Centrale Commissie Dierproeven

# Appendix
## Description animal procedures

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

**1**

## General information

| | | |
|---|---|---|
| 1.1 | Provide the approval number of the 'Netherlands Food and Consumer Product Safety Authority'. | 50100 |
| 1.2 | Provide the name of the licenced establishment. | The Netherlands Organisation for Applied Scientific Research (TNO) |
| 1.3 | List the serial number and type of animal procedure.<br><br>*Use the serial numbers provided in Section 3.4.4 of the Project Proposal form.* | Serial number: 3.4.4.1<br>Type of animal procedure: Muscle loss: optimization of the animal model |

## **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 atrophy can be induced in approx. 2 weeks in mice by caloric restriction:
Caloric restriction is well studied in mice and 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. Oct 2; 1997; 337 (14): 986-994 and references therein).

Mice will be housed individually during the study 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%).

The total study duration and subsequently the length of the study is approx. 2-3 weeks (excluding acclimatization period) 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 plastic stick/cast used for immobilization).

A study can include the following groups:
1. Control group muscle atrophy is induced using 40% caloric restriction (amount of normal chow diet will be adapted, no changes to different caloric diets).
2. Intervention groups muscle atrophy is induced and adaptations to the model (for instance regarding the induction method) to be evaluated are applied.

3. Healthy reference group (no induction of muscle atrophy: mice on *ad libitum* control diet)

Animal studies can be performed aiming at developing a novel model or to improve the existing model. **At this moment**, studies have been performed without immobilization and using a 40% caloric restriction, leading to a stabilized body weight loss of approximately -25% after 14 days. Since male adult mice between 27-30 g have been used in previous experiments, a weight loss of approximately -25% results in an average body weight of 21-22 g after 2 weeks of 40% caloric restriction, which is a relatively mild form of frailty, more reflecting the prefrailty phase in humans. Using this percentage of caloric restriction, mice behaved normally and did not display signs of discomfort. The animal welfare officer checked our mice at the end of the caloric restriction period and classified the discomfort due to the caloric restriction as mild. Despite the model mimicking more the prefrail phase in humans, on a molecular level, the underlying pathways affected were translational to the human prefrail and frail situation (comparison of the data obtained in the animal model with human data). Furthermore, the model demonstrated to be predictive, since interventions with beneficial effects in the animal model showed beneficial effects in the human situation as well (2 manuscripts in preparation; poster at conference of European Society for Clinical Nutrition and Metabolism, September 2016).
Despite the loss of body weight and individual loss of muscle mass, as well as muscle atrophy characterized by decreased myofiber diameter, muscle functionality measured via grip strength was not impaired.
Since it would be a clear benefit to the model if beneficial effects of interventions on muscle function could be evaluated as well, we would like to shift the model from the prefrail stage more towards the frail stage, using a stepwise approach:
A caloric restriction of 40-60% increases longevity (Weindruch et al. & Sohal et al., Free Radical Biology and Medicine 73 (2014): 366-382). It's currently still uncertain what the optimal level of food intake should be to induce muscle atrophy with loss of muscle function/muscle strength, but the tipping point is expected to be around 50%.
**For future studies, we would first** like to increase the caloric restriction to 50%. If with 50% we still do not see an impairment in muscle functionality (=go/no-go decision), we would **thereafter** like to increase the caloric restriction to 60%. Also assuming the discomfort of the mice does not exceed above moderate. If the discomfort of the mice is exceeding above moderate, this will lead to a no-go decision and we will then continue with the currently used 40% caloric restriction.
The added value of the improved model as compared to the current model is that instead of reflecting the prefrail stage, the model reflects the frail stage and the model can be used for studying functional readouts of muscle strength, in relation to pathogenesis, but also when evaluating novel interventions, see Appendix 2.

Since there can be synergistic beneficial effects of nutritional/pharmaceutical interventions with exercise, it would be beneficial to the model if we can discriminate between the effects of caloric restriction and immobilization on muscle atrophy. This would lead to more fundamental and mechanistic understanding of the underlying processes in muscle atrophy and helps to identify better interventions.
So therefore, **a separate step** in improving the animal model would be to add (=on top of caloric restriction) partial immobilization to the model or use partial immobilization without caloric restriction (for research questions related to muscle atrophy induced by immobilization only):
Partial immobilization of one of the hindlegs will be induced by using a plastic stick placed over and under the limb and fixation using medical adhesive bandage (Madaro et al., Basic Applied Myology 18 (5): 149-153, 2008) or by using a cast immobilization procedure of one hindleg (Frimel et al., Muscle & Nerve, 32: 672-674, 2005), resulting in fixation of the knee joint at 180°. During the immobilization procedure, appropriate anesthesia will be used and after the procedure animals will be monitored on a daily basis for chewed plaster, abrasions and problems with ambulation.
Again also assuring that the discomfort of the mice does not exceed above moderate. If the discomfort of the mice is exceeding above moderate, this will lead to a no-go decision and we will then continue with the currently used model without immobilization.
The **added** value of the improved model as compared to the current model is that the model is more suitable for fundamental research because the contribution of immobilization and malnutrition on muscle atrophy can be separately studied. When studying the underlying metabolic and molecular mechanisms of the *pathogenesis* of muscle atrophy, as well as during *efficacy studies*, when studying the

effectiveness of novel interventions, the separate and individual contributions of immobilization and malnutrition can now be studied (in contrast to human studies). For all studies, it is very important that we use translational models that reflect muscle atrophy in humans as much as possible. We are continuously improving our in vivo models, and perform comparative studies using human organ tissues and human-based plasma readouts (e.g. same biomarkers). A continuous comparison of data obtained in animals and humans and vice versa will take place.

**In addition**, in the current study proposal adaptations to the model will be evaluated in order to improve the animal welfare, i.e. tuning the food supply to the natural eating periods of the mice or offering additional food with empty calories as well as playing music during the night period to partially minimize gnawing noise of other mice and adding more than legally required environmental enrichment.

The primary outcome parameters for muscle atrophy will be individual muscle masses (measured weights at sacrifice) and body composition (body weight, lean body mass and fat mass). In addition, functional readouts for muscle function or muscle strength will be added, like grip strength, latency to let go of the grid during inverted screen test 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 secondary research question.
(Remark: It is an intrinsic feature of metabolic dysfunction that several organs become diseased, often requiring the analysis of a primary endpoint in context of a secondary endpoint.)

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

As mentioned above, the precise study design depends on the primary and secondary research question 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 every study muscle atrophy is induced either using malnutrition via caloric restriction (the currently used 40% restriction will be used as control) or other novel to be evaluated induction methods (like 50%-60% caloric restriction and/or partial immobilization, 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 stick coverage/cast). If novel induction methods are introduced, the recovery period also needs to be re-evaluated before this type of study design can be used again for evaluating novel interventions.

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.

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. Blood samples can be taken:
- via tail vein after 4-5 hours fasting
- via tail vein after overnight fasting (in particular cases it may be necessary to collect blood after

prolonged fasting, e.g. when ketone bodies or free fatty acids or other markers of fasting are measured or if we need blood samples that do not contain chylomicrons or any food derived markers).
- via tail vein non-fasted (in particular cases it may be necessary to collect non-fasted blood, e.g. after postprandial challenge tests after meal or to assess intestinal absorption of a food component, metabolite, gut integrity marker or nutrient).
Maximum volume / frequency to be used are according to what is considered good practice (Diehl *et al.*, 2001).

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

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

*Running wheel* (as exercise treatment)
A running wheel will be added to the mouse cage to allow voluntary exercise. Please note that the running wheel is part of the intervention/treatment only and is not used during the induction period, hence this is not an anorexia model!

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

*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. If the mouse does not lose urine spontaneously, light bladder massage can be applied.

*Non-invasive imaging measurements*
- Body composition (fat, water and lean body mass) can be measured via placement in Echo MRI (about 1-3 minutes) without anesthesia.
- Other imaging methods relevant for metabolic disease assessment such as (f)MRI and fluorescence imaging for instance could be used for more precise measurement of abdominal fat, ectopic fat or detection of metabolic disease-related morphological changes, by imaging of reflected light or changes of electromagnetic fields under appropriate anesthesia.
- Indirect calorimetry using the TSE system. The mice are housed (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)
- 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).

*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.

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, estimated numbers, and life stages. Provide justifications for these choices.

Mice, either C57BL6/J wild-type mice obtained from a commercial breeder or ApoE*3Leiden and ApoE*3Leiden.CETP mice from our own breeding facility, approx. 10 weeks old will be used. We have knowingly chosen to use young mice because the studies will research all three underlying causes of muscle atrophy. 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 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.

For muscle atrophy studies, male mice were used in previous experiments 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 female mice we will evaluate whether the same induction procedures (caloric restriction and partial immobilization) will lead to useful levels of muscle atrophy as well.

The required number of mice will be estimated to be max. 300 for a period of 5 years. This is an estimated guess, based on historical data of last 2 years, and assuming the muscle atrophy research is a growth area. The average study will consist of 5 groups of 12 mice each, so on average 60 mice per study. This leads to a required total number of 300 mice (1 study x 60 animals / study x 5 years).

**C. Re-use**

Will the animals be re-used?

X No, continue with question D.

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

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

X No

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

**D. Replacement, reduction, refinement**

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

**Replacement:** We continuously seek to replace animal studies with other methods, preferably using human tissue or cells. For example, we validate our animal pathology and molecular observations in human biopsy material (e.g. liver, adipose tissue, muscle tissue but also human plasma and microbiota). However, fresh human tissue is scarce and often not fresh enough to quantify sensitive molecules with rapid turn-over or short half-life such as mRNAs, cytokines and hormones, bioactive lipids (see also Vital tissue whitepaper: http://mailing.tno.nl/ct/m8/k1/lG9BLvQKYu1MheK9DpQtFAFy3yeEoutjgfEr6Gj5ucJVVT1LUJPXZ6kSA7n8Q0sK4vv7T0WuHMUTxdHEBnJhNg/ZD8yND3CLIt58HW). We work with human cell and *in silico* models, where possible, since that helps us to make better predictions about how a substance, for example a drug, will affect humans. When we have discovered ways to replace animal testing, we use them ourselves and encourage others to apply these alternative tests. Non-testing techniques, such as computer modelling, are continually being developed and improved.
Before we consider to perform a novel muscle atrophy study, we will first analyse appropriate cell lines, existing patient materials or materials available from previous animal studies, which contributes to a reduction of animal numbers. The use of human material and *in vitro* cultures allows this project to comply with the 3Rs (replacement, reduction and refinement) by keeping the animal numbers to a minimum.
Some of the experiments for muscle atrophy can be performed in humans. In collaboration with other parties, we are involved in a number of human muscle atrophy studies and are taking care of some of the analyses. We use these data to evaluate whether the underlying processes in our animal model are comparable to the human situation. This leads to more insight whether we indeed have a translational model. However, evaluation of new interventions still requires the use of animal models. Immediate testing of new interventions in humans, especially in this vulnerable patients group, can be quite dangerous. In addition, taking muscle biopsies is not always possible in these vulnerable patients. Furthermore, animal studies are currently unavoidable to study the complex organ-organ interactions that are intrinsic to all metabolic diseases, including muscle atrophy. The development of muscle atrophy is a complicated multifactorial process in which multiple organs interact in an orchestrated way (gut, liver, adipose tissue, muscle, vascular system, brain) to provide energy from food and distribute it to the other organs. 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 and there are currently no established alternative methods to investigate these metabolic processes.
**Reduction:** TNO aims to reduce the number of animals involved in testing. We regularly review our testing methods and implement integrated testing strategies. This helps us to determine whether animal testing is needed or whether the same information can be obtained in other ways. 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. The partial immobilization procedure of one hindleg also leads to reduction since the other hindleg can be used as control.
**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. For the current study proposal individual housing is required. In future studies we will investigate whether it is possible to use only temporary individual housing with interim group housing. In addition, in the current study proposal other adaptations to the model with the aim of improving the animal welfare will be evaluated: tuning the food supply to the natural eating periods of the mice or offering additional food with empty calories as well as playing music during the night period to partially minimize gnawing noise of other mice and adding more than legally required environmental enrichment.

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

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

## Repetition and duplication

**E. Repetition**

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

N.a.

## Accommodation and care

**F. Accommodation and care**

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

☐ No

X Yes > If this may adversely affect animal welfare, describe how the animals will be housed and provide specific justifications for these choices.
At this moment the individual measurements of caloric intake during the acclimatization procedure and caloric restriction requires individual housing. We will explore the possibilities for temporary individual housing combined with interim group housing and/or use of more advanced technological options for individual measurement of food intake and caloric restriction that allows group housing.

**G. Location where the animals procedures are performed**

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

X No > Continue with question H.

☐ Yes > Describe this establishment.

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

## Classification of discomfort/humane endpoints

**H. Pain and pain relief**

Will the animals experience pain during or after the procedures?

☐ No > Continue with question I.

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

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

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

For more invasive procedures, such as placement sc minipumps, 1-leg-immobolization procedure or removal of immobilization, 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.

**I. Other aspects compromising the welfare of the animals**

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.
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 21-22 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.
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 plaster, abrasions and ambulation can occur and therefore animals will be monitored on a daily basis and if necessary intervene by removing or renewing of plaster.
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.

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

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

Upon start of treatment/intervention mice will be closely monitored and upon signs of discomfort or adverse effects, the situation will be discussed with the animal welfare officer or veterinarian and if possible proper measurements will be taken to relief the animal discomfort or animals will be taken out of experiment and sacrificed.

**J. Humane endpoints**

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

☐ No > Continue with question K.

X 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). Interventions 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.

Indicate the likely incidence.

<1%.

**K. Classification of severity of procedures**

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

In general, the cumulative discomfort for 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:** |
|---|---|
| Individual housing | moderate |
| Caloric restriction 40% | mild |
| Caloric restriction 50 or 60% | moderate |
| Immobilization (casting tape/plastic stick coverage) | moderate |
| Removal of immobilization (casting tape/plastic stick coverage) | mild |
| Diet interventions | mild |
| Drinking water interventions | mild |
| $D_2O$ drinking water | mild |
| Voluntary exercise (running-wheel) | mild |
| Grip strength and inverted screen test | mild |
| Blood sampling (unfasted, after 4-5 hours fasting or after overnight fasting) | mild |
| Multiple blood sampling via tail vein within good practice | mild |
| Urine collection | mild |
| Feces collection or fecal swabs | mild |
| Indirect calorimetry (TSE systems) | mild |
| EchoMRI | mild |
| Non-invasive imaging | mild |
| Challenge tests | mild |
| Euthanasia | mild |

## End of experiment

**L. Method of killing**

Will the animals be killed during or after the procedures?

☐ No

X 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 (and other) 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.

X Yes