| | **Inventaris Wob-verzoek W16-08S** | | | | | | | | |
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| | | **wordt verstrekt** | | | | **weigeringsgronden** | | | |
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| | **NTS2015250** | | | | | | | | |
| 1 | Aanvraagformulier | | | | x | | x | x | |
| 2 | Niet-technische samenvatting | x | | | | | | | |
| 3 | Projectvoorstel | | | | x | | x | x | |
| 4 | Bijlage beschrijving dierproeven 1 | | | x | | | | | |
| 5 | Bijlage beschrijving dierproeven 2 | | | x | | | | | |
| 6 | Bijlage beschrijving dierproeven 3 | | | x | | | | | |
| 7 | Bijlage beschrijving dierproeven 4 | | | x | | | | | |
| 8 | Bijlage beschrijving dierproeven 5 | | | x | | | | | |
| 9 | Bijlage beschrijving dierproeven 6 | | | x | | | | | |
| 10 | DEC-advies | | | | x | | x | x | |
| 11 | Appendix I | | | | x | | x | x | |
| 12 | Appendix II | | | x | | | | | |
| 13 | Overzicht aantallen dieren | | | x | | | | | |
| 14 | Ontvangstbevestiging | | | | x | | x | x | |
| 15 | Advies CCD | | x | | | | | | x |
| 16 | Beschikking en vergunning | | | | x | | x | x | |

Betreft:
AVD
80100 2015
250

20 OKT. 2015

# 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.zbo-ccd.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* 80102 (Hubrecht Instituut-KNAW) 80101 NIN
☐ Nee > *U kunt geen aanvraag doen*

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

| | |
|---|---|
| Naam instelling of organisatie | KNAW |
| Naam van de portefeuillehouder of diens gemachtigde | [redacted] |
| KvK-nummer | 5 4 6 6 7 0 8 9 |

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.*

| | |
|---|---|
| Straat en huisnummer | |
| Postbus | Postbus 19121 |
| Postcode en plaats | 1000GC Amsterdam |
| IBAN | NL94 |
| Tenaamstelling van het rekeningnummer | Hubrecht Instituut |

1.4 Vul de gegevens in van de verantwoordelijke onderzoeker.

| | |
|---|---|
| (Titel) Naam en voorletters | [redacted] ☐ Dhr. ☑ Mw. |
| Functie | Group Leader |
| 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<br>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<br>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 | Wat is de geplande start- en einddatum van het project? | Startdatum | 0 1 - 1 1 - 2 0 1 5 |
| | | Einddatum | 0 1 - 1 1 - 2 0 2 0 |
| 3.2 | Wat is de titel van het project? | Factors involved in cardiac function and repair | |
| 3.3 | Wat is de titel van de niet-technische samenvatting? | De moleculaire basis van hartziekten | |
| 3.4 | Wat is de naam van de Dierexperimentencommissie (DEC) aan wie de instellingsvergunninghouder doorgaans haar projecten ter toetsing voorlegt? | Naam DEC | DEC-KNAW |
| | | Postadres | [redacted] Amsterdam |
| | | E-mailadres | [redacted] |

# 4 Betaalgegevens

4.1 Om welk type aanvraag gaat het?

- ☑ Nieuwe aanvraag Projectvergunning € 741,00 Lege
- ☐ 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
- ☐ Na ontvangst van de factuur

# 5 Checklist bijlagen

5.1 Welke bijlagen stuurt u mee?

Verplicht

- ☒ Projectvoorstel
- ☒ Niet-technische samenvatting

Overige bijlagen, indien van toepassing

- ☒ flow chart
- ☒ Table with experimental groups en Bijlagen I en II

# 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 1.6). 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 | [redacted] |
| Plaats | Amsterdam |
| Datum | 14-10-2015 |
| Handtekening | [redacted] |

Centrale Commissie Dierproeven

# Form
## Project proposal

- This form should be used to write the project proposal for animal procedures.
- The appendix 'description animal procedures' is an appendix to this form. For each type of animal procedure, a separate appendix 'description animal procedures' should be enclosed.
- For more information on the project proposal, see our website (www.centralecommissiedierproeven.nl).
- Or contact us by phone (0900-2800028).

## 1 General information

| | | |
|---|---|---|
| 1.1 | Provide the approval number of the 'Netherlands Food and Consumer Product Safety Authority'. | 80102 |
| 1.2 | Provide the name of the licenced establishment. | Hubrecht Institute KNAW |
| 1.3 | Provide the title of the project. | The molecular basis of cardiac diseases and recovery |

## 2 Categories

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

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

Heart failure [1] is a major public health issue in the industrialized world, associated with high morbidity and mortality rates and posing a major burden on the healthcare system. HF can be viewed as a progressive disease resulting from an *acute event*, such as myocardial infarction (MI), that results in the

loss of functional cardiac myocytes due to the inability of the heart to **regenerate and repair** the injured tissue [2]. HF can also be caused by a *gradual event* such as increased hemodynamic pressure (hypertension) or by a genetic mutation all resulting in a **pathological remodeling** response, characterized by cardiomyocyte hypertrophy and fibrosis, that induces a decline in cardiac pump function[3].
In our lab aim to understand the molecular and cellular mechanisms that are involved in cardiac regeneration and repair and pathological remodeling by using animal models that allow for detailed analysis of the different aspects of heart disease. See **appendix I** for a brief overview of our results obtained so far with studies related to heart regeneration and pathological remodeling. Often the same mechanisms are involved in both types of heart failure and it is therefore of scientific value to study the involvement of a particular gene, microRNA, or (stem)cells, in experimental models of regeneration and repair and in models for pathological remodeling.

**1. Cardiac regeneration and repair**
Ischemic heart disease (IHD) is a form of congestive heart failure and is the leading cause of death worldwide [4]. IHD is induced by an insufficient blood supply of the heart muscle typically due to coronary artery disease or **myocardial infarction (MI)**. During an acute MI, the complete occlusion of coronary vessels impedes a sufficient oxygen supply to the heart muscle and the resulting hypoxia can induce loss of viable cardiac tissue. Currently, the most effective strategy for reducing the size of an infarct and improving the clinical outcome after an acute MI, is early myocardial reperfusion by either thrombolytic therapy or primary percutaneous coronary intervention. However, although restoration of the blood flow in response to an ischemic event has been shown to be beneficial, the ischemic event still leads to the loss of viable cardiac tissue and the infarct size is often correlated with an impairment of cardiac contractility. Cardiomyocytes are terminally differentiated cells that have lost the capability to divide. Consequently, the loss of contracting tissue is permanent and is only replaced by scar tissue. While the heart is notoriously resistant to regeneration in response to ischemic injury, considerable evidence suggest that the fundamental biology of the myocardium provides multiple therapeutic opportunities to enhance **cardiac regeneration and repair**. This can be achieved by stimulating cardiomyocyte survival, triggering myocyte proliferation, or by improving the recruitment and homing of stem cells, parameters which are all looked at in our lab. Improving heart repair by the replacement of lost cardiomyocytes will result in a better maintenance in cardiac function, that we can measure by echocardiography or other functional measurements (**see appendix I**).
Tissue surrounding the actual ischemic area is also affected, and typically shows a remodeling process that is characterized by hypertrophy of cardiac myocytes and fibrosis. This remodeling process further adds to the deterioration of the pump function, which can ultimately lead to heart failure and sudden death. Due to the high incidence and severity of IHD, much effort has been directed towards the search for strategies that can limit ischemic injury or secondary remodeling. Because an effect on regeneration (and the resulting infarct size) will influence the level of pathological remodeling are these 2 processes directly linked. Cardiomyocyte renewal, hypertrophy and fibrosis is successfully being studied in our lab as a read out for heart regeneration and pathological remodeling secondary to the infarct (**see appendix I**) [5-7].

To study the biological mechanisms involved in cardiac regeneration and repair our lab currently uses the following two models of ischemic injury to the heart [5, 7]:

- **Myocardial infarction (MI)**: permanent occlusion of a coronary artery
- **Ischemia Reperfusion (IR)**: occlusion of a coronary artery, followed by reperfusion

**2. Pathological remodeling**
Cardiomyocyte hypertrophy is the dominant cellular response of the heart not only after MI, but occurs in response to virtually all forms of hemodynamic overload, endocrine disorders, or inherited mutations in a variety of structural and contractile proteins. These disease drivers activate a pathological remodeling response that causes the heart to become hypertrophic and fibrotic, decompensate and ultimately go into failure. As such, there has been intense interest in deciphering the fundamental molecular mechanisms

that drive cardiomyocyte hypertrophy and fibrosis, so we can explore opportunities to protect the heart against these changes [3]. Cardiomyocyte hypertrophy and fibrosis is currently being studied in our lab as a read out for heart pathological remodeling (**see appendix I**) [5, 8-12] Because all disease drivers activate different signaling cascades, depending on the specific research question our lab makes use of the following models to induce this pathological remodeling (**see appendix I**).

- **Transverse Aortic Banding [13]:** A surgical model, in which the aorta is partially occluded with a trans-aortic band [13]. This mimics the effect of atherosclerosis, causing a permanent narrowing of the vasculature because of plaque deposition. This narrowing of the aorta by the aortic constriction increases the workload on the heart by an increase in pressure, which results in pathological cardiac remodeling and dysfunction that is characterized by cardiomyocyte hypertrophy and fibrosis that results in a worsening in cardiac function [12].
- **High-salt diet [8, 10]:** Since hypertension is an important driver of cardiac disease and HF a lot of research is dedicated to study exactly what happens in the heart during this increase in blood pressure. In the lab we can elevate blood pressure by increasing the salt-content of the diet (high-salt diet) of mice.
- **Delivery of remodeling agent [8]:** Remodeling agents, like angiotensin II (AngII), phenylephrine (PE) or cardiotrophin (CT-1) can be administered to induce a pathological remodeling response in the heart [8, 10].
- **High-fat diet [9]:** Another important cause of heart disease that is rapidly gaining ground is the Western diet. Because of unhealthy dietary habits there is an increasing incidence of people that are diagnosed with type II diabetes mellitus (DM), and these patients often die from cardiovascular disease. Heart disease in patients with diabetes is called diabetic cardiomyopathy and is characterized by myocyte hypertrophy, prominent interstitial fibrosis and diastolic dysfunction. To study the remodeling response of the heart in during diabetic cardiomyopathy and the underlying molecular changes we make use of an animal model of type II diabetes, which is based on a high-fat diet.
- **Exercise to trigger heart disease:** Inherited mutations in a variety of structural and contractile proteins can also be the underlying cause of specific forms of heart disease, like Arrhythmic Right Ventricular Cardiomyopathy (ARVC) or Hypertrophic Cardiomyopathy (HCM)[14]. These mutations induce a specific remodeling response, with all different underlying causes of heart disease. While the mouse models often appear resistant to developing the disease, we can use exercise to increase the workload on the heart and thereby triggering the disease. This mimics the situation in people, where people suddenly expose cardiac issues under conditions of intense physical activity.

Several groups, including our own, identified microRNAs (miRNAs) as crucial gene regulators during heart repair and pathological remodeling [5, 7, 10-12]. At the same time it is well known that physical exercise can decrease infarct size and pathological remodeling by enhancing cardiac regeneration and inducing a physiological remodeling response that is beneficial for the heart [15]. Studying the effects of therapeutic interventions (such as microRNA-based treatments) or exercise on the heart will help us to better understand the potential beneficial effects of physical activity on heart disease.

Gender has a big influence on heart disease. Women are generally less susceptible for HF which is thought to be due to a cardioprotective effect of estrogen. Women show more replacement of cardiomyocyte after myocardial infarction, less hypertrophy and less fibrosis than men. The presence of estrogen influences the severity of heart disease and changes with age [16]. Our group previously studied the impact of the contribution of estrogen, which appeared to be very significant in the setting of heart disease [6]. While this is a very interesting and relevant observation, this is currently not a topic of investigation in our lab. To exclude this highly impactful variable, we (first) aim to understand the molecular mechanism of cardiac regeneration and remodeling only in male mice. While we are aware that this choice increases the required number of animals not used in our breeding schemes, we strongly believe that using only males will provide more reliable and consistent, less variable, data. Including females will introduce more variation, which will increase the number of animals required to get a significant result, and will increase the number of mice exposed to discomfort.

The overall theme in the [redacted] is to unravel the underlying molecular and cellular mechanisms of cardiac repair (cardiomyocyte renewal) and pathological remodeling (cardiomyocyte hypertrophy and fibrosis) by using animal models that allow for detailed analysis of the different aspects of heart disease. Based on previous experiments, literature, interactions with other scientists and data obtained during the course of our studies we identify a novel mechanism that could potentially be relevant for heart disease. If possible we first test our hypothesis in isolated cardiomyocytes [5, 11], a well validated in vitro model to study heart biology, before we move to in vivo experiments. We use a genetic mouse model or other way of gene manipulation to understand the molecular mechanisms that are important for the heart. These mice are studied in the absence or presence of myocardial infarction or another type of cardiac stress to examine the molecular effects both under basal or cardiac stress conditions. This can be done in the absence or presence of a therapeutic intervention like an microRNA drug or exercise

Our work so far has formed the basis for the foundation of a biotechnology company called miRagen Therapeutics (**see appendix I**), that aims to develop new therapies for cardiovascular disease. Our work opens new avenues for a variety of new and/or improved therapeutic strategies for heart disease.

**References**


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### 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 overall theme of our research is to unravel the underlying molecular and cellular mechanisms of cardiac repair (cardiomyocyte renewal) and pathological remodeling (cardiomyocyte hypertrophy and fibrosis).
Our aim is to understand the molecular mechanisms that are involved in cardiac regeneration and remodelling.

**Specific research aims are:**
1. Understand the function of specific genes, microRNAs or (stem) cells in **heart regeneration** under basal and stress conditions by looking at the effect on cell death, proliferation and differentiation (Animal Procedures 1, 2, 3, 4, and 5)
2. Understand the function of specific genes, microRNAs or (stem) cells in **pathological remodeling** under basal and stress conditions by looking at their effect on fibrosis and cardiomyocyte hypertrophy (Animal Procedures 1, 2, 3, 4, and 6)
3. Understand the influence of specific drug compounds, microRNA therapeutics or exercise on **heart regeneration** and **remodeling** by looking at the effect of cell death, proliferation, differentiation, fibrosis and cardiomyocyte hypertrophy (Animal Procedures 1- 6)
4. Can we enhance cardiac delivery of therapeutic compounds? (Animal Procedures 2-6)

There are several reasons why we think that we can achieve our aims:
All required techniques (except for one – the transplantation of cells or their derivatives in mice) are currently being performed in our lab under the appropriate DEC protocols. A summary of the currently ongoing studies, approved by the DEC and that will be part of this project after the license is obtained, is listed in **Appendix II**. Most of the Animal Procedures as described in Appendices 1-6 are currently being performed and are part of the ongoing projects.
Work from our group on heart regeneration and pathological remodeling has resulted in seminal papers that described the required techniques and even resulted in the foundation of miRagen therapeutics , a biotechnology company focused on the development of microRNA based therapies, of which I am a founder (**Appendix I**).
Our research and experiments are continuously being evaluated by our group and by various other groups within our institute. Moreover, our research is positively judged by national and international funding agencies including the ERC and the Hartstichting. Our group is also a member of a Leducq consortium, which is a prominent cardiovascular research network of seven research institutions in both Europe and the US. Our ambition is to significantly improve life expectancy and quality of life for patients suffering from heart disease and to provide multidisciplinary training for the next generation of cardiac researchers and specialists. The scientists working in our group are selected based on their excellence and their commitment to the mission of the program.
Over the last few years, we have built up a repertoire of genetic and surgical models that allow us to determine the functional and molecular effects of cardiac remodeling, disease and therapeutic interventions. Our embedding in an excellent scientific environment, our expertise in cardiac remodeling, disease and mouse genetics, unique techniques and approaches, and our previous achievement makes it very likely that with the experiments described in this project we will make large contributions to our main research questions.
Our group is embedded in the Hubrecht Institute, which is a center-of-excellence on developmental biology, and stem cell and cancer research. The Hubrecht Institute provides core facilities for various high-end techniques such as deep sequencing, histology, fluorescent imaging, mRNA expression array, and flow cytometry. Moreover, the Hubrecht Institute has just renovated their animal facility, and now it can compete with the best animal facilities that can be found internationally. Dedicated staff takes care of regular housing of the animals and support the scientist in their experiments. Within our group, we

have two dedicated in vivo technicians and very experienced scientists that oversee the breeding of all mouse lines, experiments and procedures, and new people are trained when required. This guarantees that only experienced people perform experiments.

### 3.3 Relevance

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

Heart disease is a modern world epidemic for which there currently are no effective therapies that could stop - let alone reverse- disease progression with the exception of heart transplantation or assist devices. Unfortunately, these treatment options are only available to a minute fraction of the population in need of treatment due to donor scarcity, and are accompanied by incredibly high costs. Given the global burden of heart disease and its increasing prevalence, the development of novel regenerative approaches is of the utmost importance.
Our lab has unveiled mechanism by which we can enhance heart regeneration and/or block pathological remodeling. We have an ongoing sponsored research agreement with miRagen Therapeutics, a biotechnology company focused on the development of microRNA based therapies, of which I am a founder and special advisor. The enthusiasm for developing new therapeutics for heart disease is further underscored by the $352M partnership between miRagen and Servier for Research development and commercialization of microRNA-targeting drugs for cardiovascular disease (http://www.miragentherapeutics.com/4/News/).
This is a clear example of how fundamental discoveries can help to better understand biology and thereby support the development of better therapies for patients that are suffering from heart disease to improve their quality of life.

### 3.4 Research strategy

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

**1. Workflow of experiments:**
See flow charts in attachment 1
Based on data generated in previous and ongoing studies, available literature, interactions with other scientists and preliminary data, we generate a hypothesis about a molecular and/or cellular mechanism that can contribute to cardiac regeneration and remodeling. The identified hypothesis surrounding the function of a gene, microRNA or cell type in aspects of heart disease (cardiomyocyte renewal, hypertrophy or fibrosis) will initially be carefully tested in tissues from previous cardiac stress studies, human heart samples and cell lines, including neonatal rat cardiomyocytes. **The specific hypotheses we are working on at present are listed in Appendix II**, but new hypothesis are constantly added and will be tested using the same approaches.
Once our preliminary tests on available material provides enough support for our original hypothesis or research question, we will consider the extensive and careful analysis of the mechanism in wildtype or GM mice. For our in vivo experiments we will use wildtype, GM mice or mice in which a gene or microRNA has been modified with a compound or a virus. If the desired genotype is not available we will create the mouse line ourselves. We will only use well-established reagents and protocols to induce expression or deletion of the candidate gene/s. Our considerations and findings that support the use of mice will be part of our application that we submit to the IvD. In the rare occasion that genetic interventions that modulate our gene, microRNA or cell type of interest induce a phenotype under baseline conditions, we will not expose these mice to any additional cardiac stresses like myocardial infarction or transverse aortic banding.
Once we decide our hypothesis is supported enough to pursue a gene, microRNA or cell type using animal experiments (go/no go), we will first set up a pilot studies with the minimum amount of animals possible to test the validity of our research question in vivo. For every experiment, we design the experiment with clear go-no-go decisions, to reduce the amount of cumulative discomfort and/or the number of animals. For every experiment, the best trade-off will be made. For example: based on elaborate time-response studies, we have RNA material from cardiac tissue available at many different time points after myocardial infarction or transverse aortic banding. This material should help us to identify the time point at which it is most logical for a gene, microRNA or cell type to be relevant for either cardiac repair or pathological remodeling. Based on these data we will perform a pilot study in the appropriate mouse lines and collect tissue at the time when this gene, microRNA or cell type should have the greatest effect. In case we do not observe an effect, we will go back to our original hypothesis and

determine what might explain the lack of effect, before we move forward with a second pilot experiment. Depending on the result of this second experiment we might opt to not further investigate the function of the gene, microRNA or cell type under the stress conditions tested (go/no go).
We might consider a therapeutic intervention only after we validate that the pathway our compound or microRNA-therapeutic is involved in is activated in the hearts of the mice that we are studying. We can test this by exploring either historical sample sets or by testing samples obtained from pilot studies. Only if we are able to confirm the activation of a certain pathway relevant to our compound or microRNA, we will test the therapeutic effect of intervening with the activation of this pathway (go/no go). Swimming-induced exercise might have a beneficial effect on heart repair and regeneration and the effect will therefore only be tested in unstressed mice or mice that received an MI or IR).

**2. Strategy in relation to the main scientific research questions**

To answer our **research question 1** regarding the function of a gene, microRNA or specific cell type in **heart regeneration**, we can use mice in which a gene, microRNA or (stem) cell component has been modified after which we expose the animals to no intervention or ischemic injury (MI or IR) (Animal Procedures 2, 3, 4 and 5). The readout will be an effect on cardiac function and cell death, proliferation and differentiation.

To answer our **research question 2** regarding the function of a gene, microRNA or specific cell type in **pathological remodeling**, we can use mice in which a gene, microRNA or (stem) cell component has been modified after which we expose the animals to no intervention or a stress that induced pathological remodeling (TAB, high-salt diet, remodeling agent, high-fat diet or exercise) (Animal Procedures 2, 3, 4, and 6). The choice of stress depends on the specific research questions and the proposed function of the gene or microRNA that is being studied. The readout will be an effect on cardiac function, hypertrophy and fibrosis.
To answer our **research question 3** in vivo we will study the effect of drug, microRNA therapeutic or swimming in mice that received no intervention or mice that were exposed to ischemic injury or a stress driving pathological remodeling (Animal Procedures 2, 3, 4, 5, and 6). The readout will be an effect on cardiac function, cell death, proliferation, differentiation, fibrosis and cardiomyocyte hypertrophy.
To answer our **research question 4** regarding cardiac delivery of therapeutic agents in vivo, we will study the effect of using different delivery vehicles (like hydrogel) to enhance cardiac delivery of compounds or microRNA therapeutics. This will be done in mice that received no intervention or mice that were exposed to ischemic injury or a stress driving pathological remodeling (Animal Procedures 2, 3, 4, 5, and 6). The readout will be an effect on cardiac function, cell death, proliferation, differentiation, fibrosis and cardiomyocyte hypertrophy.

**3. In planning our studies we base our experimental design on the following choices:**

**- In vitro experiments using rat neonatal cardiomyocytes:**

To test a research hypothesis in vitro we will in some cases isolate neonatal rat cardiomyocytes. These primary cells are collected from neonatal rats and provide a much used model system for studying heart muscle cell biology. We can only use neonatal cells since adult heart muscle cells cannot be taken in culture. This procedure is routinely carried out in our lab.

**- Mouse model (indicated on flowchart with A):**

To test a research hypothesis in vivo we will use an appropriate mouse model. This can be done in wild type mice, genetically modified mice, or mice in which gene or microRNA levels have been modulated in vivo by a virus (adeno associated virus or lentivirus). In some cases we might need to use mice that got transplanted with cells to study the in vivo effect of specific cells or cellular derivatives.
Considerations for choosing a mouse model:

- Wild type mouse

  Wild type mice will be used if we want to study the effect of myocardial infarction or a different pathological stress in the absence or presence of a therapeutic intervention.
- GMM
- Genetically modified mice will be used to study the cardiac function of a specific gene, microRNA or type of (stem) cell. In case a mouse line with the required genetic modification is not available we will generate new mouse line via standard oocyte injection, blastocyst injection, or via the CRISPR/Cas9 system (outlined in **animal procedure 3.4.4.2**)

- Mouse injected with virus to increase or inhibit a gene or microRNA

  In case genetic approaches fail to provide appropriate mouse lines, we will revert to viral vectors to increase or inhibit a gene or microRNA by systemic dosing. Viral vectors can also be used to manipulate a gene or microRNA more locally via intracardiac injection
- Wild type mice or GMM transplanted with cells or derivatives

  In case we want to study the cardiac effect of cell-based treatments, we will transplant (stem) cells or their derivatives in either wild type or genetically modified mice.

**- Cardiac stress / induction of heart disease (indicated on flowchart with B):**

We can choose to study these mice under basal conditions or under conditions of cardiac stress (like myocardial infarction or pathological stress). Considerations for choosing a cardiac stress:

- No intervention

  Mouse lines will receive no intervention in case we want to study the cardiac function of a gene, microRNA or (stem) cell under basal conditions.
- Myocardial infarction (MI or IR)

  Mice will receive a myocardial infarction (permanent occlusion (MI or ischemia followed by reperfusion (IR)) when we want to study the function of a gene, microRNA or (stem) cell in cardiac repair / regeneration.
- Pathological stress to induce remodeling (TAB, high-salt diet, remodeling agent, high-fat diet, exercise to trigger disease)

  Mice will receive a pathological stress when we want to study the function of a gene, microRNA or (stem) cell in pathological remodeling. The differences between the different stress models are explained in section **3.1** and our choice between the different model will depend on the specific research aim.

**- Intervention (indicated on flowchart with C):**

After introducing a cardiac stress or leaving the animals untreated, we can opt to study the effect of a therapeutic intervention either by the delivery of a compound

- No intervention

  Mouse lines will receive no intervention in case we want to study the cardiac function of a gene, microRNA or (stem) cell under basal conditions.
- Deliver a drug compound or microRNA therapeutic
- Exercise for cardioprotection.

- **Readouts /endpoint (indicated on flowchart with D)**

- Cardiac function
- Behavior
- Ex vivo analysis of cells and tissues

To study the molecular and/or cellular mechanisms of cardiac regeneration and remodeling, we need to analyze tissues and cells from the mice ex vivo to perform cellular, molecular and histological analysis.

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.

The specific protocols that we apply to achieve our research goals are outlined below. All these Animal Procedures and their components are currently already ongoing in our lab, with the exception of transplanting cells or their derivatives to study their function in heart disease.

**1. Isolation of neonatal rat cardiomyocytes (described in detail in 3.4.4.1)**

To study aspects of regeneration or remodeling in vitro we will isolate neonatal rat cardiomyocytes. These primary cells will be collected from neonatal rats and provide a much used model system for studying heart muscle cell biology.

**2. Generation of genetically modified mice (described in detail in 3.4.4.2)**

To study the function or behavior of a gene or a cell type relevant for cardiac we will use appropriate mouse lines that are either already available or that need to be generated. New mouse line(s) will be generated via standard oocyte injection, blastocyst injection, or via the CRISPR/Cas9 system. The CRISPR/Cas9 system will especially be used as highly efficient tool for simultaneously multi-gene editing.

This prevents the generation and breeding of multiple homozygotes from individually targeted ES cells (Reduction of the 3Rs).
In contrast to conventional gene-targeting strategy, the use of the Cre/LoxP recombination system in conjunction with gene targeting allows us study the consequence of gene manipulation in a cell type specific manner. By incorporating Cre recombinase recognition sites (LoxP) into the genome, Cre expression from a specific promoter can drive gene disruption, activation or tracing in a cell type specific manner. New transgenic lines and/or KO lines generated via classical methods and/or novel combinations and used for breeding of these aforementioned lines will be monitored for 2 generations to determine the absence or presence of constitutional discomfort.
Cre recombinase expression can also be activated in an inducible manner by the addition of tamoxifen. To this end the Cre is flanked by 2 mutated estrogen receptors (Creert2, or merCremer) and will only allow for Cre activation when tamoxifen is administered.

3. **Collecting (stem) cells or tissue for ex vivo experiments (described in detail in 3.4.4.3)**

In case we only collect cells or tissues for ex vivo experiments we will use wild type or genetically modified mice without any further intervention. In some cases we might need to use mice that received a

cellular transplant to study the in vivo effect of specific cells or cellular derivatives.

4. **Study the effect of drug delivery or exercise in mouse model under basal conditions (described in detail in 3.4.4.4)**

To study the cardiac function of a gene, microRNA or (stem) cell under **basal conditions**, the appropriate mouse line will be left untreated (no intervention). These mouse models can be used in studies without further intervention or we add an additional therapeutic intervention (drug or exercise) to determine the effect on heart function, behavior or molecular readouts.

5. **Study the function of a gene, microRNA or specific cell type in heart regeneration by using a model for myocardial infarction (MI or ischemia reperfusion (IR) (described in detail in 3.4.4.5).**

In case we want to study the function of a gene, microRNA or specific cell type in **heart regeneration**, we can use mice in which a gene, microRNA or (stem) cell component has been modified after which we expose the animals to ischemic injury (MI or IR). These mouse models can be used in studies without further intervention or we add an additional therapeutic intervention (drug or exercise) to determine the effect on heart function, behavior or molecular readouts.

6. **Study the function of a gene, microRNA or specific cell type in heart remodeling by using a model for pathological remodeling (described in detail in 3.4.4.6).**

In case we want to study the function of a gene, microRNA or specific cell type in **pathological remodeling**, we can use mice in which a gene, microRNA or (stem) cell component has been modified after which we expose the animals to a stress that induces a pathological remodeling response. These mouse models can be used in studies without further intervention or we add an additional therapeutic intervention (drug or exercise) to determine the effect on heart function, behavior or molecular readouts.

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.

See Flow Chart in attachment 1.
All experiments start with the identification of a candidate gene, microRNA or cell type. The identified gene, microRNA or cell type will initially be carefully tested in tissues from previous cardiac stress studies, human heart samples and cell lines, including neonatal rat cardiomyocytes (**3.4.4.1**). If the identified gene, microRNA or cell type shows an interesting phenotype in these in samples / in vitro experiments, we will consider the extensive and careful analysis of (compound) GM mice. For our in vivo experiments we will use genetically modified mice. If the desired genotype is not available we will create them ourselves. We will only use well-established reagents and protocols to induce expression or deletion of the candidate gene/s (**3.4.4.2**).

Whenever possible, for all our in vivo experiments we will perform a pilot studies with the minimum amount of animals possible. For every experiment, we design the experiment with clear go-no-go decisions, to reduce the amount of cumulative discomfort and/or the number of animals. For every experiment, the best trade-off will be made. See section 3.4.1 for a more detailed workflow.

In some cases we will use our mouse lines without any further intervention for the collection of tissues or cells for ex vivo analysis (**3.4.4.3**).
Our mice will either receive no intervention (**3.4.4.4**), a stress to study regeneration (**3.4.4.5**) or a stress to study pathological remodeling (**3.4.4.6**). Per mouse line we might switch between these 3 options, depending on the condition we want to study the function of a gene, microRNA or cell type under. Since the process of regeneration and repair also involves aspects of cardiac remodeling we will want to expose most of our genetic mouse models to both kind of stresses to study the exact function in the different aspects of heart disease.

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 neonatal rat cardiomyocytes |
| 2 | Generation, welfare assessment and breeding genetically modified mice (GMM) |
| 3 | Collecting (stem) cells or tissue for ex vivo experiments |
| 4 | Drug delivery or exercise |
| 5 | Myocardial infarction / Ischemia reperfusion |
| 6 | Models for cardiac pathological remodeling |
| 7 | |
| 8 | |
| 9 | |
| 10 | |

# 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'. | 80102 |
| 1.2 | Provide the name of the licenced establishment. | Koninklijke Nederlandse Akademie van Wetenschappen (Hubrecht Institute) |

1.3 List the serial number and type of animal procedure.

*Use the serial numbers provided in Section 3.4.4 of the Project Proposal form.*

| Serial number | Type of animal procedure |
|---|---|
| 3.4.4.1 | Isolation of neonatal rat cardiomyocytes |

## 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 general design of the experiments in this appendix is shown in the attached flowchart (**3.4.4.1**).

In some cases we will use cardiomyocytes in an attempt to validate a research hypothesis.The general design of the experiments in this appendix is shown in the attached flowchart. The rat is the most validated species to obtain cardiomyocytes from and give the biggest yield. Neonatal hearts are isolated because neonatal cardiomyocytes can be kept in culture longer and better than adult cardiomyocytes. This procedure is standardized and well validated in many different labs and the applicant as well as lab members are familiar with the isolation technique.

Pregnant rats will be used to deliver the pups required for the neonatal rats. From these pups cardiac tissue will be collected for the isolation of primary cardiomyocytes between postnatal day 1-4.

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

<u>Animal procedure in rats</u>

- Pregnant female rats ordered from an establishment licensed breeder by the NVWA or from a registered commercial company and delivered around E11 of their pregnancy.
- Heart cells of neonatal rat pups (P1-P4) will be collected for cell culture.
- Pups will be removed from their mother's cage and sedated by hypothermia before sacrifice after

which the heart will be collected.
- The females (mothers) will be will be euthanized by O2/CO2 method.

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

Whenever possible we strive for a situation to express the outcome of our experiments in quantitative terms. To estimate the number of cells to be required for an experiment, we use the effect size (if known, e.g., from data in the literature, from our own historical data or if unknown from small scale pilots) to estimate the sample size needed to achieve a certain power (usually around 0.8) with appropriate statistical test like the t test with a $p<0.05$.
Qualitative analysis is based on literature and/or on years of experience with similar type of experiments. Moreover, the in vitro experiments will be performed sequentially via which we ensure that we will use the minimum number of rats per group needed to get a conclusive result.

**B. The animals**

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

Rat wildtype
Age; adult (mothers), pups (P1-P4)
Origin; external licensed breeders
Number of animals: 360 pregnant rats, 2700 pups

To perform in vitro experiments in cardiomyocytes we need to isolate primary neonatal rat ventricular cardiomyocytes. Rat is the most validated species to obtain these cells from and give the biggest yield. Neonatal hearts are isolated because neonatal cardiomyocytes can be kept in culture longer and better than adult cardiomyocytes. This procedure is standardized and well validated in many different labs and the applicant as well as lab members are familiar with the isolation technique.

We will analyze the effect of modulating our gene, factor or microRNA of interest, in cardiomyocytes. Based on our experience during the last 2 years we expect to isolate neonatal rat cardiomyocytes once every 3 weeks (= 18 x per year * 5 years = 90 isolations). This is to keep a continuous supply of cells for continuity and reproducibility of the results.
We isolate cardiomyocyte cells from 25-30 neonatal rat pups per isolation. The number of pups and expected yield is based on years of experience with working with these cells.

4 (pregnant rats) * 90 (isolations) = maximum of **360** pregnant rats
30 (pups) *90 (isolations) = maximum **2700** neonatal rat pups

**C. Re-use**

Will the animals be re-used?

☒ 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’?

☒ 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.

The experiments described in this protocol allow us to perform in vitro testing of our hypothesis in an

appropriate cells line. For some projects, using this cell line will enable us to assess whether there is enough support to consider the import or generation and subsequently analysis of GGM to study the function of a gene or microRNA in the heart. The use of in vitro cultures allows this project to comply with the 3Rs (replacement, reduction and refinement) by keeping the mice numbers to be used to a minimum. Based on our experience, cardiomyocyte-like cells (like HL-1 cells) do not provide reliable data regarding a biological function in true cardiomyocytes, so there is no good alternative using no experimental animals for these types of in vitro experiments.

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

Appropraite possibilities to reduce pain, fear or suffering will be used. There are no negative environmental effects; all rats will be housed under strict D1 conditions.

## Repetition and duplication

**E. Repetition**

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

The experiments in this project are not carried out due to legal requirements.

## 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

☒ 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?

☒ 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.

☒ 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.

- Pups will be removed from their mother's cage and placed on melting ice (but not in direct

contact with).
- After sedation by hypothermia pups will be sacrificed by decapitation after which the heart will be collected.
- Heads will immediately be put in liquid nitrogen.
- The mothers will be will be euthanized by O2/CO2 method.

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

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

none

Explain why these effects may emerge.

-

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

-

**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.

☐ 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').

Mothers: mild discomfort 100%
Pups: mild discomfort 100%

## End of experiment

**L. Method of killing**

Will the animals be killed during or after the procedures?

☐ No

☒ Yes > Explain why it is necessary to kill the animals during or after the procedures.

The mother rats will be sacrificed to prevent discomfort from removing her litter and the pups will be sacrificed to collect cardiomyocytes for in vitro experiments.

Is the proposed method of killing listed in Annex IV of Directive 2010/63/EU?

☐ No > Describe the method of killing that will be used and provide justifications for this choice.

☒ Yes

# Appendix
## Description animal procedures

- This appendix should be enclosed with the project proposal for 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'. | 80102 |
| 1.2 | Provide the name of the licenced establishment. | Koninklijke Nederlandse Akademie van Wetenschappen (Hubrecht Institute) |

1.3 List the serial number and type of animal procedure.

*Use the serial numbers provided in Section 3.4.4 of the Project Proposal form.*

| Serial number | Type of animal procedure |
|---|---|
| 3.4.4.2 | Generation, welfare assessment and breeding genetically modified mice (GMM) |

## 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 general design of the experiments in this appendix is shown in the attached flowchart (**3.4.4.2**).

Generate new mouse lines by injecting DNA/RNA into oocyte, injection of genetically modified ES cells into blastocysts and/or via the CRISPR/Cas9 system. The CRISPR/Cas9 system will especially be used as a highly efficient tool for simultaneously multi-gene editing. We will do zygote injections with Cas9 and sgRNA to create a genetic deletions or zygote injection with Cas9, sgRNA and DNA template to create a knock in mice.

Welfare assessment according to the Consensus documents on genetically altered animals. New compound mouse models and new created transgenic lines and/or KO lines generated via classical methods and/or novel combinations of these aforementioned lines will be monitored for 2 generations to determine the absence or presence a phenotype with constitutional discomfort. We will daily check the mice on several parameters like overall appearance, size, growth, coat conditions, behaviour and clinical signs.

We breed our own transgenic and knock out mouse lines. In some mouse lines we make use of the Cre/Loxp recombination system. For that we had to cross transgenic or knock out mouse lines, who are caring LoxP site in there genome, with a Cre or tamoxifen inducible Cre mouse line. We have 3-8 breeding pairs per mouse line that will be retain for a maximum of 6 month. The offspring will be used for experiments.

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

Generation new lines

1. Superovulation; Administration of gonadotropin's (2x) in female mice by subcutaneous or intraperitoneal injections followed my mating. Females will be killed for the isolation of early embryos.
2. Embryo recipients; Recipients for embryo transfer will be rendered pseudo-pregnant by mating with a sterile (vasectomized) male. Genetically modified embryos will be implanted surgically or non-surgically into the reproductive tract. Embryo recipients, not as part of an experiment, will be killed after weaning of the pups at three weeks of age.
3. Weaned pups at 3 weeks of age; tissue sampling for genotyping and/or identification via tail, toe and ear cut, respectively, under isoflurane anesthesia.

Welfare assessment
We will daily check the mice on several parameters (overall appearance, size, growth, coat conditions, behaviour, clinical signs, relative size and numbers) as has been described in the Directive 2010/63/EU: corrigendum of 24 Jan. 2013.

Breeding genetically modified mice
Mice will be housed under normal conditions with free access to food and water. Start breeding with a minimum of 8 weeks old mice. Breeding will be retaining for a maximum of 6 months. Depended on the amount of experimental animals, 3-10 breeding pairs per genotype will be needed.

Killing animals
In case of discomfort or surplus animals (mice who don't have the right genotype), animals will be euthanized by O2/CO2 method or by isoflurane and will be confirmed by cervical dislocation.

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

Statistical analysis doesn't play a role for these type of experiments. We will use state of art techniques. All techniques are proven to be effective in generating genetic modified mice with a minimum number of mice possible.

**B. The animals**

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

Mus musculus; WT or genetically modified
Age; adult
Origin; All vasectomized males which will be obtained from a registered commercial company, all other mice are obtained from our own Institute, an establishment licensed breeder by the NVWA, or from a registered commercial company.

Generation of genetic modified mice; We expect to generate a maximum of 40 new mouse lines over the next 5 years. For the creation of new mouse line we will use on average max 150 mice (according to the 'besluit biotechnologie'). Therefore, a total of max 150 (mice) x 40 (mouse lines) = **6000** mice is requested.

Welfare assessment;
We expect to generate over the next 5 years 40 new genetically modified lines for which we have to perform the welfare assessment for 2 generations. We therefore need in total: 40 (new (compound) lines) * 2 (generations) * 32((8 male +8 female =16 GM mice) + (8 male +8 female = 16 control mice))= 2560 mice.
These 40 lines will on average be bred to 3 other different lines for 2 generations (in general this will entail breeding with different Cre lines to specifically manipulate a gene or microRNA or cells type). We therefore need in total: 40 (new (compound) lines) * 3 (lines) * 2 (generations) * 32((8 male +8 female

=16 genotype 1) + (8 male +8 female = genotype 2)) = 7680 mice**.**
Total number for welfare assessment is **10.240** mice

The offspring of these breeding pairs will be used in appendix 3.4.4.3 t/m 3.4.4.6. The number of animals are described in these appendixes.

**C. Re-use**

Will the animals be re-used?

☒ 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'?

☒ 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.

Before we consider the generation of a new (compound) GM mice we first will extensively analyze in samples from previous in vivo studies, human cardiac samples that are available or we will use in vitro experiments to determine whether our research hypothesis is valid. Only if the tissue analysis and/or in vitro experiments are insufficient to completely address the research question/hypothesis, we will consider the generation of a novel genetically modified mice.
The CRISPR/Cas9 system allows us, if required, to genetically modify up to 5 different genes at the same time. This strongly reduce the number of mice used for the generation and/or breeding of these compound mice.
Since we are dealing with complex systems where all different cell types contribute to the outcome, it is not possible to appropriately study heart biology without using animals.

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

All possibilities to reduce pain, fear or suffering will be used. There are no negative environmental effects; all mice will be housed under strict D1 conditions

## Repetition and duplication

**E. Repetition**

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

The experiments in this project are not carried out due to legal requirements.

## 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

☒ 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?

☒ 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.

☒ 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.

If required, isoflurane and/or a mixture of ketamine and xylazine will be used for general anaesthesia.

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

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

Some of the animal lines might experience discomfort under basal conditions due to the genetic modification of a gene or microRNA. This is expected in less than 5% of the animal lines that are being studied.

Explain why these effects may emerge.

These effects might occur due to genetic modification of a gene or microRNA

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

Daily monitoring of the mice combined with an experimental design aiming at reducing the discomfort of the animals.

**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.

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

Weight loss (>20% in comparison with control or 15% weight loss in two days), changes in behavior or body posture, signs of general sickness and/or discomfort.

Indicate the likely incidence.

Expected in <5% of the new lines generated.

**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').

Donors: Moderate 100%
Fosters: Moderate 100%
Genetically modified mice: 100% moderate. This is likely an overestimation of the discomfort, but

Welfare assessment: Mild 100%
the true discomfort will be assessed for each individual experiment.

## End of experiment

**L. Method of killing**

Will the animals be killed during or after the procedures?

☐ No

☒ Yes > Explain why it is necessary to kill the animals during or after the procedures.

Animals are no longer needed anymore (surplus or fosters) or animals are killed

Is the proposed method of killing listed in Annex IV of Directive 2010/63/EU?

☐ No > Describe the method of killing that will be used and provide justifications for this choice.

☒ Yes

# 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'. | 80102 |
| 1.2 | Provide the name of the licenced establishment. | Koninklijke Nederlandse Akademie van Wetenschappen (Hubrecht Institute) |

1.3 List the serial number and type of animal procedure.

*Use the serial numbers provided in Section 3.4.4 of the Project Proposal form.*

| Serial number | Type of animal procedure |
|---|---|
| 3.4.4.3 | Collecting (stem) cells or tissue for ex vivo experiments |

## 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 general design of the experiments in this appendix is shown in the attached flowchart (**3.4.4.3**).

**Animal model (indicated on flowchart with A):**

To test a research hypothesis we will use wild type mice, genetically modified mice, or mice in which a specific gene or microRNA level has been modulated in vivo by a virus (such as adeno associated virus or lentivirus).

Considerations for choosing a mouse model:

- Wild type mouse
  Wild type mice will be used if we need normal tissues or cells to study our research hypothesis.
- GMM
  Genetically modified mice will be used to study the cardiac function of a specific gene, microRNA or type of (stem) cell. In case a mouse line with the required genetic modification is not available we will generate new mouse line via standard oocyte injection, blastocyst injection, or via the CRISPR/Cas9 system (outlined in Appendix 3.4.4.2)
- Mouse injected with virus to increase or inhibit a gene or microRNA
  In case genetic approaches fail to provide appropriate mouse lines, we will revert to viral vectors to increase or inhibit a gene or microRNA by systemic dosing. Viral vectors can also be used to manipulate a gene or microRNA more locally via intracardiac injection.

**Cardiac stress (indicated on flowchart with B):**

- No intervention

**Readout parameters / endpoint (indicated on flowchart with D):**

- Ex vivo analysis of cells and tissues

To study the molecular and/or cellular mechanisms of the heart, we need to analyze tissues and cells from mice ex vivo. In some cases we will need to collect cells with a certain genetic constitution to transplant back into wildtype or genetically modified mice.

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

Animal model (indicated on flowchart with A):

- Mice are ordered from a commercial NVMA licensed breeder or from a registered commercial company, or are generated by ourselves under protocol 3.4.4.2.
- Tissue sampling for genotyping and identification will be done via ear, toe and/or tail biopsy carried out under anaesthesia (4% (isoflurane/oxygen).
- In some cases we will administer transgene inducing or deleting agents alone or in combination, continuously or intermittently by one or maximally 2 of the following routes:
    - in diet or drinking water (max. 1 time, < 2 wks)
    - subcutaneous injection (max. 3 times)
    - intraperitoneal injection (max. 7 times)
    - implantation of a slow release pellet subcutaneously under adequate anesthesia and analgesia
    - implantation of a slow release pump subcutaneously under adequate anesthesia and analgesia
    - oral (max. 10 times)
- In some cases we will inject a virus or microRNA therapeutic by one or maximally 2 of the following routes:
    - Intravenous injection (max. 10 times)
    - Subcutaneous injection (max. 10 times)
    - Intraperitoneal injection (max. 10 times)
- In some cases WT or GM mice will be transplanted with cells under adequate anaesthesia and analgesia.
-

Readouts /endpoint (indicated on flowchart with D)

- Ex vivo analysis of cells and tissues

All animals will be killed for ex vivo analysis or use of cells and or tissues.
Adult mice will be killed via CO2/O2 method, via cervical dislocation under isoflurane anesthesia, or via perfusion fixation under lethal dose of Nembutal.
Neonates (< 4 days) will be put on melting ice water for 10 min. (but not in contact with) after which they will be decapitated and the head immediately frozen.
Tissue will be collected for histological and molecular analysis to look at aspects of cell size, survival, proliferation, differentiation, hypertrophy or fibrosis.

The cumulative discomfort in the genetic models will maximally consist of discomfort due to:

- the genetic modification (< 5% of lines)
- tissue sampling for genotyping
- maximally 2 routes of administration of transgene inducing or deleting agents
- transplantation of cells under adequate anaesthesia and analgesia
- sacrifice

The cumulative discomfort in the models injected with a virus or microRNA therapeutic will maximally consist of discomfort due to:

- inject a virus or microRNA therapeutic by maximally 2 routes of administration
- sacrifice

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

Whenever possible we strive for a situation to express the outcome of our experiments in quantitative terms. To estimate the number of animals to be used in an experiment, we use the effect size (if known, e.g., from data in the literature, from our own historical data or if unknown from small scale pilots) to estimate the sample size needed to achieve a certain power (usually around 0.8) with appropriate statistical test like the t test with a $p<0.05$.
Qualitative analysis (most of the animal procedures described in this appendix): the number of animals (group size) is based in literature and/or years of experience with similar type of experiments. Moreover, these types of experiments will be performed sequentially via which we ensure that we will use the minimum number of mice per group needed that will be informative.

**B. The animals**

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

Mus musculus; WT, genetically modified or injected with a virus or microRNA therapeutic
Age; neonates p1-p4 and adults
Origin; Hubrecht institute or external licensed breeders
Number of animals: 4800

All animal studies in this project will exclusively involve mice. Up to today, there are no alternative methods to fully understand how the heart functions as an organ and how it remodels during disease. For the majority of the proposed studies, the mouse is the most appropriate animal model because: (1) like humans, mice are mammals; (2) physiology is more extensively characterized; (3) mice are amenable to transgenic modification; (4) a large number of relevant transgenic and knock out lines are already available.

To study the effect of increasing or deleting a gene or microRNA on the heart, on average we use 20 mice per genotype that we want to study (which is usually WT and GM mice). Based on our experience we will maximally analyze 40 different mouse lines over the next 5 years and we will study these mice at different time points (3 time points on average).

Since cardiac biology and pathology is really different in males and females because of estrogen signaling in females we only use males in our functional studies to exclude gender based variation (see also project proposal).

The maximum required number of animals. 20*2 (WT and GM mice) *3 (different time points) * 40 (mouse lines) = **4800 male mice**

**C. Re-use**

Will the animals be re-used?

☒ 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’?

☒ 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.

Before we consider the import or generation and subsequently analysis of GGM to study the function of a gene or microRNA in the heart, we first will analyze appropriate cell lines, existing patient material or materials available from previous animal studies, which extensively reduces the animal numbers. The use human material and in vitro cultures allows this project to comply with the 3Rs (replacement, reduction and refinement) by keeping the mice numbers to be used to a minimum. Also trying to use the newly developed CRISPR/Cas9 method to create new mouse lines will significantly reduce the number of animals needed to generate a new line.

However, animal studies are unavoidable if we seek for the fundamental insight into the molecular and cellular mechanisms in cardiac biology and disease. Whenever possible, we will perform pilot studies with the minimum number of animals possible. Experiments will be done sequential, where on basis of the results, decisions will be made on the next steps.

If homozygous mice do not show any discomfort we will keep the mice on a homozygous background for breeding, thereby reducing the number of mice.

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

All possibilities to reduce pain, fear or suffering will be used. There are no negative environmental effects; all mice will be housed under strict D1 and/or D2 conditions.

## Repetition and duplication

**E. Repetition**

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

The experiments in this project are not carried out due to legal requirements.

## 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

☒ 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?

☒ 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.

☒ 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.

If required, adequate anesthesia and analgesia will be used.

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

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

Some of the animal lines might experience discomfort under basal conditions due to the genetic modification of a gene or microRNA. This is expected in less than 5% of the animal lines that are being studied.

Explain why these effects may emerge.

These effects might occur due to genetic modification of a gene or microRNA

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

Daily monitoring of the mice combined with an experimental design aiming at reducing the discomfort of the animals. If we unexpectedly observed weight loss (>20% in comparison with control or 15% weight loss in two days), changes in behavior or body posture, signs of general sickness and/or discomfort animals will be taken out of experiment and sacrificed.

**J. Humane endpoints**

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

☐ No > Continue with question K.

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

Weight loss (>20% in comparison with control or 15% weight loss in two days), changes in behavior or body posture, signs of general sickness and/or discomfort.

Indicate the likely incidence.

Expected in <5% of the new lines generated

**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 cumulative discomfort in the genetic models will maximally consist of moderate discomfort due to:

- the genetic modification (< 5% of lines)
- tissue sampling for genotyping
- maximally 2 routes of administration of transgene inducing or deleting agents
- transplantation of cells under adequate anaesthesia and analgesia
- sacrifice

The cumulative discomfort in the models injected with a virus or microRNA therapeutic will maximally consist of moderate discomfort due to:

- inject a virus or microRNA therapeutic by maximally 2 routes of administration
- sacrifice

Neonatal mice: 100% moderate. This is likely an overestimation of the discomfort, but the true discomfort will be assessed for each individual experiment.
Adult mice: 100% moderate. This is likely an overestimation of the discomfort, but the true discomfort will be assessed for each individual experiment.

## End of experiment

**L. Method of killing**

Will the animals be killed during or after the procedures?

☐ No

☒ Yes > Explain why it is necessary to kill the animals during or after the procedures.

All animals are killed to use their organs for ex vivo analysis

Is the proposed method of killing listed in Annex IV of Directive 2010/63/EU?

☐ No > Describe the method of killing that will be used and provide justifications for this choice.

☒ Yes

# 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'. | 80102 |
| 1.2 | Provide the name of the licenced establishment. | Koninklijke Nederlandse Akademie van Wetenschappen (Hubrecht Institute) |

1.3 List the serial number and type of animal procedure.

*Use the serial numbers provided in Section 3.4.4 of the Project Proposal form.*

| Serial number | Type of animal procedure |
|---|---|
| 3.4.4.4 | Drug delivery or exercise |

## 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 general design of the experiments in this appendix is shown in the attached flowchart (**3.4.4.4**).

To study the cardiac function of a gene, microRNA or (stem) cell under **in the absence of cardiac stress conditions**, the appropriate mouse line will be left untreated (no intervention). These mouse models can be used in studies without further intervention or we add an additional therapeutic intervention (drug or exercise) to determine the effect on heart function, behavior or molecular read outs

The different components of the proposed experiments are:

**Mouse model (indicated on flowchart with A):**

To test a research hypothesis in vivo we will use an appropriate mouse model. This can be done in wild type mice, genetically modified mice, or mice in which gene or microRNA levels have been modulated in vivo by a virus (such as adeno associated virus or lentivirus). In some cases we might need to use mice that got transplanted with cells to study the in vivo effect of specific cells or cellular derivatives.

Considerations for choosing a mouse model:

- Wild type mouse

  Wild type mice will be used if we want to study the effect a therapeutic intervention.
- GMM

  Genetically modified mice will be used to study the cardiac function of a specific gene, microRNA or type of (stem) cell. In case a mouse line with the required genetic modification is not available

we will generate new mouse line via standard oocyte injection, blastocyst injection, or via the CRISPR/Cas9 system (outlined in Appendix 3.4.4.2)

- Mouse injected with virus to increase or inhibit a gene or microRNA

  In case genetic approaches fail to provide appropriate mouse lines, we will revert to viral vectors to increase or inhibit a gene or microRNA. This can also serve to manipulate a gene or microRNA more locally via intracardiac injection
- Wild type mice or GMM transplanted with cells or derivatives

  In case we want to study the cardiac effect of cell-based treatments, we will transplant (stem) cells or derivatives in either wild type or genetically modified mice.

**Cardiac stress (indicated on flowchart with B):**

- No intervention

  Mouse lines will receive no intervention in case we want to study the cardiac function of a gene, microRNA or (stem) cell under basal conditions.

**Intervention (indicated on flowchart with C):**

- No intervention

  Mouse lines will receive no intervention in case we want to study the cardiac function of a gene, microRNA or (stem) cell under basal conditions.
- Deliver a drug compound
- Exercise

**Readouts / endpoint (indicated on flowchart with D)**

- Cardiac function (by for example echocardiography or pressure catheters)
- Behavior
- Ex vivo analysis of cells and tissues

In all experiments, we will determine cardiac function and determine the general behavior of the animals. All mice will be sacrificed for detailed analysis of the heart and other tissues involved in aspects of heart disease (lungs, kidneys, liver). Analysis will include histology and molecular analysis. Also, cells from organs might be isolated by FACS for subsequent analysis.

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

Animal model (indicated on flowchart with A):

- Mice are ordered from a commercial, NVWA licensed breeder or from a registered commercial company, or are generated by ourselves under protocol 3.4.4.2.
- Tissue sampling for genotyping and identification will be done via ear, toe and/or tail biopsy under anaesthesia (4% (isoflurane/oxygen).
- In some cases we will administer transgene inducing or deleting agents alone or in combination, continuously or intermittently by one or maximally 2 of the following routes:
  - in diet or drinking water (max. 1 time, < 2 wks)
  - subcutaneous injection (max. 3 times)
  - intraperitoneal injection (max. 7 times)
  - implantation of a slow release pellet subcutaneously under adequate anesthesia and analgesia
  - implantation of a slow release pump subcutaneously under adequate anesthesia and analgesia
  - oral (max. 10 times)
- In some cases we will inject a virus or microRNA therapeutic by one or maximally 2 of the following routes:
  - Intravenous injection (max. 10 times)
  - Subcutaneous injection (max. 10 times)
  - Intraperitoneal injection (max. 10 times)
- In some cases WT or GM mice will be transplanted with cells under adequate anaesthesia and analgesia

- In some cases we will administer a labelling agent (e.g. BrdU) via one of the following routes:
    - intraperitoneal injection (1 time)
    - implantation of a slow release pump subcutaneously under adequate anaesthesia and analgesia (1 time)
    - intravenous injection (1 time)

Intervention (indicated on flowchart with C):

- No intervention
- Deliver a drug compound
    - Systemic delivery of compound or microRNA therapeutic by
        - Subcutaneous injection once weekly or every two weeks for maximally 12 weeks
        - intraperitoneal injection once weekly or every two weeks for maximally 12 weeks
        - implantation of a slow release pump subcutaneously under adequate anaesthesia and analgesia (1 time)
    - Intracardiac delivery of compound or microRNA therapeutic
        - Mice will be anesthetized with appropriate anesthesia.
        - Hair will be removed from the ventral surface of the neck and thorax
        - A tracheal tube will be placed and the mouse connected to a ventilator
        - A surgical plane of anesthesia as judged by lack of pain reflex (toe pinch)
        - The surgical site will be cleaned with iodine and 70% ethanol.
        - Using aseptic technique with sterile instruments, the skin will be incised left of midline to allow access to the third intercostal space. Pectoral muscles will be retracted and the intercostal muscles cut caudal to the third rib. A rib spreader will be placed to allow access to the heart.
        - Drug or microRNA therapeutic compound will be delivered via intracardiac injection.
        - The rib cage will be closed with a suture and the skin closed with tissue adhesive.
        - The animal will be disconnected from the ventilator the tracheal tube removed and the animal placed unrestrained on a nose cone with 100% oxygen in a warm recovery cage until fully ambulatory, at which point the oxygen will be turned off
- Exercise
  Swimming exercise starting with 10 minutes 2 times daily with 10 minutes increase each day until 90 minutes, 2 times per day is reached. The 2 x 90 minutes a day will continue for 2 to max 10 weeks (depending on how long it takes to observe cardiac remodeling by echo). The mice will be observed at all times to avoid mice submerging under the water surface.

Readouts /endpoint (indicated on flowchart with D)

- Cardiac function
  Cardiac function will be evaluated by echocardiography, ECG, or pressure catheters on sedated, adult mice
- Behavior
  Animals will be monitored for general signs of sickness and/or discomfort by looking for immobility, significant weight loss or lack of grooming and/or symptoms of heart failure (like dyspnoea and edema). If these signs are unexpectedly observed the animals will be euthanized prematurely
- Ex vivo analysis of cells and tissues
  Tissue will be collected for histological and molecular analysis to look at aspects of cell size, survival, proliferation, differentiation, hypertrophy or fibrosis.

The choice of intervention depends on the specific research questions and the proposed function of the gene or microRNA that is being studied.

The cumulative discomfort in the genetic models will maximally consist of discomfort due to:

- the genetic modification (< 5% of lines)

- tissue sampling for genotyping
- maximally 2 routes of administration of transgene inducing or deleting agents
- administration of a labelling agent
- transplantation of cells under adequate anaesthesia and analgesia
- systemic or intracardiac delivery of a drug or exposure to exercise
- sedation to determine cardiac function by echo
- sacrifice

The cumulative discomfort in the models injected with a virus or microRNA therapeutic will maximally consist of discomfort due to:

- inject a virus or microRNA therapeutic by maximally 2 routes of administration
- administration of a labelling agent
- transplantation of cells under adequate anaesthesia and analgesia
- systemic or intracardiac delivery of a drug or exposure to exercise
- sedation to determine cardiac function by echo
- sacrifice

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

Whenever possible we strive for a situation to express the outcome of our experiments in quantitative terms. To estimate the number of animals to be used in an experiment, we use the effect size (if known, e.g., from data in the literature, from our own historical data or if unknown from small scale pilots) to estimate the sample size needed to achieve a certain power (usually around 0.8) with appropriate statistical test like the t test with a $p<0.05$.
Qualitative analysis (most of the animal procedures described in this appendix): the number of animals (group size) is based in literature and/or years of experience with similar type of experiments. Moreover, these types of experiments will be performed sequentially via which we ensure that we will use the minimum number of mice per group that will be informative.

**B. The animals**

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

Mus musculus; WT, genetically modified or injected with a virus or microRNA therapeutic
Age; neonates p1-p4 and adults
Origin; Hubrecht institute or external licensed breeders
Number of animals: 3200 male mice

All animal studies in this project will exclusively involve mice. Up to today, there are no alternative methods to fully understand tissue development/homeostasis and cancer in the context of the whole organism. For the majority of the proposed studies, the mouse is the most appropriate animal model because: (1) like humans, mice are mammals; (2) physiology is more extensively characterized; (3) mice are amenable to transgenic modification; (4) a large number of relevant transgenic and knock out lines are already available.

For the type of experiments described in this procedure, it is difficult to calculate the exact number of animals, since for various experimental setups require different numbers of mice, and the variation and outcome of the experiments are unknown. The number of mice required depends on the intervention (e.g no intervention, drug or microRNA therapeutic delivery or exercise), and the goal of the experiment. For these reasons we have provided a total number of mice based on experience over the past 5 years with these types of experiments in our group. Before we will start an experiment we will write an application to the IVD. In this application we will exactly describe which considerations, facts and results have led to the proposition of the number of animals needed for these experiments.

To study the effect on the heart of no intervention, drug or microRNA therapeutic delivery or exercise after increasing or deleting a gene or microRNA we use on average 20 mice per genotype that we want to study (which is usually WT and GMM). We want to study the effect of either no intervention, drug or microRNA therapeutic delivery or exercise on mouse lines used in our lab. We expect to generate a

maximum of 40 new mouse lines over the next 5 years and each mouse line to receive around 2 interventions.
Since cardiac biology and pathology is really different in males and females because of estrogen signaling in females we only use males in our functional studies to exclude gender based variation.

The maximum required number of animals. 20*2 (WT and GM mice) * 2 (different interventions) * 40 (mouse lines) = **3200 male mice**

**C. Re-use**

Will the animals be re-used?

☒ 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'?

☒ 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.

Before we consider the import or generation and subsequently analysis of GGM to study the function of a gene or microRNA in the heart, we first will analyze appropriate cell lines, existing patient material or materials available from previous animal studies, which extensively reduces the animal numbers. The use human material and in vitro cultures allows this project to comply with the 3Rs (replacement, reduction and refinement) by keeping the mice numbers to be used to a minimum. Also trying to use the newly developed CRISPR/Cas9 method to create new mouse lines will significantly reduce the number of animals needed to generate a new line.

However, animal studies are unavoidable if we seek for the fundamental insight into the molecular and cellular mechanisms in cardiac biology and disease. Whenever possible, we will perform pilot studies with the minimum number of animals possible. Experiments will be done sequential, where on basis of the results, decisions will be made on the next steps.

If homozygous mice do not show any discomfort we will keep the mice on a homozygous background for breeding, thereby reducing the number of mice.

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

All possibilities to reduce pain, fear or suffering will be used. There are no negative environmental effects; all mice will be housed under strict D1 and/or D2 conditions.

## Repetition and duplication

**E. Repetition**

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

The experiments in this project are not carried out due to legal requirements.

## 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

☒ 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?

☒ 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.

☒ 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.

Adequate anaesthesia and analgesia will be used.

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

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

Some of the animal lines might experience discomfort due to the genetic modification of a gene or microRNA. This is expected in 5% of the animal lines that are being studied.

Explain why these effects may emerge.

These effects might occur due to genetic modification of a gene or microRNA

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

Daily monitoring of the mice combined with an experimental design aiming at reducing the discomfort of the animals. If we unexpectedly observed weight loss (>20% in comparison with control or 15% weight loss in two days), changes in behavior or body posture, signs of general sickness and/or discomfort animals will be taken out of experiment and sacrified.

**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.

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

Weight loss (>20% in comparison with control or 15% weight loss in two days), changes in behavior or body posture, signs of general sickness and/or discomfort.

Indicate the likely incidence.

Expected in <5% of the new lines generated

**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 cumulative discomfort in the genetic models will maximally consist of moderate discomfort due to:

- the genetic modification (< 5% of lines)
- tissue sampling for genotyping
- maximally 2 routes of administration of transgene inducing or deleting agents
- administration of a labelling agent
- transplantation of cells under adequate anaesthesia and analgesia
- systemic or intracardiac delivery of a drug or exposure to exercise
- sedation to determine cardiac function by echo
- sacrifice

The cumulative discomfort in the models injected with a virus or microRNA therapeutic will maximally consist of moderate discomfort due to:

- inject a virus or microRNA therapeutic by maximally 2 routes of administration
- administration of a labelling agent
- transplantation of cells under adequate anaesthesia and analgesia
- systemic or intracardiac delivery of a drug or exposure to exercise
- sedation to determine cardiac function by echo
- sacrifice

Neonatal mice: 100% moderate. This is likely an overestimation of the discomfort, but the true discomfort will be assessed for each individual experiment.
Adult mice: 100% moderate. This is likely an overestimation of the discomfort, but the true discomfort will be assessed for each individual experiment.

## End of experiment

**L. Method of killing**

Will the animals be killed during or after the procedures?

☐ No

☒ Yes > Explain why it is necessary to kill the animals during or after the procedures.

All animals are killed to use their organs in ex vivo analyses.

Is the proposed method of killing listed in Annex IV of Directive 2010/63/EU?

☐ No > Describe the method of killing that will be used and provide justifications for this choice.

☒ Yes

# 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'. | 80102 |
| 1.2 | Provide the name of the licenced establishment. | Koninklijke Nederlandse Akademie van Wetenschappen (Hubrecht Institute) |

1.3 List the serial number and type of animal procedure.

*Use the serial numbers provided in Section 3.4.4 of the Project Proposal form.*

| Serial number | Type of animal procedure |
|---|---|
| 3.4.4.5 | Myocardial infarction / Ischemia Reperfusion |

## 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 general design of the experiments in this appendix is shown in the attached flowchart (**3.4.4.5**).

To study the cardiac function of a gene, microRNA or (stem) cell in **heart regeneration**, the appropriate mouse line will be exposed to ischemic injury (MI or IR). These mouse models can be used in studies without further intervention or we add an additional therapeutic intervention (drug or exercise) to determine the effect on heart function, behavior or molecular read outs

The different components of the proposed experiments are:

**Animal model (indicated on flowchart with A):**

To test a research hypothesis in vivo we will use an appropriate mouse model. This can be done in wild type mice, genetically modified mice, or mice in which gene or microRNA levels have been modulated in vivo by a virus (such as adeno associated virus or lentivirus). In some cases we might need to use mice that got transplanted with cells to study the in vivo effect of specific cells or cellular derivatives.

Considerations for choosing a mouse model:

- Wild type mouse

  Wild type mice will be used if we want to study the effect of myocardial infarction or a different pathological stress in the absence or presence of a therapeutic intervention.
- GMM

  Genetically modified mice will be used to study the cardiac function of a specific gene, microRNA

or type of (stem) cell. In case a mouse line with the required genetic modification is not available we will generate new mouse line via standard oocyte injection, blastocyst injection, or via the CRISPR/Cas9 system (outlined in Appendix 3.4.4.2)

- Mouse injected with virus to increase or inhibit a gene or microRNA

  In case genetic approaches fail to provide appropriate mouse lines, we will revert to viral vectors to increase or inhibit a gene or microRNA by systemic dosing. Viral vectors can also be used to manipulate a gene or microRNA more locally via intracardiac injection
- Wild type mice or GMM transplanted with cells or derivatives

  In case we want to study the cardiac effect of cell-based treatments, we will transplant (stem) cells or derivatives in either wild type or genetically modified mice.

**Cardiac stress (indicated on flowchart with B):**

- Myocardial infarction (MI or IR)

  Mice will receive a myocardial infarction (permanent occlusion (MI or ischemia followed by reperfusion (IR)) when we want to study the function of a gene, microRNA or (stem) cell in cardiac repair / regeneration.

**Intervention (indicated on flowchart with C):**

- No intervention

  Mouse lines will receive no intervention in case we want to study the cardiac function of a gene, microRNA or (stem) cell under basal conditions.
- Deliver a drug compound
- Exercise

**Readouts / endpoint (indicated on flowchart with D)**

- Cardiac function
- Behavior
- Ex vivo analysis of cells and tissues

In all experiments, we will determine cardiac function and determine the general behavior of the animals. All mice will be sacrificed for detailed analysis of the heart and other tissues involved in aspects of heart disease (lungs, kidneys liver). Analysis will include histology and molecular analysis. Also, cells from organs might be isolated by FACS for subsequent analysis.

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

Animal model (indicated on flowchart with A):

- Mice are ordered from a commercial, NVWA licensed breeder or from a registered commercial company, or are generated by ourselves under protocol 3.4.4.2.
- Tissue sampling for genotyping and identification will be done via ear, toe and/or tail biopsy under anaesthesia (4% (isoflurane/oxygen).
- In some cases we will administer transgene inducing or deleting agents alone or in combination, continuously or intermittently by one or maximally 2 of the following routes:
  - in diet or drinking water (max. 1 time, < 2 wks)
  - subcutaneous injection (max. 3 times)
  - intraperitoneal injection (max. 7 times)
  - implantation of a slow release pellet subcutaneously under adequate anesthesia and analgesia
  - implantation of a slow release pump subcutaneously under adequate anesthesia and analgesia
  - oral (max. 10 times)
- In some cases we will inject a virus or microRNA therapeutic by one or maximally 2 of the following routes:
  - Intravenous injection (max. 10 times)
  - Subcutaneous injection (max. 10 times)
  - Intraperitoneal injection (max. 10 times)

- In some cases WT or GM mice will be transplanted with cells under adequate anaesthesia and analgesia
- In some cases we will administer a labelling agent (e.g. BrdU) via one of the following routes:
    - intraperitoneal injection (1 time)
    - implantation of a slow release pump subcutaneously under adequate anaesthesia and analgesia (1 time)
    - intravenous injection (1 time)

Cardiac stress (indicated on flowchart with B):

- **Myocardial infarction (MI):** We will perform MI by permanent ligation of the LAD if we want to induce a large insult causing a more severe stress than when giving mice an ischemic insut and reperfusion the vessel later on (as happens during IR).
    - Mice will be weighed and anesthetized with a mixture of ketamine and xylazine by IP injection. Toe pinch will be done to determine whether the pain reflection is absent.
    - A tracheal tube will be placed and the mouse connected to a ventilator.
    - Hair will be removed from the ventral surface of the neck and thorax. The surgical site will be cleaned with iodine and 70% ethanol.
    - Using aseptic technique with sterile instruments, the skin will be incised left of the midline to allow access to the third intercostal space.
    - Pectoral muscles will be retraced and the intercostal muscles cut caudal to the third rib. Wound hooks will be placed to allow access to the heart.
    - The pericardium will be incised longitudinally and the left anterior descending coronary artery (LAD) identified.
    - In case of **MI** a 7-0 silk suture will be placed beneath the LAD.
    - In case of **sham** the rib case will be open and closed.
    - The rib cage will be closed with 5-0 silk suture and the skin closed with a wound clip.
    - The animal will be disconnected from the ventilator, the tracheal tube removed and the animal placed unrestrained on a nose cone with 100% oxygen till he wakes up.
    - The whole surgery is done on a 39 degrees heat mat.
    - To alleviate pain or distress adequate anesthesia is provided.
    - When needed, adequate analgesia will be given 1-3 days after surgery.
- **Ischemia Reperfusion (IR):** We will perform IR by a temporal ligation of the LAD if we want to induce Ischemic injury that is less severe and more closely mimics the clinic where the occluded vessels are reopened in patients that suffer an infarct.
    - Mice will be weighed and anesthetized with an adequate cocktail of anesthesia. Toe pinch will be done to determine whether the pain reflection is absent.
    - A tracheal tube will be placed and the mouse connected to a ventilator.
    - Hair will be removed from the ventral surface of the neck and thorax with nair. The surgical site will be cleaned with iodine and 70% ethanol.
    - Using aseptic technique with sterile instruments, the skin will be incised left of the midline to allow access to the third intercostal space.
    - Pectoral muscles will be retraced and the intercostal muscles cut caudal to the third rib. Wound hooks will be placed to allow access to the heart.
    - The pericardium will be incised longitudinally and the left anterior descending coronary artery (LAD) identified.
    - In case of **IR** a 7-0 silk suture will be placed beneath the LAD. A 2-3 mm piece of PE 10 tubing will be placed over the LAD and the ligature secured around the LAD and PE tube. Following by 1hr ischemic period, the ligature will be cut and the PE tubing removed to allow for reperfusion via the LAD.
    - In case of **sham** the rib case will be open and closed.
    - The rib cage will be closed with 5-0 silk suture and the skin closed with a wound clip.
    - The animal will be disconnected from the ventilator, the tracheal tube removed and the animal placed unrestrained on a nose cone with 100% oxygen till he wakes up.
    - The whole surgery is done on a 39 degrees heat mat.
    - To alleviate pain or distress appropriate analgesic is given at completion of surgery.

- When needed, adequate analgesia will be given 1-3 days after surgery..

Intervention (indicated on flowchart with C):

- No intervention
- Deliver a drug compound
  - Systemic delivery of compound or microRNA therapeutic by
    - Subcutaneous injection once weekly or every two weeks for maximally 12 weeks
    - intraperitoneal injection once weekly or every two weeks for maximally 12 weeks
    - implantation of a slow release pump subcutaneously under adequate anaesthesia and analgesia (1 time)
  - Intracardiac delivery of compound or microRNA therapeutic
    - Mice will be anesthetized with appropriate anesthesia.
    - Hair will be removed from the ventral surface of the neck and thorax
    - A tracheal tube will be placed and the mouse connected to a ventilator
    - A surgical plane of anesthesia as judged by lack of pain reflex (toe pinch)
    - The surgical site will be cleaned with iodine and 70% ethanol.
    - Using aseptic technique with sterile instruments, the skin will be incised left of midline to allow access to the third intercostal space. Pectoral muscles will be retracted and the intercostal muscles cut caudal to the third rib. A rib spreader will be placed to allow access to the heart.
    - Drug or microRNA therapeutic compound will be delivered via intracardiac injection.
    - The rib cage will be closed with a suture and the skin closed with tissue adhesive.
    - The animal will be disconnected from the ventilator the tracheal tube removed and the animal placed unrestrained on a nose cone with 100% oxygen in a warm recovery cage until fully ambulatory, at which point the oxygen will be turned off
- Exercise
  Swimming exercise starting with 10 minutes 2 times daily with 10 minutes increase each day until 90 minutes, 2 times per day is reached. The 2 x 90 minutes a day will continue for 2 to max 10 weeks (depending on how long it takes to observe cardiac remodeling by echo). The mice will be observed at all times to avoid mice submerging under the water surface.

Readouts /endpoint (indicated on flowchart with D)

- Cardiac function
  Cardiac function will be evaluated by echocardiography, ECG or pressure catheters, on sedated, adult mice
- Behavior
  Animals will be monitored for general signs of sickness and/or discomfort by looking for immobility, significant weight loss or lack of grooming and/or symptoms of heart failure (like dyspnea and edema). If these signs are unexpectedly observed the animals will be euthanized prematurely
- Ex vivo analysis of cells and tissues
  Tissue will be collected for histological and molecular analysis to look at aspects of cell size, survival, proliferation, differentiation, hypertrophy or fibrosis.

The choice of ischemic injury model depends on the specific research questions and the proposed function of the gene or microRNA that is being studied.

The cumulative discomfort in the genetic models will maximally consist of moderate discomfort due to:

- the genetic modification (< 5% of lines)
- tissue sampling for genotyping
- maximally 2 routes of administration of transgene inducing or deleting agents
- administration of a labelling agent
- transplantation of cells under adequate anaesthesia and analgesia
- MI or IR surgery

- discomfort due to surgery
- discomfort due to the development of heart disease
- systemic or intracardiac delivery of a drug or exposure to exercise
- sedation to determine cardiac function by echo
- sacrifice

The cumulative discomfort in the models injected with a virus or microRNA therapeutic will maximally consist of moderate discomfort due to:

- inject a virus or microRNA therapeutic by maximally 2 routes of administration
- administration of a labelling agent
- transplantation of cells under adequate anaesthesia and analgesia
- MI or IR surgery
- discomfort due to surgery
- discomfort due to the development of heart disease
- systemic or intracardiac delivery of a drug or exposure to exercise
- sedation to determine cardiac function by echo
- sacrifice

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

Whenever possible we strive for a situation to express the outcome of our experiments in quantitative terms. To estimate the number of animals to be used in an experiment, we use the effect size (if known, e.g., from data in the literature, from our own historical data or if unknown from small scale pilots) to estimate the sample size needed to achieve a certain power (usually around 0.8) with appropriate statistical test like the t test with a $p<0.05$.
Qualitative analysis (most of the animal procedures described in this appendix): the number of animals (group size) is based in literature and/or years of experience with similar type of experiments. Moreover, these types of experiments will be performed sequentially via which we ensure that we will use the minimum number of mice per group that will be informative.

**B. The animals**

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

Mus musculus; WT, genetically modified or injected with a virus or microRNA therapeutic
Age; adult
Origin; Hubrecht institute or external licensed breeders
Number of animals: 7680 male mice

All animal studies in this project will exclusively involve mice. Up to today, there are no alternative methods to fully understand how the heart functions as an organ and how it remodels during disease. For the majority of the proposed studies, the mouse is the most appropriate animal model because: (1) like humans, mice are mammals; (2) physiology is more extensively characterized; (3) mice are amenable to transgenic modification; (4) a large number of relevant transgenic and knock out lines are already available.

For the type of experiments described in this procedure, it is difficult to calculate the exact number of animals, since for various experimental setups require different numbers of mice, and the variation and outcome of the experiments are unknown. The number of mice required depends on the intervention (e.g no intervention, drug or microRNA therapeutic delivery or exercise), and the goal of the experiment. For these reasons we have provided a total number of mice based on experience over the past 5 years with these types of experiments in our group. Before we will start an experiment we will write an application to the IVD. In this application we will exactly describe which considerations, facts and results have led to the proposition of the number of animals needed for these experiments.

To study the effect of ischemic injury on the heart after increasing or deleting a gene or microRNA in the combination with no intervention, drug or microRNA therapeutic delivery or exercise on average we use 24 mice per genotype per study. Per line on average 2 different time points will be studied. These groups

of mice will either receive no intervention after surgery or will receive a therapeutic intervention by delivery of a compound or exercise.
We expect to use a maximum of 40 mouse lines for either MI or IR studies over the next 5 years for which we will collect materials at 2 different time points on average.

Since cardiac biology and pathology is really different in males and females because of estrogen signaling in females we only use males for our studies to exclude gender based variation.

24 mice x 2 genotypes x 2 studies x 2 interventions x 40 mouse lines

The **maximum** required number of animals = **7680 male mice**

**C. Re-use**

Will the animals be re-used?

☒ 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'?

☒ 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.

Before we consider the import or generation and subsequently analysis of GGM to study the function of a gene or microRNA in the heart, we first will analyze appropriate cell lines, existing patient material or materials available from previous animal studies, which extensively reduces the animal numbers. The use human material and in vitro cultures allows this project to comply with the 3Rs (replacement, reduction and refinement) by keeping the mice numbers to be used to a minimum. Also trying to use the newly developed CRISPR/Cas9 method to create new mouse lines will significantly reduce the number of animals needed to generate a new line.

However, animal studies are unavoidable if we seek for the fundamental insight into the molecular and cellular mechanisms in cardiac biology and disease. Whenever possible, we will perform pilot studies with the minimum number of animals possible. Experiments will be done sequential, where on basis of the results, decisions will be made on the next steps.

If homozygous mice do not show any discomfort we will keep the mice on a homozygous background for breeding purposes, thereby reducing the number of mice.

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

All possibilities to reduce pain, fear or suffering will be used. There are no negative environmental effects; all mice will be housed under strict D1 and/or D2 conditions.

## Repetition and duplication

**E. Repetition**

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

The experiments in this project are not carried out due to legal requirements.

## 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

☒ 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?

☒ 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.

☒ 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.

Adequate anaesthesia and analgesia will be used.

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

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

Some of the animal lines might experience unexpected discomfort after ischemic injury

Explain why these effects may emerge.

This may occur if the gene or microRNA that has been modulated in the mice has a crucial function during in heart regeneration that can now no longer be fulfilled.

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

Daily monitoring of the mice combined with an experimental design aiming at reducing the discomfort of the animals. If we unexpectedly observed weight loss (>20% in comparison with control or 15% weight loss in two days), changes in behavior or body posture, signs of general sickness and/or discomfort, the absence of grooming or symptoms of heart failure like dyspnea and edema, the animals will be taken out of experiment and sacrificed.

**J. Humane endpoints**

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

☐ No > Continue with question K.

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

Weight loss (>20% in comparison with control or 15% weight loss in two days), changes in behavior or body posture, signs of general sickness and/or discomfort, the absence of grooming or symptoms of heart failure like dyspnoea and edema.

Indicate the likely incidence.

<0.1%

**K. Classification of severity of procedures**

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

The cumulative discomfort in the genetic models will maximally consist of moderate discomfort due to:

- the genetic modification (< 5% of lines)
    - Mild discomfort: >95%
    - Moderate discomfort: <5%. Animals that do show a phenotype under basal conditions will not receive a cardiac stress.
- tissue sampling for genotyping
- maximally 2 routes of administration of transgene inducing or deleting agents
- administration of a labelling agent
- transplantation of cells under adequate anaesthesia and analgesia
- MI or IR surgery
    - Sham surgery: 100% mild discomfort,
    - MI surgery:
        - animals that die during surgery: 15% mild discomfort
        - animals that die during first few days after surgery: 10% mild discomfort
        - animals that survive till sacrifice: 75% moderate discomfort
    - IR surgery:
        - animals that die during surgery: 10% mild discomfort
        - animals that die during first few days after surgery: 10% mild discomfort
        - animals that survive till sacrifice: 80% moderate discomfort
- systemic or intracardiac delivery of a drug or exposure to exercise
- sedation to determine cardiac function by echo
- sacrifice

The cumulative discomfort in the models injected with a virus or microRNA therapeutic will maximally consist of moderate discomfort due to:

- inject a virus or microRNA therapeutic by maximally 2 routes of administration
- administration of a labelling agent
- transplantation of cells under adequate anaesthesia and analgesia
- MI or IR surgery
    - Sham surgery: 100% mild discomfort,
    - MI surgery:
        - animals that die during surgery: 15% mild discomfort
        - animals that die during first few days after surgery: 10% mild discomfort
        - animals that survive till sacrifice: 75% moderate discomfort
    - IR surgery:
        - animals that die during surgery: 10% mild discomfort
        - animals that die during first few days after surgery: 10% mild discomfort
        - animals that survive till sacrifice: 80% moderate discomfort
- Discomfort due to surgery
- systemic or intracardiac delivery of a drug or exposure to exercise
- sedation to determine cardiac function by echo
- sacrifice

**Cumulative level of discomfort:**
100% moderate. This is likely an overestimation of the discomfort, but the true discomfort will be assessed for each individual experiment.

## End of experiment

**L. Method of killing**

Will the animals be killed during or after the procedures?

☐ No

☒ Yes > Explain why it is necessary to kill the animals during or after the procedures.

All animals are killed to use their organs in ex vivo analyses.

Is the proposed method of killing listed in Annex IV of Directive 2010/63/EU?

☐ No > Describe the method of killing that will be used and provide justifications for this choice.

☒ Yes

# 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'. | 80102 |
| 1.2 | Provide the name of the licenced establishment. | Koninklijke Nederlandse Akademie van Wetenschappen (Hubrecht Institute) |

1.3 List the serial number and type of animal procedure.

*Use the serial numbers provided in Section 3.4.4 of the Project Proposal form.*

| Serial number | Type of animal procedure |
|---|---|
| 3.4.4.6 | Models for pathological remodeling |

## 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 general design of the experiments in this appendix is shown in the attached flowchart (**3.4.4.6**)

In case we want to study the function of a gene, microRNA or specific cell type in **pathological remodeling**, we can use mice in which a gene, microRNA or (stem) cell component has been modified after which we expose the animals to a stress that induces a pathological remodeling response. These mouse models can be used in studies without further intervention or we add an additional therapeutic intervention (drug or exercise) to determine the effect on heart function, behavior or molecular read outs.

The different components of the proposed experiments are (see also the flowchart in appendix 1):

**Mouse model (indicated on flowchart with A):**

To test a research hypothesis in vivo we will use an appropriate mouse model. This can be done in wild type mice, genetically modified mice, or mice in which gene or microRNA levels have been modulated in vivo by a virus (such as adeno associated virus or lentivirus). In some cases we might need to use mice that got transplanted with cells to study the in vivo effect of specific cells or cellular derivatives.

Considerations for choosing a mouse model:

- Wild type mouse

  Wild type mice will be used if we want to study the effect of a pathological stress in the absence or presence of a therapeutic intervention.

- GMM
  Genetically modified mice will be used to study the cardiac function of a specific gene, microRNA or type of (stem) cell. In case a mouse line with the required genetic modification is not available we will generate new mouse line via standard oocyte injection, blastocyst injection, or via the CRISPR/Cas9 system (outlined in Appendix 3.4.4.2)
- Mouse injected with virus to increase or inhibit a gene or microRNA
  In case genetic approaches fail to provide appropriate mouse lines, we will revert to viral vectors to increase or inhibit a gene or microRNA by systemic dosing. Viral vectors can also be used to manipulate a gene or microRNA more locally via intracardiac injection
- Wild type mice or GMM transplanted with cells or derivatives
  In case we want to study the cardiac effect of cell-based treatments, we will transplant (stem) cells or derivatives in either wild type or genetically modified mice.

**Cardiac stress (indicated on flowchart with B):**

- Mice will receive a pathological stress when we want to study the function of a gene, microRNA or (stem) cell in pathological remodeling. Pathological stress models to induce remodeling:
  - TAB
  - high-salt or high-cholesterol diet
  - remodeling agent
  - exercise to trigger disease

The choice of stress depends on the specific research questions and the proposed function of the gene or microRNA that is being studied.

**Intervention (indicated on flowchart with C):**

- No intervention
  Mouse lines will receive no intervention in case we want to study the cardiac function of a gene, microRNA or (stem) cell under basal conditions.
- Deliver a drug compound
- Exercise

**Readouts / endpoint (indicated on flowchart with D)**

- Cardiac function
- Behavior
- Ex vivo analysis of cells and tissues

In all experiments, we will determine cardiac function and determine the general behavior of the animals. All mice will be sacrificed for detailed analysis of the heart and other tissues involved in aspects of heart disease (lungs, kidneys liver). Analysis will include histology and molecular analysis. Also, cells from organs might be isolated by FACS for subsequent analysis.

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

Animal model (indicated on flowchart with A):

- Mice are ordered from a commercial, NVWA licensed breeder or from a registered commercial company, or are generated by ourselves under protocol 3.4.4.2.
- Tissue sampling for genotyping and identification will be done via ear, toe and/or tail biopsy, under anaesthesia (4% (isoflurane/oxygen).
- In some cases we will administer transgene inducing or deleting agents alone or in combination, continuously or intermittently by one or maximally 2 of the following routes:
  - in diet or drinking water (max. 1 time, < 2 wks)
  - subcutaneous injection (max. 3 times)
  - intraperitoneal injection (max. 7 times)
  - implantation of a slow release pellet subcutaneously under adequate anesthesia and analgesia
  - implantation of a slow release pump subcutaneously under adequate anesthesia and

analgesia
    - oral (max. 10 times)
- In some cases we will inject a virus or microRNA therapeutic by one or maximally 2 of the following routes:
    - Intravenous injection (max. 10 times)
    - Subcutaneous injection (max. 10 times)
    - Intraperitoneal injection (max. 10 times)
- In some cases WT or GM mice will be transplanted with cells under adequate anaesthesia and analgesia
- In some cases we will administer a labelling agent (e.g. BrdU) via one of the following routes:
    - intraperitoneal injection (1 time)
    - implantation of a slow release pump subcutaneously under adequate anaesthesia and analgesia (1 time)
    - intravenous injection (1 time)

<u>Cardiac stress (indicated on flowchart with B):</u>

- TAB
    - Hair over the site of incision will be removed with clippers and nair
    - Animal will be sedated using ketamine xylazine mix
    - Ketamine will be supplemented with isoflurane (nosecone) using 100% oxygen as a carrier gas
    - Skin will be disinfected with surgical iodine using standard surgical sterile preparative techniques
    - The appropriate plane of anesthesia will be determined based on observations of spontaneous respiration and pain reflexes (toe pinch). Level of anesthesia will be continually monitored throughout the surgery
    - Sterile instruments, supplies and suture material will be used. Instruments used on the surface of the skin will be segregated from those used later in the procedure
    - The transverse thoracic aorta will be accessed via a left lateral thoracotomy. Suture material will be used to ligate the transverse aorta and an overlying needle. After ligation, the needle will be removed immediately leaving a discrete region of stenosis in the aorta. In published studies, this degree of stenosis is associated with a 40•0 mm Hg gradient.
    - The chest will be closed and the animals will be observed during recovery from anesthesia. The surgical site will be monitored daily for signs of infection or wound dehiscence. Appropriate analgesia will be administered postoperatively when needed.

- High-salt or high-cholesterol diet
  Animals will be put on a high-salt or high-fat content diet for the duration of 2-6 months to establish a pathological effect on the heart as determined by echocardiography.

- Remodeling agent
    - Systemic delivery of remodeling agent by
        - Subcutaneous injection
        - intraperitoneal injection once weekly or every two weeks
        - implantation of a slow release pump subcutaneously under adequate anaesthesia and analgesia (1 time)

- Exercise to trigger disease
  Swimming exercise for 2 x 90 minutes a day will continue for 2 to max 10 weeks (depending on how long it takes to observe cardiac remodeling by echo) in mouse line that is genetically predisposed to develop cardiac disease. The mice will be observed at all times to avoid mice submerging under the water surface.

Intervention (indicated on flowchart with C):

- No intervention
- Deliver a drug compound
    - Systemic delivery of compound or microRNA therapeutic by
        - Subcutaneous injection once weekly or every two weeks for maximally 12 weeks
        - intraperitoneal injection once weekly or every two weeks for maximally 12 weeks
        - implantation of a slow release pump subcutaneously under adequate anaesthesia and analgesia (1 time)
    - Intracardiac delivery of compound or microRNA therapeutic
        - Mice will be anesthetized with a mixture of ketamine and sodium pentobarbital by ip injection. Hair will be removed from the ventral surface of the neck and thorax.
        - A tracheal tube will be placed and the mouse connected to a ventilator
        - A surgical plane of anesthesia as judged by lack of pain reflex (toe pinch)
        - The surgical site will be cleaned with iodine and 70% ethanol.
        - Using aseptic technique with sterile instruments, the skin will be incised left of midline to allow access to the third intercostal space. Pectoral muscles will be retracted and the intercostal muscles cut caudal to the third rib. A rib spreader will be placed to allow access to the heart.
        - Drug or microRNA therapeutic compound will be delivered via intracardiac injection.
        - The rib cage will be closed with 5-0 silk suture and the skin closed with tissue adhesive.
        - The animal will be disconnected from the ventilator the tracheal tube removed and the animal placed unrestrained on a nose cone with 100% oxygen in a warm recovery cage until fully ambulatory, at which point the oxygen will be turned off
- Exercise to induce cardioprotection
  Swimming exercise starting with 10 minutes 2 times daily with 10 minutes increase each day until 90 minutes, 2 times per day is reached. The 2 x 90 minutes a day will continue for 2 to max 10 weeks (depending on how long it takes to observe cardiac remodeling by echo). The mice will be observed at all times to avoid mice submerging under the water surface.
  This intervention will not be used in the mouse lines where we use swimming exercise to trigger disease.

Readouts /endpoint (indicated on flowchart with D)

- Cardiac function
  Cardiac function will be evaluated by echocardiography, ECG or pressure catheters on sedated, adult mice
- Behavior
  Animals will be monitored for general signs of sickness and/or discomfort by looking for immobility, significant weight loss or lack of grooming and/or symptoms of heart failure (like dyspnea and edema). If these signs are unexpectedly observed the animals will be euthanized prematurely
- Ex vivo analysis of cells and tissues

The choice of stress depends on the specific research questions and the proposed function of the gene or microRNA that is being studied.

The cumulative discomfort in the genetic models will maximally consist of discomfort due to:

- the genetic modification (< 5% of lines)
- tissue sampling for genotyping
- maximally 2 routes of administration of transgene inducing or deleting agents
- administration of a labelling agent
- transplantation of cells under adequate anaesthesia and analgesia
- pathological stress
- discomfort due to the development of heart disease

- systemic or intracardiac delivery of a drug or exposure to exercise
- sedation to determine cardiac function by echo
- sacrifice

The cumulative discomfort in the models injected with a virus or microRNA therapeutic will maximally consist of discomfort due to:

- inject a virus or microRNA therapeutic by maximally 2 routes of administration
- administration of a labelling agent
- transplantation of cells under adequate anaesthesia and analgesia
- pathological stress
- discomfort due to the development of heart disease
- systemic or intracardiac delivery of a drug or exposure to exercise
- sedation to determine cardiac function by echo
- sacrifice

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

Whenever possible we strive for a situation to express the outcome of our experiments in quantitative terms. To estimate the number of animals to be used in an experiment, we use the effect size (if known, e.g., from data in the literature, from our own historical data or if unknown from small scale pilots) to estimate the sample size needed to achieve a certain power (usually around 0.8) with appropriate statistical test like the t test with a $p<0.05$.
Qualitative analysis (most of the animal procedures described in this appendix): the number of animals (group size) is based in literature and/or years of experience with similar type of experiments. Moreover, these types of experiments will be performed sequentially via which we ensure that we will use the minimum number of mice per group that will be informative.

**B. The animals**

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

Mus musculus; WT, genetically modified or injected with a virus or microRNA therapeutic
Age; adult
Origin; Hubrecht institute or external licensed breeders
Number of animals: 4800 male mice

All animal studies in this project will exclusively involve mice. Up to today, there are no alternative methods to fully understand how the heart functions as an organ and how it remodels during disease. For the majority of the proposed studies, the mouse is the most appropriate animal model because: (1) like humans, mice are mammals; (2) physiology is more extensively characterized; (3) mice are amenable to transgenic modification; (4) a large number of relevant transgenic and knock out lines are already available.

For the type of experiments described in this procedure, it is difficult to calculate the exact number of animals, since for various experimental setups require different numbers of mice, and the variation and outcome of the experiments are unknown. The number of mice required depends on the intervention (e.g no intervention, drug or microRNA therapeutic delivery or exercise), and the goal of the experiment. For these reasons we have provided a total number of mice based on experience over the past 5 years with these types of experiments in our group. Before we will start an experiment we will write an application to the IVD. In this application we will exactly describe which considerations, facts and results have led to the proposition of the number of animals needed for these experiments.

To study the effect of a pathological stress on the heart after increasing or deleting a gene or microRNA in the combination with no intervention, drug or microRNA therapeutic delivery or exercise we use on average 20 WT and GM mice. Our extensive experience has thought us that this number is usually sufficient to detect a significant effect. In most lines 2 different time points will be studied. These groups of mice will either receive no intervention after surgery or will receive a therapeutic intervention by delivery of a compound or exercise.

We expect to use a maximum of 30 different mouse lines over the next 5 years for pathological stress studies.

Since cardiac biology and pathology is really different in males and females because of estrogen signaling in females we only use males in our in vivo studies to exclude gender based variation.

20 mice x 2 genotypes x 2 time points x 2 interventions x 30 mouse lines

The **maximum** required number of animals = **4800 male mice**

**C. Re-use**

Will the animals be re-used?

☒ 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'?

☒ 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.

Before we consider the import or generation and subsequently analysis of GGM to study the function of a gene or microRNA in the heart, we first will analyze appropriate cell lines, existing patient material or materials available from previous animal studies, which extensively reduces the animal numbers. The use human material and in vitro cultures allows this project to comply with the 3Rs (replacement, reduction and refinement) by keeping the mice numbers to be used to a minimum. Also trying to use the newly developed CRISPR/Cas9 method to create new mouse lines will significantly reduce the number of animals needed to generate a new line.

However, animal studies are unavoidable if we seek for the fundamental insight into the molecular and cellular mechanisms in cardiac biology and disease. Whenever possible, we will perform pilot studies with the minimum number of animals possible. Experiments will be done sequential, where on basis of the results, decisions will be made on the next steps.

If homozygous mice do not show any discomfort we will keep the mice on a homozygous background for breeding purposes, thereby reducing the number of mice.

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

All possibilities to reduce pain, fear or suffering will be used. There are no negative environmental effects; all mice will be housed under strict D1 and/or D2 conditions.

## Repetition and duplication

**E. Repetition**

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

The experiments in this project are not carried out due to legal requirements.

## 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

☒ 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?

☒ 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.

☒ 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.

Adequate anaesthesia and analgesia will be used.

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

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

Some of the animal lines might experience unexpected discomfort after a pathological stress

Explain why these effects may emerge.

This may occur if the gene or microRNA that has been modulated in the mice has a crucial function during in pathological remodelling that can now no longer be fulfilled.

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

Daily monitoring of the mice combined with an experimental design aiming at reducing the discomfort of the animals. If we unexpectedly observed weight loss (>20% in comparison with control or 15% weight loss in two days), changes in behavior or body posture, signs of general sickness and/or discomfort, the absence of grooming or symptoms of heart failure like dyspnea and edema, the animals will be taken out of experiment and sacrificed.

**J. Humane endpoints**

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

☐ No > Continue with question K.

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

Weight loss (>20% in comparison with control or 15% weight loss in two days), changes in behavior or body posture, signs of general sickness and/or discomfort, the absence of grooming or symptoms of heart failure like dyspnea and edema.

Indicate the likely incidence.

Although some of our new mouse lines are intended to cause a cardiac disease phenotype it is unlikely that the severity is such that it would introduce humane endpoints.

**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 cumulative discomfort in the genetic models will maximally consist of moderate discomfort due to:

- the genetic modification (< 5% of lines)
  - Mild discomfort: >95%
  - Moderate discomfort: <5%. Animals that do show a phenotype under basal conditions will not receive a cardiac stress.
- tissue sampling for genotyping
- maximally 2 routes of administration of transgene inducing or deleting agents
- administration of a labelling agent
- transplantation of cells under adequate anaesthesia and analgesia
- pathological stress
  - Control animals: 100% mild discomfort due to procedure
  - Mice exposed to cardiac stress: 100% moderate discomfort due to the development of cardiac disease
- systemic or intracardiac delivery of a drug or exposure to exercise
- sedation to determine cardiac function by echo
- sacrifice

The cumulative discomfort in the models injected with a virus or microRNA therapeutic will maximally consist of moderate discomfort due to:

- inject a virus or microRNA therapeutic by maximally 2 routes of administration
- administration of a labelling agent
- transplantation of cells under adequate anaesthesia and analgesia
- pathological stress
  - Control animals: 100% mild discomfort due to procedure
  - Mice exposed to cardiac stress: 100% moderate discomfort due to the development of cardiac disease
- systemic or intracardiac delivery of a drug or exposure to exercise
- sedation to determine cardiac function by echo
- sacrifice

**Cumulative level of discomfort:**
100% moderate.

## End of experiment

**L. Method of killing**

Will the animals be killed during or after the procedures?

☐ No

☒ Yes > Explain why it is necessary to kill the animals during or after the procedures.

All animals are killed to use their organs for ex vivo analysis

Is the proposed method of killing listed in Annex IV of Directive 2010/63/EU?

☐ No > Describe the method of killing that will be used and provide justifications for this choice.

☒ Yes

# Format DEC-advies

*Maak bij de toepassing van dit format gebruik van de bijbehorende toelichting, waarin elke stap in het beoordelingsproces wordt toegelicht*

## A. Algemene gegevens over de procedure

1. Aanvraagnummer: AVD/801002015250
2. Titel van het project: The molecular basis of cardiac diseases and recovery.
3. Titel van de NTS: De moleculaire basis van hartziekten.
4. Type aanvraag:
   - ✓ nieuwe aanvraag projectvergunning
   - ☐ wijziging van vergunning met nummer
5. Contactgegevens DEC:
   - naam DEC: KNAW
   - telefoonnummer contactpersoon: [redacted]
   - mailadres contactpersoon: [redacted]
6. Adviestraject (data dd-mm-jjjj):
   - ✓ ontvangen door DEC: 22-09-2015
   - ✓ aanvraag compleet: 09-10-2015
   - ✓ in vergadering besproken: 01-10-2015
   - ✓ anderszins behandeld: n.v.t.
   - ✓ termijnonderbreking(en): n.v.t.
   - ☐ besluit van CCD tot verlenging van de totale adviestermijn met maximaal 15 werkdagen:
   - ☐ aanpassing aanvraag:
   - ✓ advies aan CCD: 13-10-2015
7. Eventueel horen van aanvrager
   - Datum: n.v.t.
   - Plaats:
   - Aantal aanwezige DEC-leden:
   - Aanwezige (namens) aanvrager:
8. Correspondentie met de aanvrager:
   - Datum 02-10-2015
   - Strekking: completering van de aanvraag
   - Datum antwoord: 09-10-2015
   - Strekking van de antwoorden: de aanvraag is gecompleteerd
9. Eventuele adviezen door experts (niet lid van de DEC): geen

## B. Beoordeling (adviesvraag en behandeling)

**1.** Het project is vergunningplichtig. Het omvat dierproeven in de zin der wet.

**2.** De aanvraag betreft een nieuwe aanvraag. Er is overlap met een aantal al van een positief advies voorziene DEC-protocollen. De experimentele groepen van

deze DEC protocollen zullen worden geclassificeerd als Type Dierproeven zoals beschreven in de bijlagen van de aanvraag en zullen bij verlening van de vergunning onderdeel uitmaken van de vergunning (Zie bijlage II van de aanvraag).

3. De DEC is competent om over deze projectvergunningsaanvraag te adviseren. De benodigde expertise op dit wetenschappelijk terrein is aanwezig binnen de DEC. Geen van de DEC-leden is betrokken bij het betreffende project.
4. Vanwege betrokkenheid bij het betreffende project is een aantal DEC-leden, met het oog op onafhankelijkheid en onpartijdigheid, niet betrokken bij de advisering: n.v.t.

## C. Beoordeling (inhoud):

1. Het project is wetenschappelijk verantwoord. De indiener heeft een duidelijk overzicht gegeven van de reeds behaalde resultaten (Bijlage I), van het nu lopende onderzoek en welke specifieke factoren en cellulaire processen onderwerp van de huidige studies zijn (Bijlage II). Het lopende onderzoek maakt reeds gebruik van de reeks samenhangende dierproeven zoals die beschreven zijn in de verschillende bijlagen “Type Dierproeven” van de aanvraag. De DEC beschouwt de beschreven werkwijze, inclusief de dierproeven, als een coherent geheel en het geeft een goed beeld van de wetenschapsstrategie van de onderzoeksgroep: van hypothese tot resultaat (bijlage “Flow chart”). De groep werkt nu, met succes, volgens de beschreven strategie en wil die de komende 5 jaar op vergelijkbare manier voortzetten. Het overkoepelend doel is het verkrijgen van wetenschappelijke kennis over de biologische mechanismen die betrokken zijn bij het ontstaan en het herstel van hartziekten. *Op basis hiervan acht de DEC het project een toetsbare eenheid.* De toekomstige experimenten op basis van *nieuwe specifieke hypotheses* over de betrokkenheid van een specifiek moleculair-biologisch of cellulair mechanisme, zullen aan de IvD-Hubrecht Instituut worden voorgelegd.

2. De in de aanvraag aangekruiste doelcategorie fundamenteel onderzoek is in overeenstemming met de hoofddoelstelling.

3. De doelstelling, in relatie tot de uitvoering, is helder omschreven; te weten het verkrijgen van fundamenteel wetenschappelijke inzichten in het identificeren en bestuderen van de moleculair-biologische en cellulaire mechanismen (genen, microRNAs, factoren, (stam)cellen, fysieke inspanning) die belangrijk zijn voor het ontstaan en het herstel van hartziekten. Als onderdeel van de betrokkenheid van een moleculair-biologisch of cellulaire mechanisme wordt ook onderzocht of het mechanisme een mogelijke therapeutische waarde heeft. Op termijn kunnen de resultaten leiden tot nieuwe behandelingsmethoden voor patiënten met hartziekten. De projectleider heeft banden met een bedrijf dat zich richt op het ontwikkelen van op microRNA-gebaseerde therapieën voor hartziekten in patiënten; een concreet voorbeeld van hoe de resultaten kunnen dienen als

uitgangspunt voor het opzetten van nieuwe behandelingsmethoden voor patiënten met hartziekten.

Het project richt zich specifiek op de moleculair-biologische en cellulaire mechanismen die betrokken zijn bij de regeneratie en herstel na permanente of langdurige afsluiting van de kransslagaders en de mechanismen die leiden tot pathologische veranderingen van het hartweefsel gekenmerkt door een hypertrofie van de hartspiercellen en fibrose van het hartspierweefsel (remodellering). In beide gevallen is het resultaat een verlies van de pompfunctie van het hart. Voor het onderzoek naar de mechanismen die een rol spelen bij het ontstaan en herstel van hartziekten worden verschillende experimentele modellen in de muis gebruikt. Uitgangspunt is telkens de hypothese dat een specifiek gen, microRNA, of (stam)celtype, zoals geïdentificeerd in een aantal screeningsexperimenten (reeds uitgevoerd of als onderdeel van Type Dierproef 3.4.4.5 en 3.4.4.6) of op basis van literatuurgegevens, betrokken is bij bovengenoemde hartziekten en dat door modulatie van de functie van het specifiek gen, microRNA, (stam)celtype of fysieke inspanning het verloop van de hartziekte kan worden beïnvloed in positieve of negatieve zin. De onderzoekers laten zien dat veelal dezelfde mechanismen betrokken zijn in beide vormen van hartziekte en volgen daarom de strategie om diermodellen voor zowel regeneratie en herstel en voor remodellering van hartweefsel te bestuderen. De DEC ziet deze integrale benadering als een belangrijk kenmerk van het project.

Het fundamenteel wetenschappelijke belang acht de DEC substantieel.

Het verkrijgen van deze fundamenteel wetenschappelijke kennis is essentieel voor het ontwikkelen van nieuwe en/of verbeterde therapeutische strategieën voor de behandeling van hartziekten en dit is naar de mening van de DEC een substantieel belang. Het project dient hiermee een belangrijk maatschappelijk belang, mede gezien de grote groep patiënten met hartziekten waarvoor een effectieve behandeling op dit moment niet beschikbaar is.

**4.** De gekozen strategie en experimentele aanpak in combinatie met de infrastructuur op het Hubrecht Instituut en de expertise van de betrokken onderzoeksgroep bieden een realistisch uitzicht op het behalen van de beoogde doelstellingen binnen gevraagde looptijd van het project. Het project bouwt voort op een langlopende lijn van onderzoek van een grote groep onderzoekers. Over de afgelopen jaren zijn met een vergelijkbare strategie en integrale aanpak belangrijke wetenschappelijk resultaten behaald, resulterend in vele publicaties in vooraanstaande tijdschriften. Het onderzoek in dit zeer competitieve veld wordt financieel gesteund door verschillende onafhankelijke subsidiegevers. Er zijn internationale samenwerkingsverbanden met andere laboratoria actief in dit onderzoeksveld.
Naar de mening van de DEC is een projectduur van 5 jaar reëel overwegende de strategie waarbij een telkens nieuwe mechanismen worden onderzocht op een

manier vergelijkbaar met het onderzoek van deze groep zoals dat over de afgelopen jaren is uitgevoerd. De publicatielijst en de huidige plannen (Bijlage I en II) ondersteunen dit.

**5.** Alle dieren worden gefokt voor het gebruik in dierproeven, er is geen sprake van afwijkende huisvesting en/of hergebruik. Er is geen sprake van bedreigde diersoorten, niet-menselijke primaten, zwerfdieren en/of dieren in/uit het wild. De toegepaste methoden voor anesthesie/euthanasie zijn conform de Richtlijn.

**6.** Het cumulatieve ongerief gepaard gaand met de dierproeven, zoals beschreven in de verschillende type dierproeven, is naar inschatting van de DEC licht (Type dierproef 1) of matig (Type dierproef 2-6). Er een beperkt risico op onbedoelde bijwerkingen. Deze inschatting van de DEC is in overeenstemming met het niveau van cumulatief ongerief zoals dat is geclassificeerd door de onderzoekers. Dit is gebaseerd op hun ruime ervaring met de gebruikte modellen in vergelijkbare dierproeven.

**7.** Binnen het project wordt maximaal gebruik gemaakt van methoden die de voorgestelde dierproeven geheel of gedeeltelijk **vervangen.** Een belangrijk onderdeel van de experimentele strategie is de gefaseerde opzet. In de eerste fase vindt een uitgebreid literatuuronderzoek plaats en onderzoek op materiaal verzameld bij eerder uitgevoerde proeven, op patiëntmateriaal en op cellijnen. Na deze fase is er go/no-go-beslissingsmoment voor het onderzoek met neonatale cardiomyocyten (type Dierproef 3.4.4.1 – licht ongerief). Vervolgens is er een beslismoment voorafgaand tot de uitvoering van dierproeven met meer ongerief. Nieuwe inzichten in de moleculair-biologische en cellulaire processen betrokken bij hartziekten kunnen op dit moment alleen maar verkregen worden in een intact levend organisme. Deze processen, waarbij verschillende typen cellen betrokken zijn binnen een gecompliceerde anatomische context, zijn zeer complex en kunnen niet met cellijnen worden bestudeerd. Naar het oordeel van de DEC zijn er geen alternatieven beschikbaar voor het voorgestelde gebruik van intacte levende dieren om te doelstelling van dit project te realiseren.

**8.** In het project wordt optimaal tegemoet gekomen aan de vereisten van **vermindering** van dierproeven. De onderzoeksgroep heeft een jarenlange ervaring opgebouwd met dit soort experimenten en door een veelal gefaseerde opzet wordt per experiment niet meer dan het minimaal benodigde aantal dieren ingezet. Voorafgaand aan de kwantitatieve experimenten wordt op basis van literatuurgegevens, eigen historische data of een specifiek hiertoe uitgevoerd pilot experiment de groepsgrootte bepaald. Technieken en procedures worden zorgvuldig toegepast. Het aantal te gebruiken dieren is realistisch geschat.

**9.** De uitvoering van het project is in overeenstemming met de vereisten van **verfijning** van dierproeven en is zo opgezet dat de dierproeven met zo min mogelijk ongerief worden uitgevoerd.
Bij de opzet wordt rekening gehouden met dierenwelzijn en wel op de volgende manieren: 1) het gebruik van adequate anesthesie en analgesie waar nodig, 2) een intensieve monitoring van de proefdieren na de inductie van hartfalen, 3) het gebruik van weefselspecifiek genetisch-gemodificeerde muizen, 4) een monitoring op het optreden van onverwacht constitutioneel ongerief van nieuwe gecreëerde genotypes.
Er is geen sprake van belangwekkende milieueffecten.

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

# D. Ethische afweging

De centrale vraag voor de ethische afweging is of het belang van het doel van dit project opweegt tegen het ongerief dat de dieren ondergaan (geclassificeerd voor het merendeel van de dieren als matig). Het doel van het project is het vergroten van de wetenschappelijke kennis. Centraal staat het identificeren en bestuderen van de moleculair-biologische en cellulaire mechanismen (genen, microRNAs, factoren, (stam)cellen, fysieke inspanning) die belangrijk zijn voor het ontstaan en het herstel van hartziekten.
Het onderzoek is primair fundamenteel wetenschappelijk van karakter. De verwachting is dat de resultaten op den duur kunnen bijdragen aan nieuwe of verbeterde therapieën voor hartpatiënten.

Het fundamenteel wetenschappelijke onderzoek in dit project is van aangetoonde en excellente kwaliteit. De onderzoeksgroep beschikt over ruime ervaring met de gekozen onderzoeksstrategie en met de beschreven type dierproeven.

De classificatie van het ongerief van de dieren in de verschillende typen dierproeven is merendeel matig. Bij de 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. De DEC onderschrijft dat de doelstellingen niet zonder het gebruik van proefdieren kunnen worden behaald.

De DEC is van mening dat de resultaten van dierproeven zullen bijdragen aan het behalen van het geformuleerde doel en schat de kans op het realiseren van de fundamenteel wetenschappelijke doelstellingen in als hoog. Het project is uit wetenschappelijk oogpunt verantwoord. De verkregen fundamenteel wetenschappelijke kennis is essentieel om te kunnen komen tot nieuwe therapeutische benaderingen of van een verbetering van bestaande therapieën in patiënten met hartziekten. De experimenten geven hiertoe een eerste aanzet. Het gaat om een grote groep patiënten met in veel gevallen een slechte prognose. Het

maatschappelijk belang is daarom groot.

De DEC komt tot de conclusie dat de doeleinden van het project het voorgestelde gebruik van de proefdieren en het daarmee samenhangende ongerief van de proefdieren rechtvaardigt.

# E. Advies

1. Advies aan de CCD
   - ✓ **De DEC adviseert de vergunning te verlenen**
2. Het uitgebrachte advies is gebaseerd op consensus.
3. Er zijn geen knelpunten of dilemma's gesignaleerd tijdens het beoordelen van de aanvraag of het formuleren van het advies.

**Appendix I: Results obtained so far with studies related to heart regeneration and pathological remodeling (publication list).**

**Heart regeneration and repair**

*These following papers describe mouse studies in which we either induce myocardial infarction or ischemia reperfusion to study aspects of heart regeneration and pathological remodelling. The factors or cellular mechanisms are **indicated**.*

1. [redacted] Lillian B. Sutherland, Jeffrey E. Thatcher,J. Micheal DiMaio, Rao H. Naseem, William S. Marshall, Joseph Hill and Eric N. Olson. (2008) Dysregulation of microRNAs after myocardial infarction reveals a role of **miR-29** in cardiac fibrosis. Proc Natl Acad Sci U S A. 105(35):13027-32. Epub 2008 Aug 22.

2. [redacted] Jens Fielitz, Lillian B. Sutherland, Victor L. Thijssen, Harry J. Crijns, Michael J. Dimaio, John Shelton, Leon J. De Windt, Joseph A. Hill, Eric N. Olson. **Myocyte Enhancer Factor 2 and Class II Histone Deacetylases** Control a Gender-Specific Pathway of Cardioprotection Mediated by the Estrogen Receptor. (2010) Circ Res. 106(1):155-65. Epub 2009 Nov 5.

3. Thomas G. Hullinger, Rusty L. Montgomery, Anita G. Seto, Brent A. Dickinson, Joshua M. Lynch, Christianna Stack, Paul Latimer, Christina M. Dalby, Katie H. Robinson, Joshua Hare, Eric N. Olson and [redacted]**.** (2012) LNA-mediated inhibition of **miR-15** protects against cardiac ischemic injury. Circ Res. 110(1):71-81. Epub 2011 Nov 3.

4. Porrello ER, Johnson BA, Aurora AB, Simpson E, Nam YJ, Matkovich SJ, Dorn GW 2nd, [redacted], Olson EN. (2011) The **miR-15 Family** Regulates Post-natal Mitotic Arrest of Cardiomyocytes Circ Res. 109(6):670-9.

5. Aurora AB, Mahmoud AI, Luo X, Johnson BA, [redacted] Matsuzaki S, Humphries KM, Hill JA, Bassel-Duby R, Sadek HA, Olson EN. (2012) **MicroRNA-214** protects the mouse heart from ischemic injury by controlling Ca2+ overload and cell death. J Clin Invest. 122(4):1222-32. doi: 10.1172/JCI59327. Epub 2012 Mar 19.

**Pathological remodelling**

*These following papers describe mouse studies in which we induce stress on the heart to study aspects of pathological remodelling.*

1. [redacted] Sutherland LB, Liu N, Williams AH, McAnally J, Gerard RD, Richardson JA, Olson EN. (2006) A signature pattern of **stress-responsive microRNAs** that can evoke cardiac hypertrophy and heart failure. Proc Natl Acad Sci U S A. 103(48):18255-60. Epub 2006 Nov 15

2. [redacted] Lillian B. Sutherland, Xiaoxia Qi, James A. Richardson, Joseph Hill and Eric N. Olson. (2007) Control of stress-dependent cardiac growth and gene expression by **a microRNA**. Science. 316(5824):575-9. Epub 2007 Mar 22.

3. David Patrick, Rusty L. Montgomery, Xiaoxia Qi, Sakari Kauppinen, Joseph Hill, [redacted] and Eric N. Olson. (2010) Stress-dependent cardiac remodeling in the absence of **microRNA-21**. J Clin Invest. 120(11):3912-6. *(Shared last author)*

4. Montgomery RL, Hullinger TG, Semus HM, Dickinson BA, Seto AG, Lynch JM, Stack C, Latimer PA, Olson EN, [redacted] (2011) Therapeutic inhibition of **miR-208** improves cardiac function and remodeling during heart failure. Circulation.124(14):1537-47. Epub 2011 Sep 6.

5. Grueter CE, [redacted] Johnson BA, Deleon SM, Sutherland LB, Qi X, Gautron L, Elmquist JK, Bassel-Duby R, Olson EN. (2012) A Cardiac **MicroRNA** Governs Systemic Energy Homeostasis by Regulation of MED13. Cell. 149(3):671-83.

6. Brent A. Dickinson, Hillary M. Semus, Rusty L. Montgomery, Christianna Stack, Paul A. Latimer, Steven M. Lewton, Joshua M. Lynch, Thomas G. Hullinger, Anita G. Seto, and [redacted] (2013) Plasma **miRNAs** serve as biomarkers of therapeutic efficacy and disease progression in hypertension-induced heart failure. Eur J Heart Fail. 15(6):650-9

**Societal relevance and societal impact**

Heart disease is a modern world epidemic for which there currently are no effective therapies that could stop -or even reverse- disease progression with the exception of heart transplantation or assist devices. Unfortunately, these treatment options are only available to a minute fraction of the population in need of treatment due to donor scarcity, and are accompanied by incredibly high costs. Given the global burden of heart disease and its increasing prevalence, the development of novel therapies is of the utmost importance.

Our work triggered the foundation of miRagen Therapeutics, a biotechnology company focused on the development of microRNA based therapies, of which I am a founder and special advisor. The enthusiasm for developing new therapeutics is further underscored by the $352M partnership between miRagen and Servier for Research development and commercialization of miRNA-targeting drugs for cardiovascular disease (http://www.miragentherapeutics.com/4/News/).

This underscores the importance and relevance of our fundamental studies and how our findings can lead to the development of new therapies for heart disease.

**Appendix II: Approved DEC protocols related to cardiac regeneration and pathological remodelling which contain the procedures described in the project proposal.**

**HI1323.01 The function of miR-208 during cardiac remodelling**
Procedures involved:
- antimiR injections
- angiotensin II supplementation
- High salt diet
- Echo and pressure measurements to determine cardiac function

**HI1323.05 Cardiac function of miR-208 target genes**
Procedures involved:
- Generation transgenic lines
- Angiotensin II supplementation
- High salt diet
- Echo measurements to determine cardiac function

**HI1323.07 The role of ZEB2 in cardiac disease**
Procedures involved:
- Neonatal rat cardiomyocyte isolation

**HI1423.01 The cardiac function of miR-19b in cardiomyocyte survival**
Procedures involved:
- Neonatal rat cardiomyocyte isolation

**HI1423.02 The cardiac function of miR-499 during physiological hypertrophy**
Procedures involved:
- Swimming induced exercise
- Echo measurements to determine cardiac function

**HI1423.03 The cardiac function of miR-19b in cardiomyocyte proliferation**
Procedures involved:
- Generation transgenic lines
- Myocardial infarction
- Ischemia reperfusion
- Echo measurements to determine cardiac function

**HI1423.05 Identification of stem cells in heart regeneration**
Procedures involved:
- Generation transgenic lines
- Tamoxifen injections
- Myocardial infarction
- Echo measurements to determine cardiac function
- Isolation cardiac stem cells

**HI1423.06 Wnt signalling during heart regeneration.**
Procedures involved:

- Generation transgenic lines
- Myocardial infarction
- Echo measurements to determine cardiac function

**HI1423.07 MicroRNAs that regulate cardiac Adenylyl Cyclase 6**
Procedures involved:

- Neonatal rat cardiomyocyte isolation

**HI1423.08 SDF1 in cardiac regeneration**
Procedures involved:

- intracardiac injection of drugs
- ischemia reperfusion
- Echo measurements to determine cardiac function

**HI1423.09 Cardiac specific delivery of microRNA therapeutics by UPy-hydrogel-based intramyocardial delivery.**
Procedures involved:

- intracardiac injection of microRNA drugs
- ischemia reperfusion
- Echo measurements to determine cardiac function

**HI1423.11 The cardiac function of Zeb2**
Procedures involved:

- Myocardial infarction (in transgenic line)
- Echo measurements to determine cardiac function

**HI1423.12 The cardiac function of Zeb2**
Procedures involved:

- Myocardial infarction (in cardiac knockout line)
- Echo measurements to determine cardiac function

**HI1423.13 Using CRISPR/Cas to mutate DSP to create a mouse model for Arrhythmic Right Ventricular Dysplasia (ARVD)**
Procedures involved:

- Generation transgenic lines
- Exercise to trigger disease
- ECG and Echo measurements to determine cardiac function

**HI1423.14 The uptake and intracellular distribution of antimiRs during cardiac disease.**
Procedures involved:

- inject mice with labelled rugs
- ischemia reperfusion

**HI1423.15 Identification of stem cells in heart regeneration.**

Procedures involved:

- Generation transgenic lines
- Tamoxifen injections
- Myocardial infarction
- Isolation cardiac cells

**HI1423.16 Using CRISPR/Cas to mutate PKP2 to create a mouse model for Arrhythmic Right Ventricular Dysplasia (ARVD)**

Procedures involved:

- Generation transgenic lines
- Exercise to trigger disease
- ECG and Echo measurements to determine cardiac function

**HI1423.17 Determine clonality of stem cell progeny during heart regeneration**.

Procedures involved:

- tamoxifen injections

**HI1423.18 Gene expression in response to ischemia reperfusion**

Procedures involved:

- Ischemia reperfusion
- Echo measurements to determine cardiac function

**HI1423.19 Regenerative capacities of cardiomyocytes after ischemic damage.**

Procedures involved:

- Ischemia reperfusion
- Isolation cardiac cells

**HI1423.20 Identification of cKit positive progenitor cells in heart regeneration.**

Procedures involved:

- Generation transgenic lines
- Tamoxifen injections
- Myocardial infarction
- Isolation cardiac cells

**HI1423.22 MicroRNAs in cardiac regeneration**

Procedures involved:

- inject mice with antimiR
- ischemia reperfusion
- Echo measurements to determine cardiac function

**HI1423.26 Cell types in the heart that are relevant for repair.**

Procedures involved:

- ischemia reperfusion
- collect cardiac cells

With the exception of HI1324.08 all studies are currently still ongoing.

Summary of genes, microRNAs and cell types that are currently being studied in our lab for their function in heart disease:

| **Gene** | **microRNA** | **Cell type** |
|---|---|---|
| Zeb2 | miR-208 | Cardiomyocyte |
| Dynlt | miR-499 | Fibroblast |
| Purbeta | miR-19b | c-kit + cell |
| DSP | miR-15 | Lgr5+ cell |
| PKP2 | miR-1 | Cardiac stem cell |
| AC6 | miR-23 | |
| SDF1 | | |
| Axin2 | | |
| Lgr5 | | |
| Lgr6 | | |
| Troy | | |

**AVD-801002015250**
**Overzicht aantal muizen, groepen en ongerief**

Total numbers:
Rats: newborn 2700 and adults 360: 100% mild discomfort
Mice: newborns 400 and adults 36.320: 28% mild discomfort and 72% moderate discomfort

| | Procedure | Group | Animals | mild | moderate | Total group |
|---|---|---|---|---|---|---|
| 3.4.4.1 | Gene (in) activation interventions (ex vivo analysis) | Group 1a | Rat mothers | 360 | | |
| | | Group 1b | Rat neonates | 2700 | | 3060 *rats* |
| 3.4.4.2 | Generation Welfare assessment and Breeding of GMM | Group 2 | Mice adults 100% | 10240 | 6000 | 16240 |
| 3.4.4.3 | Gene (in) activation | Group 3a | Mice pups 5% | | 240 | |
| | | Group 3b | Mice adults 95% | | 4560 | 4800 |
| 3.4.4.4 | Gene (in) activation | Group 4a | Mice pups 5% | | 160 | |
| | | Group 4b | Mice adults 95% | | 3040 | 3200 |
| 3.4.4.5 | Gene (in) activation interventions (myocardial infarction / ischemia reperfusion) | Group 5 | Mice adults 100% | | 7680 | 7680 |
| 3.4.4.6 | Gene (in) activation intervention (pathological cardiac stress) | Group 6 | Mice adults 100% | | 4800 | 4800 |
| | | | Rats total | 3060 | | |
| | | | Mice total | | | 36.720 |

Centrale Commissie Dierproeven

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[redacted]
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**Onze referentie**
Aanvraagnummer
AVD801002015250
**Bijlagen**
2

Datum 15 oktober 2015
Betreft Ontvangstbevestiging Aanvraag projectvergunning Dierproeven

Geachte [redacted]
Wij hebben uw aanvraag voor een projectvergunning dierproeven ontvangen op 13 oktober 2015.
Het aanvraagnummer dat wij aan deze aanvraag hebben toegekend is AVD801002015250. 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).

Met vriendelijke groet,

Centrale Commissie Dierproeven

Deze brief is automatisch aangemaakt en daarom niet ondertekend.

Bijlagen:
- Gegevens aanvraagformulier
- Factuur

**Gegevens aanvrager**

| | |
|---|---|
| Uw gegevens | |
| Deelnemersnummer NVWA: | 80100 |
| Naam instelling of organisatie: | Kon. Ned. Academie van Wetenschappen |
| Naam portefeuillehouder of diens gemachtigde: | [redacted] |
| KvK-nummer: | 54667089 |
| Postbus: | Postbus 19121 |
| Postcode en plaats: | 1000 GC AMSTERDAM |
| Tenaamstelling van het rekeningnummer: | Hubrecht Instituut |

| | |
|---|---|
| Gegevens verantwoordelijke onderzoeker | |
| Naam: | [redacted] |
| Functie: | Group Leader |
| Afdeling: | [redacted] |
| Telefoonnummer: | [redacted] |
| E-mailadres: | [redacted] |

**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 november 2015 |
| Geplande einddatum: | 1 november 2020 |
| Titel project: | Factors involved in cardiac function and repair |
| Titel niet-technische samenvatting: | De moleculaire basis van hartziekten |
| Naam DEC: | DEC-KNAW |
| Postadres DEC: | [redacted] Amsterdam |
| E-mailadres DEC: | [redacted] |

**Betaalgegevens**

| | |
|---|---|
| De leges bedragen: | € 741,- |
| 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] DEC-advies |

**Ondertekening**

| | |
|---|---|
| Naam: | [redacted] |
| Functie: | [redacted] |
| Plaats: | Amsterdam |
| Datum: | 12 oktober 2015 |

Centrale Commissie Dierproeven

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**Onze referentie**
Aanvraagnummer
AVD801002015250

**Bijlagen**
2

Datum 15 oktober 2015
Betreft Factuur aanvraag projectvergunning Dierproeven

**Factuur**
Factuurdatum: 15 oktober 2015
Vervaldatum: 14 november 2015
Factuurnummer: 15700250

| **Omschrijving** | **Bedrag** |
|---|---|
| Betaling leges projectvergunning dierproeven<br>Betreft aanvraag AVD801002015250 | € 741,00 |

Wij verzoeken u het totaalbedrag vóór de gestelde vervaldatum over te maken op rekening NL28RBOS 056.99.96.066 onder vermelding van het factuurnummer en aanvraagnummer, ten name van Centrale Commissie Dierproeven, Postbus 20401, 2500 EK te 's Gravenhage.

Centrale Commissie Dierproeven

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**Onze referentie**
Aanvraagnummer
AVD801002015250

Datum 23 NOV 2015
Betreft Aanvraag projectvergunning Dierproeven

Geachte [redacted]
Op 13 oktober 2015 hebben wij uw aanvraag voor een projectvergunning dierproeven ontvangen. Het gaat om uw project "Factors involved in cardiac function and repair" met aanvraagnummer AVD801002015250. 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. De IvD moet betrokken worden bij go/no go momenten. Overlap van door de DEC goedgekeurde protocollen en deze vergunning moet voorkomen worden. Daarvoor zijn voorwaarden opgenomen in de vergunning. De algemene voorwaarde betreffende artikel 10, lid 1a van de wet wordt gesteld bij vergunningen met een langere looptijd. Dit om te voldoen aan datgene wat volgt uit dit artikel. U kunt met uw project "Factors involved in cardiac function and repair" starten. De vergunning wordt afgegeven van 23 november 2015 tot en met 1 november 2020.
Overige wettelijke bepalingen blijven van kracht.

**Procedure**
Bij uw aanvraag heeft u een advies van de Dierexperimentencommissie DEC-KNAW gevoegd. Dit advies is opgesteld op 13 oktober 2015. 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. De CCD stelt wel algemene voorwaarden aan dit project. Wij nemen dit advies van de commissie over, inclusief de daaraan ten grondslag liggende motivering.

Dit 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).

Met vriendelijke groet,

Centrale Commissie Dierproeven
namens deze:

[redacted]

ir. G. de Peuter
Algemeen Secretaris

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

**Projectvergunning**

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

Verleent de Centrale Commissie Dierproeven aan

| | |
|---|---|
| Naam: | Kon. Ned. Academie van Wetenschappen |
| Adres: | Postbus 19121 |
| Postcode en plaats: | 1000 GC AMSTERDAM |
| Deelnemersnummer: | 80100 |

deze projectvergunning voor het tijdvak 23 november 2015 tot en met 1 november 2020, voor het project "Factors involved in cardiac function and repair" met aanvraagnummer AVD801002015250, volgens advies van Dierexperimentencommissie DEC-KNAW.
De functie van de verantwoordelijk onderzoeker is Group Leader .
De aanvraag omvat de volgende bescheiden:

1 een aanvraagformulier projectvergunning dierproeven, ontvangen op 13 oktober 2015
2 de bij het aanvraagformulier behorende bijlagen:
a Projectvoorstel, zoals ontvangen per digitale indiening op 13 oktober 2015;
b Niet-technische Samenvatting van het project, zoals ontvangen per digitale indiening op 13 oktober 2015;
c Advies van dierexperimentencommissie d.d. 13 oktober 2015, ontvangen op 13 oktober 2015.

| Naam proef | Diersoort/ Stam | Aantal dieren | Ernst | Opmerkingen |
|---|---|---|---|---|
| 1 Isolation of neonatal rat cardiomyocytes | Ratten (Rattus norvegicus) / wildtype; adult (mothers); pups (P1-P4) | 3060 | Licht / mild | 2700 neonatal pups, 360 pregnant rats |
| 2 Generation, welfare assessment and breeding genetically modified mice (GMM) | Muizen (Mus musculus) / WT or genetically modified; adults | 6000 | Matig / moderate | |
| 3 Collecting (stem) cells or tissue for ex vivo experiments | Muizen (Mus musculus) / genetically modified or injected with a virus or microRNA therapeutic; neonates p1-p4 and adults | 4800 | Matig / moderate | 240 pups, 4560 adults |
| 4 Drug delivery or exercise | Muizen (Mus musculus) / WT, genetically modified or injected with a virus or microRNA therapeutic; neonates p1-p4 and adults | 3200 | Matig / moderate | 160 pups, 3040 adults |
| 5 Myocardial infarction / Ischemia Reperfusion | Muizen (Mus musculus) / WT, genetically modified or injected with a virus or microRNA therapeutic; adult | 7680 | Matig / moderate | |
| 6 Models for pathological remodeling | Muizen (Mus musculus) / WT, genetically modified or injected with a virus or microRNA therapeutic, adult | 4800 | Matig / moderate | |

**Voorwaarden**

**Op grond van artikel 10a1 lid 2 Wod zijn aan een projectvergunning voorwaarden te stellen**

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

Daar waar er sprake is van overlap tussen de in deze vergunning vergunde dierproeven en eerder goedgekeurde DEC protocollen zullen de dieren en experimenten na het verlenen van deze vergunning

formeel onder deze vergunning gaan vallen, zoals in Appendix II bij uw aanvraag ook heeft aangegeven. Hierdoor is er geen sprake meer van overlap.

In artikel 10, lid 1a 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 overleg met de IvD te melden bij de CCD. De CCD kan in een dergelijke situatie aan de vergunning nieuwe voorwaarden verbinden en gestelde voorwaarden wijzigen of intrekken.

# 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.