Centrale Commissie Dierproeven

Appendix
Description animal procedures

- This appendix should be enclosed with the project proposal for animal procedures.
- A different appendix 'description animal procedures' should be enclosed for each type of animal procedure.
- For more information, see our website (www.centralecommissiedierproeven.nl).
- Or contact us by phone (0900-2800028).

1 General information

1.1	Provide the approval number of the 'Netherlands Food and Consumer Product Safety Authority'.	50100
1.2	Provide the name of the licenced establishment.	The Netherlands Organisation for Applied Scientific Research (TNO)
1.3	List the serial number and type of animal procedure. *Use the serial numbers provided in Section 3.4.4 of the Project Proposal form.*	Serial number: 3.4.4.1 Type of animal procedure: Metabolic disorder(s) study

2 Description of animal procedures

A. Experimental approach and primary outcome parameters

Describe the general design of the animal procedures in relation to the primary outcome parameters. Justify the choice of these parameters.

The different metabolic disorders are not separate diseases but are related: the metabolic disorders coincide and the occurrence of one metabolic disorder enhances the risk for others. In addition, similar common pathways play a role in the different metabolic disorders. To study these metabolic disorders, we use a whole set of different mouse models that for instance differ in which aspect of the metabolic disorders is most prominently present (see also paragraph B). The general design of metabolic disorder studies is a prolonged treatment of mice to a high caloric diet in order to induce metabolic disorder(s). The detailed study design being used depends on specific metabolic disorder(s) under investigation: **obesity**, **Type 2 Diabetes**, **hyperlipidemia, atherosclerosis**, non-alcoholic fatty liver disease (NAFLD) progressing towards non-alcoholic steatohepatitis (**NASH**), as well as **nephropathy** or **retinopathy** will be studied (the exception to studies with induction of the metabolic disorder via a high caloric diet, is when ob/ob or db/db mice are used (obesity or diabetes studies); These genetically modified models are already obese and insulin resistance and do not require induction via a high fat diet).

The specific diet being used will depend on the research question(s) to be studied in order to induce metabolic disorder(s): high fat diet for induction of obesity, NASH, nephropathy or retinopathy; high cholesterol diet to induce hyperlipidemia/atherosclerosis or a high fat and cholesterol diet to induce NASH. The study duration and subsequently the length of the study ranges from approx. 4-40 weeks and will depend on the diet being used, the sensitivity of a mouse strain towards this diet, and stage of metabolic disorder development under investigation (as an example: in ApoE*3Leiden.CETP mice NAFLD develops from simple liver steatosis at 4-6 weeks after dietary intervention to steatosis with hepatic inflammation/NASH (around 10 weeks) to NASH with early (around 15 weeks) and subsequently advanced

fibrosis (at 25 weeks); the study duration will then be dependent on the stage to be investigated and mouse strain and diet being used).
Importantly, the specific combination of animal model + diet (e.g. the type of fat) determines which characteristics of metabolic disease will predominantly be induced and via which pathways the metabolic disorder(s) will be evoked.

A study can include the following groups:
1. Negative control group (metabolic disorder is induced, but no intervention).
Also an additional negative control group can be added that reflects the metabolic disease stage at the start of the treatment (if intervention starts after metabolic disorder(s) have been established it might be needed to add this additional control group. The additional control group then allows discrimination between: 1) whether the treatment can stop further progression of the disease development or 2) whether the treatment leads to reversal of metabolic disorder).
2. Positive control group or reference group (metabolic disorder is induced, but also intervention with known beneficial (positive control) or well-established effect (reference control) on the metabolic disorder).
3. Intervention groups (metabolic disorder is induced and interventions to be evaluated are applied)
Interventions may be given as prevention design (administration simultaneously with disease induction) or as treatment design (administration after metabolic disorder has been established) and may be directed to the early phase of metabolic disorder development, the later established phase of metabolic disorder development or the advanced stage. This will also determine the timing and duration of the interventions to be applied.
4. Healthy reference group (no induction of metabolic disorder: mice on control diet: low fat or chow diet)

Additional groups can be added as well: additional groups for PK (pharmaco-kinetic) analysis could be added if an additional research question is also to obtain more PK information on the administered compounds.
Furthermore, it can also be decided to first perform a pilot experiment if crucial information is lacking (for example, first a dose-finding pilot study could be performed to find the optimal dose).

Besides directly testing the efficacy of different prevention- or intervention-therapies (e.g. by lifestyle, nutrition and pharmaceuticals), also more basic research studies are performed aiming at understanding the sequence of events or underlying mechanisms of the disease development. For understanding the development of metabolic disorders, time-course studies might be performed in which groups of animals are compared after a different time period of diet administration (for instance to unravel whether metabolic dysfunction of one organ precedes metabolic dysfunction of another organ).
Also animal studies can be performed aiming at developing a novel model or to improve existing models. For all studies, it is very important that we use translational models that reflect the metabolic disorder(s) in humans as much as possible. We are continuously improving our in vivo models, and perform comparative studies using human organ tissues and human-based plasma readouts (e.g. same biomarkers). An improvement of the model could be for instance a) an adaptation of the diet being used to induce the metabolic disorder, or b) use of a more translational mouse strain.

The primary outcome parameters will depend on the specific research question:

- For **obesity** studies primary outcome parameters will be body composition (body weight and fat mass)
- For **Diabetes** studies, the primary outcome parameters will be insulin resistance, measured via blood glucose/plasma insulin levels, oral glucose tolerance or insulin tolerance tests or hyperinsulinemic euglycemic clamps.
- For **lipid** studies, the primary outcome parameters will be plasma lipids.
- For **atherosclerosis** studies the primary outcome parameters will be histological analysis of plaque size and severity in aortic root area or aorta.
- For **NASH** studies the primary outcome parameters will be the histological analysis of NASH and fibrosis, similarly as is currently done in human patients to diagnose NASH. Also biochemical measurements of liver lipids or collagen can be added.
- For **nephropathy** studies the primary outcome parameters will be histological analysis of kidneys, as well as kidney function measured by creatinine/albumin ratio in urine or glomerular filtration rate.

- For **retinopathy** studies the primary outcome parameters will be the measurement of structural changes of the retina for instance with a retinal imaging camera.

In general, body weight and food intake will be monitored and blood/plasma measurements will be performed to analyse risk factors for the metabolic disorder. At the end of each study, tissues will be collected for analysis of primary outcome parameters. Typically, histopathological analysis of tissues other than mentioned in the list of primary outcome parameters such as gut and adipose tissue (as secondary endpoint) can be performed as well because they constitute key drivers of the primary endpoint and are investigated as secondary research question.
(Remark: It is an intrinsic feature of metabolic dysfunction that several organs become diseased, often requiring the analysis of a primary endpoint in context of a secondary endpoint.)

Describe the proposed animal procedures, including the nature, frequency and duration of the treatment. Provide justifications for the selected approach.

As mentioned above, the precise study design depends on the primary and secondary research question and type of metabolic disorder (or combinations thereof) being studied. In most (<95%) of our studies metabolic disorder is induced via diet manipulations. The exception is when ob/ob or db/db mice are used (obesity or diabetes studies). These genetically modified models are already obese and insulin resistance and do not require induction via a high fat diet.
During every study body weight and food intake are monitored regularly, different blood samples will be taken and at the end mice will be sacrificed and tissues will be collected. Depending on the research questions, additional procedures, either for induction of metabolic disorder or for measurement of additional parameters could be added to the study. These additional procedures are described below for the different metabolic disorders.

For all metabolic disease studies applies that interventions can be performed in a prevention design (simultaneously with induction of disease) or as treatment design (after disease induction). There are different possibilities for administration routes of compounds:
- Oral administration via diet admix of via drinking water
- Oral administration (p.o.) by gavage
- Intraperitoneal injection (i.p.)
- Subcutaneous injection (s.c.)
- Intravenous injection (i.v.)
- Intramuscular injection (i.m.)
- Via osmotic mini-pump (s.c.): Using appropriate anesthesia and analgesia, osmotic minipumps suitable for mice will be placed subcutaneously. During an experiment, it might be necessary to replace the minipump. Maximal 4 minipumps will be placed sequentially.

Maximum volume / frequency to be used are according to what is considered good practice (Diehl *et al.*, J Appl Toxicol. 2001 Jan-Feb; 21(1):15-23).
In addition to an intervention with diets, nutrients or pharmaceutical drugs, lifestyle interventions (e.g. exercise via running wheel) can be performed (alone or in combination), essentially as it is common practice for patients with metabolic disease.

During the study mice will be checked daily and body weight and food intake will be monitored regularly by weighing the mice individually and weighing food per cage.

During the study at several time-points blood samples will be taken to measure risk factors for the metabolic disease being studied. Blood samples can be taken:
- via tail vein after 4-5 hours fasting
- via tail vein after overnight fasting (in particular cases it may be necessary to collect blood after prolonged fasting, e.g. when ketone bodies or free fatty acids or other markers of fasting are measured or if we need blood samples that do not contain chylomicrons or any food derived markers).
- via tail vein non-fasted (in particular cases it may be necessary to collect non-fasted blood, e.g. after postprandial challenge tests after meal or to assess intestinal absorption of a food component, metabolite, gut integrity marker or nutrient).

Maximum volume / frequency to be used are according to what is considered good practice (Diehl *et al.*, 2001).

At the end of the experiment mice will be euthanized, and plasma and different tissues will be collected.

During the study the following additional induction or intervention procedures could be added:

Obesity studies
Fat removal or transplantation (to study the fat-fat or fat-other organ interaction)
To gain more insight into the metabolic function of the different fat depots and the disease-promoting effect of inflamed adipose tissue on other metabolic disorders (e.g. NAFLD development), it is possible that in some (fundamental) studies we would like to transplant white or brown fat from one mouse to another or remove a specific fat depot (mimicking liposuction of excess fat in humans). Appropriate anesthesia and analgesia will be used.

Generic fat loss/redirection to healthy weight
A generic loss of adipose tissue mass (unspecific and not limited to a particular depot) can be achieved with restricted or alternate diet feeding (switch from high fat diet to chow/low fat diet). This type of dietary challenge is a specific form of lifestyle intervention and advised to patients suffering from metabolic disease. It may be applicable in some of the studies, alone or in combination with other therapies (nutritional and pharmaceutical). Generic weight loss may also be achieved with voluntary exercise (e.g. running wheels) as it is commonly advised in humans to strengthen muscles and burn excess energy/calories through intensified movement. In some of our studies these generic lifestyle interventions for metabolic disease will be employed and the calories burnt will be determined (e.g. via indirect calorimetry, analysis of the resultant of exercise (e.g. muscle strength).

Lipid and atherosclerosis studies
Drinking water interventions
- Fructose in drinking water and other fructose-containing liquids stimulate VLDL production, which will result in a more atherogenic lipoprotein profile

Nephropathy studies
Drinking water interventions
- Blood pressure increases by administering a vasoconstrictor in drinking water. L-NAME and L-NNA (N (G) -nitro-L-arginine methyl ester) are NOS inhibitors that increases the blood pressure. Nitric oxide (NO) is a gas molecule which plays an important messenger role in various biological processes. In blood vessels NO relaxes the smooth muscle cells which results in vasodilatation and in this way, it can reduce blood pressure. NO can be made from endogenous L-arginine and oxygen by means of various nitric oxide synthase (NOS) enzymes.

Partial nephrectomy (to augment nephropathy in the remaining kidney)
With respect to kidney function, all organisms have an overcapacity and one kidney is typically sufficient to maintain metabolic health (therefore it is allowed to donate one kidney to a patient). To augment the development of metabolic kidney disease and induction of e.g. diabetic nephropathy in the remaining kidney, in some nephropathy studies, renal mass will be removed during the experiment. Appropriate anesthesia and analgesia will be used.

Furthermore, during the study additional parameters could be measured. Depending on the metabolic disorder being studied and the research question, a combination of parameters will be chosen that best answers the research question. During the study the following procedures could be added for measurement of additional parameters:

Obesity studies
Feces collection (for measurements of metabolites, proteins, for example, to be able to make an energy balance or microbiota analysis)
- By collecting feces after several days from the cage bed (on group level) or after lifting mice (individual

feces collection)
- By taking rectal swabs

Non-invasive imaging measurements
- Body composition (fat, water and lean body mass) can be measured via placement in Echo MRI (about 1-3 minutes) without anesthesia.
- Other imaging methods relevant for metabolic disease assessment such as (f)MRI and fluorescence imaging for instance could be used for more precise measurement of abdominal fat, ectopic fat or detection of metabolic disease-related morphological changes, by imaging of reflected light or changes of electromagnetic fields under appropriate anesthesia.
- Indirect calorimetry using the TSE system. The mice are temporarily housed individually (max. one week) in sealed cages, which are comparable in terms of bedding and size with the normal housing cages. There is a constant air flow through the cage. The air goes into the cage and the air cage again is measured by VO_2 and VCO_2. From this the energy consumption and the oxidation of the substrate can be measured as a measure of metabolic performance. In addition, the continuous activity is measured by infra-red light streams around the cage.

Challenge measurement (to measure metabolic resilience after application of acute metabolic stressor)
- Administration of inflammatory (e.g., bolus of interleukin; i.p. or s.c. or orally) or dietary challenge (bolus of dietary fat, lipids, carbohydrates, dextran, cholesterol) to assess the metabolic or inflammatory response after set time points.
- Challenges to measure gut permeability (with administration of FITC-dextran, 14C-PEG200 or heat-inactivated bacteria).
Blood samples can be taken before, during and after the challenge (one or more blood samples, depending on the specific research question).

Grip strength and inverted screen test (to measure muscle strength)
Grip strength will be determined by placing mice with two or four limbs on a grid attached to a force gauge and steadily pulling the mice by their tails. Grip strength is defined as the maximum strength produced by the mouse before releasing the grid. On each occasion five trials will be performed for each mouse with a 1-minute resting period between the trials.
Inverted screen test will be performed by placing the mouse in the center of a wire mesh screen and then rotating the screen to an inverted position within 2 seconds. The time when the mouse lets go of the grid is noted or the mouse is removed when the criterion time of 60 seconds is reached. If a mouse lets go of the grid within 10 seconds, another trial will be performed with a maximum of three trials.

Diabetes studies
Glucose tolerance test (indicative measurement of insulin sensitivity)
After 4-5 hours or overnight fasting a first blood sample is taken via tail vein (t=0 minutes). Thereafter, the glucose tolerance test will be started. For this purpose, the mice receive a glucose bolus (2 g / kg via oral gavage or via ip injection), after which small blood samples will be taken after 5, 15, 30, 45, 60 and 120 minutes for the measurement of blood glucose (and optional plasma insulin). As compared to the hyperinsulinemic euglycemic clamp this method is less accurate, but easier and not lethal.

Insulin tolerance test (measurement of insulin clearance)
After 4-5 hours or overnight fasting a first blood sample is taken via tail vein (t=0 minutes). Thereafter, the insulin tolerance test will be started. For this purpose, the mice receive an insulin bolus (0.75-1U / kg g / kg, via ip injection), after which small blood samples will be taken after 15, 30, 60 and 120 minutes for the measurement of plasma insulin (and optional blood/plasma glucose).

Hyperinsulinemic euglycemic clamp (measurement of insulin sensitivity; non-recovery)
Mice are fasted overnight, and then anesthetized with appropriate injection anesthesia. During this clamp procedure insulin will be administered via a primed continuous intravenous infusion for 2 to 3 hours to attain steady-state circulating insulin levels. A variable glucose infusion will also be started and adjusted to maintain euglycemia, measured at 10-minutes intervals via tail bleeding. Both hepatic and peripheral

insulin sensitivity as well as tissue-specific insulin sensitivity (in heart, fat, and muscle) can be determined (using a 3 H-glucose label to measure peripheral and hepatic insulin resistance, and in addition, 14 C-deoxyglucose label for assessment of tissue-specific insulin resistance). Blood samples will be taken during the procedure and at the end mice will be sacrificed via cervical dislocation and heart puncture will be performed and tissues will be collected.

Hyperglycemic clamp (measurement of insulin secretion; non-recovery)
Mice are fasted overnight, and then anesthetized with appropriate injection anesthesia. During this clamp procedure glucose will be administered via a primed continuous intravenous infusion for 2 to 3 hours to attain hyperglycemia. Blood samples will be taken after 0, 2, 5, 10, 15, 40, 50 and 60 minutes for measurement of plasma insulin and at the end mice will be sacrificed via cervical dislocation and heart puncture will be performed and tissues will be collected.

Lipid and atherosclerosis studies
Deuterated water administration (D_2O)
To be able to trace newly formed proteins (lipids for instance) within a given period, D_2O can be given for a short period (number of days) or long (several weeks) period in our studies. Labeling can take place at various times of the study and depends on the specific question. D_2O will be built into all the newly synthesized proteins and in this way newly formed protein can be traced. On first day, the mice receive a single i.p. injection with body warm D_2O 100% / 0.9% NaCl to label the body water around 2-5% of the mouse. Then the D_2O body water levels will be maintained by adding D_2O in the drinking water (containing 4-8% D_2O) until sacrifice.

Feces collection (for measurements of fatty acids, sterols, for example, to be able to make an energy balance or microbiota analysis)
- By collecting feces after several days from the cage bed (on group level) or after lifting mice (individual feces collection)
- By taking rectal swabs

VLDL production (measurement of VLDL production; non-recovery)
Mice are fasted for 4 hours, and then anesthetized with appropriate injection anesthesia. Under anesthesia Tran 35S-label is injected intravenously in the tail for measurement of the apoB novo synthesis and 30 minutes thereafter the mice are injected with Triton WR 1339 (iv, tail) for complete blocking of VLDL clearance. 0, 15, 30, 60 and 90 minutes after Triton injection, a blood sample (40 µl) is taken via the tail for measurement of plasma triglycerides (VLDL production measurement). After 90 minutes, the mice are sacrificed via cervical dislocation and the remaining blood is collected via cardiac puncture for VLDL isolation and determination of the ApoB novo synthesis and lipid composition VLDL. Different tissues can be isolated as well.

VLDL clearance (measurement of VLDL clearance; non-recovery)
For this purpose, mice are anesthetized intraperitoneally with appropriate injection anesthesia. Mice will receive VLDL-like particles labeled with 3 H-triolein and 14C-cholesteryl oleate via an injection into the tail. After 2, 5, 10 and 15 minutes, a blood sample is taken via the tail (40 µl per time point). The mice are sacrificed by cervical dislocation and blood is collected at the end of the experiment by means of a heart puncture and different tissues are isolated as well. 3H and 14C activity is measured in tissues and plasma samples.

Blood pressure measurement
Carried out using non-invasive tail cuff measurement, in a restrainer without anesthesia.

Gall bladder cannulation (to measure bile production; non-recovery).
This experiment is carried out under appropriate anesthesia and implies that the gall bladder is cannulated, then every 15 minutes bile is collected for 1 hour (4 time points). At the end of the experiment mice are euthanized by means of cervical dislocation, followed by section and tissue removal.

Challenge measurement (to measure metabolic resilience after application of acute metabolic stressor)
- Administration of dietary challenge (bolus of dietary fat, lipids, bile acids, fatty acids, cholesterol) to assess the metabolic response after set time points.
- Challenges to measure gut permeability (with administration of FITC-dextran, 14C-PEG200 or heat-inactivated bacteria).
- Challenges with heparin administration for determination of lipoprotein lipase (LPL) activity
- Challenges using injection of thioglycolate (1 mL, 3% thioglycolate i.p.) for the isolation of peritoneal macrophages. This creates an inflammatory response. After 4 days, the mice are sacrificed by CO2 inhalation and the macrophage will be isolated by peritoneal lavage.

Blood samples can be taken before, during and after the challenge (one or more blood samples, depending on the specific research question).

NASH studies
Deuterated water administration (D_2O)
To be able to trace newly formed proteins (hepatic collagen for instance) within a given period, D_2O can be given for a short period (number of days) or long (several weeks) period in our studies. Labeling can take place at various times of the study and depends on the specific question. D_2O will be built into all the newly synthesized proteins and in this way newly formed protein can be traced. On first day, the mice receive a single i.p. injection with body warm D_2O 100% / 0.9% NaCl to label the body water around 2-5% of the mouse. Then the D_2O body water levels will be maintained by adding D_2O in the drinking water (containing 4-8% D_2O) until sacrifice.

Non-invasive imaging measurements
- Body composition (fat, water and lean body mass) can be measured via placement in Echo MRI (about 1-3 minutes) without anesthesia.
- Other non-invasive imaging methods relevant for metabolic disease assessment such as (f)MRI, FibroScan and fluorescence imaging for instance could be used for more precise measurement of hepatic fat or fibrosis or detection of metabolic disease-related morphological changes for example by imaging of reflected light or changes of electromagnetic fields under appropriate anesthesia.

Removal of a liver lobe or liver biopsy (for interim NASH and fibrosis measurement)
In clinical settings, liver biopsies are taken to diagnose liver disease such as NAFLD and to estimate the efficacy of a treatment. To mimic the situation in patients, the NASH development over time during the experiment within one animal is measured in some studies. Appropriate anesthesia and analgesia will be used and one liver lobe will be ligated and removed or a small liver biopsy will be taken.

Challenge measurement (to measure metabolic resilience after application of acute metabolic stressor)
- Administration of inflammatory (e.g., bolus of interleukin; i.p. or s.c. or orally) or dietary challenge (bolus of dietary fat, lipids, bile acids, fatty acids, carbohydrates, dextran, cholesterol) to assess the metabolic or inflammatory response after set time points.
- Challenges to measure gut permeability (with administration of FITC-dextran, 14C-PEG200 or heat-inactivated bacteria).

Blood samples can be taken before, during and after the challenge (one or more blood samples, depending on the specific research question).

Nephropathy studies
Urine sampling (for measurements of proteins and metabolites)
- Spontaneous excretion caused by lifting the mouse and collection of urine. If the mouse does not lose urine spontaneously, light bladder massage can be applied.
- Urine sampling overnight (for detailed measurement of excretion of proteins and metabolites). Mice will be housed individually in an excretion collection cage overnight (16 hours). Urine and feces can be sampled.

Glomerular filtration rate (=GFR) measurement

As a measure for kidney function/microvascular function by determining the rate of loss and re-absorbance of vital factors such as protein and carbohydrates. This is the golden standard measurement for kidney function. This can be done in 2 different ways:
1) Via iv bolus injection of 5% FITC-inulin, followed by small blood samples via tail cut after 3, 7, 10, 15, 35, 55 and 75 minutes to measure clearance of 5% FITC-inulin.
2) Via an osmotic minipump. Blood samples will be collected at start and end of the pumping period. In between, urine will be sampled.

Non-invasive imaging measurements
- Non-invasive imaging methods relevant for metabolic disease assessment such as (f)MRI and fluorescence imaging for instance could be used for more precise measurement of blood vessel function or detection of metabolic disease-related morphological changes for example by imaging of reflected light or changes of electromagnetic fields under appropriate anesthesia.
- Blood pressure measurement using non-invasive tail cuff measurement, in a restrainer without anesthesia.

Retinopathy studies
Non-invasive imaging measurements
- Other non-invasive imaging methods relevant for metabolic disease assessment such as (f)MRI, Raman and fluorescence imaging for instance could be used for more precise measurement of blood vessel function, detection of metabolic disease-related morphological changes, retinopathy for example by imaging of reflected light or changes of electromagnetic fields under appropriate anesthesia.

If the research question of the study extends beyond one metabolic disorder and is also a combination of one of the above mentioned metabolic disorders, also a merged study design could be used and a combination of procedures described, dedicated to a specific metabolic disorder in this appendix could be combined. However, the number of different procedures in total will be considered and the cumulative discomfort will not exceed above moderate.

Describe which statistical methods have been used and which other considerations have been taken into account to minimise the number of animals.

Whenever possible we strive for a situation to express the outcome of our experiments in quantitative terms. To estimate the number of animals to be used in an experiment, we use the effect size (if known, e.g., from data in the literature, from our own historical data or years of experience with similar type of experiments or if unknown from pilot studies) to estimate the sample size needed to achieve a certain power (usually between 0.8-0.9) with appropriate statistical tests like the t-test with a $p<0.05$.
As an indication: usually n=8 mice will be used for lipid studies, n=12 mice per group for obesity, Diabetes and nephropathy studies and n=15 mice per groups for NASH , atherosclerosis and retinopathy studies, based on variation in the respective primary outcome parameters.

In addition, there could be low-responders / outliers. For ApoE*3Leiden and ApoE*3Leiden.CETP mice it is well known that a certain percentage of the mice does not respond to a cholesterol containing diet with respect to the development of hyperlipidemia. For lipid and atherosclerosis studies, these mice will be excluded in the beginning of the study to reduce the variation and thus the number of animals required per group. Although for NASH parameters it is not very clear yet to predict which mice develop NASH or not, recent data has suggested that mice (both ApoE*3Leiden.CETP mice as well as LDLR-/-.Leiden mice) with relatively low blood glucose levels at the start of the study have a higher propensity to develop extremely high levels of hepatic fibrosis after several weeks on certain diets. In future NASH studies, we might decide to exclude these mice in the beginning of the study, to achieve a more homogenous induction of fibrosis. For diabetes studies using ob/ob, db/db or KKA^y mice, outliers with respect to glucose and insulin levels can be excluded at the beginning of the study to start the treatment with more homogenous groups.
If possible different study groups will be combined, so that different treatment groups can share the same control groups.

Several research questions focus on therapeutic interventions in animal model where metabolic diseases and its complications have been established. To this end mice have to be put on the high caloric diets first to induce the metabolic disease. Mice are not useful anymore in starting new studies from the age of 22 weeks. To reduce breeding surplus and extend the usability of our mice, several cohorts of the mouse strains we breed ourselves (ApoE*3Leiden(.CETP) or LDLR-/-.Leiden mice) will be put on a high caloric diet (e.g. high fat or high fat with cholesterol diet) without yet being assigned to a definite study protocol. In this way, the usability of these mice is extended because these mice can be used in yet to be agreed future studies that require a run-in period on a high fat or high fat and cholesterol diet. This will extend the period that mice can be assigned to studies well beyond the age of 22 weeks.
During this period of high fat or high fat and cholesterol diet, 2-3 times a blood sample can be taken for the measurement of cholesterol, triglycerides, glucose, insulin, or other parameters. At t=0 for baseline and 1-2 additional time points to monitor in-life the development of metabolic disease and to eliminate non-responders to diet or biological outliers (to homogenize the study population). This possibility is described in our breeding policy to ensure optimal breeding and use of animals and reduction of surplus animals.

B. The animals

Specify the species, origin, estimated numbers, and life stages. Provide justifications for these choices.

Mice, ApoE*3Leiden, ApoE*3Leiden.CETP mice or LDLR-/-.Leiden mice from our own breeding facility, 8-22 weeks old will be used. In addition, C57BL6/J wild-type mice, ob/ob, db/db, KKA^y, ApoE-/-, LDLR-/- mice obtained from commercial breeders, 8-22 weeks old can be used.

Obesity studies & **Diabetes** studies: C57BL6/J mice can be used, when put on a high fat diet these mice become obese and insulin resistant. In addition, ob/ob, db/db, KKA^y mice can be used. The latter genetic models are all obese and insulin resistant/diabetic mice and can also be used without additional induction of high fat diet.

For **lipid** studies & **atherosclerosis** studies the following mice can be used:
*ApoE*3Leiden*: Mice carrying a human APOE*3Leiden transgene that leads to a defective clearance of triglyceride-rich lipoproteins. While normal wild-type mice have a very rapid clearance of triglyceride rich lipoproteins, ApoE*3Leiden (E3L) mice have an impaired clearance and are thereby mimicking the slow clearance observed in humans. APOE*3-Leiden transgenic mice are highly responsive to fat, sugar and cholesterol feeding with respect to the effects on plasma cholesterol and triglyceride levels.
APOE*3Leiden animals have proven to be responsive to the most of the drugs that are also used in the clinic, and therefore extremely suitable in combination / comparison studies. The animals also respond to lifestyle interventions, dietary supplements, anti-oxidants, omega-3 PUFAs, hormones and pre / probiotics. Males and females can be used for studies of lipids, only females are suitable for atherosclerosis research. Male mice do not/hardly develop atherosclerosis but do in turn develop insulin resistance and liver disease (NAFLD).
*APOE*3Leiden.CETP*: In contrast to humans, wild type mice express no CETP (which transfers cholesterol from HDL to (V)LDL). The double transgenic ApoE3*Leiden.CETP mouse brings CETP to expression and therefore this model is translational to the human situation regarding HDL metabolism. Furthermore, this mouse has the same characteristics as the APOE*3Leiden mouse regarding its (V)LDL metabolism.
LDLR-/- and LDLR-/-.Leiden mice: both males and females can be used for lipids and atherosclerosis research. The mice lack a specific receptor (Ldlr) and reflect a particular group of patients that have the same genetic impairment (patients with defective or absent Ldlr). LDLR-/-.Leiden mice are an established substrain of LDLR-/- mice that are susceptible to become obese on energy dense diets and that activate proinflammatory and profibrotic pathways in response to diets with human-like composition of macronutrients.

ApoE-/-mice: both males and females can be used for lipid and atherosclerosis research. This is a more severe model than all the above models, with higher lipid levels and more atherosclerosis.

For **NASH** studies the following mice can be used:
Mice, ApoE*3Leiden with or without CETP (ApoE*3Leiden.CETP) mice or LDLR-/-.Leiden mice, from our own breeding facility, 8-22 weeks old.

*ApoE*3Leiden(.CETP) mice* are mice that have a human-like lipoprotein metabolism and when put on a high fat and high cholesterol diet these mice develop obesity, dyslipidemia, mild insulin resistance and several characteristics of NASH (steatosis, inflammation and hepatic fibrosis).
LDLR-/-.Leiden mice develop hyperlipidemia when treated with all sorts of high caloric diets. These mice develop pronounced obesity and insulin resistance and several characteristics of NASH (steatosis, inflammation and hepatic fibrosis).
Both models develop diet-induced NASH, but the underlying mechanisms differ: in ApoE*3Leiden(.CETP) mice hepatic cholesterol crystals are being formed, leading to hepatic inflammation and lipotoxicity, which probably plays a role in the NASH induction. In both models the increase in white adipose tissue is thought to be involved in the NASH induction, but in the LDLR-/-.Leiden mice this mechanism plays a more prominent role.

At this moment, we know for all our strains of mice that male mice develop NASH and fibrosis when put on the high fat or high fat and cholesterol diet. Male mice are more susceptible to become obese in response to high caloric diets and accumulate fat in the abdominal cavity (essentially as it is also the case in humans with metabolic disease). For ApoE*3Leiden.CETP and LDLR-/-.Leiden mice we are currently evaluating whether the female mice also develop NASH and fibrosis (*in vivo* studies ongoing). Depending on the outcome, female mice might be used as well.

Nephropathy studies: ob/ob, db/db, KKA^y mice are all obese and insulin resistant/diabetic mice and will be used for nephropathy studies.

Retinopathy studies: ApoE*3Leiden(.CETP) mice and LDLR-/- mice on a high fat diet develop retinopathy and will therefore be used for retinopathy studies.

The required number of mice will be estimated to be 10.000 for a period of 5 years. This is based on an expected number of metabolic disorder studies of approx. 25 per year (based on historical data of last 2 years). The average study will consist of 5-6 groups of 12-15 mice each, so on average 80 mice per study. This leads to a required total number of 10.000 mice (25 studies x 80 animals / study x 5 years).

C. Re-use

Will the animals be re-used?

☒ No, continue with question D.

☐ Yes > Explain why re-use is considered acceptable for this animal procedure.

Are the previous or proposed animal procedures classified as 'severe'?

☒ No

☐ Yes> Provide specific justifications for the re-use of these animals during the procedures.

D. Replacement, reduction, refinement

Describe how the principles of replacement, reduction and refinement were included in the research strategy, e.g. the selection of the animals, the design of the procedures and the number of animals.

Replacement: We continuously seek to replace animal studies with other methods, preferably using human tissue or cells. For example, we validate our animal pathology and molecular observations in human biopsy material (e.g. liver, adipose tissue, muscle tissue but also human plasma and microbiota). However, fresh human tissue is scarce and often not fresh enough to quantify sensitive molecules with rapid turn-over or short half-life such as mRNAs, cytokines and hormones, bioactive lipids (see also Vital tissue whitepaper:
http://mailing.tno.nl/ct/m8/k1/lG9BLvQKYu1MheK9DpQtFAFy3yeEoutjgfEr6Gj5ucJVVT1LUJPXZ6kSA7n8Q0sK4vv7T0WuHMUTxdHEBnJhNg/ZD8yND3CLIt58HW). We work with human cell and in silico models, where possible, since that helps us to make better predictions about how a substance, for example a drug, will affect humans. When we have discovered ways to replace animal testing, we use them

ourselves and encourage others to apply these alternative tests. Non-testing techniques, such as computer modelling, are continually being developed and improved.
Before we consider to perform a novel metabolic disease study, we will first analyse appropriate cell lines, existing patient materials or materials available from previous animal studies, which contributes to a reduction of animal numbers. The use of human material and in vitro cultures allows this project to comply with the 3Rs (replacement, reduction and refinement) by keeping the animal numbers to a minimum.
However, animal studies are currently unavoidable to study the complex organ-organ interactions that are intrinsic to all metabolic diseases and the resulting metabolic disease complications (for example cardiovascular or hepatic endpoints). TNO has launched a program Organ-on-a-Chip (see also Organ-on-a-Chip whitepaper: http://publications.tno.nl/publication/34622299/rkPoAJ/grootaers-2016-three.pdf) that aims at mimicking organs and combining them via a fluidic system and we are involved as partner in many international consortia. These tools do currently not mimic the organ-organ interactions and the elementary features of metabolic disease (e.g. ectopic fat accumulation, vascular dysfunction) and are still in their infancy. The development of metabolic diseases is a too complicated multifactorial process in which multiple organs interact in an orchestrated way (gut, liver, adipose tissue, muscle, vascular system) to provide energy from food and distribute it to the other organs. Another layer of complexity is the involvement of multiple different cell types (e.g. immune cells) most of which cannot be cultured in vitro without activating them. Hence, given the complexity of the glucose and lipid metabolism and metabolic health in general, the effect of compounds or nutritional interventions on metabolism can be examined only in intact animal models with intact vascular system and there are currently no established alternative methods to investigate these metabolic processes.
Reduction: TNO aims to reduce the number of animals involved in testing. We regularly review our testing methods and implement integrated testing strategies. This helps us to determine whether animal testing is needed or whether the same information can be obtained in other ways. Data simulations are performed to determine the optimal study design that will provide the most valuable information with the smallest number of animals in each experiment. Whenever possible, we will perform pilot studies with the minimum number of animals possible. Experiments will be done sequentially, where on basis of the results, decisions will be taken for the next steps. If possible different study groups from different studies will be combined, so that they can share the same control groups.
Refinement: We continuously aim to develop, validate and adapt (preferentially non-invasive) test methods in such a way that the test animals are exposed to as little discomfort and stress as possible (e.g. use of echoMRI as a non-invasive method to measure body composition within one animal at multiple time-point during a study, so leads to both refinement as well as reduction). All animal handlings will be carried out by authorized and qualified persons. Where possible, all animals will be group housed and environmental enrichment will be provided.

Explain what measures will be taken to minimise 1) animal suffering, pain or fear and 2) adverse effects on the environment.

Appropriate possibilities to reduce pain, fear or suffering will be used. All work will be carried out by a small number of highly trained bio-technicians. Injection fluids will be brought to room or body temperature before injection. In consultation with the attending veterinarian, surgery appropriate peri-operative care, including appropriate anaesthesia and analgesia, is determined and frequently reviewed and updated towards best practices. The welfare of the animals will be daily observed by different people. If there are clear signs of unexpected severe discomfort, the animals will be euthanized.
There are no negative environmental effects; all mice will be housed under strict D1 conditions.

Repetition and duplication

E. Repetition

Explain what measures have been taken to ensure that the proposed procedures have not already been performed. If applicable, explain why repetition is required.

N.a.

Accommodation and care

F. Accommodation and care

Is the housing and care of the animals used in experimental procedures not in accordance with Annex III of the Directive 2010/63/EU?

☒ No

☐ Yes > If this may adversely affect animal welfare, describe how the animals will be housed and provide specific justifications for these choices.

G. Location where the animals procedures are performed

Will the animal procedures be carried out in an establishment that is not licenced by the NVWA?

☒ No > Continue with question H.

☐ Yes > Describe this establishment.

Provide justifications for the choice of this establishment. Explain how adequate housing, care and treatment of the animals will be ensured.

Classification of discomfort/humane endpoints

H. Pain and pain relief

Will the animals experience pain during or after the procedures?

☐ No > Continue with question I.

☒ Yes > Will anaesthesia, analgesia or other pain relieving methods be used?

☐ No > Justify why pain relieving methods will not be used.

☒ Yes > Indicate what relieving methods will be used and specify what measures will be taken to ensure that optimal procedures are used.

For more invasive procedures appropriate anesthesia / analgesia will be used or procedure will be terminal. In consultation with the attending veterinarian, surgery protocols, including appropriate anaesthesia and analgesia is determined and frequently reviewed and updated towards best practices.

I. Other aspects compromising the welfare of the animals

Describe which other adverse effects on the animals' welfare may be expected?

The induction of the metabolic disorders obesity, hyperlipidemia, atherosclerosis and NASH do not lead to discomfort. Similarly as in humans, that are often unaware of these disturbances until a very late stage. For most Diabetes studies (>95%) we will also not reach the very severe diabetic stage which is accompanied by discomfort to the mice. Only in long-lasting Diabetes and in nephropathy studies using db/db or KKA^y mice, severe Diabetes, characterized by polyuria, can develop and can lead to (moderate) discomfort.
With respect to discomfort caused by interventions: it is possible that novel compounds have an adverse effect in these animal models, especially during the disease induction in NASH studies when normal liver function gets compromised.

Explain why these effects may emerge.

Although most novel compounds to be tested are expected to have a beneficial effect, we cannot exclude the possibility that the combination of the novel compound with our models leads to unexpected adverse effects. The likelihood (based on historical data of last 5 years) is <0.1%.

Indicate which measures will be adopted to prevent occurrence or minimise severity.

Upon start of treatment/intervention mice will be closely monitored and upon signs of discomfort or adverse effects, the situation will be discussed with the animal welfare officer or veterinarian and if

possible proper measurements will be taken to relief the animal discomfort or animals will be taken out of experiment and sacrificed.

J. Humane endpoints

May circumstances arise during the animal procedures which would require the implementation of humane endpoints to prevent further distress?

☐ No > Continue with question K.

☒ Yes > Describe the criteria that will be used to identify the humane endpoints.

In general the induction of the metabolic disorder(s) do not lead to discomfort. Treatments of the animals can have adverse effects and may lead to (unexpected) discomfort. Mice will be monitored daily and in case of discomfort, cages will be labeled and the affected mice will be closely monitored to observe whether the health status is improving or deteriorating. Deterioration of the health status with unexpected weight loss severe wounds and/or signs of general sickness and/or discomfort, will lead to the decision that mice will be euthanized.

Indicate the likely incidence.

For obesity, Diabetes, lipid, atherosclerosis, NASH, nephropathy and retinopathy studies: <1%.

K. Classification of severity of procedures

Provide information on the expected levels of discomfort and indicate to which category the procedures are assigned (‘non-recovery’, ‘mild’, ‘moderate’, ‘severe’).

In general, the cumulative discomfort for metabolic disorder(s) studies will be mild to moderate and will not extend beyond moderate. The different procedures that can be used, will all be within good practice and are assigned to the following categories:

Animal procedure:	Level of discomfort:
Diet interventions	mild
Drinking water interventions	mild
D_2O drinking water	mild
Exercise (running-wheel)	mild
Grip strength and inverted screen test	mild
4 or 5 hours fasting	mild
Overnight fasting	mild
Multiple blood sampling via tail vein within good practice	mild
Single or multiple gavage or injections (iv, ip, im, sc)	mild
Urine collection	mild
Feces collection or fecal swabs	mild
Indirect calorimetry (TSE systems)	mild
EchoMRI	mild
Non-invasive imaging	mild - moderate
Glucose tolerance test	moderate
Insulin tolerance test	moderate
Hyperinsulinemic euglycemic clamps	non-recovery
Hyperglycemic clamp	non-recovery
Glomerular filtration rate	moderate
VLDL production test	non-recovery
VLDL clearance test	non-recovery
Blood pressure measurements	mild - moderate
Challenge tests	mild - moderate
Surgery: s.c. osmotic minipumps	moderate
Surgery: liver lobe dissection / liver biopsy	moderate
Surgery: fat removal / transplantation	moderate
Surgery: kidney mass removal	moderate
Surgery: gall bladder cannulation	non recovery
Euthanasia	mild
Long-lasting studies using db/db mice	mild - moderate

Long-lasting studies using KKA^y mice	mild - moderate

End of experiment

L. Method of killing

Will the animals be killed during or after the procedures?

☐ No

☒ Yes > Explain why it is necessary to kill the animals during or after the procedures.

Metabolic disease manifests within tissues and cannot be analyzed only via plasma. Therefore, the tissues are needed for measurements: heart, aortic root or aorta for atherosclerosis analysis; livers for NASH and fibrosis measurements; kidneys for analysis of nephropathy; eyes for analysis of retinopathy.

Is the proposed method of killing listed in Annex IV of Directive 2010/63/EU?

☐ No > Describe the method of killing that will be used and provide justifications for this choice.

☒ Yes