"MULTICENTRIC, PROSPECTIVE, OPEN, RANDOMISED, CONTROLLED, AND INTERVENTIONAL STUDY PROTOCOL THAT DOES NOT INVOLVE THE USE OF MEDICINAL PRODUCTS"

Study Code: **KETONASH**

ClinicalTrial.gov (NCT) number: **TBD**

Study title:

"The Role of Very Low-Calorie Ketogenic Diet (VLCKD) in Patients Affected by Non-Alcoholic Steatohepatitis (NASH) with Fibrosis"

Author(s):	Federico Ravaioli
Study Code:	KETONASH
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Version Date:	23.11.2023
Version:	4.0

SIGNATURE PAGE

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Author(s) signature(s):

This protocol has been written and will be conducted in accordance with good clinical practice and applicable national regulations.

Dr. Federico Ravaioli (Author) _____ Date: 24.11.2023

Principal Investigator's and Collaborators' signatures:

I declare that I have read the protocol and consent to conduct this clinical study in accordance with all protocol requirements and Good Clinical Practice Guidelines.

Prof. Fabio Piscaglia (Principal Investigator) _____ Date: 24.11.2023

Dr. Federico Ravaioli (Scientific Supervisor) _____ Date: 24.11.2023

Prof. Maria Letizia Petroni (Scientific Supervisor) _____ Date: 24.11.2023

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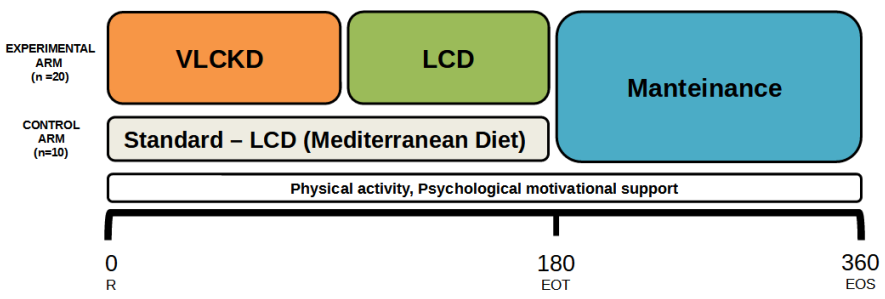
PROTOCOL SYNOPSIS

Study Code	KETONASH
NCT Number	TBD
Principal Investigator	Prof. Fabio Piscaglia: Internal Medicine Unit for Hepatobiliary and Immunoallergological Diseases, IRCCS Hospital-University of Bologna, Sant'Orsola Polyclinic
Study Purpose	The purpose of the KETONASH study is to evaluate, in patients with metabolic-associated fatty liver disease (MAFLD) with non-alcoholic steatohepatitis (NASH) and significant liver fibrosis (F1-F2-F3), the effect of a very-low-calorie ketogenic diet (VLCKD) compared to that of a standard low-calorie diet (standard Mediterranean LCD - in accordance with the European Association for the Study of the Liver/European Society for Clinical Nutrition and Metabolism guidelines on MAFLD/NAFLD).
Primary Objective	• Histological improvement in terms of liver fibrosis without worsening of NASH • Histological improvement of NASH without worsening of liver fibrosis
Study Type	Multicentric, Prospective, Open, Randomised, Controlled, and Interventional, with no medicinal use
Study Phase	N/A
Study Design	

Study Coordinator	Internal Medicine Unit for Hepatobiliary and Immunoallergological Diseases, IRCCS Hospital-University of Bologna, Sant'Orsola Polyclinic
Study Duration per Patient	The study has a duration of 12 months from patient enrollment and randomisation.
Planned Follow-up	N/A
Population	Patients with a current or past diagnosis of metabolic-associated fatty liver disease (MAFLD/NAFLD/MAFLD) formulated in accordance with the European Association for the study of the Liver guidelines, with significant liver fibrosis (F2-F3) and non-alcoholic steatohepatitis (NASH)
Sample Size Estimate	42 patients (2:1 randomisation)
Number of Study Centers	3 centers (including Coordinator)
Randomisation	Two arms, 2:1 randomisation stratified by diabetes mellitus and gender
Participants	Inclusion Criteria:
	• Patients aged ≥18 years with histological diagnosis of NASH with evidence of stage 1, 2, or stage 3 fibrosis (F1/F2/F3) (defined according to NASH CRN[1]) obtained no more than 6 months before enrollment; • Stable weight for more than 6 months with BMI between 30-40 kg/m2; • Patients in whom it is safe and feasible to proceed with liver biopsy and who consent to undergo liver biopsy after 12 months of enrollment to assess the effect of dietary treatment; • Obtained informed consent.
	Exclusion Criteria:

	• BMI <30 or BMI >40 • Presence of evolved chronic liver disease into cirrhosis (histological F4 or elastometric LSM >14 kPa) • Type 1 diabetes mellitus • Model for End-stage Liver Disease (MELD) score >12, AST or ALT ≥5× ULN, HbA1c >9.5%, INR ≥1.4, creatinine >1.5 mg/dl, platelets <100,000/mm3, and total bilirubin >1.5 mg/dl. • Concurrent presence of any other known chronic liver disease beyond MAFLD/NAFLD, such as alcoholic liver disease, viral (HCV/HBV), cholestatic-autoimmune (PBC/PSC/AIH), Wilson's disease, hemochromatosis, drug-induced liver injury (DILI), or the presence or suspicion of hepatocellular carcinoma (HCC); • Average alcohol consumption exceeding 4/2 units/day (males/females) in the preceding 6 months and a history of excessive alcohol consumption in the last 5 years; • Previous or planned liver transplant, bariatric surgery, ileal resection, or biliary diversion; • History of acute cholecystitis and biliary obstructions (cholangitis); • Recent (in the last 12 months) or concurrent use of agents known to cause hepatic steatosis (long-term systemic corticosteroids [>10 days], amiodarone, methotrexate, tamoxifen, tetracyclines, high-dose estrogens, valproic acid); • Recent (in the last 3 months) change in the dose/regimen or introduction of Vitamin E (at doses ≥400 IU/day), ursodeoxycholic acid (UDCA), betaine, S-adenosyl methionine, silymarin, or pentoxifylline; • Presence of psychiatric disorders and/or diagnosis of any eating disorder; • Life expectancy <6 months.
Interim Analysis	No interim analyses are planned.

LIST OF ABBREVIATIONS AND ACRONYMS

AASL	American Association for The Study Of Liver Diseases	ICW	Intra Cell Water
AGA	American Gastroenterology Association	IgA	Immunoglobulin A
AIH	Autoimmune Hepatitis	IgG	Immunoglobulin G
ALT	Alanine Amino Transferase	IgM	Immunoglobulin M
AST	Aspartate Amino Transferase	BMI	Body Mass Index
BCM	Body Cellular Mass	INR	International Normalized Ratio
BDI	Beck Depression Inventory	IPAQ	International Physical Activity Questionnaire
BES	Binge Eating Scale	LCD	Low Calorie Diet
BIA	Body Impedance Analysis	LDL	Low-Density Lipoprotein
BIVA	Body Impedance Vectorial Analysis	LSM	LiverStiffnessMeasure
BMI	Body Mass Index	MAFLD	Metabolic Associated Fatty Liver Disease
BMR	Basal Metabolic Rate	MELD	Model for end-stage Liver diseases
CAP	Controlled Attenuation Parameters	MM	Muscle Mass
CONSORT	Consolidating Standards Of Reporting Trials	NAFLD	Non-Alcoholic Fatty Liver Disease
DILI	Drug-InducedLiverInjury	NASH	Non-AlcoholicSteatohepatitis
DNA	Deoxyribonucleic acid	NFS	NAFLD Fibrosis Score
EASL	European Association for The Study of The Liver	NHANES	National Health and Nutrition Examination Survey
ECW	Extra Cell Water	PBC	PrimaryBiliaryCholangitis
EDTA	Ethylenediaminetetraacetic acid	PSC	PrimarySclerosingCholangitis
EFSUMB	European Federation of Societies for Ultrasound in Medicine and Biology	RCT	RandomizedControled Trial
ESPEN	European Society for Clinical Nutrition and Metabolism	RNA	Ribonucleic acid
FFT	Free Fat Mass	STAI	State-Trait Anxiety Inventory
FLI	Fatty Liver Index	T2D	Type 2 Diabetes
FM	Fat Mass	TBW	Total Body Water
Hb1Ac	glycated haemoglobin	TSD-OC	SIO Obesity-Related Disability Test
HBV	Hepatitis B Virus	TSH	ThyroidStimulatingHormone
HCC	Hepato Cellular Carcinoma	UDCA	Ursodesossicolic Acid
HCV	Hepatitis C Virus	VLCD	Very Low-Calorie Diet
HDL	High-Density Lipoprotein	VLCKD	Very Low-Calorie Ketogenic Diet
HRQoL	Health-Related Quality Of Life		

INTRODUCTION AND RATIONALE

Non-Alcoholic Fatty Liver Disease (NAFLD/MAFLD)

Non-alcoholic fatty Liver Disease (NAFLD) is a spectrum of diseases characterised by the accumulation of lipids in the liver parenchyma. It includes simple steatosis but can progress to non-alcoholic steatohepatitis (NASH) and advanced fibrosis, cirrhosis, and hepatocellular carcinoma [2]. Since it is widely associated with insulin resistance and related metabolic disorders, NAFLD has recently been renamed Metabolic Associated Fatty Liver Disease (MAFLD) [3]. MAFLD/NAFLD is also an independent risk factor for type 2 diabetes, cardiovascular diseases, and mortality [4].

MAFLD/NAFLD is one of the most common chronic liver disorders [5]. The global prevalence is estimated to be increasing, currently around 25%, highest in the Middle East (32%) and South America (31%), followed by Asia (27%), the United States (24%), Europe (23%), and Africa (14%) [3]. The prevalence in the United States is expected to increase from 83.1 million in 2015 to 100.9 million in 2030, with increasingly early diagnoses in the adolescent population (estimated prevalence from 3% to 18%) [6].

The aetiology of MAFLD/NAFLD is predominantly related to dietary habits but also to endocrine or pharmacological causes that lead to the accumulation of lipids in the liver, promoting metabolic alterations that contribute to hyperglycemia, dyslipidemia and hyperinsulinemia[7]. These alterations further increase liver damage by regulating adipose tissue lipolysis, hepatic lipid absorption, lipogenesis, and beta-oxidation [8].

A cohort study reported that the major independent predictors of the severity of fatty liver are poor adherence to the Mediterranean diet, high body mass index (BMI), and the homeostasis model assessment of insulin resistance (HOMA-IR) [9].

Therefore, MAFLD/NAFLD considered the hepatic component of the metabolic syndrome, is closely associated with insulin resistance, obesity, and type 2 diabetes (T2D). Approximately 70% - 80% of obese and diabetic patients with MAFLD/NAFLD are positively correlated with a sedentary lifestyle, promoting hyperglycemia and hypertriglyceridemia, which in turn promote T2D and cardiovascular diseases[10, 11].

The pathogenesis to progress assumes the presence of lipid accumulation, insulin resistance, and inflammation, but the underlying mechanisms, including those driving disease progression, still need to be fully understood to date.

Fatty acids derived from lipogenesis and de novo synthesis circulate in peripheral tissues, including the liver and adipose tissue, where they accumulate, causing insulin resistance. Adipose tissue acts as a mediator of systemic lipid storage and secretes hormones and adipokines. Adiponectin is a specific adipokine that regulates fatty acid oxidation and inhibits lipid accumulation in both adipose tissue and the liver, maintaining glucose homeostasis, including liver insulin sensitivity[12]. Recent studies have shown that serum adiponectin levels are lower in patients with MAFLD/NAFLD than in healthy individuals. Hypoadiponectinemia compromises fatty acid metabolism, promoting a chronic inflammatory state in the liver. Therefore, maintaining adiponectin levels may prevent patients with MAFLD/NAFLD from developing inflammation and fibrosis[13].

The progression of MAFLD/NAFLD to NASH and, therefore, the pathogenesis of the latter is based on the two-stage hypothesis: The first stage is insulin resistance associated with high blood lipid levels, leading to fatty liver. The second stage involves oxidative stress, lipid peroxidation, and mitochondrial dysfunction essential for the progression to non-alcoholic steatohepatitis (NASH). With the identification of more advanced mechanisms, it has been demonstrated that NASH develops through a multifactorial process that includes not only insulin resistance, oxidative stress, and genetic factors but also nutritional factors, lifestyle, and changes in the intestinal microbiota. Therefore, it becomes important to treat MAFLD/NAFLD to prevent its progression and the associated metabolic diseases[14–17].

MAFLD/NAFLD is the negative consequence of ectopic hepatic lipid accumulation in the presence of insulin resistance, characterised by the deposition of triglycerides in hepatocytes. A close connection exists between visceral adiposity and a Western diet high in carbohydrates and fats[18–20]. Visceral fat stimulates the release of pro-inflammatory cytokines and maintains insulin resistance. Diets rich in saturated fats and simple carbohydrates release fatty acids into the liver, stimulating lipogenesis, oxidative stress, and lipotoxicity[21].

Ketogenic Diets with Very Low Caloric Intake (VLCKD)

Since the 1960s, low-carbohydrate diets (LCKD) have been utilised for obesity treatment. While the origins of the ketogenic diet as a medical therapy for managing drug-resistant epilepsy date back to the early 1900s, the very-low-calorie ketogenic diet (VLCKD) has emerged as a promising option for achieving significant weight loss in a short period[22]. It has been observed that diet-induced ketosis positively influences metabolic and

inflammatory markers (HbA1c, fasting glucose, insulin levels, blood lipid levels, liver enzymes, and advanced fibrosis) in type 2 diabetic (T2D) patients, aiding in weight management as well[22–25].

Ketogenesis, a physiological process occurring through hepatic beta-oxidation of fatty acids, leads to the production of energy in the form of ketone bodies. These water-soluble molecules are produced when hepatic glucose and glycogen reserves are minimised (during fasting, pathological states, excessive physical exercise, etc.). The onset of ketosis stabilises insulin levels, and in recent decades, the ketogenic diet has shown promising results for related pathological conditions[23].

Stored energy as fat in adipose tissue is released as acetyl-CoA and converted into intermediate substrates. When insulin levels are low, stored fat in adipose tissue undergoes lipolysis through hormone-sensitive lipase. Once released, free fatty acids undergo beta-oxidation in hepatic mitochondria to produce acetyl-CoA for ketone body generation: acetoacetate, beta-hydroxybutyrate, and - in lesser amounts - acetone, inducing a state of nutritional ketosis. This can lead to a metabolic shift. Reduced insulin release promotes a metabolic shift towards lipid oxidation, utilising excess fatty acids and ketones to produce energy, sparing muscle mass, and improving insulin sensitivity[22, 23].

A systematic review and meta-analysis were conducted to assess the efficacy and safety of VLCKD in overweight and obese patients[26]. The reported studies associated VLCKD with weight loss when the ketogenic phase lasted at least four weeks, and the lost weight was maintained in the subsequent follow-up for up to two years. No changes were observed in LDL cholesterol, HDL cholesterol, serum creatinine, serum uric acid, and serum potassium, while serum sodium increased. Current nutritional strategies are only sometimes effective in long-term weight loss and maintenance. Very low-calorie ketogenic diets (VLCKD), although requiring support from further studies, are emerging as a successful nutritional model for obesity management. The rapid weight loss enhances patient motivation, and increased satiety promotes dietary compliance[26].

Additionally, VLCKDs preserve lean mass, which is crucial for glucose metabolism and resting energy expenditure. Bezerra, Bueno et al. examined 13 randomised controlled trials (RCTs) involving a total of 1569 subjects, comparing VLCKD (<50 g carbohydrates/day) with a low-fat diet (<30% kcal) in overweight and obese adults. The included RCTs had a minimum follow-up of 12 months. The primary outcome was body weight, and secondary goals included lipid profile (triglycerides, HDL, LDL), systolic and

diastolic blood pressure, fasting glucose, insulin, HbA1c, and CRP. Analyses showed that VLCKD achieved more significant long-term reductions in body weight, triglycerides, and diastolic pressure, along with more significant increases in HDL and LDL cholesterol compared to a low-fat diet[27, 28].

It is, therefore, crucial to identify recommendations for the correct use of this therapeutic approach[29, 30]. Recently, the consensus of the Italian Society of Endocrinology (SIE) has indicated the use of VLCKD as a dietary treatment for overweight/obese patients with MAFLD/NAFLD[31].

VLCKD in NAFLD

Currently, all the latest guidelines from the European Society for Clinical Nutrition and Metabolism (ESPEN)[32, 33], as well as those from the European Association for the Study of the Liver (EASL), American Association for the Study of Liver Diseases (AASLD), and American Gastroenterology Association (AGA)[2, 6, 30, 34], conclude that the therapeutic goal in patients with MAFLD/NASH is significant weight loss through a hypocaloric diet of 1200-1500 kcal/day. Among the diets with more substantial evidence, patients with MAFLD/NAFLD should follow the Mediterranean Diet, minimising the intake of saturated fatty acids and red and processed meats and eliminating consuming commercial products containing fructose[34].

However, how much weight loss should be targeted in NAFLD therapy? Vilar-Gomez E. et al., conducting a prospective study on nearly 300 patients with histological diagnosis of NASH, demonstrated that a weight loss of ≥5%, ≥7%, and ≥10% maintained over time (52 weeks) leads to the improvement of hepatic steatosis, resolution of NASH, and regression of hepatic fibrosis, respectively[24, 35]. However, despite the use of best practices in our centre, only 28.1% of patients manage to lose at least 5% of their weight, and just over 13% achieve a weight loss of at least 10% of the initial weight at 12 months[36].

Given the significant weight loss in obese patients, VLCKD could also positively impact MAFLD/NAFLD due to its shallow carbohydrate content. Although it is not known whether ketosis itself plays an additional role, various mechanisms could be directly connected to ketosis, leading to a direct improvement in NAFLD[23]. Currently, few studies considering the role of VLCKD in the context of MAFLD/NAFLD have ever evaluated hepatic histological changes after this dietary approach. The available data show improved surrogate parameters in uncontrolled studies[37].

In light of such limited evidence regarding the role of VLCKD diet therapy in improving histological parameters in patients with MAFLD/NAFLD, we aimed to assess the role of the VLCKD diet in patients with MAFLD/NAFLD complicated by significant hepatic fibrosis (F2-F3) and NASH compared to a standard hypocaloric dietary approach.

STUDY OBJECTIVES

The purpose of the KETONASH study is to evaluate, in patients with metabolic liver disease (MAFLD) presenting with non-alcoholic steatohepatitis (NASH) and significant hepatic fibrosis (F1-F2-F3), the effect of a very-low-calorie ketogenic diet (VLCKD) compared to that of a standard low-calorie diet (standard Mediterranean LCD) - in accordance with the most recent guidelines on MAFLD/NAFLD[2, 6, 30, 34].
The primary objectives of the study are to assess patients with MAFLD/NAFLD/MAFLD treated with VLCKD compared to standard LCD:
1. Improvement in histological terms of hepatic fibrosis without worsening of NASH.
2. Improvement in NASH without worsening of hepatic fibrosis.

The secondary objectives include the evaluation of changes in histological, biochemical, and imaging parameters in patients treated with VLCKD compared to standard LCD, specifically assessing:
1. Individual histological components that comprise the NIH NASH CRN Score and/or FLIP SAF score (steatosis, ballooning, lobular inflammation, fibrosis, etc.).
2. Liver biochemical tests (transaminases, GGT, alkaline phosphatase, total proteins, albumin, etc.).
3. Composite liver biochemical biomarkers such as Fibrosis-4 (FIB-4), MAFLD/NAFLD fibrosis score (NFS), Fatty Liver Index (FLI), and FAST Score.
4. Physical liver fibrosis biomarkers through Transient Elastography (Fibroscan, Echosens, France).
5. Physical biomarkers of hepatic steatosis evaluated by
   a) qualitative method (mild, moderate, severe) hepatic ultrasound.
   b) quantitative method using Controlled Attenuation Parameter (CAP, Echosens, France).

6. Anthropometric parameters (BMI, circumferences), strength (handgrip), and body composition (bioimpedance and skinfold thickness).
7. Questionnaires on quality of life (Health-related quality of life, HRQoL), lifestyle (physical exercise/sedentary habits), and liver disease-related events (NASH-CHECK, CLDQ).
8. Tolerance, occurrence of side effects, and compliance of patients undergoing diet therapy with VLCKD.

STUDY POPULATION

The study population consists of patients referred to the Coordinating Center (including satellite centers) with a current or past diagnosis of metabolic liver disease (MAFLD/NAFLD) formulated in accordance with the most recent guidelines on MAFLD/NAFLD [2, 6, 30, 34]. The inclusion and exclusion criteria align with the most recent trials [38–40] in patients with MAFLD/NASH and international expert recommendations [41, 42].

Inclusion criteria:

- Patients aged ≥18 years with histological diagnosis of MAFLD/NAFLD/NASH with evidence of stage 1, 2, or stage 3 fibrosis (F1/F2/F3) (defined according to the NASH Clinical Research Network - NASH CRN criteria [1]) obtained no more than 6 months before enrollment.
- Stable weight for over six months with a BMI between 30-40 kg/m2.
- Patients in whom it is safe and functional to proceed with liver biopsy and who consent to undergo liver biopsy after 12 months of enrollment to assess the effect of diet therapy.
- Informed consent obtained.

Exclusion criteria:

- BMI <30 or BMI >40.
- Presence of evolved chronic liver disease into cirrhosis (histological F4 or elastometric LSM >14 kPa).
- Type 1 diabetes mellitus.

- Model for End-stage Liver Disease (MELD) score >12, AST or ALT ≥5× ULN, HbA1c >9.5%, INR≥1.4, creatinine >1.5 mg/dl, platelets <100,000/mm3, and total bilirubin >1.5 mg/dl.
- Concomitance of any other known chronic liver disease beyond MAFLD/NAFLD, such as alcoholic liver disease, viral (HCV/HBV), cholestatic-autoimmune (PBC/PSC/AIH), Wilson's disease, hemochromatosis, alpha-1-antitrypsin deficiency, drug-induced liver injury (DILI), or the presence or suspicion of hepatocellular carcinoma (HCC).
- Average alcohol consumption exceeding 4/2 units/day (males/females) in the 6 months preceding and a history of excessive alcohol consumption in the last 5 years.
- Previous or planned liver transplant, bariatric surgery, ileal resection, or biliary diversion.
- History of acute cholecystitis and biliary obstructions (cholangitis).
- Recent (within the last 12 months) or concurrent use of agents known to cause hepatic steatosis (long-term systemic corticosteroids [>10 days], amiodarone, methotrexate, tamoxifen, tetracyclines, high-dose estrogens, valproic acid).
- Recent (within the last 3 months) change in dose/regimen or introduction of Vitamin E (at doses ≥400 IU/day), ursodeoxycholic acid (UDCA), betaine, s-adenosyl methionine, silymarin, or pentoxifylline.
- Presence of psychiatric disorders and/or a diagnosis of any eating disorder.
- Life expectancy <6 months.

Withdrawal criteria from the study:

Subjects previously enrolled can withdraw at any time upon their request or at the request of their legal representative. The principal investigator may withdraw a subject from the study at any time for the following reasons:
- Serious adverse events related to the study intervention.
- Presence or appearance of exclusion criteria.
- Development of pathological conditions that make it impossible to continue the study.
- Non-compliance of the participant with study visits and/or procedures.
- A serious protocol violation determined by the PI or investigators.

STUDY DESIGN

The KETONASH study is a multicenter, open-label, randomised, controlled clinical trial that will be consecutively proposed to all patients with histological diagnosis of non-alcoholic

steatohepatitis (NASH) and hepatic fibrosis (F1-F2-F3) in the context of chronic metabolic liver disease (MAFLD/NAFLD).

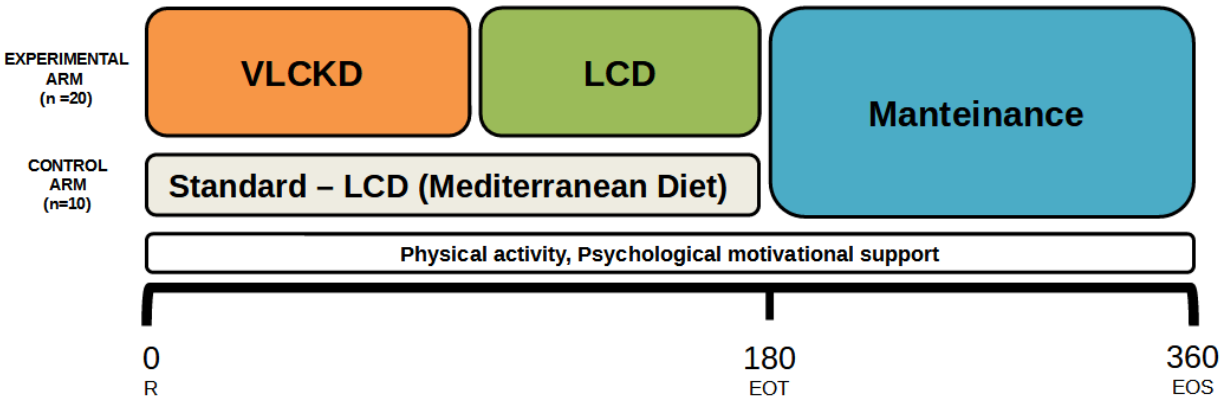
Once the inclusion criteria are confirmed and the exclusion criteria are ruled out, patients will be subsequently randomly assigned (randomisation) with a 2:1 ratio to one of the two study arms:

- VLCKD Study Arm → will receive experimental diet therapy with very low-calorie ketogenic meals (VLCKD) consisting of 5 successive phases (600 – 1500 kcal/day).
- LCD Control Arm → will receive standard low-calorie diet therapy, a Mediterranean-type diet in accordance with the most recent guidelines [2, 6, 30, 34] on MAFLD/NAFLD (1200-1500 kcal/day).

The KETONASH study consists of an initial 4-month diet intervention phase (Visits 1-8), followed by a second 8-month weight maintenance phase (Visits 9-15). This structure, particularly in the intervention arm, has been validated in previously published studies [43–46]. In both study arms, the intervention will be conducted through a standardised multidisciplinary approach (Physician/Dietitian/Nurse/Psychologist) aimed at weight loss through changes in dietary regimen, exercise program, and emotional support techniques. The two study arms differ in nutritional composition, types of foods, and caloric intake.

IDENTIFICATION, SCREENING, AND RANDOMIZATION (Visit 1)

Potential participants will be identified during routine outpatient visits or scheduled hospital appointments at the study centre. Eligible participants will be invited to join the study by their specialist or a member of the clinical and/or research team, who will also explain the study. A specially created information sheet detailing the study will be provided, and the patient will have sufficient time to review it and make their considerations. If new scientific evidence that could affect a subject's decision to continue participating in the study becomes available, it will be discussed with the enrolled patient.



A screening register will be established to retain details of patients invited to participate in the study. This is in accordance with CONSORT recommendations to ensure that available data are not influenced by unexpected biases from those who refuse to participate in the study. For subjects who decline participation, the register will keep track of each reason and also ensure that potential participants are involved only once. The screening register will store pseudonymised data containing the presence/absence of inclusion/exclusion criteria for the study.

After assessing the patient's eligibility for the study, using a dedicated application on the REDCap UNIBO platform and stratifying the patient by the presence of type 2 diabetes and gender, the patient will be randomised into one of the two study arms. The following assessments will be collected and recorded on the study's electronic case report form (eCRF):

- Age
- Average alcohol intake (units/week) in the last six months
- Alcohol intake – history of abuse?
- Smoking status – Yes/No/Ex
- Tea/Coffee consumption – cups/day
- Co-morbidities:
- Hypertension, dyslipidemia, type 2 diabetes
- Obstructive sleep apnea
- Malignant neoplasms
- Cardiovascular diseases/Stroke

- Other relevant diseases
- Recent/ongoing therapy (including over-the-counter medications, herbs, homoeopathic remedies)
- Results of recent haematological/biochemical/immunological/virological analyses such as:
- Haematological (complete blood count, coagulation)
- Clinical biochemistry (urea and electrolytes, liver function [Alb, Bili, ALP, ALT, AST, gGT], Ferritin/transferrin saturation, A1AT, copper/ceruloplasmin, glucose, HbA1C, Insulin, C-peptide, TSH, lipid profile, etc.)
- Autoimmunity screening, Immunoglobulins (IgG, IgA, IgM)
- Viral serology (HBV, HCV)
- Liver biopsy outcomes reported within six months
- Electrocardiogram (ECG)
- Report of abdominal ultrasound evaluation

DIETARY INTERVENTION (Visits 2-8)

VLCKD Study Arm:

The VLCKD dietary intervention consists of five successive phases involving the consumption of meal replacements and nutritional supplementation (vitamins, trace elements, and omega-3 fatty acids), organised as follows:

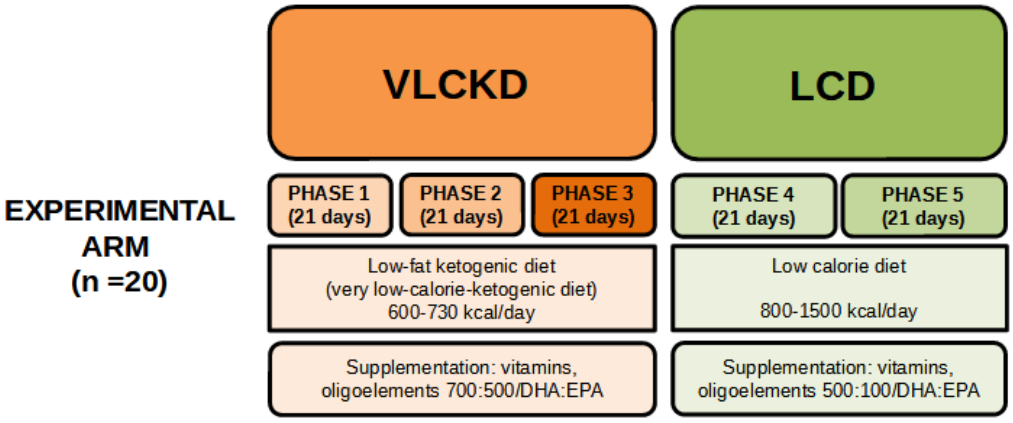
Ketogenic Low-Calorie Period (2 months):

- Phase 1 (30 days – Visits 1-3): Ketogenic diet with low-fat content, 600 kcal/day (with low glycemic index products and vegetables).
- Phase 2 (15 days – Visit 5): Ketogenic diet with low-fat content, 660 kcal/day (with four products as a high-protein food source [meat or fish] and vegetables with a low glycemic index).
- Phase 3 (15 days – Visit 5): Ketogenic diet with low-fat content, 730 kcal/day (with two products as high-protein food sources [meat or fish] and vegetables with a low glycemic index).

Low-Calorie Period (2 months):

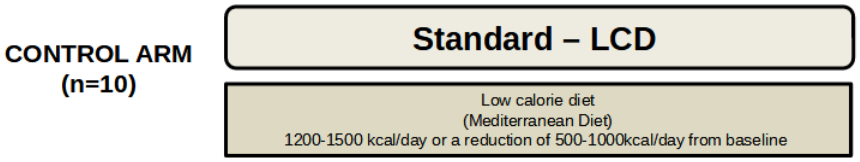
- Phase 4 (30 days – Visits 6-7): Hypocaloric diet with the reintroduction of different foods, 1,050 kcal/day (with products).
- Phase 5 (30 days – Visits 7-8): Hypocaloric diet with the reintroduction of different foods, 1,400 kcal/day (with products).

During these phases, the patient will receive nutritional supplementation with vitamins, trace elements, and omega-3 fatty acids with a DHA: EPA ratio of 700:500 during phases 1-3 and 500:100 during phases 4 and 5. Throughout the very low-calorie ketogenic period, the patient will have three interim dietetic consultations (Visits 2-4) and a medical visit at the end of the ketosis period (Visit 5). During the low-calorie period, the patient will receive alternating two dietetic consultations (Visits 6 and 7) and one medical visit every 30 days (Visit 8).



Control LCD Study Arm:

The control LCD arm consists of a diet with natural low-calorie foods (1200-1500 kcal/day or a reduction of 500-1000 kcal/day compared to baseline) and a low glycemic index based on the "Mediterranean Diet" model, following the most recent guidelines on MAFLD/NAFLD [2, 6, 30, 34]. Similar to the VLCKD arm, for the entire duration of the dietetic treatment, the patient will alternately receive dietetic consultations and medical visits.



Maintenance Phase (Visits 9-13):

At the end of the dietary intervention, patients from both study arms will continue with a controlled, low glycemic index diet tailored to the patient's basal metabolic rate (BMR) (estimated with bioimpedance assessment) for an additional six months. According to the current clinical practice of the study centre, all patients will receive alternating dietetic consultations and/or medical visits every 30 days (Visits 9-15). Depending on patient availability, some visits/consultations during the maintenance phase will occur via teleconsultation.

End of Study (Visit 15):

At the end of the 12-month study, the patient will undergo an evaluation of the primary and secondary study objectives. Following the end of the study, the patient, based on the achieved BMI and existing comorbidities, will resume the diagnostic and care pathway planned by the Investigator Center in accordance with current clinical practice.

Physical Activity:

Throughout the study, regardless of the patient's randomisation arm, the physician will prescribe structured and standardised physical exercises specifically tailored to the patient's abilities and physical conditions, progressively increasing in frequency and intensity, following the latest evidence on physical activity in patients with MAFLD/NAFLD [34, 47]. During the dietary intervention phases, the prescribed physical activity will include muscle toning exercises (resistance); conversely, during the maintenance period, the patient will alternate muscle toning exercises with cardiovascular training.

Psychological-Motivational Support:

Throughout the study, regardless of the patient's randomisation arm, the multidisciplinary team, including a clinical psychologist and a pedagogue with specific motivational coaching skills, will support the study participants. They will utilise techniques currently used at the centre for psycho-emotional support and provide the patient with a set of easily applicable tools to help make lasting lifestyle changes, following the experience of the Investigator Center [48] and the latest evidence in MAFLD/NAFLD patients [47].

STUDY PROCEDURES

Medical Visits and Dietetic Consultations:

Throughout the study, there will be a total of 15 alternating visits between medical assessments and dietetic consultations. These evaluations will occur at variable intervals during different study phases and can be conducted in person or via teleconsultation (phone/online).

During the in-person visits, an anthropometric assessment will be conducted, and in particular, the following variables will be evaluated:

Anthropometric Assessment:

- Height (cm)
- Current weight (kg)
- BMI (m/kg^2)
- Waist circumference (cm)
- Hip circumference (cm)
- Neck circumference (cm)
- Arm circumference (MAC)
- Hand grip test
- Skinfold thickness measurements (triceps, suprailiac, abdominal, anterior thigh)
- Blood pressure
- Heart rate

These assessments will follow the National Health and Nutrition Examination Survey (NHANES) 2017-2018 guidelines.

The visits V1, V5, V8, V12, and V15 will be of medical relevance; consequently, the others will be of a dietary nature.

During these visits, patients will complete translated transcultural versions of the following validated questionnaires:

- Chronic Liver Disease Questionnaire for MAFLD/NAFLD/NASH, CLDQ-MAFLD/NAFLD-NASH (HRQQL)
- Food Frequency Questionnaire (FFQ)
- Mediterranean Diet Score (MDS)
- International Physical Activity Questionnaire (IPAQ)

- SIO-Obesity correlated Disability Test (TSD-OC)
- STOP-Bang questionnaire
- Binge Eating Scale (BES)
- Beck Depression Inventory (BDI)
- State-Trait Anxiety Inventory Forma Y (STAY-Y)

Body Composition Assessment via Bioimpedance (BIA):

At visits V1, V5, V8, and V15, body composition will be evaluated using Bioelectrical Impedance Vector Analysis (BIVA) following European Society for Clinical Nutrition and Metabolism (ESPEN) guidelines. Bioelectrical impedance analysis (BIA) is one of the most useful techniques for assessing body composition because of its simplicity, quick execution, non-invasiveness, and cost-effectiveness, and requires minimal participation from the subject. Vectorial BIA (BIVA) is based on the principle that biological tissues behave as conductors, semiconductors, or dielectrics. It involves administering an alternating current at a fixed frequency of 50Hz through the skin, using two surface electrodes as injectors. A second pair of electrodes (sensors) are tasked with recording the body's resistance to the applied current. For the KETONASH study, a BIA 101 bioimpedance analyser (Akern s.r.l., Florence, Italy) will be used. By measuring Resistance (Rz, representing the ability of all biological structures to resist the passage of electrical current) and Reactance (Xc, also known as capacitive resistance - the force opposing the passage of an electric current due to capacitance, i.e., a capacitor), the following estimated parameters will be obtained:

- Total Body Water (TBW)
- Extracellular Water (ECW)
- Intracellular Water (ICW)
- Body Cell Mass (BCM)
- Fat-Free Mass (FFM)
- Fat Mass (FM)
- Muscle Mass (MM)
- Basal Metabolism related to cell mass

Liver Biopsy:

The transcutaneous liver biopsy with a needle (using, for example, a Trucut or Menghini needle) will be performed as standard clinical practice and under ultrasound guidance [62, 63] for the diagnostic purposes of non-alcoholic steatohepatitis (NASH) and repeated solely for study purposes to assess the effectiveness of the intervention after 12 months from randomisation. For an accurate histological evaluation/diagnosis, it will be necessary to obtain tissue of a minimum length of 1-1.5 cm. If the total length of the biopsy is less than indicated, the operator may decide to take a second sample to ensure there is sufficient material for diagnostic purposes. Given the nature of the procedure, commonly, a tissue longer than necessary for diagnostic purposes is collected at the time of the biopsy. The excess tissue beyond the length necessary for diagnostic purposes of 1-1.5 cm will be considered surplus for diagnostic requirements.

Before performing the liver biopsy, the patient will be explained the indications for the biopsy, and it will be emphasised that the risks to the patient do not outweigh the potential benefits. The procedure will be performed after checking the platelet count and coagulation indices according to the standards and procedures of the IRCCS Azienda Ospedaliera Universitaria Policlinico di Sant'Orsola, Bologna.

Samples that meet diagnostic requirements can be collected at the time of the procedure for research purposes. The samples will be processed for histological evaluation and/or frozen in liquid nitrogen (or fixed in a suitable preservative) for subsequent analysis and stored at -80°C. After histological analysis, the excess tissue, fixed in formalin and mounted in wax blocks, is kept indefinitely in the archive.

Histological evaluations will be centralised and carried out by a single pathologist experienced in liver diseases, following the NASH CRN criteria[1] and blinded to the patient's clinical, laboratory, and imaging characteristics, as well as randomisation for study purposes.

Abdominal ultrasound with Transient Elastography (FibroScan®) and Controlled Attenuation Parameter (CAP™)

In accordance with clinical practice and the European Federation of Societies for Ultrasound in Medicine and Biology (EFSUMB) guidelines [65, 66], the study-specific abdominal ultrasound will be performed concurrently during visits V1, V8, and V15 under fasting conditions of at least 12 hours. Specifically, the following variables will be assessed and recorded:

- Hepatomegaly (yes, no)
- Echostructure (homogeneous, bright, granular, nodular)
- Surface (regular, irregular, nodular)
- Hepatic steatosis (absent, mild/Grade I, moderate/Grade II, severe/Grade III)
- Gallbladder (alithiasic, lithiasis, ectopic)
- Focal lesions (type)
- Vascular diameters (PV, SV, UMV)
- Portal flow (hepatopetal/hepatofugal)
- Portal vein velocity
- Suprahepatic vein flow (triphasic, biphasic, monophasic, reversed)
- Splenic diameter/area
- Presence of collateral circulation
- Free abdominal fluid (mild, moderate, severe)

Simultaneously, measurement of liver elasticity will be performed using the Transient Elastography method with FibroScan® (Echosens, Paris, France), and quantification of hepatic fat using Controlled Attenuation Parameter (CAP™).

The physical principle, known as the "Young's Modulus," on which these indirect techniques for quantifying liver fibrosis and steatosis are based, is the ability of a mechanical wave to propagate at different speeds through a propagation medium, depending on the elasticity of the medium itself.

The probe of the ultrasound device used for elastography is placed on the skin at the anatomical location of the liver. It consists of a small transducer that produces a mechanical wave of constant frequency, amplitude, and shape, emitting a minimal amount of energy. The propagation speed of the wave in the medium, which in this case is the liver parenchyma, is studied by emitting ultrasound from the probe itself. The acoustic energy required to follow the propagation of the mechanical wave is also very low and not harmful. Data related to the propagation of the wave in the liver parenchyma are analysed by the software included in the device through sophisticated algorithms, leading to the determination of the liver elasticity value. The execution procedures of elastometric methods using FibroScan® will be in accordance with the operators' experience [67, 68], the manufacturer's recommendations, and the EFSUMB European guidelines [65, 66].

Laboratory Tests and Study-Specific Biomarkers

During visits V1, V5, V8, and V15, the patient will undergo a blood draw and chemical-physical analysis of urine, where the following tests will be assessed:

Procedure	VLCKD		LCD	Maintenance
	Standard – LCD (Mediterranean Diet)			
Phase	B	F2	EOT	EOS
Week	0	4-6	16-18	40-44
Visit	V1	V4	V8	V15
BLOOD COUNT	X	X	X	X
PROTHROMBIN TIME (PT)	X		X	X
CREATININE	X	X	X	X
SODIUM	X	X	X	X
POTASSIUM	X	X	X	X
TOTAL CALCIUM	X	X	X	X
CHLORIDE	X	X	X	X
TOTAL MAGNESIUM	X	X	X	X
PHOSPHORUS	X	X	X	X
TOTAL AND FRACTIONATED BILIRUBIN	X	X	X	X
TOTAL PROTEINS	X		X	X
ALBUMIN	X	X	X	X
ALANINE AMINOTRANSFERASE (ALT) (GPT)	X	X	X	X
ASPARTATE AMINOTRANSFERASE (AST) (GOT)	X	X	X	X
GAMMA GLUTAMYL TRANSPEPTIDASE (gamma GT)	X	X	X	X
ALKALINE PHOSPHATASE	X		X	X
GLUCOSE	X	X	X	X
GLYCATED HEMOGLOBIN (Hb)	X		X	X
INSULIN	X	X	X	X
URATE	X		X	X
UREA	X	X	X	X
HDL CHOLESTEROL	X		X	X
LDL CHOLESTEROL	X		X	X
TOTAL CHOLESTEROL	X		X	X
TRIGLYCERIDES	X	X	X	X
C REACTIVE PROTEIN	X		X	X
URINE CHEMICAL, PHYSICAL AND MICROSCOPIC EXAMINATION	X	X	X	X

Concomitantly, the following biomarkers based on laboratory, elastometric, and clinical data of the patient will be evaluated for the risk stratification of liver fibrosis (FIB4 & NFS), liver steatosis (FLI), and NASH with significant fibrosis (FAST SCORE - Fibrotic-NASH) [64, 69]. The formulas for the mentioned scores are summarised in the table.

The table below summarises the various study procedures in different phases of the protocol.

Procedure	VLCKD				LCD				Manteinance						
	Standard – LCD (Dieta Mediterranea)														
Phase	B	F1	F1	F2	F3	F4	F5	EOT							EOS
Week	0	0-2	2-4	4-6	6-8	8-12	12-16	16-18	18-22	22-28	28-32	32-26	36-40	40-44	48
Visit	V1	V2	V3	V4	V5	V6	V7	V8	V9*	V10	V11*	V12	V13*	V14	V15
Randomisation	X														
Ketonemia		X													
Questionnaires	X				X			X							X
Liver biopsy															X
Laboratory exams	X*				X			X							X
BMI	X				X			X							X
Abdominal ultrasound + LSM&CAP	X							X							X
Note	*via teleconsultation (phone/online) Red: medical assessments; Blu: dietetic consultations														

MEASUREMENT OF STUDY OBJECTIVES

The measurement of primary objectives will occur at the end of the study (EOS - Visit 15) 12 months after enrollment and will be based on the evaluation of liver biopsy using NASH CRN criteria[1]. Specifically, the following will be assessed:

- Reduction ≥1 stage of hepatic fibrosis without an increase in hepatocellular ballooning, lobular inflammation, or steatosis compared to baseline biopsy.
- Improvement in NASH through histopathological assessment defined as a) "absence of fatty liver," or b) "simple or isolated hepatic steatosis in the absence of steatohepatitis" and NAS 0 for ballooning and 0-1 for inflammation without an increase in fibrosis stage compared to baseline biopsy.

The measurement of secondary objectives will take place at the end of treatment (EOT - Visit 8) and at the end of the study (EOS - Visit 15) 12 months after enrollment, through the comparison of biochemical, biomarker, and imaging parameters compared to baseline evaluation.

Expected Results

At the end of the study, it is expected that the group of patients who underwent VLCKD intervention will show an improvement of at least one stage of hepatic fibrosis with improvement in NASH compared to the group of patients treated with LCD. Additionally, alongside weight reduction and improvement in anthropometric measurements, a simultaneous improvement in other histological, biochemical, and biomarker parameters and an improvement in fibrosis and steatosis grade through non-invasive imaging evaluation (TE, CAP, RM-PDFF) is expected, especially in the VLCKD study arm. Patients treated with VLCKD will demonstrate a better quality of life at the end of the study compared to the control group. Finally, greater tolerance and compliance are expected in the VLCKD study arm.

Study Duration

Scheduled start date of the study (enrollment of the first patient): 01/05/2021

Protocol-specified last date for the end of enrollment: 31/12/2024

Data access will, however, be possible for ten years from the conclusion of the study.

STATISTICAL ANALYSIS PLAN

Sample Size Determination

The study is designed to demonstrate that the effect of VLCKD is superior to LCD after 12 months from randomisation. It has been calculated that, to have a 90% probability of finding, with a significance level of 0.05%, a difference between nutritional treatments of 1 stage of fibrosis (assessed by liver biopsy) with a statistical power of 80% and an effect size (Cohen's d) equal to 80%, a total of 42 patients need to be randomised, with a 2:1 ratio in the two study arms (VLCKD vs LCD).

Statistical Analysis

Parametric variables will be compared using the Student's T-test, while non-parametric categorical variables will be analysed using Fisher's test, Chi-square; continuous non-parametric variables will be analysed using the Mann-Whitney test. Continuous variables will be reported as median ± interquartile range (IQR), while categorical variables as numbers and percentages; predictors of events occurring in a specific time interval will be tested using logistic regression or Cox methods. Subsequently, to confirm their independent predictive value, variables with $p < 0.1$ will be studied in a multivariate logistic

or Cox model; comparison groups will be studied using Student's t-test and the Chi-square test. Associations will be considered statistically significant if they present a p-value < 0.05. The incidence of events will be calculated using the Kaplan-Meier method and compared through the log-rank test. The aforementioned analyses will be performed using STATA15 or another statistical analysis software.

Interim Analysis

There is no planned interim analysis of primary objectives.

DATA COLLECTION

Electronic Data Capture Form (eCRF)

Structured data collection necessary for evaluating objectives will occur through an electronic data capture form. Designated personnel will collect information obtained from clinical records, outpatient visits, and instrumental diagnostics within the normal care pathway. The collected data will be recorded with the help of the REDCap UNIBO platform. Data processing will be carried out through procedures for collection, recording, storage, and processing using electronic tools suitable for processing them according to the law and in compliance with security and confidentiality standards, respecting applicable regulations.

Data Management and Data Quality

The study sponsor and the experimentation centre are autonomous data controllers, each for their competence. Data will be collected through the REDCap UNIBO platform. Access to the REDCap platform occurs via the https protocol, ensuring a secure connection protected by SSL algorithms and a certificate guaranteeing encrypted data transmission.

Access to the archive on the REDCap platform will be granted only to registered users (researchers belonging to the KETONASH study research group) who will access it through their credentials (self-chosen username and password): each researcher user will be able to view, and modify, and update the data of enrolled patients from the experimenting centre.

Server-level management of the REDCap platform, such as installation, periodic monitoring of hardware resources, resolution of any blocking events, and issues related to direct server access, is guaranteed by CESIA - AREA SISTEMI E SERVIZI INFORMATICI,

University of Bologna. Access to the hardware and software resources of the REDCap platform is regulated by the Personal Data Protection Authority regulations.

Regarding administrative operations at the application level, such as creating and configuring the study and creating users authorised to participate in the study, these are the responsibilities of the sponsor.

To protect the identity of individuals involved in the study, the REDCap platform generates a unique identification code for each subject associated with their data collection forms, allowing each centre to maintain the association with the patient's personal data. Additionally, the REDCap platform requires an external list outside the application containing the association of these codes with the nominative data of patients, kept exclusively at the experimentation centre as a confidential document, carefully preserved, and concerning only their patients.

The patient data required for the conduct of this research study, while not directly or indirectly identifying the patient, are recorded within the REDCap database with asymmetric SSL double-key encryption combined with AES 256 encryption.

As outlined in the Personal Data Protection Authority regulations "Code on the protection of personal data," the REDCap platform provider guarantees the execution of data saving with a weekly frequency on archive files protected by passwords.

The main experimenter and their collaborators, who will be bound by an obligation of confidentiality for this data, will have access to the data in pseudonymised form in Excel or another data processing format (exported from the REDCap platform after the end of active enrollment). However, access to pseudonymised data will be possible for ten years beyond the final enrollment deadline for patients.

The data resulting from the analysis will be processed with statistical methods in pseudonymised form to obtain information constituting the purpose of the study. The study results may be material for scientific publication, where the data will be presented in an equally anonymous form.

STUDY COSTS

The KETONASH study is a non-profit study aimed at improving clinical practice according to Article 1 of the Ministerial Decree of 17.12.2004. The ketogenic dietary regimen with substitute foods will be provided at no additional cost by companies producing commercial VLCKD products.

REGULATORY AND ETHICAL ASPECTS

Statements of Compliance

The conduct of this study will be in accordance with the recommendations for physicians involved in research on human subjects adopted by the 18th World Medical Assembly in Helsinki in 1964 and its subsequent revisions. The process of obtaining informed consent at the recruiting centres involved is conducted according to the Declaration of the World Medical Association of Helsinki 2000, respecting the Charter of Fundamental Rights of the European Union (2000/C 364/01) and the Belmont Report. Before commencing the study, favourable ethical opinions will be obtained from an appropriate Ethics Committee. Information sheets and written informed consent will be provided to all eligible subjects.

Ethics Committee

The study protocol, any protocol amendments, informed consent, and any other information for patients must be approved by the relevant Ethics Committee. Any protocol amendments can be immediately implemented by the Principal Investigator, in agreement with the Study Scientific Committee, without waiting for approval if patient safety is at stake. If, for safety reasons, an immediate protocol modification is deemed necessary by the Principal Investigator in agreement with the Study Scientific Committee, the Ethics Committee must be informed within ten working days.

Informed Consent Management

For enrolled patients, Informed Consent and adherence to the study will be obtained during a visit as part of the normal care pathway at the centre. Informed consent discussions will be conducted by appropriate personnel involved in the study (as indicated in the delegation log) at the reference centre, including medical and research nursing staff, with the opportunity for participants to ask any questions. Once provided with information about the study, candidates will be given adequate time to decide on their participation. Those wishing to participate will provide a signed and dated written informed consent, which will be counter-signed and dated by a research team member with documented responsibility for this task. Written informed consent will always be required before any study-related procedures. The right to refuse participation without providing any explanation will be respected. At the time of consent or any subsequent time, each individual may decide to discontinue their participation without any consequences. Non-

participation in this study will not prejudice the patient's therapeutic course. The original signed copy of the informed consent will be kept in the patient's medical record, a copy will be kept in the investigator's study file, and a copy will be provided to the patient.

The data resulting from the analysis will be processed using statistical methods in an anonymous form to extract the information constituting the purpose of the study. The Principal Investigator and their collaborators, bound by confidentiality obligations, will have access to the data. Access to the data will be possible for ten years after the completion of the study. The study results may be used for scientific publication, where the data will be presented in an equally anonymous form.

Confidentiality and Data Protection

The operations of collecting, recording, storing, and modifying personal data will take place through manual and computerised tools with logic strictly related to the above purposes. The data will be processed to ensure the confidentiality and security of information, per Articles 25 and 32 of the GDPR, only by the Principal Investigator or their collaborators.

Insurance

A liability insurance policy for trial-related risks and the ethics committee will be taken out, with a limit of €1,500,000 per event for the entire duration of the study.

Data Use and Results Publication

Information about the study and study data will be made available through publication on clinicaltrials.gov.

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