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 (0800-7890789).

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: Muscle loss study – applied science

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.

Muscle loss or atrophy can be induced in approx. 2 weeks in mice by caloric restriction and/or partial immobilization:

1) Caloric restriction is well studied in mice and prolonged caloric restriction has been reported to have beneficial health effects: lifespan is increased and the onset of age-associated diseases like cancer, but also diabetes, hyperlipidemia and hypertension is delayed (Weindruch et al., N Engl J Med. 1997, PMID: 9309105 and references therein). A caloric restriction of 40-60% increases longevity (Weindruch et al. N Engl J Med. 1997, PMID: 9309105 & Sohal et al., Free Radical Biology and Medicine. 2014, PMID: 24941891). At 40% restriction, we previously found that although lean body mass and muscle mass was reduced in C57BL6 mice, muscle strength was not found to be impaired. Caloric restriction (by decreasing the amount of normal chow diet with 40% as compared to the food intake as measured before this intervention, not via adaptations to caloric content of the diet) will be performed for 2 weeks
2) Partial immobilization of one of the hindlegs will be induced by using adhesive bandage (Madaro et al., Basic Applied Myology 18 (5): 149-153, 2008, no PMID nr). This partial immobilization will be performed for 2 weeks. During the immobilization procedure, appropriate anesthesia will be used and after the procedure animals will be monitored on a daily basis for chewed bandage, abrasions and problems with ambulation or blood flow to toes. Partial immobilization leads to decreased muscle mass and decreased muscle strength, but total lean body mass is not decreased.
3) Combination of 1 + 2 leads to loss of muscle mass, lean body mass and loss of muscle strength (de Jong et al., Aging Disease. 2023, PMID: 37191430.

During the study period mice will be housed individually and during the acclimatization period of 2-3 weeks individual *ad libitum* food intake will be assessed during 3 periods of approx. 2 days. For each mouse, average normal food intake will be calculated (=100%). Individual housing is required to be able to measure the normal caloric intake for each mouse separately and based on this calculation the amount of food for the caloric restriction period is determined for each mouse. Individual housing during caloric restriction phase is required since otherwise the most dominant animal of the cage would eat all diet available and other animals of the same cage would starve.

Study duration
The total study duration and subsequently the length of the study is approx. 2-3 weeks (excluding acclimatization period and excluding aging period for aged mice) and will depend on the study design being used:
1) prophylactic design with approx. 2 weeks induction period
2) therapeutic design with approx. 2 weeks induction period followed by approx. 1 week of recovery (100% calorie intake and/or removal of adhesive bandage used for immobilization) and treatment period.

Groups
A study usually includes the following groups:
1. Negative control group muscle atrophy is induced, but no intervention.
2. Positive control group or reference group muscle atrophy is induced, but also intervention with known beneficial (positive control) or well-established effect (reference control) on muscle atrophy.
3. Intervention groups muscle atrophy is induced and interventions to be evaluated are applied. Interventions may be given as prevention design (administration simultaneously with muscle atrophy induction) or as treatment design (administration after muscle atrophy has been established).
4. Healthy reference group (no induction of muscle atrophy: mice on *ad libitum* control diet)

Furthermore, it can also be decided to first perform a pilot experiment. This is a study with less mice per group than in general studies. In these pilot studies the primary endpoints such as bodyweight or muscle mass will be similar to the main experiments. With these pilots we will get crucial information about different experimental conditions such as optimal dosing regimen or administration routes in order to optimize the subsequent main study.

Type of study
The applied science studies are studies that are focused on new interventions and that include efficacy studies in which we will test the efficacy of different prevention- or intervention-therapies (e.g. by lifestyle, nutrition and pharmaceuticals) or proof-of-concept studies in which we will test the hypothesis that a certain (type of) intervention will indeed have the expected effect on muscle atrophy.

Animal handlings/parameters
The primary outcome parameters for muscle atrophy will be individual muscle masses (measured weights at experimental end-points) and body composition (body weight, lean body mass and fat mass via echoMRI). In addition, functional readouts for muscle function or muscle strength can be added, like grip strength, latency to let go of the grid during inverted screen test, voluntary movement etc.
In general, body weight and food intake will be monitored and blood/plasma measurements will be performed to analyse parameters related to metabolic dysfunction. At the end of each study, tissues will be collected for analysis of primary outcome parameters. Histopathological analysis of muscle tissue to analyse myofiber diameter or fiber type can be added. 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 additional or secondary research question (for instance after proven efficacy) providing more insight on the mechanistic effects.
(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 being studied. Each study design though will consist of first an **acclimatization period** of 2 weeks with individual housing and during this period for each mouse the average *ad libitum* food intake will be calculated.

Thereafter the **induction period** of 2 weeks will follow: during each study muscle atrophy is induced either using malnutrition via caloric restriction (40% restriction) and/or immobilization (via adhesive bandage, as described under A).

Depending on the study design (prophylactic or therapeutic intervention), a **recovery period** can be added: caloric restriction and/or immobilization will be removed (via 100% or ad libitum food administration and removal of adhesive bandage).

In summary, for all studies, the experimental period is short: 2 weeks acclimatization + 2 weeks of caloric restriction and/or immobilization in case of prophylactic/preventive study design or 2 weeks acclimatization + 2 weeks of caloric restriction and/or immobilization + max. 1 week recovery in case of therapeutic/treatment design. In some cases, there might be an additional period before the experimental period starts, in which mice will receive the normal chow diet with tracer amounts of 14C labelled amino acid (in order to follow protein decline in muscle).

A representative study example is shown here for prophylactic/preventive study design:

	caloric restriction phase														
days of treatment	**0**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**
1) Control group, chow ad lib	x	→	→	→	→	→	→	→	→	→	→	→	→	→	x
2) Caloric restriction (60%, chow) control group	x	⋯	⋯	⋯	⋯	⋯	⋯	⋯	⋯	⋯	⋯	⋯	⋯	⋯	x
3) Caloric restriction (60% chow/ingredient 1)	x	-	-	-	-	-	-	-	-	-	-	-	-	-	x
Matching	x														
Body weight and food intake	x			x				x			x				x
EchoMRI	x			x				x			x				x
Blood sampling: blood glucose & plasma chol, TG, protein, albumin	x							x							x
Voluntary movement during 24h												x	x	x	
Sacrifice															x
Muscle histology															x
Muscle transcriptomics															x
Muscle protein analysis															x

Or for therapeutic/treatment design:

	caloric restriction phase														refeeding phase						
days of treatment	**0**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**
1) Control group, chow ad lib	x	→	→	→	→	→	→	→	→	→	→	→	→	→	x	→	→	→	→	→	→	x
2) Caloric restriction (60%, chow) control group	x	⋯	⋯	⋯	⋯	⋯	⋯	⋯	⋯	⋯	⋯	⋯	⋯	⋯	x	⋯	⋯	⋯	⋯	⋯	⋯	x
3) Caloric restriction (60% chow/ingredient 1)	x	-	-	-	-	-	-	-	-	-	-	-	-	-	x	-	-	-	-	-	-	x
Matching	x														x							
Body weight and food intake	x			x				x			x				x	x	x	x	x	x	x	x
EchoMRI	x			x				x			x				x		x		x		x	x
Blood sampling: blood glucose & plasma chol, TG, protein, albumin	x														x							x
Voluntary movement during 24h																			x	x	x	
Sacrifice																						x
Muscle histology																						x
Muscle transcriptomics																						x
Muscle protein analysis																						x

In all studies mice will be housed individually and 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 as intervention or for measurement of additional parameters could be added to the study. These additional procedures are described below.

Interventions can be performed in a prevention design (simultaneously with induction of muscle atrophy) or as treatment design (after induction of muscle atrophy). 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.
Maximum volume / frequency to be used are according to what is considered good practice (Proefdieren, zorg, kwaliteit en biotechniek, van Buiten *et al.*, ISBN: 9789090363974).
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.

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 parameters related to (muscle specific) metabolic dysfunction, e.g. glucose, CA-3, CK-M, specific muscle biomarkers. Blood samples can be taken:
- via tail vein non-fasted (in most muscle loss studies it is sufficient to collect blood non-fasted)
- via tail vein after 4-5 hours fasting (this can be used as well, 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).
Maximum volume / frequency to be used are according to what is considered good practice (Proefdieren, zorg, kwaliteit en biotechniek, van Buiten *et al.*, ISBN: 9789090363974).

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

During the study the following additional intervention procedure could be added:

Running wheel (as exercise treatment)
A running wheel will be added to the mouse cage to allow voluntary exercise.

During the study the following procedures could be added for measurement of additional parameters:

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.
- Voluntary movement using the apparatus of manufacturer TSE systems. The mice are housed in their normal housing cages and continuous activity is measured by infra-red light streams around the cage.
- 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.

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.

Deuterated water administration (D_2O)
To be able to trace newly formed proteins 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 the 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-alanin (or other protein); 14C-acetate; 14C-amino acids; 14C-fatty acids) ingested by diet (during study or prior during acclimatization/run-in phase) 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, muscle and the vasculature towards other organs) via blood and urine measurements to assess e.g. protein synthesis, de novo lipid synthesis, fiber fermentation, bile acid production, fructose metabolism etc.

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 or after lifting mice
- By taking rectal swabs (swiping with a cotton swab along (not in) the anus)

Urine sampling (for measurements of proteins and metabolites)
- Spontaneous excretion caused by lifting the mouse and collection 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.

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

Challenge measurement (to measure metabolic resilience after application of acute metabolic stressor)
- Challenges to measure gut permeability (with oral (gavage) 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).

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 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=12 mice will be used for muscle atrophy studies, based on variation in the respective primary outcome parameters in model validation studies previously performed. The partial immobilization procedure of one hindleg only will allow within animal comparison of the effect of immobilization (by comparison with the other hindleg that is not partly immobilized).

If possible different study groups will be combined, so that different treatment groups can share the same control groups.

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	For young mice 8-20 weeks For old mice up to 25 months	800	Males and females	Yes and no (depending on strain)	C57BL/6J wild-type ApoE*3Leiden ApoE*3Leiden.CETP

Provide justifications for these choices	
Species	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
Origin	ApoE*3Leiden, ApoE*3Leiden.CETP mice from our own breeding facility. C57BL/6J wild-type mice from commercial breeders.
Life stages	8-20 weeks.
Number	We previously estimated for the last 5 years to use 1200 animals in total. At that time we predicted to have approx. 4 studies per year, with an average study of 5 groups of 12 mice each, so on average 60 mice per study. This led to a required total number of 1200 mice (4 studies x 60 animals / study x 5 years). In hindsight, we used approx. 460 animals (total number at July 2023, so last year not finalized yet). Taking into account that muscle atrophy is a growing research area and we had less studies during 2 years of COVID-19 pandemic, we would like to apply for 1000 animals in total, consisting of 800 animals for applied science studies (this appendix) and 200 for basic science studies (appendix 2)
Gender	For muscle atrophy studies, young/adult male mice (with induction of muscle atrophy via caloric restriction and/or immobilization) have been used, since male mice are slightly heavier with more lean body mass and muscle mass than female mice. The induction of muscle atrophy therefore allows a larger window of difference in male mice. For aged mice studies both males and females were used and we found gender specific differences (in deterioration of voluntary activity, loss of muscle mass, myofiber type etc). For future studies we might be interested to investigate the male/female differences in young mice as well.
Genetic alterations	No for C57BL/6J mice, yes for ApoE*3Leiden, ApoE*3Leiden.CETP mice

Strain	The choice of mouse model depends on the research question. Many different animal models are used in general to study muscle atrophy, but we would like to focus on translational models and therefore chose to mimic the underlying causes of muscle atrophy, malnutrition and immobilization, in mice. In many cases, normal *C57BL/6J wild-type mice* will be sufficient. However, if for the research question a more humanized lipid metabolism is important, ApoE*3Leiden or ApoE*3Leiden.CETP mice might be preferred. ApoE*3Leiden and ApoE*3Leiden.CETP mice have a more humanized lipid metabolism: since for skeletal muscle mitochondrial function is very important, a similar lipid metabolism may be preferred. *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 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. *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.

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. 5 weeks) to allow measurement of individual calorie intake or caloric restriction or in case of fighting for aged mice. At this moment the individual measurements of caloric intake during the acclimatization procedure and caloric restriction requires individual housing. We previously have explored the possibilities for temporary individual housing combined with interim group housing but this led to increased corticosterone (stress) levels and was therefore not implemented.
Calorie restriction is part of the induction of the model.
In addition, short term fasting during the daytime when mice are naturally not eating (4h/5h) may be performed as well for blood sampling 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).

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, such as placement sc minipumps, 1-leg-immobolization procedure or removal of immobilization, appropriate anesthesia / analgesia will be used. In consultation with the designated 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 muscle atrophy does not lead to discomfort. Similarly as in humans, that are often unaware of these disturbances until a very late stage. The induction method, either via caloric restriction or 1-leg-immobilization as well as the individual housing leads to moderate discomfort. With 40% caloric restriction mice will become hungry. The extent of the weight loss will be closely monitored during the study and can be individually adapted (via the % caloric restriction) during the study. We previously found that with 40% caloric restriction body weight loss stabilizes to -25% after 14 days (resulting in a prefrail body weight of 20-22 g), without loss of muscle strength and with no signs of deteriorated health status. If necessary we can intervene by individual adjustment of the caloric restriction (lower limit of 17 g for males and 16 g for females will be used).
Immobilization procedure leads to hampering of normal behaviour, but this effect is minimal: after an apparent adjustment period of 1-2 days, animals with 1-leg-immobilizaton procedure maintain good mobility and normal grooming behaviours. After the procedure, problems with chewed adhesive bandage, abrasions and ambulation or diminished blood supply to toes can occur and therefore animals will be monitored on a daily basis and if necessary intervene by removing or renewing of adhesive bandage.
With respect to discomfort caused by interventions: although the interventions are expected to have a beneficial effect, it is possible that the interventions have an unexpected adverse effect.

Explain why these effects may emerge.

When adhesive bandage for immobilization is too tight, problems with diminished blood supply to toes can occur. Procedures for applying adhesive bandage have been adapted to decrease the likelihood of this occurrence.
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 muscle atrophy does not lead to discomfort. The caloric restriction obviously will lead to weight loss. The weight loss is well-controlled though with daily monitoring of mice and the severity of caloric restriction can be adjusted individually on a daily basis if required. Furthermore, a lower limit of body weight will be used (we will not exceed body weight <17 g for males and <16 g for females). Immobilization and 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 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 within 24 hours at a combination of scores ≥2:

- Body weight <17 g for males <16 g for females (2)
- Abnormal behaviour, such as:
  - Shortness of breath/panting (2)
  - 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.

<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 muscle atrophy studies will be 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:**	**Max frequency:**
Nutritional interventions		
Caloric restriction (40%)	moderate	Max. 2 wks
Diet feeding	mild	Max. 6 wks
Drinking water interventions	mild	Max. 6 wks
D_2O drinking water	mild	Max. 4 wks
Administration of 14C tracer (via diet or drink)	mild	Max. 8 wks
Immobilization procedures		
Immobilization (adhesive bandage)	moderate	Max. 2 wks
Removal/renewal of adhesive bandage	mild	Max. 14x
Functional tests/interventions		
Exercise (running-wheel)	mild	Max. 2 wks
Grip strength and inverted screen test	mild	1-4 x per study
Fasting		
4 or 5 hours fasting	mild	1-5x per study
Overnight fasting	mild	1-2x per study
Blood/feces/urine collection		
Multiple blood sampling via tail vein within good practice	mild	1-2x per study
Urine collection	mild	1-5x per study
Feces collection or fecal swabs	mild	1-5x per study
Administration		
Single or multiple gavage or injections (iv, ip, im, sc)	mild	Max. twice a day
Individual housing		
Individual housing	mild	Max. 5 wks
Measurements (mild discomfort)		
Body temperature measurement via rectal probe	mild	1-2x per study
Indirect calorimetry (TSE)	mild	1-2x per study 3 days
Non-invasive imaging without anaesthesia (e.g. EchoMRI)	mild	1-5x
Measurements (moderate discomfort)		
Challenge tests	mild - moderate	2x
Surgical interventions		
Surgery: s.c. osmotic minipumps	moderate	Max. 1x
Euthanasia	mild	1x

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 including muscle atrophy. 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. 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, environmental enrichment will be provided. Recent example of the engagement of our technicians was that 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

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.

Muscle atrophy manifests within tissues and cannot be analyzed via plasma. Therefore, the muscle tissues are needed for measurements (e.g. mass, histological analysis, gene expression, protein levels).

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 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.