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 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	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.	x Basic research
		x Translational or applied research
		☐ Regulatory use or routine production
		☐ Research into environmental protection in the interest of human or
		☐ 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.

- For legally required animal procedures, indicate which statutory or regulatory requirements apply (with respect to the intended use and market authorisation).
- For routine production, describe what will be produced and for which uses.
- For higher education or training, explain why this project is part of the educational program and describe the learning targets.

Muscle atrophy or loss of muscle mass can be a partial or complete wasting away of muscle tissue or muscle strength. Muscle atrophy can be caused by:

- **Temporary disabling circumstances** such as when persons are being restricted in movement for instance due to bone fractures.

- After **short periods of illnesses**, for instance when patients are confined to bed when hospitalized.
- Due to severe **chronic illnesses (cachexia)**.
- **Age-related** loss of muscle mass and strength (**sarcopenia**).

On a global scale, the prevalence of muscle loss is currently rapidly growing, due to the increasing proportion of elderly. Therefore, sarcopenia is now probably the most general form of muscle loss and muscle loss or frailty represents a huge potential public health issue worldwide with high impact on healthcare costs. Due to the aging population, the other causes of muscle atrophy are also more likely to occur. The implications of muscle wasting on the individual level are often underestimated: muscle wasting is associated with many comorbidities, like osteoporosis, increased fracture risk, potentially directly via crosstalk between muscle and bone tissues and indirectly via increased risk of falling, as well as endocrine diseases like Diabetes, obesity and chronic kidney diseases, all together resulting in a greater risk of hospitalization or mortality. On the individual level, muscle wasting leads to inability to manage daily activity, which has a major impact and translates directly into poor quality of life.

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. Frailty does not occur 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. In Europe alone, 33 million people suffer from malnutrition. 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 and are involved in a number of human muscle atrophy studies, having the responsibility over taking several analyses. We use these data for further refinement of our animal model which is suitable to the human situation, allowing a more detailed study of the physiological processes than in patients.

In the literature, 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, and 4) Models using peripheral nerve damage to induce muscle atrophy. 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**. 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, Nature 554,293-295). 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**. At this moment we are using an animal model with 40% caloric restriction that 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). In order to improve the model the specific stepwise approach that we would like to follow to obtain an even more translational model (by increasing the caloric restriction and by introducing immobilization) are described in appendix I.

3.2 Purpose

Describe the project's main objective and explain why this objective is achievable.

- If the project is focussed on one or more research objectives, which research questions should be addressed during this project?
- If the main objective is not a research objective, which specific need(s) does this project respond to?

Our aim for the next 5 years is to gain better 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 and 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.

In order to do so, the following sub-goals are integrated into this project:
- Developing knowledge about the **process of muscle loss in general** (eg following loss of muscle function in time or comparing the processes between animal and human to allow optimal translation).
- Continuous validation and further optimization of the models (for example by adding additional read-out capabilities or integrating different inducers (malnutrition and partial immobilization) in one model.

The project has a high feasibility:
Within the strategy of TNO, Healthy Living is one of the nine 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 as well as healthy aging. The research described in this project is embedded within the theme of predictive health technologies that aims to have a better understanding of health and diseases and better predict effects of new interventions such as nutrition's or therapeutics. Muscle atrophy is a relatively new and fast growing research area. 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, the researchers within the group have more than 20 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 150 peer-reviewed international publications. For the research described in this project, focusing on muscle function, we have now performed a number of studies, to first explore a relevant animal model. In addition, the effect of different nutritional supplements or new therapeutics as well as lifestyle interventions like exercise were evaluated. We have now conducted several animal studies in the field of muscle atrophy for different parties and we work together in a number of human studies in the field of muscle atrophy. So far, the data obtained via animal studies led to new insights that could be used in human studies and the data seemed to be highly predictive and translational to the human data. 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.
In general, 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 over the past 15 years more than 150 cooperation projects (both bilaterally and in larger consortia) that were dedicated to metabolic research. Our previous achievements make it very likely that we can exploit this network and experience and convert this to muscle atrophy related research as well.

3.3 Relevance

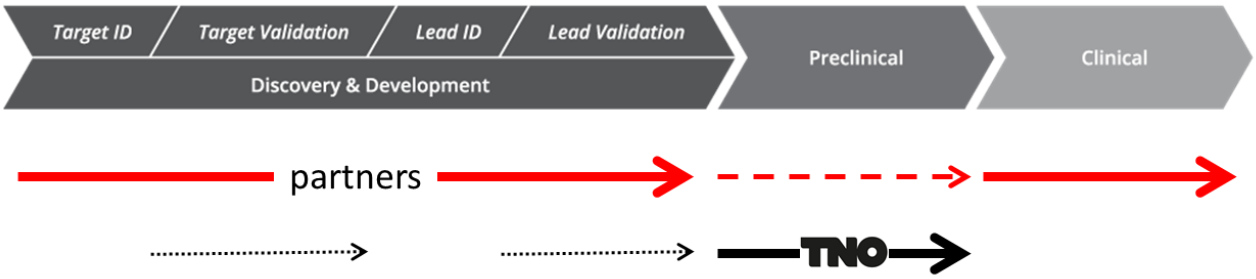
What is the scientific and/or social relevance of the objectives described above?

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 one study has currently reported the healthcare costs of sarcopenia in United States. The direct costs, due to hospitalization, nursing home admissions and home healthcare expenditure in United States, were estimated to be $18.5 billion/year (Janssen I et al., J Am Geriatr Soc. 2004; 52(1): 80–85). Due to the aging population, **the other two causes of muscle atrophy 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. This projects contributes to the development of new insights into **the underlying mechanisms of muscle atrophy in general** and ultimately contributes 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.

3.4 Research strategy

3.4.1 Provide an overview of the overall design of the project (strategy).

The development of new interventions such as nutrition's or therapeutics follows a fixed pattern:



An essential step in testing novel therapeutic interventions is to demonstrate the efficacy in a preclinical setting before being tested in a clinical phase. TNO has a broad portfolio of *in-vivo* models and an

extensive experience in metabolic research. Via our large network, consisting of various medical centers, academic groups, as well as pharmaceutical and nutritional companies, we strive to identify the underlying mechanisms of muscle loss and frailty and the effects thereof on whole-body metabolism and to evaluate novel therapeutic interventions.
Because the research into muscle loss and function is still a relatively young field of research, improvements to models and readouts are continuously taking place. We strive to incorporate them into our portfolio to offer the most optimal pre-clinical research.

The first step within the development of new interventions is to identify suitable targets on the basis of, for example, literature, 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, leads will be developed that can affect the target (eg, nutritions, inhibitory antibodies, small molecules, RNA therapy) and those leads will be validated (which substance is most potent, what's the solubility or specificity?). 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). The ultimate goal is of course that candidates successfully complete the clinical phase and will be admitted as a new therapy.

TNO as Research and Technology Organization (RTO) supports for years both nutritional or pharmaceutical industry and academic partners in implementing and optimizing preclinical research in the field of metabolic health and disease. The majority of work under this project is in the preclinical phase and involves fundamental and applied research in support of human studies for instance, but work in the area of target validation (for example, show that a pathway is involved) or lead validation (eg comparing nutritions or different variants of a new compound in order to determine which variant will continue further development) also occurs.

3 R developments in metabolic research: possibilities and limitations
TNO has set up a research program to refine, reduce and replace animal testing. In this program, TNO collaborates with others to accelerate the process of developing better alternatives. TNO is constantly looking for new insights and technologies that can reduce animal experiments. For example, human muscle samples are used to analyze the effect of frailty on a molecular level. However, the development of muscle loss is a complicated multifactorial process that develops in time and not only affects muscle but the metabolism of the whole organism (since multiple metabolic active organs interact). Therefore at present it is unavoidable to use animal models to study the development of muscle loss in time and the metabolic effects thereof on other tissues. We study the metabolic organ-organ interactions using mouse models and are currently evaluating whether it is possible to link specific organ dysfunction to markers in blood/plasma. By continuous comparison of data obtained in human studies with data obtained in mouse studies, our ultimate goal is to use blood/plasma biomarkers (instead of taking invasive muscle biopsies) as rendering of organ specific metabolic (dys)function.

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

The majority (>70%) of the studies that will be conducted in this project will be fundamental and applied efficacy studies to evaluate novel nutritional interventions or new therapeutics and the effects thereof on different metabolic organs/whole-body metabolism. This will 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 instance, a dose-finding pilot study could first be performed to find the optimal dose).

The remaining studies that will be carried out under this project application, will be aimed at improvement of the animal models (including improvements on animal welfare as well) or to obtain more knowledge about the underlying mechanisms of the development of muscle atrophy in general, and are therefore of a more fundamental nature. The currently used, and validated, model is a model without immobilization but is using 40% caloric restriction. This model has been proven to be representative and predictive of the human situation and is therefore currently used for intervention studies. However, we would like to improve the model 1) in order to allow testing of interventions on muscle functionality as well and 2) in order to allow discrimination of the effects of malnutrition and immobilization on muscle atrophy (mechanistic insight) and the effects of interventions thereof. The optimization of the animal model will be performed using a stepwise approach which is described in appendix I. Since in this project we focus on the underlying causes of muscle atrophy **in general**, we chose to use a model that is representative for all 3 forms of muscle atrophy (disabling circumstances, short or long periods of illnesses, age-related muscle loss) and is based on the shared similarities. Therefore. we use young mice with caloric restriction and would like to add immobilization.**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. Malnutrition is one of the important causes of muscle atrophy and can be mimicked in animal models via a limited calorie intake. A sedentary lifestyle or limited movement is another important factor in the development of muscle atrophy. This limited movement can be mimicked in our animal models as another inducer of muscle atrophy. 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.

3.4.2 Provide a basic outline of the different components of the project and the type(s) of animal procedures that will be performed.

For all studies (both fundamental research as well as the applied or translational research), first muscle atrophy will be induced in mice via 2 weeks of malnutrition and/or partial immobilization. For the applied/translational studies a preventive or therapeutic intervention will be applied as well.
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 fundamental or applied/translational 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 can be measured during the study and are described in more detail in the appendices.

3.4.3 Describe the coherence between the different components and the different steps of the project. If applicable, describe the milestones and selection points.

All components of this project application are designed to assess whether new therapies show preclinical effectiveness with respect to maintenance of muscle function and prevention or treatment of muscle loss and frailty. This assessment allows evaluation whether these therapies are suitable for further clinical development. In all studies we use animal models in which muscle atrophy will be induced via malnutrition and/or partial immobilization. Through a process of progressive validation and further development, we strive to offer the best possible study designs at all times.
The currently used, and validated, model is a model without immobilization but using 40% caloric restriction. This model has been proven to be representative and predictive of the human situation and can therefore already be used for intervention studies (2 manuscripts in preparation; poster at conference of European Society for Clinical Nutrition and Metabolism, September 2016). However, we would like to improve the model 1) in order to allow testing of interventions on muscle functionality as well and 2) in order to allow discrimination of the effects of malnutrition and immobilization on muscle atrophy (mechanistic insight) and the effects of interventions thereof. The optimization of the animal model will be performed using a stepwise approach and is described in Appendix I. In short: the currently used condition without immobilization but using a 40% caloric restriction, is 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. 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). At 40% restriction, we previously found that although muscle mass was reduced in C57BL6 mice, muscle strength was not impaired. 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 first 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 would exceed 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.

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 40% 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). Again also assuring 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 in general, 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).

In addition, 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.

After evaluation of these optimization steps, the adapted animal model might be used as well for intervention studies, described in Appendix 2. Please note that in Appendix 2, possible adaptations to the model that might be performed in the next 5 years are already included, but will not be performed in efficacy studies (Appendix 2) until proven to be indeed beneficial to the model (Appendix 1).

3.4.4 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: optimization of the animal model
2	Muscle loss: intervention study
3	
4	
5	

6	
7	
8	
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10	
11	