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 on the project proposal, see the Guidelines to the project licence application form for animal procedures on our website (www.centralecommissiedierproeven.nl).
- Or contact us by phone (0800-789 0789).

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 numbers provided at 3.4.3 of the project proposal.*	Serial number: 3.4.3.1 — 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.

Metabolic disorder(s)
The different metabolic disorders are not separate diseases but are mechanistically connected: often 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 metabolic disorders, we use a whole set of different mouse models that for instance differ in which aspect of the metabolic disorder develops most prominent (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 **neurodegenerative disease/neuroinflammation** will be studied. An exception (to studies with induction of the metabolic disorder via a high caloric diet) are studies using ob/ob or db/db mice. These genetically modified models have intrinsic metabolic derangements and are already obese and insulin resistance and therefore do not require induction via a high fat diet).

Diet & study duration
In general diets will be used to induce a specific metabolic disorder. The specific diet being used will depend on the research question(s) to be studied and the type of metabolic disorder(s) according to the table below:

Diet:	**Disease induction:**
High fat diet	Obesity
	NASH
	Nephropathy
	Neurodegenerative diseases/neuroinflammation
High cholesterol diet	Hyperlipidemia
	Atherosclerosis
High fat- and cholesterol diet	NASH
Fructose diet	Mitochondrial stress

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.

Groups
A general study design includes 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 evaluated in a prevention design (i.e. intervention simultaneously with induction of disease) or in a treatment design (i.e. intervention after disease induction). The intervention may be directed to intercept in an early phase of metabolic disorder development, in a later more advanced phase of metabolic disorder development or a late 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).

Type of study
Besides directly testing the efficacy of different prevention- or intervention-therapies (e.g. by lifestyle, nutrition and pharmaceuticals or injections of AAV or siRNA), 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 (see Zadelaar et al. ATVB 2004, PMID: 17541027; Morrison et al. Hepatol Communic 2018, PMID: 30556039; van den Hoek et al, Cells 2021, PMID: 32883049). 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; e.g. Morrison et al., Frontiers Physiol, 2018, PMID: 29527177; Martinez-Arranz et al Hepatology, 2022, PMID: 35220605). 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, or c) expression of additional human genes/proteins/metabolites using AAV vectors or siRNA.

Animal handlings/parameters
In general, body weight and food intake will be monitored and blood/plasma measurements will be performed to analyse dedicated risk factors for the different 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, skeletal muscle 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 in a distant organ.)

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

- For **obesity** studies primary outcome parameters will be body composition (body weight, fat mass and inflammation in adipose tissue)
- 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 lipid levels and lipoprotein profiles.
- 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 (Liang et al PLoS ONE 2014, PMID: 25535951). Also biochemical measurement of liver lipids/collagen turnover 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 **neurodegenerative diseases/neuroinflammation** studies the primary outcome parameters will be the measurement of structural changes of the brain using MRI or by histology complemented by brain lipids.

(During the last 5 years we noticed a novel tendency that instead of more conventional plasma/tissue parameters as primary outcome parameter, it's also possible that a gene signature for a certain metabolic characteristic (e.g. fibrosis) is used instead. We expect that in the next 5 years, this trend will continue and gene signatures or (plasma/tissue) biomarkers can be the primary outcome parameter. This might replace the more conventional plasma/tissue parameters in some studies (for instance screening studies of compounds) and may have the benefit that studies can be shortened, since instead of long diet induction studies to get end-point disease, early gene signatures/biomarkers can be used.

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. A typical study, treatment design (prevention design with treatment from the start is also possible), is shown here:

	Disease induction							Treatment period										
week of treatment:	**0**	**1**	**…**	**…**	**7**	**…**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**	**23**	**24**	**25**
Healthy reference group (standard chow diet)	x	→	→	→	→	→	→	x	···	···	···	···	···	···	···	···	···	···	x
Control group: HFD to induce disease	x	→	→	→	→	→	→	x	···	···	···	···	···	···	···	···	···	···	x
Treatment group 1	x	→	→	→	→	→	→	x	···	···	···	···	···	···	···	···	···	···	x
Treatment group 2	x	→	→	→	→	→	→	x	···	···	···	···	···	···	···	···	···	···	x
Treatment group 3	x	→	→	→	→	→	→	x	···	···	···	···	···	···	···	···	···	···	x
Treatment group 4	x	→	→	→	→	→	→	x	···	···	···	···	···	···	···	···	···	···	x
Stratification								x										
Treatment								x	x	x	x	x	x	x	x	x	x	x
Body weight and food intake (cage)	x							x		x			x			x		x
Blood sampling (4/5h fasted)	x							x					x					x
Blood glucose, plasma lipids	x							x					x					x
Sacrifice + collection plasma and tissues																		x
Histological analysis																		x

In most (<95%) of our studies metabolic disorder is induced via diet manipulations akin to humans. The exception is when ob/ob or db/db mice are used (in specific obesity or diabetes studies). These genetically modified models cannot control appetite and overeat irrespective of the type of diet which is why they are already obese and insulin resistant at young age 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/parameters 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.)
- Retro orbital injection (r.o).
- 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 3 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, PMID: 11180276).
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 (this is most often used, short period of fasting during the daytime when mice are naturally not eating is performed to exclude large variations in food derived markers)
- 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, PMID: 11180276).

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

Furthermore, as mentioned during the study additional induction or intervention procedures or additional parameters could be measured. Please note that for most studies (>90%), these additional procedures/parameters are not included, and certainly not altogether! Except for nephropathy studies, which are or most severe studies (moderate discomfort) in which partial nephrectomy, urine sampling and GFR measurements are done in almost all studies.
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/parameters could be added:

Obesity studies
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).

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

Body temperature
Using a rectal probe body temperature can be measured in unanesthetized mice.

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. Elasticity of organs can be measured by sound waves (e.g. echo or opto-acoustic technologies).
- 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 or the CaloBox 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., i.p. bolus of interleukin; or s.c. other inflammatory stressor) or dietary challenge (e.g. a bolus of dietary fat, lipids, carbohydrates, dextran, acetate, 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.

Fat removal (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 remove a specific (abdominal) fat depot (mimicking liposuction of excess fat in humans). Appropriate anesthesia and analgesia will be used.

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
Drinking water interventions
- Fructose in drinking water and other fructose-containing liquids stimulate de novo lipogenesis and subsequent triglyceride and VLDL production, which will result in a more atherogenic lipoprotein profile.

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.

Flux measurements using microdose of 14C tracers and Accelerated Mass Spectrometry (AMS)
- Metabolic fluxes can now easily and minimal-invasively be measured using sophisticated AMS technology in combination with microdose of 14C-labeled metabolites (e.g. 14C-acetate; 14C-amino acids; 14C-fatty acids) ingested by diet or administered by gavage. The method is extremely sensitive and unique in Europe at TNO-Leiden. (For reference, the radiolabel dose is less than the exposure of a passenger on an international flight). AMS allows tracing metabolic fluxes between gut (microbiota-derived molecules), liver and the vasculature towards other organs) via blood and urine measurements to assess e.g. de novo lipid synthesis, fiber fermentation, bile acid production, fructose metabolism etc.

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

Body temperature
Using a rectal probe body temperature can be measured in unanesthetized mice.

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.

Vasodilatation measurement
Measurement of blood flow via Laser Doppler in ears of mice using appropriate injection 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 (e.g. a 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 CO_2 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.

Flux measurements using microdose of 14C tracers and Accelerated Mass Spectrometry (AMS)
- Metabolic fluxes relevant for NAFLD/NASH can be analyzed with 14C labeled metabolites quantified in blood, urine or tissue using AMS technology. The method is extremely sensitive and TNO has the only facility in in Europe (at Leiden). AMS allows tracing metabolic fluxes that affect liver homeostasis including microbiota/gut-derived molecules, adipose and muscle derived molecules as well as bioactive lipids and proteins that can be detected in blood/urine. NAFLD/NASH relevant fluxes include de novo lipid synthesis, fiber fermentation processes and fatty acid formation, bile acid production, fructose breakdown, cytokine production.

Body temperature
Using a rectal probe body temperature can be measured in unanesthetized mice.

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. Elasticity of organs can be measured by sound waves (e.g. echo or opto-acoustic technologies).
- Other non-invasive imaging methods relevant for metabolic disease assessment such as (f)MRI, FibroScan, opto-acoustic (echo) 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.
In the liver biopsies or liver tissue in general, fibrosis gene expression signatures (e.g. van Koppen et al, CMGH 2017, PMID: 29276754) that inform on fibrosis progression or effectivity of an intervention can be analyzed.

Challenge measurement (to measure metabolic resilience after application of acute metabolic stressor)
- Administration of inflammatory (e.g., i.p. bolus of interleukin; or s.c. other inflammatory stressor) 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

In almost all nephropathy studies performed:

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.

Urine sampling (for measurements of proteins and metabolites)

- Spontaneous excretion can be collected by either lifting the mouse and collection of spontaneous loss of urine or to house the mouse on hydrophobic labsand for certain time period. If the mouse does not lose urine spontaneously, light bladder massage can be applied.

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 is done using a transdermal GFR device which is attached to the shaved flank of a freely moving mice. A r.o. bolus injection of FITC-inulin is given and every second a measurement is taken by the device. Using software, the filtration rate can be calculated.

Additional can be added:

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.

Non-invasive imaging measurements

- Non-invasive imaging methods relevant for metabolic disease assessment include (f)MRI, CT and PET 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.

Deuterated water administration (D_2O) or isotope administration for in vivo labeling

To be able to trace newly formed proteins (hepatic collagen for instance) within a given period, D_2O or other label 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 or other label 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 or other label 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.

Neurodegenerative disease/neuroinflammation studies

Non-invasive imaging measurements

- Other non-invasive imaging methods relevant for neurodegenerative disease assessment such as (f)MRI, PET scan, Raman and fluorescence imaging for instance could be used to assess the brain and the vasculature that nourishes the brain cells. MRI for instance allows to assess non-invasively blood vessel function (constriction/relaxation), detection of disease-related morphological brain changes and connectivity between brain regions and white matter integrity. MRI measurements are conducted under appropriate anesthesia to optimize image collection by preventing the animal from moving.
- Functional cognition and behavior tests can be measured using the open field test, the social interaction test, Morris water maze test, novel object recognition test & and balance test (Rotarod).

Combined metabolic disorders studies
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 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 (sigma of approx. 15% and minimal difference of 25%,), n=10 mice per group for obesity, Diabetes and nephropathy studies (sigma of approx. 20% and minimal difference of 30%,) and n=15 mice per groups for NASH, atherosclerosis and neuroinflammation studies (sigma of approx. 25% and minimal difference of 30%), all 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 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. Number of outliers are different per animal model and dependent on specific research question/parameters measured and are therefore determined specifically for each study protocol.
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 diet, fast food diet 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 diet, fast food diet 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, life stages, estimated numbers, gender, genetic alterations and, if important for achieving the immediate goal, the strain.

Serial number	Species	Origin	Life stages	Number	Gender	Genetically altered	Strain
1	Mice	Own breeding facility or commercial breeder	8-22 weeks	10.000	Males and females	Yes and no (depending on strain)	ApoE*3Leiden, ApoE*3Leiden.CETP, Ldlr-/-.Leiden, C57BL/6J wild-type, ob/ob, db/db, KKA^{y}, ApoE-/-, Ldlr-/-, MS-NASH mice, FRG® KO mice

Provide justifications for these choices

Species	Mice
Origin	ApoE*3Leiden, ApoE*3Leiden.CETP mice or LDLR-/-.Leiden mice from our own breeding facility. C57BL/6J wild-type mice, ob/ob, db/db, KKA^{y}, ApoE-/-, Ldlr-/- mice, MS-NASH mice, FRG® KO mice from commercial breeders.
Life stages	8-22 weeks
Number	10.000
Gender	Males and females
Genetic alterations	ApoE*3Leiden, ApoE*3Leiden.CETP, Ldlr-/-.Leiden, db/db, ApoE-/-, Ldlr-/- and FRG® KO. (Ob/ob and KKA^{y} mice are spontaneous mutations, MS-NASH is polygenic inbred model). The genetic alterations do not lead to intrinsic discomfort.
Strain	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-/-, MSNASH and FTO mice obtained from commercial breeders, 8-22 weeks old can be used.

Justification:
Mice are used since within the pharmaceutical industry there is a large preference for the use of mouse models since for these small mammals less pharmaceutical compounds need to be produced. Furthermore, there is great overlap between metabolic processes and organ-organ interactions between mice and men.

Obesity studies & **Diabetes** studies: C57BL/6J 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 (with the disease developing from birth on chow) and can thus also be used without additional induction of high fat diet. MS-NASH mouse, a polygenic mouse model that develops obesity and metabolic syndrome in presence of an intact leptin pathway, might be used as well.

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 more 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. LDLR-/-.Leiden mice will particularly be used if atherosclerosis has to be studied in the context of obesity with insulin resistance, adipose inflammation and gut leakage.
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, insulin resistance and gut leakage, and ultimately several characteristics of NASH (steatosis, inflammation and hepatic fibrosis). In a head-to-head comparison with humans, they were shown to mimic NASH patients with high risk of cardiovascular disease (Martinez-Arranz et al. Hepatology, 2022, PMID: 35220605) and they express fibrosis genes identified to characterize humans with liver fibrosis.

The two above 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.
FGT KO mice: A triple knock-out mouse model (in which the liver has been repopulated for 70-90% with human primary hepatocytes, thereby mimicking the human liver. We might would like to use this model in future NASH studies.

At this moment, we know for all our strains of mice that male mice are to be preferred for NASH and fibrosis studies, since 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) and therefore are providing greater windows for measuring disease endpoints and also surrogate endpoints and blood risk factors (Jacobs et al., Nutrients, 2019, PMID: 31405127).

Nephropathy studies: ob/ob, db/db, KKA^{y} mice are all obese and insulin resistant/diabetic mice and will be used for nephropathy studies. MS-NASH mouse, a polygenic mouse model that develops obesity and metabolic syndrome in presence of an intact leptin pathway, might be used as well.

Neurodegenerative disease/neuroinflammation studies: LDLR-/-.Leiden mice on a high fat diet develop irregularities in cerebral blood flow, high blood pressure and other histological characteristics (e.g. white/grey matter changes; increased Iba1 expression) that are indicative of onset of neuroinflammation and neurodegenerative diseases/dementias. They will therefore be used for neuroinflammation studies.

We previously estimated for the last 5 years to use 10.000 animals in total. At that time based on the historical data of the last 2 years, we predicted to have approx. 25 metabolic disorder studies per year, with an average study of 5-6 groups of 12-15 mice each, so on average 80 mice per study. This led to a required total number of 10.000 mice (25 studies x 80 animals / study x 5 years). In hindsight, we used approx. 8.250 animals (total number at August 2022, so last year not finalized yet). Taking into account that we had less studies during 2 years of COVID-19 pandemic, we would like to apply again for 10.000 animals.

C. Accommodation and care

Is the housing and care of the animals used in experimental procedures in accordance with Annex III of the Directive 2010/63/EU?

☐ Yes

☒ No > If this may adversely affect animal welfare, describe how the animals will be housed and provide specific justifications for these choices

Individual housing (max. 1 month) in case of fighting or for KKA^{y} mice (to prevent fighting and diminish stress KKAy mice are single housed using half-heated cages). Short term fasting during the daytime when mice are naturally not eating (4h/5h) is most often used, and performed to exclude large variations in food derived markers. In some cases overnight fasting for blood sampling is used (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). Urine collection via labsand is performed via individual housing without addition of food and water (since this will distirb the collection) but is also performed for a short period (3h) during daytime when mice are normally not eating. Calobox measurements are also performed via individual housing with/without addition of food (depending on research question).

D. Pain and compromised animal welfare

Will the animals experience pain during or after the procedures?

☐ No

☒ 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 invasive procedures and terminal procedures appropriate anesthesia / analgesia will be used. In consultation with the attending veterinarian, surgery protocols, including appropriate anaesthesia and analgesia is determined and frequently reviewed and updated towards best practices. After surgical procedures increased monitoring of mice is performed and soaked food or solid water may be offered at the bottom of the cage.

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 and diabetic ulcers 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.

E. 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 F

☒ 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. The following criteria are used for this decision and mice are euthanized the same day within 4 hours at a combination of scores ≥2:

- Weight loss >20% (2)
- Abnormal behaviour, such as:
  - Shortness of breath/panting (1)
  - Salivation (1)
  - Not responding to stimuli (2)
  - Persistent shivering (tremors) (2)
  - Persistent convulsions (2)
- Light pilo erection (1)
- Self-mutilation (2)
- Heavy pilo erection (2)
- Abnormal posture (1)

Indicate the likely incidence.

For obesity, Diabetes, lipid, atherosclerosis, NASH, nephropathy and neurodegenerative disease/neuroinflammation studies: <1%.

F. Classification of severity of procedures

Provide information on the experimental factors contributing to the discomfort of the animals and indicate to which category these factors are assigned ('non-recovery', 'mild', 'moderate', 'severe'). In addition, provide for each species and treatment group information on the expected levels of cumulative discomfort (in percentages).

In general, the cumulative discomfort for metabolic disorder(s) studies will be mild to moderate and will not extend beyond moderate. In general all obesity, diabetes, lipid, atherosclerosis and NASH studies (all models/treatment groups) have discomfort level mild and nephropathy and neurodegenerative studies have discomfort level moderate. During the last 5 years approx. 88% of the mice had discomfort level mild and 12% had discomfort level moderate (mostly nephropathy and neurodegenerative studies) and we expect similar percentages for the next 5 years. 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:**	**Max frequency / duration:**
Nutritional interventions		
Diet feeding	mild	Max. 40 wks
Drinking water interventions	mild	Max. 40 wks
D_2O drinking water	mild	Max. 2 wks
Administration of 14C tracer (via diet or drink)	mild	Max. 20 wks
Functional tests/interventions		
Exercise (running-wheel)	mild	Max. 40 wks
Grip strength and inverted screen test	mild	1-4x per study
Fasting		
4 or 5 hours fasting	mild	3-15x
Overnight fasting	mild	1-2x per study
Blood/feces/urine collection		
Multiple blood sampling via tail vein within good practice	mild	3-15x
Urine collection	mild	1-20x
Feces collection or fecal swabs	mild	3-9x
Administration		
Single or multiple gavage or injections (iv, ip, im, sc)	mild	Max. twice a day
Individual housing		
Individual housing (short period, e.g. during urine sampling on labsand)	mild	1-20x
Individual housing (max. 1 month, e.g. due to fighting)	moderate	Max. 1 month
Measurements (mild discomfort)		
Body temperature measurement via rectal probe	mild	1-2x
Indirect calorimetry (TSE or CaloBox systems)	mild	1-2x
Non-invasive imaging without anaesthesia (e.g. EchoMRI)	mild	1-5x
Measurements (moderate discomfort)		
Non-invasive imaging with anaesthesia (e.g. MRI, opto-acoustic (echo) imaging such as FibroScan, and fluorescence imaging)	moderate	1-2x
Glucose tolerance test	moderate	1-2x
Insulin tolerance test	moderate	1-2x
Functional behaviour and cognition tests	moderate	1-10x
Hyperinsulinemic euglycemic clamps	moderate	1x
Hyperglycemic clamp	moderate	1x
Glomerular filtration rate	moderate	Max. 1x per 2 weeks
VLDL production test	moderate	1x
VLDL clearance test	moderate	1x
Blood pressure measurements	mild - moderate	1-4x
Vasodilatation measurements	moderate	1-2x
Challenge tests	mild - moderate	2x
Surgical interventions		
Surgery: s.c. osmotic minipumps	moderate	Max. 3x

Surgery: liver lobe dissection / liver biopsy	moderate	1x
Surgery: fat removal	moderate	1x
Surgery: kidney mass removal	moderate	1x
Surgery: gall bladder cannulation	moderate	1x
Euthanasia	mild	1x
Long-lasting studies using db/db mice	mild - moderate	Max. 20 weeks
Long-lasting studies using KKA^{y} mice	mild - moderate	Max. 20 weeks

Based on the number of mice used in the last 5 years, we expect for the coming 5 years the following numbers of mice per metabolic disorder/discomfort level:

	Study	Number	Maximal cumulative Discomfort	Discomfort is sum of:
1	Obesity/diabetes	200	mild	Diet feeding Functional test/intervention Fasting Blood/feces/urine collection Administration Measurements (mild discomfort)
2	Lipid/atherosclerosis	5000	mild	As above
3	NASH	2200	mild	As above
4	Obesity/diabetes	200	moderate	Diet feeding Functional test/intervention Fasting Blood/feces/urine collection Administration Measurements (moderate discomfort)
5	Lipid/atherosclerosis	1000	moderate	As above
6	NASH	500	moderate	As above
7	Nephropathy	700	moderate	As above + Surgical intervention
8	Neurodegenerative diseases	200	moderate	As above, without surgical intervention + functional behaviour and cognition tests

Of all our studies approximately 10% of the mice are used for developing a novel model or to improve existing models. For the next 5 years we therefore expect approx. 1000 mice will be used for model improvement, and for the coming years we expect this will primarily be for the metabolic disorder atherosclerosis (mild discomfort) and nephropathy (moderate discomfort).

G. 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) and we study processes that do not require an intact organism in alternative in vitro systems, in biobanked tissues (from previous mouse studies or human tissue if available). 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:Een supply chain voor vitaal humaan weefsel | TNO 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 consider 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), the more so because very recent evidence shows a huge impact of the sympathicus/parasympathicus on these endpoints (Mohanta et al., Nature, 2022, PMID: 35477759). For research where physiological integrity is not of importance, food processing (microbiota/gut) does not play role, and metabolic fluxes are not critical, TNO has launched a program Organ-on-a-Chip (see also Organ-on-a-Chip whitepaper: A change in drug development: organ-on-a-chip | TNO This program 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 real organ-organ interactions and the elementary features of metabolic disease (e.g. ectopic fat accumulation, vascular dysfunction, defective lymphatic drainage and compromised innervation) 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 & microbiota, liver, adipose tissue, muscle, vascular system) to provide energy from food and distribute it to the other organs via well-operating vessels and capillaries of the blood stream. Also the very basic homeostasis mechanisms via the lymphatic system, blood pressure and, most importantly, the autonomous nervous system e.g. nervous vagus) cannot be mimicked yet. 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 and lymphatic system and there are unfortunately 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. Recent examples of the last years are the use of wood sticks in our NASH studies after investigation whether this did not affect the dietary induced NASH induction, published in: Biotechniek 4 augustus 2021_ZAI.pdf (dalas.nl). In addition, in 2021 TNO technicians received the DALAS price for excellent culture of care and for implementation of 3R principles in animal research that were done on handlings, housing, pain relieving methods etc in KKA^{y} mice studies (a.o. reduction of handling and measurements, improved housing on half-heated cages, improved handling using tubes, urine collection using labsand and improved skin and wound care): https://1drv.ms/v/s!Amz-cLGZ3F3YjoAxBsfyvUR4Z-geKA Another example how we refined studies is the use of predictive gene expression signatures that inform on the efficacy of an intervention and the pathways being affected after only a few weeks of treatment with a test compound. This allows to shorten treatment time and thereby animal handling and thus discomfort (e.g. shorter gavage period).

Are adverse environmental effects expected? Explain what measures will be taken to minimise these effects.

☒ No

☐ Yes > Describe the environmental effects and explain what measures will be taken to minimise these effects.

H. Re-use

Will animals be used that have already been used in other animal procedures ?

☒ No > Continue with question I.

☐ 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.

I. Repetition

Explain for legally required animal procedures what measures have been taken to ensure that the proposed procedures have not already been performed. If applicable, describe why duplication is required.

N.a.

J. 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 K.

☐ 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.

End of experiment

K. Destination of the animals

Will the animals be killed during or after the procedures?

☐ No > Provide information on the destination of the animals.

☒ 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; small blood vessels for nutritional and oxygen supply of organs, brain for neurodegenerative diseases/neuroinflammation

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 > Will a method of killing be used for which specific requirements apply?

☒ No > Describe the method of killing.

Mice will mostly be sacrificed via gradual fill CO_2 suffocation or via cervical dislocation or via deep anesthesia after which blood via cardiac puncture is taken/animals are perfused.

☐ Yes > Describe the method of killing that will be used and provide justifications for this choice.

If animals are killed for non-scientific reasons, justify why it is not feasible to rehome the animals.