PROTOCOL TITLE:

'Acute Nutritional Ketosis in VLCAD Deficiency: testing the metabolic basis for therapeutic use'

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LIST OF ABBREVIATIONS AND RELEVANT DEFINITIONS

31 P MRS	**^{31}Phosphorus magnetic resonance spectroscopy**
$\tau HHb_{recovery}$	**Time constant of HHb during recovery from exercise**
ABR	**ABR form, General Assessment and Registration form, is the application form that is required for submission to the accredited Ethics Committee (In Dutch, ABR = Algemene Beoordeling en Registratie)**
AE	**Adverse Event**
AR	**Adverse Reaction**
ATP	**Adenosine triphosphate**
ATT	**Adipose tissue thickness**
CA	**Competent Authority**
CCMO	**Central Committee on Research Involving Human Subjects; in Dutch: Centrale Commissie Mensgebonden Onderzoek**
CK	**Creatinine Kinase**
CV	**Curriculum Vitae**
DPF	**Differential pathlength factor**
DSMB	**Data Safety Monitoring Board**
EU	**European Union**
EudraCT	**European drug regulatory affairs Clinical Trials**
FAO	**Fatty Acid Oxidation**
FEV_1	**Forced expiratory volume in one second**
GCP	**Good Clinical Practice**
HAES	**Habitual Activity Estimation Scale**
HMP	**Hexose monophosphate**
IB	**Investigator's Brochure**
IC	**Informed Consent**
IMCL	**Intra myocellular lipids**
IMP	**Investigational Medicinal Product**
IMPD	**Investigational Medicinal Product Dossier**
KO	**Knock Out**
METC	**Medical research ethics committee (MREC); in Dutch: medisch ethische toetsing commissie (METC)**

MRS	**Magnetic Resonance Spectroscopy**
NK	**Nutritional Ketosis**
Nm	**Nanometer**
O_2	**Oxygen**
PCr	**Phosphocreatine**
Pi	**Inorganic phosphorus**
PPE	**Physical performance enhancement**
(S)AE	**(Serious) Adverse Event**
SPC	**Summary of Product Characteristics (in Dutch: officiële productinfomatie IB1-tekst)**
Sponsor	**The sponsor is the party that commissions the organisation or performance of the research, for example a pharmaceutical company, academic hospital, scientific organisation or investigator. A party that provides funding for a study but does not commission it is not regarded as the sponsor, but referred to as a subsidising party.**
SUSAR	**Suspected Unexpected Serious Adverse Reaction**
VLCADD	**Very Long Chain Acyl-CoA Dehydrogenase Deficiency**
Wbp	**Personal Data Protection Act (in Dutch: Wet Bescherming Persoonsgevens)**
WMO	**Medical Research Involving Human Subjects Act (in Dutch: Wet Medisch-wetenschappelijk Onderzoek met Mensen**
WOB	**Work of breathing**

SUMMARY

Rationale: Very Long-Chain Acyl-CoA Dehydrogenase deficiency (VLCADD) is a rare inborn error of fatty acid metabolism with a broad clinical presentation ranging from infant fatality to exercise intolerance and elevated risk of exertional rhabdomyolysis with onset in childhood or adolescence. These episodes of rhabdomyolysis can result in permanent muscle damage and deterioration of activity level of adult patients. We recently obtained evidence that these symptoms are in part due to a lower energetic efficiency of upper leg muscle fibers that aggravates reliance on carbohydrate stores in this disease, rendering the organ vulnerable to an exertional energy crisis (Diekman et al, PloS One 2016). While no effective treatment has been available for VLCADD, it has long been proposed that nutritional ketosis (NK) could be highly beneficial to patients. Amongst others, ketone bodies could take on the role of primary energy source in exercising muscle. The problem has been that, until now, no vehicle for establishing NK in humans without undesired side effects has been available. A breakthrough has finally been achieved by collaborator Kieran Clarke in Oxford whose team has recently produced an edible ketone ester that can achieve acute NK in human subjects via oral ingestion without any undesired side-effects. It was found that the ketone ester produced significant physical performance enhancement in rodents and human athletes (Cox et al., *Science* (in review)).
The effect has been attributed to enhanced muscle mitochondrial function in addition to glycogen sparing. Here, we will investigate if acute NK in adolescent and adult symptomatic VLCAD deficient patients can boost muscle mitochondrial function in vivo. If so, a rational basis will have been established for therapeutic use in this metabolic myopathy. This study will include patients from the age of 16 years that could benefit from NK as well. Since rhabdomyolysis often has an onset in adolescence, confirmation of our hypothesis would lead to therapeutic use in that age group as well. Moreover, older patients often suffer from severe exercise intolerance and loss of muscle function due to earlier episodes of rhabdomyolysis, leaving most of them unable to complete our study protocol. Due to the rarity of the disease and earlier argumentation, it's vital to include adolescent subjects to reach sufficient power in this study. As such, this study constitutes a vital first step towards possible validation of an effective treatment for patients with VLCADD that may improve the quality of life including an active lifestyle with overall health benefits.

Objective: To test if acute NK boosts muscle mitochondrial function in vivo in patients with VLCADD in order to establish a rational basis for therapeutic use in this disorder. A secondary objective is to gather data to test the working hypothesis that the positive effect of acute NK on muscle mitochondrial function results from enhanced substrate supply to the tricyclic acid (TCA) cycle.

Study design: randomised, blinded, placebo controlled, 2-way cross over trial.

Study population: Six Patients with VLCADD between 16 and 65 years of age, seen for evaluation in the VLCADD expertise centre of the UMC Utrecht.

Intervention (if applicable): Oral intake of nutritional drinks; prolonged moderate-intensity exercise on bicycle ergometer; in vivo Magnetic Resonance Spectroscopy; muscle microbiopsy; venipuncture.

Main study parameters/endpoints:

- Whole body:
  Physical performance during a 45 bicycle exercise test at individual FATMAX workload (typically of the order of 40%MVO_2). Specifically:
  - completion of 40 min upright exercise bout at FATMAX workload (yes/no; if no, #minutes)
  - completion of 5 min in-magnet exercise bout at FATMAX workload (yes/no; if no, #minutes)
  - subjective fatigue and muscle ache score after each exercise bout (scale 0-10) and 24 hours after session (telephone consultation)
  - HAES score questionnaire
- Muscle:
  - resting quadriceps phosphocreatine (PCr) and inorganic phosphate concentrations; pH
  - exercise quadriceps phosphocreatine (PCr) and inorganic phosphate concentrations; pH
  - post-exercise rates of recovery of quadriceps PCr, Pi and pH
  - optional: resting and post-exercise muscle levels of glycogen, lactate, 3-OH butyrate, and (very) long (C18-C14) to medium (C12-C8) to short chain (C6-C2) acetyl-carnitines (on a patient-voluntary basis)
- Blood:
  levels of 3-OH butyrate, glucose, bloodgas, lactate, acylcarnitines and creatine kinase at rest
  and post-exercise
- Urine: myoglobine

Nature and extent of the burden and risks associated with participation, benefit and group relatedness: In clinical practice, many patients with VLCADD wonder to which extent they are able to exercise safely. Any means of boosting this capacity will greatly improve the quality of life and long-term health in this patient population. Acute nutritional ketosis after ingestion of a novel edible ketone ester is a very promising novel therapy towards this aim and has already been tested with positive effects in human athletes. This study will gain insight whether a sound metabolic basis exists for its therapeutic use in this patient population.

Patients will be asked to participate in three study sessions:
session I (site: nearest Academic Medical Center, Pediatric test ward).

- standard maximal cardiopulmonary exercise test (CPET; bicycle ergometry with indirect calorimetry)

  objective: determination of VO_{2max} in order to set the desired exercise workload of maximal individual rate of fat oxidation (FATMAX; typically ~40% MVO_2) in sessions 2 and 3.

- venipuncture
  objective: measure basal metabolic profile and rise in blood lactate and CK levels in response to CPET.

sessions II and III (site: Neuroimaging Magnetic Resonance Center UMC Groningen).

- venipuncture + cannulation

  objective: prepare subject for serial (every 10 min) blood sampling for off-site metabolic profiling

- oral intake of nutritional drink A or B (ketone and placebo drinks, respectively)
  Each subject will receive both drinks over the course of the trial in a blinded fashion – i.e., if drink A in session II, then drink B in session III, and vice versa.

- microbiopsy from the quadriceps muscle (optional)

  1st biopsy: 20 min after oral drink; 2nd biopsy: immediately following end of exercise bout 1.
  objective: metabolomic profiling of the state of the energy metabolic network in muscle and mapping of individual phenotypic muscle properties (fiber type, mitochondrial density, capillary density).Biopsies will be obtained from the m. vastus lateralis of the left leg. Hereto, a preparatory superficial incision of the skin and fascii will be made under local sedation and bandaged, allowing fast and reproducible biopsy sampling prior to and immediately following exercise bout 1 below.

- 40 min upright bicycling exercise at FATMAX outside MR scanner (exercise bout 1)

  Blood samples are collected every 10 minutes.

- microbiopsy from the quadriceps muscle (optional)

  Biopsy will be obtained from the m. vastus lateralis of the left leg (see above).

- 5 min supine bicycling exercise at 50%MVO2 inside MR scanner (exercise bout 2)

  *serial in vivo 31P MR spectra will be recorded non-invasively from the m. vastus lateralis of the right leg as previously (protocols **12-211/K** (VLCAD; UMC Utrecht)**; NL41313.042.12** (MCAD; UMG) to determine energy and proton balance in the muscle during exercise and the kinetics of metabolic recovery following exercise. A blood sample will be taken after the subject has exited the scanner room. Final blood samples including on-site analysis of CK levels are taken up to 3 hr post-session III to monitor any rhabdomyolytic event.*

- *24 hours after session II and III, co-investigator Bleeker will call each subject to evaluate subjective fatigue and muscle complaints after the subjet left the test facility.*

Time between study sessions I and II will be at least 7 days; time between study sessions II and III will be 7 days. For session I, patients will be asked to abstain from food 3 hrs prior to the test. For sessions II and III, participants will be asked to keep a 'food diary' for the week prior to the test in order to monitor the caloric intake and macronutrient content of each participant. For sessions II and III, patients will travel to Groningen the evening prior to the study and will be housed in a hotel across the street from UMCG. Patients are able to take a

late evening snack or meal of their choice in correspondence with their individual record of maximal fasting duration, given a start time of the study at 08:00 am the following day. Patients will be allowed the same light breakfast according their normal diet before they will receive the caloric drive before the test. The breakfast will be the same for session II and III.

No adverse effects of this study are expected. Of the listed interventions that are proposed here, all but one – i.e., ingestion of the ketone caloric drink - have previously been included and approved in clinical investigations of metabolic myopathy patients by members of the investigative team in Amsterdam, Utrecht and/or Groningen: venipuncture and microbiopsy of the quadriceps muscle prior to and after exercise (protocol 04/1984 (MCADD; AMC Amsterdam); protocol **NL41313.042.12 (MCADD; UMCG)**); bouts of prolonged bicycle exercise outside and inside a MR scanner including in vivo MR spectroscopic data collection (protocol **12-211/K (VLCADD; UMC Utrecht;** protocol **NL41313.042.12 (MCADD; UMCG)**).

Of the interventions, only the **burden** of collecting tissue samples from the quadriceps muscle by microbiopsy is rated as moderate (as opposed to ratings of nil to minor burden for all other interventions[1]). As such, this intervention will only be performed on a voluntary basis; it will not affect enrollment in the study. Muscle tissue sampling is, however, needed – and therefore desirable - to test the secondary hypothesis that NK boosts muscular mitochondrial function specifically at the level of substrate supply to the TCA cycle. Without the biopsy data, this secondary hypothesis cannot be validated. It would be highly desirable to know the outcome of that test since the information is needed to develop a proper mechanistic understanding of any therapeutic value of ketone administration in human metabolic myopathies such as fatty acid oxidation defects. On these grounds, the burden of microbiopsy is considered justifiable.

The **safety** of the nutritional drinks that will be administered and the expected acute, transient mild ketosis (concentrations of ketone bodies < 3 mM) has been thoroughly tested and documented in healthy subjects (see references 9,10). The drink has been designated as ‘GRAS’ (= Generally Recognized as Safe) by the Federal Drug Administration of the

[1] the short, maximum exercise test in session I will use anaerobic derived energy. As patients with VLCADD do not have a problem in glycolysis, the burden for this test will be nihil. The endurance test at submaximal level might induce muscle pain temporarily. the burden for patients is classified as minimal and patients will be monitored accordingly during the study period. Blood will be drawn intravenously from patients; this implies a minimal burden.

United States allowing it to be used as a foodstuff in the USA. No particular risk for VLCADD patients is expected with respect to oral ingestion of the ketone ester since its metabolites are natural, organic compounds released without any caution or proton load that are readily oxidizable by cellular mitochondria irrespective of enzymatic defects in fatty acid oxidation.

The **benefit** to VLCADD patients of participation in the study is potentially enormous. In our previous applications for exercise and MRS testing in metabolic myopathy (protocols **12-211/K (VLCAD;** UMC Utrecht) and **NL41313.042.12 (MCAD)**, respectively), we put forward that the benefit of those studies would be that it would help establish a platform to conduct studies on the effectiveness of medication. Here, we now exploit this validated platform to test the metabolic effects of a nutritional drink that may revolutionize therapy of human metabolic myopathies due to defects in carbohydrate or fat metabolism. Ultimately, an effective treatment for VLCADD may be available for the first time to improve the quality of life of patients, including the opportunity to engage in a more active lifestyle with generic (including cardiovascular and anti-diabetic) health benefits.

1. INTRODUCTION AND RATIONALE

Disorders of long-chain fatty acid beta-oxidation (FAO) comprise genetic deficiencies of fatty acid transport from the cytosol to the mitochondrial matrix and mitochondrial beta-oxidation of acyl-CoA compounds with a chain length of 14-20 carbon atoms. One of the most common long-chain fatty acid oxidation defects in humans is very long-chain acyl-CoA dehydrogenase deficiency VLCADD (OMIM 201475, EC 1.3.99.13; [1]). Impairment of energy production from exogenous or endogenous fatty acids is the most prominent feature of VLCADD [1]. Clinical symptoms, therefore, arise or are exacerbated during catabolic situations e.g. during exercise, illness or fasting when lipids are released from endogenous stores and have to be used as energy substrate. Organs most frequently involved are those using long-chain fatty acids as primary energy source, such as the heart and skeletal muscles. Age at onset, manifestation patterns and clinical severity differ between patients. The most severe presentations are Reye-like symptoms, cardiomyopathy and SIDS (sudden infant death syndrome) in the neonatal period. Milder clinical phenotypes manifest with hypoketotic hypoglycaemia during intercurrent illness and exercise-induced skeletal myopathy as well as episodic rhabdomyolysis. Patients may present with different symptoms at different ages.

Rhabdomyolyis

One of the most prominent symptoms of VLCADD patients is myopathy [1,2]. It most often involves the breakdown of muscle (rhabdomyolysis). Myopathy is provoked by fasting, fever, cold, medication and exercise from infancy to adulthood, but often has its onset in adolescence. In clinical practice, parents of pediatric patients and adult patients themselves wonder therefore to which extent they will be able to perform exercise. The pathophysiology of myopathy and rhabdomyolysis in general is not completely understood. The likely mechanisms behind the exercise-induced rhabdomyolysis and myopathy in VLCADD are a failing compensatory mechanism during requirement of extra energy (eg during exercise, fever, fasting). The symptoms are thought to result from one or more different pathophysiological mechanisms: (i) energy shortage in (exercising) muscle due to the FAO defect; (ii) toxicity caused by the accumulation of FAO intermediates. Reported muscle biopsies show a range from normal muscle fibre structure, to diffuse lipid accumulation and aspecific abnormalities. No ragged red fibres have been reported. Based on the recurrent episodes of rhabdomyolysis, myopathy in VLCADD might also be caused by meta-flammation: inflammation caused by abnormal metabolites. The accumulation of long chain acylcarnitine esters has been implicated in muscle pathology in VLCADD, particularly in the heart. But, there is also evidence that ischemic changes in heart

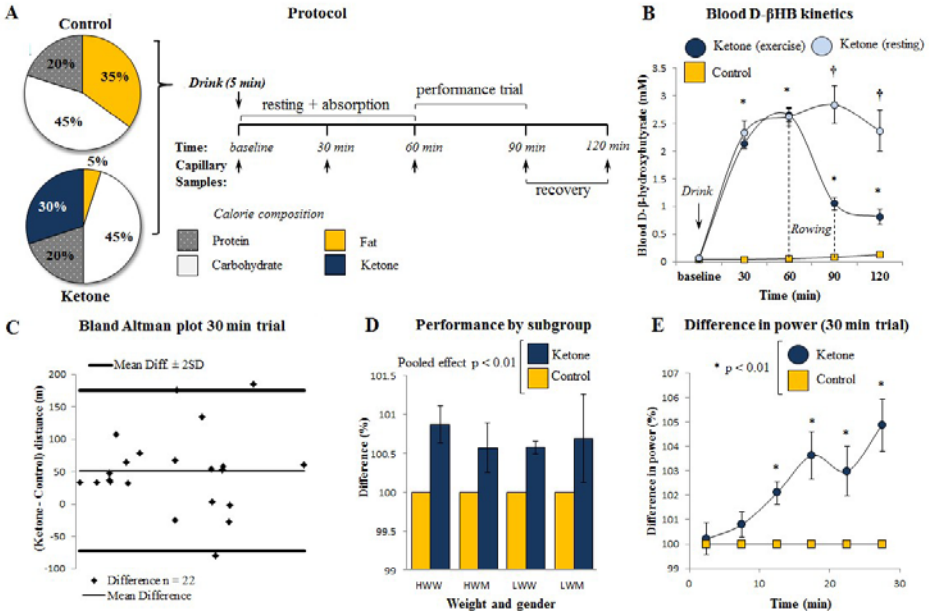
might be due to impaired energy production rather than from long chain acylcarnitines accumulation, emphasizing the importance of available free carnitine to recycle CoA.

Therapy in VLCADD.

Clinical management of VLCADD patients poses a major challenge. At present, there is no effective therapy to alleviate the debilitating condition of severe exercise intolerance and myalgia with risk of episodic rhabdomyolysis [3]. Patients are typically advised to engage only in light physical work in combination with timely and ample intake of carbohydrates and/or medium-chain triglycerides (MCT). It has, however, long been hypothesized that nutritional ketosis (NK) could be highly beneficial to patients with metabolic myopathies such as VLCADD [4]. Amongst others, ketone bodies could take on the role of primary energy source in exercising muscle. The problem has been, however, that, until now, no vehicle for establishing NK in humans without undesired side effects has been available [4].

Ketone bodies the evolutionary adaptation to power metabolism

Ketone bodies, acetoacetate and D-β-hydroxybutyrate, are the metabolic response to energy crisis, representing a powerful evolutionary mechanism to sustain life by altering oxidative fuel selection [5]. The teleological advantages of ketone body metabolism are clear during conditions where survival of the organism depends upon optimal mitochondrial efficiency, and conservation of fuel reserves Ketosis may also provide thermodynamic advantages over other carbon substrates by increasing the free energy conserved in ATP (ΔG_{ATP}) by the oxidation of ketones during mitochondrial oxidative phosphorylation [6]. The combination of improved energetic efficiency and fuel sparing is vitally important not only during famine, but could also provide clues to new methods of sustaining human performance. Recent work within the group of collaborator Kieran Clarke at University of Oxford on the effects of oral administration of ketone bodies during exercise has demonstrated a significant, 4% improvement in the power output of elite athletes over 30 minutes (seated rowing ergometer; Figure below; [7]). This degree of improvement in this population of athletes is of significant competitive advantage.



The effects of acute nutritional ketosis on physical performance in athletes.
(A). Protocol and nutritional composition of ketone and control drinks. (B). blood concentrations of ketone body over time showing increased ketosis after ingestion of ketone drink and oxidation of ketone from blood stream during exercise. (C). Bland-Altman plot of performance. (D) Uniform erformance improvement over all weight and gender classes of athlete. (E). Power profiles over 30 min of maximal exercise, with a 4% greater power output in the ketone arm at the end of exercise vs. control. (from [7]).

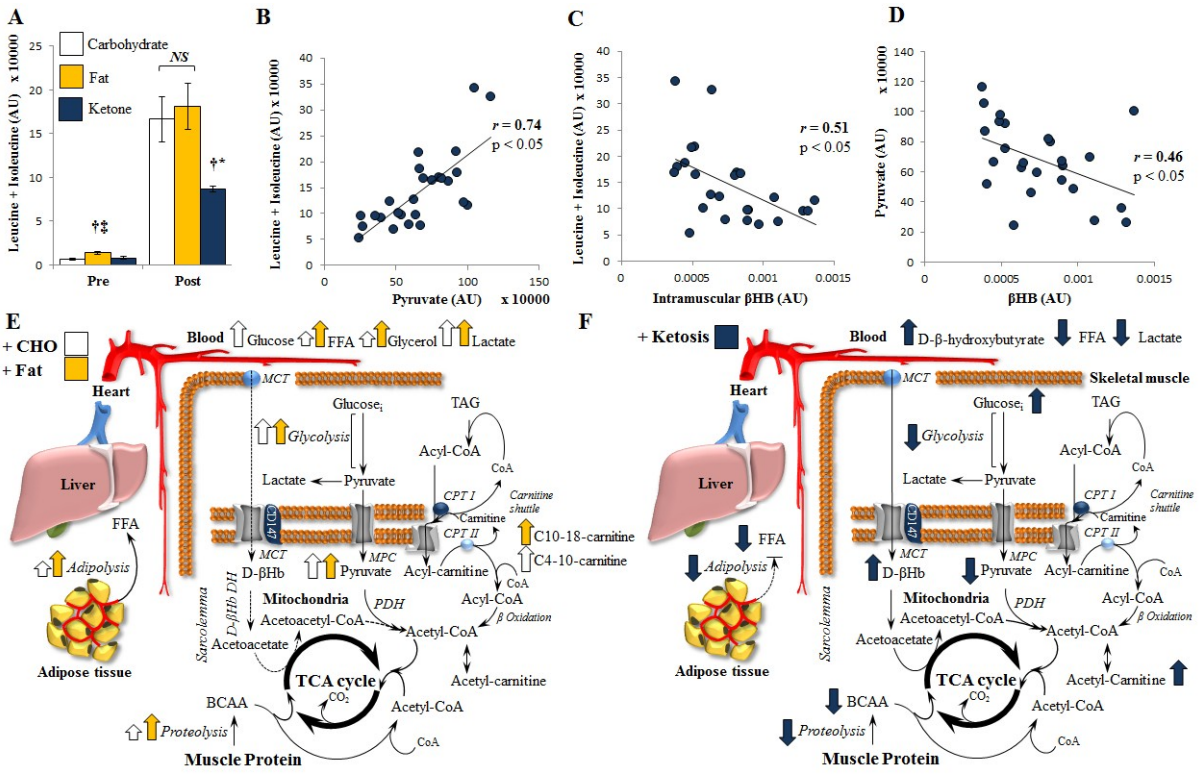
Harnessing ketosis for the preservation of muscle performance

In order to harness the physiological advantages of ketosis, a ketone ester was developed to deliver nutritional ketosis without elevations in fatty acids, or depletion of carbohydrate reserves (as is seen in starvation or endogenous ketosis) [8]. This exogenous form of ketosis enables the creation of a novel physiological condition with which to re-evaluate human performance, and metabolic regulation. Developed by TDeltaS Ltd. in association with the University of Oxford, this nutritional ketone body is not commercially available and to this point has been tested on over 200 people without adverse effects The safety, tolerability and toxicology profile of this ester has been published previously [9]. The drink has been designated as *GRAS* by the FDA allowing it to be used as a foodstuff in the USA. The ketone ester has previously been used in clinical research by Dr Pete Cox at the University of Oxford; LREC reference numbers O7/Q1605/37, 10/H0605/10, 11/SC/0223, 14/EE/0063, and 14/LO/0288 as well as in previous work conducted in partnership with the UK military MODREC 298/PPE/11. In accordance with University of Oxford policy, the Intellectual property rights to this ketone ester are linked to an investigator (prof dr Kieran Clarke) who has a declared interest.

Ketosis alters muscle fuel metabolism in working muscle

Strong supportive evidence for the importance of ketosis in altering working muscle metabolism dates back in excess of 60 years. Most notably the work of Randle in 1963, who demonstrated that the presence of ketone bodies inhibits glycolytic flux and increases the quantity of glycogen in both rat cardiac muscle and diaphragm [10] . The thermodynamic potential of ketosis was shown by Kashiwaya and colleagues, who demonstrated a dramatic 28% increase in hydraulic efficiency over glucose by the addition of ketones to the perfusate of a working heart model [11].

We have recently characterised the effects of nutritional ketosis on skeletal muscle metabolism, showing the clear evolutionary advantages of ketosis in sparing not only carbohydrate and fat metabolism, but in preserving muscle protein breakdown. The metabolic alterations induced by nutritional ketosis on the major energy pathways before and after exercise in humans are shown below.



The effects of nutritional ketosis on muscle metabolism before (pre) and after (post) 1 hour of exercise at 75% of W_{Max}. **(A).** Branched chain amino acid (BCAA) concentrations are significantly reduced following ketosis vs. other nutritional substrates. (**B**). Branched chain amino acid levels in muscle are related to pyruvate levels as the end products of glucose breakdown. (**C**). Conservation of muscle BCAA is proportional to the muscle ketone concentration, as a result of ketone bodies reducing muscle glucose use (**D**). (**E**). Schematic representation of the differences between fat and glucose metabolism in exercise are shown vs. ketosis (**F**). (From: [7])

Nutritional intervention in metabolic myopathy

Enzymatic deficiencies affecting the metabolism of fats are multiple, albeit rare disorders characterized by consistent clinical phenotypes. The primary symptoms of defective metabolism of fat vary in severity depending on the metabolic location of the defect, and the degree of expression. However common to almost all conditions are an intolerance of exercise, skeletal or cardiac muscle myopathy, and a reliance on exogenous dietary fuels to sustain muscular energy demands.

Whilst the mainstay of therapy for these patients is the maintenance of blood borne substrates via adherence to various diets [12-14], these regimes are often inadequate to allow a normal physical lifestyle. In severe cases muscle damage, and other organ dysfunction reduces life expectancy and can render patients severely incapacitated. An alternative nutritional strategy to supply fuels in the form of ketone bodies may offer a new approach in the management of these conditions, without the unwanted effects of bolus glucose intake [3], or unpalatable dietary intervention.

Ketosis in metabolic myopathy

Previously, inducing a physiological ketosis required unpleasant Atkin's style high fat, low carbohydrate diets [15], or infusions of dissolved ketones salts [16]. Neither of these methods are applicable in VLCAD deficiency for obvious reasons. Now it is possible to deliver nutritional ketone bodies in drink form, making the oral nutritional delivery of ketones possible for the first time in humans [7]. This proposal seeks approval for a first clinical investigation into the possible benefits of acute NK delivered via the ketone ester drink in human VLCADD patients, specifically the metabolic basis for any enhanced muscle function.

Collaboration with the Dutch VLCADD expertise centre

Since 2007 Very Long Chain Acyl-CoA Dehydrogenase Deficiency (VLCADD) is included in the Dutch neonatal screening program (NSP). VLCADD is a rare disease (1:80000) and has only been discovered recently in 1992. Knowledge about disease course and long term effect of therapy is scarce. Therefore, the UMC Utrecht in collaboration with the Laboratory Genetic Metabolic Diseases (Academic Medical Center Amsterdam) and RIVM started an expertise centre in which all patients with VLCADD in the Netherlands can be seen. The primary goal of this expertise center is to document the clinical and biochemical phenotype and disease course of all diagnosed VLCADD patients in the Netherlands (METC nr 10-430/C). The Center partakes in the present investigative team to investigate the clinical benefits of acute NK in human VLCADD (dr Visser). The PI of the present application (dr Jeneson) has

previously collaborated with the center in a clinical investigation into muscle ATP metabolism in VLCADD patients (protocol **12-211/K** (UMC Utrecht)).

Inclusion of adolescent patients

Due to the rarity of the disease this study will include patients from the age of 16 years that could benefit from NK as well. Since rhabdomyolysis often has an onset in adolescence, confirmation of our hypothesis would lead to therapeutic use in that age group as well. Moreover, older patients often suffer from severe exercise intolerance and loss of muscle function due to earlier episodes of rhabdomyolysis, leaving most of them unable to complete our study protocol. Originally, all adult VLCADD patients known by the Dutch expertise center were provided with information on the study. Unfortunately, 1.5 years after the first inclusion only four patients participated. The main reason the other patients did not want to participate was the intensity of the protocol. Although the exercise test is at moderate intensity, the physical condition of these patients withholds them from riding a bike. Therefore an amendment was made to the original protocol to lower the minimum age to 16 years to reach sufficient power for this study. Unfortunately not many adolescent patients are known in The Netherlands, lowering the minimum age to 16 years will make participation possible for one patient. Lowering the minimum age even further won't make a difference, since the patients between 10-16 years are both rare and severely affected.

In vivo ^{31}P MRS read-out of muscle mitochondrial performance

Phosphorus magnetic resonance spectroscopy (^{31}P MRS) allows for non-invasive read-out of intramuscular pH and concentrations of Pi, PCr and ATP in human tissues (17). As such, dynamic 31P MRS recording of these metabolic variables in muscle offers a window onto the integrated *in vivo* performance of the cellular metabolic networks that produce ATP from the breakdown of fat and carbohydrates to maintain energy balance during exercise. 31P MRS-derived parameters such as the rate constant of PCr and Pi recovery post-exercise offer unsurpassed indices of in vivo mitochondrial function that have proven most useful in the clinical investigation of human metabolic myopathy (17). Recently, we applied this method to investigate energy balance in quadriceps muscle of VLCADD patients during an endurance exercise task (Diekman, manuscript in preparation). We found no evidence for impaired mitochondrial ATP synthetic function. However, changes in Pi and PCr during a standardized in-magnet bicycling exercise test were threefold larger than in healthy controls suggesting lower contractile efficiency of VLCADD muscle. Such a muscle phenotypic adaptation would aggravate the problem in VLCADD of exclusive dependence on turnover of the finite muscle carbohydrate store for ATP production, in turn explaining the known elevated risk for exertional rhabdomyolysis in VLCADD (Diekman, manuscript in preparation). Conversely, these results suggest that any boost of muscle mitochondrial function from acute NK should

be an effective therapy to improve exercise tolerance and lower the risk of exertional rhabdomyolisis in VLCADD.

2. OBJECTIVES

1. To investigate the potential of a novel dietary substrate preparation to enhance muscle mitochondrial function in VLCADD via acute nutritional ketosis.

2. To investigate if tri-citric acid cycle substrate supply in muscle of patients with VLCADDis boosted by acute nutritional ketosis.

3. STUDY DESIGN

This is a randomised, blinded, placebo controlled, two-way cross over trial. Patients with VLCADD will be recruited by the VLCADD Expertise Center at UMC Utrecht. Nutritional ketosis will be compared to a placebo, delivered as an isocaloric drink before exercise. An individualised, standardised bicycle exercise protocol will be completed by each participant with collection of blood metabolites, respired gases, and subjective ratings of fatigue and muscle complaints. In addition in vivo ^{31}P MR Spectroscopic recordings will be made from the quadriceps muscle before (resting), during and post-exercise. An optional muscle biopsy will be obtained from the quadriceps before and after exercise.
Patients will be asked to participate in three study sessions:

session I (site: nearest Academic Medical Center, Pediatric test ward).

- standard maximal cardiopulmonary exercise test (CPET; bicycle ergometry with indirect calorimetry)

  objective: determination of VO_{2max} in order to set the desired exercise workload of maximal individual rate of fat oxidation (FATMAX; typically ~40% MVO_2) in sessions II and III below.

- venipuncture
  objective: measure rise in blood lactate and creatine kinase (CK) levels in response to CPET

sessions II and III (site: Neuroimaging Magnetic Resonance Center UMC Groningen).

- venipuncture + canulation

  objective: prepare subject for serial (every 10 min) blood sampling for off-site metabolic profiling

- oral intake of nutritional drink A or B (ketone and placebo drinks, respectively)
  Each subject will receive both drinks over the course of the trial in a blinded fashion – i.e., if drink A in session II, then drink B in session III, and vice versa.

- microbiopsy from the quadriceps muscle (optional)

  1st biopsy: 20 min after oral drink; 2nd biopsy: immediately following end of exercise bout.
  objective: metabolomic profiling of the state of the energy metabolic network in muscle and mapping of individual phenotypic muscle properties (fiber type, mitochondrial density, capillary density).Biopsies will be obtained from the m. vastus lateralis of the left leg. Hereto, a preparatory superficial incision of the skin and fascii will be made under local sedation and bandaged, allowing fast and reproducible biopsy sampling prior to and immediately following exercise bout 1 below.

- 40 min upright bicycling exercise at FATMAX outside MR scanner (exercise bout 1)

  Blood samples are collected every 10 minutes.
  Subjects state subjective fatigue and muscle ache following exercise bout.

- microbiopsy from the quadriceps muscle (optional)

  Biopsies will be obtained from the m. vastus lateralis of the left leg (see above).

- 5 min supine bicycling exercise at FATMAX inside MR scanner (exercise bout 2)

  *serial in vivo 31P MR spectra will be recorded non-invasively from the m. vastus lateralis of the right leg as previously (protocols **12-211/K** (VLCAD; UMC Utrecht)**; NL41313.042.12** (MCAD; UMG) to determine energy and proton balance in the muscle during exercise and the kinetics of metabolic recovery following exercise. Subjective fatigue and muscle ache scores and a blood sample will be taken after the subject has exited the scanner room. Final blood samples including on-site analysis of CK levels are taken up to 3 hr post-session III to monitor any rhabdomyolytic event.*

- *24 hours after session II and III, co-investigator Bleeker will call each subject to evaluate subjective fatigue and muscle complaints after the subjet left the test facility.*

Time interval between study sessions I and II will be at least 7 days; between study sessions II and III: 7 days. The night prior to sessions II and III, patients will be housed in close vicinity to the UMCG. Patients will be instructed to take a late evening snack or meal according to their individual record of maximal fasting duration, given a start time of the study at 08:00 am the following day. Patients will be allowed a light breakfast according their normal diet before they will receive the caloric drive before the test. The breakfast will be the same for session II and III.

The total duration of the first session will be around 45 minutes, with 30 minutes of preparation and 8-12 minutes of cycling (exact time length dependent on the maximal endurance of the participant). The total duration of the second and third session will be around 5 hours including 45 minutes of preparation, 45-60 minutes of bicycling for the submaximal endurance exercise test and 3 hours of post-exercise resting where after the last blood and urine sample will be collected. During this 3 hour period, participants will be asked to fill out a HAES diary and can watch a DVD to pass time.

4. STUDY POPULATION

4.1 Population (base)

Six patients with VLCADD between 16 and 65 years of age, seen by dr. G. Visser for evaluation in the VLCADD expertise center of the UMC Utrecht.

4.2 Inclusion criteria

- Confirmed VLCADD by genetic profiling.
- age 16-65.

4.3 Exclusion criteria

- contraindications for MRI studies (assessed by standardised questionnaire as previously used in METC 08-267/K; see UMCG section F METC documents)
- inability to perform bicycle exercise.
- recent episode of rhabdomyolysis, or treatment for acute renal failure in the past 2 months.
- intercurrent illness which may influence exercise tolerance (anaemia, musculoskeletal injury, or other undiagnosed illness under investigation).
- known coronary artery disease, positive history for angina, or changes on ECG suggestive of previous ischaemia without a negative stress test.
- insulin-dependent diabetes mellitus.
- loss of, or an inability to give informed consent.
- pregnancy or current breastfeeding, or females not taking the oral contraceptive pill (this is due to the variability in hormonal patterns and substrate levels with different parts of the menstrual cycle).
- any other cause which in the opinion of the investigators, may affect the volunteers ability to participate in the study

4.4 Sample size calculation

Exact sample size calculation for this study is difficult for several reasons:

1. VLCADD is a rare metabolic disorder. The UMCU Center currently has 6 patients that may qualify for this particular study.
2. The proposed study is the very first of its kind. No equivalent means of imposing acute NK has previously been available. As such, there are no data available on the magnitude of any enhancement effect of the nutritional intervention on metabolic performance in this particular group of patients.

As mentioned in Section 3, this study is a randomised, blinded, placebo controlled, two-way cross over trial. In previous case-control design studies, it has been well established that ≥30% differences in the two categories of MRS-derived metabolic parameters – i.e., steady-state muscle pH, Pi and PCr levels, and kinetic rate constants of metabolic recovery post-exercise - can be reliably and reproducibly identified by the proposed investigational methods (e.g. [18]). We previously successfully detected statistically significant abnormalities in muscle MRS parameters (P<0.05; two-sided) in a case-control study of VLCADD featuring 5 patients (Diekman et al, PloS One 2016).

5. TREATMENT OF SUBJECTS

5.1. Investigational product/treatment
Nutritional drink and placebo ("sports drinks").

5.2. Use of co-intervention
Prolonged moderate-intensity exercise.

5.3. Escape medication
In case of physical complaints, the exercise will be terminated prematurely. Paracetamol is available if needed by the patient. Additionally, dextrose tablets will be available to administer when clinical symptoms of hypoglycaemia (e.g. headache, or light headedness) occur.

6. INVESTIGATIONAL MEDICINAL PRODUCT
This section is not applicable for this study.

7. NON-INVESTIGATIONAL PRODUCT
This section is not applicable for this study

8. METHODS

8.1 Study parameters/endpoints

8.1.1 Main study parameter/endpoint

(i) steady-state in vivo intramuscular levels of Pi, PCr, and pH during exercise versus rest.
(ii) kinetic rate constants of metabolic recovery post-exercise.

8.1.2 Secondary study parameters/endpoints

- completion of 40 min upright bicycling bout at FATMAX (yes/no; if no, #minutes)
- completion of 5 min supine bicycling bout at FATMAX in scanner (yes/no; if no, #minutes)
- subjective fatigue and muscle ache score after each exercise bout (scale 0-10) and 24 hours after exercise
- blood levels of 3-OH butyrate, acetoacetate, acylcarnitines, glucose, lactate and pH prior to and post-exercise.
- optional: muscle levels of glycogen, lactate, 3-OH butyrate, and (very) long (C18-C14) to medium (C12-C8) to short chain (C6-C2) acetyl-carnitines prior to and immediately post-exercise (on a patient-voluntary basis)

8.2 Randomisation, blinding and treatment allocation

Patients will be handed an unlabeled caloric drink (placebo and ketone ester drink) prior to the endurance exercise bout in each of the two study sessions at UMCG. All patients will receive both drinks over the course of the study. Co-investigator Bleeker, MD, who will

conduct the studies at UMCG together with PI Jeneson, will plan, randomize and record the order of placebo and ketone ester administration to the patients. Jeneson, who will analyze all MRS data, will not have, nor seek access to, these records thus ensuring blinded MRS data analysis.

8.3 Study procedures

The study is introduced to the patient by co-investigator Bleeker, MD, of the VLCADD expertise center in Utrecht in correspondence with their individual metabolic paediatrician/internist. The study will be announced during the 'patient information day' that's annually held by the the lcFAO expertise centre and by electronic newsletter to which most VLCADD patients or their caretakers are subscribed. Eligible patients will receive an information letter about the study. After a two week consideration period co-investigator Bleeker will send a reminder letter to subjects who did not respond to the inquiry. This letter will repeat the invitation to participate in the study. If after one more week the subject fails to reply, it is assumed that the subject is not interested and he or she will not be contacted any further. If the subject is interested and has given informed consent, the patient is included in the study, and the subject will be checked for the inclusion- and exclusion criteria The investigator informs the subject about final inclusion, or possible exclusion of the study on basis of the criteria.

Session I: Determination of the VO2max (CPET) and FATMAX.
Participants are asked to cycle at maximal speed on an ergometric bicycle on a functional test ward at the nearest UMC location (AMC, UMC Utrecht or UMC Groningen). Prior to this session they will be asked to complete a HAES questionnaire containing questions on exercise frequency and the presence of physical complaints during or after exercise.

Sessions II and III.
In the week prior to testing, participants will be asked to keep a 'food diary' for the week prior to the test in order to monitor the caloric intake and macronutrient content of each participant. For sessions II and III, patients will travel to Groningen the evening prior to the study and will be housed in a hotel across the street from UMCG. Patients will be instructed to take a late evening snack or meal in correspondence with their individual record of maximal fasting duration, given a start time of the study at 08:00 am the following day. Patients will be allowed the same light breakfast according their normal diet before they will receive the caloric drive before the test. The breakfast will be the same for session II and III.

Venipuncture and blood sampling:
Blood samples of 1-2 ml will be drawn via a 3-way tap from a 22 G venous canula (for subject comfort) inserted into a forearm vein at the start of each study visit. Blood samples

for free fatty acids, glucose and other assays will be taken and stored on ice, subsequently centrifuged, and stored at -80 until further processing. Up to 7 samples will be taken from the canula (~15 ml of blood total): #1 and #2 at baseline (in duplo), #3-5 during exercise (every 10 min), #6 after 40 minutes of moderate-intensity exercise, and #7 after the final MRS-analysis when the participant has recovered from exercise.

Muscle biopsy:
Participants are given the option at the start of the trial of undergoing muscle biopsy. If the patient has given informed consent to muscle biopsy, samples will be taken before and after exercise testing protocol during one or both study visits (2 samples/run; maximal of four biopsies for the complete study). 0.25 gm. of muscle tissue will be collected using percutaneous needle biopsies from the lower third of the Vastus Lateralis muscle with a biopsy gun (Bard Monopty, Bard Biopsy Systems, USA; a sterile standard biopsy technique (SOP).Muscle biopsy will be obtained from new incisions under local anaesthesia. Tissue samples will be frozen immediately in liquid nitrogen and stored at -80 ^{o}C until further analysis. Assays include glycogen and the metabolites of the glycolytic, TCA cycles. If sufficient tissue is available, enzymatic activity of mitochondrial and cytosol proteins, muscle fibre types, amino acids, and fat stores will also be determined.

Storage of tissue samples
All samples will be frozen immediately and stored at -80^{o} C in the laboratory for Liver, Digestive and Metabolic diseases of the department of Paediatrics at UMCG.

31P MRS
MR data gathering will be performed using a 3.0 T whole-body MR-scanner (Intera; Philips Medical Systems, Best, The Netherlands). Subjects are introduced to the scanner and exercise apparatus and instructed. MRS data gathering will be performed with a surface coil wrapped over the quadriceps muscle. Conventional anatomical MRI scans will be acquired for positioning and scanner preparation. 31P MR spectra will be acquired serially to measure dynamics in intramuscular PCr, ATP, Pi, and pH using standard 31P-MRS protocols.

8.4 Withdrawal of individual subjects
Subjects can leave the study at any time for any reason if they wish to do so without any consequences. Subjects can withdraw from the study by writing the coordinating investigator a letter, stating that they would like to withdraw from the study. The investigator can decide to withdraw a subject from the study for urgent medical reasons.

8.4.1 Specific criteria for withdrawal (if applicable)
Not applicable.

8.5 Replacement of individual subjects after withdrawal
In case a patient decides to withdraw from the study, a new patient will be recruited if

possible. If a patient decides to withdraw from the study prematurely because of clinical complaints during the prolonged moderate-intensity exercise, no new participant will be recruited. The data that have been obtained before termination will then be used as much as possible during data analysis.

8.6 Follow-up of subjects withdrawn from treatment

Patients who decide to withdraw from the study will be followed at the outpatient clinic at regular intervals. The follow-up scheme will remain similar to the scheme that was followed before participation in the study. Withdrawal from the study has no consequences for treatment and follow-up of the patients.

8.7 Premature termination of the study

Patients will have to terminate the study prematurely in the unlikely event of (symptoms of) hypoglycaemia during exercise. Dextrose tablets will be available to increase the blood glucose concentration in case of such a hypoglycaemia.

9. SAFETY REPORTING

9.1 Section 10 WMO event

In accordance to section 10, subsection 1, of the WMO, the investigator will inform the subjects and the reviewing accredited METC if anything occurs, on the basis of which it appears that the disadvantages of participation may be significantly greater than was foreseen in the research proposal. The study will be suspended pending further review by the accredited METC, except insofar as suspension would jeopardise the subjects' health. The investigator will take care that all subjects are kept informed.

9.2 Adverse and serious adverse events

Adverse events are defined as any undesirable experience occurring to a subject during the study, whether or not considered related to prolonged moderate-intensity exercise or MRS-analysis. All adverse events reported spontaneously by the subject or observed by the investigator or his staff will be recorded.

A serious adverse event is any untoward medical occurrence or effect that at any dose:

- results in death;
- is life threatening (at the time of the event);
- requires hospitalisation or prolongation of existing inpatients' hospitalisation;
- results in persistent or significant disability or incapacity;
- is a congenital anomaly or birth defect;
- is a new event of the trial likely to affect the safety of the subjects, such as an unexpected outcome of an adverse reaction, lack of efficacy of an IMP used for the treatment of a life threatening disease, major safety finding from a newly completed animal study, etc.

All SAEs will be reported through the web portal *ToetsingOnline* to the accredited METC that approved the protocol, within 15 days after the sponsor has first knowledge of the serious adverse reactions.

SAEs that result in death or are life threatening should be reported expedited. The expedited reporting will occur not later than 7 days after the responsible investigator has first knowledge of the adverse reaction. This is for a preliminary report with another 8 days for completion of the report.

9.2.1 Suspected unexpected serious adverse reactions (SUSAR)

Chapter 9.2.2 is not applicable for this study.

Adverse reactions are all untoward and unintended responses to an investigational product related to any dose administered.

Unexpected adverse reactions are adverse reactions, of which the nature, or severity, is not consistent with the applicable product information (e.g. Investigator's Brochure for an unapproved IMP or Summary of Product Characteristics (SPC) for an authorised medicinal product).

The sponsor will report expedited the following SUSARs through the web portal *ToetsingOnline* to the METC:

– SUSARs that have arisen in the clinical trial that was assessed by the METC;

– SUSARs that have arisen in other clinical trials of the same sponsor and with the same medicinal product, and that could have consequences for the safety of the subjects involved in the clinical trial that was assessed by the METC.

The remaining SUSARs are recorded in an overview list (line-listing) that will be submitted once every half year to the METC. This line-listing provides an overview of all SUSARs from the study medicine, accompanied by a brief report highlighting the main points of concern.

The expedited reporting of SUSARs through the web portal ToetsingOnline is sufficient as notification to the competent authority.

The sponsor will report expedited all SUSARs to the competent authorities in other Member States, according to the requirements of the Member States.

The expedited reporting will occur not later than 15 days after the sponsor has first knowledge of the adverse reactions. For fatal or life threatening cases the term will be maximal 7 days for a preliminary report with another 8 days for completion of the report.

9.3 Annual safety report

In addition to the expedited reporting of SUSARs, the sponsor will submit, once a year throughout the clinical trial, a safety report to the accredited METC, competent authority, Medicine Evaluation Board and competent authorities of the concerned Member States.

This safety report consists of:

– a list of all suspected (unexpected or expected) serious adverse reactions, along with an aggregated summary table of all reported serious adverse reactions, ordered by organ system, per study;

– a report concerning the safety of the subjects, consisting of a complete safety analysis and an evaluation of the balance between the efficacy and the harmfulness of the medicine under investigation.

9.4 Follow-up of adverse events

All adverse events will be followed until they have abated, or until a stable situation has been reached. Depending on the event, follow up may require additional tests or medical procedures as indicated, and/or referral to the general physician or a medical specialist.

9.5 Data Safety Monitoring Board (DSMB)

Due to the short duration of the study, no Data Safety Monitoring Board will be appointed.

10 STATISTICAL ANALYSIS

10.1. Descriptive statistics

Data will be expressed both quantitatively (values of PCr, ATP, Pi, pH, rate constants tau_PCr and tau_Pi, expressed as median and range), and qualitatively (MRS-spectra).

10.2 Univariate analysis

Results will be presented by means of confidence intervalsThe significance level will be set at $p<0.05$. Statistical analysis of the results will be performed using standard two-sided Mann-Whitney testing and will be conducted using Origin software (Caltech, USA, version 6.1).

10.3 Multivariate analysis

not applicable

10.4 Interim analysis

Not applicable.

11. ETHICAL CONSIDERATIONS

11.1 Regulation statement

The study will be conducted according to the principles of the Declaration of Helsinki Brazil, October 2013, and in accordance with the Medical Research Involving Human Subjects Act (WMO).

11.2 Recruitment and consent

Patients will be informed about the study during follow-up visits to the outpatient clinic, or during the patient information day that is organized every year in the UMC Groningen. They will be recruited by means of information that is administered both orally, and written in a patient information letter. After information has been administered orally, potential participants will receive written patient information. The physician will inform the patients and supply the written patient information. Patients will be asked to reply to the request within 14 days after oral and written information has been provided. This period can be extended 14 days extra. If after 14 days patients didn't reply after the provided written information, they will receive a recall letter. If subjects don't respond after 14 days they wil not be contacted any further. In order to approve of participation in the study, participants have to return the signed informed consent form by mail to the coordinating investigator. After the informed consent form has been received, the coordinating investigator will contact the participant by phone to provide further details if needed. All participants will be asked to sign a written consent form.

11.4 Benefits and risks assessment, group relatedness

In clinical practice, many patients with VLCADD wonder to which extent they are able to exercise safely. Any means of boosting this capacity will greatly improve the quality of life and long-term health in this patient population. Acute nutritional ketosis after ingestion of a novel edible ketone ester is a very promising novel therapy towards this aim and has already been tested with positive effects in human athletes. This study will gain insight whether a sound metabolic basis exists for its therapeutic use in this patient population.

No adverse effects of this study are expected. Of the listed interventions that are proposed here, all but one – i.e., ingestion of the ketone caloric drink - have previously been included and approved in clinical investigations of metabolic myopathy patients by members of the investigativce team in Amsterdam, Utrecht and/or Groningen: venipuncture and microbiopsy of the quadriceps muscle prior to and after exercise (protocol **04/1984** (MCADD; AMC Amsterdam); protocol **NL41313.042.12 (MCADD; UMCG)**); bouts of prolonged bicycle exercise outside and inside a MR scanner including in vivo MR spectroscopic data collection (protocol **12-211/K (VLCADD; UMC Utrecht;** protocol **NL41313.042.12 (MCADD; UMCG)**). Of the interventions, only the **burden** of collecting tissue samples from the quadriceps muscle by microbiopsy is rated as moderate (as opposed to ratings of nil to minor burden for

all other interventions[2]). As such, this intervention will only be performed on a voluntary basis; it will not affect enrollment in the study. Muscle tissue sampling is needed to test the secondary hypothesis that NK boosts muscular mitochondrial function specifically at the level of substrate supply to the TCA cycle; therefore, the burden of microbiopsy is considered justifiable.

The **safety** of the nutritional drinks that will be administered and the expected acute, transient mild ketosis (concentrations of ketone bodies < 3 mM) has been thoroughly tested and documented in healthy subjects (see references 9,10). The drink has been designated as 'GRAS' (= Generally Recognized as Safe) by the Federal Drug Administration of the United States allowing it to be used as a foodstuff in the USA. No particular risk for VLCADD patients is expected with respect to oral ingestion of the ketone ester since its metabolites are natural, organic compounds released without any cation or proton load that are readily oxidizable by cellular mitochondria irrespective of enzymatic defects in fatty acid oxidation.

The **benefit** to VLCADD patients of participation in the study is potentially enormous. In our previous applications for exercise and MRS testing in metabolic myopathy (protocols **12-211/K (VLCAD;** UMC Utrecht) and **NL41313.042.12 (MCAD)**, respectively), we put forward that the benefit of those studies would be that it would help establish a platform to conduct studies on the effectiveness of medication. Here, we now exploit this validated platform to test the metabolic effects of a nutritional drink that may revolutionize therapy of human metabolic myopathies due to defects in carbohydrate or fat metabolism. Ultimately, an effective treatment for VLCADD may be available for the first time to improve the quality of life of patients, including the opportunity to engage in a more active lifestyle with generic (including cardiovascular and anti-diabetic) health benefits.

11.5 Compensation for injury

The sponsor/investigator has a liability insurance which is in accordance with article 7, subsection 6 of the WMO.

The sponsor (also) has an insurance which is in accordance with the legal requirements in the Netherlands (Article 7 WMO and the Measure regarding Compulsory Insurance for Clinical Research in Humans of 23th June 2003). This insurance provides cover for damage to research subjects through injury or death caused by the study.

[2] the short, maximum exercise test in session I will use anaerobic derived energy. As patients with VLCADD do not have a problem in glycolysis, the burden for this test will be nihil. The endurance test at submaximal level might induce muscle pain temporarily. the burden for patients is classified as minimal and patients will be monitored accordingly during the study period. Blood will be drawn intravenously from patients; this implies a minimal burden.

1. € 450.000,-- (i.e. four hundred and fifty thousand Euro) for death or injury for each subject who participates in the Research;
2. € 3.500.000,-- (i.e. three million five hundred thousand Euro) for death or injury for all subjects who participate in the Research;

3. € 5.000.000,-- (i.e. five million Euro) for the total damage incurred by the organisation for all damage disclosed by scientific research for the Sponsor as 'verrichter' in the meaning of said Act in each year of insurance coverage.
The insurance applies to the damage that becomes apparent during the study or within 4 years after the end of the study.

11.6 Incentives (if applicable)
Not applicable.

12. ADMINISTRATIVE ASPECTS AND PUBLICATION

12.1. Handling and storage of data and documents
Each participant will obtain a code, and data will be handled according to this code. The code cannot be directly traced back to the participant. The key to the code is stored in a file on the computer of the principal investigator, which is secured by a login code and password of which only the coordinating investigator knows. Only the principal investigator, the coordinating investigator and the Monitor Board have access to the source data.
Signed informed consent forms will be stored in a separate research file of the patient. These files will be stored in a cabinet with a lock, together with the signed informed consents of the control participants. Only the coordinating investigator has the key to this lock. The data are kept until 20 years after termination of the study.

12.2 Monitoring and Quality assurance
Quality assurance will be performed by an independent monitor. The monitor will be a colleague who is independent of the current study. The members of the research team will meet with the monitor before advent of the study, and after the study has been completed ("close-out visit"). The meeting before the advent of the study is used to discuss how monitoring will take place, and to analyse what both the monitor and the members of the research team expect of the monitoring. The close-out visit will be combined with a monitoring visit. Furthermore, the monitor will monitor the study after analysis of the first 3 participants.
Monitoring consists of:
- Checking the presence and correctness of 100% of the informed consent forms.
- 100% monitoring of in- and exclusion criteria of first 3 participants who are included

for the study, and monitoring of 10% of the other participants who are included.

- Analysis of agreement between the data in the database, and the data in the source documents. 10% of all data will be monitored.
- 100% verification of reported SAEs and SUSARs. Data from 10% of the participants will be checked for possible missed SAEs and SUSARs.

The monitor will write a report about each visit, which will be discussed with the principal investigator and the coordinating investigator. The principal investigator will store the reports.

12.3 Amendments

Amendments are changes made to the research after a favourable opinion by the accredited METC has been given. All amendments will be notified to the METC that gave a favourable opinion.

12.4 Annual progress report

The investigator will submit a summary of the progress of the trial to the accredited METC once a year. Information will be provided on the date of inclusion of the first subject, numbers of subjects included and numbers of subjects that have completed the trial, serious adverse events/ serious adverse reactions, other problems, and amendments.

12.5 End of study report

The investigator will notify the accredited METC of the end of the study within a period of 8 weeks. The end of the study is defined as the last patient's last visit.

In case the study is ended prematurely, the investigator will notify the accredited METC, including the reasons for the premature termination.

Within one year after the end of the study, the investigator/sponsor will submit a final study report with the results of the study, including any publications/abstracts of the study, to the accredited METC.

12.6 Public disclosure and publication policy

Results from the study will be presented at the patient information day that is annually hosted by the lcFAO expertise center in the UMCU. Additionally, a manuscript will be written about the obtained results. This manuscript will subsequently be offered for publication in a peer-reviewed journal.

13. STRUCTURED RISK ANALYSIS

This chapter is not applicable for this study

14. REFERENCES

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