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Protocol

Investigation of hepatic and cardiac fatty acid metabolism in patients with type 2 diabetes mellitus with and without non-alcoholic fatty liver disease

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Time Schedule
Patient inclusion: 01.12.2020
End of study: 01.12.2022

Collaboration departments
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Lever- Mave- og Tarmsygdomme, Aarhus Universitetshospital
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List of abbreviations:

CT, computed tomography

DXA, dual-energy X-ray absorptiometry

DNL, de novo lipogenesis

EF, ejection fraction

FF %, fatty fraction

FFA, free fatty acids

GLP-1-receptor agonist, glucagon-like peptid 1 agonist

IHTG, intrahepatic triglyceride

HCC, hepatocellular carcinoma

LPL, lipoprotein lipase

MRS, magnetic resonance spectroscopy

MS, metabolic syndrome

NAFLD, non-alcoholic fatty liver disease

NAFL, non-alcoholic fatty liver

NASH, non-alcoholic steatohepatitis

PET, positron emission tomography

BG, blood glucose

T2DM, type 2 diabetes mellitus

TG, triglyceride

VLDL, very low-density lipoproteins

Background

NAFLD

Type 2 diabetic patients and obese subjects with upper-body fat distribution have increased risk of heart disease (ischemia and heart failure), insulin resistance (1,2), dyslipidemia (high plasma triglycerides (TG) and low HDL-cholesterol concentrations), abnormal metabolism of free fatty acids (FFA) and very often, increased liver fat content (3). The latter, non-alcoholic fatty liver disease (NAFLD) is considered the hepatic manifestation of the Metabolic Syndrome (MS). NAFLD covers a spectrum from simple reversible hepatic steatosis (i.e. non-alcoholic fatty liver (NAFL) to inflammation and fibrosis, termed non-alcoholic steatohepatitis (NASH). The latter is considered a state of "burned out" NAFLD with gradual loss of hepatocytes with increased risk of progression to cirrhosis and end-state liver disease (4,5, 43-46). NASH and especially NASH with fibrosis, is associated with excess mortality (43-46). Several studies also indicate that NAFLD is associated with development of cardiac steatosis, reduced left ventricular function and cardiac arrhythmia (6-11).

NAFLD is the most common chronical liver disease in the Western world with a prevalence between 17-45 % (47). The estimated prevalence of NAFLD has been shown to mirror the increasing population prevalence of obesity and MS with prevalence rates of NAFL corresponding to 40 %, 30-50 % and 50-90 % in upper-body obese, insulin resistant and dyslipidemic subjects, respectively (with even greater prevalence in type 2 diabetic patients), whereas the prevalence of NASH is reported to be somewhat lower (12). It is not clear whether steatosis leads to NASH or if NASH is developed independently of steatosis – a study however has showed that steatosis progress to NASH in 25 % of patients with NAFL over 3 year (48). The mechanisms behind why some subjects progress from NAFL to NASH are poorly understood, but may involve differences in hepatic fatty acid (FA) trafficking, resulting in an unhealthy balance between FA uptake and increased FA synthesis (*de novo lipogenesis*, DNL) in liver vs. oxidation and export of FA from liver to plasma in "very low-density lipoproteins"-triglyceride (VLDL-TG) particles. The changed hepatic metabolism of fatty acids may in turn promote pro-inflammatory and oxidative stress conditions with degeneration and inflammation of the normal liver architecture (51, 52).

NAFLD and risk of cardiovascular disease

Mediators linking NAFLD with myocardial dysfunction are not known but are presumed to include both pre-hepatic (e.g. ectopic/cardiac fat) and hepatic mechanisms with potential direct

impact on cardiac function and mirrored at the whole body level by metabolic inflexibility. Metabolic inflexibility is the inability to shift efficiently between glucose and lipid oxidation due to a chronic increase in plasma FFA caused by insulin resistant anti-lipolysis and hepatic VLDL-TG hypersecretion (13).

The metabolic dysfunction of the myocardium in NAFLD and T2DM includes 1) reduced basal and insulin stimulated glucose oxidation, 2) reduced phosphate creatinine/adenosine triphosphate (PCr/ATP) ratio (a marker of the myocardial fuel utilization) and 3) a potential reduced FFA oxidation (9,19). Both intrahepatic TG and TG secreted into the bloodstream as lipoproteins (VLDL-TG) are derived from plasma FFA and released into the bloodstream via lipolysis in peripheral adipose tissue (21). Human studies suggest that the circulating FFA in combination with insulin resistance determine the amount of VLDL particles produced by the liver and, hence the quantity of TG released into the blood (22, 27). During basal fasting, the amount of visceral fat correlates directly to the delivery of liver FFA from the circulation (yet with significant difference between men and women) (2).

Insulin resistance and its role in the pathogenesis of NAFLD

Immediately after a meal, insulin increases and, in healthy people, reduces blood glucose (BG) and results in an almost immediate halt in VLDL secretion. The effect of insulin is, however, severely impaired in NAFL, NASH and type 2 diabetic patients both in the liver (23,28), adipose tissue (24-26), and skeletal muscle (27). The overall effect is an increased amount of VLDL-TG in the blood with subsequent risk of atherosclerosis and ectopic fat accumulation in e.g. the myocardium (10). Although the effect of reduced insulin sensitivity on increased FFA and VLDL-TG levels are well-documented (28), it is uncertain to what extent each factor contributes quantitatively to NAFLD due to the liver's anatomical localization and the lack of robust tracer methods to assess VLDL-TG production rate. Even though NAFLD is recognized as a strong predictor of CVD risk factors associated with MS (5), this is, however, not always the case, and has led researchers to question whether NAFLD is the result of insulin resistance or whether insulin resistance is secondary to NAFLD, or both (29). Studies have shown that the degree of NAFLD in obese, insulin resistant subjects correlates with cellular and intracellular insulin resistant manifestations in many metabolic processes. This is the case in both liver, muscle, and adipose tissue, such as FFA uptake in liver and muscle, hepatic de novo lipogenesis, altered lipogenic enzyme activity in liver and adipose tissue, muscle, mitochondrial/TCA cycle activity, and cardiac steatosis with impaired ventricular function (10, 31, 32). The multiple direct and sometimes temporary relationships between NAFLD and the

heart and peripheral tissues indicate that insulin resistance and metabolic inflexibility is a key component of NAFLD, but also that significant integrated pathophysiological cross-talk exists between the heart, liver, skeletal muscle and adipose tissue. Thus, insulin resistance in combination with hyperinsulinemia and increased hepatic FFA delivery may promote liver fat accumulation by increasing hepatic TG production and de-novo lipogenesis (12, 21, 24, 33). In addition, the development of NAFLD is also assumed to be an imbalance between hepatic FA delivery (FA uptake and/or de-novo synthesis) and hepatic lipid export (i.e hepatic FA oxidation and/or VLDL-TG secretion) as previously described.

In this study we aim at identifying potential differences between patients with T2DM with and without NAFLD, when compared to FA oxidation of the heart, hepatic FA metabolism (uptake, oxidation and re-esterification), and hepatic VLDL-TG kinetic. In addition, muscle and adipose tissue biopsies will be taken out to determine the content of lipid and lipolytic and lipogenic enzymes, as insulin resistant conditions potentially have an substantial effects on the cellular regulation of lipolysis and substrate oxidation of the skeletal and adipose tissue.

We have developed a technique to radioactive label VLDL-TG. The technique involves isolating of the VLDL particles from plasma by ultracentrifugation and incorporation of [1-^{14}C]-triolein in the particles. The labelled VLDL-TG can hereafter be infused to determine the turnover of VLDL-TG (23,58). We have similarly implemented a method to determine the organ-specific FA uptake and oxidation by PET (positron emission tomography) scan with [^{11}C]-palmitat. The method with PET scan has previously been used to determine FA uptake and oxidation of the heart (20) as well as of the liver (21-23)
The combination of the tracers makes it possible to determine the turnover of FA and VLDL-TG simultaneously.

Hypothesis

We hypothesizes that the heart fuel utilisation and FA turnover and hepatic lipid kinetic (FA and VLDL-TG) in relation to NAFL and NASH are abnormal in a coordinated manner in patients with T2DM and is related to myocardial function, intrahepatic triglyceride (IHTG) content and adipose and skeletal lipid metabolism.

Specific aims

To determine differences in subjects with T2DM with and without NAFLD in relation to:

- FA uptake and oxidation in the heart
- Hepatic FA uptake and oxidation
- Secretion and oxidation of VLDL-TG
- Identification of potential coordinated changes in the metabolism in heart, liver, skeletal and adipose tissue.

Materials and Method

Inclusion criteria

- 20 study participants with T2DM (definition (49)) will be recruited:
- Group 1: 10 men/women with NAFL and/or NASH, confirmed fat fraction (FF) % > 5,6 assessed using MR spectroscopy (MRS)
- Group 2: 10 men/women matched for age and BMI without NAFLD (definition (47))
- Age of 30-70 years
- Non-smokers
- Written consent before the study start

Exclusion criteria

- Comorbidity other than hypertension and dyslipidaemia
- Fixed medical drug consumption (including insulin) except statins and anti-diabetic medications. However, statins and weekly based GLP-1 agonist must be paused 1 week before the examination date and other antidiabetic medication 3 days before the study date.
- Patients with cancer or former cancer patients
- Blood donation within the last 3 months prior to the study
- Participation in experiments involving radioactive isotopes within the last 3 months
- Alcohol abuse (over 21 items per week for men and over 14 for women) or other substance abuse
- Pregnancy

Study participants will be omitted from the study if

- They choose to
- There are serious or intolerable side effects during the study
- Study participants develop conditions mentioned under ”exclusion criteria”.

Recruitment

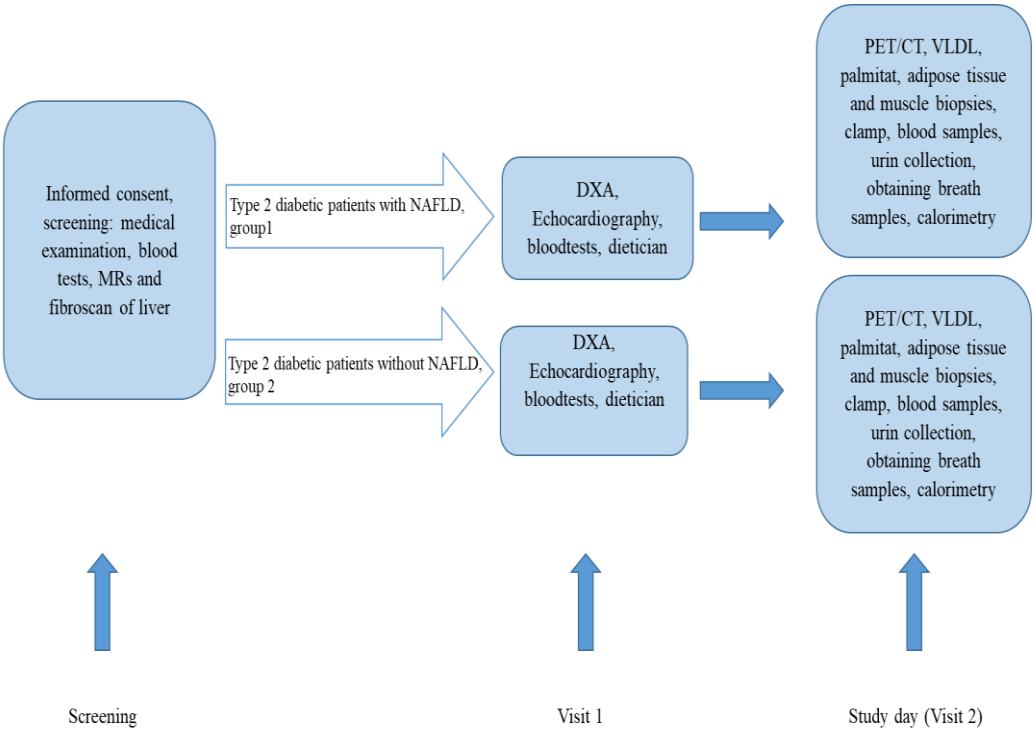
Recruitment will be done via notice at Aarhus Universitetshospital (www.auh.dk), Aarhus Universitet (www.au.dk) and other educational institutions in Aarhus as Aarhus Business College (www.aabc.dk), Aarhus teknisk Skole (www.aarhustech.dk), VIA University College Aarhus (www.via.dk), by announcing in patient association (www.diabetes.dk) and in newspapers such as Jyllands-Posten, Aarhus Stiftstidende and Lokalavisen, via social media (Facebook) and via homepage www.forsøgsperson.dk.

Recruitment and information about the study will also be done via medical doctors from outpatient clinics at The Department of Endocrinology and Internal Medicine and The Department of Hepatology and Gastroenterology, AUH.

The candidate participants will be informed about the project either by telephone or orally in the outpatient clinics. In addition, they will receive the ”Project Information” and ”Information material from The Denmark Central Region Committees on Health Research Ethics”. Candidate participants will be contacted after prior agreement by telephone and will be invited to an information meeting. The participant can bring an assessor to the meeting.

All information about the study will be given by investigator Indumathi Kumarathas and if the participant accepts final participation, a declaration of consent is signed.

Flow chart



Screening

The purpose of the visit is to evaluate inclusion and exclusion criteria. Prior to the visit, the potential candidate participant has already received written information material about the project. The potential candidate will be informed orally. If the participant accepts final participation and is eligible to participate in the study, a declaration of consent is signet. Furthermore, during this visit following will be obtained:

- Medical history and examination (incl. Weight, height, blood pressure and pulse), blood tests (plasma glucose, hemoglobinA1C, lipid profile, SD163, haematological parameters, TSH, ALAT, ASAT, alkaline phosphatase, eGFR, creatinine, sodium and potassium).

- Magnetic resonance spectroscopy (MRS)

MRS is used to quantify the hepatic and cardiac fat content and ATP/AMP ratio. A fat content of more than 50 mg/g corresponding 5 % of the wet weight is diagnostic for hepatic steatosis,

measured by MRS as FF % > 5,6 (23). MRS is used to distribute the participants into group 1 and group 2.

- Fibroscan

Fibroscan is used to asses if NAFL is progressing into NASH. Values < 1 kPa is a sign of absence of NASH. Values > 8 kPa is a strong predictor for NASH with fibrosis. Fibroscan is based on ultrasound technology and is an non-invasive examination.

Visit 1

The purpose of visit 1 is to take blood samples to isolate and label VLDL-TG and to screen for genetic susceptibility (64). Furthermore we wish to determine body composition by performing DXA (dual-energy X-ray absorptiometry) scan and determine the cardiac contractility and pump function via echocardiography and finally to advise the participant concerning physical activity and diet up to the study day.

- Blood tests

- VLDL-TG will be labelled with 20 μCi [1-^{14}C]triolein and isolated via centrifugation. Labelled VLDL particles were tested for bacterial growth to ensure sterility and stored at 5 °C for reinfusion on the study day.

- DXA-scan

- Is performed to asses total body fat and lean body mass.

- Echocardiography (”Echo”)

Is a non-invasive test that uses high frequency sound waves (ultrasound) to make pictures of the heart, and it can give information about the size and shape of the heart and about the function of the heart valves and pumping performance. In atherosclerosis, where the blood supply to the heart muscle is reduced, the echocardiography can show reduced muscle strength in parts of the heart, where the blood supply is reduced.

Echocardiography will be performed by a chief physician from the Department of Cardiology, AUH.

- Interview by a dietician

The participants will be interviewed by a dietician regarding physical activity and diet up to the study day. The volunteers were requested to avoid vigorous exercise and were provided a isocaloric (weight maintaining) diet (55 % carbohydrate, 15 % protein, and 30 % fat) delivered from the Clinical Trial Unit, AUH, for the 3 days preceding the study day.

The Study Day (Visit 2)

The participant will be admitted to the Clinical Trial Unit, CTU, at 22.00 the evening before the study. The participants are only allowed to ingest tap water after the admission to CTU.

The study can be divided into a 3 hours basal period (0-180 min) and a 5 hours clamp period (180-450 min). A hyperinsulinemic euglycaemic clamp is used to determine insulin sensitivity. During insulin clamp, hyperinsulinemia, is achieved by infusing constant intravenous insulin of 0,6 mU/kg/min (Actrapid, Novo Nordisk) and euglycemia is maintained via concomitant intravenous 20 % isotonic glucose infusion, so the plasma glucose is constantly at 5 mM. Plasma glucose levels are monitored via frequent intervals during the study.

Flow sheet over the study day

Time	06.30	07.00	7.30	8.00	8.30	9.00	9.30	10.00	10.30	11.00	11.30	12.00	12.30	13.00	13.30	14.00
Minutes	-30	0	30	60	90	120	150	180	210	240	270	300	330	360	390	420
Period		Basal						Clamp (insulin+glucose infusion)								
Infusions																
Glucose								Isotonic 20 %								
Actrapid								0,6 mU/kg/min								
TRACERS																
^{14}C-VLDL			14C-VLDL-TG infusion, 20 % bolus initially, 80 % constant infusion													
^{11}C-palmitate																
9,10-^{3} H-palmitate																
BLOOD SAMPLES																
^{14}C-VLDL		x					xxxx								xxxx	
9,10^{-3} H-palmitate						x	xxxx					x			xxxx	
Plasma glucose	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
FFA		x		x		x		x		x	x	x		x		x
Insulin		x		x		x		x		x		x		x		x
Plasma lipid		x		x		x		x		x		x		x		x
Plasma Li-hep		x		x		x		x		x		x		x		x
Plasma EDTA		x		x		x		x		x		x		x		x
Serum		x		x		x		x		x		x		x		x
EXAMINATIONS																
Insertion of catheter	x															
PET/CT																
Muscle biopsy		x												x		
Adipose tissue biopsy		x												x		
Calorimetry			x										x			
Breath sample		x					xxxx									
Urin sample							x								x	

At t= - 30, catheters were placed in an antecubital vein and a contralateral heated hand vein in order to obtain arterialized blood. If it is difficult to extract blood from a hand vein in rare occasions, an artery cannula can be inserted with help from a nurse or a doctor from the Department of Anaesthesiology, AUH.

At t=0, a primed-constant intravenous infusion of [1-^{14}C]triolein-labelled VLDL-TG (priming with bolus of 20 % of labelled VLDL, 80 % is given as continues infusion) was initiated to determine VLDL-TG kinetics. Blood samples are obtained to measure VLDL-TG secretion rate.

At t=0 and at t=360, muscle and adipose tissue biopsies are obtained under sterile conditions. The samples will be quick-frozen for subsequent analysis.

At t=60 and t=240 an intravenous bolus injection of IV ^{11}C-palmitat will be administered and PET/CT scans and blood samples will be obtained after 50 minutes. Blood samples are obtained to determine uptake, oxidation and re-estification of FFA in heart and in liver.

At t=180 the insulin clamp period is initiated with IV insulin infusion and IV glucose infusion.

FFA turnover is examined via a constant infusion of [9,10-^{3}H] palmitate (1 hour) in basal and clamp periods. Blood samples to determine palmitate turnover, lipogenesis, and specific activity in blood and FA derivates will be obtained in the beginning of the [9,10-^{3}H]-palmitate infusion and after 30, 40, 50 and 60 minutes of infusion.

Breath samples will be obtained during the study to examine the $^{14}CO^{2}$ specific activity to determine VLDL-TG FA oxidation.

Indirect calorimetry will be done to examine the energy metabolism and substrate oxidation rate.

Blood samples are taken at different intervals to determine glucose, FFA, insulin and tracer concentrations.

After blood sampling at t=450 the catheters will be removed and the particpants will be offered a meal.

Procedures

Biopsies

Muscle biopsies are taken under local anaesthesia with 6 ml 10 mg/ml lidocaine under sterile conditions from the vastus lateral muscle (approx. 0.2 g). Adipose tissue biopsies are taken from the subcutaneous adipose tissue of the abdomen and femur by a modified liposuction technique with a 2.1 mm cannula, also under local anaesthesia.

PET / CT scans

PET scans is a minimal invasive nuclear medicine examination, often used to diagnose cancers, but can also be used to diagnose cardiovascular diseases. . A modern PET/CT scan can make images of the whole body within 10-20 minutes (56,57). Scans are performed using an integrated Siemens Biograph 64 PET / CT with 21 centimetres of field of view which covers the heart and liver. In connection with all PET scans, a simultaneous low-dose CT scan is performed to co-locate the PET-positive areas as well as to correct for tissue attenuation. The ^{11}C palmitate tracer will be given as an IV bolus injection through a catheter into an elbow vein.

^{11}C-palmitate:

A total dose of 400 MBq will be administered to participants while scanning the heart and liver. Metabolite correction (^{11}CO2 and ^{11}C-TG) will be performed using our own validated population mean (36,37). In-put function is obtained from images covering the lumen of the left ventricle. FFA turnover in liver and heart is determined using ^{11}C palmitate PET technique.

Echocardiography

The ability of the heart to contract and fill is measured by echocardiography.

VLDL-triglyceride turnover

Turnover is determined by isotope dilution technique with continuous infusion of ex-vivo labelled VLDL triglyceride (23, 58). One week before the examination, a 60 ml venous blood sample is taken under sterile conditions. VLDL triglyceride will be labelled with 20 μCi [1-

14C] triolein and subsequently isolated by ultracentrifugation (40,000 rpm for 18 hours at 4 ° C). Sterility will be ensured by culturing before the sample is used for re-infusion. The radiolabelled VLDL will be stored at 5 ° C until the day of the study. On the day of the examination, the patient's own [1-14C] triolein labelled VLDL triglyceride will be infused and the sample now contains only about 10% of the initial triolein dose used, due to decay during the week. Blood samples will be taken on the day of the study as indicated to determine VLDL triglyceride concentration and substrate activity.

Oxidation of VLDL-TG

Determined by measuring CO2 concentration and specific activity in the exhaled air (determined by hyamine) as previously described (41, 58).

VLDL-TG-FA uptake into muscle and adipose tissue

Determined by measuring FFA concentration and specific activity in muscle biopsies and fat biopsies.

FFA turnover in the liver and heart

Measured using PET technique after bolus administration of ^{11}C palmitate and subsequently determining the fatty acid oxidation, re-esterification and secretion of the liver and heart.

Lipoprotein lipase activity

Heparin-releasable lipoprotein (LPL) activity in adipose tissue is measured in fat biopsies after incubation in 5 U / ml heparin for 45 minutes at room temperature. The solution is then analysed for LPL activity using the "glycerol-stabilized substrate" method (59). For each tissue analysis, "post-heparin plasma" (PHP) is included as an internal standard to correct for substrate variation. Different dilutions of PHP are used to validate a linear response. Results are given as U / µmol FFA / hour / g.

FFA turnover

Turnover is determined by isotope dilution technique with 2 times 1-hour continuous infusion of [9,10-3H] palmitate (0.3 µCi / min total corresponding to 36 µCi) (60). Plasma FFA (palmitate) concentration and specific activity are determined by HPLC (high pressure liquid

chromatography) using [2H31] palmitate as internal standard. We have shown that our sample preparation does not cause in vitro hydrolysis of TG fatty acids or phospholipids. A quality control standard will be included with each assay to ensure minimal variation in the preparation of standard curves.

Resting energy expenditure (REE) and respiratory quotient (RQ)

Determined by indirect calorimetry (Deltatrack Metabolic Monitor, Datex, Helsinki, Finland). From these measurements, net lipid and glucose oxidation can be calculated. Protein oxidation will be estimated from the excretion of urea in the urine (61).

Body composition

Total body fat and fat-free mass are determined by DXA scan (Hologic QDR 2000).

Primary Endpoint

• FFA uptake and oxidation as well as energy utilization in the heart ([11C] palmitate PET)

Secondary Endpoints

• Pump function of the myocardium and left ventricular function (via echocardiography)

• FFA uptake, oxidation and re-esterification in the liver ([11C] palmitate - PET technique)

• VLDL-TG secretion ([1-14C] VLDL - isotope dilution technique)

• VLDL-TG oxidation ($^{14}CO2$ in exhaled air)

• VLDL-TG deposition in adipose tissue (fat biopsy ~ 1 g)

• Glucose metabolic tests and other blood tests. Intracellular signalling pathways in muscle and adipose tissue (AMPK, LKB1, ACC, CD36, ATGL, HSL, perilipine, G0S1, CGI58, GLUT4, and cytochrome C) as well as study for the genotypes of PNPLA3 and TM6SF2 in subjects with NAFLD.

Statistical analysis

Sample sizes are based on estimates of cardiac palmitate from two recent studies in type 2 and nondiabetic men (28,29), as well as on prior experience regarding mean basal VLDL-TG secretion of 60 μmol/min (SD of diff: 10 μmol/min). To detect a difference in palmitate oxidation as well as on VLDL-TG secretion between 2 experimental days of 20% (12.5 (SD 6.25) μmol/min) with power 0.80 and α 5%, seven subjects are needed using two-sample t-test. Group comparisons will be done with standard statistical methods (ANOVA, t-test for parametrical data) and p-value under 0,05 will be assumed to be statistical significant. Corrections will be examined using Pearson's or Spearman's test. With 10 participants in each group, we would be able to determine a 30 % increased uptake of FFA in liver in subjects with NAFLD (power: 0.80). This is based on estimates of palmitate uptake in liver and FA oxidation in the heart from our previous studies, where we used the same ^{11}C-palmitate tracer (37,50).

The scope of the screening activity

We expects to screen 30 potential candidate participants, who full fill inclusion criteria with scans and blood samples to obtain 20 participants. The test results from the blood samples from screening of volunteers who are being included or excluded, will be available in the volunteers electronic patient journal (MidtEPJ). If the blood samples or scans gives rise to further investigation, the participant will be contacted by phone and informed to contact a medical doctor/or be referred to the relevant medical speciality.

Analysis and interpretation

Data will be kept in an anonymised form and archived in the Department of Endocrinology and Internal Medicine (DOH), AUH. Data will be handled according to guidelines appointed by the Danish Data Protection Agency. The results are expected to be published in articles in peer-reviewed international scientific papers.

Feasibility of the study

The Medical Research Lab has significant expertise in conducting clinical and metabolic studies and all the methods are well established in the lab.

Economy and insurance

None of the participating researchers has an economical interest in this project. The salary for Ph.D. student Indumathi Kumarathas is finance by Danish Diabetes Academy (DDA) and Steno Diabetes Center Aarhus, SDCA, Aarhus University Hospital. Project costs will be covered by a grant from the Novo Nordisk Foundation. Participants will receive remuneration for their participation (2500 DKK in total taxable amount) and have their transportation cost paid. The study patients will be covered by a mandatory insurance in the Danish Healthcare system (”Lov om klage- og erstatningsadgang indenfor sundhedsvæsenet”).

Ethical aspects

All studies will be conducted in accordance with the articles of the Declaration of Helsinki. The protocol will be approved by the Health Research Ethics Committee of Central Region, Denmark, the Danish Data Protection Agency. Care will be taken to administer the lowest tracer dose possible. In addition as mentioned above, the study participants will be covered by a mandatory insurance in the Danish Healthcare system (”Lov om klage- og erstatningsadgang indenfor sundhedsvæsenet”).

Informed consent

By request ”Project information” and ”Information material from The Denmark Central Region Committees on Health Research Ethics” will be sent to the potential candidate participant by post or by email. A meeting with the investigator is then arranged in order to give oral information about the project and to evaluate inclusion and exclusion criteria. The meeting will take place at the Department of Internal Medicine and Endocrinology, AUH, and if the participant wishes to, the participant can bring an assessor.

All information about the study will be given by investigator Indumathi Kumarathas. This project is a scientific trial and it will be stated clearly in both oral and written information material. Likewise, aims, risks and annoyances and potential benefit of this study will be stated. It will be emphasized that participation is on a voluntary basis and that consent can be withdrawn at any time without consequences for the participant.

If the participant accepts to participate, a declaration of consent is signed.

Safety evaluation

Advantages

We consider participation in this study to be safe and tolerable. Advantages and any potential side effects and annoyances will be described in the following.

As part of the study, participants will have measured their lipid profile, blood sugar, insulin resistance, body composition, haematological parameters, liver, and kidney function. If any of these parameters suggests an increased risk of developing liver, cardiovascular, or diabetic complications the participants will be offered counselling and guidance at the end of the study.

In addition, many patients with diabetes will see it as meaningful to contribute with new knowledge about perturbations in fatty acid metabolism that on a short term may have implications in the treatment of NAFL and NASH.

Side effects and annoyances

Participants may experience discomfort in connection with placement of a venous catheter and there is a risk of developing a bruise. Placement of arterial cannula is associated with some discomfort and there is a minimal risk of developing arterial thrombosis, however, this procedure is only used in the rare cases where the venous catheter is not usable and is performed by trained personnel. Muscle and fat biopsies is associated with some discomfort / pain as well as a small risk of developing blood clots and infection at the injection site. The participants will be asked to contact the study responsible medical doctor Indumathi Kumarathas or the Emergency Department, AUH, for signs of infection (redness, heat, soreness, swelling) or major blood clot.

The total blood loss in connection with blood sampling and biopsies will amount to approx. 400 ml (over all study days) and will therefore not be a problem in healthy non-anaemic individuals.

The long-term risk of participation in the study is only related to the radiation exposure. The total radiation load is 5.03 mSv, corresponding to 1.7 times the radiation you normally receive in Denmark during a year (background radiation approx. 3 mSv per year) (63). Theoretical calculation of the extra risk of participating in the trial will be approximately 0.03% increased risk of contracting a cancer during one's lifetime, and an average Dane will thus increase his risk from approx. 25.00% to 25.03%.

Biological material, biobank and handling of data

The following samples will be obtained from each participants:

• 2 x 2 fat biopsies, one from subcutaneous adipose tissue on the abdomen and one from femoral adipose. 2 x 1 muscle biopsy from m. vastus lateralis. All procedures are performed under sterile conditions and with local anesthesia as well as subsequent cooling of the area with ice and compression to ensure rapid hemostasis. An average amount of 200 mg pr. sample is collected. A research biobank is set up, where the samples are frozen with liquid nitrogen for later analysis.
• Blood samples of 400 ml in total are collected. A research biobank is set up, where the samples are frozen with liquid nitrogen for later analysis. From trial participants who only participate in the screening trial, approx. 30 ml of blood.

If there is biological material left at the end of the experiment, the material will be transferred to the research biobank for future use. The data protection rules continue to apply so that the Health Research Ethics Committee must approve new research in the biological material and new informed consent must be obtained. Excess material will be stored for 15 years after the end of the project. The excess biological material from subjects excluded after the screening visit will not be transferred to a biobank for future research.

Biobank for future research

Blood- and tissuesamples are expected to be used in the analysis. If there are biological material left in the ending of the investigation (blood and muscle- and adipose tissue) left, the leftover material will be transferred to the research biobank for future use and will be kept for 15 years after the ending of the investigation. We would like to keep the material in a biobank, as we in a few years to come, would investigate if NAFL and NASH is affected by other systems, that we currently do not have the knowledge of. The data protection rules continue to apply, so that the Ethics Committee must approve new research in the biological material and new informed consent must be obtained. The excess biological material from subjects excluded after the screening visit will not be transferred to a biobank for future research.

Handling of data

After informed consent, information about the medical history, comorbidity, blood sample tests, test results from scans and other information relevant for the study will be registered and will be extracted from the patient journal. Project investigator will have direct access to patient files (MidtEPJ).

Data will be kept in an anonymised version on AU's server in REDCap for 15 years after the termination of the investigation and temporarily in Department of Endocrinology and Internal Medicine, AUH. REDCap will be used as electronic CRF (case report form).

Data will be handled according to guidelines appointed by the Danish Data Protection Agency.

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