| | **Inventaris Wob-verzoek W17-07** | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | |
| | | **wordt verstrekt** | | | | **weigeringsgronden** | | | |
| **nr.** | **document NTS2017862** | **reeds openbaar** | **niet** | **geheel** | **deels** | **10.1.c** | **10.2.e** | **10.2.g** | **11.1** |
| 1 | Aanvraagformulier | | | | x | | x | x | |
| 2 | Projectvoorstel | | | x | | | | | |
| 3 | Niet-technische samenvatting | x | | | | | | | |
| 4 | Bijlage beschrijving dierproeven 1 | | | x | | | | | |
| 5 | Bijlage beschrijving dierproeven 2 | | | x | | | | | |
| 6 | Bijlage beschrijving dierproeven 3 | | | x | | | | | |
| 7 | DEC-advies | | | | x | | x | x | |
| 8 | Ontvangstbevestiging | | | | x | | x | x | |
| 9 | Advies CCD | | x | | | | | | x |
| 10 | Beschikking en vergunning | | | | x | | x | x | |

Centrale Commissie Dierproeven

# Aanvraag
## Projectvergunning Dierproeven
*Administratieve gegevens*

- U bent van plan om één of meerdere dierproeven uit te voeren.
- Met dit formulier vraagt u een vergunning aan voor het project dat u wilt uitvoeren. Of u geeft aan wat u in het vergunde project wilt wijzigen.
- Meer informatie over de voorwaarden vindt u op de website www.centralecommissiedierproeven.nl. of in de toelichting op de website.
- Of bel met 0900-2800028 (10 ct/min).

## 1 Gegevens aanvrager

1.1 Heeft u een deelnemernummer van de NVWA?
*Neem voor meer informatie over het verkrijgen van een deelnemernummer contact op met de NVWA.*

| | |
|---|---|
| ☒ Ja > *Vul uw deelnemernummer in* | 11200 |
| ☐ Nee > *U kunt geen aanvraag doen* | |

1.2 Vul de gegevens in van de instellingsvergunninghouder die de projectvergunning aanvraagt.

| | | |
|---|---|---|
| Naam instelling of organisatie | Vrije Universiteit te Amsterdam | |
| Naam van de portefeuillehouder of diens gemachtigde | [redacted] | |
| KvK-nummer | 53815211 | |
| Straat en huisnummer | de Boelelaan | 1105 |
| Postbus | | |
| Postcode en plaats | 1081HV | Amsterdam |
| IBAN | [redacted] | |
| Tenaamstelling van het rekeningnummer | [redacted] | |

1.3 Vul de gegevens van het postadres in.
*Alle correspondentie van de CCD gaat naar de portefeuillehouder of diens gemachtigde en de verantwoordelijke onderzoeker.*

1.4 Vul de gegevens in van de verantwoordelijke onderzoeker.

| | | |
|---|---|---|
| (Titel) Naam en voorletters | [redacted] | x Dhr. ☐ Mw. |
| Functie | Dr. (universitair docent) | |
| Afdeling | [redacted] | |
| Telefoonnummer | [redacted] | |
| E-mailadres | [redacted] | |

1.5 *(Optioneel)* Vul hier de gegevens in van de plaatsvervangende verantwoordelijke onderzoeker.

| | | |
|---|---|---|
| (Titel) Naam en voorletters | | ☐ Dhr. ☐ Mw. |
| Functie | | |
| Afdeling | | |
| Telefoonnummer | | |
| E-mailadres | | |

1.6 *(Optioneel)* Vul hier de gegevens in van de persoon die er verantwoordelijk voor is dat de uitvoering van het project in overeenstemming is met de projectvergunning.

| | | |
|---|---|---|
| (Titel) Naam en voorletters | | ☐ Dhr. ☐ Mw. |
| Functie | | |
| Afdeling | | |
| Telefoonnummer | | |
| E-mailadres | | |

1.7 Is er voor deze projectaanvraag een gemachtigde?

☒ Ja > *Stuur dan het ingevulde formulier Melding Machtiging mee met deze aanvraag*

☐ Nee

## 2 Over uw aanvraag

2.1 Wat voor aanvraag doet u?

☒ Nieuwe aanvraag > Ga verder met vraag 3

☐ Wijziging op (verleende) vergunning die negatieve gevolgen kan hebben voor het dierenwelzijn

Vul uw vergunde projectnummer in en ga verder met vraag 2.2

☐ Melding op (verleende) vergunning die geen negatieve gevolgen kan hebben voor het dierenwelzijn

Vul uw vergunde projectnummer in en ga verder met vraag 2.3

2.2 Is dit een *wijziging* voor een project of dierproef waar al een vergunning voor verleend is?

☐ Ja > Beantwoord dan in het projectplan en de niet-technische samenvatting alleen de vragen waarop de wijziging betrekking heeft en onderteken het aanvraagformulier

☐ Nee > Ga verder met vraag 3

2.3 Is dit een *melding* voor een project of dierproef waar al een vergunning voor is verleend?

☐ Nee > Ga verder met vraag 3

☐ Ja > Geef hier onder een toelichting en ga verder met vraag 6

## 3 Over uw project

| | | |
|---|---|---|
| 3.1 | Startdatum | 01-03-2017 |
| | Einddatum | 01-03-2022 |
| 3.2 | Wat is de titel van het project? | Delineating the function of TGF-β receptor-mediated signaling pathways in regenerating skeletal muscle and Duchenne Muscular Dystrophy |
| 3.3 | Wat is de titel van de niet-technische samenvatting? | Onderzoek naar de rol van eiwitsignaalpaden in spierherstel en het ziekteverloop van Duchenne Spierdystrofie |
| 3.4 | Naam DEC | DEC Vrije Universiteit / VU Medisch Centrum |
| | Postadres | [redacted]<br>Amsterdam \| Nederland |
| | E-mailadres | [redacted] |

Wat is de naam van de Dierexperimentencommissie (DEC) aan wie de instellingsvergunninghouder doorgaans haar projecten ter toetsing voorlegt?

# 4 Betaalgegevens

4.1 Om welk type aanvraag gaat het?

☒ Nieuwe aanvraag Projectvergunning €1541

☐ Wijziging € Lege

4.2 Op welke wijze wilt u dit bedrag aan de CCD voldoen.
*Bij een eenmalige incasso geeft u toestemming aan de CCD om eenmalig het bij 4.1 genoemde bedrag af te schrijven van het bij 1.2 opgegeven rekeningnummer.*

☐ Via een eenmalige incasso

X Na ontvangst van de factuur *
* Wanneer de factuur direct naar de financiële afdeling van de VU of het VUmc dient te gaan moet hier een inkooporder nummer en factuuradres worden toegevoegd door de onderzoekers, graag van te voren afstemmen met de financiële afdeling.

Inkooporder nummer: geen
Factuuradres: [redacted] Amsterdam, Vrije Universiteit (VU)
[redacted]

-> Graag verzoeken we de CCD om het bovenstaande inkooporder nummer aan de factuur toe te voegen en de factuur te versturen naar het factuuradres.

# 5 Checklist bijlagen

5.1 Welke bijlagen stuurt u mee?

Verplicht

☒ Projectvoorstel

☒ Niet-technische samenvatting

Overige bijlagen, indien van toepassing

☒ Melding Machtiging

☐

# 6 Ondertekening

6.1 Print het formulier uit, onderteken het en stuur het inclusief bijlagen via de beveiligde e-mailverbinding naar de CCD of per post naar:

Centrale Commissie
Dierproeven
Postbus 20401
2500 EK Den Haag

Ondertekening door de instellingsvergunninghouder of gemachtigde (zie 1.7). De ondergetekende verklaart:

- dat het projectvoorstel is afgestemd met de Instantie voor Dierenwelzijn.
- dat de personen die verantwoordelijk zijn voor de opzet van het project en de dierproef, de personen die de dieren verzorgen en/of doden en de personen die de dierproeven verrichten voldoen aan de wettelijke eisen gesteld aan deskundigheid en bekwaamheid.
- dat de dieren worden gehuisvest en verzorgd op een wijze die voldoet aan de eisen die zijn opgenomen in bijlage III van richtlijn 2010/63/EU, behalve in het voorkomende geval de in onderdeel F van de bijlage bij het bij de aanvraag gevoegde projectvoorstel gemotiveerde uitzonderingen.
- dat door het ondertekenen van dit formulier de verplichting wordt aangegaan de leges te betalen voor de behandeling van de aanvraag.
- dat het formulier volledig en naar waarheid is ingevuld.

| | |
|---|---|
| Naam | [redacted] |
| Functie | |
| Plaats | Amsterdam |
| Datum | 07 - 02 - 2017 |
| Handtekening | [redacted] |

# 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'. | 11200 |
| 1.2 | Provide the name of the licenced establishment. | Vrije Universiteit Amsterdam |
| 1.3 | Provide the title of the project. | Delineating the function of TGF-β receptor-mediated signaling pathways in regenerating skeletal muscle and Duchenne Muscular Dystrophy |

## 2 Categories

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

- X Basic research
- ☐ 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.

Duchenne Muscular Dystrophy (DMD) is the most common and severe inherited muscle wasting disease in children with an annual incidence rate between 1 in 3600-9300 males worldwide (Mah et al.

Neuromuscul. Disord. 2014; Mercuri and Muntoni Lancet 2013). DMD is a lethal recessive X-linked disease that is caused by mutations in the DMD gene, which result in loss of function of the muscle protein dystrophin. Because DMD is an X-linked disease, only males are affected and females are only carrier. DMD is hallmarked by progressive muscle weakness and decline in muscle function, which results in loss of mobility and early death of DMD patients. Improvement in standards of care, especially in cardiac and respiratory care, have resulted in higher life expectancy and DMD patients can now live up to 30 years (Bushby et al. N Engl J Med. 2010). However, no treatment currently exists that can further slow, inhibit or reverse the progressive muscle pathology in these patients.

DMD pathology is characterised by extensive muscle fiber degeneration due to the loss of function of dystrophin. Dystrophin is expressed in muscle fibers and acts as a link between the actin filaments, the sarcolemma of the muscle fiber and the extracellular matrix surrounding the muscle fiber. Loss of dystrophin affects the sarcolemma integrity and results in damage and degeneration of muscle fibers. In healthy skeletal muscle, muscle damage triggers the process of muscle regeneration by activating a resident pool of quiescent muscle stem cells called satellite cells that normally reside between the basal lamina and sarcolemma of the muscle fibers. Activated satellite cells, or myoblasts, are able to proliferate and initiate a myogenic differentiation program that allows them to fuse and repair damaged muscle fibers. Notably, several studies suggest that muscle regeneration is impaired in dystrophic muscle due to defective capacity of satellite cells to proliferate or due to loss of myogenic potential and ability to self-renew, which results in exhaustion of the satellite cell pool (Blau et al. Proc Natl Acad Sci U S A. 1983; Pessina et al., Stem cell reports 2015). Furthermore, continuous muscle fiber degeneration results in an inflammatory muscle environment that stimulates excessive deposition of fibrotic and fat tissue by (myo) fibroblasts and other cells, thereby creating a hostile environment that represses satellite cell function and regeneration. Importantly, this secondary pathology that follows muscle fiber degeneration contributes to the progressive loss of muscle function of DMD muscle.

Targeting of protein signaling cascades that play a role in the progressive pathology of DMD has emerged as a promising therapeutic opportunity to improve DMD muscle function. Potentially, such therapies would help to retain muscle tissue (i.e. muscle fibers), improve muscle function and stimulate self-sufficiency and life quality of DMD males. In addition, such treatments may be crucial to increase the efficiency of other therapies currently under scrutiny that are aimed at correcting the genetic defect in DMD by retaining muscle tissue and improving the dystrophic muscle environment. Although multiple signaling pathways have been identified as potential targets for DMD therapy, Transforming Growth Factor (TGF-β)-signaling has emerged in recent years as a central player in muscle degradation and fibrosis and has been identified as one of the more promising therapeutic targets for DMD and other muscle diseases (Sartori et al. Trends Endocrinol Metab., 2014).

The TGF-β superfamily consists of a variety of secreted signaling proteins that mediate downstream signaling cascades via specific interactions with membrane associated receptor proteins (see figure 1 and figure 2). These signaling cascades are involved in a multitude of cellular processes during embryonic development and play important roles in adult tissue homeostasis, tissue regeneration and regulation of the immune system. In addition to its physiological functions, dysregulation of TGF-β signaling has been shown to play an important role in many pathologies (Blobe et al., N Engl J Med. 2000), including muscle-wasting diseases such as DMD. Importantly, some studies suggested that repression of TGF-β and structurally related proteins such as Myostatin and Activin may improve muscle regeneration and may inhibit fibrosis in dystrophic muscle of dystrophin-deficient mdx mice, which is currently the most commonly used mouse model for DMD. However, several studies have questioned the reported beneficial effect of inhibiting these signaling pathways in skeletal muscle (Amthor et al. PNAS. 2007; Relizani et al. Mol Ther. 2014) and moreover the overlapping and distinct effects of the different ligands on muscle stem cells and muscle fibers are currently unclear. These results have highlighted the complexity of TGF-β signaling in skeletal muscle and the need to elucidate the molecular mechanism and effect of TGF-β signaling on muscle regeneration and in DMD pathology in more detail.

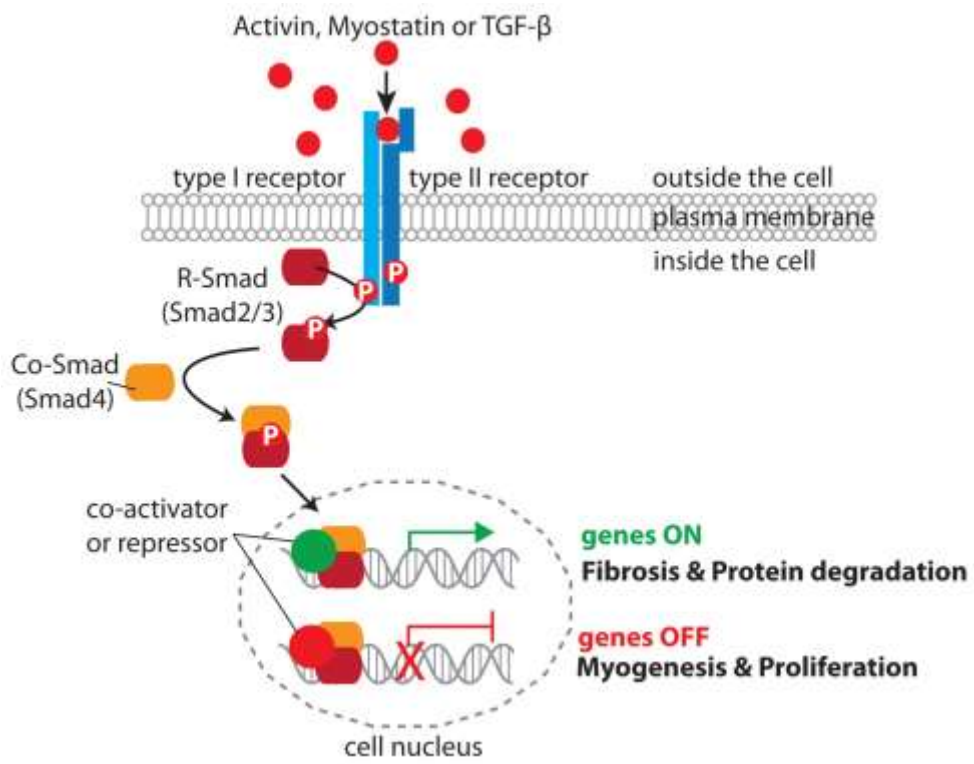



*Figure 1: Overview of TGF-β mediated signaling pathways.*

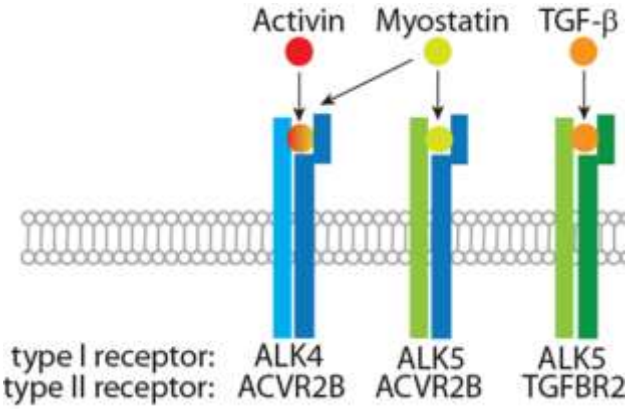



*Figure 2: Overview of different type I and type II receptors used by different TGF-β ligands.*

In this project we aim to delineate the effect of TGF-β pathways in muscle cells and healthy, injured and DMD skeletal muscle in detail and to determine if inhibiting these pathways by blocking the function of TGF-β receptors ALK4 and ALK5 (see figure 2) improves muscle regeneration and alleviates DMD pathology in mouse models of DMD.

### 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?

The **main objective** of the project is to delineate the effect of TGF-β pathways in healthy, injured and DMD muscle tissue and to determine the potential therapeutic effect of targeting these signaling pathways in muscle diseases such as DMD. Previous studies of our group suggested that targeting ALK4 and ALK5 receptors that mediate TGF-β signaling could prove to be a novel therapy to alleviate DMD pathology. Here we aim to determine the effect of inhibiting these specific TGF-β receptors on muscle regeneration and DMD pathology in more detail. Importantly, in order to understand the effect of these pathways in dystrophic muscle it is crucial to determine the function of these pathways in detail in isolated muscle cells and in healthy skeletal muscle before and after injury. Our main objective is therefore divided in **three sub-objectives**:

1) Delineate TGF-β receptor-mediated pathways in isolated muscle cells from transgenic mice and DMD mouse model.

2) Determine the effect of TGF-β receptor knockout on muscle mass, muscle regeneration and function before and after inflicting local muscle injury in mice.

3) Determine if TGF-β receptor knockout can alleviate DMD pathology in a mouse model of DMD.

Our **hypothesis** is that TGF-β receptor-mediated pathways inhibit muscle cell differentiation and regeneration and have a prominent function in DMD pathology and that inhibition of these pathways in isolated muscle cells and muscles of mice accelerates muscle regeneration after injury and alleviates the progressive nature of DMD. The **ultimate goal** is to develop novel therapeutics aimed at reducing the activity of signaling pathways that play a role in the progressive pathology of DMD in order to improve muscle function.

We expect that the main objective and sub-objectives are achievable within 5 years, which includes the time needed for import and cross(breeding) of the different mouse strains. Our department has the technical equipment and know-how to perform all the experiments as proposed in this project. Furthermore, we have an excellent track record of published papers in international renowned peer-reviewed journals (such as FASEB J, Genes Dev. Mol Ther etc.) and published multiple papers that have contributed to knowledge in this field. We have a dedicated staff for supervision, (PhD) students to perform the experiments and analysis and technicians for technical support. In addition, we have an excellent equipped laboratory, which includes among others equipment for DNA and gene expression analysis (quantitative real-time PCR equipment, analysis software, standard PCR machines, and electrophoresis equipment), protein analysis and histologic/morphologic analysis (Western blot equipment, cryostats for sectioning and state-of-the-art microscopes). Furthermore, the department has a cell culture facility with all the necessary equipment for single fiber/satellite cell isolation and there is unique, ample experience in performing *in situ* mouse muscle force measurements. The experimental animal population needed for the generation of conditional knockouts will be maintained at the animal facility by qualified and skilled personnel who are trained to adhere to the principles of replacement, reduction and refinement. The project is a collaboration with several renowned national and international institutes and is funded by an independent national funding agency.

### 3.3 Relevance

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

**Social relevance:**

In the Netherlands around 1:4000 males are affected (around 600 patients). The progressive loss of muscle fibers, impaired regeneration and replacement of functional muscle tissue by fibrotic and adipose tissue in DMD males results in immobilization and wheelchair dependency before the beginning of adolescence. Respiratory and cardiac complications emerge in their teens and results in premature death. As yet no therapy exists that can halt or further slow the progression of DMD, with the exception of glucocorticoids. However, the effect of these drugs is controversial and they have significant side-effects. Therefore, new therapies are needed that can counteract the progressive pathology of DMD. To

develop new therapies it will be crucial to better understand different pathways that are involved in DMD pathology, such as TGF-β signaling pathways, and determine if targeting these pathways can alleviate DMD pathology.

**Scientific relevance:**
In this project we will delineate the functions of TGF-β signaling pathways in healthy and dystrophic skeletal muscle and elucidate the function of TGF-β signaling receptors in skeletal muscle using transgenic animals and cell culture models. The results of this study will help us to better understand the molecular mechanism of these signaling pathways in skeletal muscle and to determine if inhibition of these pathways by suppressing TGF-β receptor function provides a potential novel therapy for DMD.

## 3.4 Research strategy

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

The project is divided in three sub-objectives that together are aimed at delineating TGF-β receptor-mediated pathways in healthy and dystrophic muscle. The strategy per sub-objective is discussed below.

**Sub-objective 1:**
**Delineate TGF-β receptor-mediated pathways in isolated muscle cells from wildtype mice and a DMD mouse model.**

The aim of sub-objective 1 is to delineate the downstream pathways of TGF-β receptors and the different ligands that signal via these receptors in more detail in cell cultures of isolated myoblasts (satellite cells). After sacrifice primary myoblasts are isolated from different muscles wildtype mice, mdx mice and transgenic ALK4/5 mice using protocols we optimized in our laboratory. These cells are cultured in growth medium to study proliferation and differentiation. We will utilize these *in vitro* models to delineate different and overlapping responses to different ligands and TGF-β receptor knockout on proliferation, differentiation and self-renewal and to identify novel pathways regulated by these signaling pathways in muscle cells. ALK4/5 knockout will be induced *in vitro* in cell culture. Gene expression analysis and protein analysis will be performed to validate known pathways and identify novel genes/pathways. Specifically, these protocols will be used to determine
1) the intrinsic differences and response of muscle cells isolated from healthy or dystrophic muscles to different ligands that signal via different TGF-β receptors and
2) the effect of TGF-β receptor knockdown in muscle cells isolated from healthy or dystrophic muscles *in vitro*.

**Sub-objective 2:**
**Determine the effect of TGF-β receptor knockout on muscle mass, muscle regeneration and function before and after inflicting local muscle injury in mice.**

To elucidate the function of type I receptors ALK4 and ALK5 in skeletal muscle we will make use of existing transgenic mouse models that will be imported into our animal facility to generate muscle specific genetic knockout mice of these receptors *in vivo* using the Cre-LoxP system. For this purpose, we will cross-breed satellite cell specific and myofiber specific inducible Cre-driver mice with floxed ALK4, ALK5 and ALK4/5 mice. The mice will be injected with tamoxifen to induce nuclear Cre activity which will result in conditional knockout of these receptors in satellite cells or muscle fibers. Importantly, no negative effect on animal health is expected in knockout mice, since the knockout is specific for skeletal muscle. The effect of individual and combined conditional knockout of ALK4 and ALK5 will be determined before and after inducing local muscle injury at different timepoints. Muscle injury will be induced locally in hindlimb muscles by single intramuscular injection of cardiotoxin. After conditional knockout of ALK4 and/or ALK5 different skeletal muscles will be isolated and analyzed to determine if muscle mass, muscle fiber size, muscle fiber type is affected and to determine the effect on satellite cell numbers in different skeletal muscles before and after muscle injury. Gene expression analysis and protein analysis will be performed to validate pathways and identify novel genes/pathways. In addition, we will perform functional tests on muscles of living anesthetized mice to determine the effect of conditional knockout on muscle force generation capacity.

**Sub-objective 3:**
**Determine if TGF-β receptor knockout can alleviate DMD pathology in a mouse model of DMD.**

To determine the effect of conditional knockout of TGF-β type I receptors ALK4 and ALK5 in dystrophic muscles we will cross-breed floxed ALK4 and ALK5 mice with a mouse model for DMD (mdx mice) and induce conditional knockout *in vivo* by intramuscular injection of adeno-associated viral vectors expressing the Cre enzyme. After conditional knockout of ALK4 and ALK5 skeletal muscles will be isolated and histologically analyzed to determine if dystrophic pathology is alleviated.

To determine the effect of conditional knockout of TGF-β, activin and MSTN type I receptors ALK4 and ALK5 in dystrophic muscles we will cross-breed floxed ALK4 and ALK5 mice with a mouse model for Duchenne (mdx mice) and induce conditional knockout by intramuscular injection of adeno-associated viral vectors expressing the Cre enzyme. After conditional knockout of ALK4 and ALK5 skeletal muscles will be isolated and histologically analyzed to determine if dystrophic pathology is alleviated. Gene expression analysis and protein analysis will be performed to validate pathways and identify novel genes/pathways. In addition, we will perform functional tests on muscles of living anesthetized mice to determine the effect of conditional knockout on dystrophic muscle function.

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.

An outline of the different animal procedures and outcome measures for each sub-objective are given below:

**Animal procedure 1 (for sub-objective 1):**
Type of animal procedures: Isolation of muscle cells from wildtype, mdx and ALK4/5 floxed mice. *In vitro* recombination and knockout of ALK4/5 in cell culture. Mice will be sacrificed after which muscle cells will be isolated from different muscles and cultured to determine proliferation and differentiation potential.

Outcome parameters:
Muscle cell proliferation and differentiation upon ALK4/5 knockdown assessed by immunofluorescence. Gene expression analysis and protein analysis will be performed to validate known pathways and identify novel genes/pathways responsive to TGF-β in isolated muscle cells.

**Animal procedure 2 (for sub-objective 2):**
Type of animal procedures: Conditional *in vivo* knockout of ALK4/5 in skeletal muscle. Intraperitoneal tamoxifen injections will be applied to induce ALK4/5 knockout. *In vivo* intramuscular cardiotoxin injections will be applied to induce local muscle damage and study the *in vivo* effect of ALK4/5 knockout on muscle regeneration. To determine the force generating capacity of muscle, we will also perform *in situ* force measurements in separate animals on the tibialis anterior muscle by using experimental set-ups that are up and running at our department. In short, mice will be anaesthetized, while the skin of the lower leg is removed and muscles of the lower leg are dissected free from their insertion, leaving intact orgin, blood supply and innervation via the ischiatic nerve. The mice will be places on a heated plate and the distal tendon will be attached to a force transducer and servo motor, while the muscle and its surrounding are kept moist at physiological temperature (34-35°C). The ischiatic nerve will be stimulated supramaxially to determine length-force and force-velocity curves as well as force-frequency relations. These measurements allow to estimate optimal active muscle force and specific tension as well as maximal shortening velocity and peak power. After *in situ* force measurements mice will be sacrificed. After *in situ* force measurements mice will not wake up and will be sacrificed.

Outcome parameters:
Muscle force, muscle mass, muscle fiber size and percentage of regenerating muscle fibers determined by histology and immunofluorescence. Gene and protein analysis of muscle and regeneration markers and genome wide expression analysis to identify novel target genes of TGF-β pathways in skeletal muscle.

**Animal procedure 3 (for sub-objective 3):**
Type of animal procedures: Conditional *in vivo* knockout of ALK4/5 in skeletal muscles of mdx mice. Intramuscular *in vivo* injections with AAV viral vectors expressing the Cre transgene will be used to induce ALK4/5 knockout in this experimental animal model of DMD. To determine the force generating capacity of muscles of these mice we will use the same protocol as described for sub-objective 2.

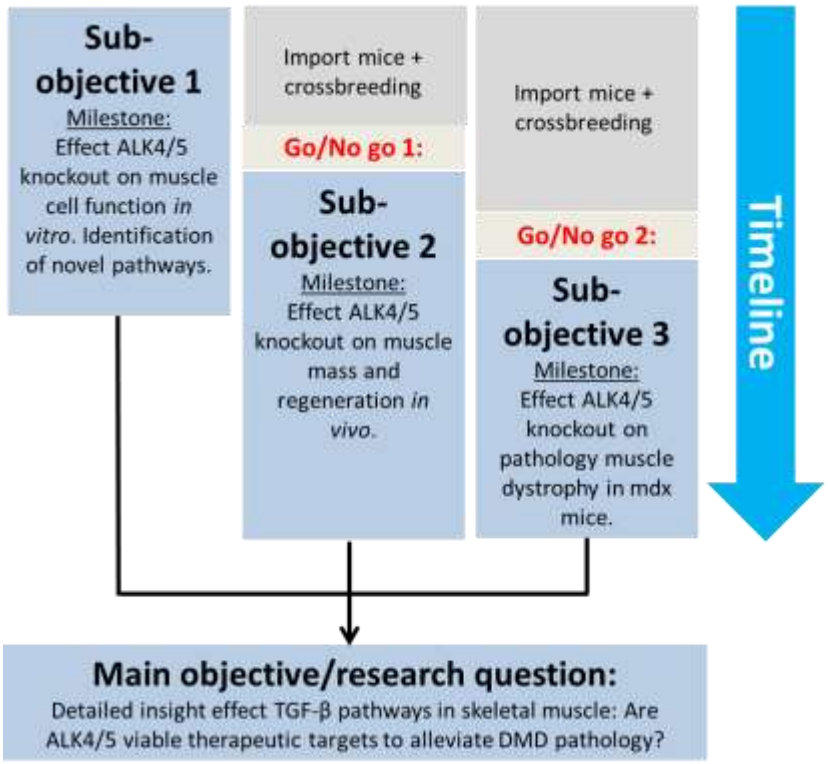
Outcome parameters:
Muscle force, muscle mass, muscle fiber size, percentage of regenerating muscle fibers, percentage of fibrotic tissue determined by histology and immunofluorescence. Gene and protein expression analysis of markers for muscle regeneration and fibrosis. Genome wide expression analysis to identify novel target genes of TGF-β pathways in skeletal muscle.

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.

We will start with isolation of muscle cells (**sub-objective 1)** to delineate the effect of individual and combined ALK4 and ALK5 knockout in more detail in muscle cells *in vitro* and to compare how healthy and dystrophic muscle cells respond to different ligands that signal via ALK4/ALK5. Importantly, *in vitro* analysis allows more detailed analysis of the effect of different doses of ligands (TGF-β, activin or MSTN) and single or combined knockdown of ALK4/5 on myoblast proliferation, differentiation and satellite cell self-renewal. In addition, *in vitro* experiments in muscle cells allow more detailed analysis of downstream canonical and non-canonical intracellular pathways involved in these signaling cascades. This will allow us to identify new genes and pathways that are regulated by these signaling pathways and to determine if there is a difference effect of different TGF-β receptors in muscle cells.

Transgenic mice for procedure 2 and 3 will meanwhile be cross-bred to obtain transgenic mice for inducible single or double knockout of ALK4 and ALK5. For **sub-objective 2 (procedure 2)** we will first determine the concentration of tamoxifen that results in efficient knockout of ALK4 and ALK5 (**go/no go 1**). The optimum dose of tamoxifen will be used to induce individual and combined knockout in skeletal muscle cells *in vivo* before and after inflicting local muscle injury and muscles will be isolated and analyzed (see 3.4.2. and animal procedure 2). If no efficient knockout is achievable after tamoxifen injections we will discontinue the experiments. For **sub-objective 3 (procedure 3)** we will first determine which serotype and concentration of AAV viral vector in dystrophic muscle results in efficient single or double knockout of ALK4 and ALK5 in dystrophic muscle (**go/no go 2**). If no efficient knockout is achievable we will discontinue the experiments. The optimal concentration will be used to generate individual and combined ALK4 and ALK5 knockout in dystrophic muscles of mdx mice and to analyze the effect on dystrophic muscle pathology. Importantly, we will validate potential novel pathways regulated by ALK4 and/or ALK5 identified in procedure 1 and 2 also in procedure 3 in dystrophic muscle of mdx mice to determine the relevance in dystrophic pathology.

The timeline and the milestones of the different sub-objectives are depicted in the following figure (see for explanation of the sub-objectives and go/no go moments text above):




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 | Isolation of muscle cells from skeletal muscle of wildtype and transgenic mice. |
| 2 | Conditional knockout of TGF-β receptors in skeletal muscle |
| 3 | Conditional knockout of TGF-β receptors in a DMD mouse model |
| 4 | |
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4

# 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'. | 11200 |
| 1.2 | Provide the name of the licenced establishment. | Vrije Universiteit Amsterdam |
| 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: 1<br>Type of animal procedure: Isolation of muscle cells from skeletal muscle of wildtype, transgenic mice and mdx mice for *in vitro* (recombination) analysis. |

## 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.

The aim of the experiments described in this appendix (animal procedure 1 in the project proposal) is to delineate the downstream pathways of the type I receptors ALK4 and ALK5 and the different ligands that signal via these receptors in detail in cell cultures of isolated myoblasts (satellite cells). Primary myoblasts will be isolated from skeletal muscles of mice, which allows us to culture these cells *in vitro* and determine the effect of the different pathways on muscle cell function. The primary outcome parameters include myoblast proliferation, differentiation and satellite cell activation/selfrenewal and are chosen to be able to determine the effect of the different ligands and ALK4/5-knockdown on satellite cell/myoblast function. These parameters will be assessed by immunofluorescent staining and gene expression analysis of markers for the different cellular processes mentioned above. In addition, detailed gene expression analysis will be performed to identify novel target genes of ALK4- and ALK5-mediated pathways.

Myoblasts and muscle fibers with satellite cells retained in their niche will be isolated for the following *in vitro* experiments:

Experiment 1: Isolation of muscle cells from wildtype and mdx mice to determine the intrinsic differences between muscle cells isolated from healthy or dystrophic muscles.

Experiment 2: Isolation of muscle cells from ALK4/5 mice and mdx: ALK4/5 receptor floxed mice to determine the effect of single and double ALK4/5 receptor knockdown.

The primary outcome parameters in relation to both sub-objectives 1 and 2 and the different procedures (described in the next section) are described in table 1 below and represent parameters of choice to determine the effect of TGF-β receptor knockout on skeletal muscle function.

| **Procedure** | **Primary outcome parameters** |
|---|---|
| - Transport to the department<br>- Killing mice<br>- Isolation primary myoblasts<br>- Isolation muscle fibers with satellite cells in niche | - Myoblast proliferation<br>- Myoblast differentiation<br>- Satellite cell activation and selfrenewal<br>- Gene expression/protein levels |

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

**Import of mice and breeding of mice:**
After import of the different mice, we will maintain colonies of each mouse line. Importantly, mdx mice and transgenic floxed mice reproduce normally and no discomfort is expected when maintaining live colonies. The following mouselines will be imported and bred:

- Wildtype mice
- Mdx mice: These mice are not transgenic but have a spontaneous mutation in exon 23 that results in a premature stopcodon in the *Dmd* gene and disruption of the *Dmd* open reading frame and dystrophin protein function.

Floxed mice:
- Tgfbr1 (ALK5) flox/flox (floxed) mice. Exon 3 deletion and functional knockout after Cre mediated recombination. Henceforth will be named ALK5 floxed mice.
- Acvr1b (ALK4) flox/flox (floxed) mice. Exon 5+6 deletion and functional knockout after Cre mediated recombination. Henceforth will be named ALK4 floxed mice.

Wildtype, mdx and transgenic floxed mice will be maintained in holding colonies. ALK4 and ALK5 floxed mice will be crossed to obtain ALK4+ALK5 floxed animals, which will be maintained in separate holding colonies. Mdx mice will be crossbred with floxed mice to obtain mdx: ALK4, ALK5 and ALK4/5 floxed mice (see appendix 3 for breeding schematic).

**Killing of mice**
To isolate muscle fibers and myoblasts from mouse muscles mice will be transported to the department and sacrificed at arrival using a method of killing listed in Annex IV of Directive 2010/63/EU.

**Isolation of muscle fibers and primary myoblasts**
Primary activated satellite cells (myoblasts) are isolated to determine the intrinsic differences between myoblasts isolated from healthy and dystrophic mouse muscles (experiment 1) and determine the effect of TGF-β receptor knockout and different ligands *in vitro* on muscle cell function (experiment 2). For the isolation of primary myoblasts we will use a protocol that has been optimized in our laboratory. For this protocol mice are transported to the department and killed, after which different muscles will be dissected and muscle fibers are isolated. Muscle fibers can then either be plated on matrigel coated plates to isolate and culture activated satellite cells, or further cultured in floating cultures to study satellite cells in their muscle fiber niche. Knockout of single receptor or both ALK4/5 receptors will be induced by infecting myoblasts/myotubes isolated from floxed mice in culture dishes with viral vectors expressing the Cre enzym.

Describe which statistical methods have been used and which other considerations have been taken into account to minimise the number of animals.

To ensure that we use the minimal amount of animals necessary for independent cell isolations and *in*

*vitro* experiments, we performed an ***a priori*** **power analysis** based on the primary outcome measures (proliferation/differentiation) and previous experience. The outcome of this analysis is n=6 animals per experiment/group. In addition, based on previous experience we expect that not every isolation is successful since it can happen that muscle are damaged and single fibers cannot be obtained due to hypercontraction after damage. Therefore we include 2 extra animals per group. This brings the total number to **n=8 animals per experiment/group**.

**B. The animals**

Specify the species, origin, estimated numbers, and life stages. Provide justifications for these choices.

**Species:** male mice. We will compare muscle cells isolated from wildtype and floxed mice with mdx mice. Since DMD is an X-linked muscle disease that mainly affects boys due to the location of the affected gene on the X-chromosome, we choose to perform the analysis on muscle cells isolated from male mice only. This is important since intrinsic differences in differentiation have been reported in male and female satellite cells as well as differences in muscle regeneration (Deasy *et al* J Cell Biol. 2007).

**Origin:**
Wildtype mice are imported from certified suppliers and the transgenic ALK4/5 floxed animals will be imported from well-established research institutes.

**Estimated numbers: n=1120** (see also table 2 below for an overview)

Experiment 1 (group 1-2; see table 2 below for an overview):
In this experiment we will compare intrinsic differences between wildtype and mdx myoblasts and satellite cells in their muscle fiber niche and determine if they respond differently to ligands that signal via ALK4 and ALK5 receptors. The number of mice is estimated based on the following numbers:
Number of mice/group (see A. statistical method): 8
Groups (Genotypes): 2 (wildtype and mdx)
Timepoint of isolation: 2 (see life stages below for explanation).
Types of analysis: 5
For cell culture of primary myoblasts it is important to realize that primary cells cannot be cultured indefinitely since they will go into senescence and will stop dividing after 6-10 passages. Therefore, we need cells from different animals for the different types of analysis to ensure we have enough cells. In primary myoblasts we will determine intrinsic differences between healthy and dystrophic muscle cells with regard to the following outcome measures (three different types of analysis); proliferation, differentiation and gene/protein expression analysis. The same protocol is used to isolate muscle fibers with satellite cells attached in their niche, however we will require extra mice to obtain enough muscle fibers for muscle fiber cultures with satellite cells in their niche to compare the following outcome measures (two different types of analysis); satellite cell activation and selfrenewal.
Taking the above numbers into account, the total number of mice estimated for experiment 1 is as follows: 8x2x2x5=160

Experiment 2 (group 3-8; see table 2 below for an overview):
In this experiment we will determine the effect of ALK4 and/or ALK5 knockout in myoblasts and satellite cells in their muscle fiber niche in healthy and dystrophic myoblasts. The number of mice is estimated based on the following numbers:
Number of mice/group (see A. statistical method): 8
Groups (Genotypes): 6 (ALK4/5 floxed and mdx: ALK4/5 floxed)
*In vitro* treatment (Cre + control adenoviral vector): 2
Timepoint of isolation: 2 (see life stages below for explanation).
Types of analysis: 5
Similar to experiment 1; see above
Taking the above numbers into account, the total number of mice estimated for experiment 2 is as follows: 8x6x2x2x5=960

| nr | Genotype | Experiment 1 | Experiment 2 |
|---|---|---|---|
| 1 | Wildtype | n=80 | X |
| 2 | Mdx | n=80 | X |
| 3 | ALK4fl/fl | X | n=160 |
| 4 | ALK5fl/fl | X | n=160 |
| 5 | ALK4+ALK5fl/fl | X | n=160 |
| 6 | mdx: ALK4fl/fl | X | n=160 |
| 7 | mdx: ALK5fl/fl | X | n=160 |
| 8 | mdx: ALK4+ALK5fl/fl | X | n=160 |
| | | n=160 | n=960 |

**Life stages: 4-6wks and 4-6 months**
The life expectancy of males with Duchenne Muscular Dystrophy is currently around 30 year. However, the progressive pathology begins at an early stage between 3-5 years. We are therefore interested in the intrinsic differences and response of cells from both young/adolescent (3-6 weeks) and adult muscles to *in vitro* knockout of TGF-β receptors and to different TGF-β ligands. Based on data at the site of the Jackson laboratories (https://www.jax.org/research-and-faculty/research-labs/the-harrison-lab/gerontology/life-span-as-a-biomarker), we consider 4-6-week-old male mice as young/adolescent and 4-6-month-old mice as adult.

**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:**
Muscle cell culture *in vitro* is used to study the responsiveness of different muscle cell types to signaling pathways and receptor knockout. Isolated primary myoblasts are able to proliferate and differentiate *in vitro* and mimic their behaviour during muscle regeneration *in vivo*. In addition, these cells can be cultured in their niche on muscle fibers to study activation and selfrenewal of these cells. Although no substitute to *in vivo* experiments, cell culture is a good model to study the effect of different signaling pathways, such as TGF-β, on these processes in more detail specifically in muscle cells. We will isolate and compare muscle cells from healthy wildtype mice and dystrophic mdx mouse muscles. Although mdx mice do not show progressive muscle dystrophy and, in contrast to DMD patients, have only a slight reduction in lifespan, they are genetically comparable to DMD patients and some of the dystrophic changes (chronic inflammation, regeneration, fibrosis, decrease in muscle force) are recapitulated. In addition, previous studies suggested that satellite cells isolated from muscles of mdx mice are impaired in their proliferative and differentiation potential. We therefore think that this model is valuable for research as a preclinical animal model of DMD.

**Reduction:**

We performed a power analysis to determine group size for the different experimental groups based on previous experience to minimize the number of animals we use per group (see A. statistical methods). In addition, standard housing/diet conditions and experienced personnel that handle the mice yields less variation between animals that may arise due to environmental conditions (for instance stress due to handling or variations in housing or diet) and thus contribute to reduction in the number of mice we need.

**Refinement:**
There are different methods used to minimize stress and discomfort in mice in our animal facility. Mice are allowed to acclimatize after import (7-14 days), housing conditions are optimized and cage enrichment is used to reduce stress for the mice. In addition, handling and killing of mice is only performed by experienced and licensed personnel.

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

The following measures are taken to minimize animal suffering, stress and fear:

- Animal handling and experimental procedures will only be performed by experienced and certified employees
- Minimizing duration of transport and acclimatization of mice
- Optimized housing conditions, use of cage enrichment

After import to the animal facility and transport, all mice will be monitored to ensure their well-being. If any signs of suffering are observed for over 3 consecutive days the mice will be sacrificed with an overdose of anaesthetic after consulting the staff of the animal housing facility and/or the animal welfare body. The following humane endpoints are used:

- Reduced activity
- Not eating and drinking
- Rapid weight loss (more than 15% in two days)
- Breathing problems

## 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.

Our research group is experienced in isolation of myoblasts and we have done extensive literature studies on the topic. Importantly, no studies were identified in scientific literature that performed similar experiments and we are therefore positive that the proposed experiments are original and have not been previously performed in other studies. In addition, the project was approved by an independent scientific committee who scrutinized both the background and content.

## 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?

X No

☐ Yes > If this may adversely affect animal welfare, describe how the animals will be housed and provide specific justifications for these choices.

**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?

X No > Continue with question I.

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

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

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

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

Describe which other adverse effects on the animals' welfare may be expected?

We will isolate cells from muscles of both wildtype mice, transgenic mice (ALK4/5 floxed mice) and mdx mice, a mouse model for Duchenne Muscular Dystrophy. Importantly, mdx mice and transgenic floxed mice reproduce normally and no discomfort is expected when maintaining live colonies. Although mdx mice show mild muscle pathology, no general health problems have been reported in literature. In addition, these mice have relatively normal lifespan and have been noted as exceptional breeders, with no problems in reproduction. The dystrophic changes in mdx muscles tend to stabilize and no progressive muscle dystrophy is observed. Adverse effects on the animals' welfare are only expected when the mice are old due to worsening of muscle dystrophy in old muscles, but the animals will not reach this age in our experiments or during breeding and animals will be sacrificed before they reach this old age. However, it has been reported that these mice may be more susceptible to stress. Therefore, mdx mice will be closely monitored to ensure their well-being and unnecessary handling of the mice will be avoided.

Explain why these effects may emerge.

Mdx mice have been reported to be more susceptible to stress, possibly due to genetic background. Transport or handling of the animals can result in some stress and hence may result in mild discomfort for the animals.

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

Animals will only be handled and killed by experienced and qualified personnel who are trained to swiftly sacrifice animals with minimum amount of stress. In addition mice will be monitored to ensure their health.

**J. Humane endpoints**

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

X No > Continue with question K.

☐ Yes > Describe the criteria that will be used to identify the humane endpoints.

Indicate the likely incidence.

**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').

Transport to the department: mild discomfort. Cumulative discomfort: mild. Killing of the mice: non-recovery

## 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.

To isolate primary myoblasts and muscle fibers for cell culture experiments whole skeletal muscles must be isolated from the mice, which cannot be done without killing the animals. In contrast to humans it is not possible to take biopsies of the muscles and isolate muscle cells from a biopsy because the skeletal muscles of mice are too small.

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

5

# Appendix
## Description animal procedures

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- 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'. | 11200 |
| 1.2 | Provide the name of the licenced establishment. | Vrije Universiteit Amsterdam |
| 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: 2<br>Type of animal procedure: Conditional knockout of ALK4 and ALK5 receptors in skeletal muscle cells |

## 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.

The objective of the animal experiments described in this appendix is to determine the effect of individual and combined ALK4/ALK5 knockout in specific muscle cell types in mouse skeletal muscle before and after muscle injury (procedure 2 in the project application). To elucidate the function of type I receptors ALK4 and ALK5 in skeletal muscle we will make use of existing transgenic mouse models that will be imported into our animal facility to generate specific genetic knockout mice of these receptors in muscle fibers and satellite cells using an inducible Cre-LoxP system. Satellite cell specific and muscle fiber specific inducible Cre-driver mice will be crossbred with floxed ALK4 and ALK5 mice. These mice will be injected with tamoxifen to induce nuclear Cre activity which will result in conditional knockout of these receptors in satellite cells or muscle fibers *in vivo*. Subsequently, the effect of ALK4/5 knockout *in vivo* will be analyzed in uninjured and injured skeletal muscle using different techniques described below.

The following experiments are described in this appendix:
Experiment 1: ALK4/5 knockout in satellite cells and muscle fibers; Pilot experiment to determine efficiency knockout different doses of tamoxifen (go/no go).
Experiment 2: ALK4/5 knockout in muscle fibers in skeletal muscle; Experiment to determine effect of knockout on muscle mass and muscle force at different timepoints after knockout.
Experiment 3: ALK4/5 knockout in satellite cells and muscle fibers in injured skeletal muscle; Experiment to determine the effect of knockout on muscle regeneration at different timepoints after muscle injury.

The primary outcome parameters in relation to the four experiments and the different procedures (described in the next section) are described in table 1 below and represent parameters of choice to determine the effect of ALK4/5 knockout on skeletal muscle function.

*Table 1. Experiments with procedures and primary outcome parameters*

| **Experiment** | **Procedure** | **Primary outcome parameters** |
|---|---|---|
| 1 | - Tamoxifen injections<br>- Transport to the department<br>- killing mice | - Optimal ALK4/ALK5 knockdown (gene/protein) after tamoxifen injection |
| 2 | - Tamoxifen injections<br>- Transport to the department<br>- *In situ* muscle force measurement<br>- killing mice | - *In situ* muscle force<br>- Muscle mass<br>- Histology: muscle fiber size<br>- Gene/protein expression analysis |
| 3 | - Tamoxifen injections<br>- Cardiotoxin injection<br>- Transport to the department<br>- Killing mice | - Histology: muscle fiber size, % regenerating fibers<br>- Immunofluorescence: satellite cell markers and regeneration markers<br>- Gene/protein expression analysis |

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

**Import, breeding and crossbreeding of mice.**

After import of the different transgenic mice, we will maintain colonies of each transgenic line. Importantly, these transgenic mice reproduce normally and no discomfort is expected when maintaining live colonies. The following Cre and floxed mouselines will be imported, bred and subsequently crossbred to obtain inducible knockout mice in skeletal muscle fibers and satellite cells:

Inducible Cre mice:

- HSA-MCM mice. HSA-MCM mice express tamoxifen-inducible Cre in both limb and craniofacial skeletal muscle fibers (McCarthy JJ et al Skelet Muscle. 2012 May 7;2(1):8).
- Pax7creER mice. Pax7creER mice can be used to generate satellite cell specific knockout mice. A CreERT2 fusion protein sequence inserted downstream of the Pax7 stop codon allows endogenous Pax7 expression in these mice while permitting specific conditional labeling, manipulation, and deletion of satellite cells.

Floxed mice:

- Tgfbr1 (ALK5) flox/flox mice, C57BL/6 (Larsson J EMBO J 2001). Exon 3 deletion and functional knockout after Cre mediated recombination.
- Acvr1b (ALK4) flox/flox mice, C57BL/6 (Ripoche Genesis 2013). Exon 5+6 deletion and functional knockout after Cre mediated recombination.

Transgenic mice will be maintained in holding colonies and will be crossbred (F0) to obtain heterozygous Cre/flox mice (F1). These mice will be crossed with floxed mice to obtain inducible knockout mice and control mice (F2). ALK4, ALK5 and ALK4/ALK5flox/flox mice will be crossbred with either HSA-MCM mice or Pax7CreER mice to obtain inducible knockout mice for these receptors in skeletal muscle fibers or satellite cells respectively (see figures 1 and 2 below). ALK4/5 double knockout mice will be generated by crossbreeding ALK4flox/flox and ALK5 flox/flox mice and crossbreeding the ALK4/ALK5flox/flox mice with the inducible cre mice. No health problems are expected in knockout mice since knockout of ALK4/5 is muscle-specific.

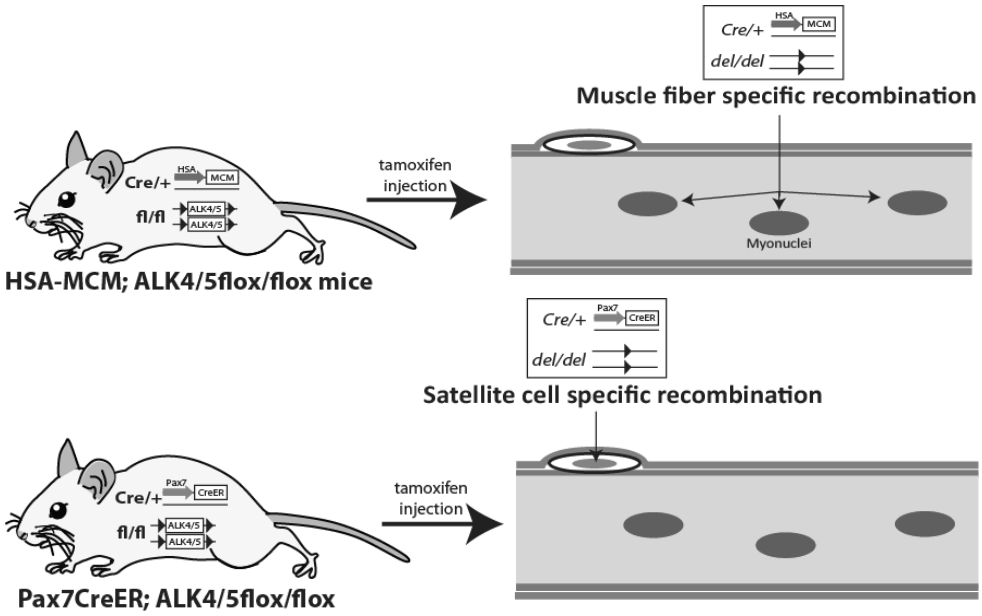


*Figure 1: Picture showing the mechanism of cell-specific inducible knockout in skeletal muscle. Top panel: Muscle fiber specific knockout. Lower panel: Satellite cell-specific knockout. fl/fl: gene of interest flanked by loxP sites; del/del: gene deleted after Cre-mediated recombination.*

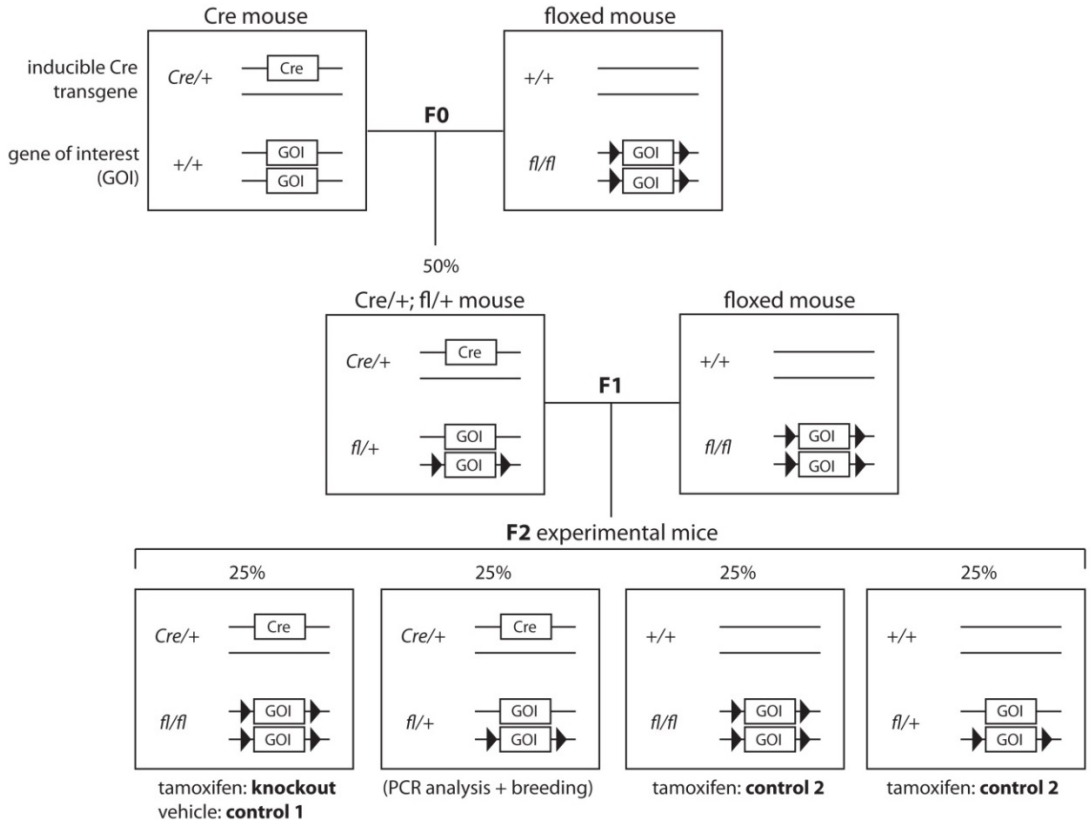


*Figure 2: General schematic of the protocol for crossbreeding and injections to obtain inducible knockout mice.*

**Experiment 1 (pilot in vivo experiment: go/no go)**
Procedures: Tamoxifen injections (i.p.), transport to the department, killing mice, muscle isolation.
Frequency/duration: Intraperitoneal tamoxifen injections 1x/day for 5 consecutive days.

General description:
We will first optimize individual and combined knockout of ALK4 and ALK5 in muscle fibers and satellite cells using an established protocol for tamoxifen injections that yielded high recombination efficiency in other comparable studies. Tamoxifen binds to the Cre-ER ligand-binding domain fusion protein and transfers Cre to the nucleus, where it mediates recombination of the loxP sites and deletion of our genes of interest in the specific cell types (see Figure 1). Three different groups are analyzed and compared: knockout mice (Cre+;flox/flox mice injected with tamoxifen), control 1 mice (Cre+;flox/flox injected with vehicle, control for knockout) and control 2 mice (+/+; flox/flox or flox/+ mice, injected with tamoxifen, control for tamoxifen) (see Figure 2). Tamoxifen is a hazardous substance and adverse effects have been reported (weight loss, scrotal enlargement in male mice). Since different tamoxifen concentrations are reported in literature (and tamoxifen can be toxic), we will test 3 different concentrations to determine which dose yields the highest knockout efficiency in our hands. A week after the last injection, the mice will be transported to the department where we will sacrifice the mice and isolate muscles, muscle fibers or satellite cells for ALK4 and ALK5 gene and protein expression analysis.

**Experiment 2**
Procedures: Tamoxifen injections (i.p.), transport to the department, surgery, *in situ* force measurements, sacrificing mice. Frequency/duration: Tamoxifen injections 1x/day for 5 consecutive days. *In situ* force measurements (under anesthesia) are non-recovery.

General description:
To determine the effect of ALK4 and/or ALK5 knockout on muscle mass and function we will crossbreed ALK4 and/or ALK5 floxed animals with HSA-MCM mice to generate muscle fiber specific inducible knockout mice and induce knockout with i.p. injections of the optimal tamoxifen dose established in experiment 1 This will be performed only in the muscle fiber specifc knockout mice, since we only expect a potential effect on muscle mass and muscle force if we knockout ALK4 and/or ALK5 in muscle fibers and not in satellite cells. Knockout mice will be compared with vehicle control and tamoxifen control groups, comparable to experiment 1. We will determine the effect of ALK4 and/or ALK5 knockout at 3 different timepoints after the last tamoxifen injection on muscle force, muscle mass and gene/protein expression. The timepoints will be chosen to determine the short term, mid term and long term effect of single and double ALK4/5 knockout.

Surgery and protocol *in situ* force measurements:
To determine the force generating capacity of muscle, we will perform separate *in situ* force measurements on the TA muscle by using experimental set-ups which are up and running at our department. We will use extra mice since these measurements can take a few hours and may affect histology and gene/protein expression. Mice will be anaesthetized by using isoflurane, while the skin of the lower leg is removed and muscles of the lower leg are dissected free from their insertion leaving the muscle, blood supply and innervation via the ischiatic nerve intact. The mice will be placed on a heated plate and the distal tendon will be attached to a force transducer and servo motor, while the muscle and its surrounding are kept moist at physiological temperature (34-35°C). The ischiatic nerve will be stimulated supramaxially to determine length-force and force-velocity curves as well as force-frequency relations. These measurements allow to estimate optimal active muscle force and specific tension as well as maximal shortening velocity and peak power. These measurements are non-recovery.

**Experiment 3**
Procedures: Tamoxifen injections (i.p.), cardiotoxin injections (i.m.), transport to the department, sacrificing mice, isolation of muscles. Frequency/duration: Tamoxifen injections 1x/day for 5 consecutive days. Local intramuscular cardiotoxin injection 1x.

Description:
To determine the effect of ALK4 and/or ALK5 knockout on muscle mass and function we will crossbreed

ALK4 and/or ALK5 floxed animals with HSA-MCM and Pax7CreER mice to generate muscle fiber specific and satellite cell specific inducible knockout mice, respectively, and induce knockout with i.p. injections of the optimal tamoxifen dose established in experiment 1. Next, muscle damage will be locally induced under anesthesia (to reduce discomfort due to pain) with one intramuscular injection of cardiotoxin (CTX) in hindlimb muscles. At five different timepoints after CTX injection, mice will be transported to the department and sacrificed. These timepoints will be chosen to determine the effect of knockout at different timepoints during the regeneration process. At the last timepoint we will additionally measure *in situ* force generated by injected muscles (see description experiment 2) to determine if single or combined knockout of ALK4/5 in muscle fibers or satellite cells during regeneration has an effect on muscle force. This method of local intramuscular CTX injection has been shown to induce muscle regeneration and satellite cell activity and is used to study efficiency of regeneration *in vivo* (Garry GA *et al* Methods Mol Biol. 2016; Hardy D *et al* PLoS One. 2016). Knockout mice will be compared with vehicle control and tamoxifen control groups, comparable to experiment 1 and 2. Muscles will be isolated at the department for histological and expression analysis, which alllows us to determine the effect of ALK4/ALK5 knockout in muscle fibers and satellite cells on muscle regeneration.

Describe which statistical methods have been used and which other considerations have been taken into account to minimise the number of animals.

To ensure that we use the minimal amount of animals necessary, we perform an ***a priori* power analysis** for each experiment based on expectations and previous knowledge of similar outcome measures in comparable experiments. Based on this power analysis we expect that we need approximately **n=5** animals/group for experiment 1 and **n=8** animals/group for experiment 2 and experiment 3. This includes extra animals to account for potential loss of animals/samples (20%) due to potential damage inflicted to muscles during the isolation.

**B. The animals**

Specify the species, origin, estimated numbers, and life stages. Provide justifications for these choices.

**Species & gender:** Male mice. The effect of ALK4/5 knockout in skeletal muscle before and after muscle injury will be compared with knockout of these proteins in a mouse model of DMD (see appendix 1+3). DMD is an X-linked muscle disease that mainly affects boys due to the location of the affected gene on the X-chromosome. To be able to compare the experiments described in this appendix with experiments in appendix 2 we will therefore also use male mice.

**Origin:** The Jackson Laboratory (inducible Cre-driver mice), established research institutes (LUMC, Leiden: ALK5flox/flox) (Centre de Recherche en Cancérologie de Lyon: ALK4flox/flox)

**Estimated numbers:** n=1422 mice

Experiment 1 (pilot experiment different tamoxifen doses)
n=5 mice/group (see A statistical methods) x 3 (three different groups; knockout, control 1 and control 2, see schematic A animal procedures) x 3 (three different concentrations, see A animal procedures) x 6 (six different genotypes, see table above). This experiment will be performed in both Pax7CreER and HSA-MCM experimental groups (see table 2; group 1-6). Total of n=270 animals

Experiment 2 (knockout ALK4/5 in skeletal muscle)
To determine the effect of knockout on muscle mass and force in skeletal muscle we will generate muscle fiber specific knockout mice of ALK4, ALK5 and ALK4+ALK5, induce knockout with tamoxifen injections and determine *in situ* muscle force and analyze muscle histology/gene expression at three different time points after the last injection. We need n=8 mice/group (see A statistical methods) x 2 (mice for histological/expression analysis + *in situ* force measurements) x 3 (three different groups; knockout, control 1 and control 2, see schematic A animal procedures) x 3 (different timepoints after tamoxifen injection) x 3 (three different genotypes, see table below). This experiment will only be performed in HSA-MCM experimental groups (see table 2; group 1-3) since we only expect an effect on muscle mass/force of skeletal muscle after inhibiting these pathways in muscle fibers. Total of n=432 animals

Experiment 3 (knockout ALK4/5 in injured skeletal muscle)
n=8 mice/group (see A statistical methods) x 3 (three different experimental groups; knockout, control 1 and control 2, see figure 2 in A. animal procedures) x 5 (five different timepoints) x 6 (six different genotypes, see table below). For the last timepoint we will include extra animals for *in situ* force measurements. This experiment will be performed in both Pax7CreER and HSA-MCM experimental groups (see table 2; group 1-6), since we expect an effect on muscle regeneration after inhibiting these pathways in both satellite cells and muscle fibers. Total of n=720 animals

*Table 2: Estimated number of experimental and breeding mice for in vivo experiments.*

| nr | Genotype | Exp 1 | Exp 2 | Exp 3 | Total |
|---|---|---|---|---|---|
| 1 | HSA-MCM x ALK4fl/fl | n=45 | n=144 | n=120 | n=309 |
| 2 | HSA-MCM x ALK5fl/fl | n=45 | n=144 | n=120 | n=309 |
| 3 | HSA-MCM x ALK4+ALK5fl/fl | n=45 | n=144 | n=120 | n=309 |
| 4 | Pax7CreER x ALK4fl/fl | n=45 | X | n=120 | n=165 |
| 5 | Pax7CreER x ALK5fl/fl | n=45 | X | n=120 | n=165 |
| 6 | Pax7CreER x ALK4+ALK5fl/fl | n=45 | X | n=120 | n=165 |
| | | 270 | 432 | 720 | **n=1422** |

**Life stages:** 4-6wks old
For *in vivo* experiments with inducible knockout mice we will use 4-6 wks old mice since tamoxifen induced Cre activity has been reported to be more efficient in mice of this age compared to older mice (Feil S *et al* Methods Mol Biol. 2009).

**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:**
Muscle regeneration and muscle growth are complicated processes that can be best studied with *in vivo* animal models such as the mouse. Although the function of satellite cells can be studied *in vitro* in cell culture, the complicated interactions between satellite cells, muscle fibers and other cell types such as immune cells and fibroblasts during muscle regeneration cannot be mimicked *in vitro*. The mouse is a suitable model since we have extensive experience and knowledge of mouse physiology and the cellular and molecular processes (including the signaling pathways we study) that regulate muscle growth and regeneration are well-conserved between mouse and human.

**Reduction:**
We performed a power analysis to determine group size for the different experimental groups based on previous experience to minimize the number of animals we use per group (see A. statistical methods). Furthermore, we will first perform a pilot experiment using a smaller number of animals to optimize the tamoxifen injections and knockout efficiency *in vivo*. In addition, standard housing/diet conditions and

experienced personnel that handle the mice yields less variation between experiments that may arise due to environmental conditions (for instance stress due to handling or variations in housing or diet) and thus contribute to reduction in the number of mice we need.

**Refinement:**
There are different methods used to minimize stress and discomfort in mice in our animal facility. Mice are allowed to acclimatize after import (seven days), housing conditions are optimized and cage enrichment is used to reduce stress for the mice. In addition, the use of suitable anaesthesia where needed is applied and handling and procedures are only performed by experienced and licensed personnel.

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

The following measures are taken to minimize animal suffering, stress and fear:

- Animal handling and experimental procedures will only be performed by experienced and certified employees
- Minimizing duration of transport and acclimatization of mice
- Optimized housing conditions, use of cage enrichment

After import to the animal facility and transport, the mice will be monitored to ensure their well-being. If any signs of suffering are observed for over 3 consecutive days the mice will be sacrificed with an overdose of anaesthetic after consulting the staff of the animal housing facility and/or the animal welfare body. The following humane endpoints are used:

- Reduced activity
- Not eating and drinking
- Rapid weight loss (more than 15% in two days)
- Breathing problems

## 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.

Our research group is very experienced in the field of signaling pathways that regulate skeletal muscle growth and regeneration and we have done extensive literature studies. Importantly, no studies were identified in scientific literature that performed similar experiments and therefore we are positive that the proposed experiments are original. In addition, the project was approved by an independent scientific committee who scrutinized both the background and content.

## 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?

X No

☐ Yes > If this may adversely affect animal welfare, describe how the animals will be housed and provide specific justifications for these choices.

**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.

Anaesthesia will be used for intramuscular cardiotoxin injections (to relieve pain of injection) and *in situ* force measurements (see A; procedures). These protocols will be performed by experienced and licenced personnel only and have been optimized in our lab before.

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

Describe which other adverse effects on the animals' welfare may be expected?

Stress resulting in mild discomfort may arise during transport of the animals. Importantly, no adverse effects in the knockout animals are expected since the knockout of ALK4/5 is satellite cell or muscle fiber-specific, which will not negatively affect the animals overall health or welfare.

Explain why these effects may emerge.

Import of mice to our animal facility and transport to our department for in situ force measurements. Tamoxifen injections are performed to induce knockout of ALK4 and/or ALK5 in skeletal muscle. Adverse effects of tamoxifen have been reported in other studies. Local cardiotoxin injection may cause discomfort due to stress and transient pain.

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

Handling and injecting of the mice by experienced and licensed personnel only. Cage enrichment and limiting transport times to minimize stress. In addition, mice will be closely monitored during and after tamoxifen and cardiotoxin injections to ensure the well-being of the mice.

**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.

Tamoxifen is a hazardous substance and adverse effects have been reported (weight loss, scrotal enlargement in male mice). The mice will be closely monitored during and after the tamoxifen injections and treatment will be stopped and mice will be killed if we observe the following major health problems/humane endpoints:

- Reduced activity
- Not eating and drinking
- Rapid weight loss (more than 15% in two days)
- Breathing problems

Indicate the likely incidence.

Although adverse effects have been reported upon tamoxifen injections, the incidince is very low. Also adverse effects mainly arise in longer term experiments using higher concentrations and not with the protocol we will use.

**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').

The table below gives an overview of the expected levels of discomfort for each procedure:

| **Procedure** | **Category** | **Expected percentage of animals** |
|---|---|---|
| Import mice and transport to facility | mild | <1% |
| Tamoxifen/vehicle injections (i.p.) | mild | 70% |
| Cardiotoxin injection (i.m.) | mild | 40% |
| Transport to department for isolation of muscles | mild | 100% |
| Cumulative discomfort | mild | 100% |
| Surgery and *in situ* muscle force measurements | non-recovery | 10% |
| Killing mice | non-recovery | 100% |

## 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.

It is necessary to kill the animals to be able to isolate and analyze skeletal muscles using histological and immunofluorescent methods and to determine gene expression and 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

# 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'. | 11200 |
| 1.2 | Provide the name of the licenced establishment. | Vrije universiteit Amsterdam |
| 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<br>Type of animal procedure: Conditional knockout of ALK4 and ALK5 receptors in dystrophic muscles of mdx mice |

## 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.

To elucidate the function of type I receptors ALK4 and ALK5 in the pathology of Duchenne Muscular Dystrophy we will generate specific genetic knockout mice of these receptors in skeletal muscles of mdx mice, the most commonly used animal model for Duchenne. We will crossbreed mdx mice with floxed ALK4 and ALK5 mice and induce knockout by injecting muscles with viral AAV vector expressing Cre recombinase. The primary outcome parameters in relation to the three experiments and the different procedures (described in the next section) are described in table 1 below and represent parameters of choice to determine the effect of ALK4/5 knockout on dystrophic muscle pathology and function *in vivo*. The following experiments are described in this appendix:

Experiment 1: ALK4/5 knockout in muscles of *mdx* mice; Pilot experiment to determine optimal dose of Cre-expressing viral vector for knockout (go/no go)
Experiment 2: ALK4/5 knockout in muscles of *mdx* mice and isolation of muscles at different timepoints

| Experiment | Procedure | Primary outcome parameters |
|---|---|---|
| 1 | - Injection AAV viral vector<br>- Transport to the department<br>- Killing mice | - Optimal ALK4/ALK5 knockdown (gene/protein) after AAV-Cre injection |
| 2 | - Injection AAV viral vector<br>- Transport to the department<br>- *In situ* muscle force Measurement<br>- Killing mice | - *in situ* muscle force<br>- Muscle mass<br>- Histology: muscle fiber size, % regenerative fibers, fibrosis<br>- Immunofluorescence: satellite cell markers fibrosis and regeneration markers<br>- Gene/protein expression analysis |

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

**Import, breeding and crossbreeding of mice.**
After import of the different transgenic mice, we will maintain colonies of each transgenic line. Importantly, both the floxed transgenic mice and mdx mice reproduce normally and no discomfort is expected when maintaining live colonies. The following mouselines will be imported, bred and subsequently crossbred to obtain inducible knockout mice in skeletal muscle fibers and satellite cells:

- Wildtype mice
- Mdx mice: These mice are not transgenic but have a spontaneous mutation in exon 23 that results in a premature stopcodon in the *Dmd* gene, disruption of the *Dmd* open reading frame and dystrophin protein.

Transgenic mice:
- Tgfbr1 (ALK5) flox/flox mice (floxed), C57BL/6 (Larsson J EMBO J 2001). Exon 3 deletion and functional knockout after Cre mediated recombination.
- Acvr1b (ALK4) flox/flox mice (floxed), C57BL/6 (Ripoche Genesis 2013). Exon 5+6 deletion and functional knockout after Cre mediated recombination.

ALK4, ALK5 and ALK4/ALK5flox/flox transgenic mice will be maintained in holding colonies and will be crossbred with mdx mice (F0) to obtain heterozygous mdx:flox/+ mice (F1). Heterozygous mdx:flox/+ mice will be crossed further to obtain homozygous mdx: flox/flox mice (F2/F3) (see figures 1 and 2 below). Mdx:ALK4/5 double knockout mice will be generated by crossbreeding ALK4flox/flox and ALK5 flox/flox mice and crossbreeding the ALK4/ALK5flox/flox mice with mdx mice. Muscle specific knockout mice are generated by AAV-Cre injections in hindlimb muscles with AAV-control as contralateral control.

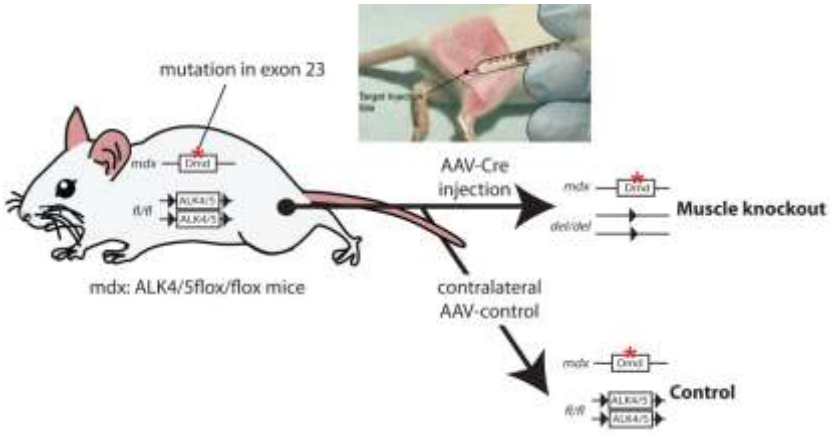

*Figure 1: Picture showing the principle of ALK4/5 knockout in mdx skeletal muscle.*

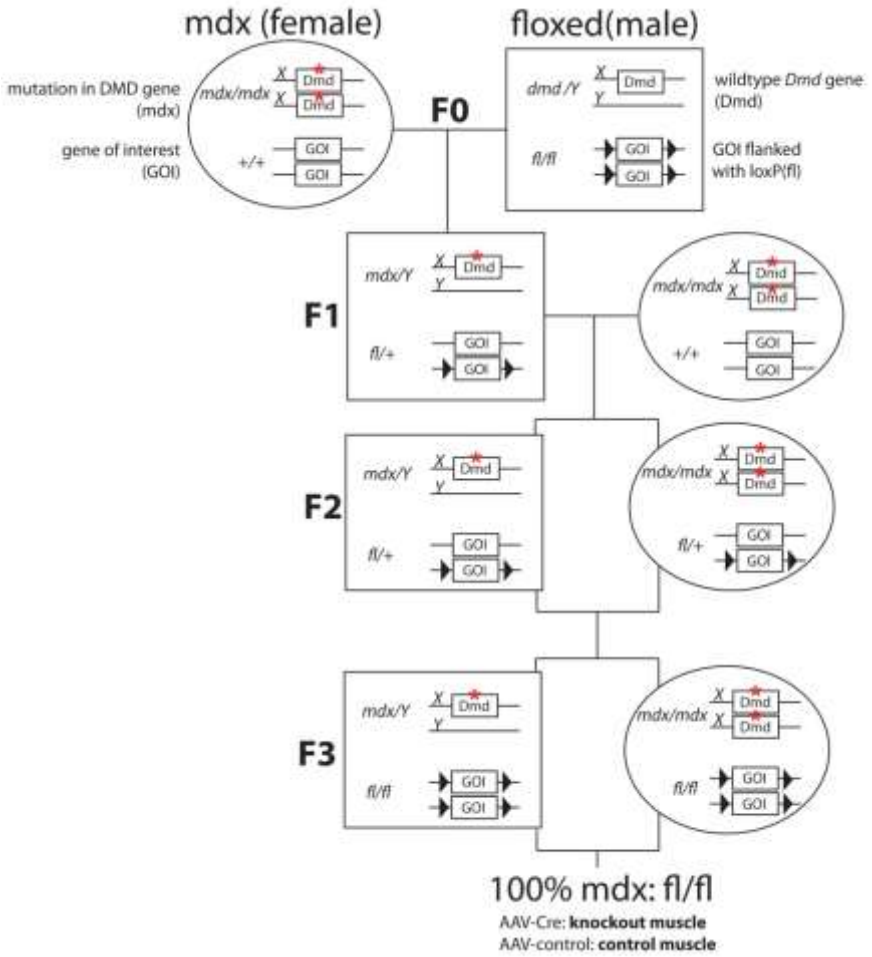


*Figure 2: General schematic of the protocol for crossbreeding to obtain Mdx: ALK4/5 fl/fl mice.*

**Experiment 1 (pilot in vivo experiment: go/no go)**
Procedures: AAV-Cre/AAV-control injections (intramuscular), transport to the department, killing mice, muscle isolation. Frequency/duration: Intramuscular AAV-Cre/AAV-control injections 1x with a maximum frequency/duration that will not exceed moderate discomfort. Blood collection for analysis of viral particles in order to comply with GGO regulations.

General description:
We will first optimize individual and combined knockout of ALK4 and ALK5 in skeletal muscle of mdx mice with intramuscular AAV-Cre injections that yielded high recombination efficiency in skeletal muscles in other comparable studies. Upon intramuscular local injection of AAV-Cre in hindlimb muscles, loxP sites are recombined and functional parts of ALK4/ALK5 genes are deleted (leakage of the viral vector in the circulation is negligible, so the effect is local). Two different groups are analyzed and compared: 1) mdx:flox/flox mice injected with AAV-Cre in hindlimb muscles on one side and AAV-control in hindlimb muscle on the contralateral side and 2) mdx mice and mdx floxed mice, not injected. Non-injected mice are included to determine the effect of AAV viral vector injection in skeletal muscles of these mice. Since different AAV doses and serotypes are reported in literature to yield efficient expression of transgenes in skeletal muscle, we will test 3 serotypes with 3 different concentrations to determine which serotype and

which dose yields the highest knockout efficiency in our hands. If the lowest dose already yields efficient knockout of ALK4/5 (determined by expression analysis) then we will use this dose in subsequent experiments. Before transport to the department, blood will be collected from the mice and will be analyzed to ensure absence of viral particles in the circulation. A month after the last injection, the mice will be transported to the department where we will sacrifice the mice and isolate muscles, muscle fibers or satellite cells for ALK4 and ALK5 gene and protein expression analysis.

**Experiment 2**
Procedures: AAV-Cre/AAV-control injections (i.m.), transport to the department, surgery, *in situ* force measurements, killing mice. Frequency/duration: Intramuscular AAV injections 1x. *In situ* force measurements once with a maximum frequency/duration that will not exceed moderate discomfort. Blood collection for analysis of viral particles in order to comply with GGO regulations.

General description:
To determine the effect of ALK4 and/or ALK5 knockout on muscle pathology and function in mdx mice we will crossbreed ALK4 and/or ALK5 floxed animals with mdx mice to generate inducible knockout mice. Knockout will be induced specifically in skeletal muscles by intramuscular injection with the optimal AAV-Cre dose established in experiment 2 in hindlimb muscles. Three different groups are analyzed and compared: 1) wildtype mice 2) mdx mice 3) mdx;flox/flox mice injected with AAV-Cre in hindlimb muscles the one side and AAV-control in hindlimb muscle on the contralateral side. We will determine the effect of ALK4 and/or ALK5 knockout on muscle pathology by histological analysis and gene/protein expression at 3 different time points (4 weeks, 4 months and 8 months) after AAV-Cre injection and compare the values with wildtype and mdx mice. The time points are chosen to determine the short term, midterm and long term effect of single and double ALK4/5 knockout. Before transport to the department, blood will be collected from the mice and will be analyzed to ensure absence of viral particles in the circulation.

Surgery and protocol *in situ* force measurements:
To determine the force generating capacity of muscle, we will also perform *in situ* force measurements in separate animals on the tibialis anterior muscle by using experimental set-ups that are up and running at our department. Mice will be anaesthetized by using isoflurane, while the skin of the lower leg is removed and muscles of the lower leg are dissected free from their insertion, leaving the muscle, blood supply and innervation via the ischiatic nerve intact. The mice will be placed on a heated plate and the distal tendon will be attached to a force transducer and servo motor, while the muscle and its surrounding are kept moist at physiological temperature (34-35°C). The ischiatic nerve will be stimulated supramaxially to determine length-force and force-velocity curves as well as force-frequency relations. These measurements allow to estimate optimal active muscle force and specific tension as well as maximal shortening velocity and peak power. After *in situ* force measurements mice will be sacrificed.

Describe which statistical methods have been used and which other considerations have been taken into account to minimise the number of animals.

To ensure that we use the minimal amount of animals necessary, we perform an ***a priori*** **power analysis** for each experiment based on expectations and previous knowledge of similar outcome measures in comparable experiments. Based on this power analysis we expect that we need approximately **n=5** animals/group for experiment 1 and **n=8** animals/group for experiment 2. This includes extra animals to account for potential loss of animals/samples (20%) due to potential damage inflicted to muscles during the isolation.

**B. The animals**

Specify the species, origin, estimated numbers, and life stages. Provide justifications for these choices.

**Species & gender:** Male mice. The effect of ALK4/5 knockout will be determined in muscles from mdx mice, which is a mouse model of DMD. DMD is an X-linked muscle disease that mainly affects boys due

to the location of the affected gene on the X-chromosome; therefore we will also use male mice.

**Origin:** Wildtype mice are imported from certified suppliers and the transgenic ALK4/5 floxed animals will be imported from well-established research institutes.

**Estimated numbers:** N=600 mice

Experiment 1 (pilot experiment different AAV vector doses)
n=5 mice/group (see A statistical methods) x 2 (two different genotypes; AAV-Cre/AAV-control injected mdx:flox/flox mice and non-injected mdx mice; see schematic A animal procedures) x 3 (three different serotypes) x 3 (three different concentrations, see A animal procedures) x 4 (four different experimental groups, see table below). Total of n=360 animals

Experiment 2 (knockout ALK4/5 in skeletal muscle of DMD mouse model)
To determine the effect of knockout on muscle mass and force in skeletal muscle we will generate muscle fiber specific knockout mice of ALK4, ALK5 and ALK4+ALK5, induce knockout with AAV-Cre and determine *in situ* muscle force and muscle mass at three different time points after the last injection. We need n=8 mice/group (see A statistical methods) x 2 (histological analysis + *in situ* force measurements) 3 (different timepoints after AAV-Cre injection) x 5 (five different groups; wildtype (group 1), mdx mice (group 2) and AAV-Cre/AAV-control injected mdx:flox/flox mice (groups 3-5), see schematic A animal procedures)). Total of n=240 animals

*Table 2: Estimated number of experimental and breeding mice for in vivo experiments.*

| Nr | Experimental group | Exp 1 | Exp 2 | Total |
|---|---|---|---|---|
| 1 | Wildtype | X | n=48 | n=48 |
| 2 | mdx | n=90 | n=48 | n=138 |
| 3 | mdx x ALK4fl/fl | n=90 | n=48 | n=138 |
| 4 | mdx x ALK5fl/fl | n=90 | n=48 | n=138 |
| 5 | mdx x ALK4+ALK5fl/fl | n=90 | n=48 | n=138 |
| | Total animals | n=360 | n=240 | **n=600** |

**Life stages:** 4-6wks old
For *in vivo* experiments with mdx knockout mice we will use 4-6 wks old mice since tamoxifen induced Cre activity has been reported to be more efficient in mice of this age compared to older mice (Feil S *et al* Methods Mol Biol. 2009).

**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:**

Muscle regeneration and muscle growth are complicated processes that can be best studied with *in vivo* animal models such as the mouse. The mouse is a suitable model since we have extensive experience and knowledge of mouse physiology and the cellular and molecular processes (including the signaling pathways we study) that regulate muscle growth and regeneration are well-conserved between mouse and human. In this appendix procedures are described to determine the effect of ALK4/5 knockout in skeletal muscles of mdx mice, a DMD mouse model. Although mdx mice do not show progressive muscle dystrophy and, in contrast to DMD patients, have only a slight reduction in lifespan, they are genetically comparable to DMD patients and some of the dystrophic changes (chronic inflammation, regeneration, fibrosis, decrease in muscle force) are recapitulated. In addition, previous studies showed that satellite cells isolated from muscles of mdx mice are impaired in their proliferative and differentiation potential. Thus, in our view, these considerations show that this model is valuable as a preclinical animal model of DMD.

**Reduction:**
We performed a power analysis to determine groupsize for the different experimental groups based on previous experience to minimize the number of animals we use per group (see A. statistical methods). In addition, standard housing/diet conditions and experienced personnel that handle the mice yields less variation between animals that may arise due to environmental conditions (for instance stress due to handling or variations in housing or diet) and thus contribute to reduction in the number of mice we need.

**Refinement:**
There are different methods used to minimize stress and discomfort in mice in our animal facility. Mice are allowed to acclimatize after import (seven days), housing conditions are optimized and cage enrichment is used to reduce stress for the mice. In addition, handling and killing of mice is only performed by experienced and licensed personnel.

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

The following measures are taken to minimize animal suffering, stress and fear:

- Animal handling and experimental procedures will only be performed by experienced and certified employees
- Minimizing duration of transport and acclimatization of mice
- Optimized housing conditions, use of cage enrichment

After import to the animal facility and transport, the mice will be monitored to ensure their well-being. If any signs of suffering are observed for over 3 consecutive days the mice will be sacrificed with an overdose of anaesthetic after consulting the staff of the animal housing facility and/or the animal welfare body. The following humane endpoints are used:

- Reduced activity
- Not eating and drinking
- Rapid weight loss (more than 15% in two days)
- Breathing problems

## 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.

Our research group is experienced DMD research and we have done extensive literature studies on the topic. Importantly, no studies were identified in scientific literature that performed similar experiments and we are therefore positive that the proposed experiments are original and have not been previously performed in other studies. In addition, the project was approved by an independent scientific committee who scrutinized both the background and content.

## 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?

X No

☐ Yes > If this may adversely affect animal welfare, describe how the animals will be housed and provide specific justifications for these choices.

**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.

Anaesthesia will be used for intramuscular AAV-Cre injections (for relieve of pain after injection) and *in situ* force measurements (see A procedures). These protocols will be performed by experienced and licenced personnel only and have been optimized in our lab before.

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

Describe which other adverse effects on the animals' welfare may be expected?

Stress resulting in mild discomfort may arise during blood collection and transport of the animals. Importantly, mdx mice and transgenic floxed mice reproduce normally and no discomfort is expected when maintaining live colonies or during crossbreeding. Although mdx mice show mild muscle pathology, no general health problems have been reported in literature. The dystrophic changes in mdx muscles tend to stabilize and no progressive muscle dystrophy is observed. In addition, these mice have relatively normal lifespan and have been noted as exceptional breeders, with no problems in reproduction. However, it has been reported that these mice may be more susceptible to stress. Therefore, mdx mice will be closely monitored to ensure their well-being and unnecessary handling of the mice will be avoided.

Explain why these effects may emerge.

Handling of the animals and blood collection will inevitably result in some stress and hence can result in mild discomfort for the animals. Importantly, mdx mice have been reported to be more susceptible to stress.

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

Handling and injecting of the mice by experienced and licensed personnel only. Cage enrichment and limiting transport times to minimize stress. In addition, mice will be closely monitored during and after injections and transport to ensure the well-being of the mice.

**J. Humane endpoints**

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

X No > Continue with question K.

Yes > Describe the criteria that will be used to identify the humane endpoints.

Indicate the likely incidence.

**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').

The table below gives an overview of the expected levels of discomfort for each procedure:

| **Procedure** | **Category** | **Expected percentage of animals** |
|---|---|---|
| Import mice and transport to facility | mild | <1% |
| AAV injections (i.m.) | mild | 45% |
| Transport to department | mild | 100% |
| Blood collection | mild | 60% |
| Cumulative discomfort | mild | 100% |
| Surgery and *in situ* muscle force measurements | non-recovery | 25% |
| Killing mice | non-recovery | 100% |

## 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.

It is necessary to kill the animals to be able to isolate and analyze skeletal muscles using histological and immunofluorescent methods and to determine gene expression and 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

7

# Format DEC-advies

## A. Algemene gegevens over de procedure

1. Aanvraagnummer:
   *NVWA nummer 11200*

2. Titel van het project:
   *Delineating the function of TGF-β receptor-mediated signaling pathways in regenerating skeletal muscle and Duchenne Muscular Dystrophy*

3. Titel van de NTS:
   *Onderzoek naar de rol van eiwitsignaalpaden in spierherstel en het ziekteverloop van Duchenne Spierdystrofie*

4. Type aanvraag:
   *Nieuwe aanvraag projectvergunning*

5. Contactgegevens DEC:
   - naam DEC: *Vrije Universiteit Amsterdam / VU medisch centrum*
   - telefoonnummer contactpersoon[redacted]
   - e-mailadres contactpersoon*:* [redacted]

6. Adviestraject (data dd-mm-jjjj):
   - ☐ ontvangen door DEC: *04-01-2017*
   - ☐ aanvraag compleet: *04-01-2017*
   - ☐ in vergadering besproken: *10-01-2017*
   - ☐ anderszins behandeld: *n.v.t.*
   - ☐ termijnonderbreking(en) van / tot: *n.v.t.*
   - ☐ besluit van CCD tot verlenging van de totale adviestermijn met maximaal 15 werkdagen: *n.v.t.*
   - ☐ aanpassing aanvraag: *13-01-2017 en 23-01-2017*
   - ☐ advies aan CCD: *07-02-2017*

7. Afstemming IvD
   -Datum advies IvD: *04-01-2017*
   -Strekking advies IvD: *De IvD geeft aan dat de aanvrager het project met de IvD heeft afgestemd en dat deze de instemming heeft van de IvD.*

8. Eventueel horen van aanvrager: *n.v.t.*

9. Correspondentie met de aanvrager

*Vraagronde 1*
- Datum: *12-01-2017*
- Strekking gestelde vragen*: Bij de NTS zijn tekstuele opmerkingen. In het projectvoorstel moet men de protein signaling cascade beter weergeven. Tekstuele en lay-out opmerkingen. Ook bloedafname toevoegen. Men moet het cumulatieve ongerief weergeven.*
- Datum antwoord: *13-01-2017*
- Strekking van de antwoord(en): *De gevraagde aanpassingen zijn doorgevoerd en de benodigde toelichting is gegeven.*

- De antwoorden hebben wel/niet geleid tot aanpassing van de aanvraag: *Ja, de antwoorden hebben geleid tot aanpassing van de aanvraag.*

*Vraagronde 2*

- Datum: *19-01-2017*
- Strekking gestelde vragen*: Nog enkele tekstuele opmerkingen.*
- Datum antwoord: *23-01-2017*
- Strekking van de antwoord(en): *De gevraagde aanpassingen zijn doorgevoerd en de benodigde toelichting is gegeven.*
- De antwoorden hebben wel/niet geleid tot aanpassing van de aanvraag: *Ja, de antwoorden hebben geleid tot aanpassing van de aanvraag.*

10. Eventuele adviezen door experts (niet lid van de DEC): *n.v.t.*

# B. Beoordeling (adviesvraag en behandeling)

1. *Is het project vergunning plichtig. Het omvat dierproeven in de zin der wet.*
2. *De aanvraag betreft een nieuwe aanvraag.*
3. *De DEC is competent om over deze projectvergunningaanvraag te adviseren. De benodigde expertise op dit wetenschappelijk terrein is aanwezig binnen de DEC.*
4. Geef aan of DEC-leden, met het oog op onafhankelijkheid en onpartijdigheid, zijn uitgesloten van de behandeling van de aanvraag en het opstellen van het advies. Indien van toepassing, licht toe waarom. *Eén van de DEC leden is betrokken bij dit project, dit lid heeft niet deelgenomen aan de beoordeling van het project.*

# C. Beoordeling (inhoud)

1. Beoordeel of de aanvraag toetsbaar is en voldoende samenhang heeft *(Zie handreiking 'Invulling definitie project'; zie bijlage I voor toelichting en voorbeeld).*

   *Deze aanvraag heeft een concrete hoofddoelstelling en kan getypeerd worden als een project. Het is helder welke handelingen individuele dieren zullen ondergaan. Hierdoor is ook duidelijk welk ongerief individuele dieren zullen ondergaan. De aanvrager heeft duidelijk de go/ no go momenten beschreven. De DEC is er daardoor van overtuigd dat de aanvrager gedurende het project op zorgvuldige wijze besluiten zal nemen over de voortgang van het project en er niet onnodig dieren gebruikt zullen worden. Gezien bovenstaande is de DEC van mening dat de aanvraag toetsbaar is en voldoende samenhang heeft.*

2. Geef aan of er aspecten in deze aanvraag zijn die niet in overeenstemming zijn met wet- en regelgeving anders dan de Wod?*: n.v.t.*

3. *De in de aanvraag aangekruiste doelcategorie fundamenteel onderzoek is in overeenstemming met de hoofddoelstelling. De doelstelling is helder omschreven.*

***Belangen en waarden***

4. *Het directe doel van deze studie het onderzoeken van de TGF-ß gemedieerde signalering in gezonde en beschadigde spieren en bij Duchenne spierdystrofie (Duchenne Musculaire Dystrofie).*

   *Het uiteindelijke doel is het ontwikkelen van nieuwe therapieën gericht op de remming van de TGF-ß receptor gemedieerde signaalketens die een rol spelen bij de progressieve pathologie van Duchenne spierdystrofie (DMD) om zo de spierfunctie van de patiënten te verbeteren.*

*Er is een reële relatie tussen deze beide doelstellingen. Het directe doel is nodig om het uiteindelijke doel in de toekomst te bereiken.*

5. Benoem de belanghebbenden in het project en beschrijf voor elk van de belanghebbenden welke morele waarden in het geding zijn of bevorderd worden.

*De belangrijkste belanghebbenden in dit project, dat gericht is op de rol van signaaleiwitten bij spierdystrofie, zijn: de proefdieren, de onderzoekers en de patiënten.*

*De waarden die voor proefdieren in het geding zijn: De integriteit van de dieren zal worden aangetast, omdat de dieren ingrepen ondergaan en omdat de dieren worden gedood. De waarde van deze proef voor onderzoekers is: Het vergroten van de wetenschappelijke kennis. Waarden die voor patiënten bevorderd worden: Het ontwikkelen van nieuwe therapieën gericht op de remming van de TGF-β receptor gemedieerde signaalketens om daarmee de spierfunctie te verbeteren.*

6. Geef aan of er sprake kan zijn van substantiële milieueffecten*: n.v.t.*

***Proefopzet en haalbaarheid***

7. Beoordeel of de kennis en kunde van de onderzoeksgroep en andere betrokkenen bij de dierproeven voldoende gewaarborgd zijn.

*Naar de overtuiging van de DEC beschikt de aanvrager over voldoende expertise en voorzieningen om de projectdoelstelling met de gekozen strategie/aanpak binnen de gevraagde termijn te realiseren. Alle technische voorzieningen die benodigd zijn voor uitvoering van het project zijn voorhanden, evenals voldoende deskundigheid en financiering om het project succesvol uit te voeren. Ervaring binnen het onderzoeksinstituut met vergelijkbaar onderzoek waarborgt het technisch succesvol uitvoeren van de dierexperimenten. Bovendien wordt er samengewerkt met de andere instituten actief binnen dit onderzoeksveld.*

8. Beoordeel of het project goed is opgezet, de voorgestelde experimentele opzet en uitkomstparameters logisch en helder aansluiten bij de aangegeven doelstellingen en of de gekozen strategie en experimentele aanpak kan leiden tot het behalen van de doelstelling binnen het kader van het project.

*De aanvraag heeft een navolgbare opbouw en is naar de mening van de DEC goed opgezet. De voorgestelde experimentele opzet en uitkomstparameters zijn logisch en helder en sluiten aan bij de aangegeven doelstellingen. De DEC acht het reëel om te veronderstellen dat op basis van de resultaten van de voorgenomen reeks experimenten beschreven in het project, nieuwe en/of aanvullende kennis zal worden verkregen. Deze inzichten kunnen in de toekomst bijdragen aan het beschikbaar komen van nieuwe therapieën gericht op het verbeteren van spierfunctie bij patiënten met Duchenne spierdystrofie (DMD). De gevraagde looptijd van 5 jaar acht de DEC reëel, gezien de gezien de opbouw en planning van de voorgestelde experimenten.*

***Welzijn dieren***

9. Geef aan of er sprake is van één of meerdere bijzondere categorieën van dieren, omstandigheden of behandeling van de dieren: *n.v.t.*

*Alle dieren worden gefokt voor het gebruik in dierproeven, er is geen sprake van hergebruik. Er is geen sprake van bedreigde diersoorten, niet-menselijke primaten, zwerfdieren en/of dieren in/uit*

*het wild. De locatie is binnen de instelling van de vergunninghouder. De dieren krijgen adequate verdoving en pijnbestrijding.*

10. *De dieren worden gehuisvest en verzorgd op een wijze die voldoet aan de eisen die zijn opgenomen in bijlage III van de richtlijn.*

11. Beoordeel of het ongerief als gevolg van de dierproeven realistisch is ingeschat en geclassificeerd.

    *Het ongerief als gevolg van de dierproeven is naar de mening van de DEC door de aanvragers realistisch ingeschat en geclassificeerd.*

    *Alle dieren ondervinden licht ongerief als gevolg van transport. Daarnaast ondervindt 40% van de dieren licht ongerief als gevolg van injecties en bloedafname. De dieren zullen terminaal ongerief ondervinden door het doden na de krachtmetingen die onder anesthesie plaatsvinden. Het cumulatieve ongerief voor alle dieren binnen dit project is licht.*

12. Geef aan op welke wijze de integriteit van de dieren wordt aangetast

    *De integriteit van de dieren zal worden aangetast omdat de dieren injecties krijgen, er bloed wordt afgenomen en de dieren uiteindelijk gedood worden.*

13. Beoordeel of de criteria voor humane eindpunten goed zijn gedefinieerd en of goed is ingeschat welk percentage dieren naar verwachting een humaan eindpunt zal bereiken.

    *De criteria voor humane eindpunten zijn goed gedefinieerd. De humane eindpunten zullen worden toegepast, wanneer er duidelijke negatieve veranderingen zijn in activiteit, gewicht, ademhaling of algehele malaise. De kans dat men de humane eindpunten toe zal passen is nihil.*

***3V's***

14. *Het project is in overeenstemming met de vereisten ten aanzien van de vervanging van dierproeven. Het gebruik van proefdiervrije methoden of minder complexe diersoorten is volgens de DEC niet mogelijk.*

    *Men zal in vitro experimenten gebruiken om de responsiviteit van verschillende spiercellen op TGF-β receptor gemedieerde signaalketens te bestuderen. Echter voor de complexe biologische processen, betrokken bij spierherstel na schade en de pathologie van Duchenne spierdystrofie, is het gebruik van diermodellen noodzakelijk.*

    *De keuze voor het gebruik van muizen is naar het oordeel van de DEC gerechtvaardigd. De onderzoekers kiezen ervoor om muizen te gebruiken, omdat muizen veel overeenkomsten vertonen met mensen betreffende biologische processen, genen/eiwitten en spierfysiologie. Het mdx muismodel is genetisch vergelijkbaar met patiënten met Duchenne spierdystrofie (Duchenne Musculaire Dystrofie). Bovendien heeft men veel ervaring met deze diersoort, waardoor de nieuwe data goed te vergelijken is met eerdere resultaten.*

15. *In het project wordt optimaal tegemoet gekomen aan de vereiste van de vermindering van dierproeven.*

*Door gebruik te maken van het gefaseerd uitvoeren van de experimenten en een poweranalyse wordt voorkomen dat er teveel of te weinig dieren worden gebruikt. Het maximale aantal proefdieren is proportioneel ten opzichte van de gekozen strategie en de looptijd. De DEC onderschrijft dat het project kan worden uitgevoerd met maximaal 3142 muizen en acht dit aantal realistisch onderbouwd. Onnodige duplicatie van experimenten wordt voorkomen doordat de onderzoekers goed bekend zijn met het onderzoeksveld en samenwerken met andere onderzoeksgroepen die vergelijkbaar onderzoek verrichten.*

16. *Het project is in overeenstemming met de vereiste van de verfijning van dierproeven en het project is zo opgezet dat de dierproeven zo humaan mogelijk kunnen worden uitgevoerd.*

    *Passende anesthesie en pijnbestrijding zullen de gevolgen van de ingrepen (injecties en krachtmetingen) minimaliseren. Alle experimenten zullen worden uitgevoerd door ervaren en bekwaam personeel. Indien het dier het humaan eindpunt bereikt zal het uit de proef worden genomen.*

17. Beoordeel, indien het wettelijk vereist onderzoek betreft, of voldoende aannemelijk is gemaakt dat er geen duplicatie plaats zal vinden en of de aanvrager beschikt over voldoende expertise en informatie om tijdens de uitvoering van het project te voorkomen dat onnodige duplicatie plaatsvindt: *n.v.t.*

### *Dieren in voorraad gedood en bestemming dieren na afloop proef*

18. Geef aan of dieren van beide geslachten in gelijke mate ingezet zullen worden. Indien alleen dieren van één geslacht gebruikt worden, beoordeel of de aanvrager dat in voldoende mate wetenschappelijk heeft onderbouwd? Geef ook aan welke maatregelen verder zijn getroffen om bij fok of aankoop van dieren het aantal in voorraad gedood te beperken.

    *Alleen mannelijke muizen zullen worden gebruikt. Men gaat de spiercellen van wildtype en floxed muizen vergelijken met die van mdx muizen. De mdx muizen worden gebruikt als model voor Duchenne Musculaire Dystrofie (DMD), dit is een X-gebonden spierziekte die bijna alleen bij jongens voorkomt door de ligging van het betrokken gen op het X-chromosoom. Daarom kiest men ervoor om alleen mannelijke dieren te gebruiken. Daarnaast zijn er intrinsieke verschillen in differentiatie gemeld tussen mannelijke en vrouwelijke satellietcellen in de spier evenals verschillen in spierregeneratie (Deasy et al. J cel Biol. 2007), hetgeen de vergelijking tussen de beide geslachten bemoeilijkt.*

19. Geef aan of dieren gedood worden in kader van het project (tijdens of na afloop van de dierproef).

    *De dieren worden gedood om hun weefsel (o.a. spieren) verder te kunnen analyseren.*

20. Indien dieren worden gedood, is adoptie of hergebruik overwogen? Licht toe waarom dit wel/niet mogelijk is.

    *Dit is niet mogelijk omdat er een post-mortem analyse nodig is om de benodigde data te verkrijgen.*

### *NTS*

21. *De niet-technische samenvatting is een evenwichtige weergave van het project en begrijpelijk geformuleerd. De NTS voldoet daarmee aan de eisen zoals gesteld in artikel 10.a.1.7 van de Wod.*

# D. Ethische afweging

1. Benoem de centrale morele vraag *(Zie Praktische handreiking ETK: Stap 3.A).*

*Rechtvaardigen de doeleinden van dit project het voorgestelde gebruik van de dieren?*

*Bij deze dierproef is de centrale morele vraag:*
*Rechtvaardigt het onderzoeken van de effecten van signaaleiwitten (TGF-β receptor-mediated pathways) op spiercellen en spierherstel het gebruik van maximaal 3142 muizen in de dierproef die daarvan maximaal licht ongerief ondervinden?*

2. Weeg voor de verschillende belanghebbenden, zoals beschreven onder C5, de sociale en morele waarden waaraan tegemoet gekomen wordt of die juist in het geding zijn ten opzichte van elkaar af.

*De waarden die voor de proefdieren in het geding zijn: De integriteit van de proefdieren wordt aangetast en de dieren ondervinden maximaal licht ongerief tijdens de proef en worden daarna gedood, dit leidt tot nadeel voor deze proefdieren. De waarden voor de onderzoekers: veel voordeel vanwege de kennisontwikkeling. De waarden die voor de patiënten bevorderd worden: mogelijk veel voordeel op de lange termijn, vanwege het beschikbaar komen van nieuwe therapieën om de spierfunctie van patiënten met Duchenne spierdystrofie (DMD) te verbeteren.*

*De DEC is van mening dat de kennisontwikkeling en de belangen van de patiënten in dit project zwaarder wegen dan de belangen van de 3142 muizen die hiervoor als proefdieren gebruikt worden. Om de complexe biologische processen betrokken bij spierherstel en Duchenne spierdystrofie (DMD) te onderzoeken is het gebruik van diermodellen noodzakelijk. Er zijn op dit moment geen alternatieven voor deze dierproeven beschikbaar waarmee men de doelstellingen kan bereiken.*

3. Beantwoord de centrale morele vraag. Maak voor het beantwoorden van deze vraag gebruik van bovenstaande afweging van morele waarden. Maak daarnaast gebruik van de volgende moreel relevante feiten: belang onderzoek (C4), kennis en kunde van betrokkenen (C7), haalbaarheid doelstellingen (C8), categorieën en herkomst dieren (C9), 3V's (C14-C18), ongerief (C10-13 en C19) en relevante wet en regelgeving (C2).

*Volgens de DEC rechtvaardigen de doeleinden van dit project het voorgestelde gebruik van dieren. Het directe doel van deze studie is het onderzoeken van de effecten van signaaleiwitten (TGF-β receptor-mediated pathways) op spiercellen en spierherstel. Het verwachte resultaat, in het kader van het beschikbaar komen van een adequate behandeling van patiënten met Duchenne spierdystrofie (DMD) is afgewogen tegen het, als maximaal licht geschatte ongerief en de aantasting van integriteit, inclusief het doden van de dieren in de proef.*

*De DEC onderschrijft dat de doelstellingen niet zonder het gebruik van proefdieren kunnen worden behaald en acht het gebruik van maximaal 3142 muizen en de daarmee samenhangende schade aan deze dieren gerechtvaardigd. Bij het uitvoeren van de dierproeven wordt een adequate invulling gegeven aan de vereisten op het gebied van de vervanging, vermindering en verfijning van de dierproeven. Het project is (1) van substantieel belang en (2) van goede kwaliteit.*

*(1) Het wetenschappelijk belang wordt door de DEC ingeschat als substantieel. De resultaten van dit onderzoek zullen bijdragen aan het beschikbaar komen van kennis over de effecten van signaaleiwitten bij spierherstel en spierdystrofie. Daarnaast is er sprake van een indirect maatschappelijk belang, door het mogelijk beschikbaar komen van nieuwe therapieën voor de behandeling van spierdystrofie.*

*(2) De DEC is van mening dat dit project verantwoord is vanuit wetenschappelijk oogpunt en acht het waarschijnlijk dat op basis van de resultaten van de voorgenomen reeks experimenten beschreven in het project, nieuwe en/of aanvullende kennis zal worden verkregen. De onderzoekers beschikken over ruime ervaring en kennis op het gebied van de te gebruiken methoden en werken samen met andere onderzoeksgroepen. Dit in combinatie met de beschikbare faciliteiten en infrastructuur betekent dat de onderzoekers goed gekwalificeerd en geoutilleerd zijn voor het uitvoeren van het in dit project beschreven onderzoek.*

*Samenvattend kan worden gesteld dat het als substantieel te kwalificeren wetenschappelijk en indirecte maatschappelijk belang van het onderzoek naar het oordeel van de DEC opweegt tegen het gebruik van maximaal 3142 muizen en het daarbij verwachte maximaal lichte ongerief.*

# E. Advies

1. Advies aan de CCD

*De DEC adviseert de vergunning te verlenen.*

2. Het uitgebrachte advies kan unaniem tot stand zijn gekomen dan wel gebaseerd zijn op een meerderheidsstandpunt in de DEC.

*Het uitgebrachte advies is gebaseerd op consensus.*

3. Omschrijf de knelpunten/dilemma's die naar voren zijn gekomen tijdens het beoordelen van de aanvraag en het opstellen van het advies zowel binnen als buiten de context van het project *(Zie Praktische handreiking ETK: Stap 4.B)*.

*Er is geen dilemma geconstateerd.*

Centrale Commissie Dierproeven

8

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Vrije Universiteit Amsterdam
T.a.v. [redacted]
[redacted]
AMSTERDAM

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centralecommissiedierproeven.nl
0900 28 000 28 (10 ct/min)
info@zbo-ccd.nl

**Onze referentie**
Aanvraagnummer
AVD112002017862

**Bijlagen**
2

Datum 8 februari 2017
Betreft Ontvangstbevestiging aanvraag projectvergunning Dierproeven

Geachte [redacted]

Wij hebben uw aanvraag voor een projectvergunning dierproeven ontvangen op 7 februari 2017. Het gaat om uw project "Delineating the function of TGF-B receptor-mediated signaling pathways in regenerating skeletal muscle and Duchenne Muscular Dystrophy". Het aanvraagnummer dat wij aan deze aanvraag hebben toegekend is AVD112002017862. Gebruik dit nummer wanneer u contact met de CCD opneemt.

**Wacht met de uitvoering van uw project**
Als wij nog informatie van u nodig hebben dan ontvangt u daarover bericht. Uw aanvraag is in ieder geval niet compleet als de leges niet zijn bijgeschreven op de rekening van de CCD. U ontvangt binnen veertig werkdagen een beslissing op uw aanvraag. Als wij nog informatie van u nodig hebben, wordt deze termijn opgeschort. In geval van een complexe aanvraag kan deze termijn met maximaal vijftien werkdagen verlengd worden. U krijgt bericht als de beslisperiode van uw aanvraag vanwege complexiteit wordt verlengd. Als u goedkeuring krijgt op uw aanvraag, kunt u daarna beginnen met het project.

**Factuur**
Bijgaand treft u de factuur aan voor de betaling van de leges. Wij verzoeken u de leges zo spoedig mogelijk te voldoen, zodat we uw aanvraag in behandeling kunnen nemen. Is uw betaling niet binnen dertig dagen ontvangen, dan kan uw aanvraag buiten behandeling worden gesteld. Dit betekent dat uw aanvraag niet beoordeeld wordt en u uw project niet mag starten.

**Meer informatie**
Heeft u vragen, kijk dan op www.centralecommissiedierproeven.nl. Of neem telefonisch contact met ons op: 0900 28 000 28 (10 ct/minuut).

**Datum:**
8 februari 2017
**Aanvraagnummer:**
AVD112002017862

Met vriendelijke groet,

Centrale Commissie Dierproeven

Deze brief is automatisch aangemaakt en daarom niet ondertekend.

Bijlagen:
- Gegevens aanvraagformulier
- Factuur

**Datum:**
8 februari 2017
**Aanvraagnummer:**
AVD112002017862

## Gegevens aanvrager

| | |
|---|---|
| Uw gegevens | |
| Deelnemersnummer NVWA: | 11200 |
| Naam instelling of organisatie: | Vrije Universiteit Amsterdam |
| Naam portefeuillehouder of diens gemachtigde: | [redacted] |
| KvK-nummer: | 53815211 |
| Straat en huisnummer: | De Boelelaan 1105 |
| Postcode en plaats: | 1081 HV AMSTERDAM |
| IBAN: | [redacted] |
| Tenaamstelling van het rekeningnummer: | [redacted] |

| | |
|---|---|
| Gegevens verantwoordelijke onderzoeker | |
| Naam: | [redacted] |
| Functie: | Dr. ( universitair docent ) |
| Afdeling: | [redacted] |
| Telefoonnummer: | [redacted] |
| E-mailadres: | [redacted] |

**Datum:**
8 februari 2017
**Aanvraagnummer:**
AVD112002017862

Gegevens gemachtigde

| | |
|---|---|
| Naam: | [redacted] |
| Adres: | [redacted] |
| Postcode en plaats: | [redacted] AMSTERDAM |
| Wilt u een nieuwe machtiging afgeven? | Nee |

**Over uw aanvraag**

| | |
|---|---|
| Wat voor aanvraag doet u? | [x] Nieuwe aanvraag<br>[ ] Wijziging op een (verleende) vergunning die negatieve gevolgen kan hebben voor het dierenwelzijn<br>[ ] Melding op (verleende) vergunning die geen negatieve gevolgen kan hebben voor het dierenwelzijn |

**Over uw project**

| | |
|---|---|
| Geplande startdatum: | 1 maart 2017 |
| Geplande einddatum: | 1 maart 2022 |
| Titel project: | Delineating the function of TGF-B receptor-mediated signaling pathways in regenerating skeletal muscle and Duchenne Muscular Dystrophy |
| Titel niet-technische samenvatting: | Onderzoek naar de rol van eiwitsignaalpaden in spierherstel en het ziekteverloop van Duchenne Spierdystrofie |
| Naam DEC: | DEC Vrije Universiteit/ VU Medisch Centrum |
| Postadres DEC: | [redacted] |
| E-mailadres DEC: | [redacted] |

**Betaalgegevens**

| | |
|---|---|
| De leges bedragen: | € 1.541,- |
| De leges voldoet u: | na ontvangst van de factuur |

**Checklist bijlagen**

| | |
|---|---|
| Verplichte bijlagen: | [x] Projectvoorstel<br>[x] Beschrijving Dierproeven<br>[x] Niet-technische samenvatting |
| Overige bijlagen: | [x] Melding Machtiging<br>[x] DEC-advies |

**Ondertekening**

| | |
|---|---|
| Naam: | [redacted] |
| Functie: | [redacted] |
| Plaats: | Amsterdam |
| Datum: | 7 februari 2017 |

Centrale Commissie Dierproeven

> Retouradres Postbus 20401 2500 EK Den Haag

[redacted]
Vrije universiteit (VU)
[redacted]

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Aanvraagnummer
AVD112002017862

**Bijlagen**
2

Datum 8 februari 2017
Betreft Factuur aanvraag projectvergunning Dierproeven

**Factuur**
Factuurdatum: 8 februari 2017
Vervaldatum: 10 maart 2017
Factuurnummer: 170862

| **Omschrijving** | **Bedrag** |
|---|---|
| Betaling leges projectvergunning dierproeven<br>Betreft aanvraag AVD112002017862 | € 1.541,00 |

Wij verzoeken u het totaalbedrag vóór de gestelde vervaldatum over te maken op rekening NL29INGB 070.500.1512 onder vermelding van het factuurnummer en aanvraagnummer, ten name van Centrale Commissie Dierproeven, Postbus 93144, 2509 AC te 's Gravenhage.

Centrale Commissie Dierproeven

> Retouradres Postbus 20401 2500 EK Den Haag

Vrije Universiteit Amsterdam
[redacted]
[redacted] AMSTERDAM

**Centrale Commissie Dierproeven**
Postbus 20401
2500 EK Den Haag
centralecommissiedierproeven.nl
0900 28 000 28 (10 ct/min)
info@zbo-ccd.nl

**Onze referentie**
Aanvraagnummer
AVD112002017862
**Bijlagen**
1

Datum 20 februari 2017
Betreft Beslissing aanvraag projectvergunning Dierproeven

Geachte [redacted]

Op 7 februari 2017 hebben wij uw aanvraag voor een projectvergunning dierproeven ontvangen. Het gaat om uw project "Delineating the function of TGF-B receptor-mediated signaling pathways in regenerating skeletal muscle and Duchenne Muscular Dystrophy" met aanvraagnummer AVD112002017862. Wij hebben uw aanvraag beoordeeld.

**Beslissing**
Wij keuren uw aanvraag goed op grond van artikel 10a van de Wet op de Dierproeven (hierna: de wet). Hierbij gelden de voorwaarden zoals genoemd in de vergunning.
Met het oog op artikel 10a, lid 1, zijn er algemene voorwaarden gesteld.

U kunt met uw project "Delineating the function of TGF-B receptor-mediated signaling pathways in regenerating skeletal muscle and Duchenne Muscular Dystrophy" starten. De vergunning wordt afgegeven van 1 maart 2017 tot en met 28 februari 2022. Deze termijn is anders dan in uw aanvraag, omdat de looptijd van de vergunning maximaal 5 jaar is.

Overige wettelijke bepalingen blijven van kracht.

**Procedure**
Bij uw aanvraag heeft u een advies van de Dierexperimentencommissie DEC Vrije Universiteit/ VU Medisch Centrum gevoegd. Dit advies is opgesteld op 7 februari 2017. Bij de beoordeling van uw aanvraag is dit advies betrokken overeenkomstig artikel 10a, lid 3 van de wet.
Wij kunnen ons vinden in de inhoud van het advies van de Dierexperimentencommissie. Dit advies van de commissie nemen wij over,

inclusief de daaraan ten grondslag liggende motivering. Er worden aanvullende algemene voorwaarde(n) gesteld.
Het DEC-advies en de in de bijlage opgenomen beschrijving van de artikelen van de wet- en regelgeving zijn de grondslag van dit besluit.

**Bezwaar**
Als u het niet eens bent met deze beslissing, kunt u binnen zes weken na verzending van deze brief schriftelijk een bezwaarschrift indienen.
Een bezwaarschrift kunt u sturen naar Centrale Commissie Dierproeven, afdeling Juridische Zaken, postbus 20401, 2500 EK Den Haag.

Bij het indienen van een bezwaarschrift vragen we u in ieder geval de datum van de beslissing waartegen u bezwaar maakt en het aanvraagnummer te vermelden. U vindt deze nummers in de rechter kantlijn in deze brief.

Bezwaar schorst niet de werking van het besluit waar u het niet mee eens bent. Dat betekent dat dat besluit wel in werking treedt en geldig is. U kunt tijdens deze procedure een voorlopige voorziening vragen bij de Voorzieningenrechter van de rechtbank in de woonplaats van de aanvrager. U moet dan wel kunnen aantonen dat er sprake is van een spoedeisend belang.

Voor de behandeling van een voorlopige voorziening is griffierecht verschuldigd. Op http://www.rechtspraak.nl/Organisatie/Rechtbanken/Pages/default.aspx kunt u zien onder welke rechtbank de vestigingsplaats van de aanvrager valt.

**Meer informatie**
Heeft u vragen, kijk dan op www.centralecommissiedierproeven.nl. Of neem telefonisch contact met ons op: 0900 28 000 28 (10 ct/minuut).

Centrale Commissie Dierproeven
namens deze:

[redacted]

ir. G. de Peute[redacted]
Algemeen Secretaris

Bijlagen:
- Vergunning
  Hiervan deel uitmakend:
  - DEC-advies
  - Weergave wet- en regelgeving

Centrale Commissie Dierproeven

# Projectvergunning

**gelet op artikel 10a van de Wet op de Dierproeven**

Verleent de Centrale Commissie Dierproeven aan

| | |
|---|---|
| Naam: | Vrije Universiteit Amsterdam |
| Adres: | De Boelelaan 1105 |
| Postcode en plaats: | 1081 HV AMSTERDAM |
| Deelnemersnummer: | 11200 |

deze projectvergunning voor het tijdvak 1 maart 2017 tot en met 28 februari 2022, voor het project "Delineating the function of TGF-B receptor-mediated signaling pathways in regenerating skeletal muscle and Duchenne Muscular Dystrophy" met aanvraagnummer AVD112002017862, volgens advies van Dierexperimentencommissie DEC Vrije Universiteit/ VU Medisch Centrum. Er worden aanvullende algemene voorwaarde(n) gesteld.
De functie van de verantwoordelijk onderzoeker is Dr. ( universitair docent ).
De aanvraag omvat de volgende bescheiden:

1 een aanvraagformulier projectvergunning dierproeven, ontvangen op 7 februari 2017
2 de bij het aanvraagformulier behorende bijlagen:
a Projectvoorstel, zoals ontvangen per digitale indiening op 7 februari 2017;
b Niet-technische Samenvatting van het project, zoals ontvangen per digitale indiening op 7 februari 2017;
c Advies van dierexperimentencommissie d.d. 7 februari 2017, ontvangen op 7 februari 2017.

**Aanvraagnummer:**
AVD112002017862

| Naam proef | Diersoort/ Stam | Aantal dieren | Ernst | Opmerkingen |
|---|---|---|---|---|
| **3.4.4.1 Isolation of muscle cells from skeletal muscle of wildtype, transgenic mice and mdx mice for in vitro (recombination) analysis.** | | | | |
| | Muizen (Mus musculus) / WT; mdx; ALK 4/5 KO | 1.120 | 100% Licht | |
| **3.4.4.2 Conditional knockout of ALK4 and ALK5 receptors in skeletal muscle cells** | | | | |
| | Muizen (Mus musculus) / spierspecifiek transgene lijnen | 1.422 | 100% Licht | |
| **3.4.4.3 Conditional knockout of ALK4 and ALK5 receptors in dystrophic muscles of mdx mice** | | | | |
| | Muizen (Mus musculus) / mdx; ALK4/5KO | 600 | 100% Licht | |

**Voorwaarden**

*Op grond van artikel 10a1 lid 2 van de Wet op de dierproeven zijn aan een projectvergunning voorwaarden te stellen*

De vergunning wordt verleend onder de voorwaarde dat go/no go momenten worden afgestemd met de IvD.

In artikel 10, lid 1 sub a van de wet, wordt bepaald dat het verboden is een dierproef te verrichten voor een doel dat, naar de algemeen kenbare, onder deskundigen heersende opvatting, ook kan worden bereikt anders dan door middel van een dierproef, of door middel van een dierproef waarbij minder dieren kunnen worden gebruikt of minder ongerief wordt berokkend dan bij de in het geding zijnde proef het geval is. Nieuwe onderzoeken naar alternatieven kunnen tot gevolg hebben dat inzichten en/of omstandigheden van het aangevraagde project in de vergunningsperiode wijzigen, gedurende de looptijd van deze vergunning. Indien bovenstaande zich voordoet dient aanvrager dit in afstemming met de IvD te melden bij de CCD.

De CCD kan in een dergelijke situatie aan de vergunning nieuwe voorwaarden verbinden en gestelde voorwaarde wijzigen of intrekken.

**Aanvraagnummer:**
AVD112002017862

# Weergave wet- en regelgeving

**Dit project en wijzigingen**
Volgens artikel 10c van de Wet op de Dierproeven (hierna de wet) is het verboden om andere dierproeven uit te voeren dan waar de vergunning voor is verleend. De dierproeven mogen slechts worden verricht in het kader van een project, volgens artikel 10g. Uit artikel 10b volgt dat de dierproeven zijn ingedeeld in de categorieën terminaal, licht, matig of ernstig. Als er wijzigingen in een dierproef plaatsvinden, moeten deze gemeld worden aan de Centrale Commissie Dierproeven. Hebben de wijzigingen negatieve gevolgen voor het dierenwelzijn, dan moet volgens artikel 10a5 de wijziging eerst voorgelegd worden en mag deze pas doorgevoerd worden na goedkeuren door de Centrale Commissie Dierproeven.
Artikel 10b schrijft voor dat het verboden is een dierproef te verrichten die leidt tot ernstige mate van pijn, lijden, angst of blijvende schade die waarschijnlijk langdurig zal zijn en niet kan worden verzacht, tenzij hiervoor door de Minister een ontheffing is verleend.

**Verzorging**
De fokker, leverancier en gebruiker moeten volgens artikel 13f van de wet over voldoende personeel beschikken en ervoor zorgen dat de dieren behoorlijk worden verzorgd, behandeld en gehuisvest. Er moeten ook personen zijn die toezicht houden op het welzijn en de verzorging van de dieren in de inrichting, personeel dat met de dieren omgaat moet toegang hebben tot informatie over de in de inrichting gehuisveste soorten en personeel moet voldoende geschoold en bekwaam zijn. Ook moeten er personen zijn die een eind kunnen maken aan onnodige pijn, lijden, angst of blijvende schade die tijdens een dierproef bij een dier wordt veroorzaakt. Daarnaast zijn er personen die zorgen dat een project volgens deze vergunning wordt uitgevoerd en als dat niet mogelijk is zorgen dat er passende maatregelen worden getroffen.
In artikel 9 staat dat de persoon die het project en de dierproef opzet deskundig en bekwaam moet zijn. In artikel 8 van het Dierproevenbesluit 2014 staat dat personen die dierproeven verrichten, de dieren verzorgen of de dieren doden, hiervoor een opleiding moeten hebben afgerond.
Voordat een dierproef die onderdeel uitmaakt van dit project start, moet volgens artikel 10a3 van de wet de uitvoering afgestemd worden met de instantie voor dierenwelzijn.

**Pijnbestrijding en verdoving**
In artikel 13 van de wet staat dat een dierproef onder algehele of plaatselijke verdoving wordt uitgevoerd tenzij dat niet mogelijk is, dan wel bij het verrichten van een dierproef worden pijnstillers toegediend of andere goede methoden gebruikt die de pijn, het lijden, de angst of de blijvende schade bij het dier tot een minimum beperken. Een dierproef die bij het dier gepaard gaat met zwaar letsel dat hevige pijn kan veroorzaken, wordt niet zonder verdoving uitgevoerd. Hierbij wordt afgewogen of het toedienen van verdoving voor het dier traumatischer is dan de dierproef zelf en het toedienen van verdoving onverenigbaar is met het doel van de dierproef. Bij een dier wordt geen stof toegediend waardoor het dier niet meer of slechts in verminderde mate in staat is pijn te tonen, wanneer het dier niet tegelijkertijd voldoende verdoving of pijnstilling krijgt toegediend, tenzij wetenschappelijk gemotiveerd. Dieren die pijn

kunnen lijden als de verdoving eenmaal is uitgewerkt, moeten preventief en postoperatief behandeld worden met pijnstillers of andere geschikte pijnbestrijdingsmethoden, mits die verenigbaar zijn met het doel van de dierproef. Zodra het doel van de dierproef is bereikt, moeten passende maatregelen worden genomen om het lijden van het dier tot een minimum te beperken.

**Einde van een dierproef**
Artikel 13a van de wet bepaalt dat een dierproef is afgelopen wanneer voor die dierproef geen verdere waarnemingen hoeven te worden verricht of, voor wat betreft nieuwe genetisch gemodificeerde dierenlijnen, wanneer bij de nakomelingen niet evenveel of meer, pijn, lijden, angst, of blijvende schade wordt waargenomen of verwacht dan bij het inbrengen van een naald. Er wordt dan door een dierenarts of een andere ter zake deskundige beslist of het dier in leven zal worden gehouden. Een dier wordt gedood als aannemelijk is dat het een matige of ernstige vorm van pijn, lijden, angst of blijven schade zal blijven ondervinden. Als een dier in leven wordt gehouden, krijgt het de verzorging en huisvesting die past bij zijn gezondheidstoestand.
Volgens artikel 13b moet de dood als eindpunt van een dierproef zoveel mogelijk worden vermeden en vervangen door in een vroege fase vaststelbare, humane eindpunten. Als de dood als eindpunt onvermijdelijk is, moeten er zo weinig mogelijk dieren sterven en het lijden zo veel mogelijk beperkt blijven.
Uit artikel 13d volgt dat het doden van dieren door een deskundig persoon moet worden gedaan, wat zo min mogelijk pijn, lijden en angst met zich meebrengt. De methode om te doden is vastgesteld in de Europese richtlijn artikel 6.
In artikel 13c is vastgesteld dat proefdieren geadopteerd kunnen worden, teruggeplaatst in hun habitat of in een geschikt dierhouderijsysteem, als de gezondheidstoestand van het dier het toelaat, er geen gevaar is voor volksgezondheid, diergezondheid of milieu en er passende maatregelen zijn genomen om het welzijn van het dier te waarborgen.
De Minister heeft vrijstelling ontheffing verleend volgens artikel 13c, die de afwijkende methode van doden op basis van wetenschappelijke motivering ten minste even humaan acht als de in de richtlijn opgenomen passende methoden.