SAFETY AND EFFICACY OF PHYTOSOMAL CURCUMIN IN NON-ALCOHOLIC SEATOHEPATITIS (NASH): A DOUBLE-BLIND, RANDOMIZED, PLACEBO-CONTROLLED TRIAL

Trial protocol

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1. Background and Rationale

1.1. NASH: epidemiology, pathogenic mechanisms, clinical relevance and unmet therapeutic needs

Non-alcoholic fatty liver disease (NAFLD) is the commonest cause of chronic liver disease, affecting up to 30% of the general population and 70–90% of obese and type 2 diabetic patients[1, 2].

NAFLD encompasses a spectrum of histological severity ranging from simple steatosis (SS) to necroinflammation and fibrosis (nonalcoholic steatohepatitis, NASH), which can differentiated only by liver biopsy. Even though simple hepatic steatosis is a benign condition, up to 30% of patients with NAFLD have the more severe, progressive inflammatory condition known as NASH.

NASH confers an increased risk of cirrhosis, liver failure and hepatocellular carcinoma, and is projected to be the commonest indication for liver transplantation in forthcoming years.

Furthermore, emerging evidence links NAFLD to chronic kidney disease(CKD), as these two conditions share common pathogenic mechanisms[3],[4],[5]: consistently, NASH poses a 2.5-fold risk of CKD than the general population[6] and is becoming the leading cause of combined liver-kidney transplantation[7,8].

Despite this, there are no universally accepted pharmacological therapies for NASH. Therefore the need for novel, safe agents in NASH is of paramount importance to prevent disease progression and the accompanying clinical burden.

1.2 Curcumin: mechanisms of action, rationale for liver, metabolic and kidney disease treatment and challenges for NASH therapeutics

Curcumin is a lipophilic polyphenol that is the primary constituent of turmeric (*Curcuma longa*), the most common popular spice in Indian cuisine and a major ingredient of curry powders. In vitro studies showed curcumin has prominent antioxidant, anti-inflammatory, antiproliferative and antifibrotic properties, and targets multiple molecular pathways involved in the pathogenesis of NASH and of chronic kidney disease(CKD)[9,10] , most prominently nuclear factor-kappa B (NF-kB)[11], a key pro-inflammatory

transcription factor involved in metabolic and nutritional inflammation[12]. Dietary curcumin supplementation ameliorated liver disease in preclinical NASH models [13, 14,15, 16, 17, 18, 19]

Preliminary human data indicate curcumin supplementation improves surrogate markers of liver disease and of metabolic risk in NAFLD patients[20,21]. The combination of curcumin with phospholipids greatly improves oral bioavailability of curcumin, allowing greater systemic concentrations and delivery to target organs[22], [23], and curcumin-phosphatidylcholine phytosome complex administration for up to 18 months was well-tolerated, safe[24] and improved clinical end-points and inflammatory parameters in patients with osteoarthritis, uveitis and chronic kidney disease (CKD)[25, 26,27].

2. Research Hypothesis and Aims

On the basis of above-mentioned data, we hypothesized that treatment with phytosomal curcumin would result in significant improvement in liver histology in patients with NASH.

Key secondary hypotheses include tolerability and safety of phytosomal curcumin and the potential to improve NASH-related CKD, systemic inflammation, glucose and lipid metabolism.

To test this hypothesis, we designed a phase II, multicentre, double-blinded, placebo-controlled randomized controlled trial (RCT).

3. Study Design setting and timeline

This is a 72-week, investigator-initiated, multicentre, randomized, double-blinded, placebo-controlled trial of treatment with phytosomal curcumin 1 g daily for adults with biopsy-proven NASH. Screening was undertaken within 14 days of randomisation to assess eligibility and collect baseline data. Patients who satisfied the eligibility criteria were randomly assigned (1:1) to receive oral phytosomal curcumin 1 g daily (experimental) or placebo (control). After which, a 12-week washout period is scheduled.

The trial will be conducted at two University hospitals (HUMANITAS Gradenigo, Turin, Italy, and Città della Salute e della Scienza, Turin, Italy) between May 25th, 2018 and Dec 30th, 2022.

The primary outcome measure will be assessed using an intention-to-treat analysis of the proportion of evaluable patients achieving an improvement in liver histology between liver biopsies at baseline (within 6 months of screening) and after 72 weeks of treatment. Histological improvement will be defined as a

combination of resolution of active steatohepatitis and no worsening in fibrosis (assessed by Kleiner Fibrosis score[28]; detailed in outcome section).

4. Ethics and regulatory approval

The study was funded by Indena Spa, Milan, was conducted in accordance with the ethical principles of the Declaration of Helsinki, was approved by A.O.U San Luigi Gonzaga hospital Ethics Committee (prot. N. 0008942) on May 25th, 2018 and is registered with ISRCTN clinical trial registry (study ID: ISRCTN27793187). All patients gave written informed consent to paricipate to the study.

End of study

Study will continue until 84 days (12 weeks) after the last enrolled patient or in any case for 6 months since June 2022, depending on levels of enrollment.

5. Methods

5.1 Eligibility criteria

Eligible adult patients (≥18 years old) were identified and recruited at the participating trial site centres starting in August 2018 and ending by June 2022, 52 patients will be recruited. Participating trial centres will include the Liver and Metabolic Units at the Humanitas Gradenigo Hospital (Turin) since August 2018), and the Metabolic Unit of Dept of Medical Sciences at the Città della Salute e della Scienza hospital(Turin) since Sept 2018).

All trial participants will give informed written consent at the beginning of the screening visit prior to undergoing any tests and procedures needed to assess eligibility.

Eligibility for the trial will be determined at screening visit 1 by standard blood tests, clinical history (including written-confirmation of drug history where necessary) and physical examination to identify other illnesses or contraindications for participation.

Patients who satisfied the eligibility criteria trial at both sites will be offered to take part in a metabolic mechanistic substudy. The substudy involves a standard Oral Glucose tolerance Test (OGTT) and a standard Oral Fat Tolerance Test (OFTT) on three admissions (at least 24 hours apart from each other) and an indirect calorimetry test at the two participating centres on visits 2 (pretreatment) and 8 (4 weeks

post-treatment). A detailed summary of the metabolic substudy will be published separately. A patient's decision to partake or withdraw from the metabolic substudy will not affect their participation in the main 72-week trial.

A schematic view of trial schedule and design is presented in **Figure 1**.

5.2 Inclusion criteria

The trial entry criteria will be based on a diagnosis of 'definite' NASH on liver biopsy obtained within 6 months of screening. Throughout the study, all liver biopsies will be read centrally by one single (RP) expertised pathologist from the central trial site (HUMANITAS Gradenigo), bilnded to clinical and laboratory data and to treatment allocation: prior to randomisation, the liver histopathologist will review all of the liver biopsies of the potential participants to assess whether a diagnosis of 'definite' NASH is present.

A 'definite' diagnosis of NASH iss defined if all of the following are present on biopsy: (1) macrovesicular steatosis (>5%); (2) hepatocyte ballooning (± Mallory Hyaline) and (3) lobular inflammation (mixed infiltrate, related to foci of ballooning). Only patients with 'definite' NASH will be classified as eligible. All participants are to be ≥18 to <70 years of age and have a BMI ≥25 kg/m2 at screening. Patients with type 2 diabetes mellitus (T2DM) at screening had to have stable glycaemic control (HbA1c <9%) and be managed by either diet and/or a stable dose of metformin/sulfonylurea/s. Patients without a history of T2D prior to the screening visit will undergo a 75-g standard OGTT and/or have their HbA1c measured at screening to determine their glycaemic status and will be labelled as 'non-diabetic' if one or more of the following was confirmed

-Impaired fasting glucose, defined using the European Criteria between 110 and 126 mg/dL.

-IGT, defined as 2 h plasma glucose levels between 140 and 200 mg/dL on the 75 g OGTT.

-Normal Fasting Plasma Glucose <110 mg/dL and normal 2 h plasma glucose levels <140 on the 75 g OGTT.

-HbA1c<6.5%

5.3 Exclusion criteria

A detailed summary of the exclusion criteria is provided in **box 1**. In brief, patients with a history or current significant alcohol consumption, poor glycaemic control (HbA1c >9.0%), Child's Pugh B or C cirrhosis or other liver disease aetiology were excluded. The latter was confirmed with a full liver aetiology screen (drug induced, viral hepatitis B/C, autoimmune and genetic) at the screening visit. Past and current alcohol consumption was ascertained by a detailed review of the patient's medical, social history and by a validated, self-administered food frequency questionnaire with reference pictures to remind participants of drink equivalents (see dietary record section). Concomitant use of drugs reported to be inducers (methotrexate, amiodarone, steroids) or potential therapies for NASH (TZDs, GLP-1 analogues, vitamin E), or other known hepatotoxins were assessed during the screening visit (**box 1**).

Box 1. Exclusion criteria

1. Refusal or lacks capacity to give informed consent to participate in the trial.
2. Participation in any clinical trial of an investigational therapy or agent within 12 months of randomisation.
3. Patient (or carer) deemed not competent at using the correct site and technique for subcutaneous injection of the trial treatment (containing dummy drug on practice).
4. NAFLD Activity Score (NAS) <3 on liver biopsy.
5. Child's B or C cirrhosis or clinical evidence of decompensated chronic liver disease: radiological or clinical evidence of ascites, current or previous hepatic encephalopathy and evidence of portal hypertensive haemorrhage or varices on endoscopy.

6. Medical history of multiple drug allergies (defined as anaphylactoid drug reactions in >2 drug groups) or allegry to curry or curcumin-based nutraceuticals
7. Presence of any acute/chronic infections or illness that at the discretion of the chief investigator might compromise the patient's health and safety in the trial.
8. Pregnancy or breastfeeding.
9. Women, of childbearing age, who are not willing to practise effective contraception (ie, barrier, oral contraceptives, impenon or past medical history of hysterectomy) for the 48-week duration of the trial and for 1 month after the last administration of the drug.
10. Liver disease of other aetiologies (ie, drug-induced, viral hepatitis, autoimmune hepatitis, PBC, PSC, haemochromatosis, A1AT deficiency, Wilsons disease).
11. Average alcohol consumption >20 g/d(males) and >10 g/d(females) (as assessed by a validated questionnaire within the last 5 years.

12. Medical/surgery history of; gastric bypass surgery, orthotopic liver transplant (OLT) or listed for OLT, hepatocellular, pancreatic, thyroid carcinoma, acute or chronic pancreatitis and total parenteral nutrition within 6 months of randomisation.
13. Diagnosis of malignancy within the last 3 years (with the exception of treated skin malignancies).
14. Hepatocellular carcinoma: dysplastic or intermediate nodules to be excluded. Regenerative and other nodules to be included at the discretion of the chief investigator.

15. Alanine aminotransferase or aspartate aminotransferase >10×upper limit of normal.

16. >5% weight loss since the diagnostic liver biopsy was obtained.
17. Recent (within 3 months of the diagnostic liver biopsy or screening visit) or significant change (as judged by the chief investigator) in dose of the following drugs: inducers of hepatic steatosis (steroids (oral/intravenous), methotrexate, amiodarone), orlistat and/or multivitamins/ vitamin E (containing >200% recommended daily amount; >30 mg/day).
18. Known positivity for antibody to HIV.
19. Currently being treated with renal replacement therapy (ie, haemodialysis or peritoneal dialysis).

Specific exclusion criteria for participants with T2D
1. Participants receiving thiazolidinediones (TZDs), GLP-1-based therapies, SGLT2 inhibitors
3. HbA1c ≥10%.

Patients who met any of the criteria (listed above) at the screening visit were excluded from trial participation.

5.4 Intervention

5.4.1 Experimental arm (phytosomial curcumin)

Phytosomal curcumin Meriva® (Indena S.p.A. Milan, Italy) 1,000 mg (active experimental group)(Indena s.p.a., Milan, Italy) was supplied was administered before meals at a dose of 1000 mg twice daily 12 hours apart (total daily dose: phytosomal curcumin Meriva 2000 mg, corresponding to 400 mg curcuminoids). Participants were encouraged to take phytosomal curcumin at the same time each day, according to the most convenient time for them. All tablets were produced and sponsored by the manufacturer who otherwise had no role in the study design, data collection, data analysis, data interpretation or publication of this manuscript. Placebo and Meriva® tablets contained the same ingredients except for curcuminoids. For simplicity, Phytosomal curcumin tablets will be referred to as curcumin in the following. Compliance will be considered acceptable if more than 75% of dosages will be ingested. Participants reported missed dosages, concomitant medication, and adverse events during the trial.

5.4.2 Phytosomal curcumin-placebo (inactive, placebo-control group)

Phytosomal curcumin-placebo (Indena S.p.A. Milan, Italy)) consist of identically appearing , colourless, tasteless pills packaged and administered in an identical manner to the active comparator,. Placebo pills and active comparator have identical composition, except for the active phytosomal curcumin substance.

5.4.3 Concomitant therapy and medications

No dose reductions of phytosomal curcumin or placebo will be allowed throughout the 72-week treatment period. Previous treatment with oral antidiabetic drugs (metformin, sulfonylureas, insulin, DPP-IV inhibitors) was continued at the same dose in participants with T2DM at randomisation. The reported rate of hypoglycaemia in literature, with phytosomial curcumin is very low and not different from placebo[3,4].
However In the event of recurrent major hypoglycaemic episodes (requiring medical or hospital intervention), the dose of the sulfonylurea will be reduced by 50% at the discretion of the investigators. In the event of recurrent major hypoglycaemic episodes in which no dose reduction could be undertaken (ie, not on a sulfonylurea) the participant will be withdrawn from treatment at the discretion of the chief investigator.
Glycaemic control will be assessed at each 12-weekly trial visit with self-measured plasma glucose readings and HbA1c. In the event that glycaemic control deteriorates, defined as HbA1c >9% (75 mmol/mol), the participant will be informed and counselled with regard to starting open labelled long-acting insulin detemir once daily (Levemir) or other allowed drugs in accordance with European guidelines (http://www.ema.europa.eu) to ensure that the participant's standard of diabetes care is not significantly compromised as a result of participating in the clinical trial.
The HbA1c cut-off >9% was based on the opinions of the TMG (GM and RG), consisting of expert endocrinologists and in accordance with previous clinical trial guidance[29].
In addition to study medications, participants will receive standard National Health Services (NHS) care recommendations concerning life-style modifications (ie, exercise, weight loss and dietary modification) and management of various coexisting illnesses throughout the trial.

Patients will be asked to limit alcohol consumption to less than 20 mg/day for women (ie, 14 units/week) and 30 mg/day for men (ie, 21 units/week), consistent with current guidelines[30]

Participants will not be allowed any new prescription or over-the-counter therapies (ie, herbal remedies, milk thistle) that may improve or worsen NASH throughout the duration of the trial, including nutraceuticals of any type containing curcumin.

Potential NASH therapies that will not be allowed during the trial duration included thiazolidinediones (TZDs), GLP-1 receptor agonists, SGLT2 inhibitors, vitamin E, orlistat or other experimental therapies for NASH. Steroids (oral or intravenous), methotrexate and/or amiodarone will also not permitted based on their ability to promote hepatic steatosis.

5.5 Randomization, stratification and blinding

Randomization and stratification

Participants meeting all the eligibility criteria and providing written informed consent will be randomized centrally on a 1:1 basis to either of the two-study treatments (phytosomal curcumin vs placebo) using computer generated randomization. To ensure even distribution between groups in key baseline covariates, randomization will be stratified into blocks of 4 according to the following variables: diabetes status (present vs. absent), obesity status(present vs. absent), fibrosis stage (stage F3-4 vs. stage 0-2), CKD(present vs. absent). Trial participants will be allocated a unique trial identification number to preserve patient confidentiality and enable the study to be double-blinded. Patients, investigators, assessors, trial site staff, and the sponsor (except for specific laboratory staff and individuals responsible for safety) remained blinded to treatment assignment throughout the trial.

Medication preparation and blinding/unblinding procedures

Both active treatment (phytosomal curcumin) and placebo control will be packaged and labelled with a unique identification number (in keeping with the European Unions Good Manufacturing Practice for Medicinal Product guidelines) by the manufacturer (Indena S.p.A.), to the extent that the receiving trial site will be blinded to the study drug throughout the duration of the trial. Sealed parcels (containing electronic information) will be sent with each drug package for the attention of the unblinded members of the central trial management group (TMG) nominated statistician, RG and database programmer, EP, to ensure (1) safe delivery of the correct drug and (2) blinding of the treatment allocation from the remainder of the TMG and the trial patient. Unblinding of treatment will only take place if the identity of the allocated study medication will be necessary for patient safety and care. If a serious adverse event (SAE) is deemed unexpected and possibly, probably or definitely related to phytosomal curcumin (ie, suspected unexpected serious adverse reaction=SUSAR), a clinical member of the TMG was unblinded to the medication to evaluate causality and subsequently label the event either as an unrelated SAE (for patients receiving placebo) or a SUSAR (for patients receiving phytosomal curcumin). The latter will be reported to the MHRA and the NRES, and only if patient safety is jeopardised the study medication will be discontinued and the treating clinician/patient informed.

6. Trial schedule and data collection

The trial will involve nine patient-related visits at their nearest trial site (**Figure 1)**.

The study comprises four stages: (1) screening, enrollment, randomisation and baseline investigations (visits 1 and 2, over a maximum period of 14 days), (2) 504 days of study treatment (visits 3–7, over 72 weeks), (3) primary endpoint assessment including liver biopsy (visit 8, within 1 day of the EOT) and (4) post-treatment follow-up assessment (visit 9, 12 weeks after EOT). If the trial investigating team or the trial participant suspected an AE, an unscheduled visit was arranged within 24 h. Each visit included patient interview, clincal examination, anthropometry and blood/urine chemistry.

The schedule for the study visits and data collection is summarised in **Figure 1**. All participants were asked to attend each visit under fasting from eating or drinking (with exception of water and due medications) for a minimum of 8 h prior to each visit. A follow-up liver biopsy (ie, primary endpoint)

was obtained under ultrasound-guidance after completion of the 72-week study treatment. Wherever possible, a 16-gauge biopsy needle and a specimen length of a minimum of 15 mm are preferred. The liver tissue will bes prepared at the local trial site in preparation for histological assessment (under light microscopy) at the central trial site at HUMANITAS Gradenigo hospital. On receipt, the 'blinded' central histopathologist (RP) will record the size and quality of the histology slides. A minimum of four unstained slides were available for each liver biopsy to enable repeat staining (H&E, haematoxylin van Gieson, Ubiquitin) to ensure adequate quality for interpretation. Liver biopsy tissue specimens will be collected, paraffin-fixed and stored at the diagnostic archive of the Department of cellular pathology (HUMANITAS Gradenigo hospital). Fasting serum and plasma samples were collected at each of the 9 visits, will be partly used for routine biochemistry and partly stored frozen in 0.5–1.0 mL aliquots at –80°C at HUMANITAS Gradenigo Hospital. Additional blood (buffer coat) will be obtained at visits 1 and 8 for future DNA extraction and stored at –80°C.

Treatment compliance

Treatment compliance will be monitored at each visit by a review of the returned sealed envelopes and pill count.

Figure 1: schematic of the trial design.

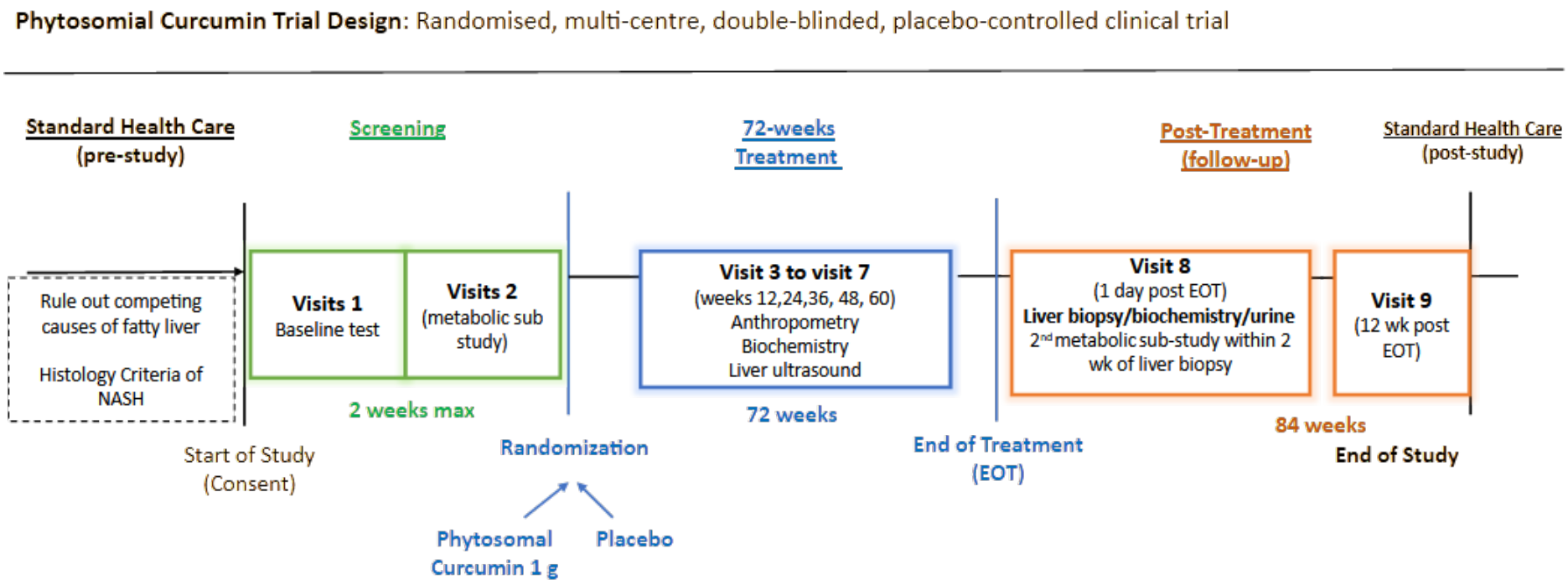

Figure 1 Schematic of phytosomal curcumin efficacy and action in non-alcoholic steatohepatitis trial design. Eligible participants are randomly assigned to a 72-week treatment of phytosomal curcumin 1 g daily or placebo control.
Both the trial investigators and the participants are blinded to drug allocation.

7. Study outcome measures: primary and secondary outcomes, adverse events(AEs) reporting and analysis

Primary outcome measure

Histological NASH resolution (defined by the NASH Clinical Research Network as no more than mild residual inflammatory cells [score of 0 or 1] and no hepatocyte ballooning [score of 0]) **and** no worsening of liver fibrosis (with worsening defined as an increase of one stage or more on the Kleiner fibrosis classification scale) after 72 weeks, in line with regulatory perspectives[31,32] .

Secondary outcome measures

Liver-centered histological outcomes:

1) a ≥1 stage improvement in fibrosis and no worsening of NASH (with worsening defined as an increase of ≥1 point in either the lobular inflammation score or the hepatocyte ballooning score according to the NASH Clinical Research Network criteria) after 72 weeks

2)improvement in clinically significant fibrosis[33], defined by the proportion of patients with baseline fibrosis stage≥2 who have fibrosis stage 0-1 at EOT, without worsening of NASH.

3)improvement in advanced fibrosis, defined by the proportion of patients with baseline fibrosis stage 3-4 who have fibrosis stage 0-2 at EOT, without worsening of NASH.

3)a ≥2-point improvement in NAFLD activity score(NAS), with at least 1 point improvement in either ballooning or lobular inflammation score;

4)individual components of the NAFLD activity score (steatosis, hepatocyte ballooning, lobular inflammation) and the Kleiner fibrosis stage. Fibrosis stages 1a, 1b, and 1c were considered stage 1 for the purposes of analysis.

Liver-centered non-histological outcomes:

4)changes from baseline to 72 weeks in serum liver enzyme concentrations, improvement in noninvasive markers of liver disease severity, including computerized sonographic hepato-renal ratio (which correlates closely with histologic and ^{1}H magnetic resonance spectroscopy steatosis [34,35, 36,,37,38], see **Analytical**

Methods subheading), serum cytokeratin-18 fragments(a marker of NASH), NAFLD fibrosis score, FIB-4 and serum lumican (as markers of fibrosis)(see supplementary material).

The extracellular matrix proteoglycan lumican was chosen because its circulating levels have been shown to be a reliable marker not only of hepatic fibrosis but also of active hepatic fibrosis deposition, its expression is upregulated by Nf-kB, and its levels have been shown to differentiate histologically progressive, fibrosing NASH from mild, nonprogressive NAFLD[39],[40],[41]

Non liver-centered outcomes

-Improvement in anthropometry, physical activity and dietary parameters (body weight, BMI, waist circumference), physical activity and daily dietary consumption.

-Improvement in glycaemic control (fasting plasma glucose, HbA1c)

-Improvement in cardiovascular risk profile: fasting plasma lipids, oxidative stress markers, inflammatory markers/adipokines and chemokine MCP-1.

-Chronic kidney disease (CKD) regression. CKD will be defined according to Kidney Disease Improving Global Outcomes (KDIGO) guidelines as sustained (i.e., documented on 2 occasions at least 3 months apart) reduction in estimated Glomerular Filtration Rate [eGFR] <60 mL/min/1.73 m^2 **and/or** sustained increase in albumin excretion rate (AER)≥30 mg/d. CKD regression will be defined as sustained (i.e., documented 3 month apart, on both follow-up visit 7 and visit 8) estimated Glomerular Filtration Rate [eGFR] ≥60 mL/min/1.73 m^2 **and** normalization of AER (i.e., <30 mg/d).

eGFR will be assessed from serum creatinine using the **CKD-EPI** (Chronic Kidney Disease Epidemiology Collaboration) equation, as recommended by KDIGO guidelines[42]. eGFR and AER will be classified according to KDIGO categories[43]

-Change in absolute values of eGFR[44,Error! Bookmark not defined.], and of albumin excretion rate(AER).

We will estimate mean annual rates of change in eGFR from baseline to the final follow-up visit (total slopes), within each treatment allocation using linear mixed model repeated measures (MMRM); adjustments were made for age, baseline eGFR, baseline urinary AER, diabetes, baseline fibrosis stage, systolic blood pressure, angiotensin-converting enzyme/angiotensin receptor blocker[45](see. **supplementary material**)

Exploratory outcomes

We explored the effect of treatment on Nuclear Factor(NF)-kB activation in the liver and in circulating mononuclear cell(MNCs). NF-kB is a key proinflammatory transcription factor involved in NASH development and progression and a known molecular target of curcumin (see Methods and supplementary material).

Safety outcomes

Safety end points include adverse events after the start of treatment, biochemical assessments, and clinical assessments. Selected events (including deaths, cardiovascular events, and acute pancreatitis) were adjudicated by an independent, external event-adjudication committee, whose members were unaware of the treatment assignments. A complete list of the trial end points is provided in protocol Table 1.

AE reporting and analysis

The reporting period for AEs will start at screening visit 1 and will continue until follow-up visit 8. SAEs were reported until day 504 (week 72) of the trial treatment and for 84 days post-EOT. All SAEs and adverse reactions will be evaluated by the investigators and recorded. The National Cancer Institute's common terminology criteria for AEs (CTCAE, V.4.02, 2010) will be used to grade each AE.

An AE will be deemed serious if it results in hospitalization or a visit to the emergency room where an intervention is made to avert hospitalization. The nature and number of all serious adverse events was adjudicated by an independent data management committee (DMC) who is unaware of treatment assignment at the time of ascertainment.

The central trial office (CRCTU, Turin) will keep detailed records of all AEs reported (nature, onset, duration,severity, outcome) and perform an evaluation with respect to seriousness, causality and expectedness.

Interim analysis of safety data will be performed and presented to the independent data management committee (DMC) on a 6-monthly basis. The unblinded DMC advises accordingly with regards to the trial conduct and specifically whether extra/new data monitoring will be required for the remainder of the trial. The DMC operated in accordance with a trial specific charter based on a predefined template.

8. Analytical Methods

8.1 Liver histopathology and immunohistochemistry

8.1.1 Liver histopathology

One liver histopathologist (RP) with specific expertise in NAFLD, blinded to the clinical, laboratory and study treatment allocation, will perform all the histopathological assessments at the central trial site (HUMANITAS Gradenigo) using an in-house designed proforma. The histological diagnosis of NASH will be established using H&E staining and haematoxylin van Gieson stains of formalin-fixed paraffin-embedded liver tissue. Both the baseline and end-of-treatment (72 weeks) biopsies will be reported as either 'definite NASH,' 'uncertain NASH' or 'not NASH.' The histological diagnosis of 'definite NASH' is defined as a combination of >5% macrovesicular steatosis, hepatocyte ballooning (± Mallory's Hyaline) and lobular inflammation (mixed infiltrate, related to foci of ballooning)[46]. The assessment of ballooning is subjective, and thus for 'uncertain' hepatocyte ballooning, a key component of the diagnosis of NASH, ubiquitin immunohistochemistry will be used to identify material compatible with Mallory's hyaline.

To validate the quality of the biopsy specimen, the core specimen length will be measured and the number of portal tracts will be recorded.

The NAS will be calculated based on the Kleiner classification[47] The NAS is scored of 8, with 8 representing the highest activity. The NAS is the sum of scores of the 3 components of the histological scoring system, namely steatosis (0=<5%, 1=5–33%, 2=>33–66%, 3=>66%), lobular inflammation (0=no foci, 1=<2 foci/×200, 2=2–4 foci/×200, 3=>4 foci) and hepatocyte ballooning (0=none, 1=few ballooned cells, 2=many cells/prominent ballooning). The Kleiner scoring system for NAFLD fibrosis (F0-F4) will be used to evaluate the stage of fibrosis in each biopsy specimen. Portal tract changes (inflammation, interface hepatitis, ductular reaction), an intrinsic feature of NASH, will also be recorded[48]. The pathologists will score each histological feature of liver biopsies and fill in separate forms.

8.1.2 Potential relevance of Nuclear Factor κB (NF-κB) activation in the liver and mononuclear cells (MNCs) for NASH pathogenesis and curcumin treatment

The pro-inflammatory transcription factor **Nuclear Factor(NF)-κB** is a master regulator at the crossroads of metabolic, oxidative, immune and inflammatory pathways and a known molecular target of curcumin(3,49). NF-κB activation in all liver cell subtypes and in peripheral mononuclear cells (MNCs) plays a central role in NASH pathogenesis and progression and in NAFLD-associated metabolic abnormalities((50, 51, 52,53, 54). We therefore assessed the effect of treatment on NF-κB activation in the liver and in circulating MNCs.

Nuclear Factor(NF)-κB activation in the liver by immunohistochemistry

The ImmunoCruz® Staining System will be used in deparaffinized liver tissue according to the manufacturer's instructions (Santa Cruz Biotechnology, Santa Cruz, CA). Briefly, slides will be soaked in 3% hydrogen peroxide for 5 min, washed, and incubated in serum blocking solution for 20 min. Specimens will be then incubated with primary antibodies for 2 h at 37◦ C. Tissue samples will be probed with mouse monoclonal antibodies reactive to NF-*κ*B p65 (Santa Cruz Biotechnology). After rinsing, specimens will be incubated with biotinylated secondary antibody and a horseradish peroxidase-streptavidin complex, for 30 min each. Tissue samples will be then colorized with DAB substrate, counterstained, mounted, and examined. NF-*κ*B immunoreactivity will be expressed as the % of positive cells/high-power field (×400)[55] and will also be semiquantitatively evaluated using a 4-point scoring system (0—no staining; 1—positive staining in <30% of cells/high-power field; 2— positive staining in 30–70% of cells/high-power field; 3—positive staining in >70% of cells/high-power field).

NF-kB activation in circulating mononuclear cells(MNCs).

Blood samples were collected in tubes containing Na-EDTA as an anticoagulant; 3.5 mL of the anticoagulated blood sample was carefully layered over 3.5 mL of the PMNL isolation medium (Robbins Scientific Corp, Sunnyvale, CA). Samples were centrifuged at 450 x *g* in a swing outrotor for 30 min at 22 °C. At the end of centrifugation, 2 bands separate out at the top of the red blood cell pellet. The top band consists of monocuclear leukocytes(MNCs), whereas the bottom band consists of polymorphumuclear leukocytes(PMNs). The MNC and PMN bands were harvested with a Pasteur pipette, repeatedly washed

with Hank's balanced salt solution, and reconstituted to a concentration of 4 x 10^5 cells/mL in Hank's balanced salt solution. This method yields> 95% pure PMN and MNC suspensions(56).

NF-kBp50/p65 transcription factor assay kit was purchased from Cayman Chemical (Ann Arbor MI, USA). The method detects specific transcription factor DNA binding activity in nuclear extracts and cell lysates. A specific double stranded DNA sequence containing the NF-kB response element is immobilized onto the bottom of wells of a 96 well-plate. NF-kB in nuclear or cytoplasmatic extract binds specifically to the NF-kB response element. NF-kBp50/65 is detected by addition of specific primary antibody directed against NF-kBp50/65. A secondary antibody conjugated to Horseradish peroxidase is added to provide a sensitive colorimetric readout at 450 nm.

Other clinical and laboratory data

Fasting blood samples will be analysed for full blood count, urea, creatinine and electrolytes, thyroid stimulating hormone (TSH), lipid profile (total cholesterol, high density lipoprotein, triglycerides), liver function tests, prothrombin time, international normalised ratio (INR), amylase, α-fetoprotein, C reactive protein, HbA1c, Monocyte chemoattractant protein-1(MCP-1), and plasma glucose using standard laboratory methods (Roche Modular system, Roche Ltd, Lewes, UK). Urinary albumin excretion rate will measured using standard routine methods.

Serum Insulin (Mercodia, Uppsala, Sweden), nonesterified fatty acids (Zen-Bio, Research Triangle Park, North Carolina, USA) and CK-18 M30 (M30 Apoptosense ELISA Kit; PEVIVA AB, Bromma, Sweden) lumican and adiponectin will be measured in-house using commercially available colorimetric ELISAs. Serum caspase-cleaved cytokeratin-18 (CK-18 M30) was performed at study entry to assess hepatic apoptosis. HOMA-IR, a marker of insulin resistance, was calculated in the standard fashion: glucose×insulin/22.5. Measurements of weight (kg), height, systolic/diastolic blood pressure and waist:hip circumferences were recorded. Waist and hip circumferences were defined as

the circumferential measurements immediately above the level of the iliac crests and at the level of the greater trochanters, respectively. BMI was defined as weight in kilograms divided by the square of the height in metres (kg/m2).

Nuclear Factor(NF)-κB activation in mononuclear cells (MNCs)

Blood samples will be collected in tubes containing Na-EDTA as an anticoagulant; 3.5 mL of the anticoagulated blood sample will be layered over 3.5 mL of the PMNL isolation medium (Robbins Scientific Corp, Sunnyvale, CA). Samples will be centrifuged at 450 x *g* in a swing outrotor for 30 min at 22 °C. At the end of centrifugation, 2 bands separate out at the top of the red blood cell pellet. The top band consists of monocuclear leukocytes(MNCs), whereas the bottom band consists of polymorphumuclear leukocytes(PMNs). The MNC and PMN bands will be harvested with a Pasteur pipette, repeatedly washed with Hank's balanced salt solution, and reconstituted to a concentration of 4 x 10^5 cells/mL in Hank's balanced salt solution. This method yields> 95% pure PMN and MNC suspensions(57).

NF-kBp50/p65 transcription factor assay kit was purchased from Cayman Chemical (Ann Arbor MI, USA). The method detects specific transcription factor DNA binding activity in nuclear extracts and cell lysates. A specific double stranded DNA sequence containing the NF-kB response element is immobilized onto the bottom of wells of a 96 well-plate. NF-kB in nuclear or cytoplasmatic extract binds specifically to the NF-kB response element. NF-kBp50/65 is detected by addition of specific primary antibody directed against NF-kBp50/65. A secondary antibody conjugated to Horseradish peroxidase is added to provide a sensitive colorimetric readout at 450 nm.

Determination of plasma curcumin and curcumin metabolites

We used a validated HPLC-MS/MS method to simultaneously measure plasma curcumin, demethoxycurcumin, bis-demethoxycurcumin, tetrahydrocurcumin and octahydrocurcumin blood samples of all included patients during the oral fat tolerance test and of the oral glucose tolerance test[58], [59],[60].

To investigate curcumin and curcumin metabolite kinetics after coingestion with fat or glucose, on the day of the oral tolerance tests patients took two pills of their curcumin/placebo formulations together with the glucoe or fat meal, and blood samples were subsequently drawn at the predefined times(see below)

Dietary and physical activity record

Dietary record with food frequency questionnaire. Subjects will be instructed to fill in a 7-day validated and reproducible dietary questionnaire during an individual training session with a nutritionist; a list of foods is designed, for each item different portion sizes are specified according to the EPIC study([61]). The di-et record was analyzed using the WinFood database (Medimatica, Teramo, Italy) according to the table of food consumption of the Italian National Institute of Nutrition and Food Composition Database for Epide-miological Study in Italy([62]). Based on FFQ data, excessive alcohol use will be defined as .30 g/d for male individuals and .20 g/d for female individuals.

The FFQ will be completed at baseline (visit 1) and at end-of-treatment (visit 7). Analysis will report the change from baseline scores to the end-of-treatment .

Physical activity record.

Physical activity (PA) will be measured with the accelerometer SenseWear Armband (SWA), which had to be worn continuously on the nondominant arm for at least 3 consecutive days (2 working days and a week-end day) at baseline and at EOT. The SenseWear Armband (SWA) is a relatively new accelerometer device that can be used to quantify and monitor physical activity and energy expenditure (EE), which has been validated against indirect calorimetry[63] and has been shown to have acceptable reliaiability and reproducibility. The SWA is designed to be worn on the upper arm over the triceps. Its internal sensors include an accelerometer, a thermal flow sensor, a galvanic sensor that records skin response, a skin temperature sensor, and an air temperature sensor. The accelerometer in the armband has two axes and uses a microelectromechanical sensor that measures movement.

Physical activity is measured relative to gravity (1 mg=9.81 mm/sec2) with an interval of 20 ms and categorized based on intensity, according to White et al.[64] , into time spent in sedentary behavior (,48 mg) and in light (48–154 mg, e.g., walking), moderate (154–389 mg, e.g., cycling), and vigorous activities (.389 mg e.g., running). The software created by the manufacturer calculates then EE using a patented algorithm that combines acceleration, heat flow, and other parameters: technical and statistical procedures have been described in detail earlier[65], [66], [67]..

PA record will be completed at baseline (visit 1) and at end-of-treatment (visit 7). Analysis will report the change from baseline scores to the end-of-treatment .

Upper abdomen ultrasound and computerized sonographic hepato-renal ratio for quantification of steatosis

All examinations will be performed at each patient visit with a GE Vivid7 ultrasound machine (GE Healthcare, Horten, Norway) equipped with a GE 4C curved array transducer (GE H4904PC). US studies will be performed by two experienced radiologists (GM and EP) who were unaware of the patient's clinical details and laboratory findings. All the instrument
settings, including "gain," "depth," and "time-gain compensation," will be preset and
fixed for each measurement. For assessment of US H/R ratio, ultrasound images with both liver and right kidney clearly visualized will be obtained in the sagittal liver/right kidney view in the lateral position. US hepatic echo-intensity attenuation rate was assessed in right intercostals
view at anterior axilla line in the supine position. All images will be reviewed by one of the two radiologists involved in scanning. Analysis of digitized ultrasound images was performed by using NIHimage software (ImageJ 1.41o, National Institutes of Health, Bethesda, MD).

US hepatic/renal echo-intensity ratio

In sagittal liver/right kidney view, a region of interest (ROI) of 1.5 × 1.5 cm (1,296 pixels) in the liver parenchyma was selected. This area is large enough to calculate an average histogram value and small enough to avoid the inclusion of vessels or bile ducts in the specimen. Several ROI parameters were displayed including circumference and area, total brightness level, mean brightness level, SD, most frequent

brightness level, and a histogram. Of all these

parameters, we used only the mean brightness level for each organ (liver and right kidney) in this study.

Technically, the echo intensity can be influenced by many factors, particularly by gain intensity.

To avoid confounding by factors that can modify the echo intensity and thus bias comparisons,

the mean brightness levels of both the liver and the right kidney cortex were obtained on the

same longitudinal sonographic plane. The ratio between the mean brightness level of the liver and the right kidney was calculated manually to determine the hepatorenal sonographic index.

In each case, the calculation of the hepatorenal sonographic index was repeated at least twice.

When the difference was less than 0.20, the average was calculated. If there was a greater discrepancy, a third measure was performed and the average of the two closest measurements was used.

The ROI had to be as uniform as possible, excluding blood vessels, bile ducts, and other focal hypo/hyperechogenicity. Another ROI of 0.5 × 0.5 cm (144 pixels) was identified in the right renal cortex with no large vessels, renal sinus or medulla. To avoid the interference of depth-depended echo-intensity attenuation and the borderline echo distorting effects, the boundary between liver and right kidney area should be placed near the center of the image, and the liver and right kidney ROIs were selected at the same depth of the ultrasound images. The gray scale mean value of the mixels within the two ROIs was used as measurement of echo intensity. Then we divided the average hepatic gray scale by the average renal cortex gray scale to calculate the US hepatic/renal ratio. US hepatic echo-intensity attenuation rate

In right intercostals view at anterior axilla line, a tangent line of the sector ultrasound image was drawn and the ultrasound wave transmission line was determined, starting from the point of tangency and perpendicular to the tangent line. Two ROIs of 1.5 × 1.5 cm (1,296 pixels) were selected in liver homogeneous regions along the ultrasound transmission line near the liver anterior margin (depth 4–6 cm) and the liver posterior margin, respectively. The linear distance

between the two ROIs was also measured.

The echo intensity of ultrasound wave was attenuated exponentially, shown as the following equation:

$$A\ A\ a\ f\ d$$

$$d = 0 \times e^{-} \cdot \cdot \quad (1)$$

Where $A0$ and Ad are ultrasound echo intensity at the sound source and the liver parenchyma at a specific

depth, respectively; *a* is the attenuation coefficient of the liver parenchyma; *f* is the frequency of the ultrasound detector; *d* is the depth of ROI. The ratio of the average echo intensity in the liver near-field ROI to liver far-field ROI was then calculated based on equation (1):

A A a f f n

n f

d d =e · ·(−) (2)

Where *A*n and *A*f are average ultrasound echo intensity in the near-field ROI and the far-field ROI, respectively; *a* and *f* have been defined in equation (1); d*n* and d*f* are the depth of liver near-field and far-field ROIs.

8.4 Metabolic substudy

ORAL FAT TOLERANCE TEST(OFTT). Within 3 months from entry, all patients will undergo a standard oral fat load test.

Participants will be encouraged to avoid strenuous physical efforts and to follow their usual diet

during the 24 hours preceding the test. The fat load consists of a mixture of 200 ml dairy cream (35% fat) and 26 g egg yolk for a total energy content of 766 kcal.: 78.3 g fat (55.6% saturated fatty acids, 29.6% monounsaturated fatty acids, 14.8% polyunsaturated fatty acids), 595 mg cholesterol, 8.8 g protein, 7 g carbohydrate. The fat load will be consumed over 5 minutes; subjects will be kept

fasting on the test morning, and strenuous activity will be forbidden.

Samples will be drawn at 0 (baseline), 2, 4, 6, and 8 hours. Plasma total cholesterol (Chol), triglyceride (Tg), and free fatty acids (FFA) will be measured by automated enzymatic methods. Very low-density lipoproteins (VLDL) will be isolated through preparative ultracentrifugation and assayed for their Tg and total Chol content. Apo E genotype will be determined by polymerase chain reaction amplification of genomic DNA using specific oligonucleotide primers.

Separation of Triglyceride-Rich Lipoprotein Subfractions.

Very-low-density lipoproteins (VLDL) will be isolated through preparative ultracentrifugation and subsequently assayed for their Tg and total Chol content. One aliquot plasma will be brought to densities of 1,006 g/l by adding a KBr solution (d_1,330 g/l) and centrifuged at 105,000g for 21 hours at 10°C in a Beckman L8-70M ultracentrifuge. Tg and total Chol concentration will be then

determined in lipoprotein fraction enzymatically. The first higher amount of blood (10 ml) will be drawn for subfractionating triglyceride-rich lipoprotein (TRLPs) by ultracentrifugation on a discontinuous density gradient. Separated plasma will be brought to a density of 1.10 g/ml by

adding solid KBr. The density gradient will be prepared by adding to 4 ml of this plasma 3 ml of a 1.065 g/ml solu-tion containing 0.05% KBr/NaCl plus EDTA (pH 7.4); 3 ml of a similar solution at 1.020 g/ml; 3 ml of physiological saline at 1.006 g/ml. The sample will be ultracentrifuged

in a Beckman L8-70M centrifuge at 20°C in stages, allowing the separation of four VLDL fractions with decreasing Sf values: A_400; B_175-400; C_100-175; D_20-100. The first centrifugation (28,300 rpm for 43 minutes) isolates fraction A in a floating volume of 0.5 ml. The volume removed will be replaced with physiological saline, and the sample centrifuged at 40,000 rpm for 67 minutes to isolate fraction B. This procedure will be then repeated at 40,000 rpm for 71 minutes and at 37,000 rpm for 18 hours to isolate fractions C and D, respectively.

The automated methods mentioned will be used to determine Chol and Tg on the 4 fractions.

ApoB48 and ApoB100 Analysis. TRLP ApoB48 and ApoB100 will be separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using 3.9% gel. Nondelipidated samples will be reduced in SDS buffer for 4 minutes at 96°C. Samples will be applied to the geland run at 40 mA in 0.025MTris, 0.192Mglycine, and 0.1% SDS. Gel will be stained with Silver Stain (Bio-Rad). Because the chromogenicity of ApoB48 is similar to that of ApoB100, a protein standard was prepared from LDL isolated by sequential ultracentrifugation and used to quantify ApoB100 and ApoB48. The bands were quantified by densitometry using Gel Doc equipment (Bio-Rad). Density values were assigned to the ApoB100 bands of the standard LDL, and a standard curve will be constructed. The values will be recalculated by linear regression.

Oxidized LDLs measurement. LDL-conjugated dienes will be determined during the fat load test, as follows. Capillary electrophoresis will be performed as described by Stocks and Miller The cathode and anode electrolytes and the capillary run buffer will be 40 mM methylglucamine-Tricine, pH 9.0. LDL samples will be injected by low pressure for 4 seconds. Dimethylformamide will be injected as an electroendosmotic flow marker for 1 second. A voltage of 24 kV was applied ramping over 0.8 minutes. Migration of LDL particles will be monitored at 200 and 234 nm. The amount of conjugated dienes will be obtained from the percentage of the height of LDL peak at 234 nm related to the height of LDL peak at 200 nm.

ORAL GLUCOSE TOLERANCE TEST (OGTT). Within 3 moths from entry into the trial, patients will undergo a standard 75-g oral-glucose-tolerance test (OGTT), with measurement of plasma glucose, insulin, C-peptide, FFA. Areas under the concentration curves (AUCs) of different parameters during the OGTT will be calculated with the trapezoidal method.

The following parameters will be calculated:

Whole body insulin sensitivity. Prehepatic insulin delivery will be estimated as the

suprabasal (D) 30-min AUC of C-peptide divided by the 30-min increase in plasma glucose. The hepatic insulin extraction, as a percentage of secreted hormone, will be estimated by [1 2 (AUC insulin/AUC C-peptide)]. Two indexes of insulin sensitivity will be calculated: the conventional quantitative insulin-sensitivity check index (QUICKI) and oral glucose insulin sensitivity (OGIS), an OGTT-derived index of whole-body insulin sensitivity[68].

Adipose tissue insulin resistance. Adipose tissue insulin resitance will be calculated (adipose IR=fasting FFA x fasting plasma insulin), as previously described and validated in diabetic and nondiabetic subjects[69, 70].

Hepatic and muscle insulin resistance. The hepatic and muscle insulin resistance index will be calculated from OGTT as proposed and validated against clamp in nondiabetic subjects[71]

Hepatic insulin extraction. The hepatic insulin extraction (He), as a percentage of secreted hormone, will be estimated using the formula [1-(AUC insulin/AUC C-pep)].

Pancreatic β-cell function. Two OGTT-derived indexes of β-cell function will be calculated[72]: the insulinogenic index (IGI), computed as the suprabasal serum insulin increment divided by the corresponding plasma glucose increment in the first 30 min (DI30/DG30), and the C-peptide–genic index, computed as DC-peptide30/DG30, which were previously validated against measures of β-cell functions derived from the frequently sampled intravenous glucose tolerance test (FIVGTT). The ability of β-cell to adapt insulin secretion to changes in insulin sensitivity will be assessed by 2 indexes, the disposition index and the adaptation index, which are calculated by multiplying OGIS times IGI and C-peptide–genic index values, respectively. These indexes relate β-cell insulin secretion to insulin resistance and represent integrated parameters of β-cell function, validated against FIVGTT minimal model variables in NAFLD subjects[73, 74]; they also accurately predict future type 2 diabetes in the general population[75], [76].

9. Statistical analyses

9.1 Power analysis

Sample size justification. There are no data on liver histological response to curcumin.

The sample size was calculated using A'Hern's single stage phase II methodology[77] with a significance level of 0.05 (type I error) and power of 0.90 (type II error).

Based on previous trials showing an improvement in surrogate markers of NAFLD with curcumin ranging 20- 50% of patients, depending on different liver disease surrogates, curcumin dose, formulations and treatment duration vs. 15% with lifestyle intervention[78], the design required 21 evaluable patients in the phytosomal curcumin group; after allowing for a 10% dropout rate, at least 25 subjects per arm were needed to detect a significant difference in the primary outcome. The study A'Hern's design stipulates that at least eight (38%) out of 21 patients in the phytosomal curcumin group have to achieve histological improvement for phytosomal curcumin to be deemed worthy of further investigation with a phase III trial [84]

9.2 Methods for analysis of primary and secondary outcomes

All evaluable patients will be analysed on an intention-to-treat basis. Evaluable patients are defined as those who have had an end-of-treatment biopsy, irrespective of the amount of treatment they have received. Patients will be categorised as either achieving the primary histological endpoint (ie, NASH resolution) or not. The proportion of patients with a reported improvement in liver histology will be presented and compared across treatments descriptively with 95% CIs.

The primary analysis will be based on a Cochran–Mantel–Haenszel test based on all randomised patients for the in-trial period with missing data handled as non-responders. The common risk ratio between phytosomal curcumin and placebo was estimated along with exact 95% CI. To test for superiority, the exact two-sided p-value was calculated as the sum of probabilities of outcomes having equal or lower probability than the observed outcome under the null hypothesis.

Adjustments for multiplicity testing in the primary analysis will be performed with the use of the Benjamini-Hochberg procedure; each active-treatment group was compared with the placebo group. Two-sided P values <0.05 were considered statistically significant.

An unpowered, preplanned secondary analysis of categorical outcome measures will be done using the $\chi 2$ test or Fisher exact test, as appropriate, of the difference between the proportions of patients with histological improvement in each treatment group.

A preplanned logistic regression analysis will be undertaken to determine the treatment effect when adjusted for the stratification variables (diabetes status, obesity status, fibrosis stage, CKD status) and trial site. We will calculate adjusted relative risks using the Mantel-Haenszel test. Two-sided P values<0.05 will be considered statistically significant in all analyses.

Continuous secondary outcome measures will be compared between treatment groups using linear

regression, adjusting for parameter baseline values and allocated treatment (as model covariates, equivalent to ANCOVA), with multilevel modelling for key continuous outcome measures to account for repeated measures within each patient.

We estimated mean annual rates of change in eGFR from baseline to the final follow-up visit (total slopes), by treatment allocation using linear mixed model repeated measures (MMRM); adjustments were made for age, sex, first baseline CKD stage, baseline eGFR; baseline urinary ACR, diabetes, history of cardiovascular disease, systolic blood pressure, angiotensin-converting enzyme/angiotensin receptor blocker use[79]

Proteinuria will be estimated using measurements of urinary protein and creatinine at baseline and at

baseline and at each visit thereafter.

Albuminuria and eGFR stage will be categorized according to the KDIGO classification system[80]

The end-stage renal disease was defined as kidney transplantation or the receipt of dialysis for at least 30 days unless death occurred.

For exploratory outcomes, we will explore the existence of a threshold in hepatic NF-kB activation change predicting histological response(NASH resolution, fibrosis improvement) using the area under receiver operating characteristic curve (AUROC) analysis and Youden index.

A sensitivity analysis will be performed in which missing data were handled by reference-based multiple imputation informed by data from placebo recipients (**see Preplanned subgroup and sensitivity analyses suheaing**).

Safety will be analysed descriptively

9.3 Methods in analysis to handle missing data

The primary imputation method for missing data will the non-responder imputation for primary and secondary dichotomous outcomes. This approach does not rely on an assumption of missing at random

and is considered conservative for estimating the treatment effect.

To investigate the sensitivity of the histological results with regard to the handling of missing

data, post-hoc sensitivity analyses using multiple imputation will be conducted. These analyses are based on the same CMH method as for the main analyses but using a multiple imputation at random method for missing data(see following Subheading)

9.5 Prespecified additional subgroup and sensitivity analyses

As previously specified, the primary imputation method for missing data will the non-responder imputation for primary and secondary dichotomous outcomes.

To investigate the sensitivity of the results of the primary analysis with regard to the handling of missing data, a sensitivity analysis using imputation based on unconditional reference was performed for the primary and secondary outcomes. The analysis is based on the same type of non-parametric method as for the primary analysis but with missing data handled by a method of multiple imputation which assumes that the unobserved outcomes are well described by the observed outcomes of the subjects in the placebo arm with similar baseline characteristics. The imputation is done by random sampling of observed outcomes from placebo subjects with the same baseline diabetes status, obesity status, fibrosis stage and CKD status. Multiple (100) replicates of a complete data set will be generated and, for each complete data set, the log risk ratios will be estimated together with the asymptotic standard errors. The estimates and standard errors for the 100 complete data sets were pooled using Rubin's rule to obtain a single estimate of the risk ratio and a 95% confidence interval. The analysis differs from the primary analysis in that it assumes that subjects with missing week 72 data had the same chances of NASH resolution as subjects with week 72 data in the placebo group. If there will be more missing week 72 data in the phytosomal curcumin group than in the placebo group, this analysis would be less conservative than the primary analysis and probably give a better estimate of the treatment effect. The handling of missing data is analogous to the "copy reference" approach for longitudinal normal data presented by Carpenter et al.[81]

We also plan to perform the following subgroup analyses to assess the effect of the following characteristics on main efficacy and safety outcomes:

-trial site

-diabetes status, obesity status, CKD status, fibrosis stage (F3-4 vs F0-2)

-per protocol analysis (only patients who completed treatment, had EOT liver biopsy and no major protocol deviations will be included).

All analyses will be carried out with Easy R ver1.61, Saitama, Japan[82].

10. Ethics and Dissemination

10.1 Data collection and patient consent and confidentiality

Data will be collected from patient paper sanitary record into an electronic case report form (CRF) consisting of an excel datasheet file, where each patient will be deidentified and coded by a unique, computer-generated number. CRFs included baseline/follow-up medical history and physical examinations to capture comorbidities and concomitant

medications in the trial's electronic database. Other CRFs incorporated in the electronic database included: laboratory tests and questionnaire results were recorded for visit 1 (eligibility criteria) through to visit 8; safety monitoring during the treatment follow-up periods; central site histopathology reports of liver biopsy specimens; specialist non-invasive markers of liver

disease; AE reporting and study drug dispensing forms for study treatment adherence and accountability.

. No patient identifiers will leave hospital unit. Patient confidentiality will be maintained by keeping data collected in the study coded. The key will be at the HUMANITAS Gradenigo Hospital, and will be managed by the principal investigator after EOT and unblinding. No Personal detail or identifying data will be transferred from the patient sanitary file to the CRF which will be available for data analysis.
Data management was undertaken according to the standard operating procedures of the CRCTU at HUMANITAS Gradenigo hospital.. The CRCTU followed the International Conference on Harmonisation Good Clinical Practice (ICH GCP). The CRCTU was responsible for monitoring the trial and providing annual reports to the sponsor. Participant identifiable data were shared only within the clinical team on a need-to-know basis to provide clinical care, and to ensure good and appropriate follow-up and the primary care practitioners. All participants provided specific written consent at trial entry to enable data to be shared

with the above. Otherwise, confidentiality was maintained throughout the trial and thereafter. On completion of the trial, data will be transferred to a secure archiving facility at HUMANITAS Gradenigo hospital, where data will be held for a minimum of 10 years and then destroyed

10.2 Sponsorship, indemnity and monitoring

HUMANITAS Gradenigo acted as the sponsor of the trial. As sponsor Humanitas was responsible for the general conduct of the study and indemnified the trial centre against any claims, arising from any negligent act or omission by the University in fulfilling the sponsor role in respect to the study. Both on-site and off-site monitoring of the trial were performed as perthe Trial Quality Management Plan.

10.3 Role of the funding source

The manifactures funding te trial (Indena SpA) had no role in study design, data collection, data analysis, data interpretation, or writing of the report. The corresponding author had access to all data in the study and had fi nal responsibility for the decision to submit for publication.

10.4 Competing interest

All participating investigators have no conflict of interest related to this study.

810.5 Dissemination

We plan to publish the results of this controlled trial to an international journal following peer review.

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Marc Colangelo,1 Thomas E. Angel,1 Carine Beysen,1 Jeffrey Cui,2 Carolyn Hernandez,2 Len Lazaro,2 David A. Brenner,2
Scott M. Turner,1 Marc K. Hellerstein,1,3 and Rohit Loomba2

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Anuradha Krishnan1, Xia Li1, Winston Whei-Yang Kao2, Kimberly Viker1, Kim Butters1,
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Protein-1 – New Insights into the Histologic Spectrum of NonAlcoholic Fatty Liver Disease
Michael Charlton1, Kimberly Viker1, Anuradha Krishnan1, Schuyler Sanderson2, Bart Veldt1, A. J. Kaalsbeek1, Michael Kendrick3, Geoffrey Thompson3, Florencia Que3, James Swain3, and Michael Sarr

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