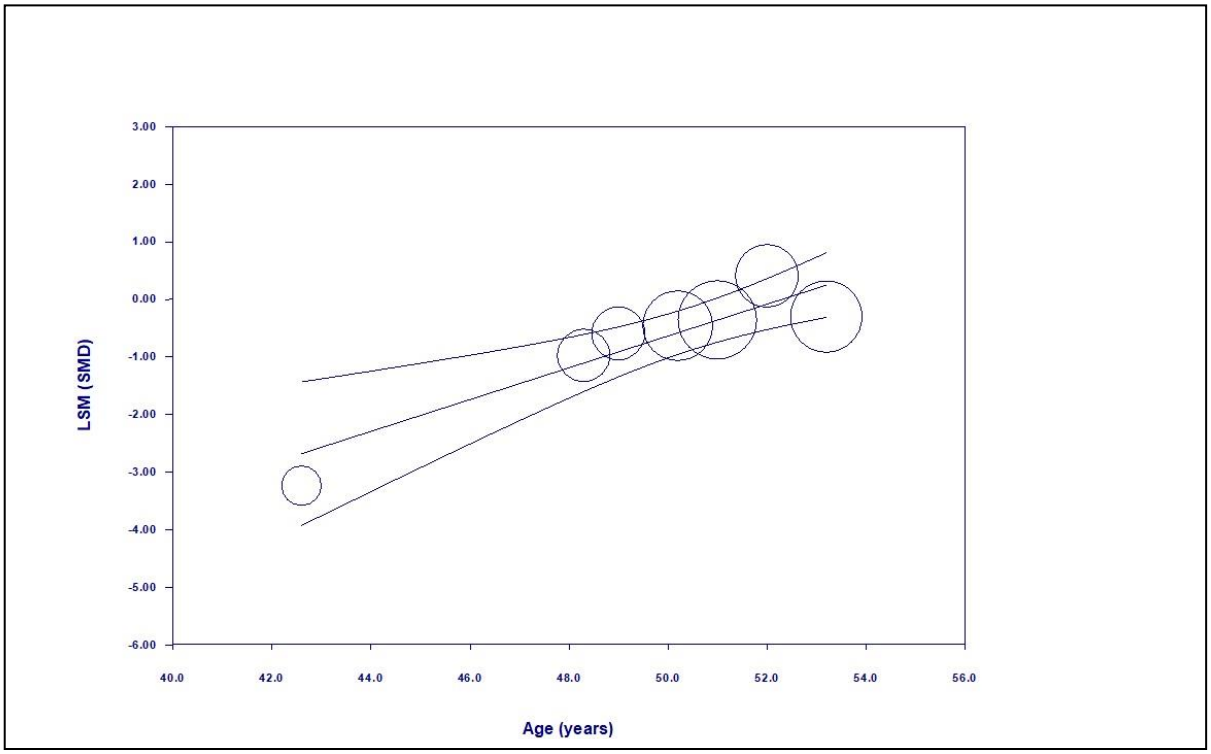



**Figure 2.10 Meta-regression of effects of age on change in measurement of liver stiffness (LSM) after dietary intervention.** SMD, standardised mean difference.

### *2.4.5.2 Secondary outcomes*

*2.4.5.2.1 Effects on dietary interventions on body weight*

Post-intervention meta-analysis found no significant effect of dietary interventions (*n* 1226 participants) [220,270,280,285,327,328,330-332,334-343] on body weight (Figure 2.11). Meta-regression analyses found no significant modifying effects for underlying NAFLD category, age, BMI, intervention duration, physical activity advice presence, sample size and attrition.

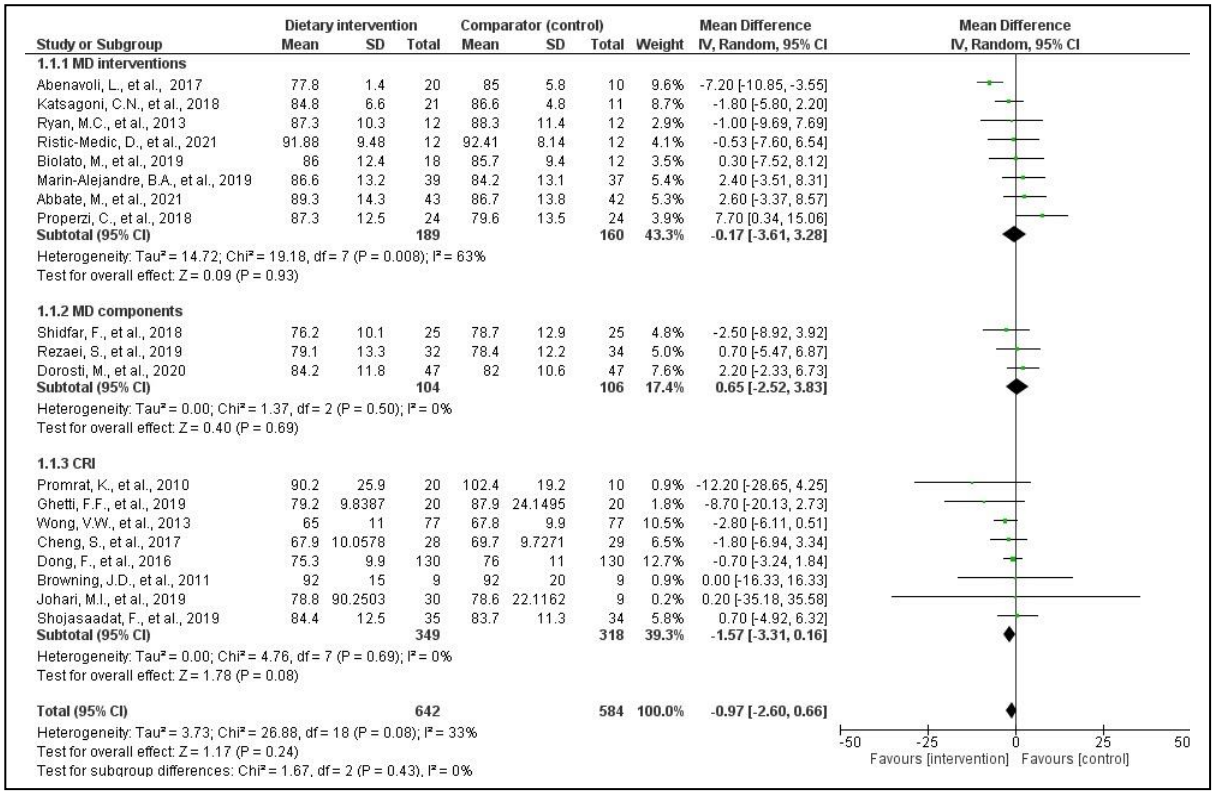



**Figure 2.11 Effects of dietary interventions on body weight.** SD, standard deviation; IV, inverse variance; CI, confidence interval; MD, Mediterranean diet; CRI, calorie-restricted interventions.

### *2.4.6 Sensitivity Analyses*

A sensitivity analyses, excluding results of two crossover trials (*n* 54 participants) [280,332], did not change the overall findings. Further sensitivity analyses were conducted, excluding results of ten trials (*n* 530 participants) that were judged as having serious [332], or high risk of bias [220,285,328,331,333,335,336,342,343]. After doing so, most of the findings were unaffected. However, excluding these trials revealed a significant effect of dietary interventions (*n* 765 participants) on post-intervention body weight ($I^2$: 26%, p = 0.03), and a significant effect of MD interventions (*n* 109 participants) on post-intervention steatosis ($I^2$: 0%, p = 0.01). There was no

significant effect of dietary interventions (*n* 670 participants) on post-intervention AST ($I^2$: 74%, p = 0.24) (Appendix J).

## Study 2: Diet Lifestyle Management of NAFLD: A Cross-sectional Survey of Clinicians.

### 2.5 Materials and Methods

#### *2.5.1 Study Design*

To gather data on current practice and perceived barriers to the effective delivery of diet lifestyle interventions, a cross-sectional survey of clinicians was conducted in two phases. The two phases targeted: The British Association for the Study of the Liver (BASL) and British Society of Gastroenterology (BSG) members (January to February 2019), and European and Israeli clinicians with an NAFLD interest (February to June 2019).

#### *2.5.2 Survey Instrument*

The e-survey instrument (using Online surveys, formerly BOS) was designed with three experts in the fields of NAFLD and nutrition and was revised through multiple rounds of feedback. The English-language 22-item e-survey questionnaire comprised 21 closed questions (mostly multiple choice) and 1 open-ended question with 10-15 minutes completion time. The e-survey instrument was piloted in December 2018, with three local dietitians, a specialist trainee and a research nurse for clarity, understanding and completion time. Subsequently, the questionnaire was simplified to 21 items. After additional input from Professor Zelber-Sagi (clinical dietitian, and nutritional epidemiologist) and two international specialist trainees, the final questionnaire was abridged to 20 items (Appendix K).

#### *2.5.3 Data Collection and Analysis*

The e-survey was circulated in two ways; i) on the BSG website, with the chance of winning a £50 amazon voucher as an incentive and ii) emailed directly to clinical groups of Professor Zelber-Sagi and the European Union IMI2-funded Liver Investigation: Testing Marker Utility in Steatohepatitis (LITMUS) biomarkers consortium (https://litmus-project.eu/). Individual clinicians were invited to share the survey with relevant others.

Upon survey completion, a quality check was performed, which indicated no requirement for clarification due to missing, inconsistent or unclear responses. The data were compiled and analysed using IBM SPSS statistics (version 24). Descriptive statistics and cross-tabulations were performed. Practice was compared across centres and professional roles and statistical significance was assessed using Pearson's $\chi^2$ test or Fisher's exact test, as appropriate. Significance was set at $p \leq 0.05$. Tables 2.1 - 2.3 presents total number percentages, which was derived by dividing the total number of responses by the total number of respondents.

## 2.6 Results

Responses were obtained from a total of 137 clinicians (senior gastroenterologists/ hepatologists (SGH), *n* 86; dietitians/nutritionists (DN), *n* 31; specialist trainees (ST), *n* 15; other, *n* 5)); from 50% specialist centres, 42% general hospitals and 8% primary care. The mean age of respondents was 42 years (range 25-69), and 55% were male.

| | |
|---|---|
| United Kingdom | 53.6% |
| Israel | 11.0% |
| Spain | 11.0% |
| Greece | 8.1% |
| Italy | 5.9% |
| Sweden | 4.4% |
| Germany | 2.9% |
| Portugal | 0.7% |
| Belgium | 0.7% |
| Estonia | 0.7% |
| Ireland | 0.7% |


**Figure 2.12 Countries represented across Europe and Israel.**

### *2.6.1 Routinely Recorded Clinical, Lifestyle and Patient Experience Data*

Data recorded routinely by clinicians are presented in Table 2.2. Recording of patient weight, BMI and alcohol intake was routine practice (99%, 91% and 85% respectively). Routine recording of diet intake and physical activity levels was

reported by only 43% and 46%, respectively. Patient satisfaction, reasons for opting out of diet lifestyle care and behaviour change readiness, were not routinely recorded. The measurement of waist circumference differed significantly between centres, with higher rates documented in specialist settings (p <0.001).

| Domain | General Hospital (%) | Specialist Centre (%) | Other (%) | Primary Care (%) | Total (%) |
|---|---|---|---|---|---|
| Weight | 57(42%) | 68 (50%) | 7 (5%) | 3 (2%) | 135 (99%) |
| BMI | 54 (40%) | 61 (45%) | 6 (4%) | 3 (2%) | 124 (91%) |
| Waist Circumference | 9 (7%) | 25 (18%) | 1 (1%) | 0 (0%) | 35 (26%)** |
| Diet Intake | 21(15%) | 29 (21%) | 6(4%) | 2 (2%) | 58 (43%) |
| Alcohol Intake | 51 (38%) | 57 (42%) | 6 (4%) | 2 (2%) | 116 (85%) |
| Physical Activity | 26 (19%) | 27 (20%) | 7 (5%) | 3 (2%) | 63 (46%) |
| Behavioural Readiness | 9 (7%) | 9 (7%) | 4 (3%) | 2 (2%) | 24 (18%) |
| Reasons for Opting Out | 7(5%) | 10 (7%) | 1 (1%) | 1 (1%) | 19 (14%) |
| Patient Satisfaction | 7(5%) | 8 (6%) | 2 (2%) | 1 (1%) | 18 (13%) |

**Table 2.2 Comparison of routinely recorded clinical, lifestyle and patient experience data by centre (n=136).** Data presented as n (%). BMI, body mass index. *P<0.5, **P<0.001.

### *2.6.2 Referrals for Diet Lifestyle Interventions*

Referral data are presented in Table 2.3. Almost two thirds of clinicians (64%) responded that all patients with NAFLD would be eligible for diet lifestyle interventions. Despite this, 68% of respondents referred less than half their patients. Around a quarter of referrals were informed by disease severity, BMI status, and the presence of two or more MetS features (26%, 25% and 18% respectively). Cardiovascular disease (CVD) risk and multi-morbidity were reported as a reason for referral by only 10% of clinicians. Respondents from general hospitals more frequently reported multi-morbidity as a criterion for referral than did those from specialist settings (9% vs. 2%) (p = 0.002).

Referrals for lifestyle services were sub-optimal; with direct access to multidisciplinary team (MDT) lifestyle services reported by only 23%. Twice as many respondents from specialist centres used this option compared with general hospitals (15% vs. 6%) (p = 0.033). Access to outsourced care providers i.e., specialist weight management (p <0.001) and surgical treatment services (p = 0.047) were more commonly reported by specialist centres.

Key barriers preventing referrals for diet lifestyle interventions included no commissioned services and short consultations/work overload (42%), limited dietetic

resources (32%) and insufficient behaviour change readiness (31%) (Figure 2.13). SGH most frequently considered lack of services a barrier (31% vs. DN 5% vs. ST 3%) (p = 0.014).

| Domain | General Hospital (%) | Specialist Centre (%) | Other (%) | Primary Care (%) | Total (%) |
|---|---|---|---|---|---|
| All NAFLD patients | 34 (25%) | 44 (32%) | 7 (5%) | 2 (2%) | 87(64%) |
| Disease Severity | 17 (13%) | 17 (13%) | 0 (0%) | 1 (1%) | 35 (26%) |
| BMI | 17 (13%) | 17 (13%) | 0 (0%) | 0 (0%) | 34 (25%) |
| ≥Two MetS Features | 15 (11%) | 10 (7%) | 0 (0%) | 0 (0%) | 25 (18%) |
| CVD Risk | 9 (7%) | 4 (3%) | 0 (0%) | 0 (0%) | 13 (10%) |
| Multi-morbidity | 12 (9%) | 2 (2%) | 0 (0%) | 0 (0%) | 14 (10%)* |
| Complex Nutritional Needs | 12 (9%) | 8 (6%) | 0 (0%) | 0 (0%) | 20 (15%) |
| Not Achieved Goals | 9 (7%) | 9 (7%) | 0 (0%) | 0 (0%) | 18 (13%) |
| Behavioural Readiness | 9 (7%) | 7 (5) | 0 (0%) | 1 (1%) | 17 (13%) |
| Other | 0 (0%) | 4 (3%) | 0 (0%) | 0 (0%) | 4 (3%) |
| <10% | 8 (6%) | 10 (7%) | 0 (0%) | 0 (0%) | 18 (13%) |
| 11-20% | 8 (6%) | 7 (5%) | 0 (0%) | 0 (0%) | 15(11%) |
| 21-30% | 8 (6%) | 8 (6%) | 0 (0%) | 1 (1%) | 17(13%) |
| 31-40% | 7 (5%) | 4 (3%) | 0 (0%) | 1 (1%) | 12(9%) |
| 41-50% | 2 (2%) | 6 (4%) | 0 (0%) | 0 (0%) | 8 (6%) |
| >50% | 13 (10%) | 25 (18%) | 3 (2%) | 2 (2%) | 43 (32%) |
| I Don't Refer | 11(8%) | 8 (6%) | 4 (3%) | 0 (0%) | 23 (17%) |
| MDT | 8 (6%) | 21 (15%) | 2 (2%) | 0 (0%) | 31 (23%)* |
| Directly | 33 (24%) | 35 (26%) | 5 (4%) | 3 (2%) | 76 (56%) |
| Via GP/ Endocrinologist | 22 (16%) | 10 (7%) | 1 (1%) | 0 (0%) | 33 (24%)* |
| Other | 5 (4%) | 13 (10%) | 0 (0%) | 1 (1%) | 19 (14%) |
| Community Management | 14 (10%) | 16 (12%) | 3 (2%) | 1 (1%) | 34 (25%) |
| Specialist Management | 24 (18%) | 51 (38%) | 5 (4%) | 2 (2%) | 82 (60%)** |
| Surgical Treatment | 11 (8%) | 25 (18%) | 2 (2%) | 1 (1%) | 39 (29%)* |
| Commercial Providers | 3 (2%) | 7 (5%) | 1 (1%) | 0 (0%) | 11 (8%) |
| Via GP/Endocrinologist | 16 (12%) | 7 (5%) | 0 (0%) | 0 (0%) | 23 (17%)* |
| Other | 11 (8%) | 9 (7%) | 0 (0%) | 2 (2%) | 22 (16%) |

**Table 2.3 Comparison of referrals for diet lifestyle interventions and perceived barriers preventing referrals by centre (n=136).** Data presented as n (%). BMI, body mass index; MetS, metabolic syndrome; MDT, multidisciplinary team. *P<0.5, **P<0.001.

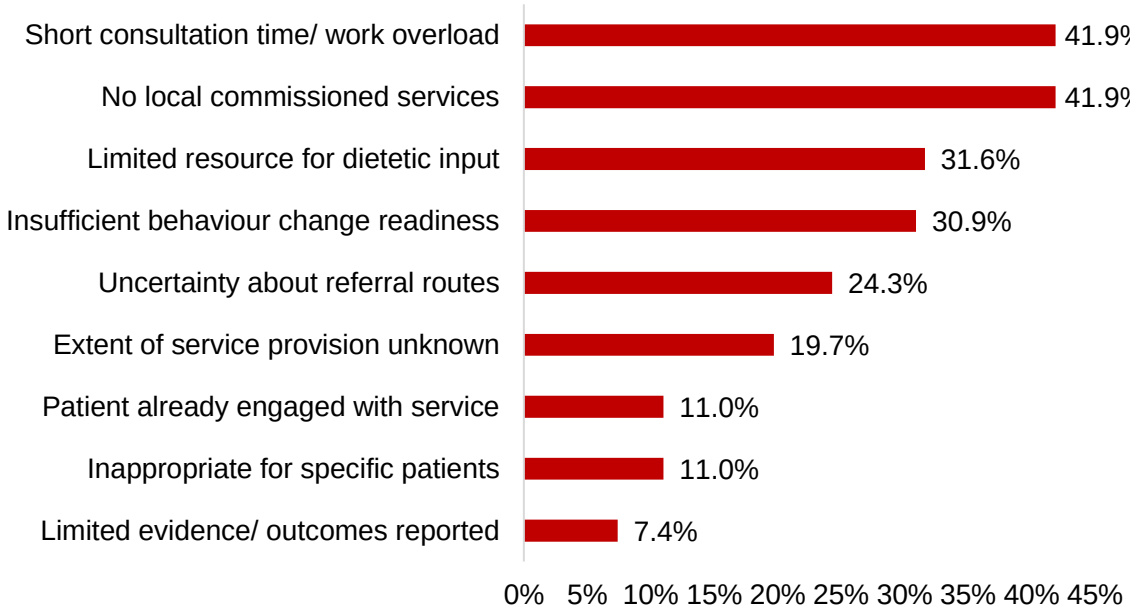



**Figure 2.13 Barriers to effective referral for diet lifestyle interventions.**

### *2.6.3 Routinely Delivered Diet Lifestyle Advice*

Lifestyle advice incorporating physical activity, weight loss and alcohol was routine (92%, 90% and 85% respectively). Around two thirds of respondents routinely advised on calorie restriction and diet composition. Specific behavioural components delivered included the impact of weight status on NAFLD outcomes (90%), and goal setting (64%) in brief/very brief interventions (43% vs. 34%) (Table 2.4). More clinicians in specialist settings explored a patient's weight and dieting history ($p = 0.033$). Only 52% advised the recommended weight loss of 7-10% (Table 2.4). More SGH and DN advised this target compared with ST (33% vs. 13% vs. 4%) ($p = 0.003$).

Perceived obstacles to lifestyle advice delivery were short consultations/work overload (80%) and inadequate behavioural training (45%) (Figure 2.14). More SGH and ST reported inadequate training as an obstacle than DN (34% vs. 6% vs. 4%) ($p <0.001$). Dietary approaches were delivered face-to-face (94%) and incorporated MD (60%). More clinicians from specialist centres advise a 600-calorie deficit approach ($p = 0.011$).

| Domain | General Hospital (%) | Specialist Centre (%) | Other (%) | Primary Care (%) | Total (%) |
|---|---|---|---|---|---|
| Weight Loss | 53 (39%) | 63 (46%) | 4 (3%) | 3 (2%) | 123 (90%) |
| Alcohol | 53 (39%) | 57(42%) | 4 (3%) | 2 (2%) | 116 (85%) |
| Calorie Restriction | 35 (26%) | 51 (38%) | 3 (2%) | 1 (1%) | 90 (66%) |
| Diet Quality/ Patterns | 40 (29%) | 43 (32%) | 6 (4%) | 3 (2%) | 92 (68%) |
| Physical Activity | 53(39%) | 62 (46%) | 6 (4%) | 4 (3%) | 125 (92%) |
| Any | 6 (4%) | 5 (4%) | 1 (1%) | 2 (2%) | 14 (10%) |
| 5% | 12 (9%) | 8 (6%) | 0 (0%) | 0 (0%) | 20 (15%) |
| 7-10% | 24 (18%) | 41 (30%) | 4 (3%) | 1 (1%) | 70 (52%) |
| >10% | 15 (11%) | 14 (10%) | 0 (0%) | 0 (0%) | 29 (21%) |
| Not Recorded | 0 (0%) | 0 (0%) | 2 (2%) | 1 (1%) | 3 (2%) |
| Weight impact on NAFLD | 52 (38%) | 60 (44%) | 7 (5%) | 3 (2%) | 122 (90%) |
| Weight/Diet History | 25 (18%) | 43 (32%) | 5 (4%) | 39 (2%) | 76 (56%)***** |
| Benefits of Change | 18 (13%) | 27 (20%) | 6 (4%) | 4 (3%) | 55 (40%) |
| Lifestyle Goals | 33 (24%) | 46 (34%) | 6 (4%) | 2 (2%) | 87 (64%) |
| Self-Monitoring Measures | 20 (15%) | 24 (18%) | 3 (2%) | 2 (2%) | 49 (36%) |
| Behavioural Counselling | 20 (15%) | 21 (15%) | 5 (4%) | 3 (2%) | 49 (36%) |
| Anti-Obesity Medications | 4 (3%) | 3 (2%) | 3 (2%) | 1 (1%) | 11 (8%) |
| Educational Materials | 13 (10%) | 18 (13%) | 5 (4%) | 3 (2%) | 39 (29%) |
| Signposting | 16 (12%) | 29 (21%) | 2 (2%) | 0 (0%) | 47 (35%) |
| Other | 0 (0%) | 0 (0%) | 1 (1%) | 0 (0%) | 1 (1%) |
| Very Brief | 23 (17%) | 23 (17%) | 0 (0%) | 0 (0%) | 46 (34%) |
| Brief | 23 (17%) | 32 (24%) | 1 (1%) | 2 (2%) | 58 (43%) |
| Extended Brief | 8 (6%) | 10 (7%) | 5 (4%) | 1 (1%) | 24 (18%) |
| Structured Programme | 3 (2%) | 3 (2%) | 1 (1%) | 1 (1%) | 8 (6%) |
| Healthy Eating Guidelines | 28 (21%) | 41 (30%) | 4 (3%) | 2 (2%) | 75 (56%) |
| Western Diet Exclusion | 15 (11%) | 24 (18%) | 5 (4%) | 3 (2%) | 47 (35%) |
| Low Fat | 18 (13%) | 23 (17%) | 2 (2%) | 1 (1%) | 44 (33%) |
| Mediterranean Diet | 30 (22%) | 42 (31%) | 5 (4%) | 4 (3%) | 81 (60%) |
| Low Glycaemic Index | 8 (6%) | 7 (5%) | 3 (2%) | 0 (0%) | 18 (13%) |
| Low Carbohydrate | 10 (7%) | 10 (7%) | 3 (2%) | 2 (2%) | 25 (19%) |
| 600 Calorie Deficit | 2 (2%) | 13 (10%) | 2 (2%) | 1 (1%) | 18 (13%)***** |
| Low Energy Diet | 3 (2%) | 11 (8%) | 2 (2%) | 0 (0%) | 16 (12%) |
| Very Low Energy Diet | 1 (1%) | 4 (3%) | 2 (2%) | 0 (0%) | 7 (5%) |
| Intermittent Fasting | 0 (0%) | 4 (3%) | 1 (1%) | 0 (0%) | 5 (4%) |
| Other | 5 (4%) | 2 (2%) | 1 (1%) | 1 (1%) | 9 (7%) |
| Face to Face | 52 (38%) | 65 (48%) | 7 (5%) | 4 (3%) | 128 (94%) |
| Online | 1 (1%) | 1 (1%) | 0 (0%) | 0 (0%) | 2 (2%) |
| Software App | 0 (0%) | 0 (0%) | 0 (0%) | 0 (0%) | 0 (0%) |
| Groups | 3 (2%) | 4 (3%) | 1 (1%) | 0 (0%) | 8 (6%) |
| Telephone | 2 (2%) | 5 (4%) | 0 (0%) | 0 (0%) | 7 (5%) |
| Other | 5 (4%) | 2 (2%) | 0 (0%) | 0 (0%) | 7 (5%) |

**Table 2.4 Comparison of routinely delivered diet lifestyle advice by centre (n=136).** Data presented as n (%). BMI, Body Mass Index. *P<0.5, **P<0.001**.**

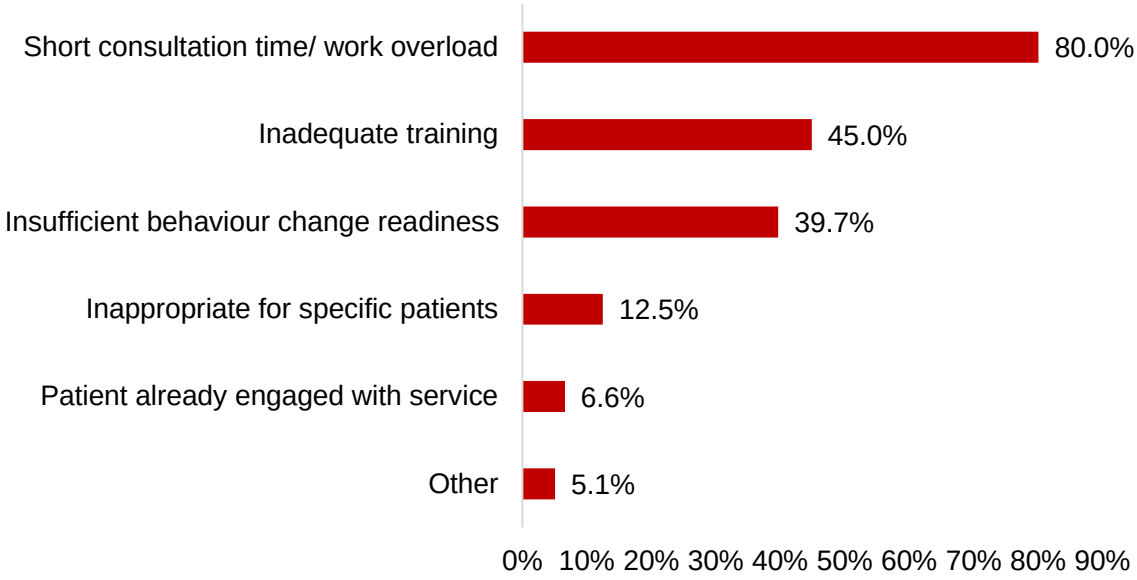



**Figure 2.14 Barriers to providing diet lifestyle advice.**

## 2.7 Discussion

The two studies described in this chapter provide up-to-date evidence on which to develop diet lifestyle care for patients with NAFLD.

The systematic review and meta-analysis (Study 1) synthesised data from 26 dietary intervention studies that reported effects on markers of liver damage, weight loss and on attrition during the intervention. The evidence from the reviewed trials suggest dietary interventions improve markers of NAFLD within two weeks and that effects can be sustained for up to two years. The meta-regression results suggest that diet-induced improvement in liver stiffness was greatest in the youngest patients and decreased with age. These results support the current clinical guidelines in NAFLD, which recommend interventions that encourage calorie (energy) restriction and healthier dietary patterns, such as the MD [6,64].

The survey (Study 2) revealed that service provision differs across centres and professional roles, and deviates from standard of care guidance [6,64]. This guidance recommends assessing anthropometrics (body weight, BMI and waist circumference), dietary patterns and physical activity levels [6]. It further recommends advising on lifestyle changes, targeting 7-10% weight loss through dietary modification (calorie restriction, excluding NAFLD-promoting components, the MD, limiting alcohol intake to national recommended limits) and increased physical activity

(aerobic exercise 150-200 minutes per week and resistance exercise on 2-3 days per week) [6,64,125]. However, there was substantial heterogeneity in reported practice patterns and in recording of key clinical, lifestyle and patient experience data. Respondents in specialist settings were more likely to recommend a 600-calorie deficit approach, utilise various lifestyle services, and document key lifestyle measures. This may be because more than half of respondents were from the United Kingdom, with marginally more from specialist settings, and where national guidelines endorse the 600-calorie deficit approach [64].

### *2.7.1 The Effectiveness of Calorie-restricted Interventions*

Calorie-restricted interventions produced improvements in liver function. The effectiveness of calorie restriction on ALT, steatosis and LSM shown in the meta-analysis findings suggests this strategy should remain the cornerstone of NAFLD management. The reviewed trials used a range of specific dietary treatments or broader healthy dietary guidance, with or without general physical activity advice, to induce from modest (e.g., 30% calorie deficit) to more severe (e.g., 1000 calories per day) calorie restriction.

There was limited evidence that over 2-weeks, a carbohydrate-restricted diet reduced liver fat more than a calorie-restricted diet [340]. Currently, there is no clear definition of a low carbohydrate diet, though <20g carbohydrate/day used in this trial could be regarded as a very low carbohydrate, potentially ketogenic, diet [344]. Studies of ketogenic diets have produced promising findings, which need to be verified in larger long-term studies [266]. There are few data on the adverse effects of these type of diets, and of their long-term outcomes in people with NAFLD. However, evidence suggests that these types of diets may have a detrimental impact on diet quality by increasing the intake of foods and nutrients linked with NAFLD pathogenesis, such as SFAs [142-144,345], while reducing the intake of nutrient-rich foods (i.e., whole grains, fruits, and some vegetables) that have been shown to be protective [345,346]. Moreover, prolonged adherence may increase the risk of deficiencies in vitamins, minerals, fibre, and phytonutrients [345]. Ketogenic diets can also have adverse effects on the gut microbiome, partly attributed to low fibre content [345,347]. The long-term health implications of ketogenic diets remain uncertain and careful consideration should be given to their implementation [345]. Responses from the survey suggests that these diets were not routinely prescribed in practice.

Low carbohydrate diets (LCDs) and LFDs are routinely delivered in obesity treatment. LCDs induce either a comparable or greater improvement in weight loss when compared with LFDs [258,348-350]. Both diets have been found to reduce the prevalence of MetS in obese adults [259]. Consistently, evidence suggests an equivalence of these diets on weight loss, liver enzymes, insulin resistance and steatosis among patients with NAFLD [222,260,261,265].

A reduction in body weight of ~5% in NAFLD and ≥7% in NASH appeared beneficial, and achievable. Two of the longest and most intensive multi-component interventions, lasting 11 and 12 months respectively, induced weight loss ≥7% [220,338]. These interventions targeted sustained weight loss, delivered nutritional/ behavioural counselling and advised ~200 minutes/week of moderate intensity physical activity, with or without resistance training. However, the dietary components of the two interventions were quite different. A community intervention advised calorie reduction with an individualized menu plan over 12-months [338]. The other intervention (lasting 48 weeks) used a more severe calorie restriction of 1000-1500cal/day with commercial portion-controlled foods, shifting to broader dietary guidance over time [220]. The more hypocaloric approach followed by flexible dietary restraint resulted in slightly higher weight loss (9.3% vs. 8.0% for the community intervention) [220,338]. More data on the cost effectiveness and acceptability of these interventions to both deliverers/patients would be beneficial. In practice the surveyed clinicians advised varying degrees of weight loss, with only 52% targeting the recommended weight loss of 7-10% [6,64]. Previous research has also revealed that weight loss advice is infrequently documented [313].

Limited evidence showed the advantageous effects of both calorie-restriction and dietary change components on cardiometabolic parameters [327,338,342,343] and abdominal obesity [338]. This may have important clinical implications for risk reduction of CVD and T2DM in patients with NAFLD. The modest average attrition rates suggests that the interventions were broadly acceptable to the participants. Moreover, limited data showed that the interventions induced diet intake modification as prescribed.

The current evidence shows mixed findings from a limited number of studies and further clarification from experimental trials is needed to determine the dietary

components associated with the greatest benefits on liver-related outcomes. In the context of moderate weight loss, a small number of trials suggested that specific dietary components, macronutrients and dietary patterns might improve liver function in those with NAFLD. Because recommended weight loss targets are difficult to achieve and to sustain, better evidence of the efficacy of different diet composition modifications could be a basis for developing personalised dietary care.

### *2.7.2 The Effectiveness of MD and MD Component Interventions*

The MD was the most widely advised dietary approach amongst surveyed clinicians. There was consistent evidence across the reviewed trials that MD interventions improved one or more measures of NAFLD activity and meta-analysis showed a significant effect on post-intervention ALT, FLI and LSM. This is consistent with recent reviews which concluded that MD interventions improved FLI [351], liver enzymes and NAFLD severity indices [352]. The summarised findings from the narrative synthesis, indicated that individual constituents of MD i.e., olive oil and wholegrains, may improve NAFLD markers [335-337], but the meta-analysis revealed no significant effects. The mechanisms through which MD exerts its NAFLD-specific effects remain poorly understood. However, the MD has been associated with several potential mechanisms that can contribute to its benefits in NAFLD including reduced inflammation [353,354], improved insulin sensitivity [280,355], decreased oxidative damage [355], and gut microbiota modulation [356].

The MD pattern, without calorie restriction, produced advantageous effects even with minimal weight loss (~2%) [280,285] which suggest that MD patterns may have potential to improve NAFLD with or without weight loss. However, calorie-restricted MD interventions resulted in weight loss ranging from >5% [270,322,330,332] to 7-10% [328,331,334], which is in line with the European Association for the Study of the Liver (EASL) clinical practice guidelines [6]. Thus, based on the available evidence [270,322,328,330-332,334] the combination of calorie restriction and MD might be an achievable strategy to induce the recommended degree of weight loss and to promote improvements in NAFLD.

Limited evidence showed that MD interventions produced favourable changes in cardiometabolic measures [324,328,330] and abdominal obesity [323,324,330,332]. Protective effects of MD on MetS features, which are strongly associated with NAFLD, have

been reported [277]. This is in line with previous reviews, which found that MD improved insulin resistance [351], lipid profile, and glycaemic indices [352]. The slightly lower attrition rates (10% in MD groups vs. 14% in comparator (control) groups; (excluding the trial with MD in both groups) [322]), supports the idea that MD is an acceptable diet therapy in this patient population. The reviewed trials suggest that, over periods of six weeks to two years, the interventions were successful in increasing MD adherence or altering intake of its components.

Current evidence of MD effectiveness comes mainly from trials conducted in Mediterranean regions/ climates with favourable food environments and cultural dietary habits. Thus, any potential impact on disease outcomes may be affected by baseline MD adherence [244]. This may affect the potential transferability of findings in populations with different habitual dietary patterns. However, previous research has shown that short-term interventions can increase MD adoption and maintenance for up to one year in non-Mediterranean countries [243,357-359].

### *2.7.3 Characteristics of Effective Interventions*

A key objective when evaluating diet lifestyle interventions is to determine whether interventions are implementable if shown to be effective [244]. Moreover, with increasing interest in personalised nutrition, the aim is to identify individuals or subgroups who may benefit from a specific intervention [360]. This is challenging in the present review, given that the interventions ranged from simple to more complex with heterogeneity in delivery mechanisms, intensity and behavioural strategies.

The interventions shown to induce the greatest weight loss were typically multi-component approaches, with most undertaken for at least six months. In practice, clinicians deliver brief or very brief interventions, focusing on physical activity, weight loss and alcohol. Brief/very brief interventions offer an opportunity to raise awareness, encourage and provide initial support for change but do not include longer term support to facilitate behaviour change. However, access to diet lifestyle interventions appears limited, with a lack of commissioned services and resources as obstacles [4,215,309]. Consistently, recent research found that the requisite support from nutrition and exercise specialists was unavailable for almost half of patients with NAFLD [308].

In most of the reviewed trials, assessment of dietary intake used self-report instruments, each of which is imperfect with risks of misreporting, errors and bias [300]. Furthermore, the inadequate recording of crucial clinical and lifestyle measures such as diet intake, physical activity and waist circumference amongst surveyed clinicians, may negatively impact decision making and on the delivery of personalised care.

The most common imaging method across the reviewed trials were MR techniques, which are the most accurate imaging for the assessment of steatosis [361], but this was used by less than half of trials. This was followed by USnd; while this is a practical tool, it is subjective and lacks precision for mild steatosis [18,84,86]. The FLI and NFS were the most commonly-used validated, non-invasive scoring systems used in the reviewed trials. However, these scoring systems were developed for risk stratification purposes with no evidence supporting their role as response biomarkers [90]. These scoring systems may not directly capture dynamic changes in NAFLD status over time. LSM was reported in less than a third of trials and performed by various methods. While invasive and subject to sampling variability, liver biopsy is the current gold standard for NAFLD assessment, but histologic examination was conducted in only one trial [220]. The need for accurate non-invasive assessment of NAFLD is yet to be addressed.

The main intervention delivery mechanism was face-to-face in individual consultations with smaller numbers of studies using group sessions and telephone contacts. These results from the systematic review are reflective of the survey responses, which indicate that face-to-face consultations are the main approach used in clinical practice. A web-based intervention that included some face-to-face interaction was as effective as a standard group intervention in NAFLD, but a higher attrition rate reported in the web-treated group [322]. The current evidence came mainly from specialist/ general settings, with fewer trials conducted in the community [323,324,338]. Nutritionists and dietitians were responsible for most of the interventions targeting dietary components. The choice of intervention setting, and the involvement of healthcare professionals, may influence the adoption/ maintenance of dietary changes [243]. Personalised approaches were utilised in some of the reviewed trials, which evidence suggests may be more effective than ‘one-size fits all’ treatments [360]. Both studies in this chapter revealed deficiencies in behavioural advice. These

deficiencies were due in part to perceived barriers of time and resource demands, as well as inadequate training. To address these limitations, identification of behavioural strategies that enhance intervention efficacy should be a priority for future research. These data would be highly relevant to the development of scalable dietary digital interventions that may reduce burden on patients and healthcare systems [243,322,362,363]. However, evidence suggests that it remains important to equip clinicians with up-to-date evidence-based behaviour change skills to support patients in practice [249,312,364].

### *2.7.4 Strengths and Limitations*

The current systematic review and meta-analysis (Study 1) focused on clinical trials, both RCTs and CCTs, that investigated the most common dietary approaches used in NAFLD and, therefore, are expected to provide robust findings. The substantial heterogeneity in the reviewed studies may impede potential transferability of findings into clinical practice. Nevertheless, the results of the sensitivity analyses did not greatly influence the overall findings and conclusions, which underlies the robustness of the observations.

More data are needed on the effects of lifestyle interventions on clinically relevant outcomes, such as hepatic cirrhosis, hepatocellular carcinoma (HCC) and mortality, which are unlikely to be noted in trials with less than five to ten years follow-up [365]. Hence, findings need to be verified in larger long-term studies, that use well-established outcomes measures and describe intervention costs and healthcare resource utilisation [365]. There was also limited evidence on the factors influencing individual patient decisions to adopt, and capacity to maintain, dietary changes. This information could support appropriate treatment adaptations [243] that optimise patient efforts at lifestyle changes and enhance intervention efficacy. Finally, other promising diet therapies remain under investigation, which are outside the scope of this review. Thus, the most effective dietary intervention for patients with NAFLD is still to be determined.

The survey of clinicians (Study 2) is unique in its explicit focus on gathering data on current practice and perceived barriers to the effective delivery of diet lifestyle interventions. Although, this survey used a convenience sample, from a consortium and societies specialising in gastroenterology and hepatology which may introduce

bias, a wide-range of clinicians were targeted producing comparable representation from general hospitals and specialist centres. Higher participation from physicians (74%) versus dietitians/nutritionists (23%) is to be expected and reflects clinical management pathways [83], although more data on the staff skill mix involved in NAFLD care delivery is needed. Limitations include a possible bias due to differential geographical responsiveness, but the magnitude of this bias is hard to determine. More than half of respondents were from the United Kingdom, which previous data has shown, scores most favourably for care management among European countries [366]. However, this score (the NAFLD preparedness index) is primarily driven by the existence of a national guideline and not on its implementation in practice [366].

The relatively small sample size means that the practice of a few specific clinicians could have potentially influenced the findings. Nevertheless, this survey provides first-hand feedback from clinicians with a NAFLD interest across a range of countries, professions and settings. To confirm the findings and reduce recall bias, an audit of real data such as medical case notes would be beneficial.

## 2.8 Conclusions

The current evidence from the reviewed trials (Study 1) suggests that most dietary interventions investigated in these trials were acceptable, and their adoption was associated with positive clinical outcomes. Calorie restriction, delivered via a range of interventions, is effective with a dose-response association between weight loss and indicators of different features of NAFLD. Changing diet composition has potential to complement diet-induced weight loss as the main therapy for NAFLD, and the limited data suggest that MD may be an effective treatment. However, further information from intervention trials that use individual participant characteristics to develop targeted diet therapies, would be advantageous. Key recommendations for future studies include robust assessment of dietary intake, intervention acceptability and sustainability, and quality of life and patient-related outcomes.

The present survey (Study 2) highlights considerable variability in diet lifestyle care for patients with NAFLD. There are deficiencies in the current lifestyle advice provided by clinicians, and access to effective diet lifestyle interventions, recognised as essential for all patients with NAFLD appears limited [6]. Given the magnitude of

the problem, these data suggest a need to improve i) access to timely and cost-effective lifestyle services with specialist expertise, and ii) training of clinicians, so that they can effectively support patients to change lifestyle-related behaviours [364]. The introduction of a specific diet lifestyle care bundle may help to standardise and optimise the advice provided by clinicians. The development of dietary digital interventions that may be outsourced and delivered at scale may address issues of resource and time demand. The combined findings from these two studies were used to inform the design of a randomised controlled feasibility trial described in Chapter 3.

# Chapter 3

# Materials and Methods

## 3.1 Introduction

Chapter 2 presented up-to-date evidence on which to develop diet lifestyle care for patients with non-alcoholic fatty liver disease (NAFLD). Importantly, these initial studies highlighted gaps in the evidence base and suggested directions for future research.

The effectiveness of current established diet therapies in NAFLD is sub-optimal [13]. Identifying therapeutic interventions that can optimise patient efforts at lifestyle modifications and maximise benefits on liver function are crucial [13]. To that end, further experimental data are needed to determine the potential of Mediterranean diet (MD) interventions to be translated in regions that traditionally consume western diets [243]. Secondly, more evidence on the factors that influence acceptability and adherence to diet therapies would be beneficial. In addition, further information on the impact of disease burden, and dietary treatments on patient-reported outcomes (PROs) such as health-related quality of life (HRQoL) is needed. Finally, given, the role of patatin-like phospholipase domain containing 3 *(PNPLA3)* variants in influencing risk of fatty liver disease and in modulating the effect of therapies, future studies should explore individual responsiveness to different diet therapies based on *PNPLA3* genotype [367]. The impact of other NAFLD-related gene variants such as in *TM6SF2, HSD17B13* in response to diet therapy also warrants further exploration [159] . In such studies, comprehensive assessment of habitual dietary intake is also warranted since this will aid development of more effective interventions [363].

The current chapter describes a protocol for a randomised controlled feasibility trial, used to determine the feasibility of a genotype-driven randomised controlled trial (RCT) in patients with NAFLD [368], and provides contextual background for the work conducted and presented in Chapters 4 to 6. The completion of feasibility studies with well-defined progression criteria informs the refinement of interventions and is an important part of planning for a successful larger scale evaluation [242].

## 3.2 Study Aims and Objectives

This project hypothesises that carriage of the *PNPLA3* variant influences MD responsiveness in NAFLD. The aim of the randomised controlled feasibility trial was to determine whether it is feasible to conduct a RCT to investigate the impact of

*PNPLA3* carriage on responsiveness to MD and NAFLD severity, and to provide preliminary data to inform the development of a definitive RCT.

The primary objective was to determine whether the protocol for a future definitive RCT is feasible, acceptable and effective:

- To determine the feasibility of recruitment and retention.
- To evaluate adherence to, and completion of, the study procedures.
- To evaluate implementation fidelity and how practicable it is to deliver the protocol in a clinical setting.
- To determine the acceptability of the diets, instruments and procedures.
- To evaluate adherence to, and completion of, the MD intervention.
- To undertake preliminary exploration of changes in key clinical and lifestyle variables.

Secondary objectives included the assessment of liver fibrosis biomarkers and the influence of *PNPLA3* genotype in response to the dietary intervention.

- To undertake preliminary exploration of changes in biomarkers of liver fibrosis.
- To undertake preliminary exploration of the influence of *PNPLA3* genotype on metabolic endpoints.

## 3.3 Materials and Methods

### *3.3.1 Study Design*

Study design and reporting were informed by the Consolidated Standards of Reporting Trials (CONSORT) extension for randomised pilot and feasibility trials [369]. This trial was a single centre, randomised controlled feasibility trial. In a crossover design, participants were randomised to either Diet 1 (MD) or Diet 2 (control) for 4-weeks, in random order, separated by a 4-weeks washout period. The intervention period (4-weeks) is relatively short and may not fully reveal the long-term effects of the dietary intervention on markers of liver health. However, it is important to note that dietary interventions have acute effects that can be observed within a few weeks, albeit very modest changes in biomarkers like cholesterol levels [370]. Observation of acute or short term effects can provide preliminary evidence of the initial responses to diet modifications. The selected time periods aimed to strike a balance between obtaining meaningful results and minimising participant dropout

rates. Moreover, this study is designed, primarily, to provide information on the acceptability and feasibility of the study protocol.

The use of a cross-over design facilitates more precise comparisons between intervention/ control diets on a within-participant basis [371]. This design is favoured in short-term trials of long-term conditions with intermediate outcomes [371]. Nevertheless, a limitation of this design is the possibility of carry-over effects from one experimental period to the next. The inclusion of a 4-week washout period sought to mitigate this issue. The required duration of washout period is influenced by the nature and duration of the intervention but, in nutritional cross-over studies, 2-4 weeks is often sufficient [368,370,372]. This is confirmed by studies of metabolomics biomarkers in urine which show that these respond rapidly to dietary change [368,373]. Importantly, the effectiveness of washout to return outcome variables to baseline after the MD intervention will be evaluated [368]. All study visits were conducted in the outpatient hepatology services, in The Newcastle upon Tyne Hospitals National Health Service (NHS) Foundation Trust, (NuTH) United Kingdom. An overview of the study design is provided in Figure 3.1.

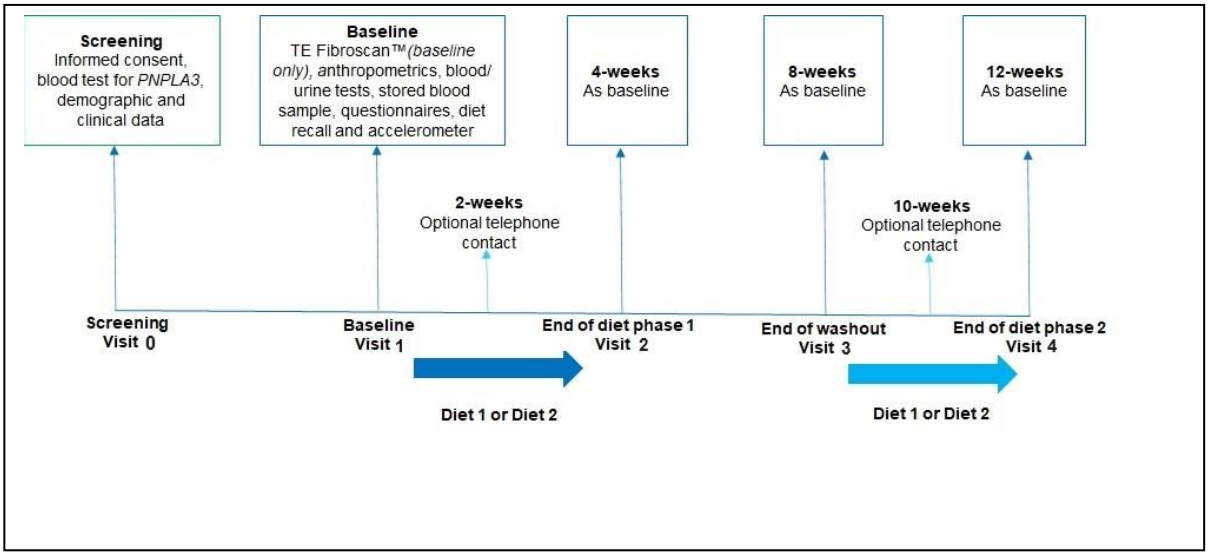



**Figure 3.1 Overview of the randomised controlled feasibility trial.** *PNPLA3,* patatin-like phospholipase domain containing 3; TE, transient elastography**.**

### *3.3.2 Sampling and Recruitment*

A recruitment target of 45-60 individuals with either imaging or histological evidence of NAFLD was established in accordance with published guidance [374]. This guidance suggests a sample of 30 individuals or greater, is sufficient to estimate a

parameter with the necessary degree of precision [374]. Adults with NAFLD were recruited from a tertiary hepatology centre, which covers a population of approximately 3 million individuals in northeast United Kingdom. Potential participants meeting the inclusion and exclusion criteria were identified from hepatology clinic lists and electronic records by members of the research and clinical care team. Potential participants were approached during an appointment in liver outpatients, or through screening of clinical notes, and invited to participate in the study. The research team contacted patients after 48 hours to answer any queries. Enrolment followed the receipt of full and written informed consent and successful screening. Recruitment and intervention delivery were anticipated to take place over 12 months.

### *3.3.3 Inclusion and Exclusion Criteria*

Participant eligibility criteria were detailed in Table 3.1. Minimal exclusions were chosen to reflect the target population for a subsequent definitive RCT.

| |
|---|
| Inclusion criteria |
| • 18-80 years old with NAFLD (confirmed on liver biopsy or by clinical diagnosis with imaging evidence of steatosis). |
| • Weekly alcohol consumption <14(women)/ <21(men) units in the last 24 months. |
| • Weight stable (+/-5%) for previous 3 months. |
| • Capacity to provide informed consent. |
| • Ability to write and converse in English without assistance of an interpreter. |
| Exclusion criteria |
| • All cancers within 5 years (except squamous cell carcinoma). |
| • Evidence of co-existent liver disease/ presence of secondary causes of NAFLD (except Gilbert's syndrome). |
| • Decompensated NASH-cirrhosis (Child Pugh >6). |
| • Uncontrolled psychiatric disorder (e.g., acute psychosis). |
| • Uncontrolled medical condition (e.g., HbA1c $\underline{\geq}$80mmol/l or acute coronary event or stroke within 12 months). |
| • Active eating disorder. |
| • Active substance misuse. |
| • Other prescribed dietary regimens, food intolerances and/or food allergies. |
| • Mediterranean diet assessment score (MEDAS) >8 (high MD consumption). |
| • Previous weight loss surgery. |
| • Taking anti-obesity medications and/ or engaged in structured, multi-component weight management interventions (specialist, community or commercial providers). |
| • Insulin use. |
| • Pregnancy/ lactation. |

**Table 3.1 Patient eligibility criteria.** NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; HbA1c, glycated haemoglobin**.**

### *3.3.4 Randomisation and Allocation Strategy*

Eligible participants were allocated in a 1:1 ratio to receive either Diet 1 or Diet 2 first using computer generated randomisation. Pre-randomisation genotyping for specific genetic variants (*PNPLA3 rs738409*) was conducted using blood samples collected at screening. Interim analysis of the genotype distribution amongst recruited cohort was conducted at intervals as recruitment proceeded (i.e., after one third and two-thirds were randomised); randomisation was stratified by *PNPLA3* status at baseline to ensure a balanced recruitment for *PNPLA3 rs738409* genetic status (wildtype, heterozygote, homozygote).

The minor allele frequency (MAF) for *PNPLA3 rs738409* is approximately 0.25 in the UK general population [181] and the MAF is greater in those with NAFLD [70]. Accordingly, it was expected that the recruitment target for wild-type cases would be met sooner than for carriers of the genetic variant. If sufficient cases of a given genotype were already enrolled. Plans were in place so that patients with a specific genotype that were not randomised would be discharged and offered a one-to-one nutrition education and counselling consultation with a dietitian. Blinding of participants, clinicians or research investigators to which diet each participant was on in each study period was not possible.

### *3.3.5 Trial Procedures*

The trial procedures are outlined in Table 3.2. At an initial screening appointment, demographic, clinical, dietary and lifestyle data were collected to determine patient eligibility and a blood sample was taken for *PNPLA3 rs738409* genotyping. In addition, at baseline, hepatic steatosis was assessed using controlled attenuation parameter (CAP) measured contemporaneously with liver stiffness (a surrogate for hepatic fibrosis) by transient elastography (TE), fibroscan.

Enrolled participants completed one-to-one diet and lifestyle consultations at baseline, end of diet phase 1 (4-weeks), end of washout (8-weeks) and end of diet phase 2 (12-weeks). At each of these time-points, the following measures were taken; anthropometry and body composition; blood biochemistry; dietary intake, urine samples and physical activity. The clinical status and medication consumption of participants was checked at each time-point. Patient-reported outcome data was captured at the end of each diet phase (4-weeks and 12-weeks).

### *3.3.6 Dietary Intervention and Control Treatments*

The experimental Diet 1 was a Mediterranean-style diet (MD) based on the traditional MD and MD pyramid [269,271]. The MD principles are described in Chapter 1. The diet was designed to be easy to follow over 4 weeks, informed by research evidence [375,376] and the findings from an earlier pilot study with patients and the public that explored barriers and facilitators to adoption of a MD intervention [377]. This body of evidence highlighted the importance of reducing the burden of dietary changes, of having diet supporters in the household, of employing frequent nutritional counselling and indicated a preference for face-to-face contacts. These ideas were incorporated into the final design.

To reduce participant burden and to facilitate appropriate changes in the food environment, intervention foods were supplied in the form of pre-packaged ready meals (FreshPrepare). Additionally, each participant received a 750ml supply of premium extra virgin olive oil intended for daily use from Filippo Berio, a renowned leader in the olive oil industry. Ten pre-packaged ready meals were home-delivered weekly, to be taken as two main meals per day (lunch and evening meal) for five days. The meals were ordered online for convenience and to streamline the process. Examples of the available options are detailed in Appendix L, which offered participants a diverse selection of more than 20 meals to choose from. Examples of the meals included red pesto salmon with vegetable medley and falafel salad with tahini, avocado and sriracha in a Mediterranean herb wrap. The meals were collaboratively designed with FreshPrepare, a chef-cooked meal plan company located in the northeast United Kingdom, where the research team specified dietary guidance and criteria for the meals. The menu was designed to appeal to a broad range of taste preferences and facilitate dietary changes.

To enhance MD adoption, nutrition counselling and education were provided one-to-one to participants during visits, by a dietitian. This included advice on selecting and preparing appropriate meals, snacks and drinks. The consultation incorporated the 'model and process for nutrition and dietetic practice', [378] which describes the components of a dietetic intervention that underpin professional practice. The consultation involved participant discussions, encouraging active engagement and clarifying any queries. Personalisation of the diet was based on the participants' current dietary habits/patterns and specific personal and sociocultural preferences.

Personalisation might have included ensuring the integration of the participants preferred Mediterranean foods and dishes; focusing on readily available local and seasonal ingredients/foods familiar to the participant; and adjustments for religious or cultural celebrations that involved specific foods, while still ensuring the participant adhered to MD principles. Advice on social interaction around meals was personalised to the individual.

A patient information booklet that explained NAFLD, MD principles, and how it can be successfully followed was given as evidence-based written material, from a credible source (http://www.livernorth.org.uk/pdfs/11%20NAFLD%20web%20&%20issuu.pdf ). Behaviour change techniques were utilised to increase intervention effectiveness. This included barrier identification, problem solving, goal setting, and action planning; social support; and instruction on how to perform a behaviour and behavioural practice [250,252,253].

Diet 2 (control) involved counselling participants to consume their habitual diet. During washout participants were asked to return to their habitual diet. Participants were asked to maintain baseline levels of physical activity and body weight (+/-3%) throughout the trial duration. The dietitian offered to contact participants by telephone mid-way through each diet phase to review progress, provide additional counselling and answer any queries.

| Variable | Instrument | Timepoint | | | | |
|---|---|---|---|---|---|---|
| | | Screening | Baseline | Phase 1 End | End of washout | Phase 2 End |
| Inclusion/ exclusion criteria | | x | | | | |
| Informed consent | | x | | | | |
| Demographic data and medical history | Self-report and clinical records | x | | | | |
| Genotyping | Venous blood sample | x | | | | |
| Anthropometrics: weight, height, waist and hip circumference<br>Whole-body composition | <br><br><br><br>Bioelectrical Impedance Analysis | | x<br><br><br><br>x | x<br><br><br><br>x | x<br><br><br><br>x | x<br><br><br><br>x |
| Cardio-metabolic measures: glucose, insulin, HbA1c, lipid profile and blood pressure | Fasting venous blood samples | | x | x | x | x |
| Liver function: LFTs, Ferritin, FBC, CRP, | Fasting venous blood samples | | x | x | x | x |
| Liver steatosis: CAP | TE Fibroscan™ | | x | | | |
| Liver fibrosis:<br>Liver stiffness, and PRO-C3 | TE Fibroscan™<br>Fasting venous blood samples | | x<br>x | x | x | x |
| Patient-reported outcomes | EQ-5D-5L, CLDQ-NASH and NASH-CHECK | | | x | | x |
| Physical activity | Accelerometer | | x | x | x | x |
| Dietary intake: dietary biomarkers, diet recall and MD questionnaire | Urine samples<br>INTAKE24<br>MEDAS | | x<br>x<br>x | x<br>x<br>x | x<br>x<br>x | x<br>x<br>x |

**Table 3.2 Schedule of study measures.** HbA1c, glycated haemoglobin; LFTs, liver function tests; FBC, full blood count; CRP, c-reactive protein; CAP, controlled attenuation parameter; TE, transient elastography; PRO-C3, N-terminal propeptide of type III collagen; EQ-5D, euroqol five dimension scale; CLDQ-NASH, chronic liver disease questionnaire for non-alcoholic fatty liver disease and non-alcoholic steatohepatitis; MEDAS, Mediterranean diet assessment score.

### *3.3.7 Primary Outcome Assessment and Process Evaluation*

The following criteria were assessed:

#### *3.3.7.1 The Feasibility of Recruitment and Retention*

The consent rate (the number of eligible participants who consented divided by the number who were eligible and invited to participate), the recruitment rate (the number of participants recruited per month), and the retention rate (the number of participants who completed follow-up data collection divided by the number randomised) were assessed using data from a trial log, between baseline and end of study.

#### *3.3.7.2 Adherence to, and Completion of, the Procedures*

Participant adherence to trial procedures was assessed by tracking the number of completed visits, and the completeness of data collection was assessed using the trial log, between baseline and 12 weeks.

#### *3.3.7.3 Implementation Fidelity and How Practicable Protocol Processes are to Deliver in a Clinical Setting*

Data collection burden was measured as the time taken to administer protocol processes. Participant processing time is the number of days from initial contact to enrolment. Both were assessed using the trial log, between baseline and end of study. To assess integrity and fidelity, a trial protocol checklist was monitored with missing, incomplete or unreliable data recorded between baseline and end of study.

#### *3.3.7.4 The Acceptability of the Diets, Instruments and Procedures*

PRO data was used to identify the impact of disease burden, diet treatments and trial procedures on HRQoL using the PRO instruments euroqol five dimension scale (EQ-5D-5L), chronic liver disease questionnaire for non-alcoholic steatohepatitis (CLDQ-NASH) and NASH-CHECK (Appendix M-O) [379-381]. The EQ-5D-5L is a widely used generic instrument that measures overall health status and HRQoL in 5 domains: mobility, self-care, usual activities, pain/discomfort, and anxiety/depression across a range of disease areas [379].

The use of disease specific instruments such as CLDQ-NASH and NASH-CHECK, are especially important as they capture the impact of the most frequent difficulties and concerns caused by NAFLD [382]. CLDQ-NASH collects data on 36 items categorised into 6 domains: abdominal symptoms, activity/energy, emotional health, fatigue, systemic symptoms, and worry [380]. NASH-CHECK comprises 31 items, used to derive six symptom scale scores (abdominal pain, bloating, fatigue, sleep,

itchy skin, cognitive symptoms), and three HRQoL scale scores (activity limitations, emotional and social impact) [381]. At 12 weeks, open-response questions captured participant perceptions of: i) the randomisation procedure, ii) the, acceptability of the MD intervention and iii) the components of the measurement protocol. These data were recorded (Appendix P), and thematic analyses performed.

The framework method was used for thematic analysis of the open-ended responses from the final questionnaire (11-items) [383]. This cross-sectional analysis combines data description and abstraction and involves data familiarisation, framework identification, indexing, charting, mapping and interpretation [383-385]. Two researchers (Laura Haigh (first researcher) and Colette Kirk (second researcher)) independently coded the same 40% of questionnaires to develop a working analytical framework. These initial findings were discussed during meetings to resolve discrepancies between researchers. This process was repeated with an additional 20% of questionnaires, producing further framework iterations. Coding involved reading and re-reading questionnaires and coding the content into themes and subthemes until no further codes emerged. Two experts in the fields of NAFLD and nutrition were consulted to refine the framework and ensure consensus was reached on themes and subthemes. This triangulation of expertise captured different perspectives and enhanced the trustworthiness of the findings. The framework then was applied by indexing the remaining questionnaire data using the existing categories and codes. The summarized abstracted data were charted into a matrix for each final theme. Direct participants quotes are reported to support themes and thus maximize confirmability. Reflexivity was fostered by recording impressions of the data and involving multiple researchers [243].

#### *3.3.7.5 Adherence to, and Completion of, the MD Intervention Protocol*

Diet adherence was assessed in self-report measures; Mediterranean diet assessment scale (MEDAS) is based on assessment of intakes of foods that are characteristic of the Mediterranean diet and measured in servings/day or servings/week (Appendix Q). Scores range between 0-14, and can be categorised as low, moderate or high consumption (<5, 6-9 and >10 points, respectively) [386]. In addition, dietary intake was quantified using INTAKE24, an open-source computerised dietary recall system based on multiple-pass 24-hour recall [387,388]. INTAKE24 data were quality checked and assessed for completeness [387,388].

Dietary recalls were removed if they were clearly under- or over-reported (e.g., calorie intakes < 400 calories or >4000 calories), or completed in a very quick time (e.g., 2 minutes), as per technical developer instructions (Open Lab, Newcastle University). The mean daily nutrient intake was calculated for 32 nutrients per participant.

Dietary biomarkers were quantified in urine to provide objective measures of dietary intake, without self-report bias (in collaboration with Aberystwyth University) [299]. A urine collection cup, transfer straw, and vacuum tubes were provided to participants with instructions on urine sampling at home (Appendix R) [299]. Three spot urine samples (first morning void) were collected at the beginning and end of each diet phase on non-consecutive days, including one weekend day, to provide estimates of habitual dietary intake, and stored in domestic fridge at 4°C. The samples were then posted back to Aberystwyth University in a Royal Mail Safebox™, which had prepaid first-class postage [299]. The three urine samples from each time-point were pooled and analysed using Ultra High-Performance Liquid Chromatography (UHPLC) [389].

A combination of non-targeted fingerprinting, which is global metabolite analysis via the generation of a spectrometric 'fingerprint' without using chromatographic separation [390] and quantitative biomarker panel assessed the feasibility of urinary intake biomarkers to: i) monitor adherence to the trial protocol and ii) investigate if there are endogenous metabolic perturbations associated with liver fibrosis and disease status. Aberystwyth University (Dr Thomas Wilson) performed the urine metabolomics and the advanced statistical analyses. The statistical analysis plan was developed in collaboration between the candidate, her supervisors and collaborators at Aberystwyth University (Dr Thomas Wilson).

*3.3.7.5.1 Urine sample preparation and adjustment*

Urine samples were thawed at room temperature and centrifuged (25,200 × g for 5 minutes at 4 °C). Then a hand-held refractometer (OpitDuo 38–53; Bellingham and Stanley) was used to measure the specific gravity of a 200-µL aliquot. Urine samples were normalised using specific gravity to ensure that all samples had the same specific gravity value prior to extraction [391-393].

*3.3.7.5.2 Flow infusion-high resolution fingerprinting (FIE-HRMS)*

Samples were analysed with FIE-HRMS, with mass spectra acquired on an Exactive Orbitrap mass spectrometer coupled to an Accela (ThermoFinnigan) ultra-performance liquid chromatography system (total assay time 3 minutes) [389]. Following data acquisition in profiling mode, raw mass spectra data files (.RAW, ThermoFinnigan) were processed to the universal mass spectrometry open file format, mzML [394]. Conversion and centroiding were conducted using msconvert from Proteowizard [394]. All further processing of mzML files was completed using the R Statistical Programming Language version 4.2 [389].

*3.3.7.5.3 Multivariate modelling and classification*

Principal component linear discriminant analysis (PC-LDA) was undertaken. For PC-LDA plots, eigenvalues (Tw) > 2.0 are generally the threshold to explanatory potential in the multivariable model. However, these heuristic thresholds often assume a certain degree of homogeneity within sample classes [395]. Given that the classes are very heterogenous when a control diet (CD) is habitual diet, the key performance indicator was the relative change between Tw. Random forest (RF) classification was implemented to determine the accuracy of each model [389]. RF is a robust, ensemble method of dimensionality reduction for high dimensional metabolomics data and is effective for determining if there are significant deviations in metabolic features by using non-linear combinations of features [396]. Overall classification accuracy is the main metric used for model evaluation, followed by area under the receiver operator characteristic curve (AUROC) [389,397]. Random forest regression modelling between total MEDAS score, MEDAS components and dietary biomarkers was undertaken to allow comparison and cross-validation of the two assessment methods.

*3.3.7.5.4 Quantification using ultra high-performance liquid chromatography (UHPLC)*

Analyses were performed on a TSQ Quantum Ultra EMR QQQ mass spectrometer (Thermo Scientific) equipped with a heated electrospray ionization source [389]. Mass spectra were obtained in multiple reaction monitoring (MRM) mode, in positive and negative ionization polarities concurrently using optimized values of collision energy and tube lens for each MRM transition [389].

Raw data (ThermoFisher) were processed to mzML using msconvert in ProteoWizard [394]. Additional processing of mzML files was completed using the R Statistical Programming Language version 4.2 [389]. Selected reaction monitoring (SRM) chromatograms were extracted from mzML files using the R library, mzR and peaks areas were calculated by extracting pre-defined chromatographic windows (based on calibration standards) around each peak apex. Absolute concentrations were calculated and for each calibration standard a quadratic equation was used to model the relationship between peak area and concentration. A squared fit of log 10-transformed values accommodated the broad concentration range for biomarkers in low and high consumers of target foods, without compromising accuracy and normal distribution requirements for regression analyses [389]. The limit of detection (LOD) and limit of quantification (LOQ) of all chemical standards were calculated as the lowest concentration of each biomarker giving a signal-to-noise ratio of 3:1 and 10:1, respectively within the linear range of each calibration curve [392].

#### *3.3.7.6 Preliminary Exploration of Changes in Key Clinical and Lifestyle Variables*

*3.3.7.6.1 Anthropometry and whole-body composition*

Height in centimetres (cm) was measured to the nearest 0.1cm without shoes using an electronic stadiometer (SECA 220; SECA, United Kingdom). Waist circumference was measured at the midpoint between the lower costal margin and the level of the anterior superior iliac crests. Hip circumference was measured at the level of the greater trochanters. Both were measured to the nearest 0.5cm. Weight in kilograms (kg) to the nearest 10g and whole-body composition were measured without shoes using bioelectrical impedance analysis (BIA) (Tanita T6360). Body mass index (BMI) was calculated as body weight (kg)/height$^2$ (metres). Waist-to-hip ratio (WHR) was calculated by dividing the waist measurement by the hip measurement.

*3.3.7.6.2 Blood pressure*

Blood pressure (BP) measurements were recorded after resting, using an automatic BP monitor (Welch Allyn, connex vital signs monitor 6000). Mean arterial pressure (MAP) was calculated as ((2 * Diastolic BP) + Systolic BP) / 3.

*3.3.7.6.3 Blood Biochemistry*

Fasting samples were analysed in a clinical pathology accredited laboratory in NuTH, Department of Clinical Biochemistry.

*3.3.7.6.4 Cardiometabolic Risk Assessment*

The QRISK3 risk prediction algorithm [398] was calculated to estimate the future risk of cardiovascular disease (CVD). The homeostatic model assessment of insulin resistance (HOMA-IR) was calculated to determine insulin resistance (IR) [399].

*3.3.7.6.5 Physical Activity*

Physical activity (PA), inactivity and sleep were measured using a triaxial accelerometer GENEActiv (ActivInsights Ltd, United Kingdom), which was worn on the wrist for seven days. R (www.cran.r-project.org) and R-package GGIR (Version 2.0-0) were used to process raw acceleration data [400]. Minimum wear time was specified as three or more days per week (including one weekend day), and 16 hours of valid data was needed daily [401]. Metric ENMO (1mg = 0.001 x gravitational acceleration) was used to calculate the average wrist acceleration per 5 second epoch [400]. Acceleration thresholds were defined as vigorous PA (VPA) (>400mg), moderate PA (MPA) (100-400mg), light PA (LPA) (40-100mg) and inactive (<40mg) [402]. Total sleep duration (in minutes) was estimated from the absence of changes in arm angle i.e., 5 degrees for 5 or more minutes, and sleep efficiency (%) was determined after the onset of sleep [402]. Data processing was conducted by experienced scientists (Dr S.J. Charman and Dr A.P. Blain in Newcastle University), who were unaware of any associated clinical data.

*3.3.7.6.6 Non-invasive tests (NITs) for liver fibrosis*

The Fibrosis-4 index (FIB-4) [97] is a validated non-invasive test (NIT) that effectively rules out advanced fibrosis if patients have a low score (using age-appropriate cut-offs), whereas indeterminate or high scores require second-line investigation. FIB-4 was calculated from liver function tests (LFTs) alongside other routine components i.e., Age × AST (IU/L)/platelet count (×109/L) × √ ALT (IU/L).

### *3.3.8 Secondary Outcome Assessment*

The following criteria were assessed:

#### *3.3.8.1 Preliminary Exploration of Changes in Biomarkers of Liver Fibrosis*

The capability of current NITs to rule in advanced fibrosis is modest [403]. There have been recent advances in biomarker development for accurate fibrosis staging and risk stratification [20] but more evidence is needed on biomarkers that predict treatment response and that have potential to achieve regulatory approval [20].

In collaboration with partners in the EU IMI2-funded Liver Investigation: Testing Marker Utility in Steatohepatitis (LITMUS) biomarkers consortium (https://litmus-project.eu/) serum concentrations of PRO-C3 (N-terminal propeptide of type III collagen) (a biomarker of active fibrogenesis), and additional biomarkers PRO-C4, PRO-C5, CTX-III (crosslinked type III collagen) (a biomarker of fibrolysis) were assessed using competitive Enzyme-Linked Immunosorbent Assay (ELISAs) (Nordic Bioscience A/S, Denmark). The measures were perfomed by experienced scientists unaware of any associated clinical data [404-406].

ADAPT [105] a combined PRO-C3, age, Type 2 diabetes (T2DM) and platelet count algorithm was calculated as: ADAPT = exp(log10((age × PRO-C3)/√(platelet count))) + T2DM.

Serum concentrations of growth differentiation factor 15 (GDF15) (a biomarker of metabolism) (Newcastle University) were measured by an experienced scientist (Dr J. Palmer) unaware of any associated clinical data. A competitive ELISA kit was used according to manufacturer's instructions [406]. Endoplasmic reticulum (ER) stress up-regulates expression of GDF15 [407]. Previous research has found that protein expression of GDF15 is associated with hepatic ballooning, inflammation and fibrosis in NAFLD serum samples [407].

#### *3.3.8.2 Preliminary Exploration of the Influence of PNPLA3 Genotype on Metabolic Endpoints*

Deoxyribonucleic acid (DNA) was extracted from whole blood samples collected with ethylenediaminetetra-acetic acid (EDTA) tubes [408] (up to 2 ml) by QIAamp Blood Midi Kit (QIAGEN, Germany), according to the manufacturer's instructions (https://www.qiagen.com/us/resources/resourcedetail?id=bf32146a-77fd-40c2-8743-c28974f7935b&lang=en). DNA concentration and 260/280 absorbance ratios were

measured using NanoDrop spectrophotometer (Thermo Fisher Scientific, USA) before proceeding to genotyping.

*PNPLA3* rs738409 (C>G I148M) and transmembrane 6 superfamily member 2 *(TM6SF2)* rs58542926 (C>T E167K) genotyping was performed by StepOne Real-Time polymerase chain reaction (PCR) system and TaqMan SNP Genotyping Analysis (Applied Biosystems, USA), as per manufacturer's instructions (https://assets.thermofisher.com/TFS-Assets/LSG/manuals/MAN0009593_TaqManSNP_UG.pdf). TaqMan genotyping assays (product codes: C_7241_10 and C_89463510_10) consist of pre-optimized PCR primer pairs and two probes for allelic discrimination.

TaqMan genotyping reactions were carried out in MicroAmp fast optical 96-well reaction plate (Applied Biosystems, USA). DNA was incorporated into a reaction mixture, comprised of TaqMan genotyping master Mix, two TaqMan minor groove binders (MGB) probes and forward/ reverse primers. Selective annealing of each TaqMan MGB probe occurs (if a corresponding sequence exists) between forward/ reverse primer sites. The nearness between the quencher dye and the reporter dye, supresses reporter fluorescence (when the probe is intact). The genotype of each sample was established by the fluorescence concentrations of the reporter dyes, and samples of the same genotype clustered together when plotted [159]. The reaction plate was set into the Real-Time PCR instrument and allelic discrimination plots generated by the proprietary StepOne software. Genotypes were successfully determined for each sample analysed.

### *3.3.9 Statistical Analysis*

All statistical analyses were performed using IBM SPSS version 27 and Microsoft Excel 2019. Descriptive summaries are reported as count and percentage, mean and standard deviation (SD), and median and range, as appropriate. Although this trial was not powered to detect significant changes in clinical and lifestyle outcomes, these were monitored, and preliminary evidence of changes are reported in Chapters 5 and 6. These data will enable the statistical power calculations for a subsequent RCT. A per protocol analysis of participants that completed the trial protocol was conducted. No imputation of missing data was undertaken.

Continuous data were tested for normality using the Shapiro-Wilks test and graphically through histograms. Continuous normally distributed variables are represented as mean (SD). Non-normal variables are summarised as median (range). Categorical variables are presented as count (percentage). Within-group changes between time points were assessed using the paired samples t-test (normally distributed variables), the Wilcoxon signed-rank test (non-normal variables) and the McNemar test (categorial variables). Fold changes were calculated to describe changes in variables between timepoints (via dividing the new value by its original value). Cross-tabulations were performed to analyse the relationship between demographic variables (age, sex, ethnicity) and missing data, and statistical significance was assessed using Pearson's $\chi 2$ test or Fisher's exact test, as appropriate.

Subgroup analyses were undertaken using the Mann-Whitney U or Student's t test, as appropriate (sex and genotype and disease stage categories), and Kruskal-Wallis tests (age and ethnicity categories). *PNPLA3* and *TM6SF2* genotypes were categorised into two groups: wildtype (CC) and risk allele (CG/ CT and GG/ TT). Disease stage categories were defined as non-advanced (fibrosis stages 0-3) and NASH cirrhosis. To explore the robustness of the primary analysis, a sensitivity analysis was conducted by excluding participants who exceeded +/-3% body weight change. Significance was set at $p \leq 0.05$.

Generalized estimating equations (GEE) were conducted to fit a repeated measures logistic regression to study the effects of the MD intervention on biomarkers of cardiovascular risk (CVR). GEE is an extension of generalised linear models, for repeated measures analysis. The within-subjects variable was time, the model type was linear, and the AR (1) correlation matrix (i.e., working correlation matrix which represents the within-subject dependencies) was set to the data. Diet, sex, T2DM and *PNPLA3* and *TM6SF2* genotypes were included as factors, and age, baseline MD adherence and baseline BMI as covariates. The selection of the factors and covariates was based on their known roles as risk factors in NAFLD and CVD, and the research question, which focuses primarily on the influence of diet. To adhere to the statistical requirements, all factors were categorical, while all covariates were scale data. Goodness of fit was assessed using the corrected quasi likelihood under

independence model criterion (QICC) (the structure that obtains the smaller QICC is superior).

### *3.3.10 Success Criteria*

The feasibility indicators are binary (successful or unsuccessful). “Successful” would indicate the protocol is sufficiently robust to advance to a fully powered definitive RCT, whilst “unsuccessful” indicates that protocol changes are required [369].

- The acceptability of diets, instruments and procedures.
- Consent rate (25% of individuals consenting).
- Recruitment rate (target 7 individuals/ month) (acceptable 5 individuals/ month).
- Retention rate (target 45 individuals completed follow-up) (acceptable 36 individuals completed follow-up)
- Protocol adherence (75% of individuals completed visits and data collection)
- Data collection burden (target 75% of individuals completed visits within 1.5 hours) (acceptable 75% of individuals completed visits within 2 hours)
- Participant processing time (<14 days between initial contact to enrolment)
- Trial protocol administration (<10% deviation from protocol checklist)

### *3.3.11 Patient and Public Involvement*

The early stages of the research process, involving the preparation of the research proposal, were supported by a national liver patient support group (LIVErNORTH). LIVErNORTH collaborated on the joint production of the patient information booklet, which is used in one-to-one diet and lifestyle consultations. To enhance the development of the MD intervention, the findings from an earlier pilot study [243] and research evidence were discussed with a patient panel (APEX) as well as patients attending clinical services in NuTH. APEX advised on the patient experience and assessed the burden of the trial instruments and procedures. This patient and public feedback was integrated into the final design. There are future plans to involve patients and the public in guiding the dissemination of trial results to participants and to relevant wider patient communities.

### *3.3.12 Ethics and dissemination*

Ethical approval was granted by East of Scotland Research Ethics Service (REC) 1 (19/ES/0112). NHS Research and Development approval was granted by NuTH (R&D8985). This trial was conducted to a high standard in accordance with the protocol, the principles of good clinical practice (GCP), relevant regulations, guidelines and with regard to patient safety and welfare.

## 3.4 Summary

This chapter describes a study protocol for a randomised controlled feasibility trial that was undertaken to determine the feasibility of a nutrigenetic therapeutic approach for patients with NAFLD. An evaluation of feasibility and acceptability of the study protocol is presented in Chapter 4. The effectiveness of the study protocol is presented in Chapter 5. Finally, Chapter 6 describes an assessment of liver fibrosis biomarkers and the influence of *PNPLA3* genotype in response to the dietary intervention.

# Chapter 4

# Randomised Controlled Feasibility Trial: Feasibility and Acceptability of The Study Protocol

## 4.1 Introduction

The current chapter presents a comprehensive evaluation of a study protocol for a randomised controlled feasibility trial, used to determine the feasibility of a genotype-driven randomised controlled trial (RCT) in patients with non-alcoholic fatty liver disease (NAFLD). This assessment provides essential data on whether the diet treatments, instruments and procedures are appropriate for further testing and likely to be implementable, if shown to be effective [244]. The qualitative and quantitative information presented indicates whether the study protocol is sufficiently robust in its current form to advance to a larger scale evaluation, or needs modification [409].

A successful dietary intervention must be acceptable to both deliverers/patients and elicit strong patient adherence [13,241,243]. A major challenge is the paucity of evidence on the factors that influence acceptability and adherence to current diet therapies. There is limited evidence on the factors influencing individual patient decisions to adopt, and capacity to maintain, dietary changes. Definitive data are also needed on which specific behaviour change strategies and intervention characteristics enhance intervention effectiveness [248,249]. Understanding the impact of dietary interventions on patient-related outcomes (PROs) such as health-related quality of life (HRQoL) in the NAFLD patient population is scarce [410]. These evidence gaps hinder the design of potential therapeutic interventions that aim to optimise patient efforts at lifestyle changes and maximise benefits on liver function.

## 4.2 Study Aims and Objectives

The overarching aims and objectives of the randomised controlled feasibility trial are outlined in Chapter 3.

The current chapter describes an assessment of the feasibility and acceptability of the study protocol.

The objectives of this current chapter were:

- To determine the feasibility of recruitment and retention.
- To evaluate adherence to, and completion of, the procedures.
- To evaluate implementation fidelity and how practicable it is to deliver the protocol in a clinical setting.
- To determine the acceptability of the diets, instruments and procedures.

## 4.3 Materials and Methods

Methods have been described elsewhere (Chapter 3).

## 4.4 Results

A total of 49 participants with NAFLD (*n* 36 biopsy-proven; *n* 13 clinical/radiologic evidence) were recruited from a specialist hepatology clinic in a tertiary care centre in the United Kingdom (UK). One participant left the study after the baseline visit for a non-related health issue, and so the final sample comprised 48 participants. A flow diagram of study participation is shown in Figure 4.1. Baseline characteristics included 46% male, with a median age of 60.0 (52.3-68.8) years and 90% white British; mean body mass index (BMI) was 35.1 (5.4) kg/m$^2$; median liver stiffness, 10.2 (6.4-14.8) kPa; 33% had advanced fibrosis (stage F3-F4). Metabolic syndrome (MetS) features of dyslipidaemia, Type 2 diabetes (T2DM) and hypertension were present in 71%, 54% and 48% of the study population (Table 4.1).

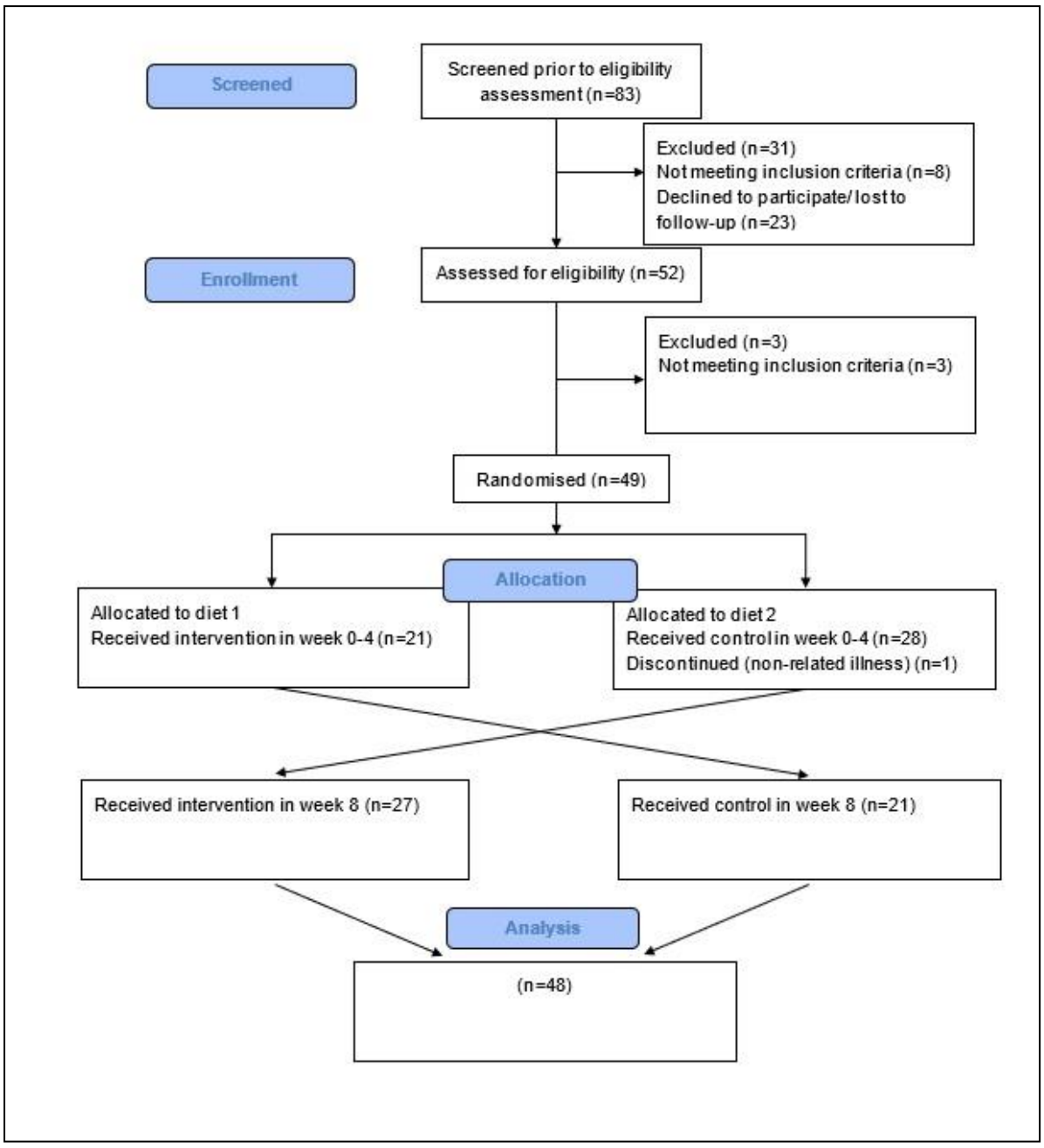



**Figure 4.1 Participant flow chart.** Summary of participant numbers at each stage of the trial. Diet 1 = Mediterranean diet; Diet 2 = control diet.

| **Demographics** | |
|---|---|
| Age (years) | 60.0 (52.3-68.8) |
| Sex (n, % male) | 22 (46%) |
| White British (n, %) | 43 (90%) |
| BMI (kg/m$^2$) | 35.1±5.4 |
| **Liver steatosis** | |
| CAP dB/m | 332 (307-382) |
| **Liver fibrosis** | |
| Liver stiffness (kPa) | 10.2 (6.4-14.8) |
| **Comorbidities and Medical History (n, %)** | |
| Dyslipidaemia | 34 (71%) |
| T2DM | 26 (54%) |
| Hypertension | 23 (48%) |
| CVD | 7 (15%) |
| OSA | 4 (8%) |
| Thyroid Disorders | 4 (8%) |
| Anxiety and Depression | 4 (8%) |
| Anxiety | 2 (4%) |
| Depression | 2 (4%) |
| Psoriasis | 2 (4%) |
| PCOS | 1 (2%) |
| **Medications (n, %)** | |
| Statins | 30 (63%) |
| Antihypertensives | 28 (58%) |
| Antiplatelet | 12 (25%) |
| Anticoagulants | 2 (4%) |
| Thyroxine | 4 (8%) |
| *Anti-diabetic Medication* | |
| Metformin | 22 (46%) |
| Sulphonylureas | 9 (19%) |
| Incretin mimetics | 5 (10%) |
| DPP-4 inhibitors | 4 (8%) |
| Thiazolidinediones | 3 (6%) |
| SGLT2 inhibitors | 2 (4%) |

**Table 4.1 Baseline characteristics of participants (n=48).** Data presented as n (%), means (standard deviation) or medians (range). BMI, body mass index; CAP, controlled attenuation parameter; T2DM, type 2 diabetes; CVD, cardiovascular disease; OSA, obstructive sleep apnoea; PCOS, polycystic ovarian syndrome; DPP-4, dipeptidyl peptidase-4; SGLT2, sodium-glucose cotransporter-2.

### *4.4.1 Mitigation of the Impact of the Coronavirus Pandemic on the Study*

The study opened for recruitment 13th January 2020 and was paused in line with the mandated National Institute for Health and Care Research (NIHR) announcement (19th March 2020) and in response to Government advice. The study re-opened 21st July 2020, which meant recruitment was behind schedule and necessitated

changes to some of the planned research activities. These changes included timely protocol amendments to allow both telephone and physical visits and to support a longer wash-out period (4 -12 weeks). For a small number of participants (*n* 7) there was a short period (up to 12 weeks) where no physical measures (e.g., anthropometry and blood samples) could be taken on-site.

### *4.4.2 The Feasibility of Recruitment and Retention and Implementation Fidelity*

Table 4.2 presents feasibility success criteria and actual outcomes. The trial protocol was feasible, as evidenced by the consent rate (i.e., *n* 49, 59% of individuals approached consented), recruitment rate (*n* 5.3 individuals per month) and retention rate (*n* 48, 98% of individuals completed the trial). The fidelity assessment, which included data collection burden (34.9±13.9 minutes) and participant processing time (12.5±9.0 days) met priori targets. There was less than 10% deviation from the protocol checklist, with only one participant missing genotype data and a small number of participants exceeding the +/- 3% weight change stipulation: on one occasion (*n* 5, 10%) and two occasions (*n* 2, 4%).

| Feasibility parameter | Priori success criteria | Target not met | Acceptable | Target met |
|---|---|---|---|---|
| Consent | 25% of individuals consented | | | ✓ |
| Recruitment | *Target*<br>• 7 individuals recruited/ month<br>*Acceptable*<br>• 5 individuals recruited/ month | | ✓ | |
| Retention | *Target*<br>• 45 individuals completed follow-up<br>*Acceptable*<br>• 36 individuals completed follow-up | | | ✓ |
| Protocol adherence | 75% of individuals completed visits and data collection | | | ✓ |
| Data collection burden | *Target*<br>• 75% of individuals completed visits within 1.5 hours<br>*Acceptable*<br>• 75% of individuals completed visits within 2 hours | | | ✓ |
| Participant processing time | • Less than 14 days between initial contact to enrolment | | | ✓ |
| Trial protocol administration | • Less than 10% deviation from protocol checklist | | | ✓ |

**Table 4.2 Feasibility criteria of the study protocol: comparison of pre-specified targets and actual outcomes.**

### *4.4.3 Adherence to, and Completion of, the Procedures*

There was high adherence to the trial protocol as illustrated by the number of completed visits with 94% completed visits and 4% partial visits respectively (Table 4.2). Partial visits were related to coronavirus (COVID-19) pandemic-related restrictions (no physical measures undertaken). The median minimum accelerometer wear time of 6 days (range 3 to 7 days) exceeded the target of > 3 days. The median self-reported Mediterranean diet (MD) meal compliance of 100% (range 80% to 100%), also exceeded the pre-specified target of 75%.

#### *4.4.3.1 The Completeness of Data Collection*

Table 4.3 presents feasibility success criteria for data collection and actual outcomes. At end-of-study, data collection for the majority of clinical and lifestyle variables (*n* 31, 82%) met the pre-specified target (defined as $\geq$90% data recorded). These data indicate high adherence to the trial procedures. The clinical and lifestyle variables, in which this target was not achieved included glycated haemoglobin (HbA1c) and controlled attenuation parameter (CAP) (by clinical/research teams); INTAKE24 and accelerometers (by participants). There were no significant associations between demographic variables (age, sex, ethnicity) and missing clinical and lifestyle data ($p > 0.05$) (Appendix S-U). However, missing data for clinical and lifestyle variables differed significantly between visits, with more missing data at visit 2 ($p < 0.05$), which coincided with the period of COVID-19 pandemic-related restrictions. Missing INTAKE24 data were more common at visit 4 (final visit) ($p = 0.003$) (Appendix V).

In addition to COVID-19 pandemic-related restrictions, the main reason for missing HbA1c results (at visit 2 and final visit ($p<0.001$)) was the laboratory refusing to process samples. In some cases, CAP results were not reported alongside liver stiffness on the participants electronic records. Missing INTAKE24 and accelerometer data were due partly to removals for invalid or poor data quality (*n* 2 and *n* 15 respectively). Accelerometer monitor failures were negligible.

| Study measures | Actual outcomes | Target not met | Target met |
|---|---|---|---|
| Genotype | 47 (98%) | | ✓ |
| **Anthropometrics** | | | |
| Weight (kg) | 184 (96%) | | ✓ |
| BMI (kg/m$^2$) | 184 (96%) | | ✓ |
| Waist-to-hip ratio | 184 (96%) | | ✓ |
| Fat (%) | 183 (95%) | | ✓ |
| FFM (kg) | 183 (95%) | | ✓ |
| **Cardiometabolics** | | | |
| Systolic blood pressure (mmHg) | 182 (95%) | | ✓ |
| Diastolic blood pressure (mmHg) | 182 (95%) | | ✓ |
| Fasting glucose (mmol/L) | 179 (93%) | | ✓ |
| Total cholesterol (mmol/L) | 181 (94%) | | ✓ |
| Triglycerides (mmol/L) | 181 (94%) | | ✓ |
| HDL-cholesterol (mmol/L) | 180 (94%) | | ✓ |
| TC:HDL ratio | 180 (94%) | | ✓ |
| Non-HDL-cholesterol (mmol/L) | 180 (94%) | | ✓ |
| Fasting insulin (pmol/L) | 149 (78%) | ✓ | |
| HbA1c (mmol/mol) | 107 (56%) | ✓ | |
| **Hepatic function** | | | |
| Platelets (x10 $^9$ /L) | 180 (94%) | | ✓ |
| Bilirubin (umol/l) | 183 (95%) | | ✓ |
| Albumin (g/L) | 184 (96%) | | ✓ |
| ALT (unit/L) | 184 (96%) | | ✓ |
| AST (unit/L) | 181 (94%) | | ✓ |
| ALP (unit/L) | 184 (96%) | | ✓ |
| Ferritin (ug/L) | 175 (91%) | | ✓ |
| CRP (mg/L) | 179 (93%) | | ✓ |
| GGT (unit/L) | 154 (80%) | ✓ | |
| **Hepatic steatosis** | | | |
| CAP dB/m *(baseline only)* | 35 (73%) | ✓ | |
| **Hepatic fibrosis** | | | |
| PRO-C3 (ng/mL) | 180 (94%) | | ✓ |
| GDF15 (pg/mL) | 181 (94%) | | ✓ |
| Liver stiffness (kPa) *(baseline only)* | 41 (85%) | ✓ | |
| **Dietary intake** | | | |
| MEDAS | 189 (98%) | | ✓ |
| Urinary intake biomarkers | 179 (93%) | | ✓ |
| INTAKE24 | 151 (79%) | ✓ | |
| **Physical Activity** | | | |
| Accelerometers | 167 (87%) | ✓ | |
| **Patient-reported outcomes** | | | |
| CLDQ-NASH | 89 (93%) | | ✓ |
| NASH-CHECK | 94 (98%) | | ✓ |
| EQ-5D-5L Utility score | 91 (95%) | | ✓ |
| EQ-5D-5L Visual analogue scale score | 90 (94%) | | ✓ |
| Open-response questionnaire | 48 (100%) | | ✓ |

**Table 4.3 Feasibility criteria of the study protocol: comparison of a priori target for data collection and actual outcomes.** Data presented as n (%). BMI, body mass index; FFM, fat free mass; HDL, high-density lipoprotein; TC: HDL, total cholesterol: HDL ratio; ALT, alanine aminotransferase; AST, aspartate aminotransferase; ALP, alkaline phosphatase; CRP**,** c-reactive protein; PRO-C3, N-terminal propeptide of type III collagen; GDF15; growth differentiation factor 15; MEDAS, Mediterranean diet assessment score; EQ-5D, euroqol five-dimension scale; CLDQ-NASH, chronic liver disease questionnaire for non-alcoholic fatty liver disease and non-alcoholic steatohepatitis; HbA1c, glycated haemoglobin; GGT, gamma-glutamyl transpeptidase; CAP, controlled attenuation parameter.

### *4.4.4 The Acceptability of the Diets, Instruments and Procedures*

The open-ended responses from the final questionnaire are described with participants identified by sex and age only. Figures 4.2 to 4.4 provide the analytical frameworks for themes, subthemes, barriers, and facilitators. Using framework analysis, three themes were identified to explain participants perceptions of the feasibility study; trial design, instruments and procedures; the MD intervention and meal supplier; and the impact of COVID-19 on diet and lifestyle behaviours.

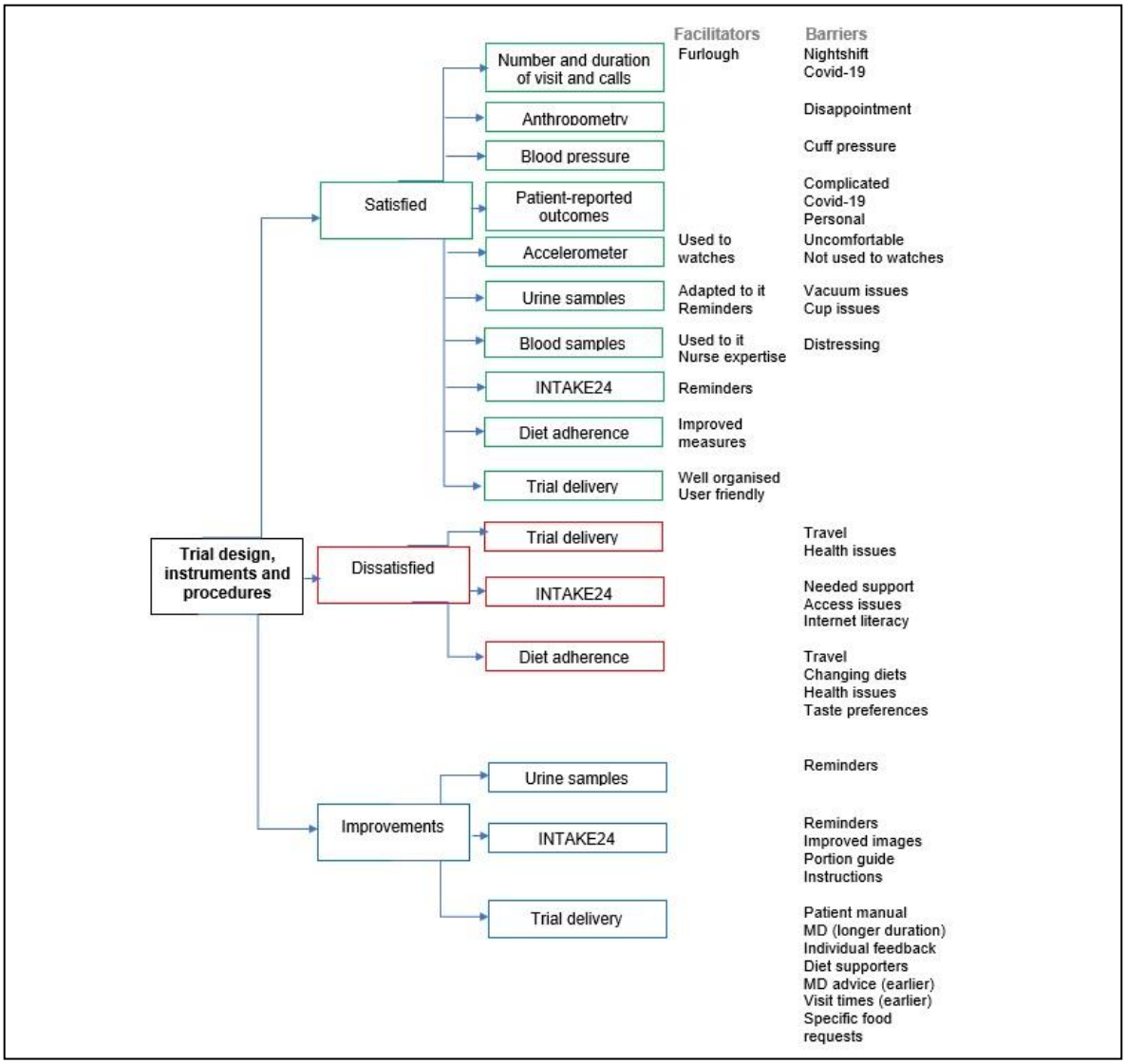



**Figure 4.2 Themes, subordinate themes, barriers, and facilitators.** Trial design, instruments and procedures.

#### *4.4.4.1 Trial Design, Instruments and Procedures*

Based on the questionnaire data, participants, endorsed most components of the trial protocol. Being on furlough was perceived to facilitate engagement with trial visits and telephone calls. In contrast, work patterns (i.e., night shift work) and COVID-19

restrictions were considered problematic. *"Since I've been on furlough it's been ok. Fasting on night shift before that was difficult"* (53-year-old man). Travel and health issues were perceived as obstacles to engagement by a few participants.

Some participants described being less willing to engage with anthropometric measurements if they interpreted their own results as disappointing. *"This was fine, but I feel disappointed with the results at times"* (59-year-old female). Similarly, engagement with the MD intervention appeared to be influenced by improvements in clinical measures. In some cases, PRO instruments were viewed as personal and complicated, with the impact of COVID-19 perceived as an aggravating factor. *"Generally, they were ok, but I found some of the questions a little uncomfortable and personal. I completed them with my wife present and found the experience challenging due to the personal nature of some questions"* (71-year-old man). A few participants reported blood samples as distressing, but also regarded nurse expertise positively. In some instances, accelerometers were perceived as uncomfortable "*they were ok, but the straps can be uncomfortable"* (30-year-old male). However, the process of becoming accustomed to an instrument (e.g., accelerometer) or procedure (e.g., urine sampling and blood sampling) was regarded as supportive to engagement *"blood sampling is fairly routine at the moment"* (49-year-old male).

Prompts and reminders intended to alert participants to perform tasks routinely (e.g., home urine sample collection and INTAKE24 data input) were described as useful. However, in some cases physical difficulties with the vacuum process for urine transfer (vacuum tube) and the urine collection cup emerged as obstacles *"I had some difficulties due to carpal tunnel, pressing down. Then I got used to it and had no problems"* (59-year-old female). Engagement with the INTAKE24 dietary recall system appeared to be hindered by technological issues of limited access, and a lack of internet literacy. The cross-over study design appeared to impact diet adherence for a small number of participants, with difficulties reported when switching diets. Travel and health issues were other reported barriers.

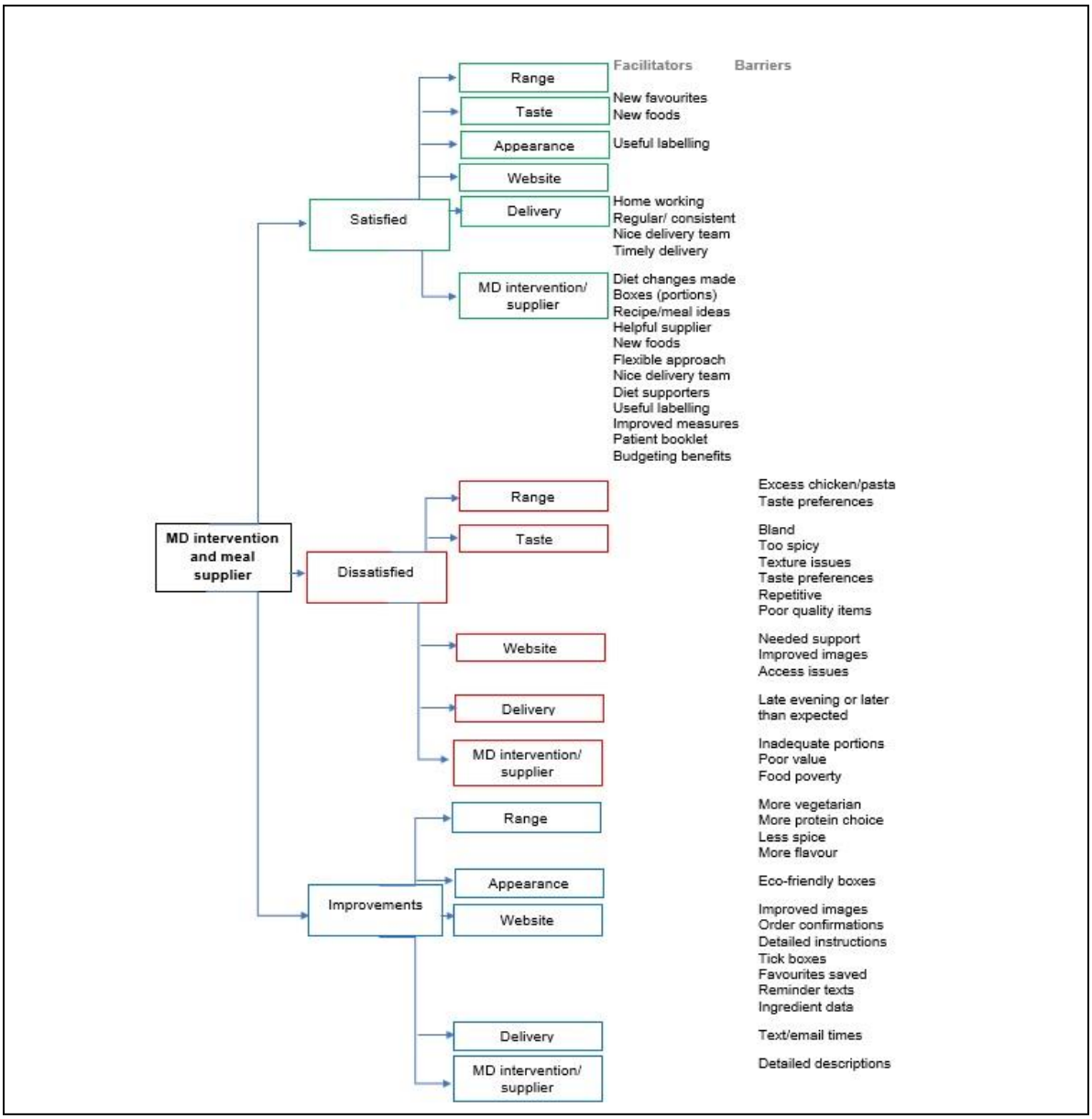



**Figure 4.3 Themes, subordinate themes, barriers and facilitators.** Mediterranean diet intervention and meal supplier.

#### *4.4.4.2 Mediterranean Diet Intervention and Meal Supplier*

The MD intervention was highly acceptable to many participants who appreciated the new experiences, flexibility and budgeting benefits. Participants shared specific dietary changes, to emphasise their engagement with the MD intervention *"we eat falafel wraps now, and batch cook. I've reduced junk food"* (55-year-old male). Having access to a patient information booklet, recipe and meal ideas as well as having diet supporters facilitated diet modifications. However, in some cases, engagement with the MD intervention appeared dependent on improvements in clinical measures.

Both the nutrition labelling and the boxes (meal containers) used by the meal supplier were viewed positively, with the latter regarded as a useful aid to achieve recommended portion sizes *"the boxes helped portion control, especially of carbohydrates. It was eye-opening"* (62-year-old female). However, taste and texture preferences were a clear obstacle in some cases, and a few participants considered the portion sizes as inadequate *"Small portions, not filing. They needed extra pulses, beans and eggs"* (70-year-old male).

Attitudes diverged on whether the meals supplied were bland, too spicy, or repetitive. A few participants questioned the quality of the food and 'value for money' (cost effectiveness) of the MD supplier and issues of food poverty were highlighted *"My daughter was out of work, so I shared the meals with her"* (57-year-old female). The meal supplier delivery process and website were broadly acceptable to most participants. Notable exceptions were the occasional late evening delivery and difficulties with access to the meal supplier's website, that required support from the research team to complete meal ordering.

Participants suggested several measures to optimise engagement with the MD intervention. These were primarily focused on the meal supplier and included access to a greater range of meals (i.e., more vegetarian and protein options) and replacement of plastic boxes (meal containers) with eco-friendly alternatives *"I was surprised there wasn't more legumes, could increase those, and vegetables/ salads"* (62-year-old female). Improved website functionality such as meal images, ingredient data as well as offering order confirmations and saved favourites were perceived to enhance future engagement *"improve the images, send order confirmations, add saved favourites and text reminders to order"* (55-year-old male). Similarly, technological improvements to the delivery process (i.e., text/ email reminders) were suggested to facilitate participant satisfaction.

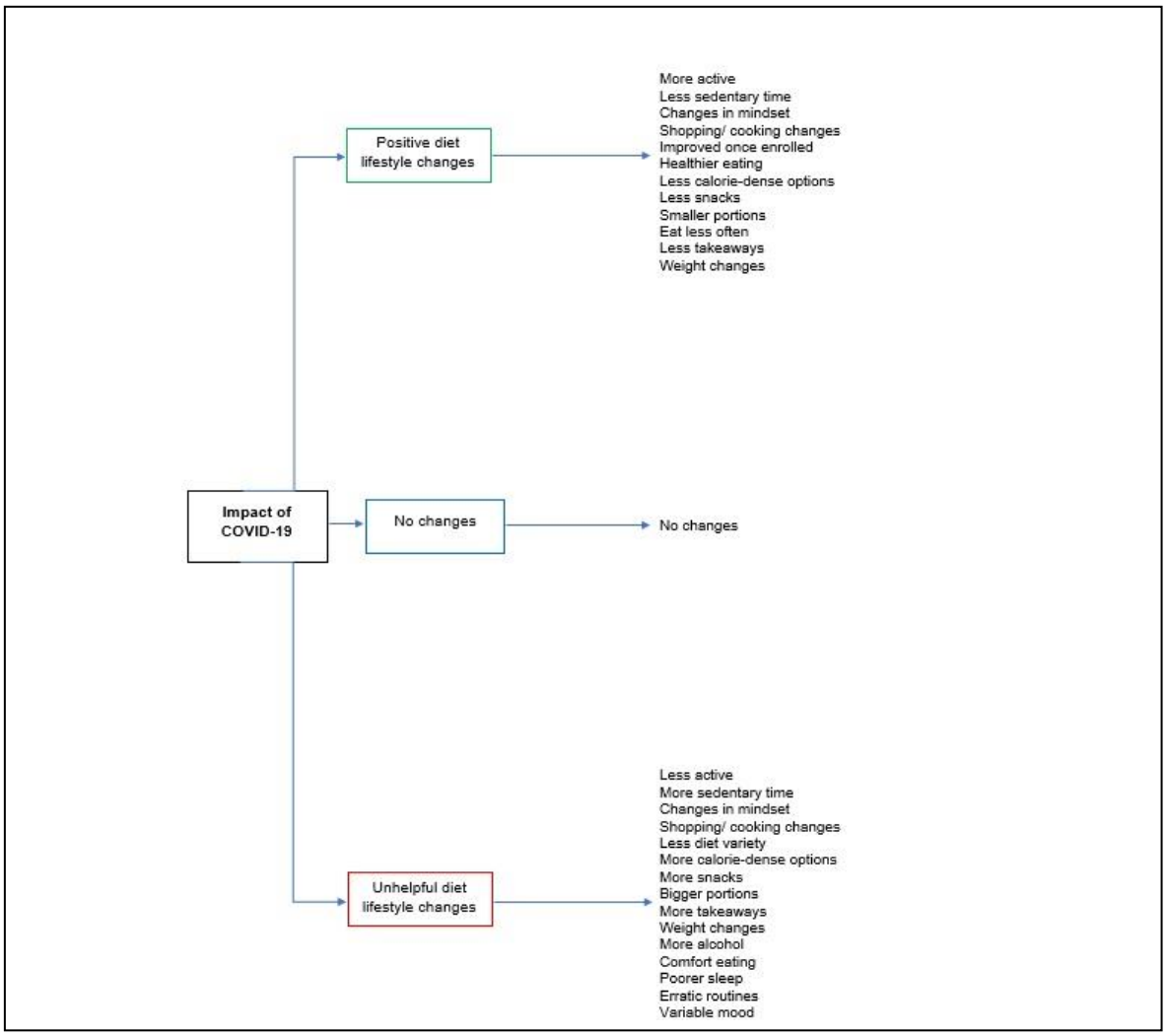



**Figure 4.4 Themes, subordinate themes, barriers and facilitators.** Impact of COVID-19 on diet and lifestyle behaviours.

#### *4.4.4.3 Impact of COVID-19 on diet and lifestyle behaviours*

There was considerable variation in participant perceptions of the impact of COVID-19 on diet and lifestyle behaviours, and weight status. In certain cases, participants experienced difficulties with erratic routines, poorer sleep and variable mood, which they perceived as related to COVID-19 pandemic-related restrictions. In addition, unhelpful diet and lifestyle changes were reported such as comfort eating, increased alcohol consumption and demand for convenience *"I was sleeping less hours overall and eating more snacks"* (30-year-old male).

Conversely, some participants highlighted the positive diet and lifestyle changes that they made during the same period. These included practical factors such as improved shopping and cooking practices, and psychological factors such as optimistic changes in mindset *"I ate more structured meals and was doing more*

*home cooking"* (58-year-old female). There were wide inconsistencies between participants regarding the consumption of calorie-dense options, snacking behaviours, consumption frequency and portion control. Participants perceptions diverged on whether they increased physical activity and reduced sedentary time. Finally, a few participants reported no changes in diet and lifestyle behaviours as a consequence of the COVID-19 pandemic.

#### *4.4.4.4 Patient-reported outcome data*

Patient-reported outcome data are presented in Figure 4.5 and Table 4.4. Lower scores for the chronic liver disease questionnaire for non-alcoholic steatohepatitis (CLDQ-NASH) and euroqol 5 dimension scale (EQ-5D-5L) indicate more severe symptoms/ worse perceived health status. Conversely, higher scores for NASH-CHECK indicates more severe symptoms.

After 4-weeks of MD the median CLDQ-NASH score was 5.60 (4.85-6.30); the subcategory's 'fatigue' and 'systemic symptoms' were the most impacted. 'Abdominal symptoms' and 'worry' were the least impaired. The median EQ-5D-5L utility and visual analogue scale scores were 0.74 (0.60-0.88) and 70.00 (50.00-85.00), respectively. The most impacted domain from the NASH-CHECK six symptom and three HRQoL scores was 'fatigue'.

The median CLDQ-NASH score after 4-weeks of CD was 5.65 (4.15-6.23); the subcategory's 'fatigue' and 'emotional functioning', were the most impaired. 'Worry' and 'abdominal symptoms' were the least impacted. The median EQ-5D-5L utility and visual analogue scale scores were 0.77 (0.44-0.88) and 75.00 (47.50-82.50) respectively. The most impacted domains from NASH-CHECK were 'fatigue', followed by 'sleep'. 'Abdominal pain' and 'social impact' were the least impaired.

When compared with MD, CD resulted in more severe symptoms for the CLDQ-NASH subcategory 'emotional functioning' ($p = 0.003$) and the domain of 'sleep' from NASH-CHECK ($p = 0.038$) (results derived from Wilcoxon signed-rank test) (Figure 4.5 and Table 4.4). Comparison of PROs using these instruments between sub-groups was carried out using the Mann-Whitney U (sex and disease stage categories) and Kruskal-Wallis tests (age and ethnicity categories) after 4-weeks of MD. The results indicate median 'social impact' from NASH-CHECK was significantly

less impaired in female participants (*n* 13) than in males (*n* 10) (Mann-Whitney U =33.5, *n* 23, p = 0.048) (Appendix W).

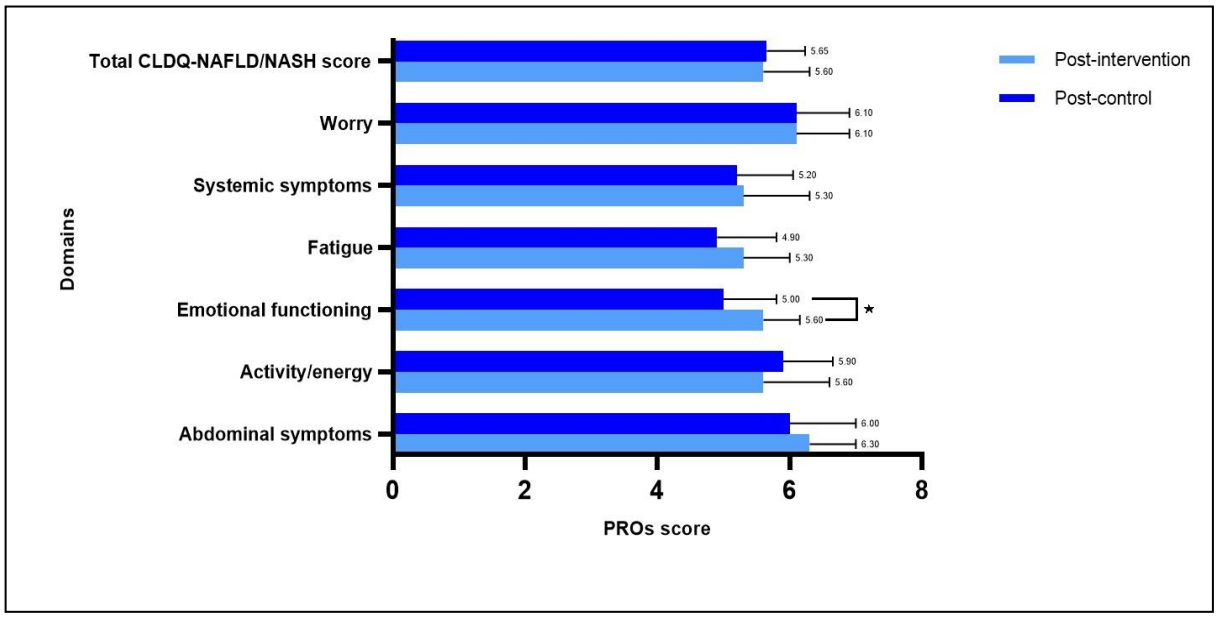



**Figure 4.5 Median values for each domain of the CLDQ-NASH questionnaire post-MD and post-control diet (n=48).** CLDQ-NASH, chronic liver disease questionnaire for non-alcoholic fatty liver disease and non-alcoholic steatohepatitis.

| | Post-MD | Post-CD | P-Value |
|---|---|---|---|
| **CLDQ-NASH** | | | |
| Abdominal symptoms | 6.30 (5.00-7.00) | 6.00 (4.30-7.00) | 0.082 |
| Activity/energy | 5.60 (4.30-6.60) | 5.90 (4.30-6.65) | 0.790 |
| Emotional functioning | 5.60 (4.90-6.15) | 5.00 (4.10-5.80) | 0.003 |
| Fatigue | 5.30 (4.10-6.00) | 4.90 (3.35-5.80) | 0.195 |
| Systemic symptoms | 5.30 (4.05-6.30) | 5.20 (4.20-6.05) | 0.929 |
| Worry | 6.10 (5.10-6.90) | 6.10 (5.05-6.90) | 0.707 |
| Total score | 5.60 (4.85-6.30) | 5.65 (4.15-6.23) | 0.209 |
| **EQ-5D-5L** | | | |
| Utility score | 0.74 (0.60-0.88) | 0.77 (0.44-0.88) | 0.916 |
| Visual analogue scale score | 70.00 (50.00-85.00) | 75.00 (47.50-82.50) | 0.733 |
| **NASH-CHECK** | | | |
| Abdominal pain | 0.00 (0.00-3.00) | 0.00 (0.00-3.75) | 0.636 |
| Abdominal bloating | 1.00 (0.000-3.25) | 1.00 (0.00-4.75) | 0.394 |
| Fatigue | 4.00 (1.00-5.00) | 4.00 (2.00-6.00) | 0.244 |
| Sleep | 1.00 (0.00-6.00) | 3.50 (1.00-6.00) | 0.038 |
| Itchy skin | 2.00 (0.00-6.00) | 2.00 (0.00-4.00) | 0.625 |
| Cognitive symptoms | 1.00 (0.00-3.30) | 1.30 (0.30-3.33) | 0.520 |
| Activity limitations | 1.45 (0.00-4.93) | 1.30 (0.08-5.00) | 0.797 |
| Emotional impact | 1.70 (0.80-3.30) | 1.70 (0.20-3.30) | 0.971 |
| Social impact | 0.50 (0.00-1.90) | 0.50 (0.00-1.90) | 0.238 |

**Table 4.4 Patient-reported outcomes for the post-MD and post-control diet (n=48).** Values are medians (range). MD, Mediterranean diet; CD, control diet; CLDQ-NASH, chronic liver disease questionnaire for non-alcoholic fatty liver disease and non-alcoholic steatohepatitis; EQ-5D, euroqol 5-dimension scale.

## 4.5 Discussion

The data presented in this chapter suggests that the study protocol was feasible, acceptable, and supportive of a future definitive RCT. High consent, recruitment and retention rates indicate that patients accessing outpatient hepatology services were interested and engaged participants in the intervention study. Importantly, this successful engagement was observed despite the concurrent COVID-19 pandemic and its related restrictions. The protocol amendments made to mitigate the impact of the COVID-19 pandemic were successful in allowing the study to proceed and to meet its original objectives with limited changes to the protocol, whilst ensuring the safety of participants and research staff.

Study protocol features that promoted participant retention included prompts and reminders and study visit characteristics (e.g., visit duration <40 minutes). Participants reported satisfaction with a well-organised research team and the provision of additional support when required [411]. Engagement with the study protocol was not perceived as too demanding, though research teams should consider the challenges that busy lifestyles, and irregular working present amongst other participant obligations and activities. Previous qualitative studies exploring participant experiences with dietary interventions have revealed similar difficulties [243,412]. One of the most significant findings was that the MD intervention was widely acceptable. There were impressions of budgeting benefits and satisfaction with enjoying new experiences, and dietary flexibility.

The current analysis suggests that the protocol adjustments that would be needed to allow scale-up to an effective RCT are likely to be relatively minor (Table 4.5). This process must be resilient and informed by current evidence of successful 'scale-up' strategies to prevent potential reductions in intervention effects [413]. Protocol adjustments include improved methods for collection of clinical data; provision of alternative accelerometer wrist straps more suitable for people living with obesity; integrating targeted dietary advice on food poverty; and improvements to the pre-packaged ready meals. Regarding the pre-packaged meals, both the nutrition labelling and portion-controlled meal containers were deemed beneficial. However, individual taste preferences, were a clear obstacle in some cases, and are a frequently reported barrier in previous research [243,414]. Some of these challenges can be overcome by conducting 'taste-test' sessions with patients and the public.

This feedback would support future intervention refinement. Additional improvements to the range of meals, meal containers, website functionality and delivery process (i.e., introduction of text/ email reminders) could enhance participant satisfaction.

Barriers and facilitators were identified that support innovative protocol adaptations (Table 4.5). Participants highlighted the importance of nutrition counselling and the patient information booklet [243]. This is in keeping with previous pilot data that explored the barriers and facilitators to MD in this patient population [243]. Consequently, in a future RCT, the development of an interactive web-based platform alongside the personalised one-to-one diet and lifestyle consultations, is likely to ensure scalability and help with cost effectiveness. Previous research has demonstrated that a web-based intervention that included some face-to-face interaction was as effective as a standard group intervention in NAFLD [322]. Leveraging this blended approach to optimise trial delivery would address an individuals need for additional guidance and provide appropriate prompts/reminders.

INTAKE24 is a validated self-completed computerised 24-hr dietary recall system, which offers the potential for increased feasibility of dietary assessment [387,388,415]. Though, in the present study, there were cases of missing and poor-quality data. This finding coupled with the greater feasibility and acceptability of MEDAS and the urinary biomarkers methodology (home urine sample collection), suggests INTAKE24 should not be included in the future definitive RCT, without major revision. Concerns were highlighted about access and internet literacy; thus, these revisions should focus on improving participation convenience and the feasible execution of the recalls.

The PROs data revealed disease burden and indicated greater symptom severity in emotional functioning and sleep at the end of the control diet. The total CLDQ-NASH score was similar at the end of both diets, and fatigue was found to be the most impacted domain. These findings are consistent with previous studies that examined CLDQ-NASH in American and Japanese patients with NAFLD [382,416]. NASH-CHECK also revealed fatigue as the most impacted symptom at the end of both diets. Fatigue appears to be a frequently reported symptom in this patient population, and correlates with worse QoL [382,417] and negative impact on well-being [247].

Abdominal symptoms/pain and worry were the least impaired domains, which is also in line with previous research [382,418]. Given the high prevalence of obesity in this cohort, the combination of a NAFLD- and obesity- specific instrument might better capture the impact of diet treatments and trial procedures on quality of life (QoL).

| Variable | Minor changes | Modest changes | Major changes | Justification |
|---|---|---|---|---|
| Study design | Increase experimental and washout periods (Chapter 5) | Consider parallel-group design (Chapter 5) | | - To minimise potential carryover and diet sequence effects<br>- To reveal the full effects of dietary intervention on liver function |
| Clinical (insulin, HbA1c, GGT, CAP and liver stiffness) | - Change the schedule of HbA1c sampling to once every six weeks<br>- Ensure the research team order all the study bloods for analysis<br>- Repeat TE, if CAP and/or liver stiffness result missing | | | - To increase the completeness of data collection and better capture the impact of diet treatments |
| BCTs | Introduce evaluation method based on reference [419] | | | - To identify effective BCTs |
| Intervention characteristics | Introduce evaluation tool based on reference [420] | | | - To detect intervention characteristics that need improvement |
| Accelerometer | Offer alternative wrist straps | | | - To increase comfortability, enhance adherence and improve validity |
| INTAKE24 | | | Do not include unless robust revisions made | - To improve accessibility and useability<br>- To reduce cases of missing and poor-quality data |
| MD intervention | - Incorporate advice on food insecurity/poverty<br>- Incorporate advice to ensure adequate protein intake (Chapter 5) | Develop an interactive web-based platform | | - To increase diet adherence and adequacy<br>- To enhance scalability and help with cost effectiveness<br>- To offer additional guidance/support and provide prompts/ reminders |
| MD meal supplier | - Conduct taste-test sessions<br>- Increase the range of meals<br>- Introduce eco-friendly meal containers | | | - To inform the future refinement of the intervention<br>- To increase participant satisfaction and adherence |

| | | | | |
|---|---|---|---|---|
| | - Improve website functionality<br>- Introduce meal delivery text/ email reminders<br>- Offer flexible meal plans to meet estimated calorie requirements | | | - To increase implementation fidelity and reduce protocol deviations |
| Urine metabolomics | More frequent urinary sample collection (Chapter 5) | | | - To improve the inter class variance observed within the treatment diets |
| PROs | - Advise participants to complete instruments alone<br>- Include baseline measurement | | | - To increase comfortability<br>- To determine the impact of diet treatments on QoL |
| Cost effectiveness | | Include cost effectiveness analysis | | - To evaluate the effectiveness of the study protocol relative to its cost |
| Final questionnaire | - Undertake patient and public involvement and engagement<br>- Revise questionnaire guide | | | - To capture participant perceptions of i) genotype-based personalised nutrition interventions and, ii) MD adherence in the second experimental period (Chapter 5) |

**Table 4.5 Proposed study protocol adjustments in preparation for a future definitive randomised controlled trial.** HbA1c, glycated haemoglobin; GGT, gamma-glutamyl transpeptidase; CAP, controlled attenuation parameter; BCTs, behaviour change techniques; MD, Mediterranean diet; PROs, patient-related outcomes; TE, transient elastography; QoL, quality of life**.**

## 4.6 Strengths and Limitations

The main strength of this trial is the use of well-defined feasibility and acceptability criteria as primary outcomes. Data derived from the patient-reported outcome and open-ended response instruments enhances understanding of the impact of disease burden on QoL and individuals' perceptions of study participation. Barriers and facilitators were identified that could guide future personalised intervention approaches. The final questionnaire also created an opportunity to explore how the COVID-19 pandemic and its related restrictions impacted upon participant diet and lifestyle patterns. However, the current analysis did not capture information about participant perceptions of genotype-based personalised nutrition interventions, which is important for the design of future trials including strategies for participant recruitment (Table 4.5). Moreover, baseline measurement of the PROs would have strengthened the study findings.

The intervention, which was broadly acceptable, was systematically developed and informed by evidence synthesis, behaviour change theory and patient and public feedback. Nevertheless, how much the behaviour change techniques (independent and interactive effects) influenced feasibility and acceptability of the study protocol merits further clarification. Furthermore, the implementation of a tool informed by the recently developed theoretical framework of acceptability might have aided the detection of intervention characteristics that require improvement [420].

The main limitation of the present study was the potential for selection bias, although minimal exclusions were chosen (to reflect the target population for a subsequent definitive RCT, and diverse clinical and demographic characteristics were represented. The sample size was relatively small in this randomised controlled feasibility trial, but it was sufficiently robust to achieve the study aims [374]. The strategy of encouraging participants to maintain baseline levels of body weight throughout trial were insufficient in some cases. Hence, to increase implementation fidelity, a subprotocol or algorithm (i.e., with energy intakes adjusted to counter changes in body weight) should be developed and flexible meal plans offered. A potential limitation of this study was the absence cost effectiveness measures. Identification of appropriate methods for assessment of cost-utility/ cost-effectiveness are required for inclusion in the future large-scale RCT.

## 4.7 Conclusion

The qualitative and quantitative information generated in this study indicates that the study protocol is sufficiently robust to advance to a larger scale evaluation, with appropriate adjustments. The data presented in this chapter lay the foundation for a future definitive RCT by informing trial design and optimising the dietary treatments, instruments and procedures. Key recommendations include robust evaluation of the acceptability and effectiveness of specific BCTs and intervention characteristics, exploration of participant perceptions of genotyping during participant recruitment and the undertaking a cost-effectiveness analysis. In addition, the development of an interactive web-based platform to support dietary change, and further patient and public involvement and engagement are advocated.

# Chapter 5

# Randomised Controlled Feasibility Trial: The Effectiveness of The Study Protocol

## 5.1 Introduction

Non-alcoholic fatty liver disease (NAFLD)-attributed rates of advanced liver disease and transplantation are increasing, but effective pharmacotherapy is unavailable [5,26]. Consequently, lifestyle interventions that focus on reduced calorie (energy) intake to induce weight loss and disease regression are the cornerstone of NAFLD management [6,218]. However, clinically significant and sustained weight loss is often unsuccessful [17]. Modifications of diet composition are important in achieving beneficial hepatic effects and could be a basis for developing personalised diet therapies [6,13,221,254]. Furthermore, evolving evidence supports the hypothesis that the Mediterranean diet (MD) induces both hepatic and extra-hepatic health benefits, with or without weight loss [266,351,352,421-423]. This has clinical relevance as patients with NAFLD have higher cardiovascular disease (CVD)-related morbidity and mortality, which is more common than liver-related mortality [5,27].

Based on the available evidence outlined in Chapter 2, the MD is the diet of choice amongst clinicians, and it is likely to be an effective therapy in this patient population. In the short and medium-term, MD interventions improved markers of NAFLD like the Fatty Liver Index (FLI), markers of liver injury and fibrosis such as alanine aminotransferase (ALT), and liver stiffness (LSM); as well as cardiometabolic parameters, and abdominal obesity. However, current evidence of MD effectiveness originates mainly from trials conducted in Mediterranean regions/ climates with favourable food environments and cultural dietary habits. Therefore, any potential impact on NAFLD severity may be affected by baseline MD adherence [244]. More evidence is needed about the effects of MD in NAFLD patients among northern European populations. In addition, further experimental data that determine the potential of MD interventions to be translated in regions that consume a habitual western diet, would be advantageous [243]. Robust assessment of dietary intake is also warranted. These findings and recommendations, as well as other research evidence, and patient and public feedback, informed the design of the study protocol described in Chapter 3. The current chapter presents data about the effectiveness of the study protocol.

## 5.2 Study Aims and Objectives

The overarching aims and objectives of the randomised controlled feasibility trial are outlined in Chapter 3.

The current chapter describes an evaluation of the effectiveness of the study protocol.

The objectives of this current chapter were:

- To evaluate adherence to, and completion of, the MD intervention.
- To undertake preliminary exploration of changes in key clinical and lifestyle variables.

## 5.3 Materials and Methods

The methods have been described in Chapter 3.

## 5.4 Results

Chapter 4 summarises the baseline characteristics, and the flow, of participants through the trial. Data from 48 out of 49 participants recruited into the feasibility trial are presented in this chapter (i.e., 1 participant left the study after the baseline visit for a non-related health issue). All the results from each time point and study arms are presented in the appendices (Appendix X1 and X3).

### *5.4.1 Mediterranean Diet Assessment Score*

At 4 weeks MD adherence significantly increased from low to moderate (mean increase, 3.3 points; 5.7±1.9 to 9.0±2.1) as assessed by total MEDAS score (Figure 5.1) [(386)]. Almost three quarters (74%) of participants achieved a 2-point increase and 60% achieved $\geq$3 point increase. The proportion of participants that reported a total MEDAS score of ≥10 points, (i.e., high consumption) increased from 2% at baseline to 45% at end of MD intervention. A comparison of total MEDAS scores between sub-groups was conducted using Mann-Whitney U (sex and disease stage categories) and Kruskal-Wallis tests (age and ethnicity categories). The results indicate non-significant difference between these sub-groups ($p > 0.05$) (Appendix Y). In contrast, at the end of CD period, there were no significant changes in MD adherence observed from moderate consumption (6.0 (5.0-8.0) to 6.5 (5.0-8.0)) as assessed by the total MEDAS score.

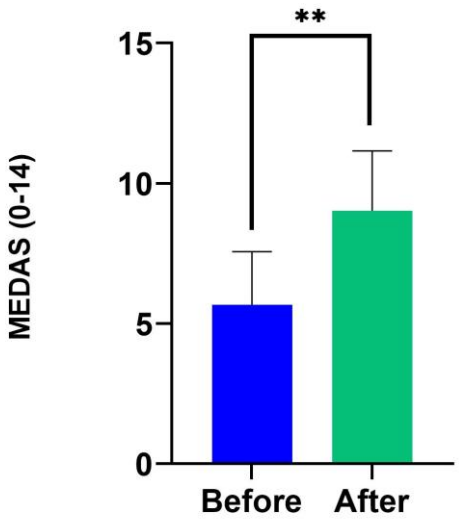



**Figure 5.1 Total MEDAS score at baseline and post-MD intervention.** MEDAS, MD assessment score. **P<0.001**.**

#### *5.4.1.1 Mediterranean diet assessment score components*

At 4-weeks, there were significant increases in the proportion of participants consuming “olive oil” (increased by 22 individuals) (p <0.001), “≥4 tablespoons/day of olive oil” (increased by 9 individuals) (p = 0.004), “≥2/day servings of vegetables” (increased by 22 individuals) (p <0.001), “≥3/day servings of fruit” (increased by 23 individuals) (p <0.001), “<1/day servings of butter, margarine, or cream” (increased by 19 individuals) (p = 0.007), “≥3/week servings of legumes” (increased by 17 individuals) (p = 0.007), “≥3/week servings of fish or shellfish” (increased by 22 individuals) (p <0.001), “≥1/week servings of nuts” (increased by 12 individuals) (p = 0.003), and “≥2/week sofrito” (i.e., cooked tomato sauce) (increased by 29 individuals) (p <0.001) (Figure 5.2) (exact McNemar's test). In contrast, at the end of the CD period, there were no significant changes in the proportion of participants consuming individual MEDAS components.

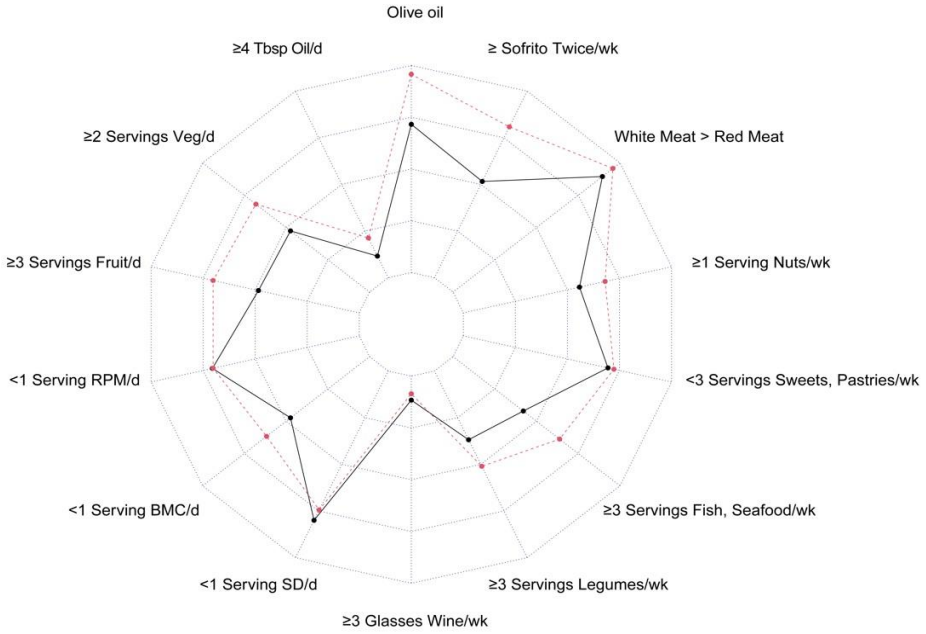



**Figure 5.2 Fold changes in intakes of MEDAS components** (black = baseline, red = 4-weeks). MEDAS, Mediterranean diet assessment score.

### *5.4.2 INTAKE24 data*

The reported changes in intake of foods translated into corresponding changes in energy and nutrient intakes. After 4 weeks, participants on MD reduced mean intakes of energy (Kcal/KJ), protein (g), trans fat (g) and sodium (mg) and median intakes of carbohydrate (g), non-milk extrinsic sugars (NMES) (g) (Table 5.1). These changes were not sustained throughout washout during which intakes of protein (g) (38.2±19.1 to 75.3±23.6) (p <0.001), saturated fat (SFA) (g) (13.5±7.5 to 23.0±12.1) (p = 0.033) and sodium (mg) (1082.0±471.0 to 1855.4±933.1) (p =0.016) increased. Participants on CD decreased mean beta-carotene levels (µg) (3249.2±3045.5 to 1723.4±1837.8) (p = 0.028).

| | **Baseline** | **Post-MD** | **P-Value** |
|---|---|---|---|
| **Energy (kcal)** | 1511.3 ± 585.8 | 1263.1 ± 434.6 | 0.038 |
| **Energy (KJ)** | 6363.2 ± 2469.8 | 5306.2 ± 1813.7 | 0.035 |
| **Carbohydrate (g)** | 170.5 (128.2-221.1) | 132.3 (103.9-162.3) | 0.001 |
| **NMES (g)** | 15.0 (5.1-39.0) | 10.3 (4.2-21.3) | 0.006 |
| **Protein (g)** | 67.0 ± 22.5 | 55.8 ± 26.5 | 0.049 |
| **Trans fat (g)** | 0.8 ± 0.6 | 0.5 ± 0.3 | 0.008 |
| **Sodium (mg)** | 1816.2 ± 882.0 | 1235.3 ± 777.7 | <0.001 |

**Table 5.1 Intakes of energy and nutrients estimates using INTAKE24 for participants at baseline and after 4 weeks MD (n=48).** Values are means (standard deviation) and medians (range). NMES, non-milk extrinsic sugars; MD, Mediterranean diet.

### *5.4.3 Urine-Based Dietary Biomarkers (Non-targeted Fingerprinting)*

Metabolome fingerprints were generated to visualise compositional differences between diets, using multivariate classification tools, i.e., random forest (RF) and principal component linear discriminant analysis (PC-LDA). Figure 5.3 presents PC-LDA plots of metabolome fingerprints after 4 weeks on the MD and CD. Models B and C illustrate the differences between the diets when diet order is considered. Each model indicates clear, but modest modifications in the urine metabolome between MD and CD with considerable inter-individual variation. The Eigenvalue (Tw) corresponds to the amount of variation explained by each principal component. The Tw for MD (first experimental period) was 1.13 compared with 0.73 for MD (second experimental period), indicating stronger discrimination between diets when MD was consumed first. Classification performance was assessed to determine the accuracy of each model from quantitative modelling output measures including area under the receiver operator characteristic (ROC) curve (AUC) [389] (Table 5.2). All models had modest classification accuracy (i.e., AUC <0.8). Model B (MD in the first experimental period) had the best classification performance.

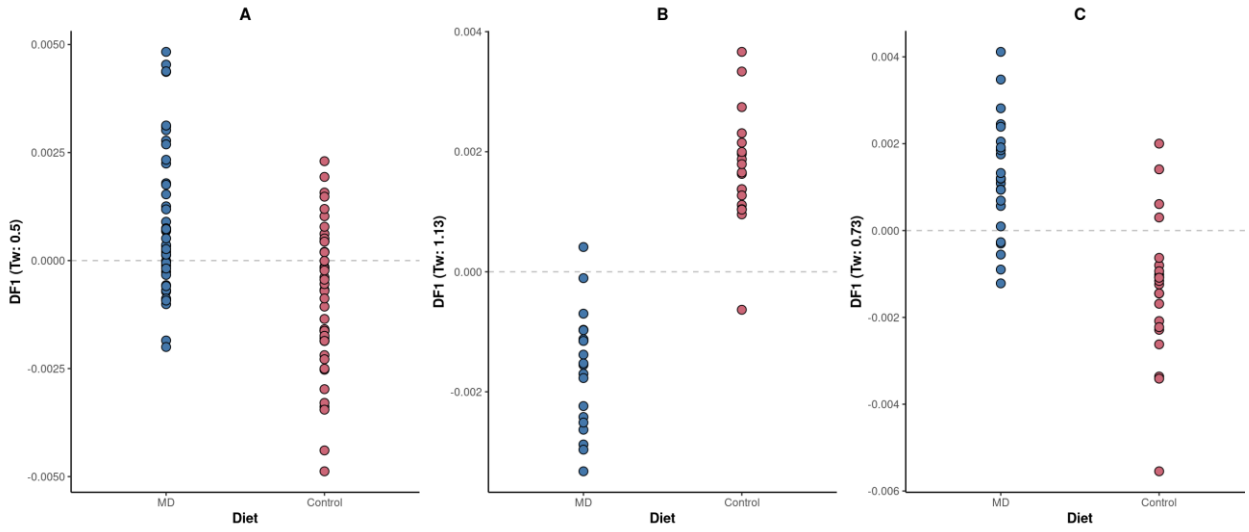



**Figure 5.3 PC-LDA of metabolite fingerprint data between MD and CD.** (A) MD versus CD. (B) MD versus CD (first experimental period = MD). (C) MD versus CD (second experimental period =MD). PC-LDA, principal component linear discriminant analysis; MD, Mediterranean diet; CD, control diet.

| | MD versus CD | | |
|---|---|---|---|
| | MD versus CD | First Experimental period (MD) | Second Experimental period (MD) |
| Accuracy | 0.42 | 0.62 | 0.38 |
| ROC-AUC | 0.35 | 0.68 | 0.38 |

**Table 5.2 Classification performance between MD and CD (non-targeted fingerprinting).** ROC, receiver operator characteristic; AUC, area under the curve. MD, Mediterranean diet; CD, control diet.

### *5.4.4 Urine-Based Dietary Biomarkers (Quantitative Biomarker Panel)*

PC-LDA plots of the targeted assays (i.e., biomarker panel) are shown in Figure 5.4 and classification performance in Table 5.3. Models B and C illustrate the differences between the diets when diet order is considered. Each model indicates clear, but modest differences in the urine metabolome between MD and CD. Again, the considerable inter-individual variation should be noted. Stronger modelling performances were found in the non-targeted data (Table 5.2) rather than the targeted data, as evidenced by the inferior classification accuracy (Table 5.3).

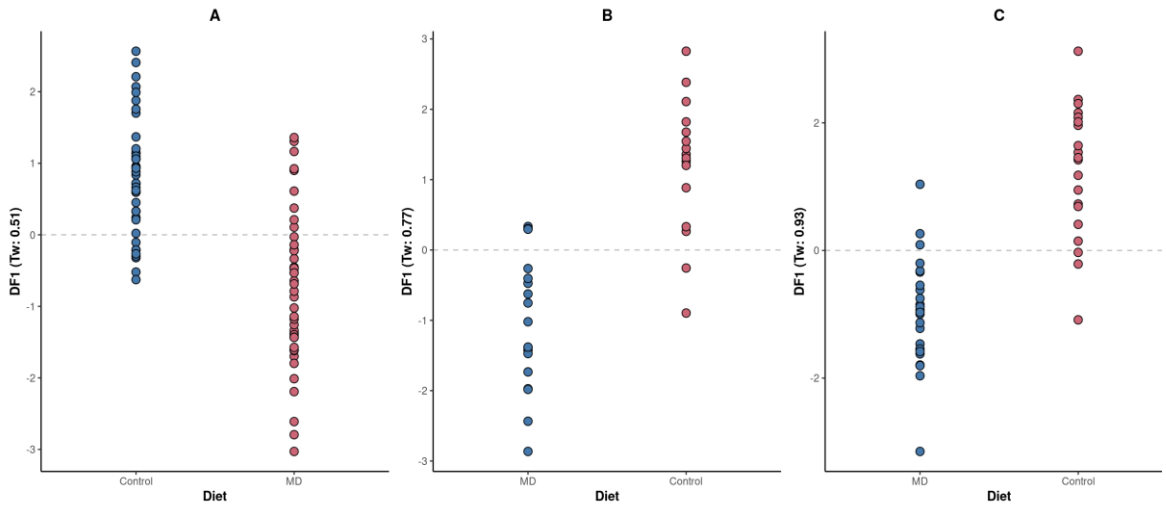



**Figure 5.4 PC-LDA of the targeted assays between MD and CD.** (A) MD versus CD. (B) MD versus CD (first experimental period = MD). (C) MD versus CD (second experimental period =MD). PC-LDA, principal component linear discriminant analysis; MD, Mediterranean diet; CD, control diet.

| | MD versus CD | | |
|---|---|---|---|
| | MD versus CD | First Experimental period (MD) | Second Experimental period (MD) |
| Accuracy | 0.48 | 0.35 | 0.49 |
| ROC-AUC | 0.45 | 0.33 | 0.49 |

**Table 5.3 Classification performance between MD and CD (targeted assays).** ROC, receiver operator characteristic; AUC, area under the curve; MD, Mediterranean diet; CD, control diet.

Next permuted feature selection of targeted biomarkers was undertaken (controlled for false positive rate*). This showed that concentrations of multiple dietary intake biomarkers in the urine were significantly different between the diets (Table 5.4). These include known biomarkers of strongly-heated foods (N-(2-Furoyl) glycine)), coffee (m-Coumaric acid), chicken (L-Anserine) and potatoes (Calystegine A) [392]. Further, there was clear differentiation when diet sequence was considered (Tables 5.5 and 5.6). After 4-weeks of MD (first experimental period), there were increased urinary concentrations of known biomarkers of poultry/ fish (3-methyl histidine) and citrus (4-hydroxyproline betaine) intake and of trimethylamine-n-oxide (TMAO) (microbiota-derived metabolite from choline, betaine and carnitine) [392]. These elevated levels were not sustained throughout washout and CD. Figure 5.5. illustrates the differences in these key food intake biomarkers during both diets.

| **Biomarker** | **P-Value*** |
|---|---|
| N-(2-Furoyl) glycine | <0.001 |
| m-Coumaric acid | <0.001 |
| L-Anserine | <0.001 |
| Calystegine A | <0.001 |
| 1-Methyl histidine | 0.002 |
| Acesulfame-K | 0.007 |

**Table 5.4 Differences in food intake biomarkers between MD and CD.** MD, Mediterranean diet; CD, control diet.

| Biomarker | P-Value* |
|---|---|
| 4-Hydroxyproline betaine | <0.001 |
| Phenyl-acetyl-L-glutamine | <0.001 |
| 3-Hydroxyhippuric acid | <0.001 |
| Naringenin | <0.001 |
| 3-Methyl histidine | <0.001 |
| Ferulic acid-4-O-β-D-glucuronide | <0.001 |
| Trimethylamine-N-oxide | <0.001 |
| BOA (1,3-Benzoxazol-2-one) | <0.001 |
| Furaneol | <0.001 |
| Hippuric acid | <0.001 |
| L-Anserine | 0.002 |
| Dihydrocaffeic acid | 0.004 |
| Sucrose | 0.004 |
| m-Coumaric acid | 0.010 |

**Table 5.5 Differences in food intake biomarkers between MD and CD.** (First experimental period = MD). MD, Mediterranean diet; CD, control diet.

| Biomarker | P-Value* |
|---|---|
| 3-Methyl histidine | <0.001 |
| N-(2-Furoyl) glycine | <0.001 |
| Calystegine A | <0.001 |
| Dihydrocaffeic acid | <0.001 |
| Furaneol | <0.001 |
| BOA (1,3-Benzoxazol-2-one) | <0.001 |
| Epicatechin (-) | <0.001 |
| L-Histidine | <0.001 |
| Quercetin-3-O-b-D-glucuronide | <0.001 |
| Calystegine B | 0.001 |
| Daidzein (4',7-Dihydroxyisoflavone) | 0.001 |
| Caffeine | 0.003 |
| DHBA-3-O-sulfate | 0.003 |
| Carnitine | 0.004 |
| m-Coumaric acid | 0.006 |

**Table 5.6 Differences in food intake biomarkers between MD and CD.** (Second experimental period = MD). MD, Mediterranean diet; CD, control diet.

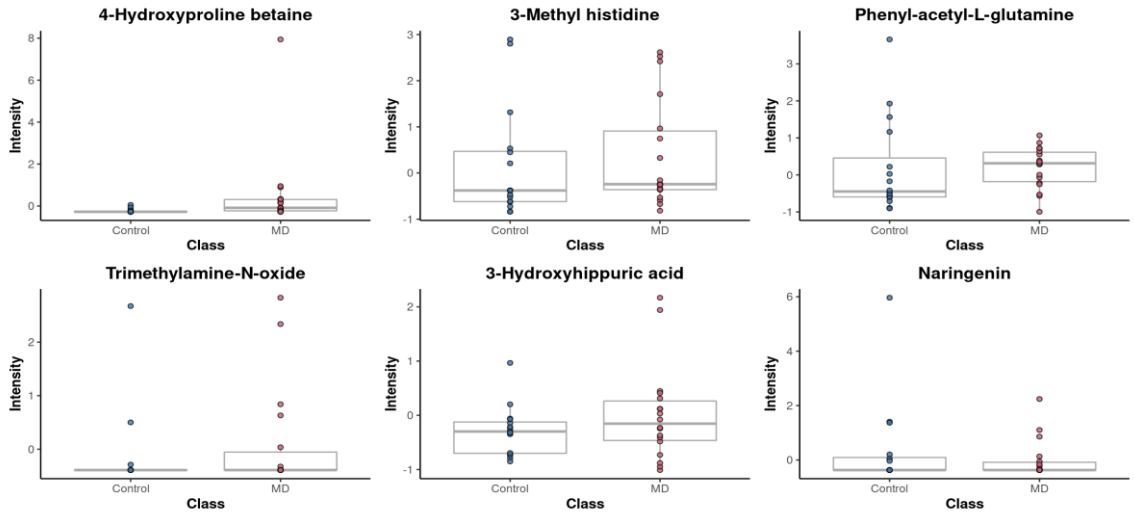



**Figure 5.5 Boxplots for key food intake biomarkers during MD and CD periods.** Note the considerable inter-individual variation during both treatments (first experimental period = MD). MD, Mediterranean diet; CD, control diet.

#### *5.4.4.1 Random forest regression modelling between total MEDAS score, MEDAS components and dietary biomarkers*

The global model results for total MEDAS score versus individual MEDAS components yielded, as expected, strong values ($R^2$ 0.93, MAE 0.57). The total MEDAS score (response variable) is the summation of individual component scores, and therefore exhibits a high degree of correlation. However, through modelling non-linear combinations of MEDAS components, random forest can determine which individual components have the most effect on changing total MEDAS score. Table 5.7 presents the rank order list of component importance (with permuted P-Values*) indicating which MEDAS components are most explanatory, and thus are most responsible for total MEDAS score change over time. Change in intakes of sofrito, legumes, and vegetables and fruit drove the differences in total MEDAS score between the diets. Note, however, these models do not demonstrate the direction of change in intakes, but only what components contributed most to change.

Urine biomarkers were modelled against total MEDAS score. The rank order list of food intake biomarker importance, which show the most explanatory biomarkers responsible for total MEDAS score change over time are summarised (Table 5.8 to 5.10). Changes in urinary concentrations of taurine and caffeine (Table 5.8); resveratrol and TMAO (MD in the first experimental period, Table 5.9); and gallic-acid and chlorogenic acid (MD in the second experimental period, Table 5.10) drove the

differences in total MEDAS score between the diets. Thus, there was clear differentiation when diet sequence was considered.

| MEDAS components | Random Forest Importance | P-Value* |
|---|---|---|
| ≥2/day servings of vegetables | 32.99≠ | <0.001 |
| ≥2/week sofrito | 31.73≠ | <0.001 |
| ≥3/week servings of legumes | 28.71≠ | <0.001 |
| ≥3/day servings of fruit | 28.60≠ | <0.001 |
| <1/day servings of red and processed meat | 25.54 | <0.001 |
| ≥3/week servings of fish or shellfish | 25.19 | <0.001 |
| Olive oil | 24.98 | <0.001 |
| <1/day servings of butter, margarine | 22.19 | <0.001 |
| ≥1/week servings of nuts | 21.53 | <0.001 |
| <3/week servings of commercial baked goods | 20.31 | <0.001 |
| <1/day servings of sugar sweetened beverages | 11.15 | 0.007 |
| White meat more than red meat | 7.87 | 0.006 |
| ≥4 Tbsp/day of olive oil | 7.61 | 0.034 |
| ≥3/week glasses wine | 2.80 | 0.245 |

**Table 5.7 Rank order list of MEDAS component importance for changes in total MEDAS score over time (MD versus CD).** ≠Most explanatory components. MD, Mediterranean diet; CD, control diet; MEDAS, MD assessment score.

| Biomarker | Random Forest Importance | P-Value* |
|---|---|---|
| Taurine | 7.09≠ | <0.001 |
| Caffeine | 5.29≠ | <0.001 |
| D, L-Sulforaphane-N-acetyl-L-cysteine | 2.87 | 0.002 |
| N-(2-Furoyl) glycine | 2.67 | 0.036 |
| Acesulfame-K | 2.43 | 0.021 |
| L-Histidine | 2.29 | 0.064 |
| 1-Methyl histidine | 2.22 | 0.049 |
| Vanillic acid | 2.07 | 0.067 |

**Table 5.8 Rank order list of food intake biomarker importance for changes in total MEDAS score over time (MD versus CD).** ≠Most explanatory components. MD, Mediterranean diet; CD, control diet; MEDAS, MD assessment score.

| Biomarker | Random Forest Importance | P-Value* |
|---|---|---|
| Resveratrol | 32.99≠ | <0.001 |
| Trimethylamine-N-oxide | 28.60≠ | <0.001 |
| Vanillic acid | 24.98 | <0.001 |
| 3-Methyl xanthine | 7.61 | 0.034 |

**Table 5.9 Rank order list of food intake biomarker importance for changes in total MEDAS score over time (MD versus CD).** (First experimental period = MD**).** ≠Most explanatory components. MD, Mediterranean diet; CD, control diet; MEDAS, MD assessment score.

| Biomarker | Random Forest Importance | P-Value* |
|---|---|---|
| Gallic-acid | 5.84≠ | <0.001 |
| Chlorogenic acid | 4.21≠ | 0.001 |
| Calystegine A | 3.31 | 0.017 |
| DHBA-3-O-sulfate | 2.51 | 0.031 |
| Pyrogallol | 2.35 | 0.074 |
| Calystegine B | 2.30 | 0.062 |
| Carnosine | 2.27 | 0.034 |
| 1-Methyl histidine | 2.06 | 0.014 |
| 4-Hydroxyproline betaine | 2.01 | 0.045 |

**Table 5.10 Rank order list of food intake biomarker importance for changes in total MEDAS score over time (MD versus CD).** (Second experimental period = MD). ≠Most explanatory components. MD, Mediterranean diet; CD, control diet; MEDAS, MD assessment score.

### *5.4.5 The Effectiveness of the MD Intervention*

Table 5.11 summarises the anthropometric and biochemical characteristics and physical activity and sleep patterns of participants before (baseline) and after the MD intervention (results derived from paired samples t-test and the Wilcoxon signed-rank test, as appropriate). At baseline, participants had elevated median fasting glucose, glycated haemoglobin (HbA1c), homeostatic model assessment of insulin resistance (HOMA-IR) and gamma-glutamyl transpeptidase (GGT). On average, participants were obese and abdominally obese and spent more time inactive or in light physical activity than in moderate or vigorous physical activity.

Randomisation to the MD intervention resulted in significantly improved cardiovascular risk (CVR) and small improvements in liver biochemistry (Table 5.11 and Figures 5.6-5.8). At end of MD intervention, fasting glucose, HOMA-IR, total cholesterol (TC), triglycerides (TG), TC: high-density lipoprotein (HDL) ratio and non-HDL all improved significantly from baseline. The MD intervention reduced blood pressure (BP), mean arterial pressure (MAP) and QRISK3, a predictor of risk of cardiovascular events over the next 10 years [(398)]. Taken together, these findings provide evidence of global improvement in CVR profile. At the end of the MD treatment, values of alkaline phosphatase (ALP) and albumin had improved.

Despite the prescriptive nature of the dietary intervention and the aim to maintain body, the intervention resulted in a small, but significant, body weight loss from baseline (-1.5%$\pm$1.7%) with corresponding improvements in waist circumference and increased fat free mass (FFM). Physical activity, sleep duration and efficiency did not

change significantly over the course of the intervention (p >0.05). In contrast, at the end of CD period, there were no significant changes in anthropometric and biochemical characteristics or in physical activity and sleep patterns (p >0.05), except for an improved QRISK3 (15.3±10.0 to 14.6±8.9) (p = 0.033) (Appendix Z).

| Participant Characteristics | Baseline | Post-MD | P-Value |
|---|---|---|---|
| **Anthropometrics** | | | |
| Weight (kg) | 93.5 (83.5-109.5) | 93.2 (82.0-109.1) | <0.001 |
| BMI (kg/m$^2$) | 35.3 ± 5.5 | 34.7 ± 5.4 | <0.001 |
| Waist circumference (cm) | 114.6 ± 13.8 | 112.7 ± 13.3 | 0.002 |
| Waist-to-hip ratio | 0.98 (0.93-1.05) | 0.97 (0.93-1.06) | 0.389 |
| Body composition (kg): Fat mass<br>Fat free mass | 44.7 (37.3-47.9)<br>53.2 (46.8-63.4) | 42.8 (36.1-49.2)<br>53.9 (45.8-63.1) | 0.919<br>0.027 |
| **Cardiometabolic measures** | | | |
| Blood pressure: Systolic (mmHg)<br>Diastolic (mmHg) | 138.9 ± 16.4<br>83.2 ± 9.8 | 132.8 ± 14.5<br>79.1 ± 8.5 | 0.013<br>0.004 |
| MAP (mmHg) | 101.7 ± 10.1 | 97.0 ± 9.3 | 0.003 |
| Fasting glucose (mmol/L) | 6.7 (5.7-9.6) | 6.5 (5.5-7.8) | 0.017 |
| Fasting Insulin (pmol/L) | 103 (79-147) | 98 (76-150) | 0.096 |
| HbA1c (mmol/mol) | 49.5 (41.0-61.8) | 50.0 (40.5-60.0) | 0.750 |
| HOMA-IR | 5.2 (3.6-10.3) | 4.3 (3.1-6.8) | 0.017 |
| Total cholesterol (mmol/L) | 4.5 ± 1.2 | 4.1 ± 1.2 | <0.001 |
| Triglycerides (mmol/L) | 1.5 (1.1-2.1) | 1.4 (1.1-2.1) | 0.011 |
| HDL (mmol/L) | 1.3 (1.1-1.5) | 1.2 (1.0-1.4) | 0.044 |
| TC:HDL ratio | 3.2 (2.7-4.1) | 3.1 (2.5-4.0) | 0.006 |
| Non-HDL (mmol/L) | 3.2 ± 1.2 | 2.8 ± 1.2 | <0.001 |
| QRISK3 | 12.4 (8.4-21.7) | 11.1 (8.1-19.1) | 0.018 |
| **Liver function** | | | |
| Platelets (x10 9 /L) | 234 (209-274) | 235 (197-268) | 0.646 |
| Bilirubin (umol/l) | 9 (7-13) | 10 (7-12) | 0.145 |
| Albumin (g/L) | 45 (43-48) | 46 (44-48) | 0.024 |
| ALT (unit/L) | 38 (28-62) | 38 (26-60) | 0.081 |
| AST (unit/L) | 31 (23-47) | 31 (23-47) | 0.257 |
| ALP (IU/L) | 89 (77-113) | 85 (71-101) | 0.014 |
| GGT (unit/L) | 68 (41-113) | 69 (39-101) | 0.015 |
| Ferritin (ug/L) | 143 (50-237) | 119 (51-211) | 0.260 |

**Table 5.11** (continued).

| **Physical Activity** | | | |
|---|---|---|---|
| Inactive | 778 ± 93 | 765 ± 104 | 0.289 |
| Light physical activity | 151 ± 54 | 153 ± 58 | 0.730 |
| Moderate physical activity | 60 ± 36 | 59 ± 36 | 0.761 |
| Vigorous physical activity | 1 (0-2) | 1 (0-2) | 0.668 |
| Sleep duration (minutes) | 394 ± 60 | 399 ± 69 | 0.538 |
| Sleep efficiency (%) | 90 (84-92) | 88 (84-92) | 0.717 |

**Table 5.11 Anthropometric and biochemical characteristics and physical activity and sleep patterns of participants at baseline and after 4-weeks of MD intervention (n=48).** Values are means (standard deviation) and medians (range). BMI, body mass index; MAP, mean arterial pressure; HbA1c, glycated haemoglobin; HOMA-IR, homeostatic model assessment of insulin resistance; HDL, high-density lipoprotein; TC: HDL, total cholesterol: HDL ratio; ALT, alanine aminotransferase; AST, aspartate aminotransferase; ALP, alkaline phosphatase; GGT, gamma-glutamyl transpeptidase.

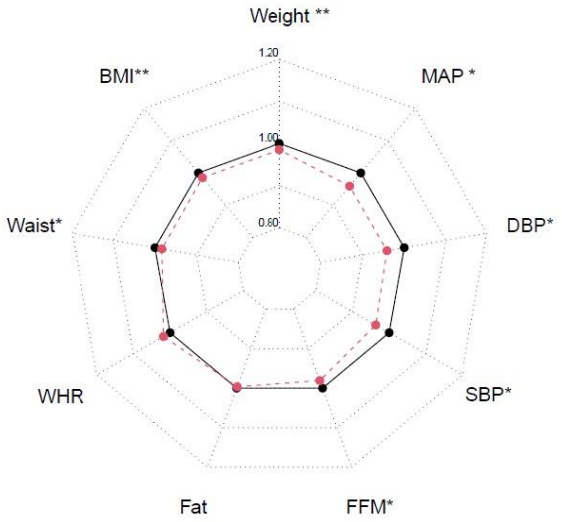



**Figure 5.6 Fold changes in anthropometry and blood pressure** (black = baseline, red = 4-weeks). BMI, body mass index; MAP, mean arterial pressure; SBP; systolic blood pressure; DBP, diastolic blood pressure; FFM, fat free mass; WHR, waist-to-hip ratio. *P<0.5, **P<0.001.

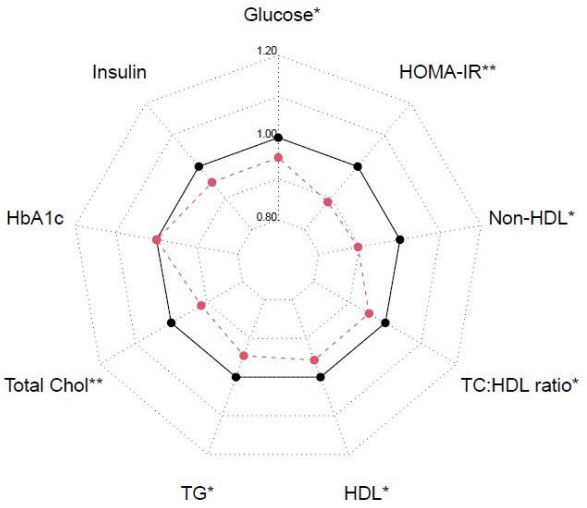



**Figure 5.7 Fold changes in cardiometabolic measures** (black = baseline, red = 4-weeks). HbA1c, glycated haemoglobin; HOMA-IR, homeostatic model assessment of insulin resistance; HDL, high-density lipoprotein; TC: HDL, total cholesterol: HDL ratio. *P<0.5, **P<0.001.

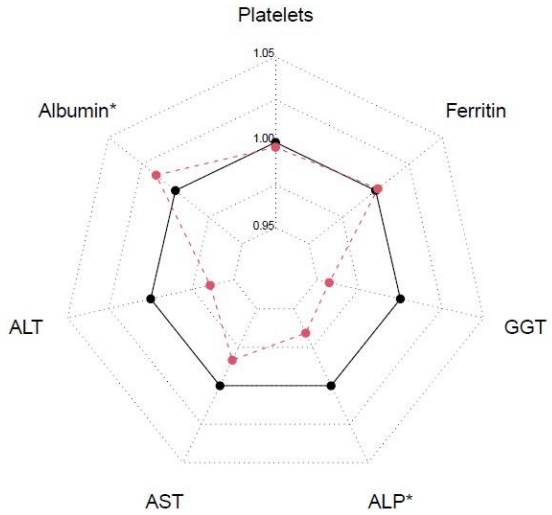



**Figure 5.8 Fold changes in liver function** (black = baseline, red = 4-weeks). ALT, alanine aminotransferase; AST, aspartate aminotransferase; ALP, alkaline phosphatase; GGT, gamma-glutamyl transpeptidase. *P<0.5, **P<0.001.

### *5.4.5.1 Sensitivity and Subgroup Analyses*

A sensitivity analysis was conducted in which results of participants (*n* 7) that exceeded +/-3% body weight change was excluded. After doing so, most of the findings were unaffected and significant improvements remained for systolic BP (p = 0.021) and diastolic BP (p = 0.002), MAP (p = 0.003), QRISK3 (p = 0.026), HOMA-IR (p = 0.008), TC (p <0.001), TG (p = 0.018), TC: HDL (p = 0.019), non-HDL (p <0.001), ALP (p = 0.048) and albumin (p = 0.009). In the sensitivity analysis,

randomisation to the MD significantly reduced gamma-glutamyl transpeptidase (GGT) (p = 0.033).

Next, sub-group analysis were undertaken in which participants were grouped according to severity of liver disease. Median body weight reduction in participants with non-advanced disease (*n* 32) was significantly less (0.9kg) than in those with NASH cirrhosis (*n* 12) (-2.4kg) (Mann-Whitney U = 107.0, *n* 44, p = 0.025). Similarly, median reductions in GGT in participants with non-advanced disease (*n* 28) (-4.5IU/L) was significantly less than in those with NASH cirrhosis (*n* 9) (-21.0IU/L) (Mann-Whitney U = 65.0, *n* 37, p = 0.030).

#### *5.4.5.2 Modelling the Effects of the MD Intervention on Cardiovascular Risk (CVR)*

Generalized estimating equations (GEE) were conducted to fit a repeated measures logistic regression to study the effects of the MD intervention on markers of CVR. GEE is an extension of generalised linear models, appropriate for use with repeated measures analysis. The within-subjects variable was time, the model type was linear, and the AR (1) correlation matrix (i.e., working correlation matrix which represents the within-subject dependencies) was set to the data. Diet, sex, T2DM were included as factors, and age, baseline MD adherence and baseline BMI as covariates. Goodness of fit was assessed using the corrected quasi likelihood under independence model criterion (QICC) (the structure that obtains the smaller QICC is superior). Early testing revealed a non-significant predictive effect of time order/ study period on CVR factors, when tested in the statistical models. This inference was reinforced through repeated analysis under the guidance of statistical support. The decision was made to exclude time order from the models presented. By doing so, the models focused on the core factors and covariates known to have associations with NAFLD and CVD, thereby providing a more precise understanding of the intervention's effects.

GEE analyses showed a significant negative interaction between the effect of MD, in males, and individuals with type 2 diabetes on lipid profile (total cholesterol and non-high-density lipoprotein) (Tables 5.12 and 5.14). The inclusion of covariates improved the model's goodness of fit, as evidenced by QICC (Tables 5.13 and 5.15). Modelling analyses also revealed that diet-induced improvement in systolic blood pressure was attenuated by participant age (Table 5.16).

| Parameter | Coefficient | 95% CI | | |
|---|---|---|---|---|
| | | Lower | Upper | P-Value |
| MD | -0.335 | -0.453 | -0.217 | <0.001 |
| Male | -0.723 | -1.255 | -0.191 | 0.008 |
| T2DM | -0.772 | -1.331 | -0.213 | 0.007 |

**Table 5.12 Generalized estimating equations model predicting effects on total cholesterol** (QICC 194.1). MD, Mediterranean diet; T2DM, Type 2 diabetes.

| Parameter | Coefficient | 95% CI | | |
|---|---|---|---|---|
| | | Lower | Upper | P-Value |
| MD | -0.335 | -0.453 | -0.217 | <0.001 |
| Male | -0.790 | -1.348 | -0.232 | 0.006 |
| T2DM | -0.664 | -1.253 | -0.075 | 0.027 |
| Age | -0.019 | -0.048 | 0.010 | 0.190 |
| MEDAS_B | 0.045 | -0.088 | 0.179 | 0.506 |

**Table 5.13 Generalized estimating equations model predicting effects on total cholesterol with covariates.** (QICC 184.8). MD, Mediterranean diet; MEDAS, MD assessment score; T2DM, Type 2 diabetes.

| Parameter | Coefficient | 95% CI | | |
|---|---|---|---|---|
| | | Lower | Upper | P-Value |
| MD | -0.291 | -0.411 | -0.172 | <0.001 |
| Male | -0.557 | -1.092 | -0.021 | 0.042 |
| T2DM | -0.609 | -1.171 | -0.047 | 0.034 |

**Table 5.14 Generalized estimating equations model predicting effects on non-high-density lipoprotein** (QICC 197.0). MD, Mediterranean diet; T2DM, Type 2 diabetes.

| Parameter | Coefficient | 95% CI | | |
|---|---|---|---|---|
| | | Lower | Upper | P-Value |
| MD | -0.291 | -0.411 | -0.172 | <0.001 |
| Male | -0.637 | -1.211 | -0.064 | 0.029 |
| T2DM | -0.498 | -1.084 | 0.088 | 0.096 |
| Age | -0.021 | -0.052 | 0.011 | 0.194 |
| MEDAS_B | 0.012 | -0.118 | 0.141 | 0.861 |

**Table 5.15 Generalized estimating equations model predicting effects on non-high-density lipoprotein with covariates** (QICC 186.4). MD, Mediterranean diet; MEDAS, MD assessment score; T2DM, Type 2 diabetes.

| Parameter | Coefficient | 95% CI | | |
|---|---|---|---|---|
| | | Lower | Upper | P-Value |
| MD | -5.345 | -9.457 | -1.234 | 0.011 |
| Age | 0.269 | 0.013 | 0.525 | 0.039 |
| MEDAS_B | 0.917 | -0.977 | 2.811 | 0.342 |
| BMI_B | -0.066 | -0.725 | 0.593 | 0.844 |

**Table 5.16 Generalized estimating equations model predicting effects on systolic blood pressure with covariates** (QICC 44777.1). MD, Mediterranean diet; MEDAS, MD assessment score; BMI, body mass index; _B, baseline.

#### *5.4.5.3 Potential Carryover Effects from One Experimental Period to the Next*

When participants were randomised to MD in the first experimental period, there was evidence of carryover effects for a small number of clinical and lifestyle variables (Table 5.17). In addition, the proportion of participants that reported consuming "olive oil" (p = 0.001), "≥2/day servings of vegetables" (p = 0.004), "<1/day servings of butter, margarine, or cream" (p = 0.016) and "≥3/week servings of legumes" (p = 0.008) did not return to baseline levels.

| **Clinical and Lifestyle Variable** | **Baseline** | **Post-Washout** | **P-Value** |
|---|---|---|---|
| **Dietary Intake** | | | |
| MEDAS | 5.5±2.0 | 8.5±2.2 | <0.001 |
| INTAKE24<br>Selenium<br>Vitamin D | <br>33.6±21.3<br>1.1 (0.6-2.7) | <br>49.0±21.1<br>3.4 (1.5-7.9) | <br>0.034<br>0.008 |
| **Anthropometrics** | | | |
| Waist circumference (cm) | 116.2±14.9 | 114.0±14.2 | 0.034 |
| **Liver Function** | | | |
| Ferritin (ug/L) | 91.0 (38.8-232.0) | 64.0 (32.5-157.0) | 0.030 |

**Table 5.17 Anthropometric and biochemical characteristics and nutrient intakes of participants at baseline and after washout (n=48).** (First experimental period = MD). MD, Mediterranean diet; MEDAS, MD assessment score.

## 5.5 Discussion

Although designed as a feasibility and pilot study, the findings from this randomised controlled clinical trial (RCT) address a gap in evidence about the effectiveness of a MD intervention in a northern European NAFLD patient population. The main findings were that a dietitian supported treatment with supplemental foods increased MD adherence and diet quality within 4 weeks. These dietary changes were accompanied with significantly improved biomarkers of cardiovascular risk with minor improvements in liver function.

Few studies have investigated the impact of MD interventions among patients with NAFLD outside the Mediterranean region. However, dietary intervention studies targeting patients with T2DM or CVR factors (NAFLD associated conditions) have induced a 1-2 point increase in MD adherence scores [424-426]. In the current analysis, the MD intervention resulted in a 3-point increase in MD adherence after 4 weeks, with almost half of participants (45%) reporting high consumption (≥10 points). These are important findings because there is consistent evidence that a 2-point increase in MD assessment scores correlates with significantly reduced CVR and overall mortality [273,427].

In the present study, randomisation to the MD resulted in significantly more participants meeting the recommendations for olive oil, vegetables, fruits, legumes, fish, nuts, sofrito as well as for butter/margarine. This is consistent with the modelling studies which found that changes in intakes of sofrito, legumes, and vegetables and fruit drove the differences in total MEDAS score between the diets. These dietary components (individual and synergistic effects) have been shown to elicit beneficial health effects [286,314,428].

Estimates of dietary intake derived using the online INTAKE24 dietary recall system showed reduced intakes of NMES, trans fat and sodium. Research has shown an association between sodium intake and increased risk of NAFLD development [429,430]. Excess intakes of trans fatty acids [140,141] and NMES [160,162] promotes disease pathogenesis. Targeting a reduction in these nutrients is recommended by European clinical guidelines [6,125]. Reduced intakes of energy (calorie), protein and carbohydrate were also observed. The energy (calorie) intake could be regarded as lower than anticipated to meet the energy intake requirements of the cohort at both

time points. The carbohydrate intake could be regarded as moderate or reduced (i.e., >130 grams carbohydrate/day) [266]. Evidence of the optimum carbohydrate prescription in NAFLD is mixed, but specific types of carbohydrate such as low glycaemic index foods and fibre, are considered beneficial in this patient population [141,167,171,266]. Based on reference nutrient intakes (median body weight x 0.75) [431], average protein consumption was below the recommended 70 grams per day. This may have clinical implications for the prevention and management of sarcopenia [432]. Incorporating targeted advice that encourages adequate protein intake in the future RCT may be a reasonable protocol adjustment (Chapter 4 Table 4.5).

The data from both the non-targeted metabolite fingerprinting and targeted urine-based biomarker approaches for dietary intake monitoring, showed that multiple intake biomarkers were altered following the MD intervention. There was clear, but modest, modifications in the urine metabolome between the diets. The strongest discrimination between the diets, and best classification performance was observed when MD was consumed in the first experimental period. Urinary concentrations of reported known biomarkers for poultry (3-methyl histidine), citrus (4-hydroxyproline betaine) intake and of TMAO [301], which are indicators of MD adherence [301] were significantly higher when the MD was consumed (Table 5.5 and Figure 5.5). These elevated levels were not sustained throughout washout, which suggests that participants returned to their habitual diet as per trial protocol. The stronger modelling performances in the non-targeted data versus the targeted data, could indicate that endogenous metabolic perturbations are a factor in adherence to dietary advice. However, potential influences on urine-based biomarkers remain poorly understood [301].

Randomisation to the MD intervention produced beneficial changes in measures of cardiometabolic risk and in abdominal obesity. This is congruent with a small number of RCTs [324,328,330] and systematic reviews which found that MD improved insulin resistance (IR) [351], lipid profile, and glycaemic indices [352]. In addition, there is evidence that the MD is protective against features of the MetS, which are strongly associated with NAFLD [277]. Given that CVD is the leading cause of mortality amongst patients with NAFLD, these data support the current clinical guidelines to improve dietary patterns in line with MD [5,6,27].

In the present study, sub-group analyses suggest that patients with NASH cirrhosis might derive greater benefit in terms of weight loss and improved liver biochemistry (as measured by GGT) when prescribed a MD. GGT is a key marker for oxidative stress [433] and has been associated with surrogate markers of IR [434]. GGT is sensitive to changes in environmental factors [433] such as BMI, waist circumference and lipid metabolism [433,435]. Note however, that these sub-group analyses were carried out post hoc and so the findings should be interpreted with caution. Modelling results showed a greater impact of the MD on total cholesterol and on non-HDL in males and individuals with T2DM. Furthermore, analyses revealed that diet-induced improvement in systolic blood pressure was attenuated by participant age. These findings require confirmation in larger intervention trials with appropriate designs.

There is good evidence that the MD reduces risk factors for age-related diseases such as CVD [436]. In addition, the potential role of MD in modulating the distinctive features of ageing such as telomere attrition, which may positively impact age-related disease risk, has been described [276]. There are multiple potential mechanisms through which the MD and its constituents can improve cardiovascular health including positive effects on IR [351] and on inflammation [353,354,437]. The MD provides high intakes of antioxidants and phenolic compounds that are associated with improved insulin sensitivity [280], and decreased oxidative damage [438]. Increased olive oil intake (10g per day) is associated with a 16% reduction in CVR [439]. Olive oil is high in monounsaturated fatty acids (MUFAs), and extra-virgin olive oil (EVOO) contains higher amounts of polyphenols, antioxidants and phytochemicals [286]. Evidence from RCTs that examined supplemental EVOO found that improvements in post-prandial glycaemic status and lipid profile were mediated by up-regulation of GLP1 [440,441]. The protective effects of nuts on CVD have been reported in numerous prospective trials [442]. Nuts contain substantial amounts of phytosterols and micronutrients such as folate and minerals that have favourable effects on IR, blood pressure, and dyslipidaemia, and influence modulation of inflammation and endothelial function [442-446]. Moreover, eating vegetables and fruits abundant in polyphenols [447,448]; fish [449], and sofrito [450] have established beneficial effects on CVR.

## 5.6 Strengths and Limitations

A unique feature of this trial was the use of urinary metabolomics as independent dietary biomarkers to assess changes in dietary intake in response to the MD. Metabolomics approaches may provide a powerful tool to monitor responsiveness to diet treatments with potential to improve the accuracy and reliability of self-reported intake [92]. In the present study, the cross-validation of the two diet assessment methods i.e., MEDAS and urine-based dietary biomarkers, demonstrates the utility of this strategy for dietary monitoring. A caveat is that some of the MEDAS components do not have corresponding urinary biomarkers i.e., sofrito. Therefore, if these components make a relatively large contribution to changes in total MEDAS scores, the urinary biomarker models may have weaker explanatory power because they are not included. Targeted assays (i.e., quantitative biomarker panel) are limited by the molecules they cover [305].

Furthermore, the urinary metabolome is a poor indicator of intake of fats, and of individual classes of fatty acids such as saturated fatty acids, because these metabolites are not sufficiently hydrophilic to be visible in urine. However, urine biomarkers have the potential to elucidate responses to these foods *via* surrogate markers. For example, artificial sweeteners are common components of ultra-processed foods, which also often contain elevated levels of saturated fatty acids. Moreover, a recent European-wide multi-centre study identified a panel of four metabolites (sucrose, hippurate, N-methylnicotinic acid, and urea), that may be predictive of ultra-processed food intake and MD adherence [302]. Nevertheless, the study findings may have been strengthened by the use of metabolomics biomarkers to capture diet-mediated effects on lipid metabolism (lipidomics) that characterises changes in the lipidome [451,452]. Additionally, these points underscore the benefits of using a combination of self-reported data and quantification of urinary dietary biomarkers to adequately assess dietary intake.

The provision of pre-packaged ready meals and of extra virgin olive oil aimed to reduce participant burden and to facilitate changes in the food environment. However, the specific foods chosen differed both between individuals, and within individuals over time. This likely contributed to the inter class variance observed within the treatment diets, which may be considered as a weakness of the study design. The data indicates that compliance with the intervention is a key potential

confounder. Therefore, it is possible that the future utility of urinary biomarkers could be best suited to food exposure strategies/ dietary approaches which are more rigid [389]. On the other hand, more frequent urinary sample collection (pooling the samples before analysis) may capture the variability in the diet over the intervention period without significant cost increase (chapter 4, Table 4.5).

Chapter 4 presented data indicating that the online INTAKE24 dietary recall system should not be included in the future definitive RCT, unless substantial revisions are undertaken. This was partly due to cases of missing and poor quality data. In the current analysis, estimated dietary intake may have been influenced by mis-reporting, despite the dataset undergoing quality checks and completeness assessments [387,388]. Dietary recalls are subject to reporting inaccuracies due to memory, social desirability and cognitive biases, leading to measurement errors [453,454].

There have been various methods proposed for the detection of misreporting, but it remains unclear which method is the optimum to apply in practice [455]. Techniques include doubly labelled water (DWL), urinary nitrogen as a biomarker to validate protein intake, energy intake: energy expenditure (EI:EE) method, and the Goldberg cut-off [455]. The Goldberg cut-off is a widely used statistical method that evaluates the plausibility of reported dietary intake, based on the ratio of EI to estimated basal metabolic rate (BMR) and applying a confidence limit [455]. Critically, each method has inherent strengths and limitations. The DWL technique objectively measures EE but it is expensive and impractical to use routinely [455]. The Goldberg cut-off is relatively inexpensive and simple to use, but it can be prone to misclassification related to the formulas used to estimate EE [456]. Additionally, it does not distinguish between varying degrees of mis-reporting [457]. In the future definitive RCT, it will be important to consider an appropriate validation procedure for identifying misreporting, to enhance the effectiveness of the dietary monitoring strategy.

The main strength of this trial is the use of explicit feasibility criteria that are presented and discussed in Chapter 4. However, the collection of, and analysis of data from, clinical and lifestyle variables (described in this chapter) provide preliminary evidence of potential effectiveness although this trial was not powered to detect significant changes in clinical and lifestyle outcomes. A methodological

strength of this study was that the observed changes in biomarkers of cardiovascular risk, and minor improvements in liver function, occurred even as physical activity levels remained unchanged, which adds to the validity of the results and conclusions. Moreover, While a small number of participants exceeded the +/- 3% weight change stipulation, the results of the sensitivity analyses showed that this did not greatly influence the overall findings which underlies the robustness of the observations. The use of a cross-over design facilitates more precise comparisons between intervention/control diets on a within-participant basis [371]. This design is favoured in short-term trials of long-term conditions with intermediate outcomes [371].

Nevertheless, a limitation of this design is the possibility of carryover effects from one experimental period to the next. Chapter 4 presented questionnaire data, which suggested that the study design appeared to impact diet adherence for a small number of participants, with difficulties reported when switching diets. This issue was mitigated by the inclusion of a washout period (4 weeks) between treatments. When participants were randomised to the MD in the first experimental period, analysis of data before and after the washout period showed that most variables e.g., cardiometabolic measures, urinary metabolomics etc., returned to (or close to) baseline whereas for others e.g., MEDAS, ferritin and waist circumference there was evidence of carryover effects. The washout period was consistent with other nutritional crossover studies, where 2-4 weeks is often sufficient [370,372]. This has been confirmed by studies of metabolomics biomarkers in urine which show that these respond rapidly to dietary change [373]. Nevertheless, there may be nuanced implications of these carry-over effects that must be carefully considered [458] . Notably, the affected variables were preliminary data, which may have a less pronounced impact on the overall study conclusions. The study involved a small sample and was conducted over a relatively short duration. Thus, while it's reasonable to suggest that understanding the true treatment effects is more challenging, the primary focus of the current study was to investigate the acceptability and feasibility of conducting a future definitive RCT.

The current analysis shows mixed findings and further clarification is needed to determine the optimum washout period, before larger scale evaluation. It may be useful to explore the underpinning mechanisms of such carry-over effects, as these effects can have various causes [459]. Additionally, caution should be exercised when

interpreting the self-reported diet intake data where there was evidence of carry over effects, as it may have been influenced by a degree of social desirability bias [460]. Alternatively, a future definitive RCT could employ a parallel group design which would require recruitment of a larger number of participants, but which would avoid potential confounding due to carry-over effects (Chapter 4, Table 4.5).

Limited evidence from the metabolomics analysis suggested that MD adherence was poorer in individuals who received this treatment in the second experimental phase. There may be some general reasons such as research participation effects [461], for example at the beginning of a study, participants may have a heightened sense of motivation and commitment to follow the study protocol. Previous research suggests that participants are more likely to drop out after the first experimental phase [462]. The pattern of when participants were randomised to MD in the first experimental period or in the second experimental period was mapped against the timeline of coronavirus (COVID-19) pandemic lockdowns in the United Kingdom (Figure 5.9). Based on this information, it appears that there was no obvious explanatory relationship between COVID-19 pandemic related restrictions and poorer MD adherence in the second experimental period.

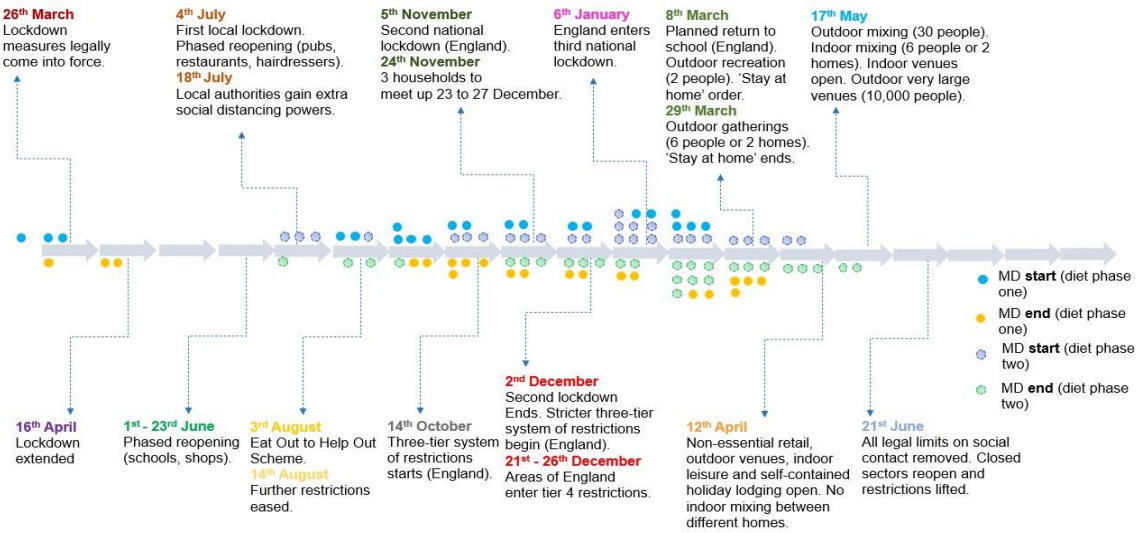



**Figure 5.9 Timeline of the participants randomisation to MD in relation to COVID-19 pandemic lockdowns, March 2020 to June 2021.** COVID-19, coronavirus; MD, Mediterranean diet

The intervention period (4-weeks) was relatively short and is unlikely to have revealed the full effects of the dietary intervention on markers of liver health. Hence, the data presented might be an underestimation of the potential benefits of the MD intervention if it were implemented over a longer period. Comparative studies

investigating MD interventions reported improved markers of NAFLD with a mean follow-up duration of 27 weeks (6 weeks to 78 weeks) [280,285,324,329,333].

However, this study was designed, primarily, to provide information on the feasibility of the study protocol. The selected timeframe sought to strike a balance between obtaining preliminary evidence of short-term benefits, assessing initial responses to dietary changes, while reducing participant burden and potential drop-outs. The MD intervention which elicited strong participant adherence builds on the evidence that brief and short-term interventions can effectively modify MD adherence in non-Mediterranean countries [357-359,377]. In the future definitive RCT, the decision on intervention duration will be informed by the wider evidence base, clinically meaningful changes in the outcomes, and the effect size associated with longer term MD interventions.

## 5.7 Conclusion

In summary, the results of this study have shown that a 4-week dietitian-led intervention with food provision produced increases in MD adherence among a northern European NAFLD patient population. These dietary changes improved CVR profile with minor improvements in liver function. These data provide further evidence of the benefits of MD and its potential to be translated in regions that consume a western diet. Important information on trial design and optimising diet treatments were identified that may inform the development of a future definitive RCT. Finally, robust assessment of dietary intake is feasible with the use of both self-reported measures and urinary metabolomics-based approaches.

# Chapter 6

# Randomised Controlled Feasibility Trial: An Assessment of Preliminary Exploratory Data

## 6.1 Introduction

Grading disease activity and staging fibrosis progression in patients with non-alcoholic fatty liver disease (NAFLD) is crucial to determine prognosis and monitor treatment response. Chapter 1 describes the intense research efforts to develop non-invasive tests (NITs) for accurate fibrosis staging and risk stratification, and the shortcomings of liver biopsy as the current reference standard [20]. The clinical application of NITs has steadily increased, including the use of the Fibrosis-4 index (FIB-4) that effectively rules out advanced fibrosis [97]. Nevertheless, the capability of current NITs to rule in advanced fibrosis is modest [403]. Recent advances in biomarker development include PRO-C3 (N-terminal propeptide of type III collagen), and the ADAPT score (an algorithm that uses a combination of PRO-C3, age, T2DM and platelet count). Preliminary data indicate that both can accurately assess clinically significant fibrosis [103-105], but neither is yet suitable for routine clinical practice [20]. More evidence is needed on biomarkers that predict treatment response and that have potential to achieve regulatory approval [20].

There is an urgent need to identify potential therapeutic interventions that can prevent NAFLD progression and induce regression [13]. However, the effectiveness of current established diet therapies in NAFLD is sub-optimal [13]. Nutrigenetics-based intervention approaches offer a promising strategy to support individuals or subgroups who may benefit from a specific intervention [360]. Differences in gene sequence can alter the activity of encoded proteins and affect the response of individuals to specific dietary components [288]. The patatin-like phospholipase domain containing 3 (*PNPLA3)* rs738409 single nucleotide polymorphism is a common modifier of disease outcome in this patient population [183,184,187,188]. Previous research has reported that the I148M variant was associated with a 1.4 fold increase in liver fat [463], and individuals homozygous for the rs738409 G allele have a 1.9-fold increased risk of cirrhosis than those with wild type PNPLA3 [291]. Importantly, a limited proportion of NAFLD pathogenesis can be explained by the role of SNP-mediated liver damage, and for substantial changes to take place synergistic interactions between the environment and these risk variants are needed [464]. However, the role of *PNPLA3* variants in influencing responsiveness to different diet therapies is largely unknown.

## 6.2 Study Aims and Objectives

The overarching aims and objectives of the randomised controlled feasibility trial are outlined in Chapter 3.

The current chapter describes an assessment of liver fibrosis biomarkers and the influence of *PNPLA3* genotype in response to the dietary intervention.

The objectives of this current chapter were:

- To undertake preliminary exploration of changes in biomarkers of liver fibrosis.
- To undertake preliminary exploration of the influence of *PNPLA3* genotype on metabolic endpoints.

## 6.3 Materials and Methods

The methods have been described elsewhere (in Chapter 3).

## 6.4 Results

Chapter 4 summarises the baseline characteristics, and the flow, of participants through the trial. Chapter 5 describes an evaluation of the effectiveness of the study protocol. Data from 48 out of 49 participants recruited into the feasibility trial are presented in this chapter (i.e., 1 participant left the study after the baseline visit for a non-related health issue). All the results from each time point and study arms are presented in the appendices (Appendix X2 and X4).

### *6.4.1 Non-Invasive assessment of Impact of the Mediterranean Diet Intervention on liver biochemistry and fibrosis biomarkers*

Table 6.1 summarises the participants characteristics before (baseline) and after the Mediterranean diet (MD) intervention (paired samples t-test and the Wilcoxon signed-rank test, as appropriate). At baseline, participants had elevated median liver stiffness (a surrogate for liver fibrosis), as well as PRO-C3 and ADAPT levels that are indicative of fibrosis stage ≥F2 [104,105,465]. Randomisation to the MD intervention resulted in significant reductions in PRO-C3 and ADAPT (Table 6.1 and Figure 6.1), suggesting reduced fibrogenesis. There were no significant changes in the other measured biomarkers, however a trend towards an increase in CTX-III was observed, suggesting that fibrolysis may have been increased. At the end of control

diet (CD) period, there were no significant changes in any of the liver fibrosis biomarkers (p >0.05) (Appendix AA).

| Participant Characteristics | Baseline | Post-MD | P-Value |
|---|---|---|---|
| Age (years) | 60.0 (52.3-68.8) | | |
| T2DM | 26 (54%) | | |
| *PNPLA3 rs738409* (n, %)<br>CC<br>CG<br>GG | <br>21 (45%)<br>14 (30%)<br>12 (26%) | | |
| *TM6SF2 rs58542926* (n, %)<br>CC<br>CT<br>TT | <br>38 (81%)<br>8 (17%)<br>1 (2%) | | |
| **Liver function** | | | |
| Platelets (x10 9 /L) | 234 (209-274) | 235 (197-268) | 0.646 |
| ALT (unit/L) | 38 (28-62) | 38 (26-60) | 0.081 |
| AST (unit/L) | 31 (23-47) | 31 (23-47) | 0.257 |
| **Liver steatosis** | | | |
| CAP dB/m *(baseline only)* | 333 ± 59 | | |
| **Liver fibrosis** | | | |
| Liver stiffness (kPa) *(baseline only)* | 9.4 (6.2-21.8) | | |
| GDF15 (pg/mL) | 1110.0 (752.0-1602.7) | 955.0 (724.1-1589.5) | 0.955 |
| PRO-C3 (ng/mL) | 15.5 (11.4-21.3) | 13.5 (10.8-20.1) | 0.032 |
| PRO-C4 (ng/mL) | 7584.7 ± 1276.1 | 7461.7 ± 1173.5 | 0.229 |
| PRO-C5 (ng/mL) | 955.1 ± 316.3 | 935.1 ± 322.6 | 0.254 |
| CTX-III (ng/mL) | 8.9 ± 4.9 | 9.4 ± 4.9 | 0.076 |
| FIB-4 | 1.2 (0.9-1.9) | 1.3 (1.0-1.8) | 0.592 |
| ADAPT | 7.27 (6.32-8.58) | 7.19 (6.39-8.28) | 0.023 |

**Table 6.1 Changes in biomarkers of liver fibrosis after 4-weeks of MD intervention (n=48).** Values are numbers (percentages), means (standard deviation) or medians (range). *PNPLA3,* patatin-like phospholipase domain containing 3; TM6SF2, transmembrane 6 superfamily member 2; T2DM, type 2 diabetes; ALT, alanine aminotransferase; AST, aspartate aminotransferase; PRO-C3, N-terminal propeptide of type III collagen; CTX-III, crosslinked type III collagen; GDF15; growth differentiation factor 15; FIB-4, Fibrosis-4 index; ADAPT, PRO-C3-based fibrosis algorithm; CAP, controlled attenuation parameter.

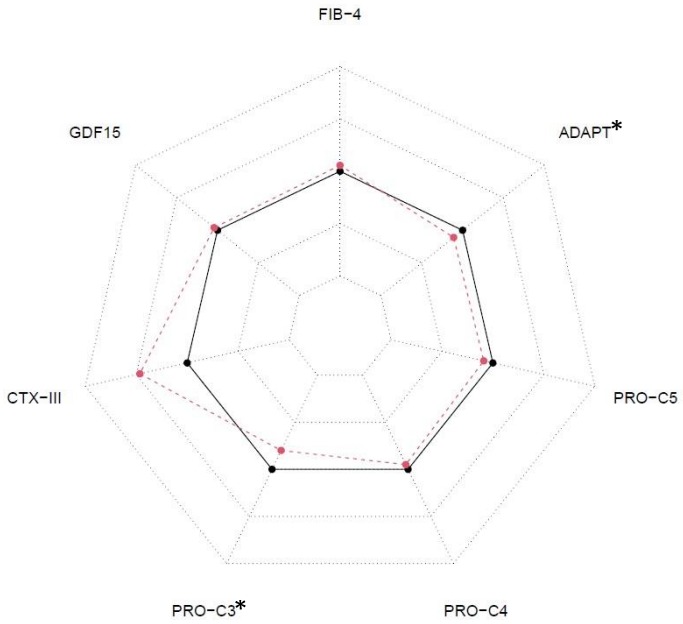



**Figure 6.1 Fold changes in biomarkers of liver fibrosis** (black = baseline, red = 4-weeks). PRO-C3, N-terminal propeptide of type III collagen; CTX-III, crosslinked type III collagen; GDF15; growth differentiation factor 15; FIB-4, Fibrosis-4 index; ADAPT, PRO-C3-based fibrosis algorithm. *P<0.5, **P<0.001.

#### *6.4.1.1 Sensitivity and Subgroup Analyses*

A sensitivity analysis was conducted in which results from participants (*n* 7) who exceeded +/-3% body weight change were excluded. The improvements in PRO-C3 (p = 0.040) and ADAPT (p = 0.016) in response to the MD intervention remained significant. A trend towards an increase in CTX-III was observed, although this remained a non-significant result. Next, sub-group analyses were undertaken in which participants were grouped according to severity of liver disease (non-advanced disease vs. NASH cirrhosis). The results indicate non-significant differences between groups (p> 0.05). (Appendix AB).

### *6.4.2 Urine-Based Dietary Biomarkers (Non-targeted Fingerprinting)*

Metabolome fingerprints were generated to determine if there were compositional differences between disease stage categories, detectable in urine. A combination of multivariate classification tools, random forest (RF) and principal component linear discriminant analysis (PC-LDA) were used to summarise high dimensional metabolome fingerprints and model multiple disease state scenarios. For the models presented in Figure 6.2, baseline samples only were considered to derive patterns of urine metabolites according to liver disease status prior to dietary intervention.

‘Model A’ is a multiple class comparison between three disease phenotypes (non-alcoholic fatty liver, NAFL; non-alcoholic steatohepatitis, NASH F1-3; NASH cirrhosis). ‘Model B’ is a binary disease model where all non-NASH cirrhosis disease phenotypes are combined and compared against NASH cirrhosis. ‘Model C’ is a multivariable disease model where all NASH F1-3 are combined and compared with NAFL and NASH cirrhosis.

Each model indicates clear discrimination between disease stage categories at baseline, based on non-targeted fingerprints. The comparisons presented in ‘Model C’ scenario show the best separation of urine metabolite patterns according to disease severity. Further, based on robust modelling output measures including area under the receiver operator characteristic (ROC) curve (AUC) [389], the greatest discrimination was observed between urinary metabolome of those participants with non-alcoholic fatty liver (NAFL) versus those with non-alcoholic steatohepatitis (NASH) (Table 6.2). In all models, NASH Cirrhosis showed the most within class variance, leading to poor classification accuracy between classes (Table 6.2).

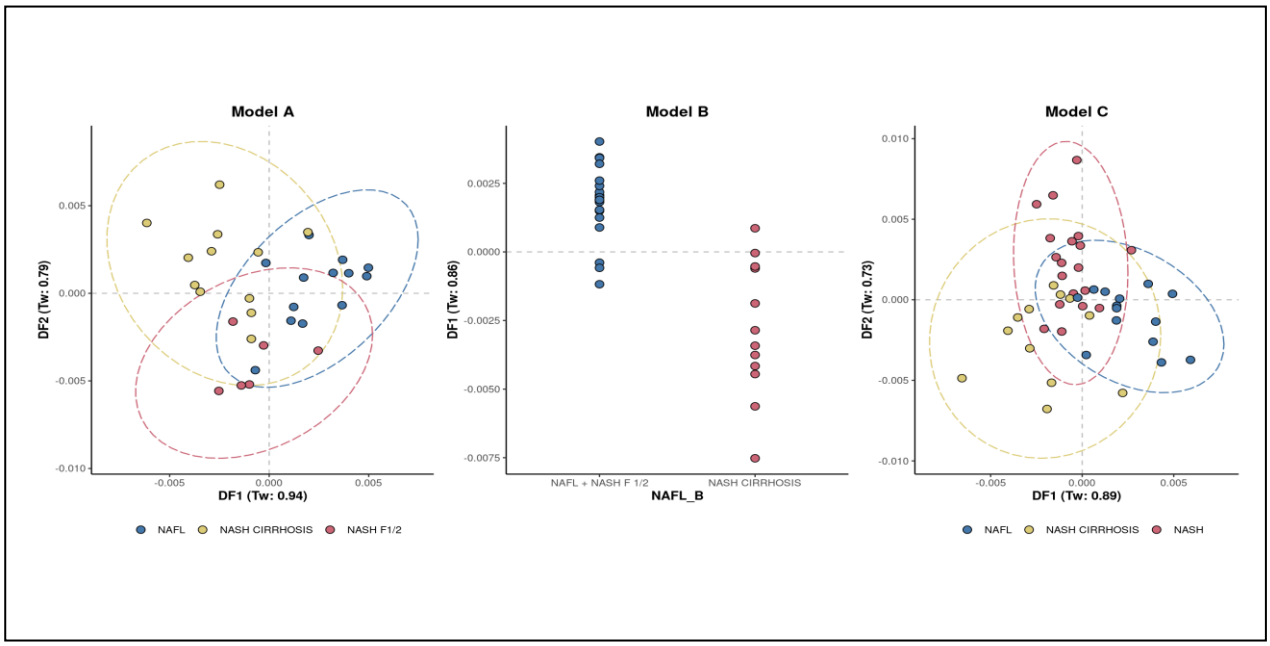



**Figure 6.2 PC-LDA of metabolite fingerprint data between disease stage categories.** (A) NAFL vs. NASH (F1-F2) vs NASH cirrhosis (B) NAFL/NASH (F1-F2) vs NASH cirrhosis (C) NAFL vs. NASH (F1-F3) vs NASH cirrhosis. NAFL, non-alcoholic fatty liver; NASH, non-alcoholic steatohepatitis.

| | Accuracy | ROC AUC |
|---|---|---|
| **Model A** | | |
| NAFL vs. NASH cirrhosis | 0.52 | 0.55 |
| NAFL vs. NASH | 0.47 | 0.39 |
| NASH vs. NASH cirrhosis | 0.44 | 0.17 |
| **Model B** | | |
| NAFL + NASH vs. NASH cirrhosis | 0.55 | 0.42 |
| **Model C** | | |
| NAFL vs. NASH | 0.64 | 0.59 |
| NAFL vs. NASH cirrhosis | 0.50 | 0.51 |
| NASH vs. NASH cirrhosis | 0.45 | 0.33 |

**Table 6.2 Classification performance between disease stage categories (non-targeted fingerprinting).** ROC, receiver operator characteristic; AUC, area under the curve; NAFL, non-alcoholic fatty liver; NASH, non-alcoholic steatohepatitis.

### *6.4.3 Preliminary Exploration of the Influence of PNPLA3 rs738409 variants on metabolic endpoints in response to the MD intervention*

Analysis of participant genotype revealed that 45% participants had *PNPLA3* rs738409 I148M CC genotype, 30% had the CG genotype and 26% had the GG genotype. At end of MD intervention, the reduction from baseline in fasting glucose concentration in participants carrying the wild type (CC) *PNPLA3* I148M (*n* 17) was significantly greater (median = -0.5mmol/L), than in those carrying either CG or GG genotypes (*n* 23), in which no reduction was observed (Mann-Whitney U = 116.5, *n* 40, p = 0.030). Similarly, the reduction from baseline in total cholesterol concentration in participants carrying the wild type (CC) *PNPLA3* I148M (*n* 17) was significantly greater (mean = -0.5mmol/L) than for carriers of the I148M variant (*n* 24) (-0.3mmol/L) (p = 0.034). Likewise, the reduction from baseline in non-high-density lipoprotein (non-HDL) cholesterol concentration in participants carrying the wild type (CC) *PNPLA3* I148M (*n* 17) was significantly larger (mean = -0.5mmol/L) than in carriers of the I148M variant (*n* 24) (-0.2mmol/L) (p = 0.033) (results derived from Student's t test).

Of note, additional genotyping for specific genetic variants of transmembrane 6 superfamily member 2 (*TM6SF2)* rs58542926 E167K, was conducted. The decision to include genotyping for the *TM6SF2* rs58543926 SNP was based on evidence suggesting that it is a promising therapeutic target in NAFLD due to its role in lipid metabolism, and its potential to mitigate cardiovascular comorbidity [(202)]. The *TM6SF2* rs58543926 SNP has been implicated in NASH and associated with

increased risk of fibrosis progression [72,197]. Analysis of the genotype distribution revealed that 81% of participants had the CC genotype, 17% had the CT genotype and 2% had the TT genotype. After the MD intervention, the reduction from baseline in systolic blood pressure in participants carrying either CT or TT genotypes was higher (mean = -15.2mmHg) than in those carrying the wildtype (CC) genotype (-4.1mmHg), although this result did not reach statistical significance (p = 0.053).

#### *6.4.3.1 Modelling the Effects of the MD Intervention and Influence of PNPLA3 and TM6SF2 genotypes on Cardiovascular Risk*

Generalized estimating equations (GEE) were conducted in Chapter 5 to fit a repeated measures logistic regression to study the effects of the MD intervention on cardiovascular risk (CVR). In the current chapter, *PNPLA3* and *TM6SF2* genotypes were included as additional factors in the models. Goodness of fit was assessed using the corrected quasi likelihood under independence model criterion (QICC) (the structure that obtains the smaller QICC is superior).

The addition of *PNPLA3* and *TM6SF2* genotypes (wildtype = 0 vs risk allele (CG or GG) = 1 in non-additive coding)) as factors in the models resulted in greater goodness of fit (Tables 6.3 to 6.5). QICC* improvements were observed for total cholesterol (184.8 to 182.8), non-high-density lipoprotein (186.4 to 181.6) and systolic blood pressure (44777.1 to 44522.5). However, there was no significant interaction between MD and the *PNPLA3* and *TM6SF2* genotypes in influencing effects of the MD intervention on these CVR factors.

| Parameter | Coefficient | 95% CI | | |
|---|---|---|---|---|
| | | Lower | Upper | P-Value |
| MD | -0.323 | -0.441 | -0.206 | <0.001 |
| Male | -0.875 | -1.424 | -0.325 | 0.002 |
| T2DM | -0.720 | -1.338 | -0.101 | 0.023 |
| *PNPLA3* wildtype (CC) genotype | 0.281 | -0.265 | 0.826 | 0.314 |
| Age | -0.020 | -0.048 | 0.007 | 0.149 |
| MEDAS_B | 0.062 | -0.076 | 0.199 | 0.380 |

**Table 6.3 Generalized estimating equations final model predicting effects on total cholesterol with covariates** (QICC 182.8*). MD, Mediterranean diet; MEDAS, MD assessment score; T2DM, Type 2 diabetes. (wildtype = 0 vs risk allele (CG or GG) = 1).

| Parameter | Coefficient | 95% CI | | |
|---|---|---|---|---|
| | | Lower | Upper | P-Value |
| MD | -0.277 | -0.396 | -0.158 | <0.001 |
| Male | -0.736 | -1.290 | -0.182 | 0.009 |
| T2DM | -0.564 | -1.162 | 0.034 | 0.065 |
| *PNPLA3* wildtype (CC) genotype | 0.318 | -0.223 | 0.858 | 0.250 |
| Age | -0.022 | -0.052 | 0.008 | 0.154 |
| MEDAS_B | 0.031 | -0.102 | 0.164 | 0.652 |

**Table 6.4 Generalized estimating equations final model predicting effects on non-high-density lipoprotein with covariates** (QICC 181.6*). MD, Mediterranean diet; MEDAS, MD assessment score; T2DM, Type 2 diabetes. (wildtype = 0 vs risk allele (CG or GG) = 1).

| Parameter | Coefficient | 95% CI | | |
|---|---|---|---|---|
| | | Lower | Upper | P-Value |
| MD | -5.675 | -9.822 | -1.527 | 0.007 |
| *TM6SF2* wildtype (CC) genotype | 0.920 | -5.514 | 7.354 | 0.779 |
| Age | 0.270 | 0.012 | 0.528 | 0.041 |
| MEDAS_B | 0.960 | -0.977 | 2.898 | 0.331 |
| BMI_B | -0.075 | -0.743 | 0.593 | 0.827 |

**Table 6.5 Generalized estimating equations final model predicting effects on systolic blood pressure with covariates** (QICC 44522.5*). MD, Mediterranean diet; MEDAS, MD assessment score; BMI, body mass index; _B, baseline. (wildtype = 0 vs risk allele (CG or GG) = 1).

## 6.5 Discussion

This initial assessment of preliminary exploratory data from the feasibility and pilot study has shown: i) the responsiveness of PRO-C3 and ADAPT to the MD intervention, ii) the potential feasibility of using urine metabolomics to investigate endogenous metabolic perturbations associated with liver fibrosis and disease severity and, iii) that carriers of the I148M variant of *PNPLA3* rs738409 appear show lower improvements in CVR factors when prescribed a MD intervention.

Few studies have investigated the effectiveness of the MD in this patient population using paired liver biopsies (before and after intervention) [266,286]. Hence, there is insufficient histological evidence of the effects of this dietary pattern on liver inflammation or on fibrosis [286]. However, meta-analytic data has indicated MD-induced improvements in NITs such as liver stiffness (a surrogate for liver fibrosis)

[314]. In addition, observational data suggests that a higher intake of hydroxybenzoic acids (a class of phenolic acid) that are abundant in MD components such as nuts, berries, and wholegrains is associated with decreased prevalence of clinically significant fibrosis (≥F2), as measured by FibroTest (liver fibrosis biomarker) [466].

In the current analysis, the MD intervention reduced circulating PRO-C3 levels and the derived ADAPT score (a PRO-C3 based algorithm) by 13% and 1% respectively, providing early evidence of benefits on liver fibrosis, and reduced fibrogenesis in particular. The early evidence of reductions could be potentially attributed to various mechanisms associated with the MD's impact on liver health and metabolism [6,280-285]. However, few comparative trials have used these biomarkers to evaluate the effects of the MD in NAFLD. Currently, there are no universally accepted thresholds or guidelines for a clinically relevant reduction in PRO-C3 levels in NAFLD [104]. Both PRO-C3 and ADAPT change in response to drug interventions and have been previously measured as exploratory endpoints in clinical trials [467,468] but the precise reduction considered clinically relevant may vary between studies. A multi-centre trial of drug intervention showed an improvement in PRO-C3 levels of up to 33% at 12 weeks [467]. Evidence suggests that it is most sensitive to active fibrogenesis, as distinct from static collagen accumulation [103,469,470].

The non-significant trend towards an increase in CTX-III, suggests that fibrolysis may have been increased, which when taken together with observed reductions in PRO-C3 and ADAPT, implies a beneficial effect on collagen turnover that might with time lead to fibrosis reduction. Although, this study was designed, primarily, to provide information on the acceptability and feasibility of the study protocol. Therefore, while the observed reductions in PRO-C3 and ADAPT are promising, the interpretation should be cautious, given the study design, duration and sample size. The clinical relevance of this result as well as its sustainability are difficult to ascertain.

In the present study, there was no significant effect of the MD intervention on FIB-4, although this is an indirect biomarker. Previous research has shown the prognostic utility of FIB-4 in predicting the long-term outcomes of patients with NAFLD (with 9-14 years follow-up), but there is limited evidence on its performance as a response biomarker [471]. Research that has conducted repeated measurements of FIB-4 within a 5 year period, found that this strategy improved the identification of

individuals at a higher risk of advanced liver disease [472]. Hence, the relatively short intervention period (4-weeks) is unlikely to have revealed the full effects of MD on FIB-4, which may take longer to reflect changes.

The findings from the non-targeted metabolite fingerprinting showed discrimination in the urine metabolome between participants grouped according to severity of liver disease. NAFLD is regarded as two pathologically distinct subtypes, NAFL and NASH [8]. The modelling results showed the strongest discrimination between NAFL and NASH groups (without the confounding effect of the NASH cirrhosis group). Additionally, Model C's composition of phenotypic groupings, which included participants with NASH F3, contributed to this distinction. This would indicate that the discrimination in Model C between NASH and NAFL is improved due to the inclusion of phenotypes further along the disease progression. Modelling data also revealed some overlap between the different disease stage categories, reflecting current knowledge of NAFLD as a complex disease metabolic phenotype [106]. In all models, NASH cirrhosis showed the most within class variance, leading to poor classification accuracy. These data provide preliminary evidence for the potential of urine metabolomic fingerprinting for patient stratification based on disease severity. In this context, a recent study that that employed urine metabolomics analysis, demonstrated its ability to distinguish the urine metabolomic profiles of patients with normoalbuminuric diabetic kidney disease compared with those with albuminuria diabetic kidney disease [473]. Metabolomics is a rapidly developing research field, and more evidence of the feasibility of urine-based biomarkers approaches to investigate potential diagnostic markers of NAFLD is needed.

In the present study, sub-group analyses suggest that the effects of the MD intervention on cardiometabolic measures i.e., fasting glucose, total cholesterol and non-HDL cholesterol were bigger in participants who were wildtype *PNPLA3* than for carriers of the I148M variant. One potential explanation for this observation is that carriers of the I148M variant may already have modest protection from CVR [474-477], and so the magnitude of benefit from the MD intervention may be diminished. As highlighted in Chapter 5, there are numerous plausible mechanisms through which the MD and its components can improve cardiovascular health. Improvements on lipid profile may be mediated by up-regulation of GLP1 [440,441]. There is strong evidence that carriage of the *PNPLA3* polymorphism increases the risk of liver fat

and progressive liver disease [183,190]. Some studies suggest that genotype for the *PNPLA3* polymorphism may attenuate cardiovascular disease (CVD) in patients with NAFLD [474-477]. In addition, there are limited data supporting an association between the *PNPLA3* risk allele and reduced blood lipids [478] i.e., triglycerides [478], possibly mediated by effects on lipoprotein metabolism [475] since *PNPLA3* genotype has been shown to affect the secretion of apoB-containing lipoproteins [479]. However, there is limited understanding of the functional consequences of *PNPLA3* rs738409 genotype and of the mechanisms by which these variants influence CVR in patients with NAFLD.

Another explanation for the influence of *PNPLA3* rs738409 genotype on response to the MD intervention might be that carriers of the I148M variant require weight loss to amplify the effects of the MD Intervention. Studies which have explored the treatment response of *PNPLA3* genotype have reported that patients with NAFLD carrying the risk allele might benefit more from weight loss but less from omega-3 supplementation [288-291]. Cross-sectional data indicates that the impact of the *PNPLA3* rs738409 SNP is amplified by obesity [188]. The variants pro-steatotic effects are triggered when surplus substrate delivery to the liver exceeds an individual's adipocyte storage capacity [184]. Thus, it can be inferred that individuals carrying the variant would derive greater benefits from weight loss [188]. Carriers of the I148M variant are more responsive to calorie and carbohydrate restriction [294,367]. Experimental data has shown that individuals homozygous for the I148M variant appear to benefit more than non-carriers from hypocaloric, low-carbohydrate diets, despite similar weight loss [296]. The limited response to omega-3 supplementation (DHA+EPA treatment) in reducing liver fat among carriers homozygous for the I148M variant could be related to lower levels of DHA [295] and pre-existing low levels of DNL [480]. Omega-3 fatty acids reduce the expression of SREBP1c, which plays a role in regulating hepatic lipogenesis [481]. Research suggests that carriers of the risk allele exhibit decreased DNL [482].

However, those with the wild type *PNPLA3* have demonstrated greater treatment response to nutraceutical therapy (silybin-phospholipids complex), with significantly improved glycemia, insulinemia, and insulin resistance [483]. Further clarification from intervention studies using prospective genotyping is needed to determine the role of *PNPLA3* variants in influencing responsiveness to different diet therapies.

In the present study, sub-group analyses showed a non-significant trend towards a greater reduction in systolic blood pressure in participants carrying the *TM6SF2* rs58542926 risk allele in response to the MD intervention. Previous research has suggested that carriers of the variant have improved lipid profile and reduced risks of atherosclerosis and myocardial infarction, indicating a cardioprotective role [72,198-200]. The *TM6SF2* rs58543926 SNP has a role in lipid metabolism and has potential to ameliorate cardiovascular comorbidity [202]. The absence of statistical significance in the current study could be attributed to complex genetic interactions (epistasis) [484], and dietary heterogeneity potentially making any influence less noticeable. Furthermore, dietary changes in sodium intake may induce a more pronounced impact on blood pressure [485]. Finally, as this study was designed, primarily, to provide information on the acceptability and feasibility of the study protocol with short intervention duration, more time may have been needed for interactions to become evident.

### 6.6 Strengths and Limitations

The results of this study have highlighted several important considerations that may inform the design of a future definitive randomised controlled trial (RCT). This assessment of preliminary exploratory data has produced encouraging findings, but they should be interpreted with caution.

There is a need to improve the sub-optimal response to current diet therapies in NAFLD. Stratified and targeted diet therapies may be an advance on the relatively ineffective 'one-size fits all' treatments. To that end, there is a need to explore the underlying mechanisms through which genotype influences responses to dietary components, and the subsequent effects on NAFLD. This trial has provided interesting data about the role of *PNPLA3* variants in modulating responsiveness to the MD, which requires further validation in larger experimental studies.

In the present study, pre-randomisation genotyping for specific genetic variant (*PNPLA3 rs738409*)) was conducted which resulted in a balanced recruitment status for *PNPLA3 rs738409* genetic status (wildtype, heterozygote, homozygote). However, a limitation of the present study is the assessment of two genes, which likely attributes a small proportion of the total variability of NAFLD [476]. A recent

genome-wide association study (GWAS) investigation using data from UK Biobank showed that at least 90 genetic variants were associated with NAFLD risk [486] so that future studies in this area would be strengthened by adopting an approach based in genetic risk score [487]. How much epistasis, the functional interaction between different genes, influenced the findings of the present study is unknown [484].

The extent to which genotype- based personalised nutrition interventions effectively facilitate behavioural changes remains uncertain [363]. The Food4Me study, a 6-month RCT involving 1270 participants across Europe, found no evidence to suggest that incorporating phenotype- and genotype- based advice within a personalised nutrition intervention increased intervention effectiveness [363]. However, further analyses revealed that incorporating genotype-based advice resulted in a more effective approach to personalisation when aiming to reduce discretionary foods and beverages [488]. A small RCT found that when compared with standard dietary advice the inclusion of personalised DNA-based dietary counselling resulted in a more pronounced decrease in sodium levels among individuals carrying the risk version of the ACE gene [489]. Current evidence shows mixed findings from phenotype- and genotype- based personalised nutrition interventions [360,363,490]. More evidence is needed to better understand the impact of genotype-based dietary advice on adherence among individuals diagnosed with complex disease traits and to determine the presence of clinically significant benefits [488]. The research team should also consider the challenges that genotype-restricted entry presents in a larger scale evaluation, including the potential need to screen many individuals, and associated cost implications [491].

Although the trial was not powered to detect significant changes in the secondary outcomes, it does provide early evidence of the effectiveness of a MD to induce benefits on liver fibrosis. However, future studies should incorporate multiple biomarkers that focus on distinct aspects of pathophysiology such as liver tissue (for metabolomic analysis), or “dry” biomarkers including magnetic resonance imaging-derived proton density fat fraction (MRI-PDFF) or transient elastography [20]. Observed improvements in such biomarkers will provide stronger evidence of disease improvement [20].

This trial provides novel information from the urinary non-targeted metabolite fingerprinting, in particular, the potential for application of this approach to support NAFLD patient stratification based on disease severity. Non-targeted metabolite fingerprinting may be more appropriate than quantitative biomarker panel when investigating if there are any endogenous metabolic perturbations associated with liver fibrosis and disease status. This unbiased comprehensive approach is fundamental for biomarker discovery, exploring unexpected associations [492], without a pre-determined specific set of metabolites of interest. In contrast, a quantitative biomarker panel with a focus on a predefined set of metabolites has more limited scope [492], potentially missing important metabolic changes.

Metabolomics use minimal invasive biofluids (e.g., urine) to offer insights into complex disease traits, and the potential to detect subtle metabolic changes as part of the pathophysiology of disease development [92]. In addition, integration of data from multiple-omics technologies might further advance mechanistic understanding of this complex disease trait [92]. Further, the use of metabolomics biomarkers to capture diet-mediated effects on lipid metabolism would have strengthened the study findings [452]. Lipidomics is a powerful method of analysis that characterises changes in the lipidome and can elucidate mechanisms underlying specific changes in lipid metabolism [451].

## 6.7 Conclusion

In summary, the results of this study provide preliminary evidence of the benefits of MD on liver fibrosis. The study also provides important information on the potential feasibility of using urinary metabolomics-based approaches to support patient stratification based on disease severity. Finally, early data appears to support the feasibility of a genotype-driven RCT in patients with NAFLD. Key recommendations for future studies include an exploration of other identified NAFLD-related genes [486], the inclusion of a wider range of liver disease biomarkers [20], and the integration of multi-omics technologies [92].

# Chapter 7

# General Discussion and Conclusion

## 7.1 Introduction

The landscape of hepatology is changing in response to the global burden of chronic liver disease (CLD) [1]. Over the past 20 years, the causes of CLD have shifted with non-alcoholic fatty liver disease (NAFLD) emerging as the major driver [2,25,40-43,493]. The rising burden of NAFLD parallels the rise in prevalence of obesity [17,33,34]. NAFLD is highly prevalent in overweight (70%) and obese (75%) populations [494]. Lifestyle interventions are the main treatment for patients with NAFLD and are recommended by clinical guidelines [6,44,64]. However, there are important evidence gaps which limit the delivery of effective diet therapies in practice. The current chapter discusses the main findings of this multi-phased project, which investigates diet lifestyle care for patients with NAFLD and explores the feasibility of a genotype-driven randomised controlled trial (RCT) investigating the differential response to a Mediterranean diet (MD) intervention of patients according to genotype for the rs738409 (I148M) variant of *PNPLA3.* The initial two studies in this project sought to provide the evidence base on which to develop diet lifestyle care for patients with NAFLD.

### *7.1.1 Effectiveness and Acceptability of Mediterranean Diet and Calorie Restriction in NAFLD: A Systematic Review and Meta-Analysis (Study 1)*

There is uncertainty about which dietary approaches are most beneficial and promote the greatest adherence in NAFLD. Systematic reviews and, if appropriate, meta-analysis provide clinicians with high-quality review evidence to answer focused clinical questions of this nature [495,496]. The current study synthesised data from randomised and clinical controlled trials describing the effects of the most common dietary approaches used in NAFLD i.e., calorie restriction and MD interventions. Evidence from the reviewed trials suggests that dietary interventions improved markers of NAFLD in as little as two weeks and that improvements were sustained for up to two years. There was a dose-response relationship between degree of calorie restriction and beneficial effects on liver function and weight loss. This parallels the dose-dependent increase in risk of NAFLD with increasing body mass index (BM) [497]. In addition, there was evidence that the MD may be an effective diet therapy.

These results support the current clinical guidelines in NAFLD, which recommend interventions that encourage calorie (energy) restriction and healthier dietary

patterns, such as the MD [6,64]. Crucially, the current study provides important directions for future research including the feasibility of implementing a genotype-based dietary intervention, the importance of robust assessment of dietary intake, information on intervention acceptability and sustainability, and on quality of life and patient-related outcomes.

### *7.1.2 Diet Lifestyle Management of NAFLD: A Cross-sectional Survey of Clinicians (Study 2)*

There is insufficient understanding about the specific weight loss or dietary strategies that are used by clinicians in patient management of NAFLD [313]. The scope and components of diet lifestyle care delivered, and the degree to which clinical practice varies between and within countries are largely unknown. The current study aimed to evaluate the current status of diet lifestyle care for patients with NAFLD. Clinicians completed an e-survey, uniquely focused on gathering data on current practice and perceived barriers to the effective delivery of lifestyle interventions.

Previous research has reported a dissonance between recommended practice and actual service provision [311,312]. The current study's results support these findings by highlighting considerable variability in the provision of diet lifestyle care and deviations from standard of care guidance [6,64]. There are deficiencies in the current lifestyle advice provided by clinicians, and access to effective diet lifestyle interventions, recognised as essential for all patients with NAFLD, appears limited [6]. Service provision differs across centres and professional roles, with substantial heterogeneity in reported practice patterns and in recording of key clinical, lifestyle and patient experience data.

Clinical practice surveys are a key method by which to facilitate targeted improvements in professional practice and service development. Figure 7.1 illustrates four actionable points that would: i) support patients to change lifestyle-related behaviours [364], ii) standardise and optimise the advice delivered by clinicians, and iii) address issues of resource and time demand.

The use of digital approaches to convey personalised dietary advice has potential to optimise intervention delivery, induce sustained dietary behaviour change and maximise benefits on liver function [322,362,363]. Promising advances include platforms,

that collect individual-level data via wearable devices and smartphones linked with artificial intelligence-based strategies, to enable ongoing tailored feedback [362]. In future, an audit of clinical data such as medical case notes will be informative in corroborating or refuting these findings.

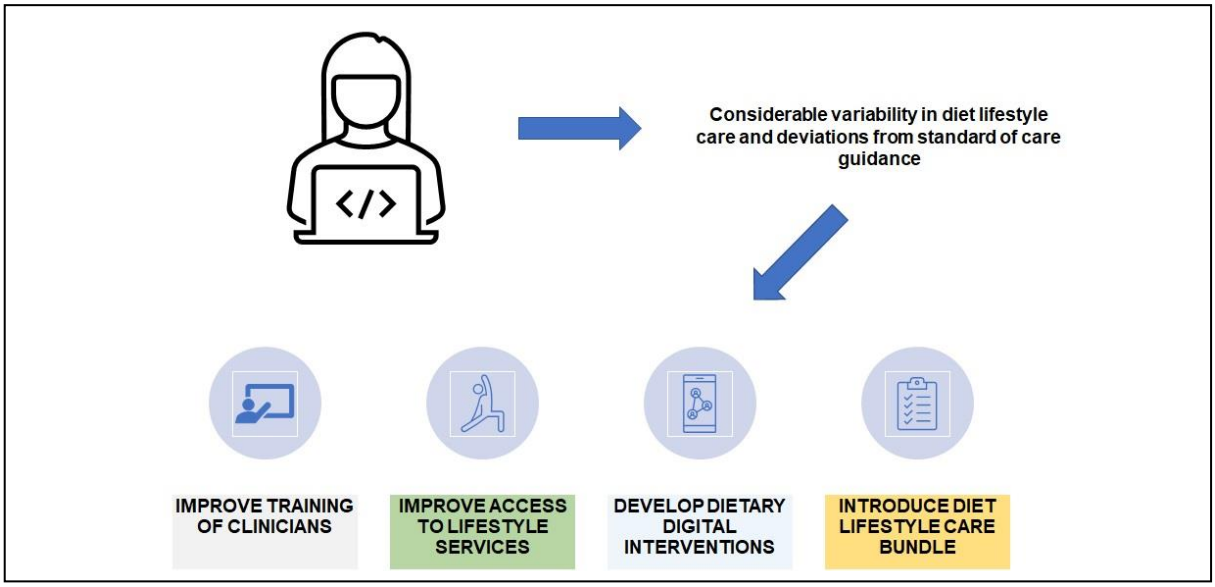



**Figure 7.1 Clinical practice survey facilitates targeted improvements in professional practice and service development.**

The combined findings from these initial two studies, as well as other research evidence, and patient and public feedback, informed the next stage of the project i.e., the design of a randomised controlled feasibility trial (Figure 7.2).

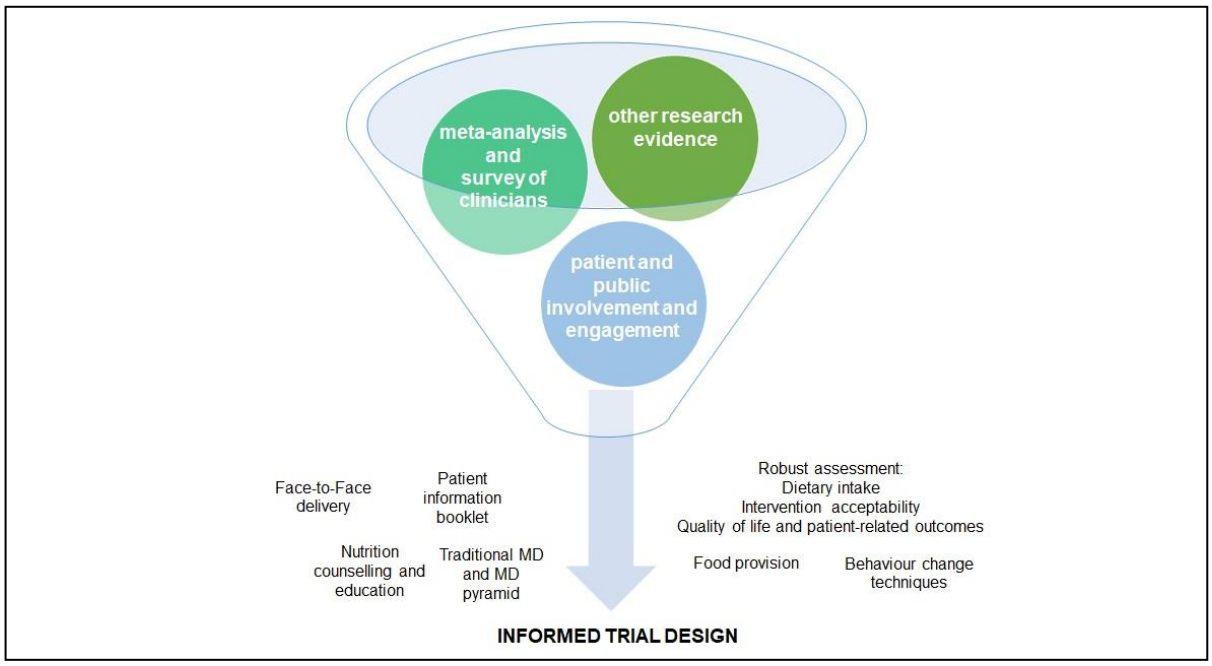



**Figure 7.2 The design process of a randomised controlled feasibility trial.** MD, Mediterranean diet.

### *7.1.3 Randomised Controlled Feasibility Trial of a Nutrigenetic Therapeutic Approach for Patients with NAFLD (Study 3)*

A randomised, crossover feasibility trial was undertaken. Participants were randomised to Diet 1 (MD) or Diet 2 (control) for 4-weeks, separated by a 4-weeks washout period. The primary outcome was the feasibility, acceptability and effectiveness of the protocol. Secondary outcomes included assessment of liver fibrosis biomarkers and the influence of *PNPLA3* genotype.

Firstly, the feasibility and acceptability of the study protocol was established. The qualitative and quantitative information generated in this study indicates that, with appropriate adjustments (Figure 7.3), the study protocol is sufficiently robust to advance to a future definitive RCT. This comprehensive evaluation used well-defined progression criteria, and integrated patient-reported outcome and open-response questionnaire data. These data provide insights into the impact of disease burden on quality of life, and individuals' perceptions of study participation. Participants in this study experienced impaired quality of life, particularly in relation to fatigue, which appears to be a frequently reported symptom in this patient population [382,416]. Barriers and facilitators were identified that could guide future personalised intervention approaches such as the development of an interactive web-based platform.

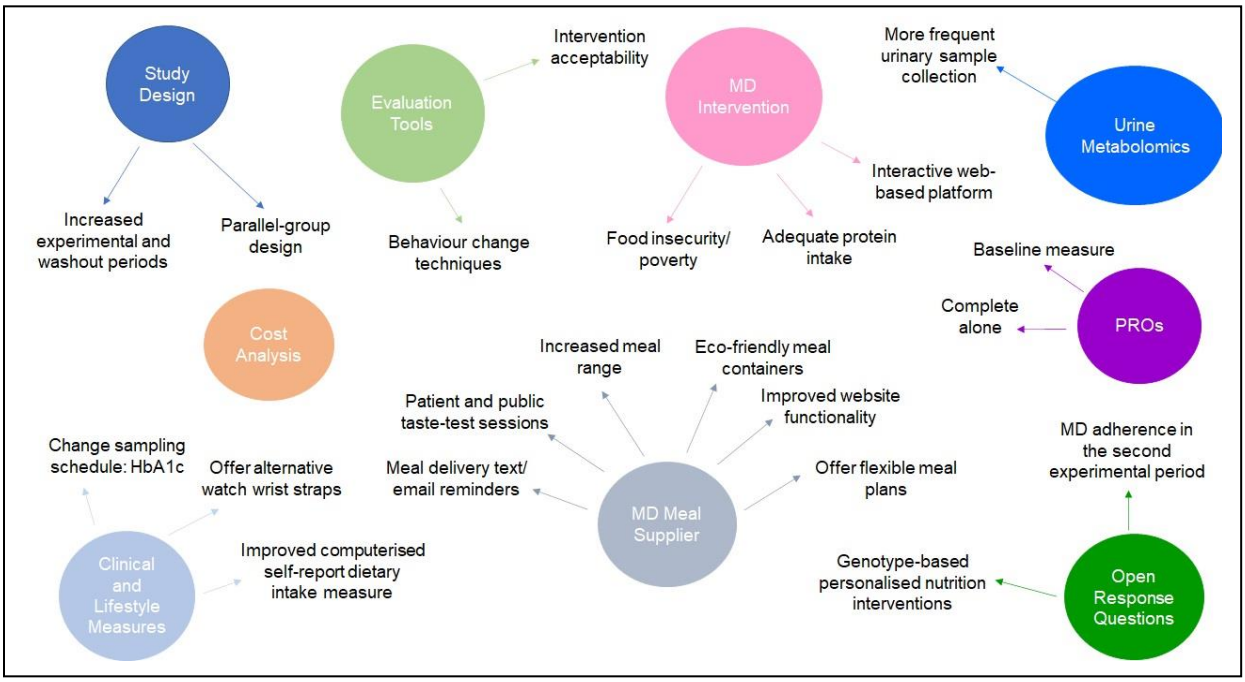



**Figure 7.3 Proposed study protocol adjustments in preparation for a future definitive randomised controlled trial.** HbA1c, glycated haemoglobin; MD, Mediterranean diet; PROs, patient-related outcomes.

Secondly, the potential effectiveness of the study protocol was established. In addition, the study showed that use of both self-reported measures and urinary metabolomics-based approaches to robustly assess dietary intake is feasible. Preliminary evidence indicates that a 4-week dietitian-led intervention with food provision produced increases in MD adherence. Previous research has reported that MD improves measures of cardiometabolic risk in this patient population [324,328,330,351,352]. The results in the current study support these findings by showing that increased MD adherence improved cardiovascular risk (CVR) profile with minor improvements in liver function (Figure 7.4). Given that cardiovascular disease (CVD) is the leading cause of mortality in this patient population, these data support the current clinical guidelines to improve dietary patterns in line with MD [5,6,27].

Observations in the current study that specific subgroups of patients might derive greater benefit from a MD intervention, has important clinical implications. Sub-group analyses suggest that patients with NASH cirrhosis might derive greater benefit in terms of weight loss and improved gamma-glutamyl transpeptidase (GGT) when prescribed a MD. Furthermore, modelling results showed a greater impact of the MD on total cholesterol and on non-high-density lipoprotein in males and individuals with Type 2 diabetes (T2DM). However, these results require confirmation in larger intervention trials with appropriate designs.

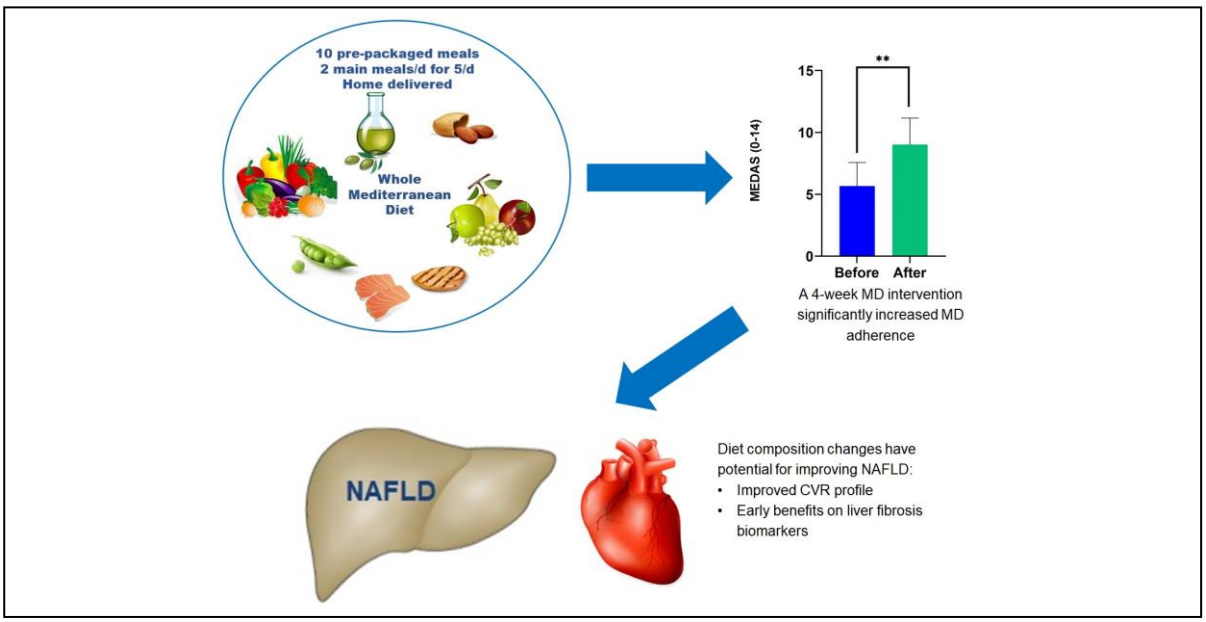



**Figure 7.4 MD intervention increases MD adherence with improvements in cardiovascular risk profile.** NAFLD, non-alcoholic fatty liver disease; MD, Mediterranean diet; MEDAS, MD assessment score.

Thirdly, an assessment of preliminary exploratory data revealed early evidence of the benefits of MD on liver fibrosis. The findings support the feasibility of a genotype-driven RCT in patients with NAFLD. In the current study, carriers of the *PNPLA3* I148M variant appear to benefit less in terms of CVR factors when prescribed a MD intervention. Further clarification from intervention studies using prospective genotyping is needed to determine the role of *PNPLA3* variants in influencing responsiveness to different diet therapies. In addition, this study provides useful information on the potential feasibility of using urinary metabolomics-based approaches to support patient stratification based on disease severity.

#### *7.1.3.1 Proposed study protocol adjustments in preparation for a future definitive randomised controlled trial*

The feasibility, acceptability and potential effectiveness of the study protocol was established. The current analysis suggests that the mostly minor protocol adjustments would be needed to allow scale-up to an effective RCT. This process should be informed by evidence of successful 'scale-up' strategies [413]. The study design of the future RCT might include increased experimental and washout periods. Alternatively, a parallel-group design could be adopted, with the final decision informed by the wider evidence base. The purpose would be to minimise potential carryover and diet sequence effects and reveal the full effects of dietary intervention on liver function. The future RCT will include a cost effectiveness analysis, to evaluate the effectiveness of the study protocol relative to its cost.

Improved methods for the collection of clinical data will be incorporated to increase the completeness of data collection and better capture the impact of diet treatments. These minor changes include changing the schedule of HbA1c sampling to once every six weeks; ensuring the research team order all the study bloods for analysis; conducting transient elastography (TE) at baseline and each time point alongside repeating measures if CAP and/or liver stiffness results are missing. To improve the inter class variance observed within the treatment diets from the urinary metabolomics, more frequent urinary sample collection will be employed. In the future RCT, the potential application of lipidomics to assess changes in the lipidome and the collection and analysis of a broader spectrum of liver disease biomarkers, involving both "wet" and "dry" biomarkers will be guided by emerging evidence.

Further consideration will be given to an analysis of the joint effects of the variants in both PNPLA3 and TM6SF2 genes, as well as exploring other NAFLD-related genes.

To identify effective BCTs and intervention characteristics in the larger scale evaluation suitable assessment methods will be incorporated [419,420]. Alternative accelerometer wrist straps will be given which are more suitable for people living with obesity to aid comfortability, enhance adherence and improve data validity. The INTAKE24 online dietary recall system will not be used unless robust revisions made to improve accessibility and useability, and to reduce cases of missing and poor-quality data. Further consideration will be given to an appropriate validation procedure to identify misreporting from self-report measures and enhance the effectiveness of the dietary monitoring strategy. To better capture the impact of diet treatments and trial procedures on QoL, the PRO's will be measured at baseline and each time point. Moreover, a combination of a NAFLD- and obesity- specific instruments will be considered. Further patient and public involvement and engagement will be undertaken to capture participant perceptions of genotype-based personalised nutrition interventions. The final questionnaire guide will be revised to capture perceptions of MD adherence in the second experimental period if a cross-over design is design is to be adopted.

Minor adjustments to the MD intervention will be made to incorporate targeted dietary advice on food poverty; and make improvements to the pre-packaged ready meals. Further patient and public involvement and engagement will be undertaken, conducting 'taste-test' sessions to overcome some issues with taste preferences and increase diet adherence. This feedback would support future intervention refinement. The range of pre-packaged ready meals, the meal containers, website functionality and delivery process (i.e., introduction of text/ email reminders) will be improved to enhance participant satisfaction. In the larger scale evaluation flexible meal plans will be offered to meet estimated calorie requirements, increasing implementation fidelity and reducing protocol deviations. An interactive web-based platform will be delivered alongside the personalised one-to-one diet and lifestyle consultations to support scalability and help with cost effectiveness. This would also offer additional guidance/support and provide prompts/ reminders.

## 7.2 Recommendations for Future Research

The identification of behavioural strategies and intervention characteristics that enhance intervention efficacy should be a priority for future research. These data would be highly relevant to the development of scalable dietary digital interventions that may reduce patient burden and healthcare resource utilisation [243,322,362,363]. The use of digital approaches to convey personalised dietary advice has potential to optimise intervention delivery, induce sustained dietary behaviour change and to maximise clinical impacts [322,362,363]. Promising advances include platforms, that collect individual-level data via wearable devices and smartphones linked with artificial intelligence-based strategies, to enable ongoing tailored feedback [362].

Further studies should investigate heterogeneity in response and the impact of participant characteristics including age; sex; disease phenotype and genotype; and Type 2 diabetes, on MD-NAFLD relationships. The findings would support the development of stratified, targeted or personalised nutrition interventions. The creation of patient registries or platforms comparable to the densely phenotyped ZOE PREDICT cohort might advance research in this area [498]. Such platforms use precision methods to collect standardised data at scale and depth and detect the most important individual characteristics for use in intervention design [498,499].

There is a need for stronger and more consistent evidence of disease improvement in experimental trials. Thus, the collection and analysis of a wider range of liver disease biomarkers that focus on distinct aspects of pathophysiology such as liver tissue (for metabolomic analysis), or “dry” biomarkers including magnetic resonance imaging-derived proton density fat fraction (MRI-PDFF), magnetic resonance elastography (MRE) or transient elastography are warranted [20]. Further comparative diagnostic accuracy studies to assess the performance of current and emergent markers, including multi-marker scores and non-invasive imaging techniques for identifying patients with NASH and fibrosis are needed [102].

In future studies, use of two or more self-reported dietary intake measures, in combination with objective urine-based biomarkers approaches, will provide a more robust strategy for dietary monitoring. The choice of self-reported measures should be based on high-quality research evidence, and may be informed by www.nutritools.org an interactive guided website for researchers [500]. The use of

objective urine-based dietary biomarkers remains important, as each subjective method has inherent limitations [389,501].

Emergent evidence has enhanced our understanding of the role of inflammation in the pathogenesis of NAFLD, and of the MD in modulating inflammation [353,354,437,502,503]. The validated empirical dietary inflammatory index (E-DII) has been used to assess the inflammatory potential of dietary patterns in multiple studies [502]. Further information from intervention trials that investigate changes in E-DII scores across different diet treatments and examine associations between E-DII scores and biomarkers of interest, would be advantageous. The findings would extend our understanding of the role of diet-related inflammation in NAFLD [502].

The use of metabolomics biomarkers to capture diet-mediated effects on lipid metabolism would have strengthened the findings of this project [452]. The application of lipidomics in future studies will assist in characterise changes in the lipidome as a first step in elucidating mechanisms underlying specific changes in lipid metabolism [451]. In the future, the integration of data from multi-omics technologies, will advance mechanistic understanding of this complex disease trait [92].

Finally, future studies should consider the joint effects of the variants in both PNPLA3 and TM6SF2 genes. Furthermore, given that at least 90 genetic variants are associated with NAFLD risk, an exploration of other NAFLD-related genes is warranted [486]. The adoption of approaches based on genetic risk score alone, or in combination with clinical or other novel biomarker data (i.e., using a clinical risk score), would advance understanding of the aetiology of NAFLD and of potential interventions for prevention and treatment [69,487]. The application of genetic risk scores in NAFLD clinical trials, and in future clinical practice have been described [504], and include risk stratification, predicting responsiveness to specific therapies and optimising personalised interventions approaches [504-507]. Personalised nutrition interventions can be tailored to individual characteristics such as habitual diet, phenotype and genotype [508,509]. The effectiveness of using habitual diet as a basis for personalisation to improve dietary choices has been demonstrated [363]. Current evidence shows mixed findings from phenotype- and genotype- based personalised nutrition interventions, and more data are needed on the most important individual

characteristics for use in such approaches as well as the associated cost implications [360,363,490].

## 7.3 Conclusion

In conclusion, this multi-phased project provides evidence on which to develop diet lifestyle care for patients with NAFLD. The findings support the current strategy of calorie restriction and diet modification, as the cornerstone of NAFLD management. In addition, the findings offer useful insights into the current status of diet lifestyle care and the targeting of improvements. The findings of the feasibility study lay the foundation for a future definitive RCT, that will address the hypothesis that carriage of the *PNPLA3* variant influences MD responsiveness in NAFLD. The data generated will inform trial design and optimise the dietary treatments, instruments and procedures. Leveraging nutrigenetics has potential to identify high-impact diet therapies for NAFLD. Long term, the outcomes of this research programme may lead to fewer patients who need intensified medical treatment, with resultant benefits in both reducing costs, and in lowering risk of premature morbidity and mortality.

# Appendices

## Appendix A: List of search terms for all databases.

| |
|---|
| "randomi*ed controlled trial" in Title Abstract Keyword OR "controlled clinical trial" in Title Abstract Keyword AND "fatty liver" in Title Abstract Keyword OR NAFLD in Title Abstract Keyword AND mediterranean OR cretan OR fruit* OR vegetable* OR fish or shellfish* OR seafood OR "olive oil" OR "red wine" OR (nut OR nuts) OR seeds OR legumes OR pulses in Title Abstract Keyword |
| (("fatty liver" OR NAFLD)) in Title Abstract Keyword AND (("randomi*ed controlled trial" OR "controlled clinical trial")) in Title Abstract Keyword AND (weight NEAR/2 (los* OR reduc* OR decreas* OR low*)) in Title Abstract Keyword OR (("body mass" OR "BMI") NEAR/4 (los* OR small* OR decreas* OR low*)) in Title Abstract Keyword OR (waist NEAR/4 (reduc* OR small* OR reduc* OR decreas*)) in Title Abstract Keyword |

**Table 1.** CENTRAL search terms.

| |
|---|
| (TITLE-ABS-KEY ("randomi*ed controlled trial") OR TITLE-ABS-KEY ("controlled clinical trial") AND TITLE-ABS-KEY ("fatty liver") OR TITLE-ABS-KEY (nafld) AND TITLE-ABS-KEY (mediterranean OR cretan OR fruit* OR vegetable* OR fish OR shellfish* OR seafood OR "olive oil" OR "red wine" OR (nut OR nuts) OR seeds OR legumes OR pulses)) |
| (TITLE-ABS-KEY (("Weight Loss" OR weight) W/2 (los* OR reduc* OR decreas* OR low*)) OR TITLE-ABS-KEY (("body mass" OR "bmi") W/4 (los* OR small* OR decreas* OR low*)) OR TITLE-ABS-KEY (waist W/4 (reduc* OR low* OR small* OR decreas*)) AND TITLE-ABS-KEY ("fatty liver" OR nafld) AND TITLE-ABS-KEY ("randomi*ed controlled trial" OR "controlled clinical trial")) |

**Table 2.** Scopus search terms.

| |
|---|
| TOPIC: (("randomi*ed controlled trial" OR "controlled clinical trial")) AND TOPIC: ((mediterranean OR cretan OR fruit* OR vegetable* OR fish OR shellfish* OR seafood OR "olive oil" OR "red wine" OR (nut OR nuts) OR seeds OR legumes OR pulses)) AND TOPIC: (("fatty liver" OR NAFLD)) |
| TOPIC: ((("randomi*ed controlled trial" OR "controlled clinical trial"))) AND TOPIC: ((("Weight Loss" OR weight NEAR/2 los* OR reduc* OR decreas* OR low*))) AND TOPIC: ((("fatty liver" OR NAFLD))) AND TOPIC: ((("body mass" OR "bmi" NEAR/4 los* OR small* OR decreas* OR low*))) AND TOPIC: (((waist NEAR/4 reduc* OR low* OR small* OR decreas*))) |

**Table 3.** Web of Science search terms.

| |
|---|
| DIET, MEDITERRANEAN/ |
| cretan diet.mp. [mp=title, abstract, original title, name of substance word, subject heading word, floating sub-heading word, keyword heading word, organism supplementary concept word, protocol supplementary concept word, rare disease supplementary concept word, unique identifier, synonyms] |
| Mediterranean.mp. [mp=title, abstract, original title, name of substance word, subject heading word, floating sub-heading word, keyword heading word, organism supplementary concept word, protocol supplementary concept word, rare disease supplementary concept word, unique identifier, synonyms] |
| exp DIET/ |
| diet.mp. [mp=title, abstract, original title, name of substance word, subject heading word, floating sub-heading word, keyword heading word, organism supplementary concept word, protocol supplementary concept word, rare disease supplementary concept word, unique identifier, synonyms] |
| exp FOOD/ |
| exp FRUIT/ |
| VEGETABLES/ |

**Table 4** (continued).

| |
|---|
| fish.mp. [mp=title, abstract, original title, name of substance word, subject heading word, floating sub-heading word, keyword heading word, organism supplementary concept word, protocol supplementary concept word, rare disease supplementary concept word, unique identifier, synonyms] |
| exp SEAFOOD/ |
| olive oil.mp. [mp=title, abstract, original title, name of substance word, subject heading word, floating sub-heading word, keyword heading word, organism supplementary concept word, protocol supplementary concept word, rare disease supplementary concept word, unique identifier, synonyms] |
| red wine.mp. [mp=title, abstract, original title, name of substance word, subject heading word, floating sub-heading word, keyword heading word, organism supplementary concept word, protocol supplementary concept word, rare disease supplementary concept word, unique identifier, synonyms] |
| 4 or 5 |
| 3 or 6 or 7 or 8 or 9 or 10 or 11 or 12 |
| 13 and 14 |
| 1 or 2 or 15 |
| randomized controlled trial.pt. |
| controlled clinical trial.pt. |
| randomized.ab. |
| placebo.ab. |
| clinical trials as topic.sh. |
| randomly.ab. |
| trial.ti. |
| 17 or 18 or 19 or 20 or 21 or 22 or 23 |
| exp animals/ not humans.sh. |
| 24 not 25 |
| Non-alcoholic Fatty Liver Disease/ |
| Non-alcoholic Fatty Liver Disease.mp. [mp=title, abstract, original title, name of substance word, subject heading word, floating sub-heading word, keyword heading word, organism supplementary concept word, protocol supplementary concept word, rare disease supplementary concept word, unique identifier, synonyms] |
| exp Fatty Liver/ |
| NAFLD.mp. [mp=title, abstract, original title, name of substance word, subject heading word, floating sub-heading word, keyword heading word, organism supplementary concept word, protocol supplementary concept word, rare disease supplementary concept word, unique identifier, synonyms] |
| 27 or 28 or 29 or 30 |
| 16 and 26 and 31 |
| weight loss.mp. |
| (weight adj2 (los* or reduc* or decreas* or low*)).mp. |
| ((body mass or bmi) adj4 (los* or small* or decreas* or low*)).mp. |
| (waist adj4 (reduc* or low* or small* or decreas*)).mp. |
| (weight loss or (weight adj2 (los* or reduc* or decreas* or low*)) or ((body mass or bmi) adj4 (los* or small* or decreas* or low*)) or (waist adj4 (reduc* or low* or small* or decreas*))).mp. |
| randomized controlled trial.mp. |
| controlled clinical trial.mp. |
| randomized.mp. |
| placebo.mp. |
| clinical trials.mp. |
| randomly.mp. |
| trial.mp. |
| (randomized controlled trial or controlled clinical trial or randomized or placebo or clinical trials or randomly or trial).mp. |
| Non-alcoholic Fatty Liver Disease.mp. |

**Table 4** (continued).

| |
|---|
| Fatty Liver.mp. |
| NAFLD.mp. |
| (Non-alcoholic Fatty Liver Disease or Fatty Liver or NAFLD).mp. |
| ((weight loss or (weight adj2 (los* or reduc* or decreas* or low*)) or ((body mass or bmi) adj4 (los* or small* or decreas* or low*)) or (waist adj4 (reduc* or low* or small* or decreas*))) and (randomized controlled trial or controlled clinical trial or randomized or placebo or clinical trials or randomly or trial) and (Non-alcoholic Fatty Liver Disease or Fatty Liver or NAFLD)).mp |

**Table 4.** MEDLINE search terms.

| |
|---|
| Mediterranean diet/ |
| cretan diet.mp. [mp=title, abstract, heading word, drug trade name, original title, device manufacturer, drug manufacturer, device trade name, keyword, floating subheading word, candidate term word] |
| Mediterranean.mp. [mp=title, abstract, heading word, drug trade name, original title, device manufacturer, drug manufacturer, device trade name, keyword, floating subheading word, candidate term word] |
| exp diet/ |
| diet.mp. [mp=title, abstract, heading word, drug trade name, original title, device manufacturer, drug manufacturer, device trade name, keyword, floating subheading word, candidate term word] |
| exp food/ |
| exp fruit/ |
| exp vegetable/ |
| exp fish/ |
| exp sea food/ |
| olive oil/ |
| olive oil.mp. [mp=title, abstract, heading word, drug trade name, original title, device manufacturer, drug manufacturer, device trade name, keyword, floating subheading word, candidate term word] |
| red wine/ |
| red wine.mp. [mp=title, abstract, heading word, drug trade name, original title, device manufacturer, drug manufacturer, device trade name, keyword, floating subheading word, candidate term word] |
| 4 or 5 |
| 3 or 6 or 7 or 8 or 9 or 10 or 11 or 12 or 13 or 14 |
| 15 and 16 |
| 1 or 2 or 17 |
| (random* or placebo*).ti,ab. |
| ((singl* or double* or triple* or treble*) and (blind* or mask*)).ti,ab. |
| controlled clinical trial*.ti,ab. |
| retracted article/ |
| 19 or 20 or 21 or 22 |
| nonalcoholic fatty liver/ |
| "nonalcoholic fatty liver disease".mp. [mp=title, abstract, heading word, drug trade name, original title, device manufacturer, drug manufacturer, device trade name, keyword, floating subheading word, candidate term word] |
| exp fatty liver/ |
| NAFLD.mp. [mp=title, abstract, heading word, drug trade name, original title, device manufacturer, drug manufacturer, device trade name, keyword, floating subheading word, candidate term word] |
| 24 or 25 or 26 or 27 |
| 18 and 23 and 28 |
| (animal$ not humans$).sh,hw. |
| 29 not 30 |

**Table 5** (continued).

| |
|---|
| Mediterranean diet/ |
| Weight Loss.mp. |
| (weight adj2 (los* or reduc* or decreas* or low*)).mp. |
| ((body mass or vmi) adj4 (los* or small* or decreas* or low*)).mp. |
| (waist adj4 (reduc* or low* or small* or decreas*)).mp. |
| (Weight Loss or (weight adj2 (los* or reduc* or decreas* or low*)) or ((body mass or vmi) adj4 (los* or small* or decreas* or low*)) or (waist adj4 (reduc* or low* or small* or decreas*))).mp. |
| nonalcoholic fatty liver.mp. |
| "non-alcoholic fatty liver disease".mp. |
| fatty liver.mp. |
| NAFLD.mp. |
| (nonalcoholic fatty liver or "non-alcoholic fatty liver disease" or fatty liver or NAFLD).mp. |
| (random* or placebo*).mp. |
| ((singl* or double* or triple* or treble*) and (blind* or mask*)).mp. |
| controlled clinical trial*.mp. |
| retracted article.mp. |
| (random* or placebo* or ((singl* or double* or triple* or treble*) and (blind* or mask*)) or controlled clinical trial* or retracted article).mp. |
| ((Weight Loss or (weight adj2 (los* or reduc* or decreas* or low*)) or ((body mass or vmi) adj4 (los* or small* or decreas* or low*)) or (waist adj4 (reduc* or low* or small* or decreas*))) and (nonalcoholic fatty liver or "non-alcoholic fatty liver disease" or fatty liver or NAFLD) and (random* or placebo* or ((singl* or double* or triple* or treble*) and (blind* or mask*)) or controlled clinical trial* or retracted article)).mp. |
| (animal$ not human$).mp. |
| (((Weight Loss or (weight adj2 (los* or reduc* or decreas* or low*)) or ((body mass or vmi) adj4 (los* or small* or decreas* or low*)) or (waist adj4 (reduc* or low* or small* or decreas*))) and (nonalcoholic fatty liver or "non-alcoholic fatty liver disease" or fatty liver or NAFLD) and (random* or placebo* or ((singl* or double* or triple* or treble*) and (blind* or mask*)) or controlled clinical trial* or retracted article)) not (animal$ not human$)).mp. |

**Table 5.** Embase search terms.

## Appendix B: Data extraction form.

| | |
|---|---|
| Reference citation<br><br>Study ID | |
| Country | |
| Study funding source<br>Conflicts of interest | |

| Characteristics | Eligibility criteria | Yes | No | Unclear | Location |
|---|---|---|---|---|---|
| Type of study | Randomised Controlled Trial | ☐ | ☐ | ☐ | |
| | Quasi-randomised Controlled Trial | ☐ | ☐ | ☐ | |
| | Clinical Controlled Trial | ☐ | ☐ | ☐ | |
| Participants | NAFLD | ☐ | ☐ | ☐ | |
| | Other (specify): | ☐ | ☐ | ☐ | |
| Interventions | Dietary/ Lifestyle Interventions | ☐ | ☐ | ☐ | |
| Outcomes | NAFLD surrogate markers | ☐ | ☐ | ☐ | |
| | Adiposity markers; cardiometabolics; quality of life measures; intervention acceptability; dietary intake modification | ☐ | ☐ | ☐ | |

**INCLUDE** ☐ **EXCLUDE** ☐

Reason for exclusion:

| Methods | Description | |
|---|---|---|
| Aim | | |
| Design | | |
| Power | | |
| Start date | | |
| End date | | |
| Total study duration | | |
| Total no of groups | | |

Notes:

| Population/Setting | Description | |
|---|---|---|
| Population *(diagnostic criteria)* | | |
| Setting/ location/ country | | |
| Inclusion criteria | | |
| Exclusion criteria | | |
| Method of recruitment | | |
| Total number | | |
| No. randomised/ included | | |
| Withdrawals/ exclusions | | |
| Subgroup reported | | |

Notes:

| Participants | Description | |
|---|---|---|
| Age | | |
| Sex | | |
| Ethnicity | | |
| NAFLD severity | | |

| | | |
|---|---|---|
| Co-morbidities | | |
| Other socio-demographics | | |
| Notes | | |
| **Intervention/Comparison** | **Description** | |
| No. randomised/ included | | |
| Treatment details/ duration | | |
| Timing | | |
| Delivery | | |
| Providers | | |
| Adherence Measures | | |
| Notes: | | |
| **Intervention/Comparison** | **Description** | |
| No. randomised/ included | | |
| Treatment details/ duration | | |
| Timing | | |
| Delivery | | |
| Providers | | |
| Adherence Measures | | |
| Notes: | | |
| **Outcome:** | **Description** | |
| Time points collected/ reported | | |
| Results and sample size | | |
| Outcome/ tool validated | ☐ Yes ☐ No ☐ Unclear | |
| Missing data and reason | | |
| Subgroup analysis | | |
| Statistics/ appropriateness | | |
| Notes: | | |
| **Outcome:** | **Description** | |
| Time points collected/ reported | | |
| Results and sample size | | |
| Outcome/ tool validated | ☐ Yes ☐ No ☐ Unclear | |
| Missing data and reason | | |
| Subgroup analysis | | |
| Statistics/ appropriateness | | |
| Notes: | | |
| Key conclusions | | |
| Notes: | | |

## Appendix C: Additional communication with authors of the included studies.

| | Additional information requested | Response from the authors |
|---|---|---|
| Abenavoli 2017, Italy | Diet intake and adherence data | Authors responded, describing good adherence to the proposed regimen, which they related to regular outpatient visits and weekly telephone contact reviews. |
| Biolato 2019, Italy | Diet adherence and acceptability data | Authors responded, directing the reviewers to the methods and results sections of the paper, which describe how diet adherence was assessed, and the corresponding results.<br><br>No response from authors regarding the requested information on diet acceptability. |
| Willmann 2019, Germany* | Diet adherence and acceptability data | **No response from authors** |
| Dorosti 2020, Iran | Diet adherence and acceptability data | **No response from authors** |
| Johari 2019, Malaysia | Diet adherence and acceptability data | Authors responded, directing the reviewers to the methods section of the paper regarding measures of diet adherence.<br><br>The authors did not measure acceptability, but proposed that diet adherence, may be related to diet acceptability to some extent. |
| Shojasaadat 2019, Iran | Diet adherence and acceptability data | No response from authors |
| Ghetti 2019, Brazil | Diet adherence and acceptability data | No response from authors |

* Study not included in meta-analyses.

## Appendix D: Characteristics of excluded full text studies.

| | **Reason for exclusion** |
|---|---|
| Cai et al., 2019, China | Ineligible intervention |
| Razmgah et al., 2017, Iran | Ineligible intervention |
| Rezaei et al., 2020, Iran | Ineligible intervention |
| Razavi Zade et al., 2016, Iran | Ineligible intervention |
| Belopolsky, et al., 2020, USA | Incorrect analysis type |
| Lim et al., 2020, Singapore | Ineligible intervention |
| Kruse, et al., 2020, Germany | Ineligible intervention |
| Cunha et al., 2020, Brazil | Ineligible intervention |
| Garousi et al, 2021 Iran | Ineligible intervention |
| Tutino et al., 2018, Italy | Incorrect outcome measures |
| Gepner et al., 2019, Israel | Incorrect analysis type |
| Krawczyk et al., 2018, Poland | Ineligible intervention |
| Haufe et al., 2011, Germany | Ineligible intervention |
| Rachakonda et al., 2017, USA | Ineligible intervention |
| Axley et al., 2018, USA | Ineligible intervention |
| Pourhassan et al., 2017, Germany | Ineligible intervention; incorrect analysis type |
| Sanguankeo et al., 2017, USA | Insufficient detail on outcome; incorrect analysis type |
| Deibert et al., 2019, Germany | Ineligible intervention |
| Markova et al., 2017, Germany | Ineligible intervention |
| Abd El-Kader et al., 2016, Saudi Arabia | Ineligible intervention |
| Al-Jiffri et al., 2013, Saudi Arabia | Ineligible intervention |
| Bozzetto et al., 2012, Italy | Ineligible intervention |
| Errazuriz et al., 2017, USA | Ineligible intervention |
| Cueto-Galan et al., 2017, Spain | Ineligible population; incorrect analysis type |
| Aller et al., 2014, Spain | Ineligible intervention |
| De Luis et al., 2010, Spain | Ineligible intervention |

## Appendix E: Characteristics of the studies included in the review.

| Author | Design | Male (n, %) | Clinical Group | Mean age (years) | Mean BMI (kg/m$^2$) | Intervention and Comparator (control) treatments | Intervention features | Duration (weeks) / contacts |
|---|---|---|---|---|---|---|---|---|
| Properzi 2018, Australia (34) | RCT | 26, 51.0% | NAFLD | 52.0 | 30.9 | **Intervention:** MD<br>Based on traditional Cretan diet (40% CHO, 20% PRO, 35-40% FAT and <10% SFA).<br>**Comparator:** Low-fat/ high CHO (LFD)<br>Based on National Health and Medical Research Council and American Heart Association guidelines (50% CHO, 20% PRO, 30% FAT and <10% SFA). | **Diet food items:** 750g nuts and 750mL olive oil (MD) and 1kg of natural muesli and 200g of low-fat snack bars, every 4-wks (LFD). | 12<br>Weekly calls (4-wks) and monthly contacts. |
| Katsagoni 2018, Greece (35) | RCT | 43, 68.3% | NAFLD | 48.3 | 31.2 | **Intervention:** MD<br>Based on MD pyramid and Greek guidelines, (45% CHO, 20% PRO and 35% FAT).<br>**Comparator:** MDL as MD, with optimal sleep (≥7 and ≤9 h/d) and mid-day rest.<br>**Control:** Standard care (healthy diet lifestyle) | **Cal restriction:** 1500cal/d (women); 1800cal/d (men) (all).<br>**Physical activity**: Moderate-vigorous intensity 30min/d/ 10,000 steps/d (MDL).<br>**Behaviour change:** Goal setting (MD/ MDL). | 26<br>7x60min every 2-wks (2m), then monthly (4m) contacts. |
| Misciagna 2017, Italy (36) | RCT | 72, 73.5% | NAFLD | - | - | **Intervention:** MD<br>Based on traditional Cretan diet and low glycaemic index foods (≤10% SFA).<br>**Control:** Standard care (healthy diet lifestyle)<br>Based on INRAN guidelines. | Recommended diets provided in brochure format with no advice given on calorie allowances, physical activity or behaviour changes (both). | 26<br>Monthly contacts. |
| Ryan 2013, Australia (37) | RCT crossover | 6, 50.0% | NAFLD | 55.0 | 32.0 | **Intervention:** MD<br>Based on traditional Cretan diet (40% CHO, 20% PRO and 40% FAT).<br>**Comparator:** Low-fat/ high CHO<br>Based on National Health and Medical Research Council and American Heart Association guidelines (50% CHO, 20% PRO and 30% FAT). | **Diet food items:** Pre-cooked meals and ingredients, every 2-wks (MD/LFD). | 18<br>Fortnightly contacts. |
| Abenavoli 2017, Italy (38) | RCT | 18, 60.0% | NAFLD | 42.6 | 30.0 | **Intervention:** MD<br>Italian RDAs, (50-60%, CHO, 15-20% PRO 50% veg PRO, <30% MUFA and PUFA, ≤10%, SFA, <300mg cholesterol and 25-30g fibre).<br>**Control:** Standard care (habitual diet) | **Cal restriction:** 1400-1600cal/d (MD).<br>**Physical activity:** General (MD). | 26<br>Weekly/monthly calls. |
| Marin-Alejandre 2019, Spain (39) | RCT | 51, 52.0% | NAFLD | 50.2 | 33.5 | **Intervention:** MD<br>Higher meal frequency (7 meals/d) (40%-45% CHO, 25% PRO (primarily veg PRO) and 30%-35% FAT)).<br>**Control:** Standard care (healthy diet lifestyle)<br>Based on American Heart Association guidelines, 3-5 meals/d (50%–55% CHO, 15% PRO and 30% FAT). | **Cal restriction:** 30% Cal deficit (both).<br>**Physical activity:** 10,000 steps/d (both). | 26<br>- |
| Biolato 2019, Italy (40) | CCT crossover | 18, 90.0% | NAFLD | 42.7 | 30.3 | **Intervention:** MD<br>40% CHO, 20 PRO and 40% FAT <10% SFA.<br>**Comparator:** Low-fat/ high CHO<br>62% CHO, 20% PRO and 18% FAT. | **Cal restriction:** 1400cal/d (both). | 48<br>Fortnightly contacts. |
| Ristic-Medic 2021, Serbia (33) | RCT | 27, 100% | NAFLD | 33.7 | 30.3 | **Intervention:** MD<br>50% CHO, 15% PRO and >30%.<br>**Comparator:** Low-fat/ high CHO<br>60% CHO, 15% PRO and <25% FAT. | **Cal restriction:** 30% or 600-800cal/d deficit (both).<br>**Physical activity:** 30 min/d (both). | 12<br>Regular contacts/ calls. |
| Nourian | RCT | 22, 32.0% | NAFLD | 48.8 | 32.2 | **Intervention:** MD | **Physical activity:** General (MD). | 8 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| 2020, Iran (41) | | | | | | Intervention based on the health belief model (HBM).<br>**Control:** Standard care | **Behaviour change:** HBM (MD). | 8x60min/wk contacts. |
| Abbate 2021, Spain (42) | RCT | 51, 60.0% | NAFLD | 53.2 | 33.9 | **Intervention:** MD<br>Higher meal frequency (7meals/d) (40-45% CHO, (low glycaemic index) 25% PRO (mainly veg PRO)) and 30%-35% FAT (mainly MUFA and PUFA).<br>**Control:** Standard care (healthy diet lifestyle)<br>Based on AASLD guidelines (45-65% CHO, 10-35% PRO and 30%-35% FAT). | **Cal restriction:** 25-30% deficit (both).<br>**Physical activity:** 10,000 steps/d (both). | 26<br>Fortnightly (6m), then monthly. |
| Yaskolka Meir 2021, Israel* (28) | RCT | 259, 88.1% | Central obesity/ dyslipidaemia NAFLD (62%) | 51.1 | 31.3 | **Intervention:** MD (green)<br><40g/d CHO (2m) then 80g/day, ~40% FAT (PUFA and MUFA) and 3 polyphenol-rich products.<br>**Comparator:** MD<br>MD as MD (green) with 1 polyphenol-rich product.<br>**Control:** Standard care (healthy diet lifestyle) | **Diet food items:** 28g/d walnuts (MD groups); 3-4 cups/d green tea and 100g/d *Wolffia globosa* (Mankai strain) as green shake replacing dinner (MD green); lunch (all).<br>**Cal restriction:** 1200-1400cal/d (women); 1500-1800cal/d (men) (MD groups).<br>**Physical activity:** Aerobic/ resistance 45-60min/3-4d/wk (all). | 78<br>90min/wk (1m), monthly (5m) then every other month (12m) contacts, text messages/ website. |
| Gepner 2018, Israel* (29) | RCT | 247, 88.8% | Central obesity/ dyslipidaemia NAFLD (53%) | 47.8 | 30.8 | **Intervention:** MD<br><40g/d CHO (2m) then ≤70g/day with 1 polyphenol-rich product (from 3m).<br>**Comparator:** Low-fat/ high CHO<br>30% FAT ≤10% SFA and ≤300mg/d cholesterol/d. | **Diet food items:** 28g/d walnuts (MD); lunch (all).<br>**Physical activity:** 60min/m educational workshop<br>Aerobic/ resistance 30-60min/3d/wk (for those randomised after 6m) (both). | 78<br>90min/wk (1m), then monthly. |
| Mazzotti 2018, Italy* (27) | CCT | 383, 53.5% | NAFLD | 50.6 | 33.5 | **Intervention:** MD<br>Web-based intervention reproduces group sessions with interactive games, tests and mail contacts.<br>**Comparator:** MD<br>Group-based multi-disciplinary intervention. | **Cal restriction:** General (both).<br>**Physical activity:** General (both).<br>**Behaviour change:** Motivational interviewing; stimulus control; and weight loss maintenance strategies (both). | 104<br>5x120min/wk contacts. |
| Willmann 2019, Germany* (30) | RCT | 46, 25.8% | Risk of T2DM | 42.0 | 31.1 | **Intervention:** MD component<br>Reduced red meat consumption.<br>**Comparator:** MD component<br>Increased wholegrain consumption (≥40 g/d).<br>**Control:** Standard care (habitual diet) | **Cal restriction:** 400cal/d deficit (all).<br>**Physical activity:** ≥3 h/wk (all). | 26<br>Monthly contacts. |
| Dorosti 2020, Iran (43) | RCT | 39, 34.8% | NAFLD | 42.8 | 32.2 | **Intervention:** MD component<br>Increased wholegrain intake (≥½ of cereal servings/d).<br>**Control:** Standard care (healthy diet lifestyle)<br>**Both groups:**<br>Based on Dietary Guidelines for Americans. | Dietary advice and education information provided with no advice given on calorie allowances, physical activity or behaviour changes (both). | 12<br>Weekly/ monthly contacts. |
| Shidfar 2018, Iran (44) | RCT | 26, 52.0% | NAFLD | 45.9 | 29.8 | **Intervention:** MD component<br>Increased olive oil intake (20% of total fat)<br>**Control:** Standard care (healthy diet lifestyle)<br>**Both groups:** 50% CHO, 20% PRO and 30% FAT. | **Cal restriction:** Personalised Cal deficit (both).<br>Olive oil dosage supplied (MD component). | 13<br>- |
| Rezaei 2019, Iran (45) | RCT | 29, 43.9% | NAFLD | 43.6 | 30.1 | **Intervention:** MD component<br>Increased olive oil intake (20g/d).<br>**Comparator:** Increased sunflower oil intake (20g/d).<br>**Both groups:** 50-55% CHO, 10-15% PRO and 30- | **Cal restriction:** 500cal/d deficit (both).<br>**Physical activity:** Moderate intensity 30-40 min/d (both).<br>Olive oil and sunflower oil dosages provided | 12<br>Fortnightly/ monthly contacts. |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | | 35% FAT. | (both). | |
| Wong 2013, Hong Kong (46) | RCT | 72, 46.8% | NAFLD | 51.0 | 25.4 | **Intervention:** Cal-restricted dietary intervention Based on American Dietetic Association guidelines. **Control:** Standard care (healthy diet lifestyle) | **Cal restriction:** General (Cal-restricted). **Physical activity:** Moderate intensity/ resistance training 30min/d (Cal-restricted). 30 min/3d/wk (control). **Behaviour change:** Coping with risky situations (Cal-restricted). | 52 Weekly (4m), monthly (8m), and every three-month contacts. |
| Dong 2016, China (32) | RCT | 280, 100% | NAFLD | 57.3 | 25.8 | **Intervention:** Cal-restricted dietary intervention 50-60% CHO, 15-20% PRO, 23-30% FAT. **Control:** Standard care (habitual diet) | **Cal restriction:** BMI dependent Cal balance (25–35cal/kg/d) (Cal-restricted). **Physical activity:** Moderate-vigorous 30-60min/3-4d/wk (Cal-restricted). | 104 Three-month calls/ annual contacts. |
| Promrat 2010, USA (51) | RCT | 22, 71.0% | NASH | 48.3 | 33.7 | **Intervention:** Cal-restricted dietary intervention Based on American Heart Association; American Diabetic Association; American College of Sports Medicine, and Food Guide Pyramid. **Control:** Standard care (healthy diet lifestyle) | **Diet food items:** Commercial portion-controlled foods (Cal-restricted). **Cal restriction:** 1000-1200cal/d <91kg BW; 1200-1500kca/d >91kg BW (Cal-restricted). **Physical activity:** Moderate intensity 200min/wk (Cal-restricted). **Behaviour change:** Stimulus control, problem solving and relapse prevention (Cal-restricted). | 48 Weekly (6m)/ fortnightly (6m) contacts. |
| Cheng 2017, China (47) | RCT | 13, 22.8% | NAFLD | 60.0 | 26.9 | **Intervention:** Cal-restricted dietary intervention Fibre-enriched diet. **Control:** Standard care (habitual diet) | **Diet food items:** Lunch as 30-40% of total energy intake/d (37–40% CHO, 9–13g fibre, 5g soluble fibre, 25–27% PRO, 35–37% FAT) (Cal-restricted). **Cal restriction:** General (Cal-restricted). | 37 - |
| Browning 2011, USA (48) | CCT | 5, 27.8% | NAFLD | 44.5 | 35.0 | **Intervention:** Low carbohydrate (<20gd). **Comparator:** Cal-restricted dietary intervention | **Diet food items:** Meals (7-14/d) (both). **Cal restriction:** ~1200 cal/d (women) and ~1500 cal/d (men) (Cal-restricted). | 2 - |
| Johari 2019, Malaysia (49) | RCT | 33, 76.7% | NAFLD | 49.0 | 29.9 | **Intervention:** Cal-restricted dietary intervention Modified Alternate-Day Cal Restriction (MACR). **Control:** Standard care (habitual diet) | **Cal restriction:** 30% of Cal needs (fast days) and ad libitum (non-fast days) (Cal-restricted). | 8 Intermittent calls/ fortnightly contacts. |
| Shojasaad at 2019, Iran (50) | RCT | 39, 51.3% | NAFLD | 41.7 | 31.2 | **Intervention:** Cal-restricted dietary intervention 52% CHO, 18% PRO and 30% FAT. **Control:** Standard care (habitual diet) | **Cal restriction:** 350-700 cal/d deficit (Cal-restricted). | 12 - |
| Ghetti 2019, Brazil (52) | RCT | 21, 47.7% | NASH | 49.5 | 31.1 | **Intervention:** Cal-restricted dietary intervention Based on American Dietetic Association guidelines and nutritional orientation (food guide Brazil). **Control:** Standard Care (healthy diet lifestyle) Nutritional orientation (food guide Brazil). | **Cal restriction:** 500-750 cal/d deficit for overweight/ obese participants (Cal-restricted). | 13 Monthly contacts. |
| Holmer 2021, Sweden* (31) | RCT | 33, 44.6% | NAFLD | 56.3 | 32.4 | **Intervention:** Cal-restricted dietary intervention (5:2). Based on Nordic Nutrition Recommendations on 5/d, (45-60% CHO, 10-20% PRO, 25% FAT). **Comparator:** Cal-restricted dietary intervention Low-carb high-fat (5-10% CHO, 15-40% PRO, 50-80% FAT). **Control:** Standard Care (healthy diet lifestyle) | **Cal restriction:** 500 cal/d (women) and 600 cal/d (men) on 2/d. 2,000 cal/d (women) and 2,400 cal/d (men) on 5/d (5:2). 1,600 cal/d (women) and 1,900 cal/d (men) (LFD). | 12 Fortnightly calls (2,4 and 8wks) and 6wk contact. |

* Study not included in meta-analyses. USA, United States of America; AASLD, American Association for the Study of Liver Disease; CCT, controlled clinical trial; RCT, randomised controlled trial; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; T2DM, Type 2 Diabetes; MD, Mediterranean diet; MDL, Mediterranean diet lifestyle; LFD, low fat diet; HBM, health belief model; INRAN, Italian National Food Consumption Survey; RDAs, Recommended Dietary Allowances; cal, calorie; CHO, carbohydrate; PRO, protein; BW, body weight; BMI, body mass index; MUFA, monosaturated fat; PUFA, polyunsaturated fat; SFA, saturated fat.

## Appendix F: Risk of bias assessment for all outcomes.

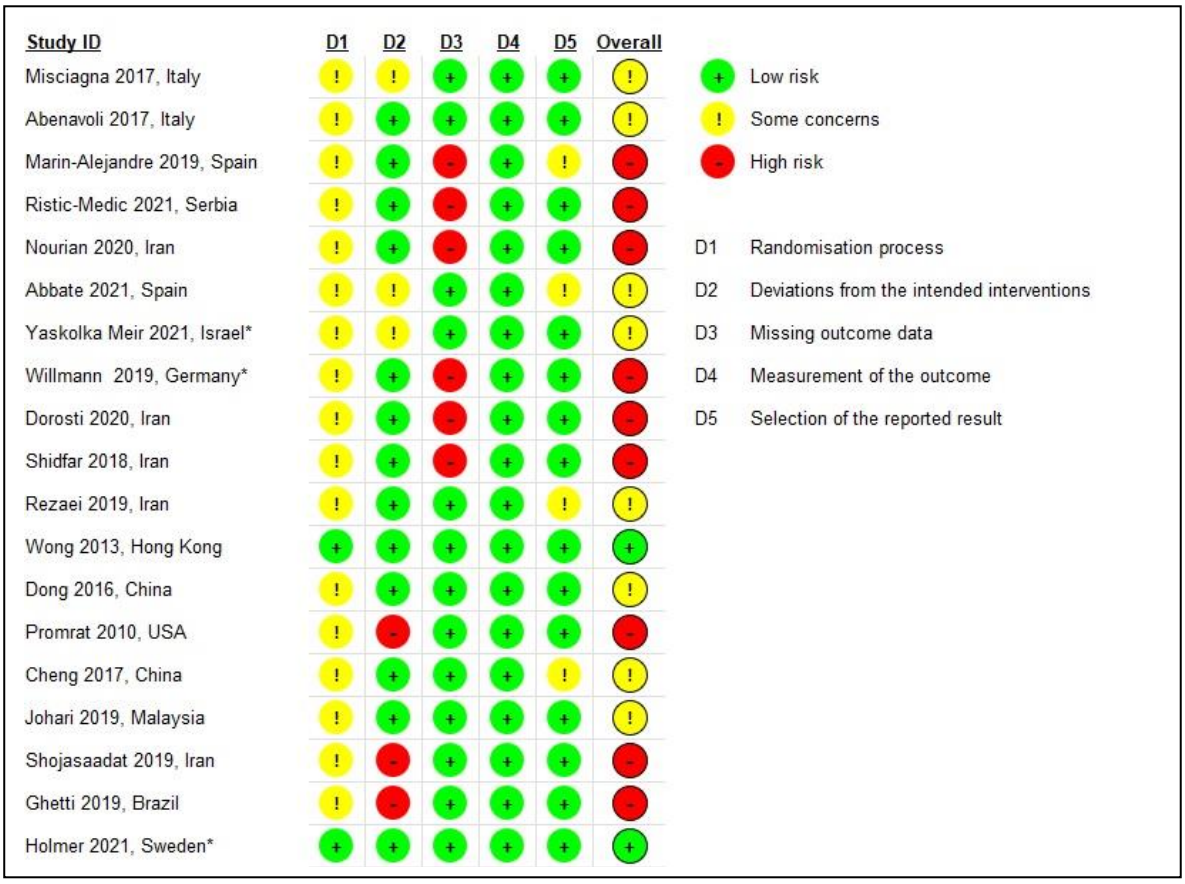
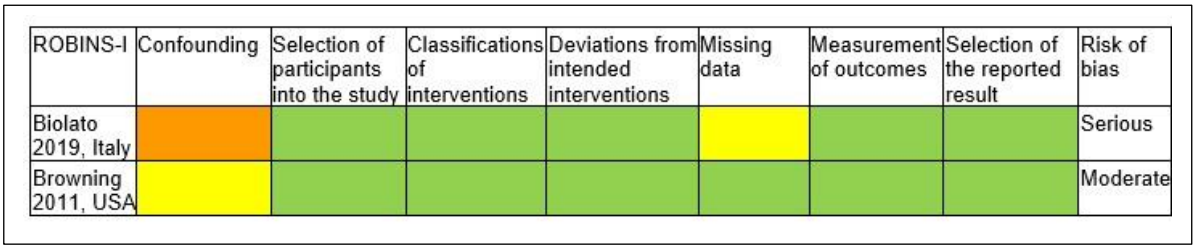



**Figure 1.** Risk of bias judgements of the included RCT studies for aspartate aminotransferase (AST). * Study not included in meta-analyses.
USA, United States of America.



| ROBINS-I | Confounding | Selection of participants into the study | Classifications of interventions | Deviations from intended interventions | Missing data | Measurement of outcomes | Selection of the reported result | Risk of bias |
|---|---|---|---|---|---|---|---|---|
| Biolato 2019, Italy | | | | | | | | Serious |
| Browning 2011, USA | | | | | | | | Moderate |

**Figure 2.** Risk of bias judgements of the included clinical controlled studies for aspartate aminotransferase (AST). Green, low risk; yellow, moderate risk; orange, serious risk; USA, United States of America.

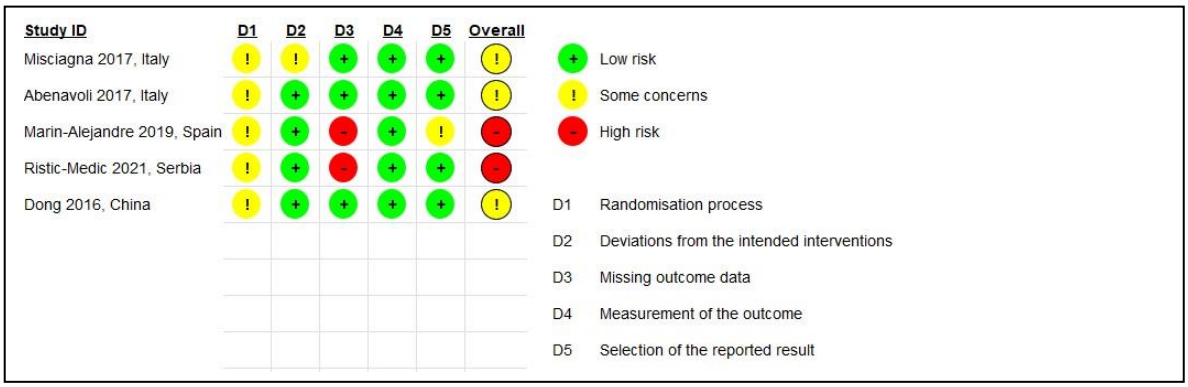



**Figure 3.** Risk of bias judgements of the included RCT studies for Fatty Liver Index (FLI).

| ROBINS-I | Confounding | Selection of participants into the study | Classifications of interventions | Deviations from intended interventions | Missing data | Measurement of outcomes | Selection of the reported result | Risk of bias |
|---|---|---|---|---|---|---|---|---|
| Mazzotti 2018, Italy* | | | | | | | | Moderate |
| Biolato 2019, Italy | | | | | | | | Serious |
| Browning 2011, USA | | | | | | | | Moderate |

**Figure 4.** Risk of bias judgements of the included clinical controlled studies for Fatty Liver Index (FLI). * Study not included in meta-analyses. Green, low risk; yellow, moderate risk; orange, serious risk; USA, United States of America.



**Figures 5 and 6.** Risk of bias judgements of the included RCT parallel and crossover studies for hepatic steatosis by imaging/histology. * Study not included in meta-analyses. USA, United States of America.

| ROBINS-I | Confounding | Selection of participants into the study | Classifications of interventions | Deviations from intended interventions | Missing data | Measurement of outcomes | Selection of the reported result | Risk of bias |
|---|---|---|---|---|---|---|---|---|
| Browning 2011, USA | | | | | | | | Moderate |

**Figure 7.** Risk of bias judgements of the included clinical controlled studies for hepatic steatosis by imaging/histology. Green, low risk; yellow, moderate risk; USA, United States of America

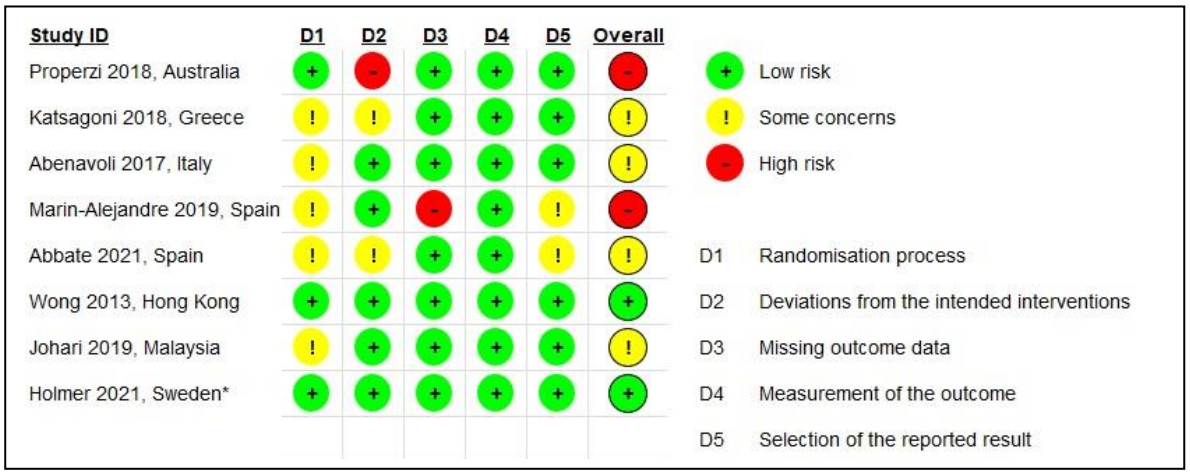



**Figure 8.** Risk of bias judgements of the included RCT studies for liver stiffness measurement (LSM). * Study not included in meta-analyses.


**Figure 9.** Risk of bias judgements of the included RCT studies for NAFLD Fibrosis Score (NFS).

| ROBINS-I | Confounding | Selection of participants into the study | Classifications of interventions | Deviations from intended interventions | Missing data | Measurement of outcomes | Selection of the reported result | Risk of bias |
|---|---|---|---|---|---|---|---|---|
| Mazzotti 2018, Italy* | | | | | | | | Moderate |

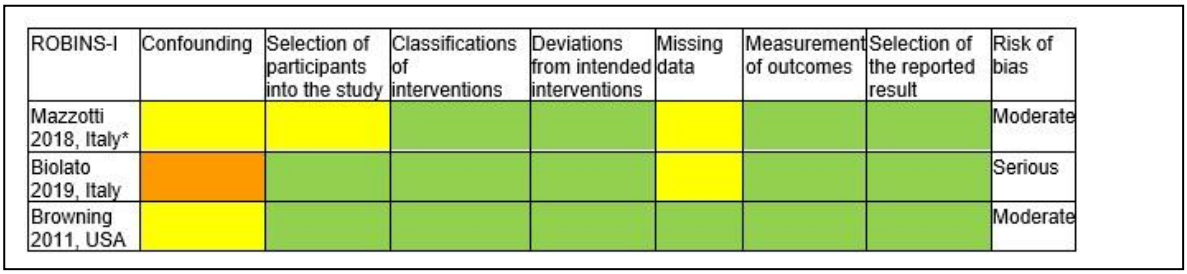
**Figure 10.** Risk of bias judgements of the included clinical controlled studies for NAFLD Fibrosis Score (NFS). * Study not included in meta-analyses. Green, low risk; yellow, moderate risk.

| ROBINS-I | Confounding | Selection of participants into the study | Classifications of interventions | Deviations from intended interventions | Missing data | Measurement of outcomes | Selection of the reported result | Risk of bias |
|---|---|---|---|---|---|---|---|---|
| Mazzotti 2018, Italy* | | | | | | | | Moderate |

**Figure 11.** Risk of bias judgements of the included clinical controlled studies for Fibrosis-4 Index (FIB-4). * Study not included in meta-analyses. Green, low risk; yellow, moderate risk.

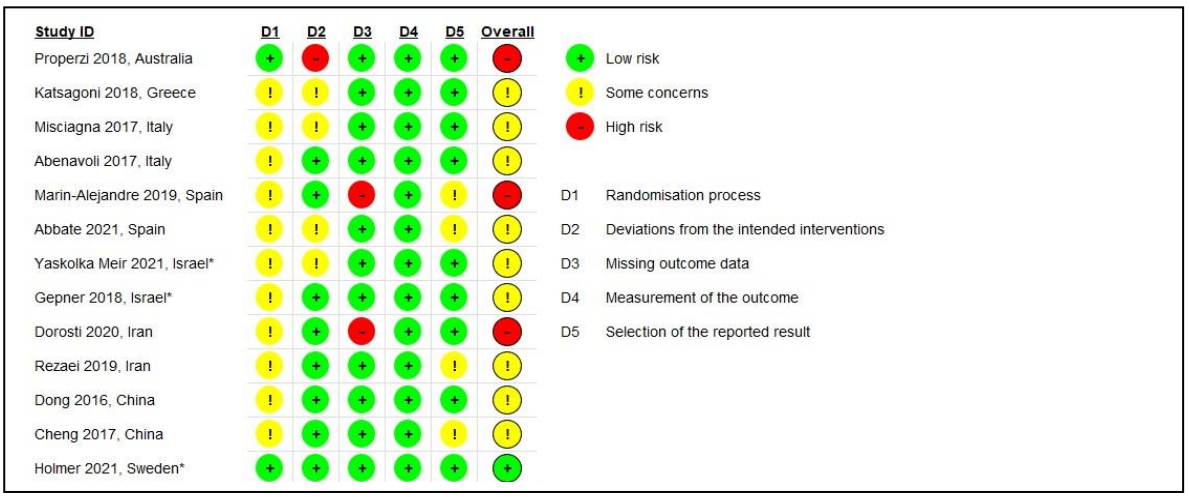

**Figure 12.** Risk of bias judgements of the included RCT studies for HepaScore.

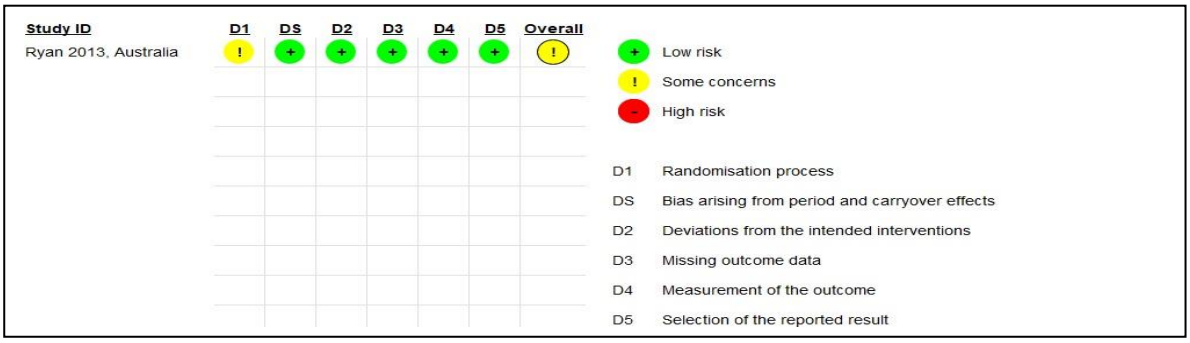

**Figures 13 and 14.** Risk of bias judgements of the included RCT parallel and crossover studies for markers of adiposity. * Study not included in meta-analyses. USA, United States of America.



| ROBINS-I | Confounding | Selection of participants into the study | Classifications of interventions | Deviations from intended interventions | Missing data | Measurement of outcomes | Selection of the reported result | Risk of bias |
|---|---|---|---|---|---|---|---|---|
| Mazzotti 2018, Italy* | | | | | | | | Moderate |
| Biolato 2019, Italy | | | | | | | | Serious |
| Browning 2011, USA | | | | | | | | Moderate |

**Figure 15.** Risk of bias judgements of the included clinical controlled studies for markers of adiposity. * Study not included in meta-analyses. Green, low risk; yellow, moderate risk; orange, serious risk; USA, United States of America.




**Figures 16 and 17.** Risk of bias judgements of the included RCT parallel and crossover studies for cardiometabolic measures (blood pressure). * Study not included in meta-analyses. USA, United States of America.

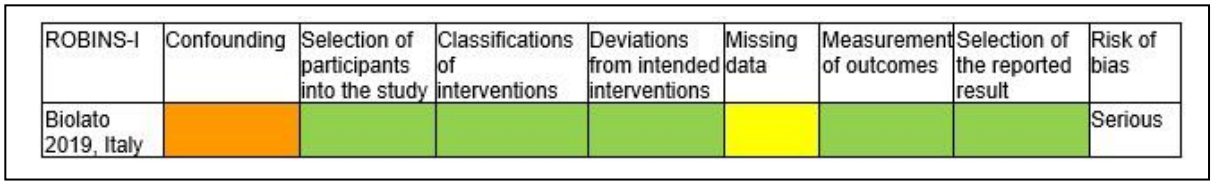
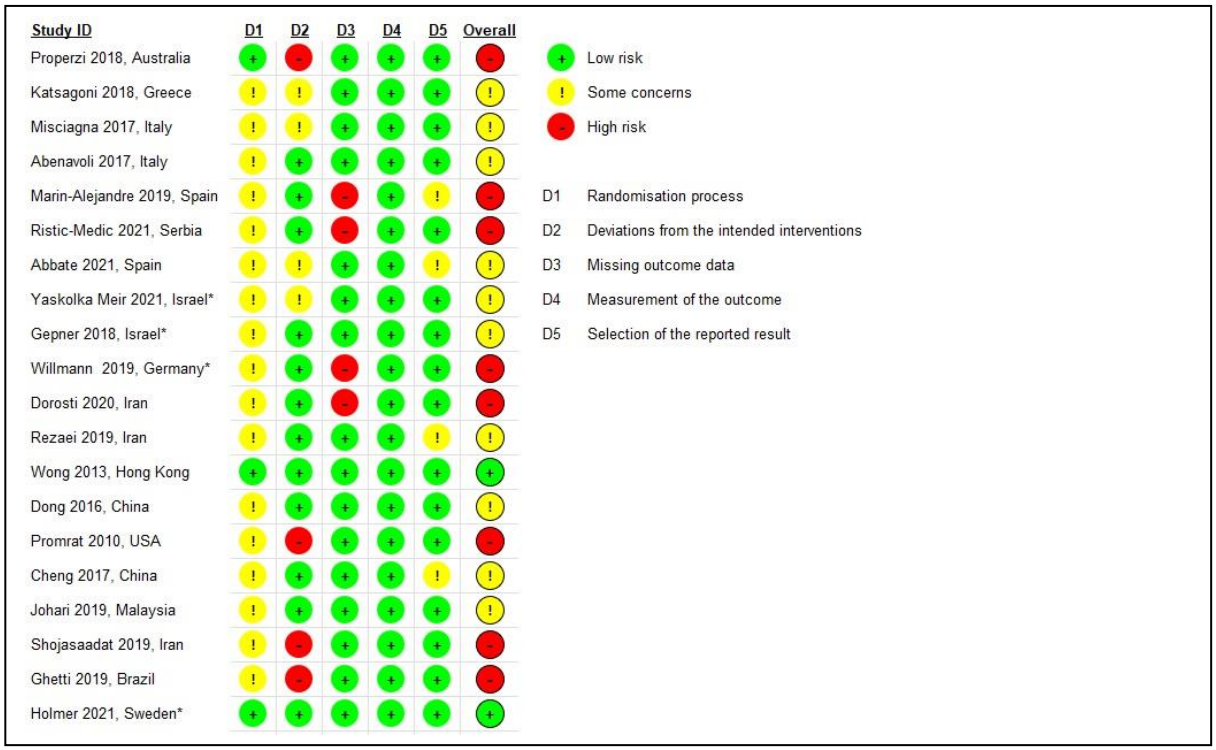
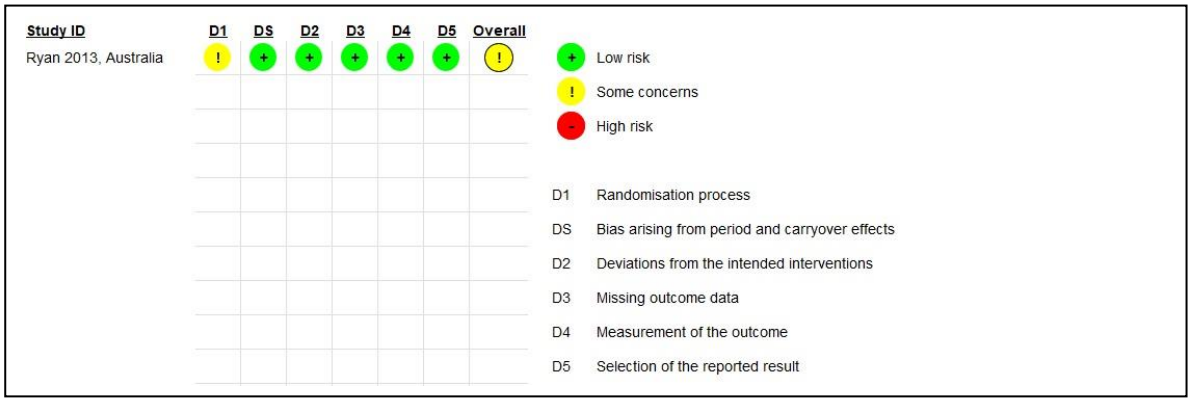



**Figure 18.** Risk of bias judgements of the included clinical controlled studies for cardiometabolic measures (blood pressure). Green, low risk; yellow, moderate risk; orange, serious risk.


**Figure 19.** Risk of bias judgements of the included RCT studies for cardiometabolic measures (Framingham risk score). * Study not included in meta-analyses.






**Figures 20 and 21.** Risk of bias judgements of the included RCT parallel and crossover studies for cardiometabolic measures (fasting glucose and fasting insulin). * Study not included in meta-analyses. USA, United States of America.

| ROBINS-I | Confounding | Selection of participants into the study | Classifications of interventions | Deviations from intended interventions | Missing data | Measurement of outcomes | Selection of the reported result | Risk of bias |
|---|---|---|---|---|---|---|---|---|
| Browning 2011, USA | | | | | | | | Moderate |

**Figure 22.** Risk of bias judgements of the included clinical controlled studies for cardiometabolic measures (fasting glucose and fasting insulin). Green, low risk; yellow, moderate risk; USA, United States of America.


**Figures 23 and 24.** Risk of bias judgements of the included RCT parallel and crossover studies for cardiometabolic measures (Homeostatic Model Assessment of Insulin Resistance). * Study not included in meta-analyses. USA, United States of America.

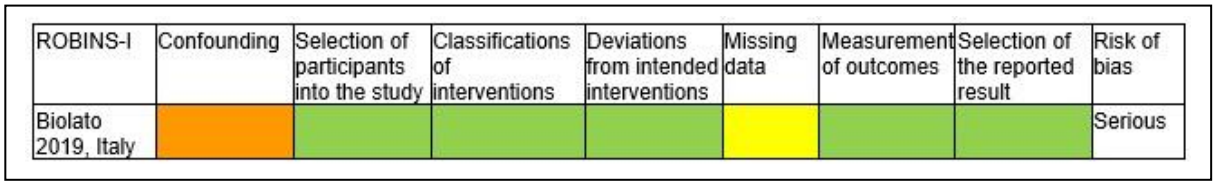
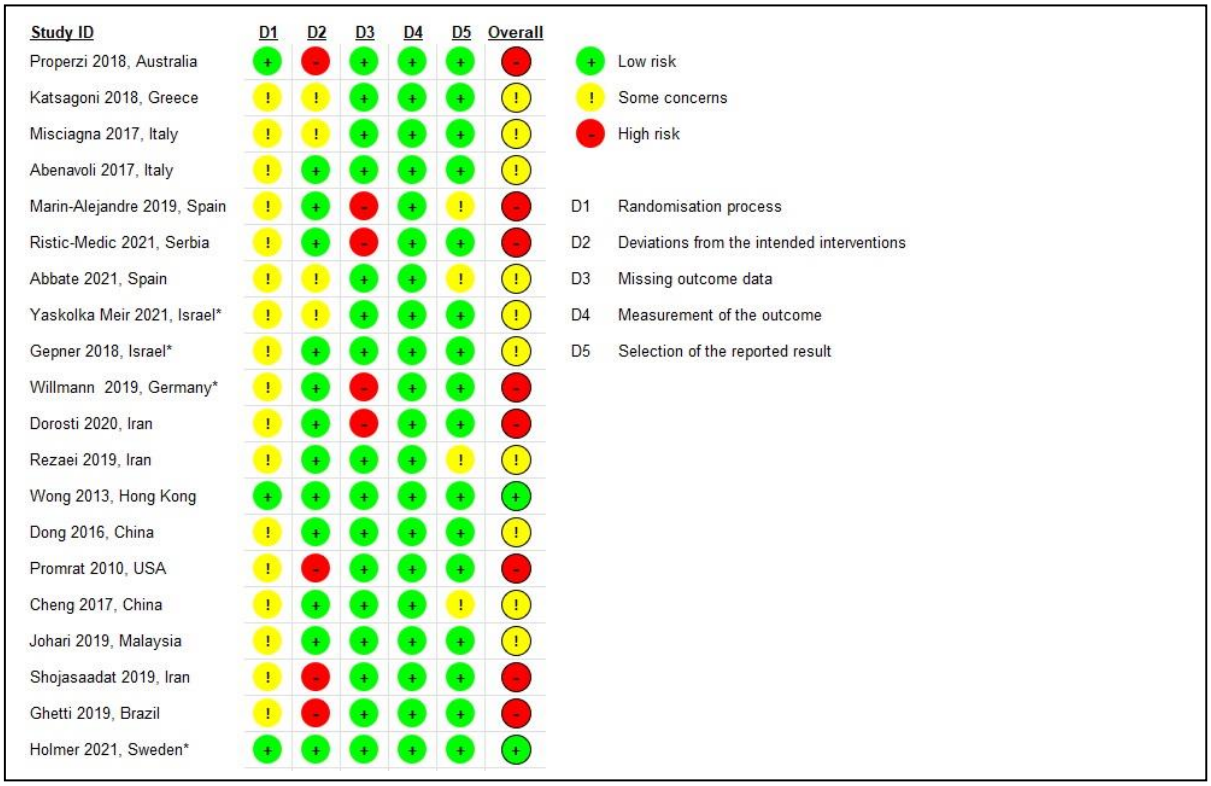
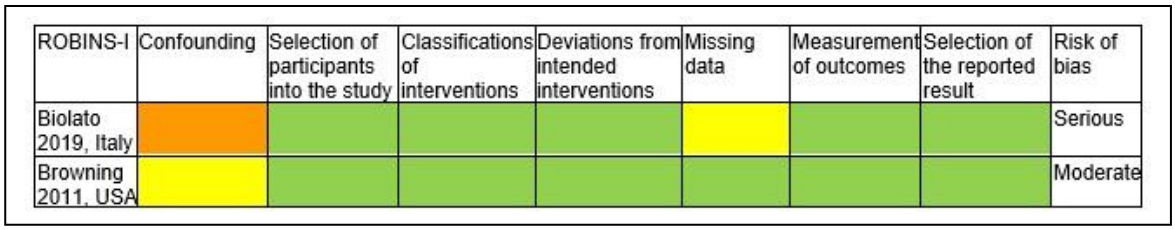



**Figure 25.** Risk of bias judgements of the included clinical controlled studies for cardiometabolic measures (Homeostatic Model Assessment of Insulin Resistance). Green, low risk; yellow, moderate risk; orange, serious risk.



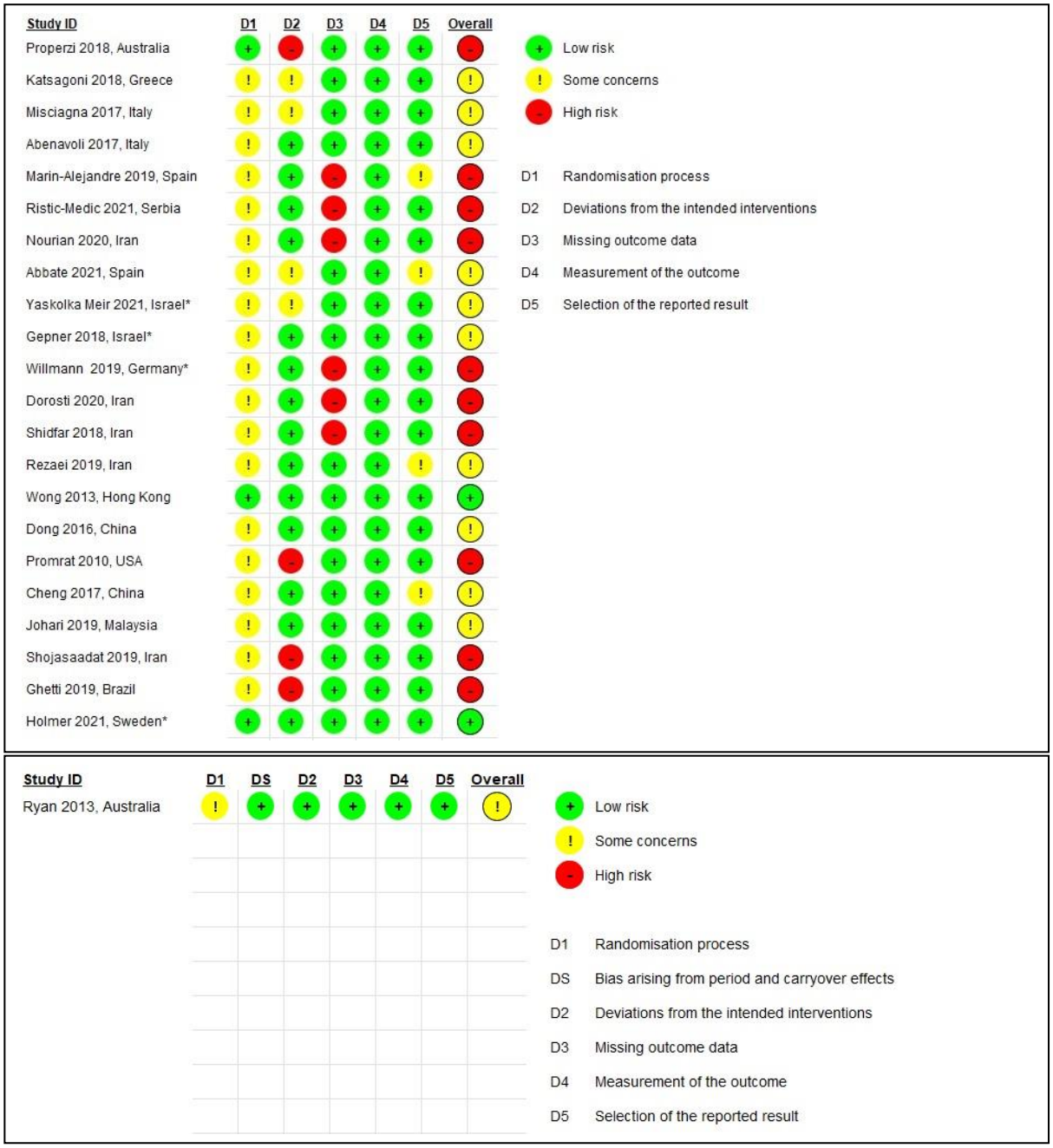

**Figures 26 and 27.** Risk of bias judgements of the included RCT parallel and crossover studies for cardiometabolic measures (Lipid profile and HbA1c). * Study not included in meta-analyses. USA, United States of America.



| ROBINS-I | Confounding | Selection of participants into the study | Classifications of interventions | Deviations from intended interventions | Missing data | Measurement of outcomes | Selection of the reported result | Risk of bias |
|---|---|---|---|---|---|---|---|---|
| Biolato 2019, Italy | | | | | | | | Serious |
| Browning 2011, USA | | | | | | | | Moderate |

**Figure 28.** Risk of bias judgements of the included clinical controlled studies for cardiometabolic measures (Lipid profile and HbA1c). Green, low risk; yellow, moderate risk; orange, serious risk; USA, United States of America.

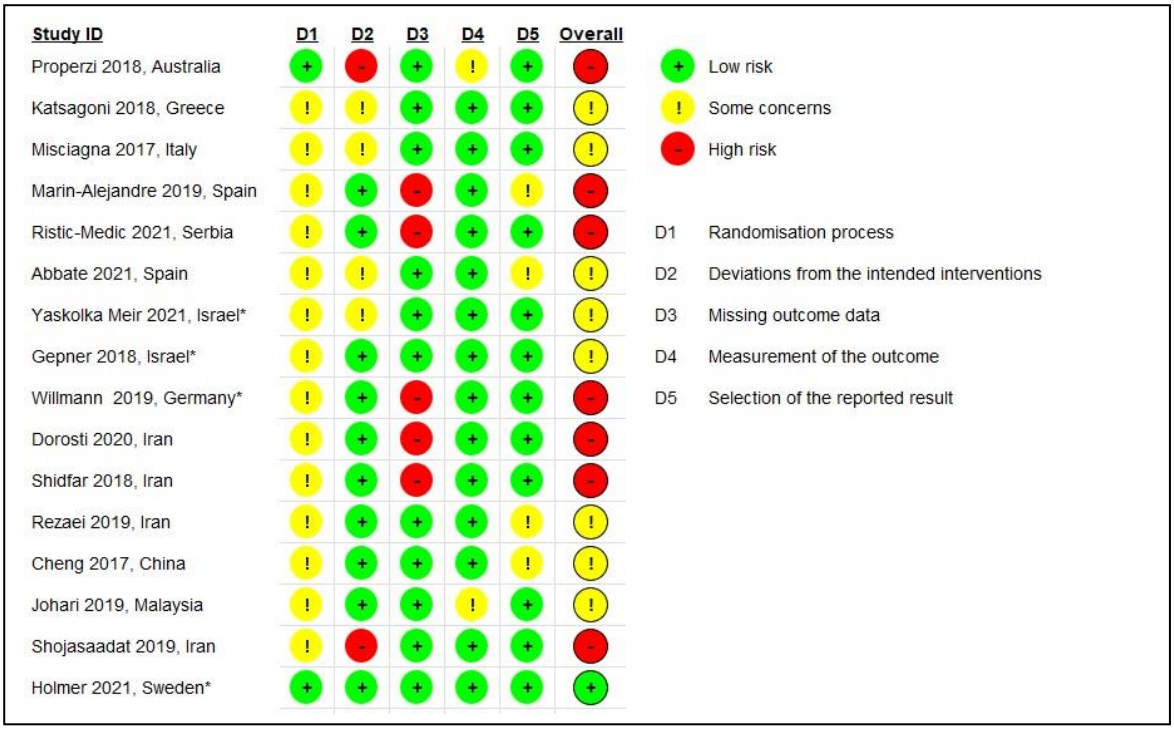

**Figure 29.** Risk of bias judgements of the included RCT studies for quality of life.




**Figures 30 and 31.** Risk of bias judgements of the included RCT parallel and crossover studies for intervention acceptability (attrition). * Study not included in meta-analyses. USA, United States of America.

| ROBINS-I | Confounding | Selection of participants into the study | Classifications of interventions | Deviations from intended interventions | Missing data | Measurement of outcomes | Selection of the reported result | Risk of bias |
|---|---|---|---|---|---|---|---|---|
| Mazzotti 2018, Italy* | | | | | | | | Moderate |
| Biolato 2019, Italy | | | | | | | | Serious |
| Browning 2011, USA | | | | | | | | Moderate |

**Figure 32.** Risk of bias judgements of the included clinical controlled studies for intervention acceptability (attrition). * Study not included in meta-analyses. Green, low risk; yellow, moderate risk; orange, serious risk; USA, United States of America.



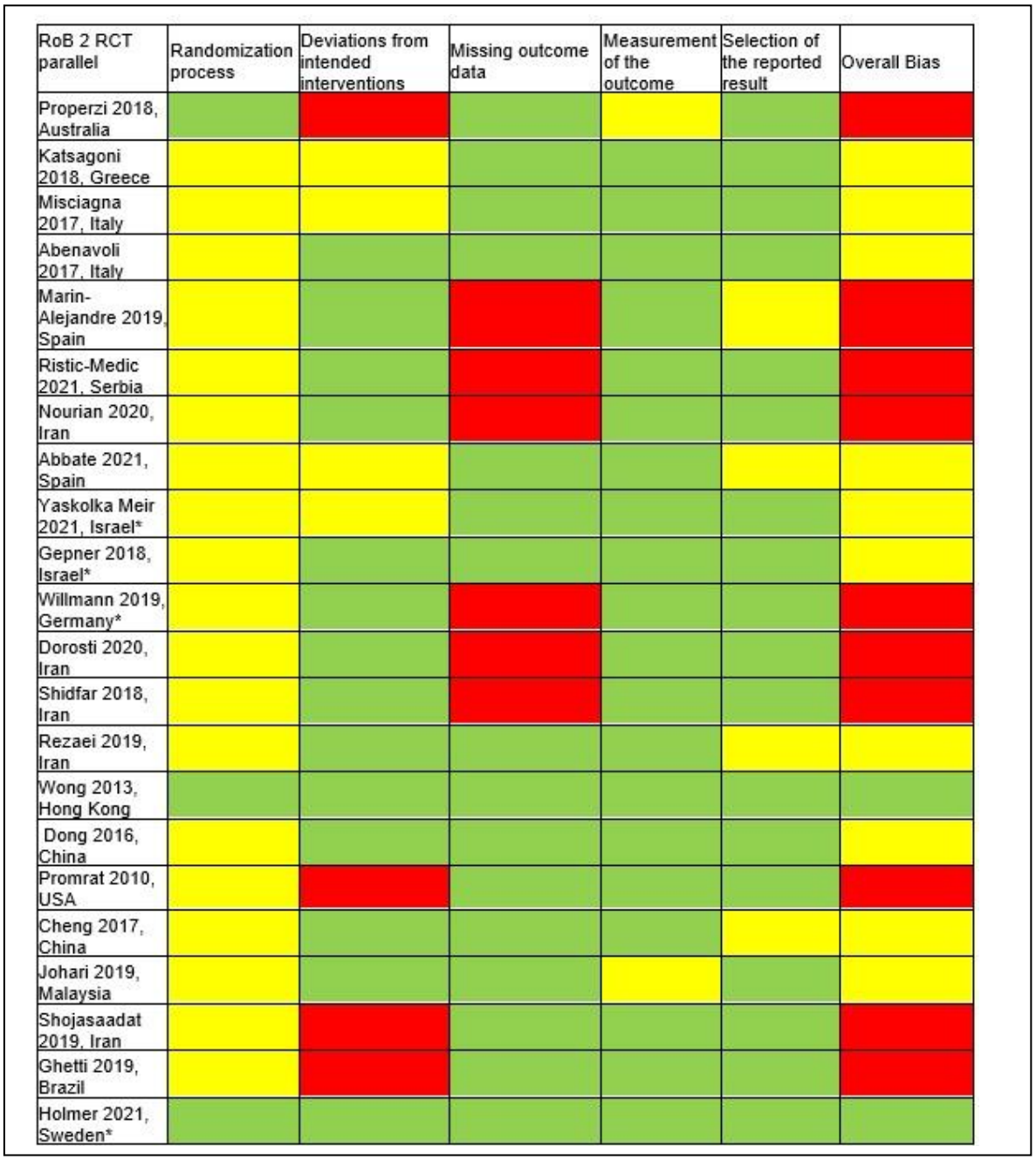


**Figures 33 and 34.** Risk of bias judgements of the included RCT parallel and crossover studies for diet intake modification. * Study not included in meta-analyses. USA, United States of America.

| ROBINS-I | Confounding | Selection of participants into the study | Classifications of interventions | Deviations from intended interventions | Missing data | Measurement of outcomes | Selection of the reported result | Risk of bias |
|---|---|---|---|---|---|---|---|---|
| Mazzotti 2018, Italy* | | | | | | | | Moderate |
| Browning 2011, USA | | | | | | | | Moderate |

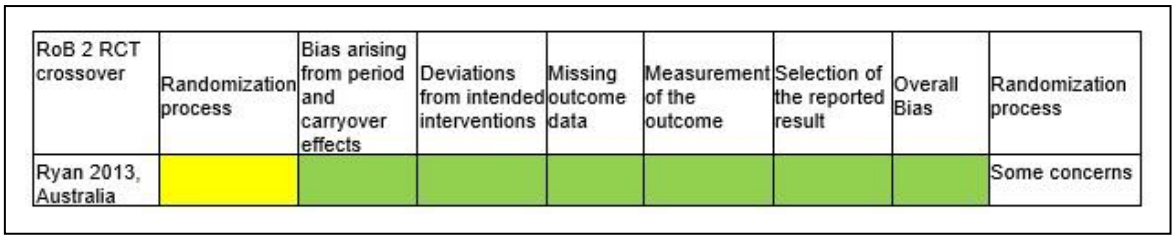
**Figure 35.** Risk of bias judgements of the included clinical controlled studies for diet intake modification. * Study not included in meta-analyses. Green, low risk; yellow, moderate risk; USA, United States of America.

## Appendix G: Overall risk of bias assessment.



| RoB 2 RCT parallel | Randomization process | Deviations from intended interventions | Missing outcome data | Measurement of the outcome | Selection of the reported result | Overall Bias |
|---|---|---|---|---|---|---|
| Properzi 2018, Australia | | | | | | |
| Katsagoni 2018, Greece | | | | | | |
| Misciagna 2017, Italy | | | | | | |
| Abenavoli 2017, Italy | | | | | | |
| Marin-Alejandre 2019, Spain | | | | | | |
| Ristic-Medic 2021, Serbia | | | | | | |
| Nourian 2020, Iran | | | | | | |
| Abbate 2021, Spain | | | | | | |
| Yaskolka Meir 2021, Israel* | | | | | | |
| Gepner 2018, Israel* | | | | | | |
| Willmann 2019, Germany* | | | | | | |
| Dorosti 2020, Iran | | | | | | |
| Shidfar 2018, Iran | | | | | | |
| Rezaei 2019, Iran | | | | | | |
| Wong 2013, Hong Kong | | | | | | |
| Dong 2016, China | | | | | | |
| Promrat 2010, USA | | | | | | |
| Cheng 2017, China | | | | | | |
| Johari 2019, Malaysia | | | | | | |
| Shojasaadat 2019, Iran | | | | | | |
| Ghetti 2019, Brazil | | | | | | |
| Holmer 2021, Sweden* | | | | | | |

**Figure 1.** Quality assessment of the included RCT studies. * Study not included in meta-analyses. Green, low risk; yellow, some concerns; red, high risk; USA, United States of America.



| RoB 2 RCT crossover | Randomization process | Bias arising from period and carryover effects | Deviations from intended interventions | Missing outcome data | Measurement of the outcome | Selection of the reported result | Overall Bias | Randomization process |
|---|---|---|---|---|---|---|---|---|
| Ryan 2013, Australia | | | | | | | | Some concerns |

**Figure 2.** Quality assessment of the included RCT crossover studies. Green, low risk; yellow, some concerns


**Figure 3.** Quality assessment of the included clinical controlled studies. * Study not included in meta-analyses. Green, low risk; yellow, moderate risk; orange, serious risk; USA, United States of America

## Appendix H: Funnel plots.

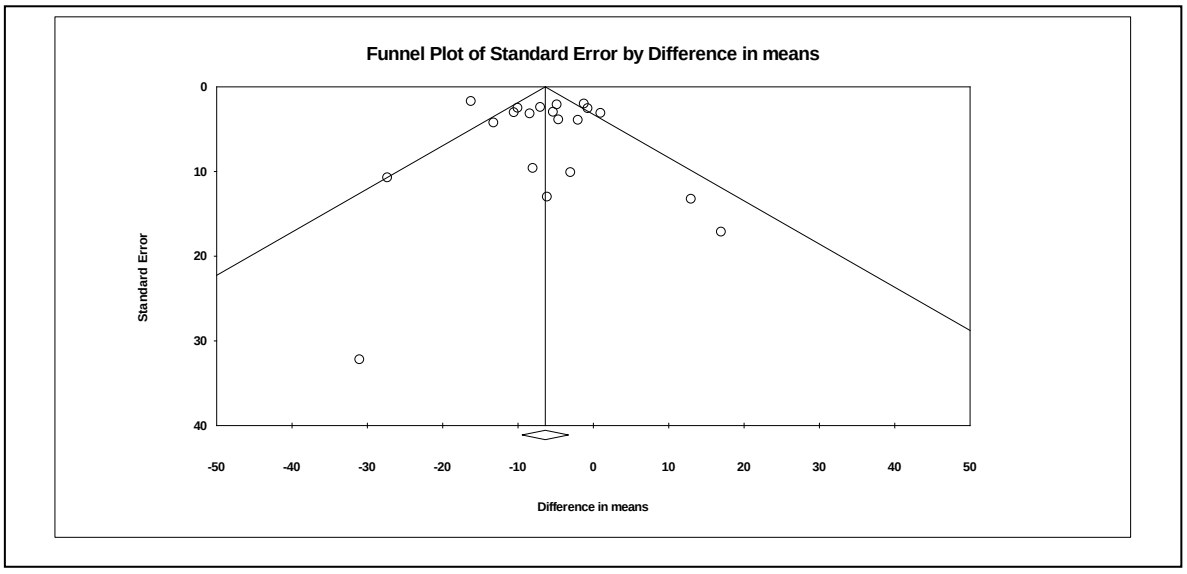



**Figure 1.** Funnel plot for alanine aminotransferase (ALT).

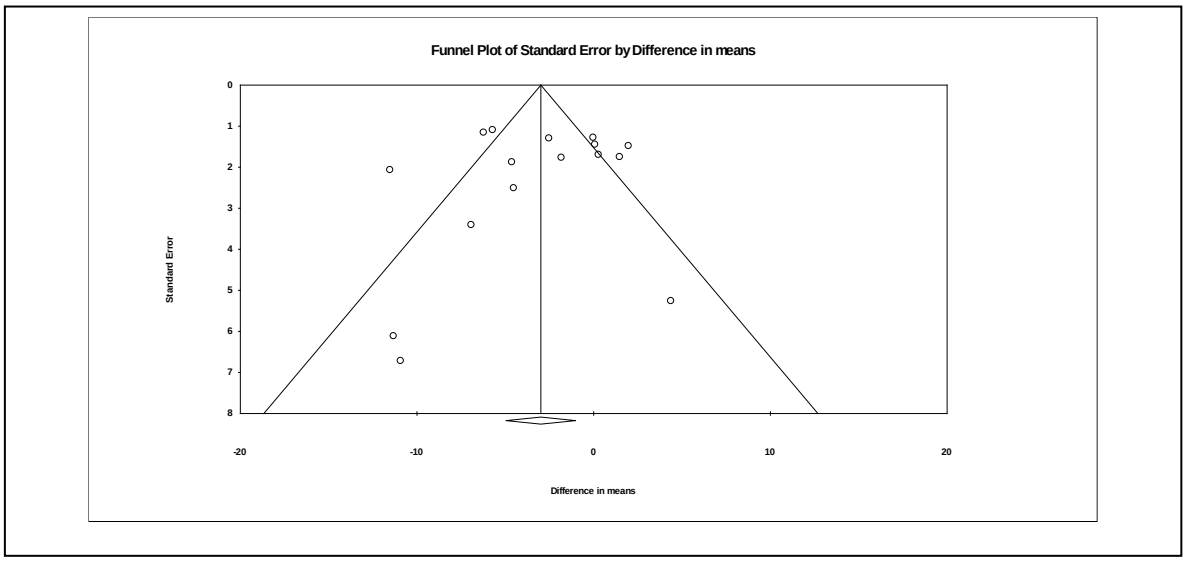



**Figure 2.** Funnel plot for aspartate aminotransferase (AST).

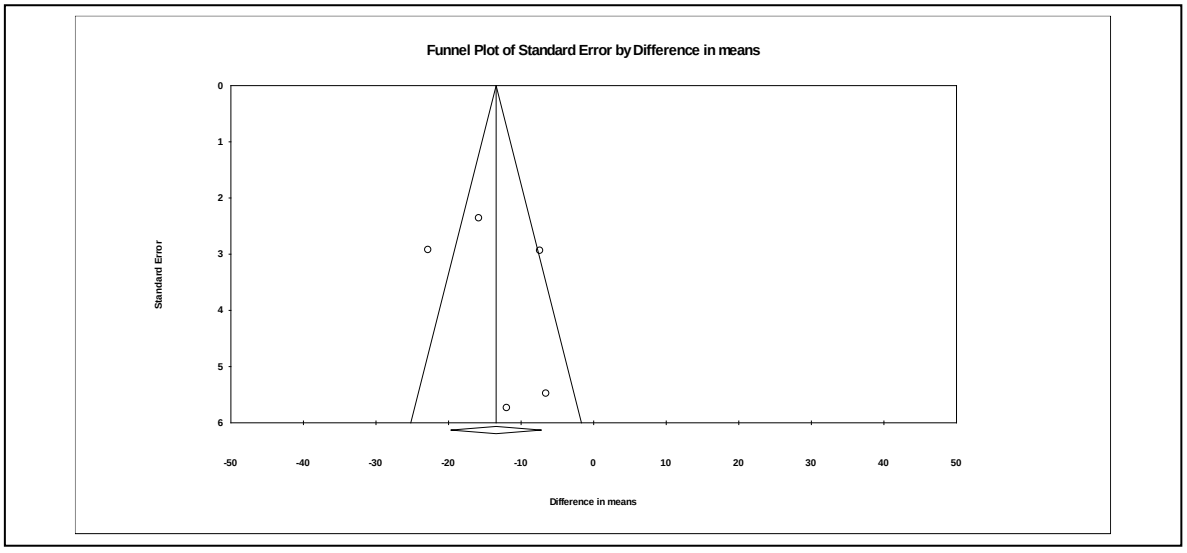



**Figure 3**. Funnel plot for Fatty Liver Index (FLI).

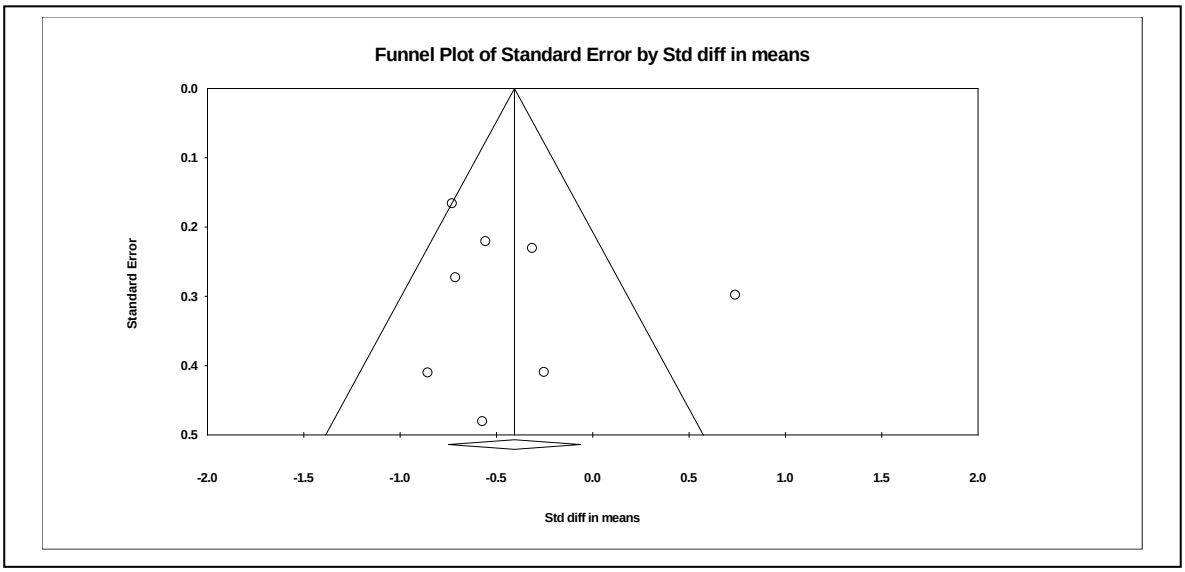



**Figure 4.** Funnel plot for hepatic steatosis.

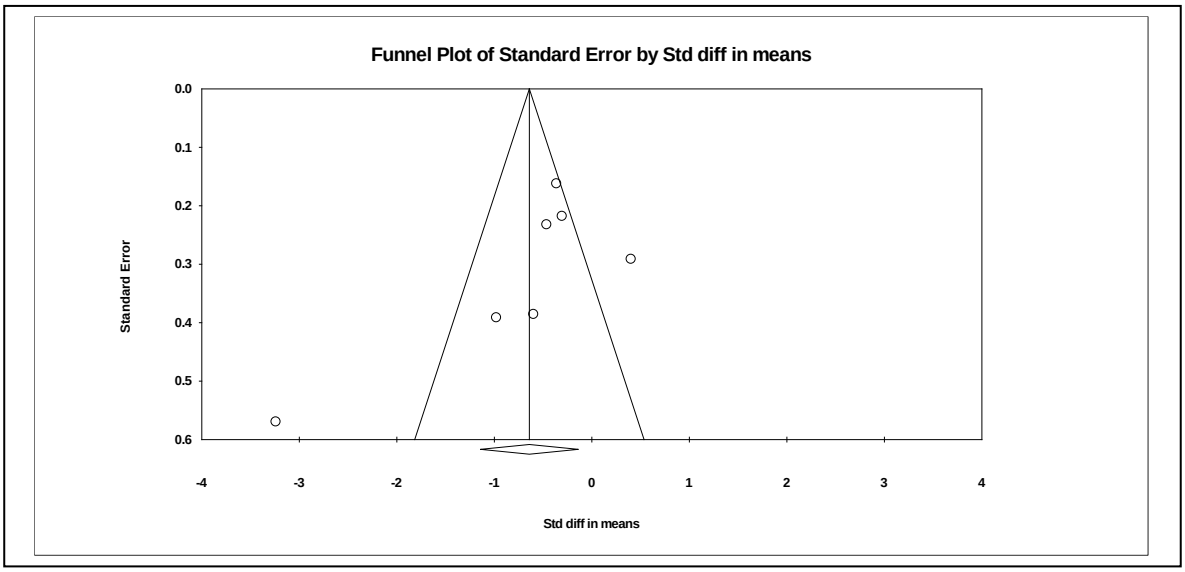



**Figure 5**. Funnel plot for liver stiffness.

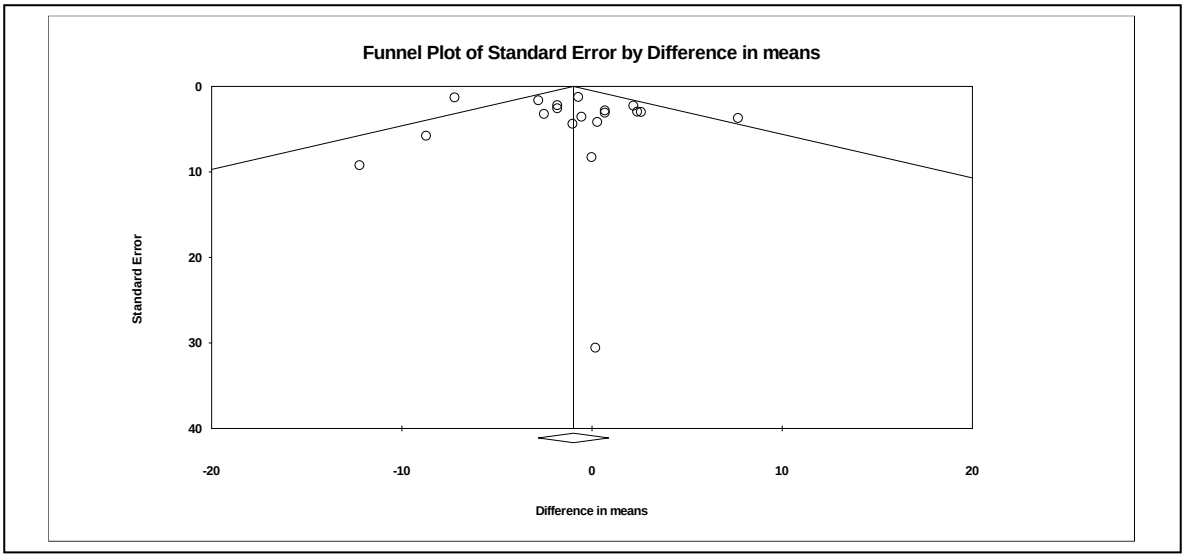



**Figure 6.** Funnel plot for body weight.

## Appendix I: Effects of dietary interventions on primary and secondary outcomes.

| Author | Outcome Measures | Results: primary outcomes (%) | Results: secondary outcomes (attrition rates n, %) | Results: secondary outcomes (cardiometabolics; anthropometry; QoL) (%) | Results: secondary outcomes (dietary intake) |
|---|---|---|---|---|---|
| Properzi 2018, Australia (34) | HS (MRS-PDFF), LSM (TE FibroScan™), HepaScore, ALT, cardiometabolics, anthropometry, QoL, and diet history. | ↓ HS and ↓ ALT (both)<br>HS RR: -32.4%±25.5% vs. -25.0%±25.3% (intervention vs. comparator) (ns) | **Intervention:** 2/26, 7.7%<br>**Comparator:** 1/25, 4.0% | ↓ GGT, HbA1c, TC, TG and FRS (intervention)<br>↓ WC and ↑ QoL (both)<br>↓ BW (both)<br>BW: -2.3% vs. -2.1% (intervention vs. comparator) (ns) | ↑ PRO, ↓ FAT, MUFA (comparator)<br>↓ CHO, sugars and sodium; ↑ FAT and MUFA (intervention)<br>↓ SFA and ↑ fibre (both)<br>↑ FAT and MUFA; ↓ CHO, sugars and sodium (intervention vs. comparator) |
| Katsagoni 2018, Greece (35) | LSM (USnd Multiwave), NFS, ALT, cardiometabolics, anthropometry, diet record diaries, FFQ and recalls. | ↓ LSM (intervention/comparator vs. control)<br>↓ ALT (comparator vs. control) | **Intervention:** 0/21, 0.0%<br>**Comparator:** 0/21, 0.0%<br>**Control:** 7/21, 33.3% | ↓ LDL and Non-HDL (intervention)<br>↓ Median BW (intervention: -5.4% and comparator: -6.3% vs. control: -2.1%) | ↓ Cal (all)<br>↑ MUFA and PUFA (intervention)<br>↑ fibre and MD adherence (intervention/comparator vs. control).<br>↑ wholegrains, fruits, vegetables and nuts; ↓ refined cereals, potatoes, red meat and sweets (intervention/comparator) |
| Misciagna 2017, Italy (36) | HS (USnd), FLI, ALT, AST, cardiometabolics, anthropometry, diet record diaries and FFQ. | NAFLD score: negative interaction between time/ intervention<br>↓ FLI and ALT (both) | **Intervention:** 6/50, 12.0%<br>**Control:** 2/48, 4.2% | ↓ GGT and HDL (intervention)<br>↓ TG and glycaemia (both) | - |
| Ryan 2013, Australia (37) | HS ($^{1}$H-MRS), ALT, cardiometabolics, anthropometry and diet record diaries. | ↓ HS (intervention)<br>HS RR: -39%±4% vs. -7%±3% (intervention vs. comparator) | **Intervention:** 0/12, 0.0%<br>**Comparator:** 0/12, 0.0% | ↓ HOMA-IR and fasting insulin (intervention)<br>↓ Systolic BP (both)<br>BW: -1.1% vs. -2.6% (intervention vs. comparator) (ns) | ↓ PRO, ↑ FAT, ↑ MUFA, fibre and ↓ SFA (intervention)<br>↑ CHO, PRO and ↓ FAT (comparator) |
| Abenavoli 2017, Italy (38) | HS (USnd), FLI, LSM (TE FibroScan™), ALT, AST, cardiometabolics and anthropometry. | ↓ FLI and LSM (intervention) (intervention vs. control)<br>↑ AST (control) | **Intervention:** 0/20, 0.0%<br>**Control:** 0/10, 0.0% | ↓ TG, BW and WC (intervention)<br>↑ GGT, ↓ TC and LDL (both)<br>↑ fasting insulin and HOMA-IR (control)<br>↓ TC, TG, fasting insulin, HOMA-IR, BW and WC (intervention vs. control)<br>Median BW: -6.0% vs. -0.5% (intervention vs. control) | - |
| Marin-Alejandre 2019, Spain (39) | HS (MRI), FLI, LSM (USnd ARFI), ALT, AST, cardiometabolics, anthropometry, diet questionnaire and FFQ. | ↓ HS, FLI and ALT (both)<br>↓ AST (control)<br>HS: -4.2% vs. -3.6% (intervention vs. control) (ns) | **Intervention:** 11/50, 22.0%<br>**Control:** 11/48, 22.9% | ↓ GGT, TG, FG, fasting insulin, HOMA-IR and WC (both)<br>↓ Systolic and diastolic BP (both)<br>↓ BW (both)<br>BW: -10.1% vs. -9.7% (intervention vs. control) (ns) | ↓ Cal and SFA; ↑ meal frequency, PRO, fibre and MD adherence (both)<br>↓ CHO and ↑ TAC and PUFA (intervention)<br>↓ Cal, CHO; ↑ meal frequency, PRO, PUFA and MD adherence (intervention vs. control) |
| Biolato 2019, Italy (40) | ALT, AST, cardiometabolics, anthropometry and diet questionnaire, FFQ and recalls. | ↓ ALT and AST (intervention)<br>↓ ALT (intervention vs. comparator) | **Intervention:** 2/20, 10.0%<br>**Comparator:** 2/14, 14.3% | ↓ WC (intervention) (intervention vs. comparator)<br>↓ BW (intervention)<br>BW: - 5.8% vs. -0.7% (intervention vs. comparator) | - |

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|---|---|---|---|---|---|
| Ristic-Medic 2021, Serbia (33) | HS (USnd), FLI, ALT, AST, cardiometabolics, anthropometry, and FFQ. | ↓ FLI, ALT and AST (both)<br>↓ FLI and AST (intervention vs. comparator) | **Intervention:** 2/14, 14.3%<br>**Comparator:** 1/13, 7.7% | ↓ GGT, TG, TC, LDL, FG, fasting insulin, HOMA-IR, BW, WC and ↑ HDL (both)<br>↓ TG and ↑ HDL (intervention vs. comparator)<br>BW: -9.1% vs. -9.5% (intervention vs. comparator) (ns) | ↓ n-6/n-3 ratio; ↑ n-3 PUFAs and DHA in serum and erythrocyte phospholipids; ↑ EPA in serum phospholipid (both).<br><br>↓ Palmitic acid and total SFAs; ↑ oleic acid, DHA, MUFA/SFA and MUFAs in serum phospholipids (intervention vs. comparator) |
| Nourian 2020, Iran (41) | HS (USnd), ALT, AST, anthropometry, HBM questionnaire, and diet record diaries. | ↓ HS (both) (intervention vs. control)<br>↓ ALT (intervention) ↓ AST (both)<br>↓ ALT and AST (intervention vs. control) | **Intervention:** 5/41, 12.2%<br>**Control:** 8/41, 19.5% | Improved knowledge and HBM variables (intervention) (intervention vs. control) | - |
| Abbate 2021, Spain (42) | HS (MRI), LSM, ALT, AST, cardiometabolics, anthropometry, FFQ and questionnaire. | ↓ HS (both) (intervention vs. control)<br>↓ ALT (both)<br>↓ AST (control)<br>HS: (intervention: -6.6% vs. control: -4.9%) | 155 randomised; 128 analysed (17.4%); 85 included.<br>**Intervention:** 43<br>**Control:** 42 | ↓ GGT, FG (control)<br>↓ TC and HbA1c (intervention)<br>↓ TG, fasting insulin, HOMA-IR, BP, serum ferritin and ↑ HDL (both)<br>↓ BW (both) ↓ WC (intervention)<br>BW: (intervention: -8.0% vs. control: -6.2% (ns) | ↑ MD adherence; ↓ CHO (both) (intervention vs. control)<br>↓ Cal, FAT, SFA, TFA, cholesterol, animal fat, meat/meat products; ↑ fibre and legumes (both)<br>↓ PRO, Vit B3, alcohol and sweets/pastries (control)<br>↑ Mg, P, K, Vit B6, folic acid, milk/dairy, fish, nuts, vegetable sources; ↓ cereals (intervention)<br>↓ Sodium; ↑ fruit (intervention) (intervention vs. control) |
| Yaskolka Meir 2021, Israel* (28) | HS ($^{1}$H-MRS), ALT, AST, cardiometabolics, anthropometry and FFQ. | ↓ HS (intervention vs. comparator/control)<br>↓ ALT (intervention/ comparator) (intervention/ comparator vs. control)<br>↓ AST (intervention/ comparator)<br>Median HS: (intervention: -2.0% vs. comparator: -1.1% and control: -0.7%) | **Intervention:** 9/98, 9.2%<br>**Comparator:** 14/98, 14.3%<br>**Control:** 7/98, 7.1% | ↓ ALP (intervention/ comparator) (intervention/ comparator vs. control)<br>↓ BW (intervention/ comparator)<br>↓ WC (all) (intervention vs. control)<br>↓ BW: (intervention: -4.0% and comparator: -2.9% vs. control: -0.4%) | ↓ CHO and ↑ walnuts (intervention/ comparator vs. control); ↓ red meat and poultry; ↑ green tea and green shake (intervention vs. comparator); ↑ serum folate (all) (intervention vs. control); ↑ plasma polyphenols (intervention/comparator vs. control) |
| Gepner 2018, Israel* (29) | HS (MRI), cardiometabolics, anthropometry and FFQ. | ↓ HS (intervention vs. comparator) | **Intervention:** 17/139, 12.2%<br>**Comparator:** 21/139, 15.1% | ↓ TG, diastolic BP and ↑ HDL (intervention vs. comparator)<br>↓ BW (both)<br>↓ WC (intervention vs. comparator) | ↓ Cal, PRO and PUFA (both)<br>↓ CHO, TFA and cholesterol (both) (intervention vs. comparator)<br>↓ FAT, MUFA and SFA (both) (comparator vs. intervention)<br>↑ nuts (intervention vs. comparator) |
| Mazzotti 2018, Italy* (27) | FLI, NFS, FIB-4, ALT, cardiometabolics, anthropometry, diet questionnaire. | ↓ FLI, FIB-4 and ALT (both)<br>↓ FLI (intervention vs. comparator) | **Intervention:** 160/278, 57.6%<br>**Comparator:** 137/438, 31.3% | ↓ BW (both)<br>BW: -5.5% vs.-4.2% (intervention vs. comparator) | ↓ Cal (both) |
| Willmann 2019, Germany* (30) | HS ($^{1}$H-MRS), ALT, AST, cardiometabolics, anthropometry and diet record diaries. | ↓ HS (intervention/comparator)<br>↓ AST (comparator/control)<br>HS RR: -29% vs. -26% vs. -10% (intervention vs. comparator vs. control) (ns) | **Intervention:** 10/58, 17.2%<br>**Comparator:** 16/60, 26.7%<br>**Control:** 20/60, 33.3% | ↓ GGT, FG, TC and BW (all)<br>↓ Fasting insulin (intervention/comparator)<br>↓TG (intervention/control)<br>BW: -4.4% vs. -3.0% vs. -3.4% (intervention vs. comparator vs. | ↓ Cal, CHO, PRO, and FAT (all)<br>↓ iron (intervention) ↑ iron (comparator); (intervention vs. comparator/control)<br>↑ fibre (comparator/ control) (comparator vs. intervention/control). |

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|---|---|---|---|---|---|
| | | | | control) (ns) | |
| Dorosti 2020, Iran (43) | HS (USnd), ALT, AST, cardiometabolics, anthropometry and recalls. | ↓ HS, ALT and AST (intervention vs. control) | **Intervention:** 9/56, 16.1%<br>**Control:** 9/56, 16.1% | ↓ GGT, systolic and diastolic BP (intervention vs. control)<br>↓ BW -1.8% vs. -1.0% (intervention vs. control) (ns) | ↓ Cal (intervention vs. control)<br>↑ fibre (intervention)<br>↑ wholegrains and FAT (intervention vs. control); ↑ grains, vegetables and meat (control vs. intervention) |
| Shidfar 2018, Iran (44) | HS (USnd), ALT, AST, anthropometry and diet questionnaire. | ↓ HS and AST (intervention)<br>↓ALT (both)<br>↓ALT and AST (intervention vs. control) | **Intervention:** 4/25, 16.0%<br>**Control:** 3/25, 12.0% | ↓ WC (both)<br>↓ BW (both)<br>BW: -4.3% vs.-3.5% (intervention vs. control) (ns) | ↓ Cal, CHO, PRO, FAT, PUFA, and SFA (both).<br>↑ MUFA and ↓ PUFA (intervention vs. control) |
| Rezaei 2019, Iran (45) | HS (USnd), ALT, AST, cardiometabolics, anthropometry and diet record diaries. | ↓ HS and AST (both)<br>↓ ALT (comparator)<br>↓ HS (intervention vs. comparator) | **Intervention:** 6/32, 18.8%<br>**Comparator:** 6/34, 17.6% | ↓ TG (intervention)<br>↓ BW, WC and systolic/ diastolic BP (both)<br>BW: -4.1% vs. -2.9% (intervention vs. comparator) (ns) | ↑ MUFA, ↑ omega-3 and ↓ PUFA (intervention vs. control) |
| Wong 2013, Hong Kong (46) | HS ($^1$H-MRS), LSM (TE FibroScan™), ALT, AST, cardiometabolics, anthropometry and diet record diaries. | ↓ HS, LSM and ALT (intervention vs control)<br>HS: -6.7%±6.1% vs. -2.1%±6.4% (intervention vs. control) | **Intervention:** 3/77, 3.9%<br>**Control:** 6/77, 7.8% | ↓ LDL, BW and WC (intervention vs. control)<br>BW: -8.0% vs. -0.9% (intervention vs. control) | - |
| Dong 2016, China (32) | HS (USnd), FLI, NFS, ALT, AST, cardiometabolics, anthropometry and diet record diaries. | ↓ HS, ALT and FLI (intervention) (intervention vs. control)<br>↓ NFS (both) | **Intervention:** 11/141, 7.8%<br>**Control:** 9/139, 6.5% | ↓ TC, LDL and ↑ HDL (both)<br>↓ TG (intervention) (intervention vs. control)<br>↓ BW and WC (intervention)<br>BW: -1.8% vs. +0.1% (intervention vs. control) (ns) | - |
| Promrat 2010, USA (51) | Histopathology, NAS, ALT, AST, cardiometabolics, anthropometry and diet record diaries. | ↓ HS, NAS, and ALT (intervention vs control)<br>↓ballooning injury and AST (both)<br>HS: -1.1%±0.8% vs. -0.3%±0.8% (intervention vs control) | **Intervention:** 1/21, 4.8%<br>**Control:** 0/10, 0.0% | ↓ BW and WC (intervention vs. control)<br>BW: -9.3% vs -0.2% (intervention vs. control) | - |
| Cheng 2017, China (47) | HS ($^1$H-MRS), ALT, AST, cardiometabolics and anthropometry. | ↓HS (intervention)<br>HS RR: -23.2% vs +20.9% (intervention vs. control) | **Intervention:** 6/28, 21.4%<br>**Control:** 11/29, 37.9% | BW: -1.2% vs. +0.3% (intervention vs. control) (ns) | ↑ fibre (intervention vs. control) |
| Browning 2011, USA (48) | HS ($^1$H-NMR), ALT, AST, cardiometabolics, anthropometry and diet record diaries. | ↓ HS and AST (both)<br>HS: -12.0% vs. -5% (intervention vs. comparator) | **Intervention:** 0/9, 0.0%<br>**Comparator:** 0/9, 0.0% | ↓ TG and ↓ BW (both)<br>BW: -4.7% vs. -4.2% (intervention vs. comparator) (ns) | ↓ CHO; ↑ PRO and FAT (intervention vs. comparator) |
| Johari 2019, Malaysia (49) | HS (USnd), LSM (USnd SWE), ALT, AST, cardiometabolics, anthropometry, diet record diaries and recalls. | ↓ HS, LSM, ALT and AST (intervention)<br>↓ HS, LSM and ALT (intervention vs. control) | **Intervention:** 3/33, 9.1%<br>**Control:** 1/10, 10.0% | ↓ FG (intervention)<br>↓ BW (intervention)<br>BW: -2.5% vs. +1.1% (intervention vs. control) | - |
| Shojasaadat 2019, Iran (50) | ALT, AST, cardiometabolics, anthropometry, FFQ and recalls. | ↓AST (intervention) | **Intervention:** 3/38, 7.9%<br>**Control:** 4/38, 10.5% | ↓ GGT, BW and WHR (both)<br>↓ FG, fasting insulin, HOMA-IR, TC, LDL and HDL (intervention)<br>↓ BW, fasting insulin and HOMA-IR | ↓ Cal, PUFA and fibre; ↑ PRO (intervention)<br>↑ PRO (intervention vs. control) |

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| | | | | (intervention vs. control)<br>BW: -3.4% vs. -1.2% (intervention vs. control) | |
| Ghetti 2019, Brazil (52) | ALT, AST, cardiometabolics, anthropometry and FFQ. | ↓ALT and ↓AST (intervention)<br>↓AST (intervention vs. control) | **Intervention:** 2/22, 9.1%<br>**Control:** 2/22, 9.1% | ↓ GGT, FG, HOMA-IR, TC, TG, BW and WC (intervention)<br>↓ GGT, HOMA-IR, and fasting insulin (intervention vs. control)<br>BW: -4.7% vs +0.7% (intervention vs. control) (ns) | - |
| Holmer 2021, Sweden* (31) | HS (MRS), LSM (TE FibroScan™), ALT, AST, cardiometabolics, anthropometry, diet record diaries and recalls | ↓ HS (all) (intervention/comparator vs. control)<br>↓ LSM (intervention/control) (intervention/control vs. comparator)<br>↓ ALT (all) ↓ AST (control)<br>HS: -6.1% and -7.2% vs. -3.6% (intervention and comparator vs. control) | **Intervention:** 1/25, 4.0%<br>**Comparator:** 5/25, 20.0%<br>**Control:** 4/24, 16.7% | ↓TC and LDL (intervention)<br>↑ HDL ↓ BP (comparator)<br>↓ TG, HbA1c and HOMA-IR (intervention/comparator)<br>↓ BW, WHR (all)<br>BW: intervention: -7.4% vs comparator: -7.7% vs. control: -2.6% (ns) | ↓ Cal and SFA (intervention/control)<br>↓ MUFA and fibre (intervention)<br>↓ CHO, fibre; ↑ ALA and LA (plasma), FAT, SFA, PUFA and MUFA (comparator)<br>↑ PRO (intervention/comparator) |

* Study not included in meta-analyses. HS and BW data presented as mean (unless indicated otherwise). USA, United States of America; HS, hepatic steatosis; RR, relative reduction; USnd, ultrasound; LSM, liver stiffness measurement; ARFI, acoustic radiation force impulse; MRS-PDFF, magnetic resonance spectroscopy-measured proton density fat fraction; $^{1}$H-MRS, proton magnetic resonance spectroscopy; MRI, magnetic resonance imaging; $^{1}$H-NMR, proton nuclear magnetic resonance; TE, transient elastography; SWE, shear wave elastography; FLI, Fatty Liver Index; NAFLD, non-alcoholic fatty liver disease; NFS, NAFLD Fibrosis Score; FIB-4, Fibrosis-4 Index; NAS, NAFLD Activity Score; ALT, alanine aminotransferase; AST, aspartate aminotransferase; ALP, alkaline phosphatase; GGT, gamma-glutamyl transferase; QoL, quality of life; health belief model; BW, body weight; WC, weight circumference; BMI, body mass index; WHR, waist-to-hip ratio; CVR, cardiovascular risk; FG, fasting glucose; HOMA-IR, Homeostatic Model Assessment of Insulin Resistance; glycated haemoglobin, HbA1c; TC, total cholesterol; LDL, low-density lipoprotein; HDL, high-density lipoprotein; Non-HDL, non-high-density lipoprotein; TG, triglycerides; FRS, Framingham risk score; BP, blood pressure; FFQ, food frequency questionnaire; Cal, calorie; CHO, carbohydrate, PRO, protein; MUFA, monounsaturated fat; PUFA, polyunsaturated fat; SFA, saturated fat; TFA, trans-fat, n-3 PUFA, omega 3 polyunsaturated fatty acid; MD, Mediterranean diet, ALA; α-linolenic acid, LA; linoleic acid; DHA, n-3 docosahexaenoic acid; EPA, n-3 eicosapentaenoic acid; TAC, total antioxidant capacity; Mg, magnesium; K, potassium; P, phosphorus; Vit, vitamin; B3, Niacin; B6, pyridoxine; ns, not significant

## Appendix J: Sensitivity analyses.

| Outcome | N | Effect Estimate (mean difference) | $I^2$ | P |
|---|---|---|---|---|
| **ALT** | 1241 | -6.28 [-9.21, -3.34] -6.34 [-9.37, -3.30] | 67% 70% | <0.001 |
| *MD* | 364 | -6.54 [-12.02, -1.05] -6.72 [-12.66, -0.79] | 81% 85% | 0.02 0.03 |
| *MDC* | 210 | -5.99 [-12.93, 0.95] -5.99 [-12.93, 0.95] | 74% | 0.09 |
| *CRI* | 667 | -5.44 [-8.01, -2.88] -5.44 [-8.01, -2.88] | 0% | <0.001 |
| **AST** | 1122 | -2.79 [-4.66, -0.91] -2.98 [-4.87, -1.09] | 75% 76% | 0.004 0.002 |
| *MD* | 284 | -3.40 [-7.18, 0.37] -4.19 [-8.09, -0.28] | 81% 83% | 0.08 0.04 |
| *MDC* | 210 | -3.21 [-7.83, 1.41] -3.21 [-7.83, 1.41] | 85% | 0.17 |
| *CRI* | 628 | -1.61 [-3.82, 0.60] -1.61 [-3.82, 0.60] | 53% | 0.15 |
| **FLI** | 488 | -13.52 [-20.05, -7.00] | 80% | <0.001 |
| *MD* | 228 | -15.60 [-22.01, -9.18] | 71% | <0.001 |
| *CRI* | 260 | -7.34 [-13.11, -1.57] | NA | 0.01 |
| **Body weight** | 1172 | -0.97 [-2.60, 0.66] -0.98 [-2.75, 0.79] | 33% 40% | 0.24 0.28 |
| *MD* | 295 | -0.17 [-3.61, 3.28] -0.00 [-4.26, 4.25] | 63% 74% | 0.93 1.00 |
| *MDC* | 210 | 0.65 [-2.52, 3.83] 0.65 [-2.52, 3.83] | 0% | 0.69 |
| *CRI* | 667 | -1.57 [-3.31, 0.16] -1.57 [-3.31, 0.16] | 0% | 0.08 |
| **Outcome** | **N** | **Effect Estimate (standardised mean difference)** | $I^2$ | P |
| **HS (imaging/ histology)** | 466 | -0.40 [-0.74, -0.06] -0.42 [-0.79, -0.05] | 66% 71% | 0.02 0.03 |
| *MD* | 209 | -0.11 [-0.66, 0.45] -0.07 [-0.77, 0.63] | 76% 84% | 0.70 0.85 |
| *CRI* | 257 | -0.72 [-0.97, -0.46] -0.72 [-0.97, -0.46] | 0% | <0.001 |
| **LSM** | 464 | -0.61 [-1.09, -0.13] | 81% | 0.01 |
| *MD* | 271 | -0.75 [-1.51, 0.00] | 87% | 0.05 |
| *CRI* | 193 | -0.39 [-0.69, -0.10] | 0% | 0.009 |

**Figure 1.** Excluding crossover trials. This resulted in the exclusion of 2 studies and 54 participants. Black, main model; blue, sensitivity analysis. ALT, alanine aminotransferase; AST, aspartate aminotransferase; FLI, Fatty liver index; HS, hepatic steatosis; LSM, liver stiffness measurement; MD, Mediterranean diet; MDC, Mediterranean diet component; CRI, calorie-restricted interventions; NA, not applicable.

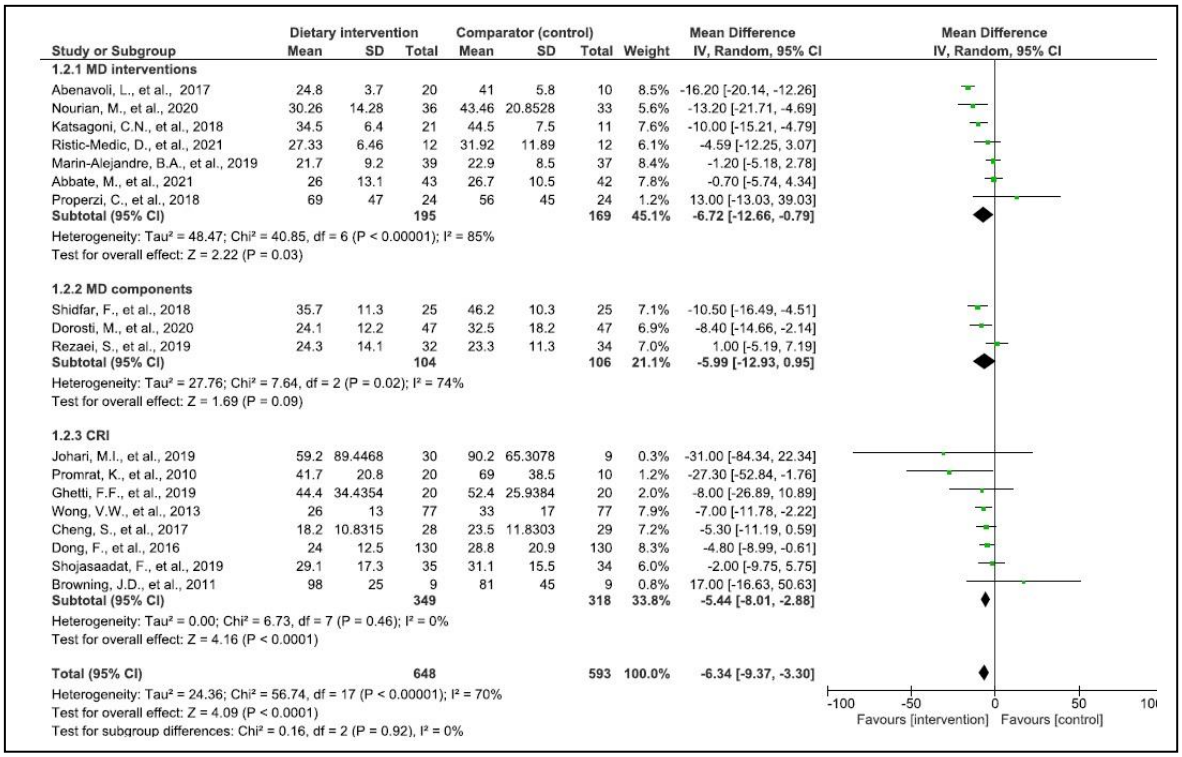



**Figure 2.** Excluding crossover trials. Effects of dietary interventions on alanine aminotransferase (ALT). SD, standard deviation; IV, inverse variance; CI, confidence interval; MD, Mediterranean diet; CRI, calorie-restricted interventions.

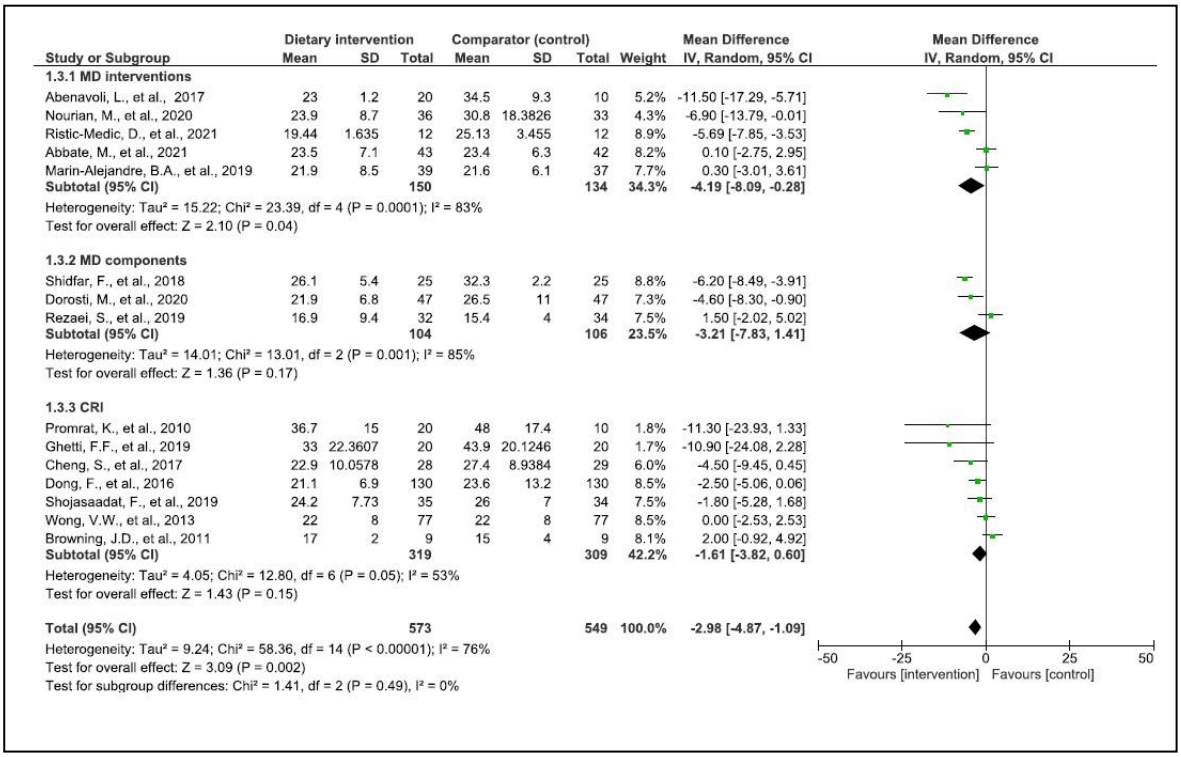



**Figure 3.** Excluding crossover trials. Effects of dietary interventions on aspartate aminotransferase (AST). SD, standard deviation; IV, inverse variance; CI, confidence interval; MD, Mediterranean diet; CRI, calorie-restricted interventions.

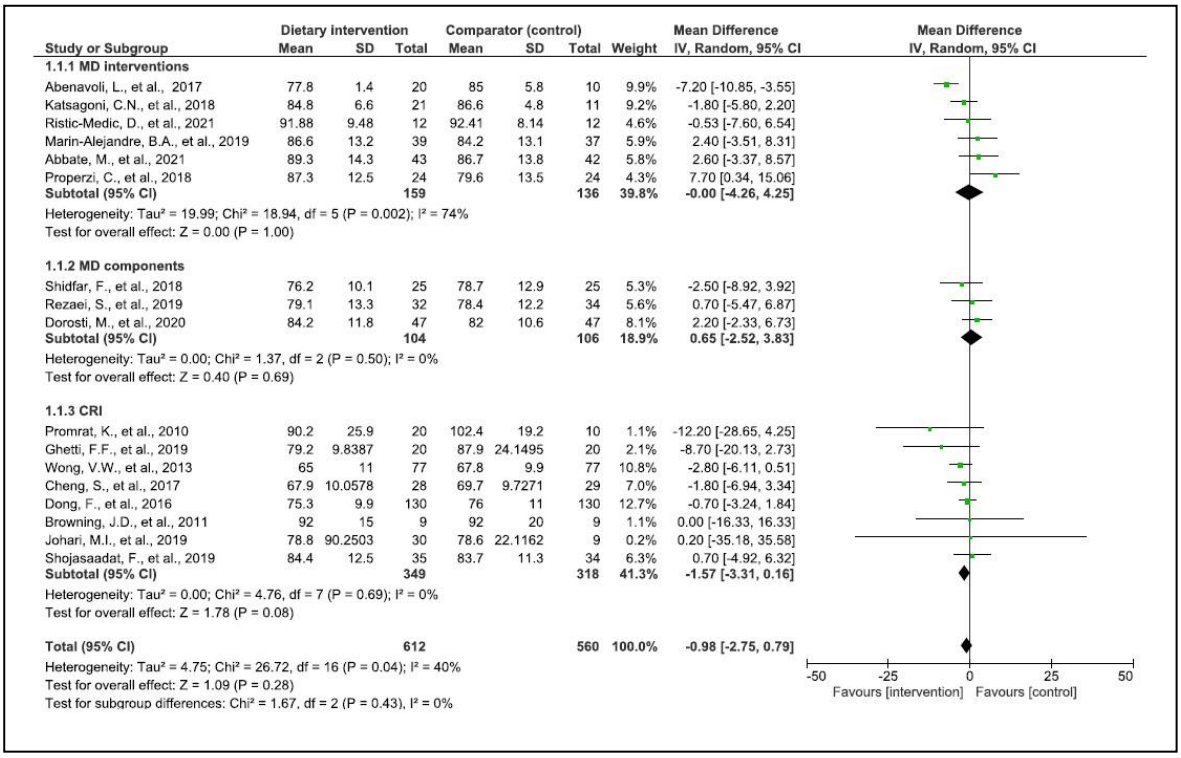



**Figure 4.** Excluding crossover trials. Effects of dietary interventions on body weight. SD, standard deviation; IV, inverse variance; CI, confidence interval; MD, Mediterranean diet; CRI, calorie-restricted interventions.

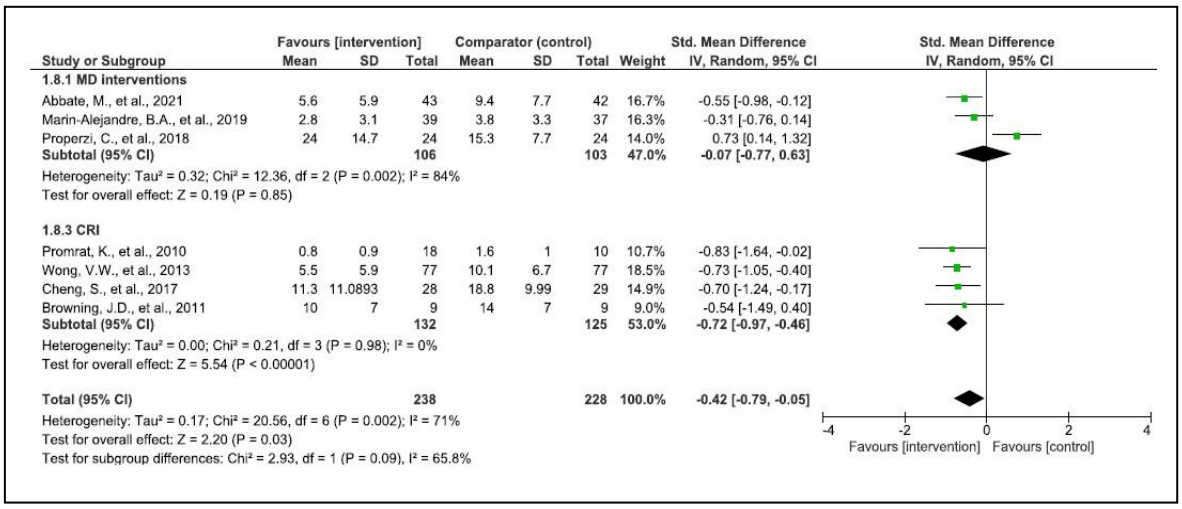



**Figure 5.** Excluding crossover trials. Effects of dietary interventions on hepatic steatosis. SD, standard deviation; IV, inverse variance; CI, confidence interval; MD, Mediterranean diet; CRI, calorie-restricted interventions.

| Outcome | N | Effect Estimate (mean difference) | $I^2$ | *P* |
|---|---|---|---|---|
| **ALT** | 765 | -6.28 [-9.21, -3.34] -6.00 [-10.38, -1.62] | 67% 77% | <0.001 0.007 |
| *MD* | 171 | -6.54 [-12.02, -1.05] -8.37 [-16.72, -0.02] | 81% 87% | 0.02 0.05 |
| *MDC* | 66 | -5.99 [-12.93, 0.95] 1.00 [-5.19, 7.19] | 74% NA | 0.09 0.75 |
| *CRI* | 528 | -5.44 [-8.01, -2.88] -5.57 [-8.33, -2.81] | 0% 0% | <0.001 <0.001 |
| **AST** | 670 | -2.79 [-4.66, -0.91] -1.46 [-3.90, 0.98] | 75% 74% | 0.004 0.24 |
| *MD* | 115 | -3.40 [-7.18, 0.37] -5.42 [-16.77, 5.94] | 81% 92% | 0.08 0.35 |
| *MDC* | 66 | -3.21 [-7.83, 1.41] 1.50 [-2.02, 5.02] | 85% NA | 0.17 0.40 |
| *CRI* | 489 | -1.61 [-3.82, 0.60] -0.89 [-3.35, 1.56] | 53% 61% | 0.15 0.48 |
| **FLI** | 388 | -13.52 [-20.05, -7.00] -15.42 [-23.74, -7.11] | 80% 88% | <0.001 <0.001 |
| *MD* | 128 | -15.60 [-22.01, -9.18] -19.24 [-26.10, -12.38] | 71% 77% | <0.001<0.001 |
| *CRI* | 260 | -7.34 [-13.11, -1.57] -7.34 [-13.11, -1.57] | NA NA | 0.01 0.01 |
| **Body weight** | 765 | -0.97 [-2.60, 0.66] -1.99 [-3.81, -0.17] | 33% 26% | 0.24 0.03 |
| *MD* | 171 | -0.17 [-3.61, 3.28] -2.35 [-6.78, 2.08] | 63% 66% | 0.93 0.30 |
| *MDC* | 66 | 0.65 [-2.52, 3.83] 0.70 [-5.47, 6.87] | 0% NA | 0.69 0.82 |
| *CRI* | 528 | -1.57 [-3.31, 0.16] -1.50 [-3.36, 0.36] | 0% 0% | 0.08 0.11 |
| **Outcome** | **N** | **Effect Estimate (standardised mean difference)** | $I^2$ | *P* |
| **HS (imaging/ histology)** | 338 | -0.40 [-0.74, -0.06] -0.63 [-0.85, -0.41] | 66% 0% | 0.02 <0.001 |
| *MD* | 109 | -0.11 [-0.66, 0.45] -0.48 [-0.86, -0.10] | 76% 0% | 0.70 0.01 |
| *CRI* | 229 | -0.72 [-0.97, -0.46] -0.70 [-0.97, -0.44] | 0% 0% | <0.001 <0.001 |
| **LSM** | 340 | -0.61 [-1.09, -0.13] -0.89 [-1.53, -0.26] | 81% 83% | 0.01 0.006 |
| *MD* | 147 | -0.75 [-1.51, 0.00] -1.38 [-2.78, 0.03] | 87% 91% | 0.05 0.05 |
| *CRI* | 193 | -0.39 [-0.69, -0.10] -0.39 [-0.69, -0.10] | 0% 0% | 0.009 0.009 |

**Figure 6.** Excluding trials judged as serious, critical or high risk of bias. This resulted in the exclusion of 10 studies and 530 participants. Black, main model; blue, sensitivity analysis. ALT, alanine aminotransferase; AST, aspartate aminotransferase; FLI, Fatty liver index; HS, hepatic steatosis; LSM, liver stiffness measurement; MD, Mediterranean diet; MDC, Mediterranean diet component; CRI, calorie-restricted interventions; NA, not applicable.

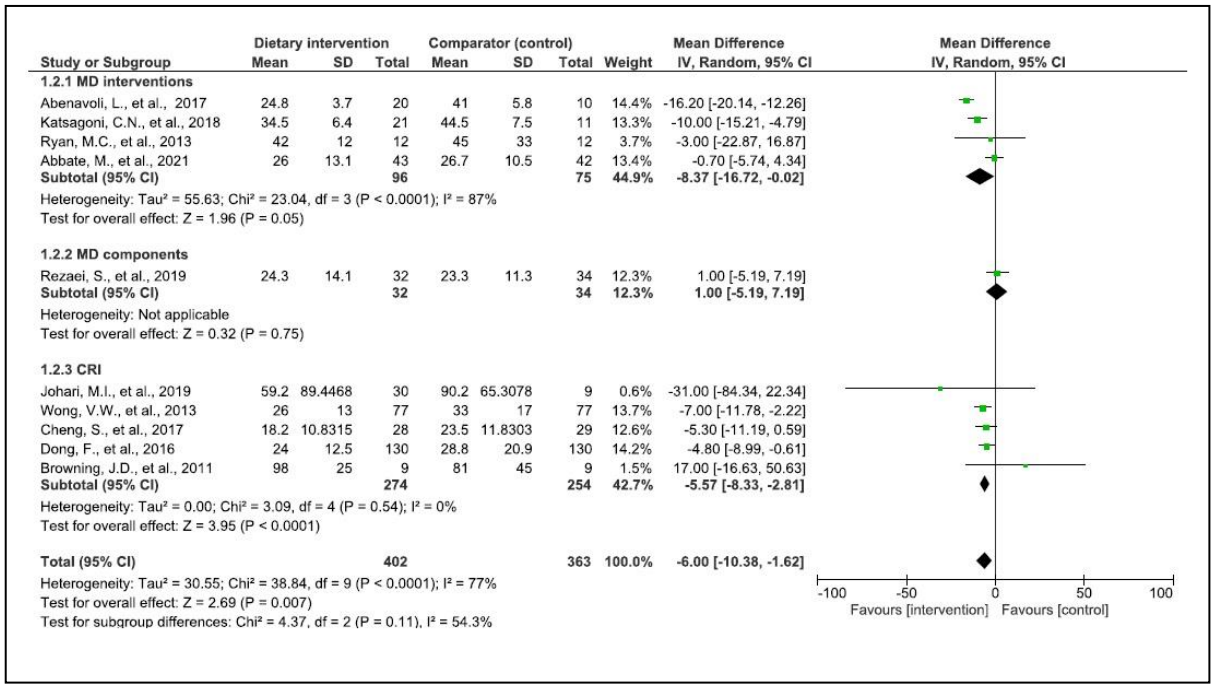



**Figure 7.** Sensitivity analyses: excluding trials judged as serious, critical or high risk of bias. Effects of dietary interventions on alanine aminotransferase (ALT). SD, standard deviation; IV, inverse variance; CI, confidence interval; MD, Mediterranean diet; CRI, calorie-restricted interventions.

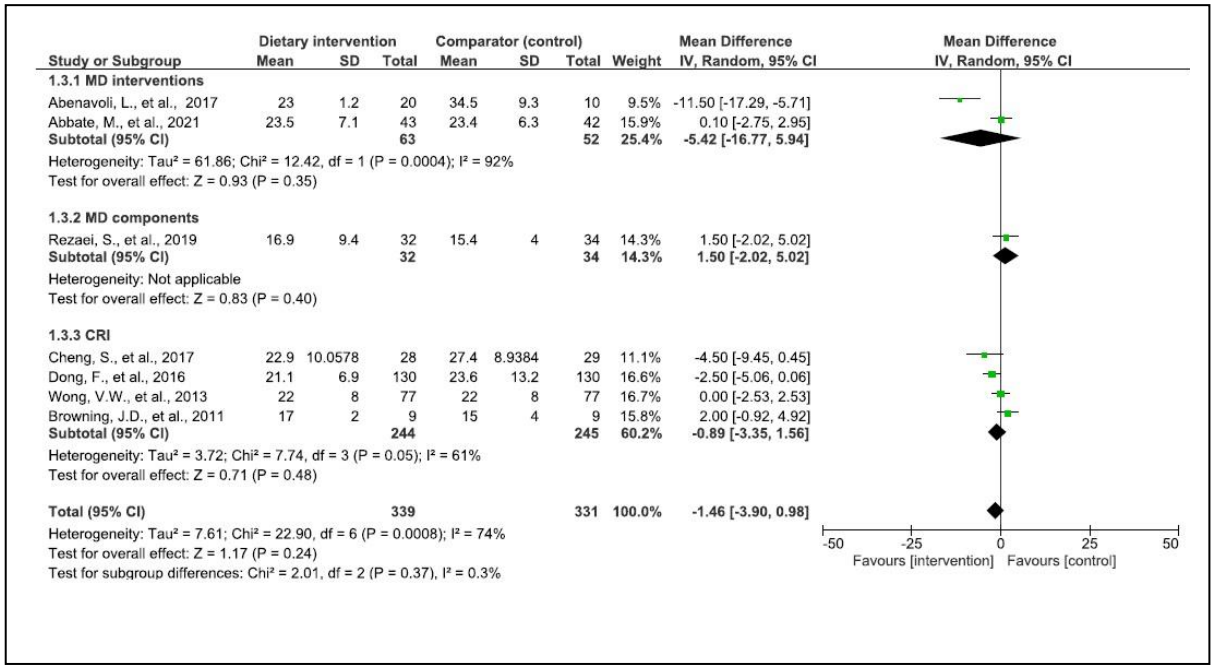



**Figure 8.** Sensitivity analyses: excluding trials judged as serious, critical or high risk of bias. Effects of dietary interventions on aspartate aminotransferase (AST). SD, standard deviation; IV, inverse variance; CI, confidence interval; MD, Mediterranean diet; CRI, calorie-restricted interventions.

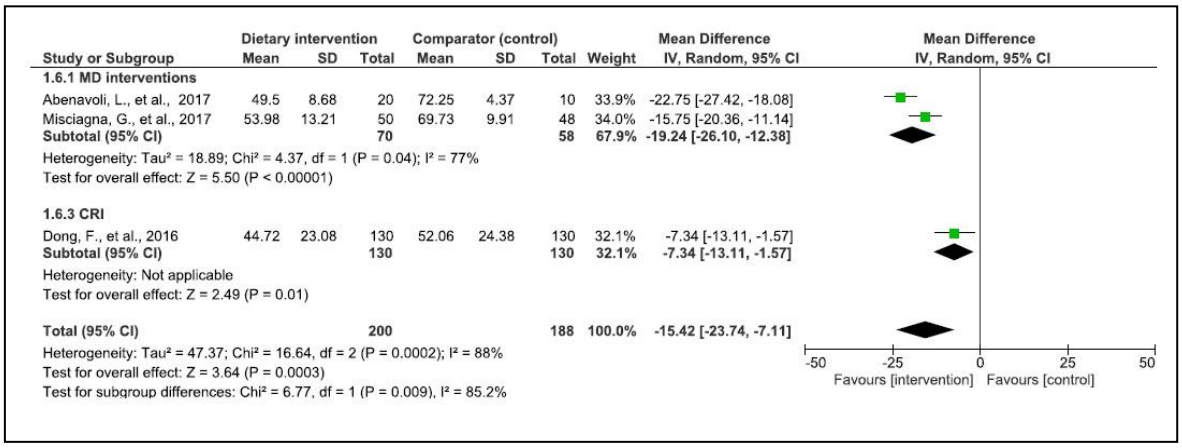



**Figure 9.** Sensitivity analyses: excluding trials judged as serious, critical or high risk of bias. Effects of dietary interventions on Fatty Liver Index (FLI). SD, standard deviation; IV, inverse variance; CI, confidence interval; MD, Mediterranean diet; CRI, calorie-restricted interventions.

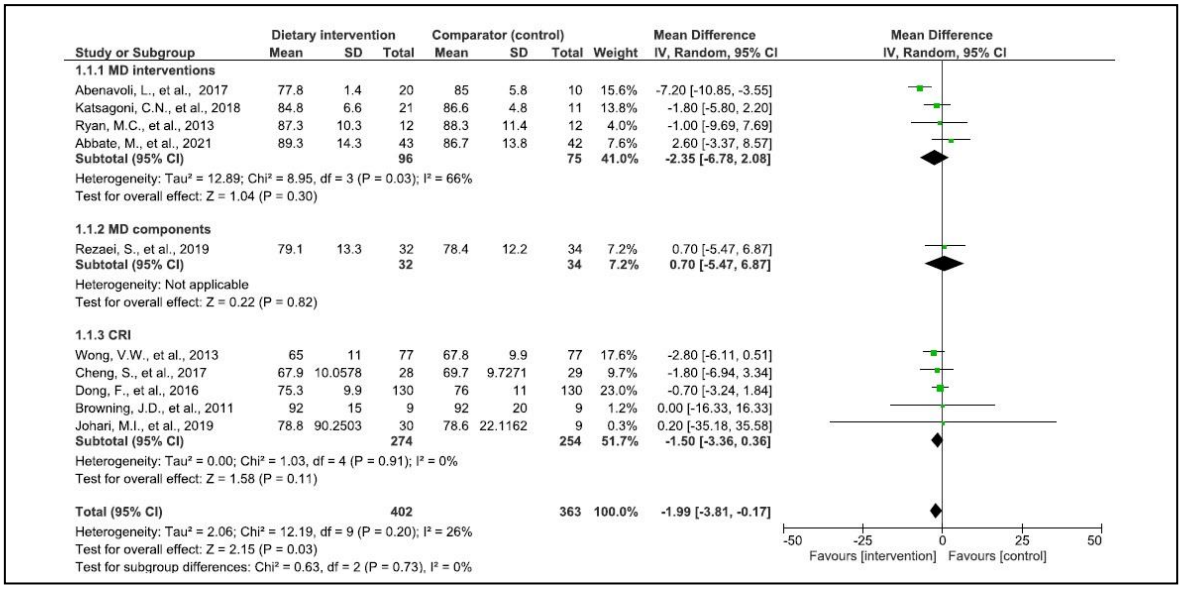



**Figure 10.** Sensitivity analyses: excluding trials judged as serious, critical or high risk of bias. Effects of dietary interventions on body weight. SD, standard deviation; IV, inverse variance; CI, confidence interval; MD, Mediterranean diet; CRI, calorie-restricted interventions.

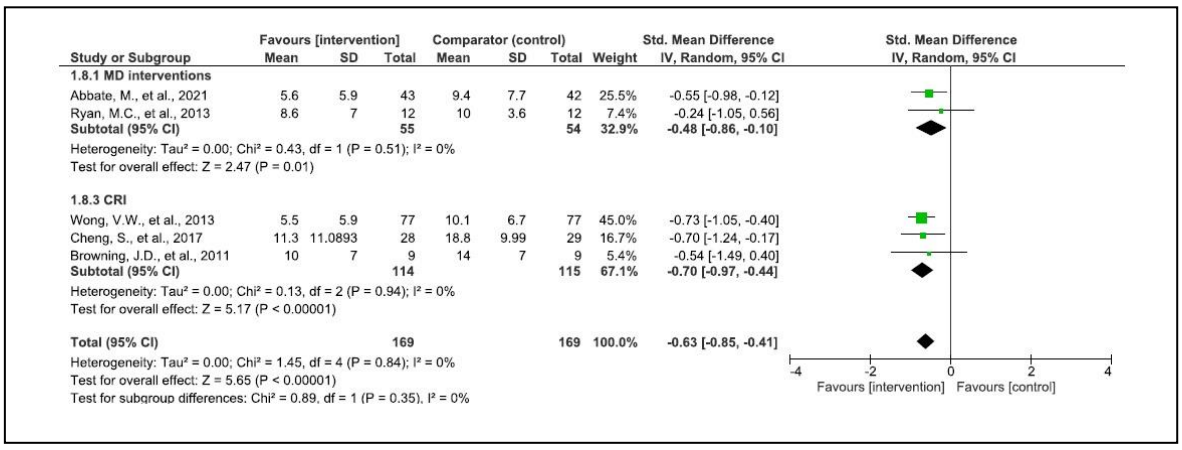



**Figure 11.** Sensitivity analyses: excluding trials judged as serious, critical or high risk of bias. Effects of dietary interventions on hepatic steatosis. SD, standard deviation; IV, inverse variance; CI, confidence interval; MD, Mediterranean diet; CRI, calorie-restricted interventions.

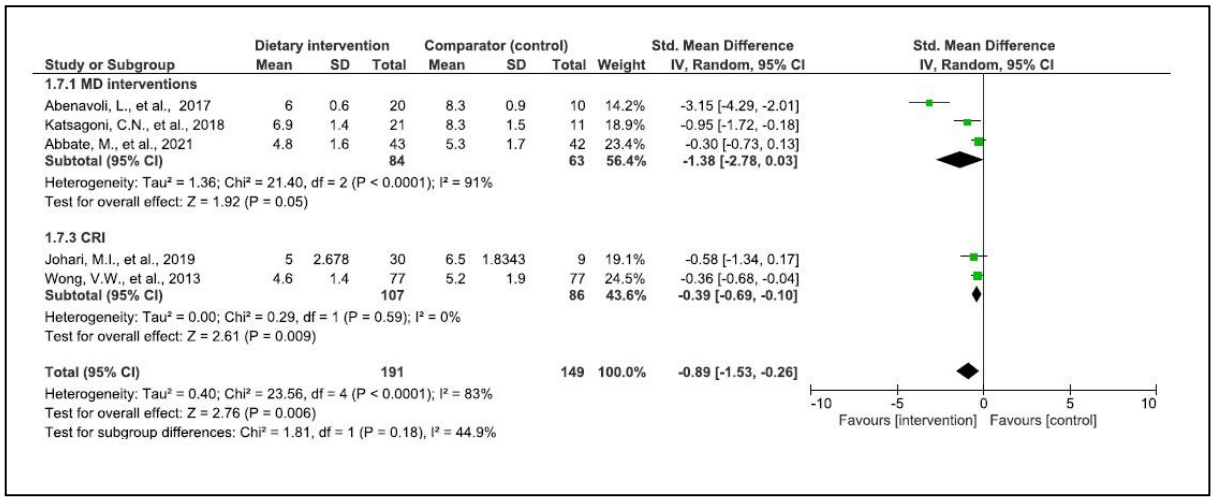



**Figure 12.** Sensitivity analyses: excluding trials judged as serious, critical or high risk of bias. Effects of dietary interventions on liver stiffness measurement (LSM). SD, standard deviation; IV, inverse variance; CI, confidence interval; MD, Mediterranean diet; CRI, calorie-restricted interventions.

## Appendix K: E-survey instrument.

1. Please tell us which organisation you are employed by:
   1a. Which European country does this correspond to?

2. Please describe your centre according to one of the following categories:
   A liver transplant unit
   An academic liver centre
   A district general hospital (community hospital)
   A public hospital
   A private hospital
   Primary care
   Other
   2a. If you selected Other, please specify:

3. Please select your professional role:
   Gastroenterologist/ hepatologist
   Specialist trainee
   gastroenterology/ hepatology
   Diabetologist
   Endocrinologist
   Non consultant grade doctor
   Specialist liver nurse
   Dietitian/ nutritionist
   General practitioner/ primary
   care practitioner
   General practice nurse/primary care nurse
   Other
   3a. If you selected Other, please specify:

4. Age:

5. Sex:

6. What is the maximum level of alcohol intake you would consider to be consistent with a diagnosis of NAFLD? (1 unit =10g)
   Abstinence
   <14 units (males and females)
   per week
   14 units (females) 21 units
   (males) per week
   22-30 units per week
   Other
   6a. If you selected Other, please specify:

7. Please indicate which lifestyle and patient experience data are routinely recorded: (select all that apply)
   Weight
   BMI
   Waist circumference
   Dietary intake
   Alcohol intake
   Physical activity levels
   Factors affecting behaviour change readiness
   Reasons for opting out
   Patient satisfaction

8. With respect to NAFLD patients, what is the main feature you use to select individuals for diet lifestyle intervention?
   All NAFLD patients are eligible
   Disease severity (moderate or advanced fibrosis)
   BMI
   Presence of 2 or more metabolic syndrome features
   Cardiovascular disease risk
   Multi-morbidity
   Presence of other
   nutrition-related illness/ complex nutritional needs
   Patients that have not
   achieved previously achieved
   lifestyle goals
   Behaviour change readiness
   Patient requested referral
   Other
   8a. If you selected Other, please specify:

9. What proportion of NAFLD patients do you refer for diet lifestyle intervention?
   <10%
   11-20%
   21-30%
   31-40%
   41-50%
   >50%
   I don't refer

10. Which of the following dietetic/ nutrition services do you access for NAFLD patients?
    Access via a multidisciplinary clinic
    Access via direct referrals to a dietetic/nutrition department
    Access via referrals made by either general practitioners or endocrinologists
    None of the above

11. Which of the following weight management services do you access for NAFLD patients? (select all that apply)
    Community-based weight management services
    Specialist medical and dietetic/ nutrition weight management services
    Bariatric surgical services
    Commercial weight management providers
    None of the above
    Other
    11a. If you selected Other, please specify:

12. What prevents the effective referral of these patients for diet lifestyle intervention? (select all that apply)
    Short consultation time/ work overload
    No locally commissioned services or gaps in service provision
    Extent of service provision unknown
    Uncertainty about referral routes, entry criteria and service details
    Limited evidence and outcomes reported
    Limited funding/ resources available for additional dietetic input
    Insufficient patient behaviour change readiness
    Diet lifestyle interventions not appropriate for specific patients
    Patient already engaged with service
    Other
    12a. If you selected Other, please specify:

13. What advice would you deliver prior to any referral for additional diet lifestyle intervention?

Weight loss
Alcohol consumption
Calorie restriction
Diet quality/ eating patterns
Physical activity

13a. If you advise weight loss, what would you target?

Any weight loss
Weight loss target 5%
Weight loss target 7-10%
Weight loss target >10%

14. Please specify what else is covered in the advice you provide for NAFLD patients: (select all that apply)

The impact of weight status on NAFLD outcomes
An exploration of weight and dieting history
An assessment of energy balance and benefits of diet/lifestyle change
Agreement of weight loss targets, diet and physical activity goals
Agreement of self-monitoring measures
Behaviour change counselling
Prescribing of anti-obesity medications
Educational materials
Signposting to relevant resources and further help
Other

14a. If you selected Other, please specify:

15. How would you describe the advice you deliver?

Very brief intervention: single session (typically last <5 minutes)
Brief intervention: single session (typically last between 5-30 minutes)
Extended brief intervention: single or multiple sessions (typically last > 30minutes)
Structured Multicomponent Programme: multiple sessions (typically last >45 minutes)

16. Which patients do you follow-up and what are the typical contact intervals?

16.1 Steatosis with no significant fibrosis (F0/F1)
16.2 NASH with no significant fibrosis
16.3 NASH with significant fibrosis (F2+)
16.4 Cirrhosis

Weekly
Fortnightly
Monthly
Three months
Six months
Annually
No Follow-up

16a. If you selected Other, please specify:

17. What are the barriers in your practice delivering lifestyle advice for NAFLD patients? (select all that apply)

Short consultation time/ work overload
Inadequate training in lifestyle and behaviour change counselling
Don't think I have a role in this
Insufficient patient behaviour change readiness
Diet lifestyle interventions not appropriate for specific patients
Patient already engaged with service
Other

17a. If you selected Other, please specify:

18. Please specify which dietary approaches are prescribed for NAFLD patients either by you or another healthcare professional? (select all that apply)
National healthy eating guidelines
Reduction in Western diet components
Low fat diet
Mediterranean diet
Low glycaemic index
Low carbohydrate (<130g/d)
600 calorie deficit diet
Low energy diet
Very low energy diet
Intermittent energy restriction/ fasting
Other

18a. If you selected Other, please specify:

19. Please indicate how these approaches are routinely delivered? (select all that apply)
Face-to-face
Online
Software app
Groups
Telephone
Other

19a. If you selected Other, please specify:

20. Is there anything else you would like to share with us?

## Appendix L: Mediterranean diet meals example options.

| Meal (one serving per container) | Protein(g) | Carb(g) | Fat(g) | Calories |
|---|---|---|---|---|
| Beef Bolognese with vegetables and whole-wheat pasta | 42 | 43 | 14 | 466 |
| Romesco chicken with vegetables and whole-wheat pasta | 38 | 45 | 17 | 485 |
| Garlic and herb chicken with vegetable medley | 36 | 27 | 16 | 396 |
| Chicken, basil and tomato stir fry | 36 | 40 | 18 | 466 |
| Mediterranean falafels with vegetable medley and tahini | 10 | 39 | 15 | 331 |
| Cod on vegetable pasta with tomato and basil sauce | 30 | 45 | 14 | 426 |
| Halloumi cashew nut and broccoli curry | 26 | 50 | 23 | 511 |
| Tomato, black bean with cashews and quinoa | 12 | 36 | 16 | 361 |
| Tomato chickpea vegetable curry | 14 | 38 | 13 | 353 |
| Chipotle sweet potato chilli with spinach | 20 | 65 | 6 | 394 |
| Chicken salad with pesto pasta | 36 | 30 | 15 | 399 |
| Garlic and herb chicken salad | 32 | 25 | 9 | 309 |
| Tuna and cheese with pesto pasta salad | 36 | 38 | 12 | 404 |
| Feta, pine nut and pesto cous salad | 16 | 40 | 17 | 377 |
| Red pesto salmon with vegetable medley | 24 | 40 | 26 | 490 |
| Honey chilli salmon salad | 23 | 25 | 20 | 313 |
| Mozzarella, roasted tomato and pesto pasta salad | 19 | 47 | 25 | 477 |
| Grilled halloumi and roasted pepper salad | 23 | 25 | 23 | 399 |
| Falafel salad with tahini, avocado and sriracha in Mediterranean herb wrap | 20 | 55 | 18 | 462 |
| Chicken with broccoli cashew nut pesto pasta | 38 | 39 | 17 | 461 |
| Lentil dahl | 23 | 75 | 5 | 437 |
| Vegetable balls and tomato pesto sauce and pasta | 12 | 56 | 16 | 416 |
| Chickpea and vegetable curry with rice | 23 | 70 | 16 | 516 |
| Butternut, lentil and spinach | 15 | 28 | 18 | 372 |

## Appendix M: Euroqol five dimension scale (5D-5L)

ED-5D-5L Health Questionnaire English version for the UK.

Under each heading, please tick the ONE box that best describes your health TODAY.

**MOBILITY**

I have no problems in walking about □

I have slight problems in walking about □

I have moderate problems in walking about □

I have severe problems in walking about □

I am unable to walk about □

**SELF-CARE**

I have no problems washing or dressing myself □

I have slight problems washing or dressing myself □

I have moderate problems washing or dressing myself □

I have severe problems washing or dressing myself □

I am unable to wash or dress myself □

**USUAL ACTIVITIES** *(e.g., work, study, housework, family or leisure activities)*

I have no problems doing my usual activities □

I have slight problems doing my usual activities □

I have moderate problems doing my usual activities □

I have severe problems doing my usual activities □

I am unable to do my usual activities □

**PAIN / DISCOMFORT**

I have no pain or discomfort □

I have slight pain or discomfort □

I have moderate pain or discomfort □

I have severe pain or discomfort □

I have extreme pain or discomfort □

**ANXIETY / DEPRESSION**

I am not anxious or depressed □

I am slightly anxious or depressed □

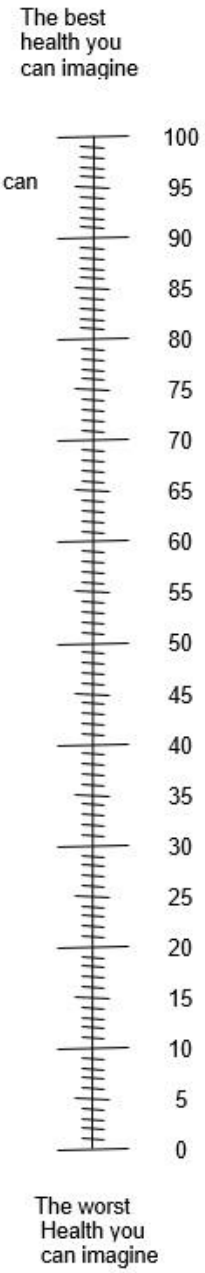
I am moderately anxious or depressed □

I am severely anxious or depressed □

I am extremely anxious or depressed □

- We would like to know how good or bad your health is TODAY.
- This scale is numbered from 0 to 100.
- 100 means the best health you can imagine. 0 means the worst health you can imagine.
- Mark an X on the scale to indicate how your health is TODAY.
- Now, please write the number you marked on the scale in the box below.

YOUR HEALTH TODAY = ☐

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## Appendix N: Chronic liver disease questionnaire for non-alcoholic-steatohepatitis (CLDQ-NASH)

### The Chronic Liver Disease Questionnaire for NAFLD NASH

### (CLDQ NAFLD-NASH)

This questionnaire is designed to find out how you have been feeling during the last two weeks. You will be asked about your symptoms related to fatty liver disease, how you have been affected when doing activities and how your mood has been. Please complete all of the questions and select only **one** response for each question.

**1. Over the last two weeks, how often have you felt troubled by a feeling of abdominal bloating?**

1 All of the time
2 Most of the time
3 A good bit of the time
4 Some of the time
5 A little of the time
6 Hardly any of the time
7 None of the time

**2. Over the last two weeks, how often have you felt tired or fatigued?**

1. All of the time
2. Most of the time
3. A good bit of the time
4. Some of the time
5. A little of the time
6. Hardly any of the time
7. None of the time

**3. Over the last two weeks, how often have you experienced bodily pain?**

1. All of the time
2. Most of the time
3. A good bit of the time
4. Some of the time
5. A little of the time
6. Hardly any of the time
7. None of the time

**4. Over the last two weeks, have you felt drowsy during the day?**

1 All of the time
2 Most of the time
3 A good bit of the time
4 Some of the time
5 A little of the time
6 Hardly any of the time
7 None of the time

**5. Over the last two weeks, how often have you experienced abdominal pain?**

1 All of the time
2 Most of the time
3 A good bit of the time
4 Some of the time
5 A little of the time
6 Hardly any of the time
7 None of the time

**6. Over the last two weeks have you experienced shortness of breath during your daily activities?**

1 All of the time
2 Most of the time
3 A good bit of the time
4 Some of the time
5 A little of the time
6 Hardly any of the time
7 None of the time

**7. Over the last two weeks, how often have you been unable to eat as much as you would like?**

1. All of the time
2. Most of the time
3. A good bit of the time
4. Some of the time
5. A little of the time
6. Hardly any of the time
7. None of the time

**8. Over the last two weeks, how often have you felt weaker than normal?**

1 All of the time
2 Most of the time
3 A good bit of the time
4 Some of the time
5 A little of the time
6 Hardly any of the time
7 None of the time

**9. Over the last two weeks, how often have you had trouble lifting or carrying heavy objects?**

1 All of the time
2 Most of the time
3 A good bit of the time
4 Some of the time
5 A little of the time
6 Hardly any of the time
7 None of the time

**10. Over the last two weeks, how often have you felt anxious?**

1. All of the time
2. Most of the time
3. A good bit of the time
4. Some of the time
5. A little of the time
6. Hardly any of the time
7. None of the time

**11. Over the last two weeks, how often have you felt a decrease in your energy levels?**

All of the time
1 Most of the time
2 A good bit of the time
3 Some of the time
4 A little of the time
5 Hardly any of the time
6 None of the time

**12. Over the last two weeks, how often have you felt unhappy?**

1. All of the time
2. Most of the time
3. A good bit of the time
4. Some of the time
5. A little of the time
6. Hardly any of the time
7. None of the time

**13. Over the last two weeks, how often have you felt drowsy?**

1. All of the time
2. Most of the time
3. A good bit of the time
4. Some of the time
5. A little of the time
6. Hardly any of the time
7. None of the time

**14. Over the last two weeks, how often has your lack of appetite been an issue?**

1 All of the time
2 Most of the time
3 A good bit of the time
4 Some of the time
5 A little of the time
6 Hardly any of the time
7 None of the time

**15. Over the last two weeks, how often have you been irritable?**

1 All of the time
2 Most of the time
3 A good bit of the time
4 Some of the time

5 A little of the time
6 Hardly any of the time
7 None of the time

**16. Over the last two weeks, how often have you had difficulty sleeping at night?**

1. All of the time
2. Most of the time
3. A good bit of the time
4. Some of the time
5. A little of the time
6. Hardly any of the time
7. None of the time

**17. Over the last two weeks, how often have you been troubled by a feeling of abdominal discomfort?**

1. All of the time
2. Most of the time
3. A good bit of the time
4. Some of the time
5. A little of the time
6. Hardly any of the time
7. None of the time

**18. Over the last two weeks, how often have you felt worried about the impact liver disease has on your family?**

1. All of the time
2. Most of the time
3. A good bit of the time
4. Some of the time
5. A little of the time
6. Hardly any of the time
7. None of the time

**19. Over the last two weeks, how often have you had mood swings?**

1 All of the time
2 Most of the time
3 A good bit of the time
4 Some of the time
5 A little of the time
6 Hardly any of the time
7 None of the time

**20. Over the last two weeks, how often have you been unable to fall asleep at night?**

1. All of the time
2. Most of the time
3. A good bit of the time
4. Some of the time
5. A little of the time
6. Hardly any of the time
7. None of the time

**21. Over the last two weeks, how often have you had muscle cramps?**

1 All of the time
2 Most of the time
3 A good bit of the time
4 Some of the time
5 A little of the time
6 Hardly any of the time
7 None of the time

**22. Over the last two weeks, how often have you felt worried that your symptoms will develop into more serious problems?**

1 All of the time
2 Most of the time
3 A good bit of the time
4 Some of the time
5 A little of the time
6 Hardly any of the time
7 None of the time

**23. Over the last two weeks, how often have you had a dry mouth?**

1. All of the time
2. Most of the time
3. A good bit of the time
4. Some of the time
5. A little of the time
6. Hardly any of the time
7. None of the time

**24. How much of the time during the last two weeks have you felt depressed?**

1 All of the time
2 Most of the time
3 A good bit of the time
4 Some of the time
5 A little of the time
6 Hardly any of the time
7 None of the time

**25. How much of the time during the last two weeks have you been worried about your condition getting worse?**

1 All of the time
2 Most of the time
3 A good bit of the time
4 Some of the time
5 A little of the time
6 Hardly any of the time
7 None of the time

**26. How much of the time during the last two weeks have you had problems concentrating?**

1 All of the time
2 Most of the time
3 A good bit of the time
4 Some of the time
5 A little of the time

6 Hardly any of the time
7 None of the time

**27. How much of the time have you been troubled by itching during the last two weeks?**

1 All of the time
2 Most of the time
3 A good bit of the time
4 Some of the time
5 A little of the time
6 Hardly any of the time
7 None of the time

**28. How much of the time during the last two weeks have you been worried about never feeling any better?**

1. All of the time
2. Most of the time
3. A good bit of the time
4. Some of the time
5. A little of the time
6. Hardly any of the time
7. None of the time

**29. How much of the time during the last two weeks have you been concerned about the availability of a liver if you need a liver transplant?**

1 All of the time
2 Most of the time
3 A good bit of the time
4 Some of the time
5 A little of the time
6 Hardly any of the time
7 None of the time

**30. How much of the time during the last two weeks have you had trouble walking down the street or climbing two flights of stairs because of your health?**

1 All of the time
2 Most of the time
3 A good bit of the time
4 Some of the time
5 A little of the time
6 Hardly any of the time
7 None of the time

**31. How much of the time during the last two weeks have you had trouble bending, lifting, or stooping?**

1 All of the time
2 Most of the time
3 A good bit of the time
4 Some of the time
5 A little of the time
6 Hardly any of the time
7 None of the time

**32. How much of the time during the last two weeks have you had a feeling like you may die early because of your fatty liver?**

1 All of the time
2 Most of the time
3 A good bit of the time
4 Some of the time
5 A little of the time
6 Hardly any of the time
7 None of the time

**33. How much of the time during the last two weeks have you felt distressed by having fatty liver?**

1 All of the time
2 Most of the time
3 A good bit of the time
4 Some of the time
5 A little of the time
6 Hardly any of the time
7 None of the time

**34.How much of the time during the last two weeks have you not enjoyed life?**

1 All of the time
2 Most of the time
3 A good bit of the time
4 Some of the time
5 A little of the time
6 Hardly any of the time
7 None of the time

**35. How much of the time during the last two weeks have you felt the need to take naps (5 minutes or longer) during the day?**

1 All of the time
2 Most of the time
3 A good bit of the time
4 Some of the time
5 A little of the time
6 Hardly any of the time
7 None of the time

**36. How much of the time during the last two weeks have you been experiencing joint pain?**

1 All of the time
2 Most of the time
3 A good bit of the time
4 Some of the time
5 A little of the time
6 Hardly any of the time
7 None of the time

## Appendix O: Non-alcoholic steatohepatitis (NASH) CHECK

### NASH-CHECK

**Assessment of symptoms and health-related quality of life in non-alcoholic steatohepatitis (NASH)**

This questionnaire asks about your experience of living with fatty liver disease. We are interested in the symptoms that you may have experienced and how these may have affected your day-to-day life and emotions.

**Symptoms**

The following questions ask about your symptoms you may have experienced related to your fatty liver disease.

**Instructions:** For each of the following questions, please choose the one response that best represents the symptom **at its worst** over the past 7 days. If you did not experience the symptom in the past 7 days, answer 0.

1. At its worst, how would you rate the severity of any pain you have had in the upper part or right side of your abdominal (stomach) area over the past 7 days?

☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐

0 1 2 3 4 5 6 7 8 9 10

**No Pain** — **Worst Possible Pain**

2. At its worst, how would you rate the severity of any abdominal (stomach) bloating you have had over the past 7 days?

☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐

0 1 2 3 4 5 6 7 8 9 10

**No Bloating** — **Worst Possible Bloating**

3. At its worst, how physically fatigued have you felt over the past 7 days?

☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐

0 1 2 3 4 5 6 7 8 9 10

**No Physical Fatigue** — **Worst Possible Physical Fatigue**

4. At its worst, to what extent have you felt the need to lie down and rest over the past 7 days?

☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐

0 1 2 3 4 5 6 7 8 9 10

**No Need** — **Extreme Need to Rest**

5. At its worst, to what extent have you had difficulty sleeping over the past 7 days?

☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐

0 1 2 3 4 5 6 7 8 9 10

**No Difficulty Sleeping** — **Extreme Difficulty Sleeping**

6. At its worst, to what extent have you had problems focussing on a task over the past 7 days?

☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐

0 1 2 3 4 5 6 7 8 9 10

**No Problems Focussing** — **Extreme Problems Focussing**

7. At its worst, to what extent have you had difficulty thinking clearly over the past 7 days?

☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐

0 1 2 3 4 5 6 7 8 9 10

**No Difficulty** — **Extreme Difficulty**

8. At its worst, to what extent have you had difficulty following a conversation over the past 7 days?

☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐

0 1 2 3 4 5 6 7 8 9 10

**No Difficulty** — **Extreme Difficulty**

9. At its worst, to what extent have you been forgetful over the past 7 days?

☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐

0 1 2 3 4 5 6 7 8 9 10

**Not at All Forgetful** — **Extremely Forgetful**

10. At its worst, how would you rate the severity of any itchy skin you have had over the past 7 days?

☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐

0 1 2 3 4 5 6 7 8 9 10

**No Itch** — **Worst Imaginable Itch**

## Day-to-Day Activities

The following questions ask about how your fatty liver disease affects your day-to-day activities. Instructions: Please select the answer that best describes the difficulty you have had with each activity listed over the past 7 days. Please select one answer for each activity.

Over the past 7 days, how much difficulty have you had with…

| | No difficulty | Mild difficulty | Moderate difficulty | Severe difficulty | Unable to do |
|---|---|---|---|---|---|
| 11. Bending over (e.g., to put on your socks and shoes or to pick something up from the ground) | ☐ | ☐ | ☐ | ☐ | ☐ |
| 12. Doing light chores around the house (e.g., dusting, cooking, light gardening) | ☐ | ☐ | ☐ | ☐ | ☐ |
| 13. Doing heavy chores around the house (e.g., changing the bed, hoovering, taking the household rubbish out, heavy gardening) | ☐ | ☐ | ☐ | ☐ | ☐ |
| 14. Lifting or carrying heavy objects (e.g., a large bag of food shopping) | ☐ | ☐ | ☐ | ☐ | ☐ |
| 15. Taking a short walk on level ground (e.g., walking for less than 5 minutes) | ☐ | ☐ | ☐ | ☐ | ☐ |
| 16. Taking a long walk on level ground (e.g., walking for more than 20 minutes) | ☐ | ☐ | ☐ | ☐ | ☐ |
| 17. Walking at a brisk pace on level ground | ☐ | ☐ | ☐ | ☐ | ☐ |
| 18. Walking up a flight of stairs | ☐ | ☐ | ☐ | ☐ | ☐ |

## Emotions and Lifestyle

The following questions ask about how you feel. Instructions: Please think about how much each statement has applied to you over the past 7 days. Please select one answer for each statement.

| | Not at All | A Little | Quite A Lot | Very Much |
|---|---|---|---|---|
| 19. I worry about my fatty liver disease | ☐ | ☐ | ☐ | ☐ |
| 20. My fatty liver disease makes me feel sad | ☐ | ☐ | ☐ | ☐ |
| 21. I get angry with myself because of my fatty liver disease | ☐ | ☐ | ☐ | ☐ |
| 22. I feel like others may judge me for my fatty liver disease | ☐ | ☐ | ☐ | ☐ |
| 23. My illness affects my relationships with my friends and family | ☐ | ☐ | ☐ | ☐ |
| 24. I feel I miss out on everyday activities with my family and friends | ☐ | ☐ | ☐ | ☐ |
| 25. I feel I miss out on family life | ☐ | ☐ | ☐ | ☐ |
| 26. I feel like I am a worry to my family | ☐ | ☐ | ☐ | ☐ |
| 27. My illness affects my intimate relationships | ☐ | ☐ | ☐ | ☐ |

Instructions: Please think about how much each statement has applied to you over the past 7 days. Please select one answer for each statement.

| | Not at All | A Little | Quite A Lot | Very Much |
|---|---|---|---|---|
| 28. I don't go out to socialise with friends | ☐ | ☐ | ☐ | ☐ |
| 29. My illness restricts the things I can do in my spare time | ☐ | ☐ | ☐ | ☐ |
| 30. My illness affects my ability to work or study | ☐ | ☐ | ☐ | ☐ |
| 31. I feel restricted in the foods I can eat | ☐ | ☐ | ☐ | ☐ |

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This questionnaire was developed by the NASH-PRO Task Force under funding from Novartis Pharma AG.

## Appendix P: Guide for open-response questions.

1. How do you feel about the number and duration of the visits and telephone contacts delivered in the trial?

2. What are your thoughts on the main tests we asked you to complete?
   prompts
   - weight, waist, hip and body composition measures
   - blood pressure
   - questionnaires
   - accelerometer
   - urine samples
   - blood samples
   - web-based diet assessment tool

3. Did you complete the main tests and follow the diets as planned? If not, what were the reasons for this?

4. Are there any aspects of the content or delivery of the trial you think we could improve upon?

5. How did your diet and lifestyle patterns change during COVID-19?

The following questions relate to the foods supplied from the Mediterranean diet supplier.

6. Can you tell me your thoughts on the range of foods available?

7. What is your opinion on the taste of the foods that you selected?

8. Can you describe how appealing or unappealing you found the appearance of the selected foods?

9. Can you share your thoughts on how easy, or difficult, was it to use the company's website to order foods?

10. What is your opinion of the delivery times?

11. Do you have any other comments about the Mediterranean meal provider you would like to share with us?

## Appendix Q: Mediterranean diet assessment scale (MEDAS).

| | Question | Yes | No |
|---|---|---|---|
| 1. | Is olive oil the main culinary fat used? | | |
| 2. | Are ≥ 4 tablespoons of olive oil used each day? | | |
| 3. | Are ≥ 2 servings (of 200g each) of vegetables eaten each day? | | |
| 4. | Are ≥ 3 servings of fruit (of 80g each) eaten each day? | | |
| 5. | Is < 1 serving (100-150g) of red meat/ hamburgers/ other meat products eaten each day? | | |
| 6. | Is < 1 serving (12g) of butter, margarine or cream eaten each day? | | |
| 7. | Is < 1 serving (330ml) of sweet or sugar sweetened carbonated beverages consumed each day? | | |
| 8. | Are ≥ 3 glasses (of 125ml) of wine consumed each week? | | |
| 9. | Are ≥ 3 servings (of 150g) of legumes consumed each week? | | |
| 10. | Are ≥ 3 servings of fish (100-150g) or seafood (200g) eaten each week? | | |
| 11. | Is < 3 servings of commercial sweets/pastries eaten each week? | | |
| 12. | Is ≥ 1 serving (of 30g) of nuts consumed each week? | | |
| 13. | Is chicken, turkey or rabbit routinely eaten instead of veal, pork, hamburger, or sausage? | | |
| 14. | Are pasta, vegetable or rice dishes flavoured with garlic, tomato, leek, or onion eaten ≥ twice a week? | | |
| **TOTAL SCORE** (total no. of 'yes' answers) | | | |

Based on/adapted from tools produced by Alison Hornby and Katherine Paterson British Association for Cardiovascular Prevention & Rehabilitation **(**BACPR 2012**)** and reference [386].

## Appendix R: Home urine sample collection guide

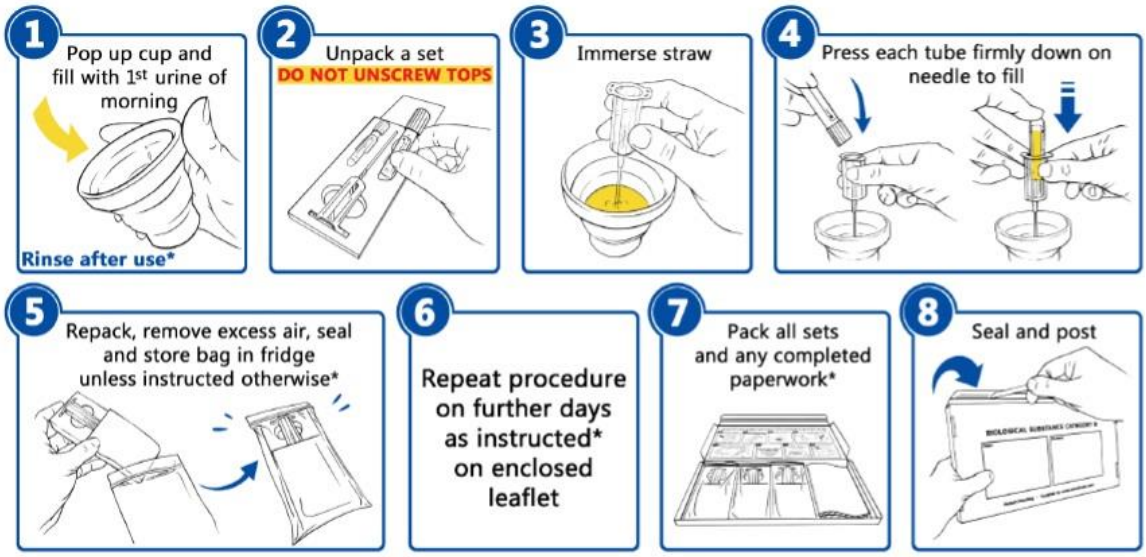
**The instructions for using the urine collection kit are printed on the inside of the box lid**. There are also printed on the reverse side of this sheet.

The collection kit has three packs. Two of the packs should be used during the week and one of the packs should be used at the weekend. All three packs should be used in one week. **You should collect your samples as soon as you get out of bed in the morning.**

The urine you collect needs to be mid-stream. This means; once you have started to urinate wait a few seconds before using the collection cup. The cup doesn't need to be filled to the top, half way is more than enough.

When you place the straw into the cup make sure it is submerged but not touching the bottom of the cup.
Once you have filled the two tubes using the collection straw, put the straw and tubes back into the foam pouch

When you have completed all your samples, remove the packs from the fridge on the day you are going to post them, and pack them in the box.



**Appendix S: Comparison of missing data for clinical and lifestyle variables by age.**

| Study measures | 30-39 | 40-49 | 50-59 | 60-69 | ≥70 | <30 | Total | P-Value |
|---|---|---|---|---|---|---|---|---|
| **Anthropometrics** | | | | | | | | |
| Weight (kg) | 1 (13%) | 0 (0%) | 4 (50%) | 3 (38%) | 0 (0%) | 0 (0%) | 8 (100%) | 0.517 |
| BMI (kg/m$^2$) | 1 (13%) | 0 (0%) | 4 (50%) | 3 (38%) | 0 (0%) | 0 (0%) | 8 (100%) | 0.517 |
| WHR | 1 (13%) | 0 (0%) | 4 (50%) | 3 (38%) | 0 (0%) | 0 (0%) | 8 (100%) | 0.517 |
| Fat (%) | 1 (11%) | 0 (0%) | 4 (44%) | 4 (44%) | 0 (0%) | 0 (0%) | 9 (100%) | 0.504 |
| FFM (kg) | 1 (11%) | 0 (0%) | 4 (44%) | 4 (44%) | 0 (0%) | 0 (0%) | 9 (100%) | 0.504 |
| **Cardiometabolics** | | | | | | | | |
| Systolic BP (mmHg) | 1 (10%) | 0 (0%) | 5 (50%) | 4 (40%) | 0 (0%) | 0 (0%) | 10 (100%) | 0.440 |
| Diastolic BP (mmHg) | 1 (10%) | 0 (0%) | 5 (50%) | 4 (40%) | 0 (0%) | 0 (0%) | 10 (100%) | 0.440 |
| Fasting glucose (mmol/L) | 1 (8%) | 1 (8%) | 5 (38%) | 5 (38%) | 1 (8%) | 0 (0%) | 13 (100%) | 0.770 |
| Total cholesterol (mmol/L) | 1 (9%) | 0 (0%) | 4 (36%) | 5 (45%) | 1 (9%) | 0 (0%) | 11 (100%) | 0.770 |
| Triglycerides (mmol/L) | 1 (9%) | 0 (0%) | 4 (36%) | 5 (45%) | 1 (9%) | 0 (0%) | 11 (100%) | 0.770 |
| HDL-cholesterol (mmol/L) | 1 (8%) | 1 (8%) | 4 (33%) | 5 (42%) | 1 (8%) | 0 (0%) | 12 (100%) | 0.753 |
| TC:HDL ratio | 1 (8%) | 1 (8%) | 4 (33%) | 5 (42%) | 1 (8%) | 0 (0%) | 12 (100%) | 0.753 |
| Non-HDL-cholesterol | 1 (8%) | 1 (8%) | 4 (33%) | 5 (42%) | 1 (8%) | 0 (0%) | 12 (100%) | 0.753 |
| (mmol/L) | 4 (9%) | 2 (5%) | 18 (42%) | 11 (26%) | 7 (16%) | 1 (2%) | 43 (100%) | 0.593 |
| Fasting insulin (pmol/L) | 5 (6%) | 5 (6%) | 29 (34%) | 29 (34%) | 16(19%) | 1 (1%) | 85 (100%) | 0.925 |
| HbA1c (mmol/mol) | | | | | | | | |
| **Hepatic function** | | | | | | | | |
| Platelets (x10 $^9$ /L) | 1 (8%) | 0 (0%) | 6 (50%) | 5 (42%) | 0 (0%) | 0 (0%) | 12 (100%) | 0.305 |
| Bilirubin (umol/l) | 2 (22%) | 0 (0%) | 4 (44%) | 3 (33%) | 0 (0%) | 0 (0%) | 9 (100%) | 0.229 |
| Albumin (g/L) | 1 (13%) | 0 (0%) | 4 (50%) | 3 (38%) | 0 (0%) | 0 (0%) | 8 (100%) | 0.517 |
| ALT (unit/L) | 1 (13%) | 0 (0%) | 4 (50%) | 3 (38%) | 0 (0%) | 0 (0%) | 8 (100%) | 0.517 |
| AST (unit/L) | 1 (9%) | 1 (9%) | 5 (45%) | 4 (36%) | 0 (0%) | 0 (0%) | 11 (100%) | 0.390 |
| ALP (unit/L) | 1 (13%) | 0 (0%) | 4 (50%) | 3 (38%) | 0 (0%) | 0 (0%) | 8 (100%) | 0.517 |
| Ferritin (ug/L) | 1 (6%) | 0 (0%) | 8 (47%) | 7 (41%) | 1 (6%) | 0 (0%) | 17 (100%) | 0.425 |
| CRP (mg/L) | 1 (8%) | 0 (0%) | 5 (38%) | 6 (46%) | 1 (8%) | 0 (0%) | 13 (100%) | 0.689 |
| GGT (unit/L) | 3 (8%) | 3 (8%) | 17 (45%) | 10 (26%) | 4 (11%) | 1 (3%) | 38 (100%) | 0.298 |
| **Hepatic fibrosis** | | | | | | | | |
| PRO-C3 (ng/mL) | 1 (8%) | 1 (8%) | 6 (50%) | 4 (33%) | 0 (0%) | 0 (0%) | 12 (100%) | 0.503 |
| GDF15 (pg/mL) | 1 (9%) | 1 (9%) | 5 (45%) | 4 (36%) | 0 (0%) | 0 (0%) | 11 (100%) | 0.390 |

**Appendix S** (continued).

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| **Dietary intake** | | | | | | | | |
| MEDAS | 0 (0%) | 0 (0%) | 2 (67%) | 1 (33%) | 0 (0%) | 0 (0%) | 3 (100%) | 0.868 |
| INTAKE24 | 4 (10%) | 2 (5%) | 15 (37%) | 15 (37%) | 4 (10%) | 1 (2%) | 41 (100%) | 0.618 |
| **Physical Activity** | | | | | | | | |
| Accelerometer | 5 (20%) | 1 (4%) | 6 (24%) | 9 (36%) | 3 (12%) | 1 (4%) | 25 (100%) | 0.063 |
| **Patient-reported outcomes** | | | | | | | | |
| CLDQ-NASH | 0 (0%) | 0 (0%) | 3 (43%) | 2 (29%) | 2 (29%) | 0 (0%) | 7 (100%) | 1.000 |
| NASH-CHECK | 0 (0%) | 0 (0%) | 2 (100%) | 0 (0%) | 0 (0%) | 0 (0%) | 2 (100%) | 0.520 |
| EQ-5D-5L Utility score | 0 (0%) | 0 (0%) | 3 (60%) | 2 (40%) | 0 (0%) | 0 (0%) | 5 (100%) | 0.751 |
| EQ-5D-5L Visual analogue scale score | 0 (0%) | 0 (0%) | 3 (50%) | 3 (50%) | 0 (0%) | 0 (0%) | 6 (100%) | 0.734 |

Data presented as n (%). (n=48). BMI, body mass index; WHR, waist-to-hip ratio; FFM, fat free mass; HDL, high-density lipoprotein; TC, total cholesterol; ALT, alanine aminotransferase; AST, aspartate aminotransferase; ALP, alkaline phosphatase; CRP, c-reactive protein; PRO-C3, N-terminal propeptide of type III collagen; GDF15; growth differentiation factor 15; MEDAS, Mediterranean diet assessment score; EQ-5D, euroqol five-dimension scale; CLDQ-NASH, chronic liver disease questionnaire for non-alcoholic fatty liver disease and non-alcoholic steatohepatitis; HbA1c, glycated haemoglobin; GGT, gamma-glutamyl transpeptidase; BP, blood pressure.

## Appendix T: Comparison of missing data for clinical and lifestyle variables by sex.

| Study measures | Male | Female | Total | P-Value |
|---|---|---|---|---|
| **Anthropometrics** | | | | |
| Weight (kg) | 5 (63%) | 3 (38%) | 8 (100%) | 0.473 |
| BMI (kg/m$^2$) | 5 (63%) | 3 (38%) | 8 (100%) | 0.473 |
| WHR | 5 (63%) | 3 (38%) | 8 (100%) | 0.473 |
| Fat (%) | 6 (67%) | 3 (33%) | 9 (100%) | 0.305 |
| FFM (kg) | 6 (67%) | 3 (33%) | 9 (100%) | 0.305 |
| **Cardiometabolics** | | | | |
| Systolic BP (mmHg) | 6 (60%) | 4 (40%) | 10 (100%) | 0.517 |
| Diastolic BP (mmHg) | 6 (60%) | 4 (40%) | 10 (100%) | 0.517 |
| Fasting glucose (mmol/L) | 7 (54%) | 6 (46%) | 13 (100%) | 0.548 |
| Total cholesterol (mmol/L) | 7 (64%) | 4 (36%) | 11 (100%) | 0.222 |
| Triglycerides (mmol/L) | 7 (64%) | 4 (36%) | 11 (100%) | 0.222 |
| HDL-cholesterol (mmol/L) | 7 (58%) | 5 (42%) | 12 (100%) | 0.369 |
| TC:HDL ratio | 7 (58%) | 5 (42%) | 12 (100%) | 0.369 |
| Non-HDL-cholesterol (mmol/L) | 7 (58%) | 5 (42%) | 12 (100%) | 0.369 |
| Fasting insulin (pmol/L) | 21 (49%) | 22 (51%) | 43 (100%) | 0.654 |
| HbA1c (mmol/mol) | 40 (47%) | 45 (53%) | 85 (100%) | 0.761 |
| **Hepatic function** | | | | |
| Platelets (x10 $^9$ /L) | 5 (42%) | 7 (58%) | 12 (100%) | 0.765 |
| Bilirubin (umol/l) | 5 (56%) | 4 (44%) | 9 (100%) | 0.735 |
| Albumin (g/L) | 5 (63%) | 3 (38%) | 8 (100%) | 0.473 |
| ALT (unit/L) | 5 (63%) | 3 (38%) | 8 (100%) | 0.473 |
| AST (unit/L) | 6 (55%) | 5 (45%) | 11 (100%) | 0.550 |
| ALP (unit/L) | 5 (63%) | 3 (38%) | 8 (100%) | 0.473 |
| Ferritin (ug/L) | 11 (65%) | 6 (35%) | 17 (100%) | 0.102 |
| CRP (mg/L) | 7 (54%) | 6 (46%) | 13 (100%) | 0.548 |
| GGT (unit/L) | 22 (58%) | 16 (42%) | 38 (100%) | 0.096 |
| **Hepatic fibrosis** | | | | |
| PRO-C3 (ng/mL) | 6 (50%) | 6 (50%) | 12 (100%) | 0.868 |
| GDF15 (pg/mL) | 6 55%) | 5 (45%) | 11 (100%) | 0.550 |

**Appendix T** (continued).

| | | | | |
|---|---|---|---|---|
| **Dietary intake** | | | | |
| MEDAS | 0 (0%) | 3 (100%) | 3 (100%) | 0.251 |
| INTAKE24 | 20 (49%) | 21 (51%) | 41 (100%) | 0.484 |
| **Physical Activity** | | | | |
| Accelerometer | 11 (44%) | 14 (56%) | 25 (100%) | 0.534 |
| **Patient-reported outcomes** | | | | |
| CLDQ-NASH | 4 (57%) | 3 (43%) | 7 (100%) | 0.705 |
| NASH-CHECK | 1 (50%) | 1 (50%) | 2 (100%) | 1.000 |
| EQ-5D-5L Utility score | 1 (20%) | 4 (80%) | 5 (100%) | 0.377 |
| EQ-5D-5L Visual analogue scale score | 2 (33%) | 4 (67%) | 6 (100%) | 0.689 |

Data presented as n (%). (n=48). BMI, body mass index; WHR, waist-to-hip ratio; FFM, fat free mass; HDL, high-density lipoprotein; TC, total cholesterol; ALT, alanine aminotransferase; AST, aspartate aminotransferase; ALP, alkaline phosphatase; CRP, c-reactive protein; PRO-C3, N-terminal propeptide of type III collagen; GDF15; growth differentiation factor 15; MEDAS, Mediterranean diet assessment score; EQ-5D, euroqol five-dimension scale; CLDQ-NASH, chronic liver disease questionnaire for non-alcoholic fatty liver disease and non-alcoholic steatohepatitis; HbA1c, glycated haemoglobin; GGT, gamma-glutamyl transpeptidase; BP, blood pressure.

**Appendix U: Comparison of missing data for clinical and lifestyle variables by ethnicity.**

| Study measures | White British | Mixed White and Asian | British Indian | Pakistani | Other Asian | Total | P-Value |
|---|---|---|---|---|---|---|---|
| **Anthropometrics** | | | | | | | |
| Weight (kg) | 8 (100%) | 0 (0%) | 0 (0%) | 0 (0%) | 0 (0%) | 8 (100%) | 1.000 |
| BMI (kg/m$^2$) | 8 (100%) | 0 (0%) | 0 (0%) | 0 (0%) | 0 (0%) | 8 (100%) | 1.000 |
| WHR | 8 (100%) | 0 (0%) | 0 (0%) | 0 (0%) | 0 (0%) | 8 (100%) | 1.000 |
| Fat (%) | 9 (100%) | 0 (0%) | 0 (0%) | 0 (0%) | 0 (0%) | 9 (100%) | 1.000 |
| FFM (kg) | 9 (100%) | 0 (0%) | 0 (0%) | 0 (0%) | 0 (0%) | 9 (100%) | 1.000 |
| **Cardiometabolics** | | | | | | | |
| Systolic BP (mmHg) | 10 (100%) | 0 (0%) | 0 (0%) | 0 (0%) | 0 (0%) | 10 (100%) | 1.000 |
| Diastolic BP (mmHg) | 10 (100%) | 0 (0%) | 0 (0%) | 0 (0%) | 0 (0%) | 10 (100%) | 1.000 |
| Fasting glucose (mmol/L) | 13 (100%) | 0 (0%) | 0 (0%) | 0 (0%) | 0 (0%) | 13 (100%) | 1.000 |
| Total cholesterol (mmol/L) | 11 (100%) | 0 (0%) | 0 (0%) | 0 (0%) | 0 (0%) | 11 (100%) | 1.000 |
| Triglycerides (mmol/L) | 11 (100%) | 0 (0%) | 0 (0%) | 0 (0%) | 0 (0%) | 11 (100%) | 1.000 |
| HDL-cholesterol (mmol/L) | 12 (100%) | 0 (0%) | 0 (0%) | 0 (0%) | 0 (0%) | 12 (100%) | 1.000 |
| TC:HDL ratio | 12 (100%) | 0 (0%) | 0 (0%) | 0 (0%) | 0 (0%) | 12 (100%) | 1.000 |
| Non-HDL-cholesterol | 12 (100%) | 0 (0%) | 0 (0%) | 0 (0%) | 0 (0%) | 12 (100%) | 1.000 |
| (mmol/L) | 40 (93%) | 1 (2%) | 0 (0%) | 2 (5%) | 0 (0%) | 43 (100%) | 0.399 |
| Fasting insulin (pmol/L) | 75 (88%) | 4 (5%) | 2 (2%) | 2 (2%) | 2 (2%) | 85 (100%) | 0.987 |
| HbA1c (mmol/mol) | | | | | | | |
| **Hepatic function** | | | | | | | |
| Platelets (x10 $^9$ /L) | 12 (100%) | 0 (0%) | 0 (0%) | 0 (0%) | 0 (0%) | 12 (100%) | 1.000 |
| Bilirubin (umol/l) | 8 (89%) | 0 (0%) | 0 (0%) | 1 (11%) | 0 (0%) | 9 (100%) | 0.477 |
| Albumin (g/L) | 8 (100%) | 0 (0%) | 0 (0%) | 0 (0%) | 0 (0%) | 8 (100%) | 1.000 |
| ALT (unit/L) | 8 (100%) | 0 (0%) | 0 (0%) | 0 (0%) | 0 (0%) | 8 (100%) | 1.000 |
| AST (unit/L) | 11 (100%) | 0 (0%) | 0 (0%) | 0 (0%) | 0 (0%) | 11 (100%) | 1.000 |
| ALP (unit/L) | 8 (100%) | 0 (0%) | 0 (0%) | 0 (0%) | 0 (0%) | 8 (100%) | 1.000 |
| Ferritin (ug/L) | 17 (100%) | 0 (0%) | 0 (0%) | 0 (0%) | 0 (0%) | 17 (100%) | 1.000 |
| CRP (mg/L) | 13 (100%) | 0 (0%) | 0 (0%) | 0 (0%) | 0 (0%) | 13 (100%) | 1.000 |
| GGT (unit/L) | 38 (100%) | 0 (0%) | 0 (0%) | 0 (0%) | 0 (0%) | 38 (100%) | 0.485 |

**Appendix U** (continued).

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| **Hepatic fibrosis** | | | | | | | |
| PRO-C3 (ng/mL) | 12 (100%) | 0 (0%) | 0 (0%) | 0 (0%) | 0 (0%) | 12 (100%) | 1.000 |
| GDF15 (pg/mL) | 11 (100%) | 0 (0%) | 0 (0%) | 0 (0%) | 0 (0%) | 11 (100%) | 1.000 |
| **Dietary intake** | | | | | | | |
| MEDAS | 3 (100%) | 0 (0%) | 0 (0%) | 0 (0%) | 0 (0%) | 3 (100%) | 1.000 |
| INTAKE24 | 35 (85%) | 3 (7%) | 0 (0%) | 1 (2%) | 2 (5%) | 41 (100%) | 0.393 |
| **Physical Activity** | | | | | | | |
| Accelerometer | 21 (84%) | 4 (16%) | 0 (0%) | 0 (0%) | 0 (0%) | 25 (100%) | 0.166 |
| **Patient-reported outcomes** | | | | | | | |
| CLDQ-NASH | 6 (86%) | 0 (0%) | 1 (14%) | 0 (0%) | 0 (0%) | 7 (100%) | 0.389 |
| NASH-CHECK | 2 (100%) | 0 (0%) | 0 (0%) | 0 (0%) | 0 (0%) | 2 (100%) | 1.000 |
| EQ-5D-5L Utility score | 5 (100%) | 0 (0%) | 0 (0%) | 0 (0%) | 0 (0%) | 5 (100%) | 1.000 |
| EQ-5D-5L Visual analogue scale score | 6 (100%) | 0 (0%) | 0 (0%) | 0 (0%) | 0 (0%) | 6 (100%) | 1.000 |

Data presented as n (%). (n=48). BMI, body mass index; WHR, waist-to-hip ratio; FFM, fat free mass; HDL, high-density lipoprotein; TC, total cholesterol; ALT, alanine aminotransferase; AST, aspartate aminotransferase; ALP, alkaline phosphatase; CRP, c-reactive protein; PRO-C3, N-terminal propeptide of type III collagen; GDF15; growth differentiation factor 15; MEDAS, Mediterranean diet assessment score; EQ-5D, euroqol five-dimension scale; CLDQ-NASH, chronic liver disease questionnaire for non-alcoholic fatty liver disease and non-alcoholic steatohepatitis; HbA1c, glycated haemoglobin; GGT, gamma-glutamyl transpeptidase; BP, blood pressure.

## Appendix V: Comparison of missing data for clinical and lifestyle variables by time.

| Study measures | Baseline | Phase 1 End | End of washout | Phase 2 End | Total | P-Value |
|---|---|---|---|---|---|---|
| **Anthropometrics** | | | | | | |
| Weight (kg) | 0 (0%) | 6 (75%) | 1 (13%) | 1 (13%) | 8 (100%) | 0.014 |
| BMI (kg/m$^2$) | 0 (0%) | 6 (75%) | 1 (13%) | 1 (13%) | 8 (100%) | 0.014 |
| WHR | 0 (0%) | 6 (75%) | 1 (13%) | 1 (13%) | 8 (100%) | 0.014 |
| Fat (%) | 1 (11%) | 6 (67%) | 1 (11%) | 1 (11%) | 9 (100%) | 0.039 |
| FFM (kg) | 1 (11%) | 6 (67%) | 1 (11%) | 1 (11%) | 9 (100%) | 0.039 |
| **Cardiometabolics** | | | | | | |
| Systolic BP (mmHg) | 0 (0%) | 6 (60%) | 3 (30%) | 1 (10%) | 10 (100%) | 0.030 |
| Diastolic BP (mmHg) | 0 (0%) | 6 (60%) | 3 (30%) | 1 (10%) | 10 (100%) | 0.030 |
| Fasting glucose (mmol/L) | 2 (15%) | 7 (54%) | 2 (15%) | 2 (15%) | 13 (100%) | 0.152 |
| Total cholesterol (mmol/L) | 1 (9%) | 7 (64%) | 1 (9%) | 2 (18%) | 11 (100%) | 0.043 |
| Triglycerides (mmol/L) | 1 (9%) | 7 (64%) | 1 (9%) | 2 (18%) | 11 (100%) | 0.043 |
| HDL-cholesterol (mmol/L) | 1 (8%) | 7 (58%) | 1 (8%) | 3 (25%) | 12 (100%) | 0.056 |
| TC:HDL ratio | 1 (8%) | 7 (58%) | 1 (8%) | 3 (25%) | 12 (100%) | 0.056 |
| Non-HDL-cholesterol (mmol/L) | 1 (8%) | 7 (58%) | 1 (8%) | 3 (25%) | 12 (100%) | 0.056 |
| Fasting insulin (pmol/L) | 16 (37%) | 10 (23%) | 11 (26%) | 6 (14%) | 43 (100%) | 0.108 |
| HbA1c (mmol/mol) | 7 (8%) | 36 (42%) | 10 (12%) | 32 (38%) | 85 (100%) | <0.001 |
| **Hepatic function** | | | | | | |
| Platelets (x10 $^9$ /L) | 2 (17%) | 7 (58%) | 2 (17%) | 1 (8%) | 12 (100%) | 0.093 |
| Bilirubin (umol/l) | 1 (11%) | 6 (67%) | 1 (11%) | 1 (11%) | 9 (100%) | 0.039 |
| Albumin (g/L) | 0 (0%) | 6 (75%) | 1 (13%) | 1 (13%) | 8 (100%) | 0.014 |
| ALT (unit/L) | 0 (0%) | 6 (75%) | 1 (13%) | 1 (13%) | 8 (100%) | 0.014 |
| AST (unit/L) | 1 (9%) | 7 (64%) | 2 (18%) | 1 (9%) | 11 (100%) | 0.043 |
| ALP (unit/L) | 0 (0%) | 6 (75%) | 1 (13%) | 1 (13%) | 8 (100%) | 0.014 |
| Ferritin (ug/L) | 4 (24%) | 6 (35%) | 2 (12%) | 5 (29%) | 17 (100%) | 0.591 |
| CRP (mg/L) | 2 (15%) | 6 (46%) | 3 (23%) | 2 (15%) | 13 (100%) | 0.355 |
| GGT (unit/L) | 11 (29%) | 11 (29%) | 7 (18%) | 9 (24%) | 38 (100%) | 0.695 |
| **Hepatic fibrosis** | | | | | | |
| PRO-C3 (ng/mL) | 0 (0%) | 7 (58%) | 3 (25%) | 2 (17%) | 12 (100%) | 0.009 |
| GDF15 (pg/mL) | 0 (0%) | 7 (64%) | 3 (27%) | 1 (9%) | 11 (100%) | 0.011 |

**Appendix V** (continued).

| | | | | | | |
|---|---|---|---|---|---|---|
| **Dietary intake** | | | | | | |
| MEDAS | 0 (0%) | 1 (33%) | 0 (0%) | 2 (67%) | 3 (100%) | 0.619 |
| INTAKE24 | 7 (17%) | 8 (20%) | 7 (17%) | 19 (46%) | 41 (100%) | 0.003 |
| **Physical Activity** | | | | | | |
| Accelerometer | 3 (12%) | 10 (40%) | 3 (12%) | 9 (36%) | 25 (100%) | 0.164 |
| **Patient-reported outcomes** | | | | | | |
| CLDQ-NASH | 0 (0%) | 2 (29%) | 0 (0%) | 5 (71%) | 7 (100%) | 0.018 |
| NASH-CHECK | 0 (0%) | 2 (100%) | 0 (0%) | 0 (0%) | 2 (100%) | 0.246 |
| EQ-5D-5L Utility score | 0 (0%) | 3 (60%) | 0 (0%) | 2 (40%) | 5 (100%) | 0.171 |
| EQ-5D-5L Visual analogue scale score | 0 (0%) | 4 (67%) | 0 (0%) | 2 (33%) | 6 (100%) | 0.057 |

Data presented as n (%). (n=48). BMI, body mass index; WHR, waist-to-hip ratio; FFM, fat free mass; HDL, high-density lipoprotein; TC, total cholesterol; ALT, alanine aminotransferase; AST, aspartate aminotransferase; ALP, alkaline phosphatase; CRP, c-reactive protein; PRO-C3, N-terminal propeptide of type III collagen; GDF15; growth differentiation factor 15; MEDAS, Mediterranean diet assessment score; EQ-5D, euroqol five-dimension scale; CLDQ-NASH, chronic liver disease questionnaire for non-alcoholic fatty liver disease and non-alcoholic steatohepatitis; HbA1c, glycated haemoglobin; GGT, gamma-glutamyl transpeptidase; BP, blood pressure.

## Appendix W: Comparison of patient-reported outcomes between sub-groups after 4-weeks of MD intervention.

| | Sex | | | Disease severity | | |
|---|---|---|---|---|---|---|
| **CLDQ-NASH** | **Male (n=10)** | **Female (n=13)** | **P-Value** | **Non-advanced (n=15)** | **NASH cirrhosis (n=8)** | **P-Value** |
| Abdominal symptoms | 5.80 (4.22-6.48) | 6.30 (5.35-7.00) | 0.427 | 5.70 (4.30-6.30) | 6.30 (5.55-7.00) | 0.261 |
| Activity/energy | 5.70 (4.60-6.05) | 5.40 (3.60-6.30) | 0.709 | 5.60 (4.80-6.20) | 5.00 (3.30-6.75) | 0.796 |
| Emotional functioning | 4.95 (3.90-5.33) | 5.60 (4.85-5.95) | 0.088 | 5.20 (4.20-5.70) | 5.20 (4.85-5.70) | 0.605 |
| Fatigue | 4.95 (3.70-5.58) | 5.00 (3.50-5.50) | 0.852 | 4.70 (3.70-5.50) | 5.20 (3.80-5.65) | 0.650 |
| Systemic symptoms | 5.50 (3.60-6.00) | 5.00 (4.00-6.45) | 0.641 | 5.00 (3.80-5.70) | 5.65 (4.10-6.20) | 0.300 |
| Worry | 5.30 (3.85-6.48) | 5.60 (4.50-6.75) | 0.901 | 5.10 (3.90-6.10) | 6.20 (5.38-6.90) | 0.106 |
| Total score | 5.20 (4.18-6.03) | 5.30 (4.50-6.15) | 0.804 | 4.90 (4.30-5.90) | 5.55 (4.83-6.07) | 0.420 |
| **EQ-5D-5L** | | | | | | |
| Utility score | 0.69 (0.50-0.88) | 0.74 (0.30-0.87) | 0.869 | 0.73 (0.59-0.88) | 0.59 (0.21-0.88) (n7) | 0.479 |
| Visual analogue scale score | 61.00 (42.50-78.50) | 70.00 (50.00-85.00) | 0.474 | 62.00 (50.00-80.00) | 74.50 (52.50-90.00) | 0.270 |
| **NASH-CHECK** | | | | | | |
| Abdominal pain | 1.00 (0.00-3.75) | 0.00 (0.00-4.00) | 0.841 | 2.00 (0.00-4.00) | 0.00 (0.00-3.00) | 0.209 |
| Abdominal bloating | 1.00 (0.00-2.25) | 2.00 (0.00-5.00) | 0.495 | 2.00 (0.00-4.00) | 0.50 (0.00-4.25) | 0.761 |
| Fatigue | 4.50 (3.75-8.00) | 4.00 (1.00-6.00) | 0.229 | 4.00 (3.00-8.00) | 4.00 (1.25-5.00) | 0.410 |
| Sleep | 2.00 (1.00-7.75) | 2.00 (0.50-6.50) | 0.548 | 1.00 (0.00-7.00) | 2.50 (1.25-7.50) | 0.157 |
| Itchy skin | 5.00 (1.00-8.50) | 2.00 (0.00-4.00) | 0.074 | 4.00 (1.00-8.00) | 1.00 (0.00-2.75) | 0.054 |
| Cognitive symptoms | 2.05 (0.75-5.60) | 1.30 (0.40-3.65) | 0.534 | 1.80 (0.80-5.30) | 1.55 (0.08-3.83) | 0.582 |
| Activity limitations | 1.30 (0.30-5.60) | 2.20 (0.00-6.40) | 0.827 | 1.60 (0.30-5.60) | 1.10 (0.08-7.03) | 0.845 |
| Emotional impact | 2.35 (0.00-5.20) | 1.70 (0.80-4.60) | 0.901 | 2.50 (0.80-5.00) | 1.25 (0.00-4.18) | 0.172 |
| Social impact | 1.90 (1.00-3.43) | 0.50 (0.00-1.65) | 0.048 | 1.40 (0.00-2.90) | 1.00 (0.50-1.78) | 0.514 |

**Table 1. Comparison of patient-reported outcomes by sex and disease severity.** Values are medians (range). MD, Mediterranean diet; CLDQ-NASH, chronic liver disease questionnaire for non-alcoholic fatty liver disease and non-alcoholic steatohepatitis; EQ-5D, euroqol 5-dimension scale.

| | Age (years) | | | | | | |
|---|---|---|---|---|---|---|---|
| **CLDQ-NASH** | **30-39 (n=1)** | **40-49 (n=2)** | **50-59 (n=5)** | **60-69 (n=9)** | **>70 (n=5)** | **<30 (n=1)** | **P-Value** |
| Abdominal symptoms | 5.70 (5.70-5.70) | 5.80 (5.30-.) | 4.00 (3.50-6.30) | 7.00 (5.00-7.00) | 6.30 (5.65-6.65) | 5.00 (5.00-5.00) | 0.382 |
| Activity/energy | 5.40 (5.40-5.40) | 5.80 (5.60-.) | 5.00 (4.00-5.70) | 4.20 (3.30-6.70) | 5.80 (5.60-6.60) | 3.60 (3.60-3.60) | 0.543 |
| Emotional functioning | 5.60 (5.60-5.60) | 4.80 (4.80-4.80) | 4.20 (3.10-5.45) | 5.30 (4.85-5.95) | 5.40 (5.10-6.45) | 4.90 (4.90-4.90) | 0.300 |
| Fatigue | 5.30 (5.30-5.30) | 4.00 (3.30-.) | 4.00 (3.50-4.85) | 5.20 (3.60-5.65) | 5.50 (5.20-6.00) | 3.50 (3.50-3.50) | 0.141 |
| Systemic symptoms | 5.00 (5.00-5.00) | 5.10 (4.20-.) | 4.00 (3.55-5.50) | 5.30 (3.70-6.45) | 6.00 (4.70-6.45) | 4.20 (4.20-4.20) | 0.704 |
| Worry | 5.10 (5.10-5.10) | 5.45 (4.60-.) | 3.90 (3.05-6.10) | 6.00 (4.50-6.50) | 6.60 (5.15-7.00) | 4.40 (4.40-4.40) | 0.411 |
| Total score | 5.30 (5.30-5.30) | 5.15 (4.80-.) | 4.40 (3.65-5.35) | 5.40 (4.25-6.30) | 6.00 (5.40-6.35) | 4.30 (4.30-4.30) | 0.256 |
| **EQ-5D-5L** | | | | | | | |
| Utility score | 0.88 (0.88-0.88) | 0.66 (0.59-.) | 0.66 (0.37-0.72) | 0.57 (0.21-0.96) | 0.88 (0.71-0.88) | 0.27 (0.27-0.27) | 0.382 |
| Visual analogue scale score | 50.00 (50.00-50.00) | 64.00 (55.00-.) | 70.00 (22.50-85.00) | 70.00 (47.50-85.00) | 62.00 (55.00-92.50) | 50.00 (50.00-50.00) | 0.853 |
| **NASH-CHECK** | | | | | | | |
| Abdominal pain | 1.00 (1.00-1.00) | 1.50 (0.00-.) | 3.00 (1.00-5.50) | 0.00 (0.00-4.50) | 0.00 (0.00-1.00) | 4.00 (4.00-4.00) | 0.391 |
| Abdominal bloating | 4.00 (4.00-4.00) | 0.50 (0.00-.) | 3.00 (1.00-5.50) | 0.00 (0.00-3.50) | 1.00 (0.00-2.00) | 5.00 (5.00-5.00) | 0.318 |
| Fatigue | 8.00 (8.00-8.00) | 4.50 (4.00-.) | 5.00 (4.00-8.00) | 4.00 (0.50-5.50) | 3.00 (1.00-4.50) | 8.00 (8.00-8.00) | 0.136 |
| Sleep | 7.00 (7.00-7.00) | 0.50 (0.00-.) | 5.00 (1.00-7.00) | 2.00 (1.00-9.00) | 1.00 (0.50-2.50) | 0.00 (0.00-0.00) | 0.191 |
| Itchy skin | 2.00 (2.00-2.00) | 2.00 (1.00-.) | 6.00 (1.50-8.00) | 1.00 (0.00-5.50) | 2.00 (0.50-7.00) | 10.00 (10.00-10.00) | 0.460 |
| Cognitive symptoms | 7.00 (7.00-7.00) | 3.25 (0.00-.) | 5.30 (0.90-7.30) | 1.80 (0.40-2.80) | 1.30 (0.15-2.55) | 0.50 (0.50-0.50) | 0.373 |
| Activity limitations | 2.20 (2.20-2.20) | 0.15 (0.00-.) | 3.40 (1.70-5.60) | 1.60 (0.45-7.80) | 0.30 (0.00-1.45) | 7.20 (7.20-7.20) | 0.160 |
| Emotional impact | 3.30 (3.30-3.30) | 1.25 (0.00-.) | 5.80 (1.50-8.75) | 1.70 (0.00-4.15) | 1.70 (0.40-3.75) | 4.20 (4.20-4.20) | 0.400 |
| Social impact | 0.50 (0.50-0.50) | 0.95 (0.00-.) | 2.90 (0.70-3.55) | 1.00 (0.25-2.40) | 1.00 (0.25-1.90) | 1.40 (1.40-1.40) | 0.767 |

**Table 2. Comparison of patient-reported outcomes by age.** Values are medians (range). MD, Mediterranean diet; CLDQ-NASH, chronic liver disease questionnaire for non-alcoholic fatty liver disease and non-alcoholic steatohepatitis; EQ-5D, euroqol 5-dimension scale.

| Ethnicity | | | | | |
|---|---|---|---|---|---|
| **CLDQ-NASH** | **White British (n=20)** | **Mixed White and Asian (n=1)** | **British Indian (n=1)** | **Pakistani (n=1)** | **P-Value** |
| Abdominal symptoms | 6.30 (4.47-6.83) | 7.00 (7.00-7.00) | 6.30 (6.30-6.30) | 5.70 (5.70-5.70) | 0.582 |
| Activity/energy | 5.60 (3.75-6.15) | 2.60 (2.60-2.60) | 6.20 (6.20-6.20) | 5.40 (5.40-5.40) | 0.301 |
| Emotional functioning | 5.05 (4.72-5.70) | 5.70 (5.70-5.70) | 5.40 (5.40-5.40) | 5.60 (5.60-5.60) | 0.679 |
| Fatigue | 4.85 (3.70-5.50) | 3.50 (3.50-3.50) | 6.30 (6.30-6.30) | 5.30 (5.30-5.30) | 0.282 |
| Systemic symptoms | 5.15 (3.73-6.15) | 5.30 (5.30-5.30) | 6.00 (6.00-6.00) | 5.00 (5.00-5.00) | 0.859 |
| Worry | 5.45 (4.03-6.53) | 7.00 (7.00-7.00) | 5.30 (5.30-5.30) | 5.10 (5.10-5.10) | 0.486 |
| Total score | 5.15 (4.33-6.07) | 5.20 (5.20-5.20) | 5.90 (5.90-5.90) | 5.30 (5.30-5.30) | 0.900 |
| **EQ-5D-5L** | | | | | |
| Utility score | 0.69 (0.27-0.84) | 0.38 (0.38-0.38) | 0.88 (0.88-0.88) | 0.88 (0.88-0.88) | 0.368 |
| Visual analogue scale score | 70.00 (51.25-87.50) | 50.00 (50.00-50.00) | 50.00 (50.00-50.00) | 50.00 (50.00-50.00) | 0.515 |
| **NASH-CHECK** | | | | | |
| Abdominal pain | 1.00 (0.00-4.00) | 0.00 (0.00-0.00) | 0.00 (0.00-0.00) | 1.00 (1.00-1.00) | 0.640 |
| Abdominal bloating | 1.50 (0.00-3.75) | 0.00 (0.00-0.00) | 0.00 (0.00-0.00) | 4.00 (4.00-4.00) | 0.381 |
| Fatigue | 4.00 (3.25-6.50) | 2.00 (2.00-2.00) | 1.00 (1.00-1.00) | 8.00 (8.00-8.00) | 0.247 |
| Sleep | 1.50 (1.00-5.75) | 8.00 (8.00-8.00) | 1.00 (1.00-1.00) | 7.00 (7.00-7.00) | 0.326 |
| Itchy skin | 2.50 (0.25-6.00) | 3.00 (3.00-3.00) | 1.00 (1.00-1.00) | 2.00 (2.00-2.00) | 0.910 |
| Cognitive symptoms | 1.55 (0.35-3.83) | 3.30 (3.30-3.30) | 1.30 (1.30-1.30) | 7.00 (7.00-7.00) | 0.489 |
| Activity limitations | 1.45 (0.08-5.60) | 5.60 (5.60-5.60) | 0.30 (0.30-0.30) | 2.20 (2.20-2.20) | 0.670 |
| Emotional impact | 1.95 (0.80-5.00) | 0.00 (0.00-0.00) | 2.50 (2.50-2.50) | 3.30 (3.30-3.30) | 0.546 |
| Social impact | 1.20 (0.13-2.65) | 1.40 (1.40-1.40) | 1.90 (1.90-1.90) | 0.50 (0.50-0.50) | 0.795 |

**Table 3. Comparison of patient-reported outcomes by ethnicity.** Values are medians (range). MD, Mediterranean diet; CLDQ-NASH, chronic liver disease questionnaire for non-alcoholic fatty liver disease and non-alcoholic steatohepatitis; EQ-5D, euroqol 5-dimension scale.

**Appendix X: All the results from each time point and study arms.**

**X.1** Anthropometric and biochemical characteristics, physical activity and sleep patterns of participants randomised to MD in the first experimental period.

| **Participant Characteristics** | **Baseline** | **Post-MD** | **Baseline** | **Post-CD** |
|---|---|---|---|---|
| **Anthropometrics** | | | | |
| Weight (kg) | 97.3 ± 22.3 | 96.1 ± 22.7 | 95.9 ± 20.1 | 95.7 ± 19.6 |
| BMI (kg/m$^2$) | 35.4 ± 6.3 | 34.7 ± 6.3 | 34.9 ± 6.0 | 34.7 ± 5.9 |
| Waist circumference (cm) | 116.2 ±14.9 | 114.4 ±15.2 | 114.0 ±14.2 | 115.8 ± 14.8 |
| Waist-to-hip ratio | 1.00 ± 0.09 | 0.98 ± 0.09 | 1.00 ± 0.09 | 1.01 ± 0.08 |
| Body composition (kg): Fat mass<br>Fat free mass | 42.3 ± 9.9<br>55.2 ± 11.2 | 42.2 ± 10.8<br>54.5 ± 14.6 | 40.5 ± 10.5<br>54.0 ± 14.1 | 40.7 ± 9.2<br>54.4 ± 12.4 |
| **Cardiometabolic measures** | | | | |
| Blood pressure: Systolic (mmHg)<br>Diastolic (mmHg) | 137.5 ±15.0<br>82.8 ± 9.2 | 136.1 ±14.0<br>79.2 ± 9.5 | 141.3 ± 21.1<br>81.8 ± 9.1 | 139.0 ±15.4<br>81.6 ± 8.1 |
| MAP (mmHg) | 101.0 ± 8.7 | 98.2 ± 9.5 | 101.6 ±10.5 | 100.7 ± 8.4 |
| Fasting glucose (mmol/L) | 6.3 (5.5-11.5) | 6.6 (5.2-9.4) | 7.1 (5.5-10.2) | 6.9 (5.6-9.8) |
| Fasting Insulin (pmol/L) | 94 (62-138) | 96 (87-121) | 96 (78-122) | 103 (86-159) |
| HbA1c (mmol/mol) | 52.0 (41.8-64.5) | 56.5 (34.8-89.5) | 44.5 (37.8-56.5) | 83.0 (38.5-111.8) |
| HOMA-IR | 7.0 ± 4.0 | 5.3 ± 4.0 | 5.4 ± 3.7 | 6.3 ± 2.7 |
| Total cholesterol (mmol/L) | 4.5 ± 1.4 | 4.3 ± 1.4 | 4.4 ± 1.3 | 4.2 ± 1.1 |
| Triglycerides (mmol/L) | 1.4 (1.0-2.6) | 1.4 (1.0-2.5) | 1.7 (1.2-3.1) | 1.4 (1.2-2.6) |
| HDL (mmol/L) | 1.4 (1.2-1.6) | 1.2 (1.1-1.4) | 1.2 (1.1-1.4) | 1.2 (1.1-1.4) |
| TC:HDL ratio | 3.2 (2.4-4.3) | 3.0 (2.4-4.1) | 3.1 (2.6-4.5) | 3.0 (2.4-3.8) |
| Non-HDL (mmol/L) | 3.2 ± 1.4 | 3.0 ± 1.4 | 3.1 ± 1.3 | 2.7 ± 0.9 |
| QRISK3 | 11.4 (7.8-19.4) | 11.1 (7.6-19.3) | 11.4 (7.7-21.5) | 11.3 (7.6-22.1) |
| **Liver function** | | | | |
| Platelets (x10 9 /L) | 221 ± 62 | 212 ± 64 | 218 ± 61 | 215 ± 60 |
| Bilirubin (umol/l) | 9 (7-14) | 10 (7-19) | 10 (6-12) | 9 (6-13) |

**Appendix X.1** (continued).

| | | | | |
|---|---|---|---|---|
| Albumin (g/L) | 45 ± 4 | 45 ± 4 | 45 ± 4 | 45 ± 4 |
| ALT (unit/L) | 34 (25-54) | 35 (24-46) | 30 (24-42) | 28 (21-43) |
| AST (unit/L) | 30 (23-41) | 31 (25-46) | 29 (24-34) | 25 (22-37) |
| ALP (IU/L) | 96 ± 29 | 90 ± 28 | 97 ± 29 | 96 ± 28 |
| GGT (unit/L) | 69 (41-110) | 72 (50-118) | 69 (49-122) | 66 (48-103) |
| Ferritin (ug/L) | 91 (39-232) | 69 (30-198) | 64 (33-157) | 82 (36-169) |
| **Physical Activity** | | | | |
| Inactive | 789 ± 62 | 775 ± 79 | 811 ± 117 | 777 ± 106 |
| Light physical activity | 138 (113-170) | 135 (116-170) | 136 (101-187) | 127 (101-207) |
| Moderate physical activity | 61 ± 31 | 58 ± 30 | 61 ± 41 | 53 ± 29 |
| Vigorous physical activity | 1 (0-2) | 1 (0-1) | 1 (0-1) | 1 (0-2) |
| Sleep duration (minutes) | 388 ± 44 | 397 ± 72 | 367 ± 68 | 400 ± 65 |
| Sleep efficiency (%) | 87 ± 5 | 86 ± 7 | 88 ± 5 | 87 ± 5 |

Values are means (standard deviation) and medians (range). (n=48). BMI, body mass index; MAP, mean arterial pressure; HbA1c, glycated haemoglobin; HOMA-IR, homeostatic model assessment of insulin resistance; HDL, high-density lipoprotein; TC: HDL, total cholesterol: HDL ratio; ALT, alanine aminotransferase; AST, aspartate aminotransferase; ALP, alkaline phosphatase; GGT, gamma-glutamyl transpeptidase. Note: The data presented in this table represents raw observations and has not undergone statistical testing.

**X.2** Biomarkers of liver fibrosis characteristics of participants randomised to MD in the first experimental period.

| **Participant Characteristics** | **Baseline** | **Post-MD** | **Baseline** | **Post-CD** |
|---|---|---|---|---|
| Age (years) | 59.0 (51.5-68.0) | | | |
| T2DM | 12 (57%) | | | |
| **Liver function** | | | | |
| Platelets (x10 9 /L) | 221 ± 62 | 212 ± 64 | 218 ± 61 | 215 ± 60 |
| ALT (unit/L) | 34 (25-54) | 35 (24-46) | 30 (24-42) | 28 (21-43) |
| AST (unit/L) | 30 (23-41) | 31 (25-46) | 29 (24-34) | 25 (22-37) |
| **Liver steatosis** | | | | |
| CAP dB/m *(baseline only)* | 333 ± 59 | | | |
| **Liver fibrosis** | | | | |
| Liver stiffness (kPa) *(baseline only)* | 9.4 (6.2-21.8) | | | |
| GDF15 (pg/mL) | 1257.7 ± 606.9 | 1217.2 ± 525.8 | 1199.1 ± 510.9 | 1215.5 ± 531.4 |
| PRO-C3 (ng/mL) | 14.0 (10.4-20.6) | 14.7 (10.9-22.4) | 14.3 (11.8-22.9) | 15.9 (13.4-21.8) |
| PRO-C4 (ng/mL) | 7502.7 ± 1062.3 | 7184.8 ± 1114.1 | 7460.8 ± 1005.8 | 7467.7 ± 1060.9 |
| PRO-C5 (ng/mL) | 1004.4 ± 223.4 | 976.0 ± 208.2 | 948.6 ± 226.8 | 988.9 ± 211.0 |
| CTX-III (ng/mL) | 6.6 (6.0-9.2) | 7.7 (5.4-9.5) | 6.5 (5.1-8.5) | 5.9 (5.1-8.0) |
| FIB-4 | 1.7 (0.9-2.4) | 1.7 (1.3-2.6) | 1.2 (1.1-2.1) | 1.4 (0.9-2.4) |
| ADAPT | 7.67 ± 1.74 | 7.89 ± 1.73 | 7.58 ± 1.40 | 7.77 ± 1.18 |

Values are numbers (percentages), means (standard deviation) or medians (range). (n=48). *PNPLA3,* patatin-like phospholipase domain containing 3; TM6SF2, transmembrane 6 superfamily member 2; T2DM, type 2 diabetes; ALT, alanine aminotransferase; AST, aspartate aminotransferase; PRO-C3, N-terminal propeptide of type III collagen; CTX-III, crosslinked type III collagen; GDF15; growth differentiation factor 15; FIB-4, Fibrosis-4 index; ADAPT, PRO-C3-based fibrosis algorithm; CAP, controlled attenuation parameter. The data presented in this table represents raw observations and has not undergone statistical testing.

**X.3** Anthropometric and biochemical characteristics, physical activity and sleep patterns of participants randomised to CD in the first experimental period.

| **Participant Characteristics** | **Baseline** | **Post-CD** | **Baseline** | **Post-MD** |
|---|---|---|---|---|
| **Anthropometrics** | | | | |
| Weight (kg) | 96.7 ± 16.7 | 95.5 ± 17.3 | 96.8 ± 17.0 | 95.8 ± 16.8 |
| BMI (kg/m$^2$) | 34.9 ± 4.7 | 35.0 ± 5.1 | 35.1 ± 4.8 | 34.6 ± 4.7 |
| Waist circumference (cm) | 114.9 ± 11.8 | 113.1 ± 13.1 | 113.9 ±12.5 | 111.7 ± 11.8 |
| Waist-to-hip ratio | 0.98 (0.94-1.04) | 1.00 (0.95-1.06) | 0.98 (0.94-1.05) | 0.97 (0.93-1.07) |
| Body composition (kg): Fat mass<br>Fat free mass | 42.7 ± 7.2<br>55.3 ± 9.8 | 43.1 ± 7.5<br>52.7 ± 8.7 | 42.7 ± 7.3<br>54.8 ± 9.9 | 42.3 ± 7.5<br>54.3 ± 10.9 |
| **Cardiometabolic measures** | | | | |
| Blood pressure: Systolic (mmHg)<br>Diastolic (mmHg) | 134.5 ± 16.3<br>79.3 ± 8.2 | 134.7 ± 15.9<br>78.0 ± 12.0 | 139.2 ±18.2<br>82.2 ± 11.0 | 129.8 ± 15.0<br>78.6 ± 8.2 |
| | | | | |
| MAP (mmHg) | 97.7 ± 9.3 | 96.9 ± 11.1 | 101.2 ± 11.3 | 95.7 ± 9.5 |
| Fasting glucose (mmol/L) | 6.5 (5.6-9.1) | 6.4 (5.9-9.3) | 6.8 (5.8-9.5) | 6.4 (5.6-7.1) |
| Fasting Insulin (pmol/L) | 135 (83-218) | 124 (90-179) | 103 (82-191) | 131 (74-188) |
| HbA1c (mmol/mol) | 45.0 (41.0-61.0) | 58.0 (42.3-82.5) | 44.5 (40.3-57.0) | 46.5 (40.5-55.8) |
| HOMA-IR | 6.1 (3.3-13.3) | 6.6 (3.6-10.0) | 4.9 (3.6-11.6) | 4.6 (3.0-9.5) |
| Total cholesterol (mmol/L) | 4.3 ± 1.0 | 4.3 ± 1.1 | 4.4 ± 1.1 | 3.9 ± 1.1 |
| Triglycerides (mmol/L) | 1.8 (1.3-2.3) | 1.9 (1.2-2.2) | 1.7 (1.3-2.0) | 1.4 (1.1-1.8) |
| HDL (mmol/L) | 1.3 ± 0.3 | 1.3 ± 0.3 | 1.3 ± 0.3 | 1.2 ± 0.3 |
| TC:HDL ratio | 3.6 ± 0.9 | 3.4 ± 1.0 | 3.5 ± 1.1 | 3.3 ± 0.9 |
| Non-HDL (mmol/L) | 3.1 ± 1.0 | 3.0 ± 1.0 | 3.1 ± 1.0 | 2.7 ± 1.0 |
| QRISK3 | 14.6 ± 8.8 | 14.7 ± 7.8 | 15.0 ± 8.6 | 13.4 ± 7.6 |
| **Liver function** | | | | |

**Appendix X.3** (continued).

| | | | | |
|---|---|---|---|---|
| Platelets (x10 9 /L) | 241 ± 54 | 246 ± 62 | 246 ± 58 | 243 ± 56 |
| Bilirubin (umol/l) | 10 (7-12) | 9 (7-13) | 9 (7-12) | 10 (7-12) |
| Albumin (g/L) | 46 ± 3 | 46 ± 3 | 46 ± 3 | 46 ± 3 |
| ALT (unit/L) | 45 (34-74) | 41 (28-73) | 43 (33-76) | 39 (27-69) |
| AST (unit/L) | 39 (23-48) | 32 (23-46) | 34 (23-50) | 31 (21-52) |
| ALP (IU/L) | 93 (76-106) | 92 (76-113) | 88 (78-114) | 86 (75-100) |
| GGT (unit/L) | 61 (26-115) | 59 (34-114) | 68 (41-122) | 59 (38-101) |
| Ferritin (ug/L) | 174 (65-237) | 185 (68-224) | 171 (58-247) | 144 (61-223) |
| **Physical Activity** | | | | |
| Inactive | 766 ± 94 | 763 ± 110 | 766 ± 108 | 757 ± 118 |
| Light physical activity | 157 ± 48 | 159 ± 63 | 159 ± 61 | 159 ± 66 |
| Moderate physical activity | 61 ± 33 | 61 ± 35 | 62 ± 38 | 60 ± 39 |
| Vigorous physical activity | 1 (0-3) | 1 (1-2) | 1 (0-3) | 1 (0-4) |
| Sleep duration (minutes) | 402 ± 61 | 399 ± 62 | 395 ± 68 | 399 ± 67 |
| Sleep efficiency (%) | 91 (86-93) | 90 (87-91) | 90 (84-93) | 88 (85-92) |

Values are means (standard deviation) and medians (range). (n=48). BMI, body mass index; MAP, mean arterial pressure; HbA1c, glycated haemoglobin; HOMA-IR, homeostatic model assessment of insulin resistance; HDL, high-density lipoprotein; TC: HDL, total cholesterol: HDL ratio; ALT, alanine aminotransferase; AST, aspartate aminotransferase; ALP, alkaline phosphatase; GGT, gamma-glutamyl transpeptidase. The data presented in this table represents raw observations and has not undergone statistical testing.

**X.4** Biomarkers of liver fibrosis characteristics of participants randomised to CD in the first experimental period.

| **Participant Characteristics** | **Baseline** | **Post-CD** | **Baseline** | **Post-MD** |
|---|---|---|---|---|
| Age (years) | 62.0 (52.0-70.0) | | | |
| T2DM | 14 (52%) | | | |
| **Liver function** | | | | |
| Platelets (x10 9 /L) | 241 ± 54 | 246 ± 62 | 246 ± 58 | 243 ± 56 |
| ALT (unit/L) | 45 (34-74) | 41 (28-73) | 43 (33-76) | 39 (27-69) |
| AST (unit/L) | 39 (23-48) | 32 (23-46) | 34 (23-50) | 31 (21-52) |
| **Liver steatosis** | | | | |
| CAP dB/m *(baseline only)* | 329 ± 52 | | | |
| **Liver fibrosis** | | | | |
| Liver stiffness (kPa) *(baseline only)* | 10.7 (7.0-14.7) | | | |
| GDF15 (pg/mL) | 1009.4 (629.1-1437.8) | 1136.7 (674.0-1436.2) | 1082.4 (678.0-1589.7) | 945.8 (710.7-1535.3) |
| PRO-C3 (ng/mL) | 16.1 (12.7-20.4) | 15.5 (11.6-21.6) | 16.1 (12.2-21.7) | 13.0 (10.2-20.4) |
| PRO-C4 (ng/mL) | 7710.8 ± 1453.3 | 7714.3 ± 1366.7 | 7716.1 ± 1410.7 | 7598.6 ± 1175.2 |
| PRO-C5 (ng/mL) | 907.6 ± 386.6 | 872.8 ± 407.9 | 888.1 ± 367.2 | 896.3 ± 373.7 |
| CTX-III (ng/mL) | 7.0 (5.6-12.5) | 8.1 (5.6-12.4) | 7.2 (5.4-12.2) | 7.6 (5.1-13.1) |
| FIB-4 | 1.3 (1.0-1.8) | 1.2 (0.9-1.7) | 1.2 (0.9-1.4) | 1.2 (0.9-1.5) |
| ADAPT | 7.47 ± 1.30 | 7.48 ± 1.40 | 7.56 ± 1.53 | 7.19 ± 1.17 |

Values are numbers (percentages), means (standard deviation) or medians (range). (n=48). *PNPLA3,* patatin-like phospholipase domain containing 3; TM6SF2, transmembrane 6 superfamily member 2; T2DM, type 2 diabetes; ALT, alanine aminotransferase; AST, aspartate aminotransferase; PRO-C3, N-terminal propeptide of type III collagen; CTX-III, crosslinked type III collagen; GDF15; growth differentiation factor 15; FIB-4, Fibrosis-4 index; ADAPT, PRO-C3-based fibrosis algorithm; CAP, controlled attenuation parameter. The data presented in this table represents raw observations and has not undergone statistical testing.

**Appendix Y: Comparison of changes in total MEDAS scores between sub-groups after 4-weeks of MD intervention.**

| Study measures | Total MEDAS scores | *n* | P-Value |
|---|---|---|---|
| **Sex** | | | |
| Male | 2.0 (1.0-6.0) | 22 | 0.367 |
| Female | 4.0 (2.0-5.5) | 25 | |
| **Disease severity** | | | |
| Non-advanced | 3.0 (1.0-5.0) | 35 | 0.351 |
| NASH cirrhosis | 4.0 (1.3-7.5) | 12 | |
| **Age (years)** | | | |
| 30-39 | 4.0 (2.0-.) | 3 | 0.675 |
| 40-49 | 5.0 (1.0-.) | 3 | |
| 50-59 | 4.0 (1.0-5.0) | 15 | |
| 60-69 | 3.0 (2.0-6.0) | 15 | |
| >70 | 2.5 (0.0-5.0) | 10 | |
| <30 | -1.0 (-1.0- -1.0) | 1 | |
| **Ethnicity** | | | |
| White British | 3.0 (1.8-6.0) | 42 | 0.445 |
| Mixed White and Asian | 5.0 (4.0-.) | 2 | |
| British Indian | 0.0 (0.0-0.0) | 1 | |
| Pakistani | 4.0 (4.0-4.0) | 1 | |
| Other Asian | 1.0 (1.0-1.0) | 1 | |

Data presented as medians (range). (n=47). MD, Mediterranean diet; MEDAS, MD assessment score; NASH, non-alcoholic steatohepatitis.

**Appendix Z: Anthropometric and biochemical characteristics, physical activity and sleep patterns of participants at baseline and after 4-weeks of CD.**

| Participant Characteristics | Baseline | Post-CD | P-Value |
|---|---|---|---|
| **Anthropometrics** | | | |
| Weight (kg) | 95.7 (83.4-109.6) | 94.2 (82.8-109.8) | 0.217 |
| BMI (kg/m$^2$) | 35.0 ± 5.5 | 34.8 ± 5.4 | 0.101 |
| Waist circumference (cm) | 114.5 (104.8-125.5) | 112.5 (104.0-125.8) | 0.964 |
| Waist-to-hip ratio | 0.99 (0.94-1.04) | 1.01 (0.96-1.06) | 0.157 |
| Body composition (kg): Fat mass<br>Fat free mass | 44.2 (35.6-47.8)<br>53.8 (46.2- 62.7) | 45.1 (36.6-48.2)<br>53.2 (44.8-60.2) | 0.747<br>0.324 |
| **Cardiometabolic measures** | | | |
| Blood pressure: Systolic (mmHg)<br>Diastolic (mmHg) | 138.7 ± 18.4<br>80.5 ± 8.8 | 137.2 ±15.7<br>79.8 ± 10.6 | 0.548<br>0.631 |
| MAP (mmHg) | 99.9 ± 9.9 | 99.0 ± 10.1 | 0.487 |
| Fasting glucose (mmol/L) | 6.6 (5.6-9.1) | 6.6 (5.8-9.3) | 0.928 |
| Fasting Insulin (pmol/L) | 113 (78-150) | 111 (89-173) | 0.972 |
| HbA1c (mmol/mol) | 62.7 ± 24.0 | 63.3 ± 24.8 | 0.708 |
| HOMA-IR | 4.9 (3.0-9.7) | 6.1 (3.8-9.2) | 0.925 |
| Total cholesterol (mmol/L) | 4.3 ± 1.1 | 4.2 ± 1.1 | 0.260 |
| Triglycerides (mmol/L) | 1.8 (1.2-2.4) | 1.7 (1.2-2.2) | 0.659 |
| HDL (mmol/L) | 1.2 (1.1-1.4) | 1.2 (1.1-1.4) | 0.494 |
| TC:HDL ratio | 3.6 (2.8- 4.1) | 3.2 (2.5-3.9) | 0.070 |
| Non-HDL (mmol/L) | 3.0 ± 1.0 | 2.9 ± 1.0 | 0.109 |
| QRISK3 | 15.3 ± 10.0 | 14.6 ± 8.9 | 0.033 |
| **Liver function** | | | |
| Platelets (x10 9 /L) | 232 ± 58 | 233 ± 63 | 0.901 |
| Bilirubin (umol/l) | 10 (6-12) | 9 (7-13) | 0.344 |
| Albumin (g/L) | 46 (43-48) | 45 (43-47) | 0.106 |
| ALT (unit/L) | 38 (26-56) | 38 (24- 56) | 0.195 |
| AST (unit/L) | 31 (24-45) | 27 (23-43) | 0.069 |
| ALP (IU/L) | 91 (75-116) | 92 (74-111) | 0.717 |
| GGT (unit/L) | 63 (35-116) | 64 (40-107) | 0.956 |
| Ferritin (ug/L) | 102 (37-210) | 126 (54-221) | 0.130 |
| **Physical Activity** | | | |
| Inactive | 782 ± 94 | 769 ± 107 | 0.365 |
| Light physical activity | 155 ± 52 | 155 ± 60 | 0.968 |
| Moderate physical activity | 58 (26-91) | 54 (27-80) | 0.480 |
| Vigorous physical activity | 1 (0-2) | 1 (1-2) | 0.067 |
| Sleep duration (minutes) | 391 ± 66 | 400 ± 62 | 0.431 |
| Sleep efficiency (%) | 89 ± 5 | 88 ± 5 | 0.061 |

Values are means (SD) and medians (range). (n=48). CD, control diet; BMI, body mass index; MAP, mean arterial pressure; HbA1c, glycated haemoglobin; HOMA-IR, homeostatic model assessment of insulin resistance; HDL, high-density lipoprotein; TC: HDL, total cholesterol: HDL ratio; ALT, alanine aminotransferase; AST, aspartate aminotransferase; ALP, alkaline phosphatase; GGT, gamma-glutamyl transpeptidase.

## Appendix AA: Changes in biomarkers of liver fibrosis after 4-weeks of CD.

| Participant Characteristics | Baseline | Post-CD | P-Value |
|---|---|---|---|
| Age (years) | 60.0 (52.3-68.8) | | |
| T2DM | 26 (54%) | | |
| **Liver function** | | | |
| Platelets (x10 9 /L) | 232 ± 58 | 233 ± 63 | 0.901 |
| ALT (unit/L) | 38 (26-56) | 38 (24- 56) | 0.195 |
| AST (unit/L) | 31 (24-45) | 27 (23-43) | 0.069 |
| **Liver steatosis** | | | |
| CAP dB/m | 329 ± 52 | | |
| **Liver fibrosis** | | | |
| Liver stiffness (kPa) | 10.7 (7.0-14.7) | | |
| GDF15 (pg/mL) | 1009.4 (696.2-1556.2) | 1256.6 (696.6- 1635.8) | 0.938 |
| PRO-C3 (ng/mL) | 17.8 ± 7.1 | 17.5 ± 7.2 | 0.656 |
| PRO-C4 (ng/mL) | 7584.1 (6629.8-8297.8) | 7329.7 (6824.7-8508.6) | 0.372 |
| PRO-C5 (ng/mL) | 923.3 (740.6-1125.2) | 890.0 (759.3-1118.0) | 0.250 |
| CTX-III (ng/mL) | 8.9 ± 5.1 | 9.1 ± 5.4 | 0.540 |
| FIB-4 | 1.2 (1.0-1.9) | 1.3 (0.9-1.8) | 0.567 |
| ADAPT | 7.55 ± 1.38 | 7.45 ± 1.23 | 0.391 |

Values are numbers (percentages), means (SD) or medians (range). (n=48). CD, control diet; T2DM, type 2 diabetes; ALT, alanine aminotransferase; AST, aspartate aminotransferase; PRO-C3, N-terminal propeptide of type III collagen; CTX-III, crosslinked type III collagen; GDF15; growth differentiation factor 15; FIB-4, fibrosis-4 index; ADAPT, PRO-C3-based fibrosis algorithm; CAP, controlled attenuation parameter.

**Appendix AB: Comparison of changes in biomarkers of liver fibrosis between sub-groups after 4-weeks of MD intervention.**

| Liver fibrosis | Non-advanced | NASH cirrhosis | *n* | P-Value |
|---|---|---|---|---|
| GDF15 (pg/mL) | 27.6 (-53.4-116.3) | -55.0 (-276.6- -7.9) | 31<br>12 | 0.091 |
| PRO-C3 (ng/mL) | -1.0 (-4.5-0.9) | -0.1 (-2.8-1.0) | 30<br>12 | 0.690 |
| PRO-C4 (ng/mL) | -182.8 ± 647.5 | 31.5 ± 695.1 | 31<br>12 | 0.346 |
| PRO-C5 (ng/mL) | -16.5 ± 116.0 | -27.1 ± 57.6 | 23<br>11 | 0.776 |
| CTX-III (ng/mL) | 0.6 ± 1.9 | 0.4 ± 1.0 | 26<br>7 | 0.780 |
| FIB-4 | 0.0 (-0.2-0.1) | 0.0 (-0.5-0.1) | 31<br>12 | 0.478 |
| ADAPT | -0.2 (-0.6-0.2) | -0.1 (-0.4-0.1) | 30<br>12 | 0.837 |

Values are means (SD) and medians (range). MD, Mediterranean diet; PRO-C3, N-terminal propeptide of type III collagen; CTX-III, crosslinked type III collagen; GDF15; growth differentiation factor 15; FIB-4, fibrosis-4 index; ADAPT, PRO-C3-based fibrosis algorithm; NASH, non-alcoholic steatohepatitis.

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