"Investigation of the effects of a synbiotic on liver fat, disease biomarkers and intestinal microbiota in non-alcoholic fatty liver disease"

Short title:

Investigation of **sy**nbiotic **t**reatm**e**nt in NAFLD
(INSYTE)

Contents

Full title: Investigation of the effects of a synbiotic on liver fat, disease biomarkers and intestinal microbiota in non-alcoholic fatty liver disease.

Short title: Investigation of synbiotic treatment in NAFLD. (Acronym: INSYTE)

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I. Summary

Objective: Non-alcoholic fatty liver disease (NAFLD) represents a spectrum of fat-related conditions ranging from simple fatty liver, to non-alcoholic steatohepatitis (NASH), fibrosis and cirrhosis. Up to 30% of the global population is affected by NAFLD and there is no licensed treatment for this condition. Serious liver diseases secondary to cirrhosis is poised to become a factor for liver transplantations. Although NAFLD is associated with obesity, approximately half of obese people are unaffected, and the development and consequences of NAFLD [chronic liver disease, type 2 diabetes and cardiovascular disease (CVD)] involve not only altered liver function, but also dysfunction of key extra-hepatic tissues, such as intestine, adipose tissue and the vasculature. Furthermore, since the gut and liver are anatomically linked, disturbance of gut microbiota can affect the liver. The objective of this study is to determine the effects of altering gut microbiota in patients with NAFLD through administration of a combination of a pre and a probiotic (termed a synbiotic) on liver fat and a range of disease biomarkers.

Aim: In NAFLD patients, we will test the effects of the combination of a prebiotic and a probiotic (termed a synbiotic) on a) liver fat, b) NAFLD fibrosis algorithm scores, c) gut microbiota, d) insulin resistance, e) classical and novel cardiovascular risk factors, and f) satiety and satiety-related factors. We will also undertake assessment of food choices and physical activity in NAFLD patients.

Methods: We will phenotype adults with NAFLD and randomise 100 participants to synbiotic or placebo (n = 50 per group). Participants will be required to attend clinic visits at study entry and at several times over the course of the 10 to 14 month intervention period. During these visits measures of liver fat, insulin resistance and satiety will be undertaken and blood samples will be collected for measurement of biomarkers of NAFLD and cardiovascular risk. We will also assess a change in gut (faecal) microbiota composition using a) the 16S ribosomal RNA gene sequence-based method (16S rRNA); b) fluorescent in-situ hybridisation (FISH) analysis; and c) quantitative polymerase chain reaction (PCR). A subgroup of patients will be studied more intensively to measure de novo lipogenesis and hepatic insulin resistance.

Outcomes: We will assess response to synbiotic compared with placebo on liver fat, biomarkers of NAFLD, biomarkers of cardiovascular risk, insulin resistance, gut microbiota, satiety and lipid metabolism.

II. Background

Non-alcoholic fatty liver disease (NAFLD) is a liver manifestation of the metabolic syndrome and is one of the risk factors for type 2 diabetes and cardiovascular disease. NAFLD is a pathologic condition in which abnormal accumulation of fatty acids in the liver exceeds 5% of the total liver weight, provided that daily alcohol consumption does not exceed 10 g/day (1). NAFLD comprises a wide spectrum of liver diseases, ranging from simple steatosis to hepatocyte inflammation, necrosis, and ultimately cirrhosis and hepatocellular carcinoma. NAFLD is strongly related to metabolic disorders such as insulin resistance, type 2 diabetes, obesity, dyslipidaemia, cardiovascular disease and microvasculature inflammation. The homeostasis of the intestinal microbiota ecosystem has a central role in several metabolic mechanisms that can lead to obesity (2), insulin resistance, type 2 diabetes, excessive adipocyte lipid storage, inflammation and NAFLD (3;4).

The liver has a central anatomic location in the gastrointestinal tract and is the major filter organ; it is linked to the gut in a bidirectional interaction: i) through a unique vasculature that converges in the portal vein and ii) through the bile and enterohepatic circulation. In this relationship, the liver is

exposed to endogenous and exogenous endotoxin and bacterial products derived from the gut microbiota.

The gastrointestinal tract is sterile at birth. Bacterial colonization begins during parturition, evolving throughout development with acquisition of a "personal profile" of gut microbiota (5). Humans harbour 10^{14} bacteria in the gut (6), comprising 150 phylotypes; Bacteroidetes and Firmicutes are the two most common bacterial phyla (7). The intestinal tract comprises a multitude of diverse anatomical sites, each characterised by a heterogeneous mucosal microbiota. Moreover, each individual has a unique community composition of mucosal microbiota (8). This inter- and intra-subject variability is influenced by numerous environmental factors such as the age, diet and health (9).

Imbalances in the gut microbiota can lead to metabolic endotoxemia, obesity, insulin resistance and inflammation, all factors implicated in NAFLD.

1. Imbalances in the gut microbiota linked to NAFLD

1.1 Energy harvest

The energy harvest from nutrients is significantly affected by the composition of the intestinal microbiota. Several studies have been conducted to analyse the relationship between gut microbiota and body fatness, resulting in divergent observations. For example, Ley et al. showed that obese individuals and genetically obese ob/ob mice have a large proportion of *Firmicutes* and a low proportion of *Bacteroidetes* compared with lean controls (10;11). In contrast, Duncan et al. did not find a relationship between a high *Firmicutes/Bacteroidetes* ratio and obesity (12).

Gut microbiota dysequilibrium can cause a reduction of *Fasting-induced adipose factor* (Fiaf, Angiopoietin-like protein 4), a protein inhibitor of lipoprotein lipase (LPL) activity synthesised in brown and white fat, liver and intestine. Fiaf is an important control factor involved in triglyceride homeostasis, as it modulates energy absorption from the diet, and energy (lipid) storage in liver, muscle and adipose tissue (13;14). Backhed et al. described that the suppression of the intestinal expression of Fiaf by gut microbiota causes increased LPL activity that leads to an increased cellular uptake of fatty acids with consequent triglyceride accumulation in the muscle, adipocytes and liver. In the same study, conducted on animals and humans, Backhed showed higher serum levels of Fiaf in healthy individuals than in patients with type 2 diabetes (15). Overall, low level of Fiaf causes an excessive energy absorption, which leads to obesity and liver lipid storage with development of NAFLD.

B. thetaiotaomicron, a Gram-negative anaerobic bacterium present in the gut significantly affects monosaccharide uptake from the intestine (16). This bacterium degrades indigestible polysaccharides and increases fermentation, contributing to produce body fat accumulation, obesity and insulin resistance (17). Moreover, in the presence of Paneth cells, *B. thetaiotaomicron* increases the capillary density of the intestinal villus favouring monosaccharide absorption. The carbohydrate uptake from the gut, promotes delivery of monosaccharides to the liver which cause the up-regulation of sterol response element binding protein 1 (SREBP-1) and carbohydrate response element binding protein (ChREBP). These two factors induce the hepatic transcription of the genes encoding lipogenic enzymes, so promoting hepatic triglyceride production (15).

1.2 Toxic effects

A change in gut microbiota can favour the overgrowth of Gram-negative bacteria, leading to an increased production of lipopolysaccharide (LPS) from the Gram-negative cell walls. Cani et al.

have identified LPS as a trigger factor for insulin resistance, obesity and diabetes; they also demonstrated that LPS causes metabolic endotoxemia and inflammation (18). LPS binds to CD14/TLR4 on innate immune cells activating nuclear factor κB (NFκB) and subsequent production of inflammatory cytokines like tumour necrosis factor (TNF-α) and interleukins (IL-1 and IL-6). The secretion of pro-inflammatory cytokines affects insulin actions. Endotoxemia and hepatic expression of TLR4 are increased in patients with NAFLD (19).

Disturbed intestinal motility and prolonged orocaecal transit time are both factors that favour the bacterial overgrowth of the small intestine. This condition causes the overabundance of Gram-negative bacteria; this in turn causes an accumulation of endotoxins, leading to the production of inflammatory cytokines (TNF-α) that have a hepatotoxic effect (20).

Liver, biliary tract, intestine, portal venous circulation, colon systemic circulation and kidney are all involved in the enterohepatic circulation of bile acids. Bile acids, water, electrolytes, phosphatidylcholine, cholesterol and bilirubin are all components of bile, an iso-osmotic micellar solution produced by the liver. Bile acids synthesis is important for lipid digestion and absorption, cholesterol catabolism, fat-soluble vitamin absorption, glucose and energy homeostasis.

Primary bile acids are synthesised from cholesterol by two pathways in hepatocytes:

i. The "classic" natural pathway (quantitatively more important) in which, firstly, cholesterol is converted by the enzyme cholesterol 7α-hydroxylase to 7α-hydroxycholesterol. Secondly, the enzyme 3β-hydroxy-Δ5-C27-steroid dehydrogenase/isomerase reduces 7α-hydroxycholesterol to 7α-hydroxy-4-cholesten-3-one. Thirdly, 7α-hydroxy-4-cholesten-3-one proceeds to form chenodeoxycholic acid (CDCA) or/and cholic acid (CA).

ii. The "alternative" acidic pathway in which cholesterol is converted to 27-hydroxy-cholesterol and proceeds via 3β,7α-dihydroxy-5-cholestenoic acid to form CDCA (21).

Before secretion into the bile canaliculi, primary bile acids are conjugated to taurine or glycine to enhance their hydrophilicity and acidic strength; after conjugation bile acids are carried through the biliary tree and small intestine. Conjugation of bile acids facilitates fat digestion and absorption of fat-soluble vitamins. In the intestine, bile acids interact with gut microbiota in three instances: i) the majority of bile acids are deconjugated by the gut microbiota to form unconjugated bile acids, that are passively or actively absorbed and returned directly to the liver for reconjugation; ii) a small portion of CDCA is modified through eperimization by gut microbiota to produce urodeoxycholic acids (UDCA); and iii) in the colon, bacterial 7α-dehydroxylase converts CA to deoxycholic acid (DCA) and CDAC in lithocholic acid (LCA); this process reduces the solubility of bile acids. Deconjugation and dehydroxylation of primary bile acids by the gut microbiota (*inter alia Eubacterium* and *Clostridium* genera) produce faecal excretion primarily of the secondary bile acids and unconjugated bile acids DCA and LCA. (22)

Secondary bile acids are metabolised by the liver and gut microbiota to form tertiary bile acids through sulfation, hydroxylation and glucuronidation. LCA activates pregnane X receptor (PXR), a nuclear receptor expressed primarily in the liver and in the intestine. PXR has an important role in the hepatic detoxification of toxic metabolites including the unmodified LCA. The activation of PXR induces the sulfotransferase enzyme that is responsible for the sulfation of LCA; after sulfation or glucuronidation, LCA can be absorbed by the intestine and rapidly excreted. These two reactions decrease the cytotoxic effect of LCA (23;24).

Bile acids are an important regulator of lipid homeostasis. They are natural ligands of farnesoid X receptor (FXR), a nuclear receptor expressed in the liver, intestine, kidney and adipose tissue. Bile acids-activated FXR represses indirectly (via induction of short heterodimer partner, SHP) SREBP-1 and ChREBP involved in the hepatic *de novo* lipogenesis. Moreover, bile acids-activated FXR activates PPAR-α which in turn induces expression of genes encoding the enzymes involved in the β-oxidation of fatty acids, that inhibit triglycerides synthesis and VLDL export (25).

A diet rich in fats is likely to result in higher levels of liver cholesterol, leading to an increased synthesis of bile acids. The consequences of this altered metabolism are the accumulation and impaired detoxification of secondary bile acids which are more hydrophobic and toxic than primary bile acids.

Secondary bile acids have the following two toxic effects:

i. Increased intestinal permeability with decreased expression of tight junctions. This allows for transfer of endotoxin products (i.e. LPS from Gram negative bacterial membrane) from the intestine to the blood stream and thus to the portal vein and into the liver, causing inflammation and cytokine production (26;27).

ii. Apoptosis of enterocytes and hepatocytes. The hydrophobicity of secondary bile acids allows for their interaction with the phospholipids in cell membranes of hepatocytes and enterocytes. Secondary bile acids can also travel to the cytosol inducing perturbations of the mitochondrial membrane. The alterations of the mitochondria stimulate the production of reactive oxygen species (ROS) that in turn enhance mitochondrial permeability and cause the release of cytochrome c and other factors that form the apoptosome, ultimately causing cellular apoptosis and necrosis (28).

1.3 Insulin resistance/satiety/intermediate metabolism

The gut microbiota modulates glycaemic homeostasis through the production of gut hormones. An imbalance in gut microbiota causes a decrease of enteroendocrine L cells, resulting in a decrease of serum levels of GLP-1, GLP-2 and PYY (29;30). This mechanism causes an impaired glucose control and an impaired satiety (31).

In the colon, dietary fibres (non-digestible carbohydrates) are fermented and converted by anaerobic bacteria (*inter alia* lactobacilli and bifidobacteria species) to form short-chain fatty acids (SCFAs). The ability of bifidobacteria to produce SCFAs is ascribed to the activity of their β-fructofuranosidase and the presence of cellular oligosaccharide uptake system (32).

Dietary fibres have the following effects:

i. Bifidogenic effect. Dietary fibres are broken down in different ways by bifidobacteria and these different degradation mechanisms are responsible for metabolic competition among intestinal bacteria. Change in the composition of the gut microbiota has been associated with: a) decreased intestinal endotoxemia and LPS production; and b) decreased translocation of pathogenic bacteria (33).

ii. Butyrogenic effect. Butyrate is one of the end products of fermentation and has an important role in regulating gene expression in colonocytes preventing colorectal serious diseases and favouring differentiation of L-cells. SCFAs can activate the L-cells promoting the secretion of glucagon-like peptide-1 (GLP-1) that in turn stimulates insulin secretion and β-cells proliferation (34). Furthermore, in the colonocytes and hepatocytes, butyrate is oxidised to

acetyl-CoA which enters into the Krebs cycle increasing ATP levels, oxidative phosphorylation and inhibits autophagy. In addition, butyrate inhibits HMG-CoA reductase activity, so decreasing intestinal and hepatic cholesterol synthesis (35).

Some members of the gut microbiota can modulate the host bioavailability of choline and can mimic a choline deficient diet, which leads to the development of NAFLD. Choline is a quaternary saturated amine; it is an essential nutrient grouped within the vitamin B complex. Choline is required for the synthesis of phosphatidylcholine (PC), an important phospholipid component of biological membranes with structural and signalling functions. PC is also fundamental for hepatic secretion of triacylglycerol in very low density lipoproteins (VLDL) and is indispensable for cholinergic neurotransmission and methylation pathways. In humans, there are two sources of choline: the diet and endogenous production in the liver.

Dietary choline can be absorbed in the small intestine or metabolised by some bacteria (*inter alia Brucella melitensis, P. aeruginosa and Borrelia burgdorferi*) via the phosphatidylcholine synthase (PCS) pathway. In this pathway, bacteria utilise the exogenous choline to form PC, also a fundamental component of the bacterial membrane. This mechanism, therefore, mimics a choline deficient diet causing a failure of supply of substrate for host PC production resulting in the subsequent accumulation of triglycerides in the liver. Moreover, in the endogenous biosynthesis of PC some bacteria (*inter alia L. pneumophila*) can add a methyl group to phosphatidylethanolamine using the methyl donor S-adenosylmethionine via the phospholipid N-methylation pathway (PMT) (36).

The above mentioned pathways of choline metabolism lead to the production of microbiota-derived methylamines: dimethylamine, trimethylamine (TMA), and trimethylamine-*N*-oxide (TMAO). High concentrations of these methylamines and low plasma levels of PC have been found to be related to NAFLD (37).

In the liver, phosphatidylethanolamine *N*-methyltransferase (PEMT) catalyses the methylation of phosphatidylethanolamine to form PC. Gene mutation of PEMT or menopausal status decreases the activity of PEMT increasing the susceptibility to NAFLD (38;39).

The process of methylation generates three molecules of *S*-adenosylhomocysteine, in the liver via PEMT and in the bacteria via PMT. In the liver, these molecules are then hydrolysed to adenosine and homocysteine. A high serum level of homocysteine has been related to NAFLD (40).

2. *(Non-alcoholic) fatty liver disease.*

The prognosis of NAFLD has been difficult to assess due to the heterogeneity of the condition and most studies are small with relatively short follow-up. Recently one small study with a follow-up of 13.7 years has reported on the natural history of the NAFLD (41). These data showed overall risk of end stage liver disease was 5.4% (7/129) and that there were differences between individuals in progression of liver disease, according to baseline pathological diagnosis. If individuals had non-alcoholic steatohepatitis (NASH), prognosis was poor, with risk of progression to fibrosis and cirrhosis. In another small population cohort in the US (n=420), survival was poorer in people with ultrasound diagnosed fatty liver compared to the general population over 7.6 years (42). However, these investigators did not assess baseline severity of fatty liver disease by liver biopsy and liver ultrasound is unable to detect liver fibrosis. Moreover, the presence of baseline liver fibrosis seems to predict outcome, and overweight, smoking and diabetes have been shown to be important independent predictors of severity of hepatic fibrosis (43;44). Data from a retrospective cohort of biopsy proven NAFLD, showed that the presence of baseline fibrosis predicted liver related serious health

consequence (45). Therefore, and relevant to the current study, it is very important to phenotype participants carefully for the measures that predict liver fibrosis, and it is important to assess whether there is already evidence of liver fibrosis at diagnosis.

Recently, it has been shown that in people with type 2 diabetes, NAFLD is associated with increased risk of cardiovascular disease. Moreover, the risk of cardiovascular disease increased with severity of NAFLD and was independent of traditional CVD risk factors (46-48), increasing risk for myocardial infarction by an additional 50% even after adjustment for all known cardiovascular risk factors. Diabetes is a significant risk factor for the development and progression of NAFLD, and up to 70% of type 2 diabetics have NAFLD. It is difficult to be certain of the true prevalence of steatohepatitis in diabetics due to lack of liver histology data, but studies have suggested it occurs in up to 80% of diabetics with NAFLD and abnormal liver enzymes.

3. *Prebiotics, probiotics and synbiotics: potential effects in NAFLD*

Etymologically, the term 'probiotic' is a combination of the Latin preposition 'pro' and the ancient Greek adjective βιωτικός and thus mean "for the living" or more loosely "favouring life". According to Havenaar and Huis In't Veld a probiotic is "a preparation of or a product containing viable, defined microorganisms in sufficient numbers, which alter the microflora (by implantation or colonization) in a compartment of the host and by that exert beneficial health effects in this host" [as referenced in "Probiotics, prebiotics, and synbiotics—approaching a definition"(49)].

Prebiotics were first considered by Gibson and Roberfroid; at present, their definition describes them as "a selectively fermented ingredient that results in specific changes in the composition and/or activity of the gastrointestinal microbiota, thus conferring benefit(s) upon host health" (50).

A synbiotic is a product derived from the combination of oligofructose as prebiotic and bifidobacteria as probiotic (17). Administration of prebiotics and/or probiotics reduce hepatic inflammation (51), insulin resistance and diabetes (52;53).

3.1 The action of probiotics

  i.   Competitive exclusion of intestinal bacteria
  ii.   Lower pH gradient in the intestine, thus promoting butyrate production and decreasing acetate concentration. This condition inhibits the growth of Gram-negative pathogenic bacteria (54).
  iii. Improvement of epithelial barrier function

3.2 The action of prebiotics
  i.   Increase of enteroendocrine L cells, resulting in increased serum levels of GLP-1, GLP-2 and PYY (29;30)
  ii.   Reduced plasma triglyceride levels, muscle lipid infiltration, adipose tissue mass and oxidative stress (30;55)
  iii. Reduced inflammation and improved immune function (56).

III.   Detection and monitoring of fatty liver disease

Key to this study will be the use of non-invasive markers of liver function and of NAFLD disease severity. These tests utilizing serum markers of fibrosis are widely used in clinical practice in Europe. Numerous studies (57-69) have evaluated the two main markers, hyaluronic acid (HA) and collagen PIIINP peptide in patients with fatty liver disease (alcohol and non-alcohol related). Studies include a total of over 6000 subjects, of whom nearly 3000 had fat related disease aetiology. In those

studies where an area under the curve analysis (AUROC) was reported (70-72), the mean value was 0.91 compared with liver biopsy – acceptable diagnostic accuracy.

Algorithms have also been developed to generate a composite liver fibrosis score generated from concentrations utilizing HA and PIIINP, in combination with another marker TIMP-1 (73). It has been suggested that this approach may lead to an increase in specificity and sensitivity for diagnosing NAFLD. Others have also very recently developed algorithms based on less expensive simple biochemical tests, used in combination with simple anthropometry. For example, the clinical algorithm developed by Newcastle/Mayo group collaboration (74) uses age, glycaemia, BMI, albumin, platelet count and the AST/ALT ratio. This algorithm also has excellent sensitivity (77%) and specificity (96%) for detecting severe fibrosis.

The use of non-invasive markers cannot yet totally replace liver biopsy. The ability of non-invasive markers to monitor the natural history of NAFLD or to follow responses to treatments needs to be tested in research studies, before biomarkers will replace liver biopsy in clinical practice. Biomarkers such as the fibrosis score are most accurate in the diagnosis of severe fibrosis and cirrhosis and are of less use with mild fibrosis.

Liver biopsy thus remains the gold standard for assessing the degree of liver damage in fatty liver disease. Liver biopsy is not without hazard, with the main complication being bleeding which requires intervention in around 1:1000 cases. Moreover, patchy liver disease and variation between reporting by histopathologists can also decrease the utility of liver biopsy. Liver biopsy does not, therefore, provide a simple diagnostic test for the testing and monitoring of new treatments for fatty liver disease. Consequently, there is a need and a priority to utilise the now well validated non-invasive diagnostic markers of fatty liver disease, to expedite testing of potential treatments.

Additional phenotyping of metabolism is crucial in people with fatty liver disease, because as stated above, important risk factors for liver fibrosis are obesity and diabetes. Furthermore, limited recent evidence to date suggests that treatments showing promise in treating fatty liver disease, act predominantly outside the liver, and thus act indirectly to improve the liver condition.

To date all of the limited studies that have tested treatments for fatty liver disease have not studied patients that have been characterized for metabolism, insulin sensitivity and body composition and many of the study results are difficult to interpret because investigators have used poor surrogates for liver disease, such as ultrasound and liver function tests. Because of these limitations, it is not possible to define the mechanism(s) of potential benefit (or harm) of such treatments. No studies to date have utilized the non-invasive algorithms, such as those described above, to monitor the effects of potential therapies.

IV.   Methods

A double blind, randomised, placebo-controlled trial will test the effects of a synbiotic intervention in over 10 to 14 months in 100 patients with NAFLD (n=50 per treatment group). (N.B It is expected that the effect of the intervention on primary and secondary end-points will be constant between 10-14 months, see below Plan of Investigation.)

The synbiotic to be used is fructo-oligosachharide with a degree of polymerization < 10 at 4 g/twice a day (two sachets a day) plus *Bifidobacterium animalis* subsp. *lactis* BB-12 at a minimum of 10 billion CFU/day (1 capsule a day). The synbiotic will be provided as a capsule for probiotics, and powder (in a sachet) for prebiotics, to stir into a cold drink (i.e. < 25 degrees). The placebo will also be provided in a capsule and as powder in a sachet, comprising a total of 4 g/twice a day of maltodextrin

daily. Consequently, the synbiotic and the placebo will be supplied in identical packaging. The synbiotic/placebo is to be consumed soon after mixing and certainly within two hours. The powder could also be spread on food.

Safety information

The following gastrointestinal manifestations have been reported after the consumption of synbiotics consumption: increased bloating, increased flatulence and increased regularity of bowel movements (53).

V. Primary outcome measures

Primary outcome measures will be:

     i. Liver fat determined by magnetic resonance spectroscopy;

     ii. Serum hyaluronic acid (HA), serum amino-terminal pro-peptide of type III collagen (PIIINP) and tissue inhibitor matrix metalloproteinase 1 (TIMP-1) concentrations; and a composite liver fibrosis score generated from concentrations of these three analytes (75).

     iii. Gut microbiota composition determined by 16S rRNA, FISH and qPCR.

VI. Secondary outcome measures

     i. Liver fibrosis determined by transient elastography

     ii. Insulin and glucose concentrations and hepatic insulin sensitivity

     iii. Microvascular function

     iv. Plasma cardiovascular risk markers

     v. Adipose tissue and blood markers of metabolism and inflammation

     vi. De novo lipogenesis

     vii. Satiety and satiety factors

VII. Power calculation for effect of treatment with a synbiotic on liver fat

There is little published literature to date upon which to base a sample size calculation to test the effects of the synbiotic treatment on the primary end point of change in liver fat.

A sample size of 50 in each group, with a 14% drop out during the study, will have 85% power to detect a difference of 40% in liver fat (in the treatment arm compared with the placebo), assuming that the common standard deviation is 62%, using a power calculation test with a 0.05 two-sided significance level. In our recent randomised controlled trial over a longer period of intervention, 5% of the randomised cohort withdrew between randomisation and end of study measurements. In this recent trial, the mean percentage fat content was 28.5% with a similar standard deviation to that presented above. Liver fat content will be measured by MRS spectroscopy and this technique can reproducibly detect liver fat content as low as 5%.

N.B. One recent publication in people with NAFLD testing the effect of a synbiotic over 24 weeks in a small study showed a 69% decrease in liver fat (53).

VIII. Plan of investigation

     1. Recruitment

At University Hospital Southampton NHS Foundation Trust, potential participants with a diagnosis of non-alcoholic fatty liver disease (NAFLD) established as part of their attendance at hospital clinics, will be contacted by the research team. Contact will occur at their hospital clinic attendance or by letter of invitation from the research team.

Outside Southampton, at Poole Hospital NHS Foundation Trust, Portsmouth Hospitals NHS Trust, Royal Bournemouth and Christchurch Hospitals NHS Foundation Trust, Hampshire Hospitals NHS Foundation Trust, and the Isle of Wight NHS Trust, our collaborators (medical doctors responsible for the care of people with NAFLD) will act as 'post boxes' and inform potential participants about the study, providing people with a patient information sheet and asking them if interested to get in touch with the research team members at Southampton listed on the patient information sheet.

Because the centres outside Southampton are acting as 'post boxes' and not undertaking any research, completion of individual SSI forms may not be required for these sites because they are classified as site specific exempt.

Participant recruitment

- There will be no payment of participants but travel costs will be reimbursed.

- After discussion of their diagnostic liver biopsy test, or CT scan, or ultrasound, or MRI results, participants will be invited to participate in the research study, by direct contact from a member of the research team, or by letter of invitation.

- Each participant will have approximately 2 weeks, or as much time as they need, to decide upon their participation after initial discussion with the research team doctor or nurse. Consent will be obtained by a participating medical doctor or nurse within the research team.. Translators are provided by the NHS Trust for non-English speaking participants. Only adults able to understand the nature of adipose tissue biopsy will be recruited.

- We will use the UHS clinical code database containing patients with a diagnosis of NAFLD. This information will be sought from the UHS Information Team in accordance with UHS NHS policy.

Detail of randomisation procedure

- Participants will be block randomized according to standardized procedures (computerized randomization) by a person from within University Hospital Southampton or the University of Southampton who is not connected to the INSYTE study. Investigators will be blinded as to treatment allocation until cessation of the trial.

- Stratified randomization to either trial medication (synbiotic or placebo) will be used.

Participant compliance

Participants will be asked to return all used and unused sachets at their next study clinic visit and their final clinic visit. Compliance will be assessed as percentage of sachets used.

- Non-compliance will be defined as missed usage of sachets for more than 6 months (53) (consecutively) during the whole study and or missed usage of sachets for 3 months (consecutively) before the end of the study.

- We will consider as compliant, participants who have had one or two courses of broad spectrum antibiotics during the study period. Participants will continue the synbiotic treatment during the antibiotic therapy and we will extend the synbiotic treatment for at least 1 month (i.e. 12+2 months of active therapy) before the final visit.

If there is indication that participants are non-compliant with trial treatment during the trial they will be withdrawn.

<u>Detail of follow-up of non-compliant subjects & withdrawal of subjects</u>

- Participants will be withdrawn if they develop a serious chronic illness that affects their liver condition.

- Participants will be withdrawn if they require treatment with a known hepatotoxic drug.

- Participants will be withdrawn if they have more than two courses of antibiotic therapy that is recognized to influence gut microflora during the study.

- Participants will be withdrawn if they develop a medical condition that would affect their consumption of trial medication.

- In all instances a member of the research team will discuss withdrawing from the trial with the participant.

- Data will be collected up to the point of withdrawal and will be held on all withdrawn participants in parallel with data collected on those who do not withdraw.

- It is envisaged that retention during the trial will be high and we do not anticipate withdrawal of many participants. However, if a participant withdraws or is withdrawn during the 18 months recruitment phase of the study, attempts will be made to find a suitable replacement.

- Withdrawn participants will be offered follow up in the liver clinic at University Hospital Southampton NHS Foundation Trust.

<u>Parameters for reporting adverse events (AEs), serious adverse events (SAEs) and suspected unexpected adverse reactions (SUSARs).</u>

A recent study in people with NASH testing the effects of a synbiotic showed the supplement is well tolerated in 100% of patients (53). In this study of 34 people treated with synbiotic for 24 weeks, one patient complained of nausea, one of moderate headache, and one of abdominal pain. However, in the placebo group of n= 32, two patients also complained of nausea, one reported fatigue, and one reported dizziness. There were no clinically relevant changes in the hematology, clinical chemistry, and renal function observed in both treatment arms. Nobody withdrew from planned treatment.

Adverse effects possibly associated with trial medication will be recorded and reported according to University Hospital Southampton research governance policy. If a volunteer reports any

untoward medical occurrences, these will be recorded on an adverse event or serious adverse event form in accordance with the most current UHS Research Related Adverse Event Reporting Policy. All adverse and serious adverse events will be reported and recorded for the duration of the study.

These effects will be classified according to whether they are AEs, SAEs and SUSARs.

2. *Inclusion and exclusion criteria*

Participants will be characterized according to:

i. Well validated panels of serum markers and simple anthropometric measurements associated with severity of liver fibrosis.
ii. Measures of insulin sensitivity, body composition and other features of the metabolic syndrome that help provide an estimate of future risk of type 2 diabetes. Presence of diabetes mellitus.
iii. Assessment of prevalent cardiovascular disease.
iv. Diet.
v. Alcohol consumption.
vi. Quality of life indicators.

Inclusion criteria

- Both men and women
- Age > 18 years
- Liver fat diagnosed on normal clinical grounds including in most cases liver assessed by Kleiner scoring system (76) to classify severity, with no known aetiological factors for underlying liver disease (e.g. exclusion of hepatitis A, B & C, primary biliary cirrhosis, autoimmune hepatitis, haemochromatosis). Last liver biopsy will be within 3 years of recruitment to the study.
- Liver fat diagnosed by ultrasound, CT or magnetic resonance imaging (MRI) within 3 years in patients who also have either diabetes and/or features of the metabolic syndrome, without evidence of known aetiological factors for underlying liver disease (e.g. exclusion of hepatitis A, B & C, primary biliary cirrhosis, autoimmune hepatitis, haemochromatosis).
- Alcohol consumption ≤ 14 units / week for women ≤ 21 units / week for men (77).

Exclusion criteria

- Alcohol consumption ≥ 15 units /week for women and ≥ 22 units /week for men.
- Decompensated acute or chronic liver disease.
- A history of viral hepatitis, diarrhoea, diverticulosis, actively symptomatic irritable bowel syndrome, inflammatory bowel diseases, coeliac disease (seropositivity for anti-endomysial immunoglobulin A antibodies; IgA EMA).
- Previous bariatric or other abdominal surgery.
- Continuous use of antibiotics that may change gut microflora, probiotics, within the 2 months preceding enrolment, or evidence of immunoglobulin A or immunoglobulin deficiency (both of which produce confounding effects during assessments of intestinal permeability and small intestinal bacterial overgrowth).
- The participants may not be able to have an MRI scan if their weight is above 155 Kg as this is the maximum weight capacity for the magnetic resonance imaging.

3. *Milestones and plan*

Months 0-18	Recruitment of cohort, baseline measurements and randomisation to active compound or to placebo (10-14 month intervention)
Months 10-12	Follow up measurements begin
Months 10-32	Follow up measurements completed
Months 32-40	Measurements and analyses of results

N.B. Since a recent publication testing the effect of a synbiotic in a small number (n=34) of people with NASH showed a 69% decrease in liver fat on liver biopsy score over 24 weeks (53), we will treat participants for a minimum of 10 months treatment. To allow for a degree of patient choice in the timing of the end of study measurement appointments, participants will undergo end of study follow tests between 10 and 12 months after randomisation. Finally, if a participant has been treated with one or two courses (two courses is the maximum allowed) of antibiotic therapy during the 12 months of the trial treatment period, we will allow for an extra 2 months of trial medication to be given to allow for the gut microbiota to return to pre-antibiotic therapy levels, Thus, although the trial intervention period is 10-12 months, there may be occasional participants who will be treated with up to 14 months (maximum) of trial medication.

4. *Data Protection & Confidentiality: policy*

- Data will be recorded on CRFs and the Data Management team will enter and manage the data on a secure UHS networked file store. Patient data (e.g. pathology results) captured directly by UHS systems will be extracted and merged with CRF data. SPSS software will be used as the primary package for managing the data prior to statistical analysis. Double entry and cross validation techniques will be used to ensure the quality of the data.

- Source data will be stored in a secure research office at the Wellcome Trust Clinical Research Facility at University Hospital Southampton NHS Foundation Trust during the trial. After cessation of the study, records will be stored in a secure room at The Institute of Developmental Sciences (IDS) Building, University of Southampton, MP 887 Southampton General Hospital, Tremona Road, Southampton SO16 6YD, in accordance with adherence to Data Protection Act 1998.

- The investigator(s)/institution will permit monitoring, audits, for REC review and provide direct access to source data and documents.

IX. Clinical Tests

1. *Liver fat measurement*

Magnetic resonance imaging

Liver fat estimation by MR will be undertaken at baseline and at the end of the study by both MR imaging and MR spectroscopy where possible, as the techniques provide an estimation of liver fat using different methodologies. Both techniques are quick and non-invasive, and the total scanning time

for both techniques combined should be ~40 minutes for each participant. Obtaining this data will provide a quantitative estimate of liver fat and will be valuable information.

A non-invasive estimate of liver fat will allow us to study relationships between diagnostic histological disease severity (as identified by the Kleiner score for NAFLD disease severity – obtained by analysis of the histological examination of the liver biopsy specimen by the pathologist at the time of diagnosis) and the MR estimate of liver fat. We will also be able to study relationships between liver fat and biomarker score, and we will be able to study the effects of the randomised intervention on liver fat quantity by repeating the MR estimation of liver fat at the end of the study. We will also undertake MR scan acquisitions that we allow assessment of pancreatic fat quantity at baseline and end of study, as there is evidence that pancreatic fat is associated with type 2 diabetes and NAFLD.

Transient elastography

We will undertake transient elastography at the beginning and at the end of the study to measure liver stiffness. This is a new non-invasive, painless method allowing the evaluation of liver fibrosis. The probe utilised for the elastography is an ultrasound transducer mounted at the end of a vibrating cylinder. The cylinder produces a vibration that is transmitted towards the tissue. The ultrasound detects the propagation of the vibration by measuring its velocity. The velocity of the wave towards the tissue is directly related to the tissue stiffness. High velocity is related to higher tissue stiffness that corresponds to an increased severity of fibrosis. The performance of the transient elastography has been assessed in a meta-analysis including fifty studies. The mean area under receiver operating characteristics curve (AUROC) was 0.84 (95% CI, 0.82-0.86) for diagnosis of significant fibrosis, 0.89 (95% CI, 0.88-0.91) for severe fibrosis and 0.94 (95% CI, 0.93-0.95) for diagnosis of cirrhosis (78).

2. *NAFLD severity biomarkers*

We will assess fatty liver disease utilising MRI, transient elastography, (or liver biopsies) together with validated algorithms utilising non-invasive measurements:
- Serum hyaluronic acid, serum amino-terminal propeptide of type III collagen
- Components of other non-invasive marker panels (e.g. Newcastle/Mayo group (74)), plasma glucose concentration, body mass index, platelet count, serum albumin, serum AST/ALT ratio
- Serum will be saved for assay using newer technologies where appropriate (e.g. composite liver fibrosis score).

3. *Bacterial identification*

We will identify the phylogenic characteristics of the gut microorganisms using fluorescent in-situ hybridisation analysis (FISH) to identify specific bacteria types (79) and the 16S ribosomal RNA sequence-based method (80) to study the composition of rRNA amplified using the quantitative polymerase chain reaction (qPCR) (81).

4. *Intestinal function tests*

Gut biomarkers

Recent studies have shown an effect of prebiotic fermentation on the production of gut hormones. We will measure plasma levels of: glucagon-like peptide 1 (GLP-1), glucose-dependent insulinotropic polypeptide (GIP), peptide YY (PYY), and ghrelin (29;31).

<u>Intestinal permeability</u>

Lactulose-Mannitol test: After an overnight fast, we will administer to each participant a solution containing 10 g of lactulose and 5 g of mannitol in 35 ml of water (1300 mOsml/L). Urine will be collected over the next 6 hours in plastic containers with 1 ml of chlorexidine, 2% as preservative, to prevent bacterial degradation of sugars. During the test participants will be offered a light lunch. A 10 ml urine sample will be stored at -20°C until assayed. Sugars will be measured by previously described methods and expressed as a percentage of urinary excretion (82).

<u>Faecal qualitative and quantitative analyses</u>

- The van de Kamer method will be used to quantify faecal output of fat over 24 hours. This method is based upon the extraction of saponified fatty acids from the stool. The amount of fat present is quantitated by titration of the fatty acid COOH group with sodium hydroxide. The mean molecular weight of fatty acids in faeces determines the quantity of fat in faeces (83).
- The acid steatocrit test will be used to quantify faecal fat excretion (84).
- Faecal bile acids (85).

5. Adipose tissue biopsy

G protein-coupled receptor 43 (GPR-43) is a protein expressed in the gut and adipose tissue and is implicated in lipolysis regulation and adipocyte differentiation. A high fat diet increases the expression of GPR-43 mRNA in the adipocytes. It has been demonstrated that prebiotic supplementation counteracts this effect and inhibits lipolysis (86).

We will take a small amount of fat (fat biopsy) from the lower abdomen at the beginning and at the end of the study. Prior to the biopsy the participant will receive an injection of local anaesthetic.

<u>Measures of insulin sensitivity</u>

We will measure fasting-induced adipose factor (Fiaf), fasting plasma glucose, plasma triglyceride, and HDL cholesterol concentrations (87-89). Metabolic syndrome will be assessed following the criteria established by the International Diabetes Federation (IDF) (87;90;91). We will estimate the homeostatic model assessment to quantify insulin resistance (HOMA-IR) with fasting insulin and glucose (92;93). A plasma sample will be taken for HbA1c measurement.

6. Cardiovascular risk

<u>Cardiovascular history</u>

Recent evidence suggests that people with newly identified fatty liver disease have fat accumulation in the epicardial area and have abnormal left ventricular metabolism, perhaps contributing to myocardial ischaemia in this condition (94-96). Self-reported history of major adverse cardiovascular events and revascularization will be recorded. Angina status (NYHA class) and cardiovascular medication will be assessed. Serum markers of vascular inflammation (e.g. hsCRP, TNF-α, IL-6, adiponectin, selected MMPs, sCD40L, IP-10 and Mig) will be measured.

Blood pressure (97) will be measured 3 times (after 5 minutes).

Cardiorespiratory fitness

Cardiorespiratory fitness will be measured in terms of maximal oxygen uptake (VO_2 peak) and determined after volunteers had avoided strenuous exercise for 24 hours. Participants will be asked to avoid alcohol on the day prior to testing. They will then be fitted with an air-tight facemask, which allows analysis of expired air, and ECG leads. To allow volunteers to become acclimatised to the facemask and also to determine resting energy expenditure, resting measurements will be taken for 3 minutes prior to commencement of activity induced measurements. We will undertake an ECG before and after the treadmill test.

Carotid Artery Ultrasound

Carotid intima-media thickness (CIMT) and carotid plaque will be measured in all subjects at the beginning and end of the study. This measure is a well-known marker of subclinical atherosclerosis (98), and is a surrogate marker for coronary disease (99). It also has independent prognostic value in cardiovascular disease (100). This is a non-invasive test, where we will measure the internal lining (intima-media layer) of the carotid arteries with B-mode ultrasound. As well as assessing baseline risk in collaboration with other markers of vascular function, we will also compare any change in CIMT and carotid plaque between active and control subjects. This is a very safe test and involves the patient lying supine for only about 15 minutes while an ultrasound probe is applied gently to the neck area while measurements are recorded.

7. *Urine samples*

Assessment of lipids and proteins in plasma and urine

In collaboration with Dr Spiros D. Garbis from the University of Southampton, we will undertake a more detailed assessment of plasma and urine lipids and proteins. We intend to analyse these samples using standardised techniques in metabonomics including Nuclear Magnetic Resonance (NMR) spectroscopy. Statistical analysis will be performed using multivariate techniques including principal component analysis and partial least squares discriminant analysis. Sample acquisition and statistical analysis will be performed at the University of Southampton.

Urine tests

We will collect urine to test: urinary albumin/creatinine ratio (as a measure of albuminuria and increased vascular risk) (101-104), hippuric acid (as measure of protein degradation), trigonelline (as measure of metabolism of niacin), 2-hydroxyisobutyrate (protein digestion in upper gastrointestinal tract) (105), bile acids and choline metabolites [dimethylamine, trimethylamine (TMA), and trimethylamine-*N*-oxide (TMAO)]. We will estimate glomerular filtration rate (eGFR) using MDRD equation as a measure of kidney function (106).

8. *Diet and lifestyle*

Micronutrient status:

Furthermore, we will measure micronutrient status. The population under investigation with NAFLD are more likely to be obese than the general population. Obesity is associated with increased risk of micronutrient deficiency which may adversely influence insulin sensitivity, energy expenditure, mitochondrial function and muscle function. We will analyse dietary micronutrient intake by

questionnaires (food frequency questionnaire and Recording your Food [24 hour recall diary]). Using the questionnaire data we will obtain an estimate of dietary intake of micronutrients. We will then compare this estimate of intake with results of plasma and serum values of: vitamin C and vitamin E, calcium, phosphorus, magnesium, iron, copper, zinc, manganese, iodine, chromium, selenium, vitamin A (107;108), vitamin B12, vitamin E, vitamin K, vitamin D (109), folic acid and lipopolysaccharide (LPS).

Dietary assessment

We will use a computer program (Tinuviel Software) to explore food preferences covering a list of regularly consumed food and drink via Food Frequency Questionnaire (FFQ) (QBuilder) and to explore food and drink consumed throughout the past 24 hours (Weighed Intake Software Package - WISP - 24 hour dietary recall), measured at baseline and 12 months.(110). Participants will complete a standard questionnaire for food and alcoholic drink recall so that we are able to derive food intake and alcohol consumption in units/week. We will assess satiety and hunger using a visual analogue scale (VAS) (29).

Satiety protocol

We will measure satiety at the beginning and at the end of the study. Participants will be instructed to abstain from alcohol and strenuous physical activity for 2 days prior to the day of the test. We will offer participants a free-choice buffet breakfast (comprising yogurt, bread, butter, cheese, jam, fruit, orange juice, and water; approximately 470 kcal(111)); participants will be instructed to complete breakfast within 15 minutes. Food and drink will be weighed before and after the meal and we will calculate energy intake. Before and after breakfast (at -5, 0, 15, 30, 60, 120, 180 minutes) we will assess appetite rating, using a 100-mm visual analogue scale; moreover, we will measure gut hormones (blood sample) (29;112).

We will use a satiety visual analogue scale with verbal descriptors expressing the most positive and the most negative ratings positioned at each end of a 100-mm line. We will ask participants to draw a vertical mark across the line corresponding to their feelings from 0 (not hungry at all) to 100 (very hungry). We will quantify the level of satiety by measuring the distance from the left end of the line to the mark (113).

Quality of life indicators

We will study the lifestyle of our participants by measuring an array of key indicators measured via the following questionnaire:

- EuroQol (EQ-5D): this questionnaire provides a descriptive profile and a single index value for health status (114;115). It consists of two parts: a) descriptive system and b) visual analogue scale. The descriptive system comprises the 5 items: mobility, self-care, usual activities, pain/discomfort and anxiety/depression. Each item has 3 levels: no problems, some problems, extreme problems. The 5 items do not have an arithmetic sum; they describe the respondent's health state. The visual analogue scale is a quantitative measure of health as perceived by the respondents. It is designed for self-completion by respondents.
- Hospital Anxiety and Depression scale (116): this questionnaire assesses psychological status such as depression and anxiety (117) as obesity is associated with psychological morbidity (118). The questionnaire has been composed using a 14 item scale, half relating to depression

and half relating to anxiety, each item is scored from 0-3 thus the score range from 0 to 21. The cut-off point for anxiety or depression is 8/21. Psychological well-being may improve as distress, anxiety and depression are often improved by exercise (119) and this improvement may be relevant to NAFLD. It is designed for self-completion by respondents.

Physical activity and energy expenditure

Physical activity will be assessed using a monitor (Sensewear Pro2) (120) worn by each participant for approximately 4 days, as well as by means of a modified Baecke questionnaire (121). As the Baecke questionnaire does not distinguish between different dimensions of exercise we will also measure total energy expenditure by SenseWear Armband Pro2 for approximately four days. Baecke's questionnaire includes 16 questions, focusing on a composite score for 3 components of physical activity in the last 12 months: (1) occupational physical activity (eight questions); (2) physical activity in leisure (four questions); and (3) leisure and locomotion physical activities (four questions). A simple formula is used that takes account of the intensity of the activity, the amount of time spent undertaking the activity and the proportion of time annually spent undertaking the activity. The SenseWear Pro2 armband is a compact and lightweight 82 g device worn around the upper arm that is well tolerated and contains sensors for 2 plane accelerometry, near body temperature, skin temperature and the galvanic skin response. The SenseWear Pro armband allows reliable measurement of physical activity levels and calculation of total energy expenditure recordings.

Fatigue assessment:

Fatigue is a common systemic feature of NAFLD though mechanisms remain poorly understood (122;122;123). We will assess the severity of fatigue (Fatigue Severity Scale) and sleepiness (Epworth Sleepiness Scale) using validated scores at baseline and after 6 and 12 months intervention. We will also explore associations between measures of NAFLD disease severity, daytime sleepiness, autonomic dysfunction, micronutrient status, muscle function via grip test, immune parameters such as IL-6 and TNF alpha and mitochondrial dysfunction via 13C-methionine breath testing. This will provide a comprehensive assessment of this symptom which can cause functional impairment in people with NAFLD.

Muscle function:

We will test for muscle function using a simple and non-invasive technique (hand grip strength). This will allow us to better understand the influence of fatigue, disease severity, micronutrient status and mitochondrial function on muscle function in NAFLD. Handgrip strength will be measured at baseline and after intervention using a Jamar dynamometer with participants seated and their arms rested on the chair arms, alternating between each arm when taking measurements. We will use for the analysis the best score out of a total of three measurements from each hand. The Jamar dynamometer is simple to use, accurate, reproducible in its measurements and the most widely used device used for assessing muscle strength. There is evidence in literature that there is association between impaired physical function, weaker muscle strength and hyperglycaemia in people with diabetes (123;124). We would like to assess whether there is similar association between other features of metabolic syndrome including NAFLD and muscle strength.

9. *Peripheral vascular function*

Ankle / Brachial Pressure Index (ABPI)

ABPI will be measured at baseline and at the end of the study. ABPI is a simple measurement for assessing peripheral macrovascular function. Low ABPI (<0.9) is an independent predictor of increased cardiovascular disease risk (125). We want to assess correlation of ABPI with other measures of macro- and microvascular function. With the participant rested and lying supine blood pressure cuffs will be placed bilaterally on the upper arm (brachial pressure) and ankle, and inflated to 20 to 30 mmHg above systolic pressure. An ultrasound Doppler probe will be placed over the brachial, dorsalis pedis and posterior tibialis arteries and used to detect return of the arterial signal at the highest systolic pressure. The ABPI is calculated by dividing the ankle pressure by the brachial systolic pressure. This is a painless and very safe procedure for the participant.

Pulse wave velocity/analysis

This will be undertaken using either Vicorder or applanation tonometry and SphygmoCor software to derive non-invasively central aortic pressure and haemodynamic indexes (90).

Microvascular function (Laser Doppler flowmetry)

Two small laser Doppler probes will be placed on the volar surface of the forearm to detect blood flow in the superficial dermal vasculature. A blood pressure cuff will be placed around the upper arm and blood flow measured before, during and after inflation of the cuff to supra-systolic pressure maintained for up to 3 minutes. The reactive hyperaemic response will be used to assess the capacity of the vasculature to dilate under rested conditions (126).

10. *Body composition*

Energy expenditure and indirect calorimetry

VO_2 max measurements will be taken since these factors affect the metabolic syndrome phenotype. Basal metabolic rate will be assessed by indirect calorimetry. Inspired oxygen consumption and expired carbon dioxide measured by indirect calorimetry at rest will be used to calculate respiratory quotient.

Hepatic mitochondrial function

13C-Ketoisocaproic (13C-KICA) breath test

The objective of this pilot study is to conduct a preliminary examination of liver function in patients with NAFLD before and after a synbiotic intervention and to characterise any changes in liver function observed using the ^{13}C-KICA breath test. This study is carried out in order to collect information that is needed to refine the assessment of liver function for the design of a large-scale study.

Study protocol

Each participant will be studied using a standard ^{13}C-KICA breath test procedure. The participant will be asked to rest for 10 minutes before the oral administration of the ^{13}C-label and throughout the breath test study period. After resting for 10 minutes, the participant will be asked to give two baseline breath samples after an overnight fast by blowing into two non-evacuated breath tubes

using a straw. This will be followed by the oral ingestion of 1mg/kg body weight of 2-keto-[1-^{13}C]-isocaproic acid (99%; ^{13}C) along with 20mg/kg body weight of L-leucine dissolved in 200ml of water. After the oral ingestion, the participant will then be asked to wait for 5 minutes followed by blowing into 2 non-evacuated breath tubes every 10 minutes for 1 hour using a straw.

The ratio of $^{13}CO_2$ to $^{12}CO_2$ in each breath sample collected will be analysed using an Isotope Ratio Mass Spectrometer (SERCON ABCA) by Dr Paul Afolabi from the Southampton NIHR Biomedical Research Centre. The results of the ^{13}C-KICA breath test will be expressed as the percentage of the administered dose of ^{13}C recovered per min (% dose/min) and as a cumulative percentage ^{13}C-dose recovered over time (cPDR). From calculating the % dose/min and cPDR for the ^{13}C-KICA breath test, we will determine the extent to which there is concordance between these pharmacokinetic variables and measurements of liver fat percentage and other measurements of NAFLD disease severity and liver function.

Body fat measurement

We will undertake a whole body DEXA scan at the beginning and at the end of the study. A DEXA scan is a special X-Ray scan to assess body fat levels. The radiation exposure from each DEXA scan is roughly the equivalent of walking around Southampton for a week during the summer.

Total body fatness will be measured with bioimpedance and regional body fat by skin fold thickness. DEXA assessment of body fat quantity and location will be supported by waist circumference, measured as waist circumference midway between the lower ribs and the iliac crests (127) also height, hip, skin fold thickness – to allow estimation of body mass index (BMI), waist hip ratio, and fat mass (128;129).

11. *DNA analyses*

Blood will be collected from participants that will include white blood cells containing DNA. All DNA samples will be anonymised and stored in -80C freezers behind locked doors in the researchers' laboratory on the Southampton General Hospital site. DNA will be prepared from white blood cells in the blood sample. Using polymerase chain technology and bisulphite sequencing, gene polymorphisms and epigenetic modification (methylation status) of gene promoters will be examined. Samples will be taken for DNA analysis to investigate potential genetic and epigenetic modifications of the phenotype. Analyses will include genome wide single nucleotide polymorphism (SNP) testing and methylation analysis at imprinted gene loci. We would like to examine the epigenetic findings at known imprinted genes in patients with NAFLD. This investigation requires DNA. We will measure levels of methylation using methylation specific PCR at known imprinted loci and compare to normal levels to investigate whether changes in expression of imprinted genes is associated with the adult phenotype. DNA will be extracted from a blood sample using standard NHS protocols at the Wessex Regional Genetics Laboratory. Methylation analysis of the known control regions of imprinted genes on chromosomes 11, 6, 7, 20, 15 and 19 will be tested. Methods successfully running in the lab such as methylation specific PCR, real-time PCR and pyrosequencing will be exploited to reveal abnormalities of methylation as per the existing testing protocols, currently used for NHS and research testing of imprinting disorders. Binary data will be collected in the form of positive (abnormal) or negative (normal) methylation levels at the imprinted loci being tested. Data generated will show that in people

with known NAFLD: a) whether gene polymorphisms for key genes relevant to NAFLD are present and b) whether the gene promoter regions that regulate whether a gene is switched on, or switched off, contains more or less methylated CpG islands for key genes relevant to NAFLD. Any data generated from these analyses, we envisage might have the potential to be used as a biomarker in the future because of a gene variant association with NAFLD. We do not envisage any clinical usefulness of the data in managing the participant. Additionally, we do not envisage any usefulness, or relevance, of the data to relatives of the participant in question. To date there are no known gene polymorphisms or epigenetic changes that have been shown to be related to NAFLD. Thus the investigation is exploratory and if we find any alteration in gene polymorphisms or in methylation status of genes, we do not foresee any ethical issues from obtaining this information, because the significance or relevance to the individual participant of obtaining this information would be uncertain and would have no bearing on any treatment or future advice given to the individual participant. The samples will be stored in the researchers' freezers behind locked gates in the freezer room at the Institute of Developmental Sciences (IDS) building at the Southampton General Hospital site.

12. *Pilot studies embedded with the main study*

A sub-group of individuals from within the main study cohort will be recruited to participate in a more detailed study of insulin sensitivity. This study is a pilot study embedded within the main randomised controlled trial to assess hepatic and whole body insulin sensitivity in people with NAFLD. The aim of this exploratory pilot study is to assess whether treatment with a synbiotic supplement improves hepatic and whole body insulin sensitivity. We hypothesise that a decrease in liver fat and inflammation induced by the synbiotic will cause an increase in insulin sensitivity that will be beneficial to the individual.

To address this scientific question we will undertake an exploratory pilot study in ~ n = 12/50 participants from the 50 participants randomised to synbiotic and ~ 12/50 participants from the group randomised to placebo. Participants will be invited to undergo a stepped hyperinsulinaemic clamp with deuterated glucose to assess hepatic and whole body insulin sensitivity. We will undertake these studies in collaboration with Professor Margot Umpleby from the University of Surrey with whom we have collaborated previously in undertaking studies of this nature.

Methods for postprandial study day

Proposed methodologies to investigate:

- fasting and postprandial hepatic DNL (fatty acid and cholesterol) in VLDL-TG using deutrated water (^2H_2O)
- bile acid and cholesterol metabolism using deutrated water (^2H_2O)
- postprandial hepatic fatty acid partitioning using [U-^{13}C]palmitate and measuring incorporation into ^{13}C-VLDL-TG, and 13C appearing in 3-hydroxybutyrate (3-OHB) in deproteinised plasma, breath CO_2 and hepatic urea (to measure $^{13}CO_2$- i.e. hepatic CO_2)

Hepatic fatty acid partitioning in NAFLD: the effect of pre- and pro-biotics

We will also investigate hepatic fat synthesis [hepatic *de novo* lipogenesis (DNL)] and hepatic fat breakdown (oxidation) (130) in a subset of individuals from the control approximately (n=12) and intervention approximately (n=12) before and after the randomised intervention. We will use stable (i.e. non-radioactive) isotopes to probe human metabolism, particularly that of the liver.

The purpose of this investigation is to obtain data in a sub-group of participants to address the question of whether there is any relationship between rate of hepatic DNL and postprandial fat oxidation, in people with a histological diagnosis of NAFLD in relation to hepatic fat quality as measured by magnetic resonance imaging (MR) and diagnostic liver biopsy. Additionally, in this sub-group study, we will test whether the therapeutic intervention has affected hepatic DNL and postprandial oxidation.

De novo hepatic fatty acid synthesis

Fasting and postprandial hepatic DNL will be measured based on the incorporation of deuterium from ^2H_2O in plasma water into VLDL-TG isolated by density gradient ultracentrifugation and immunoaffinity chromatography. Participants will be given deuterated water (^2H_2O) with detailed instructions for consumption prior to the study day. In the evening before the study day, participants will drink ^2H_2O, (3 g/kg of body water, in two equal portions) at 8 and 10 pm, in order to achieve enrichment of plasma water of 0.3 %. Participants will then continue to consume labelled water (at 0.3% enrichment) over night and also over the course of the study day.

Deuterium in plasma water will be measured using a Finnigan GasBench-II (ThermoFisher Scientific, UK) and deuterium in VLDL-TG palmitate using a well-established GC-MS and mass-isotopomer distribution analysis (MIDA) protocol (131). Ions with mass-to-charge ratios (m/z) of 270 (M+0), 271 (M+1) and 272 (M+2) will be monitored (132).

De novo cholesterol and bile acid synthesis

De novo cholesterol and bile acid synthesis will be assessed in the fasting and postprandial state(133) by measuring the incorporation of deuterium from ^2H_2O in plasma water into plasma cholesterol and the bile acids, cholic acid, and chenodeoxycholic acid. Deuterium in plasma water will be measured using a Finnigan GasBench-II (ThermoFisher Scientific, UK) and deuterium in plasma cholesterol and plasma bile acids using a well-established GC-MS and mass-isotopomer distribution analysis (MIDA) protocol (133-135). For plasma cholesterol ions with mass-to-charge ratios (m/z) of 367 (M+0), 368 (M+1) and 369 (M+2) will be monitored (131). For plasma bile acids ions with mass-to-charge ratios (m/z) of 623 (M+0), 624 (M+1) and 625 (M+2) for cholate and 535 (M+0), 536 (M+1) and 537 (M+2) for chenodeoxycholate will be monitored (135).

Postprandial fatty acid partitioning

On the study day after baseline blood and breath samples taken (time 0) participants will then consume a single test meal that is labelled with [U^{13}C]palmitate, to assess the intra-hepatic handling of dietary fatty acids. The test 'breakfast' meal will consist of 40 g rice krispies, 200 g skimmed milk and a chocolate-flavoured lipid emulsion containing 40 g olive oil with 200 mg [U-^{13}C]palmitate. The fat, protein and carbohydrate breakdown of the meal will be: fat 61% total energy (40 g), protein 7% total energy (10 g) and carbohydrate 31% total energy (40 g).

Blood and breath samples will be collected regularly throughout the study (up to 300 minutes) for the measurement of ^{13}C fatty acids in plasma-TG, plasma-NEFA, 3-OHB, breath CO_2, and CO_2 liberated from urea. ^{13}C-in plasma, CO_2 and 3-OHB will be measured by gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS) (136).

In order to assess hepatic ketone body production, we will measure isotopic enrichment from [U-^{13}C]palmitic acid appearing in 3-OHB as previously described (136). Acetyl-CoA produced from

β-oxidation of fatty acids may enter the TCA cycle and produce CO_2. Hepatic mitochondrial CO_2 is utilised in hepatic urea synthesis (137). To assess hepatic fatty acid oxidation we will measure enrichment of ^{13}C into $^{13}CO_2$ that will be liberated from urea (138). The $^{13}CO_2/^{12}CO_2$ will be measured by GC-C-IRMS. Whole body fatty acid oxidation will be assessed by measuring $^{13}CO_2/^{12}CO_2$ in breath and a group acetate correction factor will be applied (139) to allow for ^{13}C sequestered in other metabolic pathways. Indirect calorimetry will be performed (using a Gas Exchange Monitor) to determine CO_2 production.

The metabolic profile of plasma TG, NEFA, glycerol, 3-OHB, plasma urea, and plasma cholesterol will be measured using an ILAB650 clinical analyser.

Blood and breath samples will be taken at baseline (time 0), prior to consumption of the test meal and then be taken regularly (every 60 minutes) after the test meal over the next 300 minutes.

Volumes of plasma required:
Lipoprotein isolation - 3 ml (min volume 2 ml)
3-hydroxybutyrate enrichment - 1ml
Hepatic 13CO2 enrichment - 1ml
Bile acid enrichment - 1ml
Ilab (clinical chemistry), plasma water enrichment - 1ml
Spare (if at all possible to have) - 1ml

Total at each time point is then: 8ml plasma and there are 6 time points so a total of 48ml of plasma for the study day

Our collaborators at Oxford will provide

- Stable isotopes H_2O and [U-^{13}C]palmitate with the appropriated detailed instructions
- Training for preparing the test meal
- Breath bags and tubes required for the collection of the breath samples
- Transport of the fresh plasma and breath samples from Southampton to Oxford
  Transport of samples from postprandial study day: Fresh (unfrozen) plasma is required for the isolation of the lipoproteins. Plasma samples can be kept on ice in a fridge at 4°C overnight. Samples can then be sent the next day to Oxford by courier. Our collaborators in Oxford will arrange the collection of the plasma and breath samples.

Our collaborators at Belgium will provide

Our collaborator Professor Nathalie Delzenne at Catholic University of Louvain, Brussels, Belgium will analyse the bacterial species in stool samples using modern molecular biology techniques. Samples (frozen stool and/or isolated bacterial DNA/RNA from stool samples) will be sent for analysis to Professor Delzenne. The phylogenic characteristics of the gut microorganisms present in the stool samples will be determined using fluorescent in-situ hybridisation analysis (FISH) to identify specific bacteria types and the 16S ribosomal RNA sequence-based method to study the composition of rRNA amplified using the quantitative polymerase chain reaction (qPCR).

X. A summary table of participants' visits and tests during the study

Investigations and visits to the Wellcome Trust Clinical Research Facility, at University Hospital Southampton NHS Foundation Trust

Baseline measurements Visits 1 & 2 (+/- visit 3)	Visits 4 at 5-6 months	Follow-up measurements Visits 5 & 6 (+/- visit 7)
Questionnaires: quality of life, physical activity, food frequency questionnaire, 24 hour diary and satiety. FSS Epworth Sleepiness Scale	Food frequency questionnaire FSS Epworth Sleepiness Scale	Questionnaires: quality of life, physical activity, food frequency questionnaire, 24 hour diary and satiety. FSS Epworth Sleepiness Scale
Anthropometry Blood Pressure	Anthropometry Blood Pressure	Anthropometry Blood Pressure
Tests Blood tests –*up to 100 mls (approximately 5 tablespoons) Lactulose-Mannitol test Lipid profile Stool analyses Urine sample (Microalbuminuria)	Tests Blood tests – total volume 30 ml (approximately 2 tablespoons) Stool analyses	Tests Blood tests - *up to 100 mls (approximately 5 tablespoons) Lactulose-Mannitol test Lipid profile Stool analyses Urine sample (Microalbuminuria)
Indirect Calorimetry 13C-Ketoisocaproic Treadmill Hand grip Carotid Artery Ultrasound DEXA scan Fat tissue biopsy C-reactive protein & other inflammatory markers		Indirect Calorimetry 13C-Ketoisocaproic Treadmill Hand grip Carotid Artery Ultrasound DEXA scan Fat tissue biopsy C-reactive protein & other inflammatory markers
Glycated haemoglobin Pulse wave velocity/analysis Bioimpedance Fibroscan		Glycated haemoglobin Pulse wave velocity/analysis Bioimpedance Fibroscan
Microparticles Laser Doppler flowmetry Ankle-Brachial Index Cardiorespiratory fitness Physical activity energy expenditure MR scan of abdomen/liver Blood sample for DNA analyses Pulse wave velocity/analysis		Microparticles Laser Doppler flowmetry Ankle-Brachial Index Cardiorespiratory fitness Physical activity energy expenditure MR scan of abdomen/liver Blood sample for DNA analyses Pulse wave velocity/analysis
N.B. for a subgroup of participants only Incremental glucose clamp, Laser Doppler fluximetry during clamp, de-novo lipogenesis		N.B. for a subgroup of participants only Incremental glucose clamp, Laser Doppler fluximetry during clamp, de-novo lipogenesis

Please note these volumes of blood are approximate.

*100 mls is equivalent to approximately one fifth of a unit of blood given by a blood donor and will be taken over baseline visits (1, 2, +/-3) and end of study visits (5, 6 +/-7)

XI.   Appendix 1

Statistical analysis outline

Trial design: This is a randomised, single centre, double-blind clinical trial.

Trial objectives: The trial addresses the hypothesis that treatment with a synbiotic will have a beneficial effect in non-alcoholic fatty liver disease (NAFLD) compared to placebo, as measured by change in liver fat and serum biomarkers.

Participants: Patients aged >18 years with NAFLD, either biopsy-proven or confirmed by non-invasive imaging in a high-risk cohort (i.e. diabetic and/or features of metabolic syndrome). Alcohol consumption ≤ 14 units / week for women ≤ 21 units / week for men. **Intervention**: A dose of a synbiotic nutritional supplement comprising fructo-oligosaccharide with a degree of polymerization < 10 at 4 g/twice a day (two sticks a day) plus Bifidobacterium animalis subsp. lactis BB-12 at a minimum of 10 billion CFU/day (1 capsule a day) will be taken for 10-14 months.

Treatment comparison: Synbiotic versus placebo

Primary outcome:
i.   To assess change in liver fat with synbiotic treatment
ii.   To assess change in biomarkers with synbiotic treatment
iii.   To assess change in gut microbiota composition with synbiotic treatment

Secondary outcomes:
i.   Liver fibrosis determined by transient elastography
ii.   Insulin and glucose concentrations and hepatic insulin sensitivity
iii.   Microvascular function
iv.   Plasma cardiovascular risk markers
v.   Adipose tissue and blood markers of metabolism and inflammation
vi.   De novo lipogenesis
vii.   Satiety and satiety factors

Study site: University Hospital Southampton

Sample size and power

There is little published literature to date upon which to base a sample size calculation to test the effects of the synbiotic treatment on the primary end points. However, the study has been powered on the basis of the expected change in the key primary end point of a change in liver fat.

Based on the mean ± SD of liver fat content in the sample of people with NAFLD who we recruited for our current randomised trial testing the effects of a n-3 fatty acid intervention in NAFLD in the WELCOME study (the **W**essex **E**valuation of fatty **L**iver and **C**ardiovascular markers in NAFLD with **OM**acor th**E**rapy study); we estimate that the sample size would give 85% power to detect a 40% decrease in liver fat with the synbiotic intervention (allowing for 14% drop out). In the WELCOME study conducted over a longer period of intervention, 5% of the randomised cohort withdrew between randomisation and end of study measurements. In the WELCOME study the mean percentage fat content was 28.5%. Thus it is reasonable to expect that after treatment with the synbiotic, the mean percentage fat content would be 20% (giving a 30% decrease in liver fat). Liver fat content will be measured by MRS spectroscopy and this technique can reproducibly detect liver fat content as low as 5%.

One recent publication in people with NAFLD testing the effect of a synbiotic over 24 weeks in a small study showed a 69% decrease in liver fat (53).

Randomisation procedures

Patients will be randomised according to standardized procedures (computerized randomisation by a person from within University Hospital Southampton or the University of Southampton who is not connected to the INSYTE study). Stratified randomisation by age, sex and sub-study to trial medication or placebo will be used.

Data monitoring and interim analyses

Detailed analysis and publication of baseline data will be undertaken prior to trial completion by Profs, Byrne and Calder and other investigators in discussion with the research nurses and BRC statistician. An independent data monitoring committee (IDMC) has not been set up for this trial, so there are no planned interim analyses of outcome data.

Procedures for data checking

Data will be checked for transcription and validation errors. Prior to any statistical analysis, all variables will be checked for number of missing values, impossible and improbable values. Impossible and improbable values for continuous variables will be defined by clinical opinion, other improbable values, defined as being more than three standard deviations from the mean, will also be checked. Errors in categorical variables will be identified as those with values missing from given terms in the Data

Dictionary. Inconsistencies will be referred to a named investigator by the data management team. Descriptive statistics will be calculated for all variables and distributional assumptions checked.

Trial analysis

Description of baseline data

All important variables collected at baseline will be summarised by treatment group. For continuous variables; means and standard deviations will be calculated. Distributions will be assessed using histograms and the normality assumption will be tested. When the data are not normally distributed medians and percentiles will be used to summarise data. Number and percentage will be calculated for categorical and binary data.

Primary endpoint

Change in liver fat and biomarkers from randomisation to end of study.

1. Change in liver fat (measured by MRS or IPOP MRI)

2. Change in biomarkers:

a) ELF score which is calculated using the following algorithm:

Score= -7.412 + (ln(HA)*0.681) + (ln(P3NP)*0.775) + (ln(TIMP1)*0.494).

b) NAFLD fibrosis score which is calculated using age, hyperglycemia, body mass index, platelet count, albumin and AST/ALT ratio.

c) CK18 M30 and M65 as markers of apoptosis and necrosis

3. Change in gut microflora assessed by 16S rRNA, FISH and qPCR

Change in liver fat from randomisation to end of study

Measurement

Magnetic resonance spectroscopy (MRS) and/or MRI (IPOP) imaging will be used to determine the amount of liver fat as a percentage of the whole liver. This will be performed at baseline and at the end of study visit.

Change in biomarkers

Measurement

Biomarkers will be measured in plasma or serum or will be generated from an algorithm.

Change in gut microflora

Measurement

Gut microflora will be assessed by 16S rRNA, FISH and qPCR

Statistical analysis

There are three primary endpoints; change in liver fat, change in biomarkers and change in gut microflora.

For measurements of liver fat, biomarkers and gut microflora:

Multiple regression analysis will be used, with final visit measurement as the outcome, and baseline measurement and randomised treatment group as predictors.

For each measurement, a multiple regression analysis will also be performed adjusted for potential confounders.

Potential confounders include but are not limited to:
Change in body weight

For all outcomes, the treatment effect with 95% confidence interval will be reported (regardless of whether statistical significance is met). This will allow discussion of whether the trial result is compatible with a clinically important effect.

When reporting results it will be acknowledged that care should be taken in interpreting results from several outcome measures, as some statistically significant findings are likely to result from chance alone; consequently, we will allow for multiplicity of testing when interpreting our results.

Analyses by intention to treat (ITT) and Per Protocol

Analyses will be undertaken by an intent-to-treat (ITT) analysis and per protocol.

1. The ITT analysis will include all patients with complete data (i.e. having baseline and end of study measurements) in the groups to which they were randomised (regardless of whether they were later found to be ineligible, a protocol violator, given the wrong treatment allocation or never treated). This analysis assumes that any missing data is missing at random (MAR), i.e. that there is no difference between missing and observed values, once adjusted for any baseline variables which predict for missingness.

In order to explore the effect of departures from the MAR assumption, sensitivity analyses will be performed (140).

This assumes that d= the mean of the missing data minus the mean of the observed data. Under MAR, d=0.

The value of d will then be varied in order to model different scenarios (i.e. that the patients who are lost to follow up have systematically worse outcomes), and we will report whether the significance of the main analysis is maintained in the sensitivity analysis.

All randomised participants will be accounted for in these analyses.

2. Per protocol analysis will include all patients who consumed ≥50% of their supplement in the time period from randomization to final visit, and will exclude participants who were later found to be ineligible or who did not complete the study.

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