Centrale Commissie Dierproeven

Form
Project proposal

- This form should be used to write the project proposal for animal procedures.
- The appendix 'description animal procedures' is an appendix to this form. For each type of animal procedure, a separate appendix 'description animal procedures' should be enclosed.
- 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	Provide the title of the project.	Prevention and treatment of muscle loss

2 Categories

2.1 Please tick each of the following boxes that applies to your project.

- ☒ Basic research
- ☒ Translational or applied research
- ☐ Regulatory use or routine production
- ☐ Research into environmental protection in the interest of human or animal
- ☐ Research aimed at preserving the species subjected to procedures
- ☐ Higher education or training
- ☐ Forensic enquiries
- ☐ Maintenance of colonies of genetically altered animals not used in other animal procedures

3 General description of the project

3.1 Background

Describe the project (motivation, background and context) with respect to the categories selected in 2.1.

Age-related loss of muscle mass and strength, or **sarcopenia**, is a fundamental cause of frailty, functional decline and disability. Due to the rapidly increasing proportion of older people, sarcopenia represents a huge potential public health issue worldwide with high impact on healthcare costs. Since

age-related muscle loss is often seen as a natural process of aging, the impact both from a societal perspective as from the individual perspective, is often underestimated. As the total muscle mass plays a key role in the total whole body metabolic homeostasis, the loss of muscle mass during life span (often exaggerated due to the current sedentary lifestyle in Western society) has an enormous impact on the prevalence of metabolic disorders (e.g. obesity, type 2 diabetes, cardiovascular disease and neurodegenerative diseases) and therefore on societal healthcare costs as well. On the individual level the effects of muscle wasting are often underrecognized as well. Of course, muscle wasting leads to inability to manage daily activity, which has a major impact and translates directly into poor quality of life. However, the metabolic consequences of muscle wasting are often not recognized. Many comorbidities are more likely to occur, like osteoporosis, increased fracture risk, potentially directly via crosstalk between muscle and bone tissues and indirectly via increased risk of falling, as well as the above mentioned endocrine diseases like diabetes, obesity, cardiovascular diseases etc, all together resulting in a greater risk of hospitalization or mortality. Although, aging is probably the most important contributor to the prevalence of muscle loss/muscle atrophy there are other causes as well, such as **temporarily disabling circumstances** when persons are being restricted in movement for instance due to bone fractures or due to **short periods of illnesses**, for instance when patients are confined to bed when hospitalized or due to severe **chronic illnesses (cachexia)**. Due to the aging population, the other causes of muscle atrophy are more likely to occur as well.

When considering the similarities between the different causes of **muscle atrophy in general**, one notices that the underlying causes for all forms of atrophy are lack of exercise and/or malnutrition. Obviously, with a bone fracture or other forms of temporary disabling circumstances there's less exercise and also during shorter or longer (chronic) periods of illnesses there's less exercise together with a lack of appetite. However, also sarcopenic muscle atrophy is characterized by lack of exercise, in the form of a sedentary lifestyle, and malnutrition. The prevalence of frailty decreases in elderly that still exercise frequently and maintain proper nutrition, demonstrating that aging itself is not the primary cause of sarcopenia. Strikingly, malnutrition has a high prevalence among elderly and is one of the most relevant determinants that negatively affect the health of older people. A meta-analysis of the prevalence of malnutrition in Europe revealed that as many as 23% of European older adults are at high risk of malnutrition and that more than double this number (48.4%) is at some malnutrition risk, i.e. moderate and high malnutrition risk combined (Leij-halfwerk et al., Maturitas. 2019, PMID: 31239123). The main cause of malnutrition in elderly is a combination of losing appetite and reduced uptake of dietary proteins. Despite the high prevalence of malnutrition in elderly, it is still an under-recognised and under-treated condition. The role of lack of exercise and/or malnutrition within muscle atrophy in general is however also emphasized by the fact that muscle atrophy can occur in obese subjects as well. Despite the high caloric diet of these subjects, the improper balance of macronutrients within the diet in combination with the lack of exercise can lead to muscle atrophy as well.

The **muscle content** of the body is important for overall health because of the tight relation between muscle mass and physical function, strength and morbidity. The total muscle mass in the body is one of the most important factors for whole-body metabolism and is for instance the major determinant of resting energy expenditure. Muscle loss is therefore a fundamental cause of frailty, functional decline and disability, but also has large effects on whole-body metabolism since the whole metabolic chain of metabolic organs interacting together gets disturbed. Screening and appropriate, timely therapeutic intervention will help to reverse these negative effects. A combination of exercise and pharmaco-nutritional interventions might be a promising candidate in combatting muscle atrophy in general. Nevertheless, the mechanisms by which muscle atrophy can be attenuated remain elusive. A fundamental understanding of the pathogenesis of frailty, the metabolic interplay between different organs and more insight into the factors that may prevent or possibly reverse muscle loss is critical for improving the quality of life in these vulnerable populations, thereby lowering health care costs and promoting self-support.

The **development of muscle atrophy** in general 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 study of the effects of compounds or nutritional interventions on metabolism requires an intact physiology. Some aspects of muscle atrophy can be studied in human volunteers. This type of research, however, is limited due to ethical or practical constraints. Taking biopsies is often not advisable in vulnerable patients; moreover studying the mechanisms and effectiveness of new interventions in these patients leads to unacceptable health risks when tolerability and safety aspects are not well known. For these reasons, animal models are currently an important tool to study the complex organ-organ interactions that are intrinsic to all metabolic diseases, including muscle atrophy. The choice of the exact type of animal model is essential for the proper translation of the experimental results to the human situation. Therefore, we collaborate with clinical research groups that are involved in a number of human muscle atrophy studies. Since our previous project proposal (AVD5010020186844) 5 years ago, we have used available human data for further refinement of our animal model and analyzed the translatability of different models to understand which model is most suitable to the human situation, allowing in the mouse model a more detailed study of the physiological processes than in patients.

In general, various different animal models are used to investigate muscle atrophy. These are broadly divided into the following categories: 1) genetically modified animals which, for example, lead to a model with a specific muscle disease or, for example, lead to accelerated aging. 2) Cachexia models, often models for cancer cachexia, which induces cancer by injection with certain tumor cells. 3) Immobilization models. 4) Models using peripheral nerve damage to induce muscle atrophy and 5) Aged rodent models. However, when considering the similarities between the different causes of human muscle atrophy, one notices that **in general the primary underlying causes of muscle atrophy are lack of exercise and/or malnutrition** and strikingly these causes are often poorly represented in these animal models. It is recognized that suitable animal models for multi-factor diseases are needed for the development of effective interventions and are lacking at present (Bellantuono et al., Nature. 2018, PNID: 29446384). We therefore **choose to focus on these underlying causes** and implement them in our model in order to use **a translational model of muscle atrophy with similar underlying mechanisms**. In comparison to 5 years ago, we have made considerable progress in the optimalization of the animal model. Five years ago, we introduced the mouse model with 40% caloric restriction. At that time we were still considering to increase the caloric restriction to 50% or 60% but we now know that the 40% is sufficient. We have now demonstrated that the 40% model was indeed found to be predictive, since an intervention with beneficial effects in the mouse (van den Hoek et al., Metabolism. 2019, PMID: 31153978) was proven later to have beneficial effects in the human situation as well (Grootswagers et al., Aging. 2021, PMID: 33799307). In addition, we demonstrated that this model recapitulated more pathways characteristic for human muscle-aging than naturally aged (21 months old) mice (de Jong et al., Aging Disease. 2023, PMID: 37191430). Furthermore, we have now introduced partial immobilization (of one hindleg) to the model as well and we demonstrated that this combination model is a translational model for sarcopenia as well expressing key pathways of human pathology (de Jong et al., Aging Disease. 2023, PMID: 37191430).

3.2 Purpose

3.2.1 Describe the project's immediate and ultimate goals. Describe to which extent achieving the project's immediate goal will contribute to achieving the ultimate goal.

- If applicable, describe all subobjectives

Our ultimate aim is to help subjects suffering from loss of muscle mass and function and to prevent the development of muscle atrophy.

The immediate goals of this study are:

1. Test the efficacy of interventions on the prevention and reduction of muscle atrophy development.
2. Gain more insight into the processes involved in muscle atrophy and improve the models accordingly.

Our direct aim for the next 5 years is to refine our understanding of the general underlying mechanisms that drive loss of muscle function and the effect thereof on whole-body metabolism in order to contribute to the prevention and/or treatment (via nutritional or pharmacological interventions) of muscle loss. To this end, basic and applied research will be performed to study the underlying mechanisms, the difference between natural healthy muscle aging and the pathological induction of frailty, the metabolic interplay between different organs, as well as novel therapeutic strategies will be tested in our translational models to contribute as much as possible to the prediction of the ultimate clinical effectiveness. As compared to the last 5 years, we expect that optimization of the animal models will have slightly less focus, although we still foresee additional validation studies and we continuously strive to optimize the models and evaluation of the translatability to the human situation will always remain an important issue.

3.2.2 Provide a justification for the project's feasibility.

The type of work we describe in this proposal has a high feasibility and is of great interest for stakeholders for which TNO delivers translational models and studies:
Within the strategy of TNO, Healthy Living is one of the focus areas. Within the focus area of Healthy Living, research is being done that varies from the development of healthy and safe food, children growing up healthily, or working healthily to predictive health technologies. A substantial portion of the research is dedicated to education, prevention and treatment, either commissioned by the government or in collaboration with academia or industry. The research described in this project is embedded within the theme of biomedical and digital health that aims to have a better understanding of health and diseases.
For the research described in this project, we **FOCUS ON THE METABOLIC CONSEQUENCES OF MUSCLE ATROPHY IN GENERAL** and cross-talk of muscle with other organs. In the field of metabolism, researchers within the group have more than 25 years of experience (both efficacy studies as well as fundamental studies). For metabolism in general we have an extensive track record, resulting in more than 200 peer-reviewed international publications. For the research described in this project, focusing on muscle function, we have now performed a number of studies, both fundamental studies to explore the relevant animal model, as well as applied studies for different partners to test the effect of different nutritional interventions. Since sarcopenia has only relatively recently been recognized as an official muscle disease (Cao et al., J Am Med Dir Assoc. 2016, PMID: 27470918), the research on muscle atrophy is a relatively new and fast growing research area. For the next 5 years we expect that besides nutritional interventions there will be more novel (pharmaceutical) therapeutics as well. Since we have a close collaboration with academic groups and medical centers performing human studies, there's a continuous comparison of the collected human data with the data of the animals studies and vice versa. Through this collaboration with different parties (academic groups, medical centers, food and pharmaceutical companies) we have further expanded our specific knowledge of muscle atrophy.
A large network has been built within both the academic world and the pharmaceutical industry, nutritional companies, as well as biotech companies, academic medical centers, patient organizations and governments. Within this network we have conducted more than 180 cooperation projects (both bilaterally and in larger consortia) over the past decades. Animal experiments are performed by a skilled team of technicians that are very experienced with the handlings described in this application. Our previous achievements make it very likely that we with the experiments described in this project we can address the important research questions in the field of muscle atrophy that will be relevant in the future to improve patient's health and attenuate disease.

3.2.3 Are, for conducting this project, other laws and regulations applicable that may affect the welfare of the animals and/or the feasibility of the project?

☒ No

☐ Yes > Describe which laws and regulations apply en describe the effects on the welfare of the animals and the feasibility of the project.

3.3 Relevance

3.3.1 What is the scientific and/or social relevance of the objectives described above?

Currently the rapid increase of the elderly population will put pressure on hospitalization, nursing home admissions and home healthcare expenditure in the near future. Only few studies have currently reported on the healthcare costs of sarcopenia. In different studies in the United States, the direct costs, due to hospitalization, nursing home admissions and home healthcare expenditure, were estimated to be $18.-19 billion/year (Janssen I et al., J Am Geriatr Soc. 2004, PMID: 14687319; Goated et al., J Frialty Aging. 2019, PMID: 30997923). Due to the aging population, the other causes of muscle atrophy (illnesses and temporarily disabling circumstances/bone fractures) are also more likely to occur. The implementation of effective and broadly applicable preventive and therapeutic interventions has therefore become a medical and societal challenge. With our basic/fundamental studies this project contributes to the development of new insights into **the underlying mechanisms of muscle atrophy in general** and our applied studies ultimately contribute to **the development of new intervention strategies** for a large group of patients with muscle atrophy (no matter the cause) to increase self-support, mobility, quality of life, metabolic health and thereby attenuation of frailty-related disease and associated health care costs.
The development of novel insights into the underlying mechanisms involved in loss of muscle function and the consequential effects on metabolism will also have scientific relevance and will contribute to the general knowledge on muscle loss and frailty.

3.3.2 Who are the project's stakeholders? Describe their specific interests.

The experimental animals are a stakeholders. They will experience suffering and it is not in their interest to be used in these studies. Through our portfolio, we support both the nutrition & pharmaceutical industry as well as academia and medical centers in the development of new therapy options. Scientists working in this research area may benefit from the information that will be made publicly available via peer-reviewed publications. TNO scientists are stakeholders and benefit from the scientific information and published articles. In the end, patients suffering from muscle atrophy will benefit when successful therapies will become available for patients and general society will benefit with regards to public health and productivity. While TNO is an independent non-profit organization, for individual studies TNO may have a commercial interest/profit as well.

3.4 Strategy

3.4.1 Provide an overview of the overall design of the project (strategy). If applicable, describe the different phases in the project, the coherence, the milestones, selection points and decision criteria.

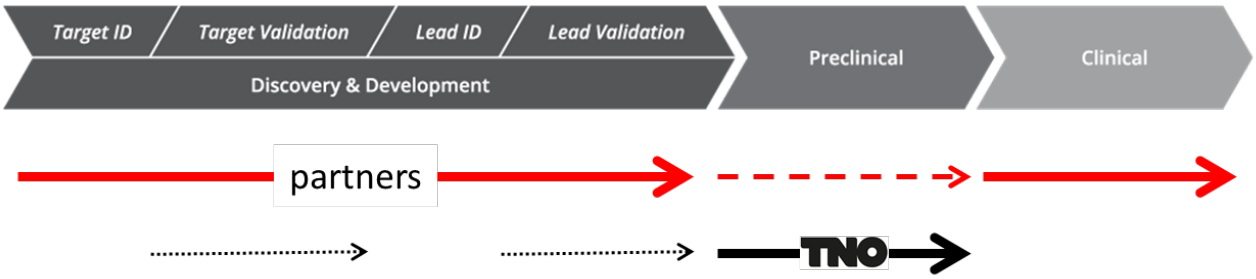
This project compasses two types of research, both basic research as applied research.

The basic research studies are all focused on gaining more insight of the models and optimalization of the models. Whereas the applied science which makes use of these models, is focused on the development of new interventions.
In the schedule below are the different phases of the project illustrated.

Applied science (max n=800)
These include:
Proof-of-concept
Efficacy studies

Basic research (max n=200)
Examples of basic research studies:
Model optimization;
Testing of novel methods of paw-fixation/inactivation;
New housing conditions (split cages to diminish discomfort due to individual housing);

In general:
The development of new interventions such as nutraceuticals or therapeutics follows a fixed pattern:



The above figure illustrates the general approach used for development of therapeutics and the position of TNO therein (for TNO/MHR primarily within preclinical phase). Fundamental research is embedded within the discovery & development part. First of all, suitable targets will be identified on the basis of, for example, an in silico search in literature, available databases on human studies and / or in vitro testing. Subsequently, it will be evaluated whether modification of the target can indeed lead to a change of pathology (for example by in vitro testing, ex vivo tests with human tissue, in vivo testing in transgenic animals). Thereafter (go/no-go decision), leads will be identified that can affect the target (e.g., nutritions, inhibitory antibodies, small molecules, RNA therapy, not all targets are suitable for internvention) and if this is indeed the case (go/no-go decision after proof of concept study) those leads will be validated (efficacy study will then be performed to evaluate which substance is most potent (go/no-go decision if there is no efficacy), but checks as well on what's the solubility or specificity?). If there is proven efficacy (go/no-go decision), mode of action can be evaluated in mechanistic study. The most promising candidates then enter the preclinical testing phase, which examines whether the candidates are good enough to be tested in humans (clinical phase). Candidates will only be tested at TNO in our preclinical models if one of our models is indeed suitable for testing this particular candidate. We will discuss the study design with the sponsor and for instance we can check whether target is indeed present in our model and for the different models we can check in our transcriptome biobank which targets/pathways are most affected in which model. If none of our models are suitable for testing a particular candidate, this will be communicated to the sponsor and preclinical study will not be performed by TNO. The ultimate goal is of course that candidates successfully complete the clinical phase and will be admitted as a new therapy.

Majority of our work in preclinical phase
TNO as Research and Technology Organization (RTO) supports both nutritional or pharmaceutical industry and academic partners in implementing and optimizing preclinical research in the field of metabolic diseases. The majority of work under this project is in the preclinical phase, but work in the area of target validation (for example, show that a pathway is involved), lead validation (e.g. comparing nutritions or different variants of a new compound in order to determine which variant will continue further development) and biomarker development also occurs.

Aim/topic of our studies
The current project describes the research that we conduct using these animal models with the overall aim to 1) either attenuate the development of muscle atrophy or 2) to treat muscle atrophy, via nutritional or pharmacological interventions.

Type of studies
The majority (>80%) of the studies that will be conducted in this project will applied studies that include proof-of-concept and efficacy studies to evaluate novel nutritional interventions or new therapeutics and the effects thereof on muscle tissue specific/whole-body metabolism. This will primarily take place in

conjunction with or on behalf of external partners. For each individual study, the partner(s) will be advised on the optimal study design. Each partner will always be asked what is already known of the therapeutic to be tested, to prevent needless animal use. Furthermore, also aspects such as the experimental design, the route of administration, treatment frequency, power analysis, the concentration of choice, the primary and secondary read-out parameters, etc. will be included. If insufficient information is available, it will be decided to first perform a pilot experiment to obtain the desired information.
For all the compounds, both pharmaceutical compounds as well as 'over-the-counter' products, they will only be tested if the compound is expected to have a significant effect on the reduction of muscle atrophy.
The remaining studies (20%) that will be carried out under this project application, will be basic research studies aimed at improvement of the animal models (including improvements on animal welfare as well (for instance testing the use of transparent partition walls for individual housed animals) or to obtain more knowledge about the underlying mechanisms of the development of muscle atrophy in general or more knowledge on the difference between natural healthy muscle aging and pathological development of muscle atrophy, and are therefore of a more fundamental nature.
We currently use, both the model with 40% caloric restriction only or the combination model with immobilization combined with 40% caloric restriction. These models have been proven to be most representative of the human situation and are therefore currently primarily used for intervention studies, however for specific research questions it is also possible to use the immobilization only model or use aged mice.

Expectation for the next 5 years:
New developments in the research field and the focus of companies that develop new compounds or nutrients will strongly affect what kind of studies will be performed the coming years. At this moment, we expect the research on muscle loss and frailty to be a growth area. As compared to our application 5 years ago, we expect that for the coming 5 years we will perform less experiments on the optimization of the model. Although we continuously strive to improve our models and there are still aspects of the model that we would like to understand better in order to obtain more insight into the translatable value of the model (for instance, a time-course of duration of caloric restriction could be performed to entangle the difference between adverse short-term muscle effects we observe and beneficial long-term effects or the effects of caloric restriction/immobilization in aged mice might be compared with the effects in young mice). Since the muscle research area is relatively new and rapidly growing, several novel therapeutics are being developed and several clinical trials are currently being performed. We therefore expect that for the coming years we will perform more studies on the evaluation of the efficacy of these novel nutritional and pharmaceutical interventions in our models. However, with the growing number of clinical trials in this research area being performed, we expect we will also obtain more insight into the translational and predictive value of our models in the coming years. In addition, also the cross-talk between organs (intestine, fat, muscle, brain and liver) and the research on biomarkers is a growing field of research, which we are expected to focus more on in the coming years.

Basic outline study
For all studies (both basic/fundamental research as well as the applied research), first muscle atrophy will be induced in mice via 2 weeks of malnutrition and/or partial immobilization. Two different study designs are possible, either a preventive study (with interventions applied during the 2 weeks of muscle atrophy induction) or a therapeutic/treatment design (with interventions applied after the 2 weeks of muscle atrophy induction period during the additional recovery period of maximum one week).
Interventions will usually be nutritional/compound administration (via admix food, admix drinking water, gavage or injections or osmotic minipumps) but can also be other interventions (exercise for instance).
For either basuc/fundamental or applied studies, parameters will be measured:
body weight and food intake will be monitored regularly during the study and blood samples will be taken regularly for the measurement of blood glucose, plasma lipids, inflammation markers etc. At the end of the study animals are sacrificed and blood and tissues will be collected for further analyses. More specific

parameters such as lean body mass via echoMRI, voluntary movement or grip strength can be measured during the study as well and are described in more detail in the appendices.

3.4.2 Provide a justification for the strategy described above.

Although in vitro models and in silico models are most helpful in studying parts of muscle atrophy, our focus on the metabolic consequences of muscle atrophy and the complex organ-organ interactions that are intrinsic to metabolism make it currently unavoidable to use animal models to study the effects of muscle atrophy and develop new therapies. TNO has set up a research program to refine, reduce and replace animal testing.

In CRO projects such as those carried out by TNO, it is difficult to predict in advance what pharmaceutical targets will be tested. Efficacy studies are often part of the preclinical phase of the sponsor and are independent studies for each sponsor (no connection to studies of other sponsors). In general, first a pilot or proof-of-concept study will be performed, followed by efficacy studies, followed by mode-of-action studies to provide more insight into the underlying mechanisms. TNO can be requested to perform all these studies sequentially for the sponsor or one specific part (for instance efficacy study will be performed at TNO, but dose finding studies have already been performed elsewhere).
For model optimalization, studies also follow a fixed pattern: first pilot or proof-of-concept study, followed by optimalization studies and validation studies.
We will not continue with the same study design to the next step if there is no effect in the previous step (e.g. if a pilot study shows no effect, main study will not be performed with this study design; mechanistic studies can only be performed after proven efficacay).

In general we always use mouse models with muscle atrophy, intervention is applied (during induction of muscle atrophy or during recovery phase thereafter) and finally the effect of this intervention is determined. Because we use already known models and interventions, the cumulative inconvenience for each experiment can be estimated well in advance and will never be more than moderate.

In the strategy we will strive to first obtain as much insight as possible with regard to target engagement, for example, by means of in vitro/ex vivo techniques. Only when in vivo models are unavoidable, animal studies will be started.

The strategy described above allows us to answer key questions such as:
- Does the intervention work on prevention or improvement of muscle loss/muscle function
- What are the mechanisms
- Are there any side effects

3.4.3 List the different types of animal procedures. Use a different appendix 'description animal procedures' for each type of animal procedure.

Serial number	Type of animal procedure
1	Muscle loss study - applied science
2	Muscle loss study – basic science
3	
4	
5	
6	
7	

8	
9	
10	