	Inventaris Wob-verzoek W23-03									
		wordt verstrekt				**weigeringsgronden**				
nr.	**document NTS 20209866**	**reeds openbaar**	**niet**	**geheel**	**deels**	**5.1, lid 1c**	**5.1, lid 2e**	**5.1, lid 2f**	**5.1, lid 2h**	**5.2, lid 1**
1	Aanvraag projectvergunning, (met natte handtekening) d.d. 07-05-2020				x		x		x	
2	Aanvraag projectvergunning, d.d. 07-05-2020				x		x		x	
3	Projectvoorstel bij aanvraag				x				x	
4	Bijlage dierproeven_1 bij aanvraag				x				x	
5	Bijlage dierproeven_2 bij aanvraag				x				x	
6	Bijlage dierproeven_3 bij aanvraag				x				x	
7	Bijlage dierproeven_4 bij aanvraag				x				x	
8	NTS bij de aanvraag			x						
9	E-mail aan DEC om advies aanvraag projectvergunning, d.d. 08-05-2020				x				x	
10	DEC-advies, d.d. 30-09-2020				x				x	
11	Projectvoorstel na DEC advies				x				x	
12	Bijlage_1 dierproeven na DEC advies				x				x	
13	Bijlage_2 dierproeven na DEC advies				x				x	
14	Bijlage_3 dierproeven na DEC advies				x				x	
15	Bijlage_4 dierproeven na DEC advies				x				x	
16	NTS na DEC advies			x						
17	Adviesnota aan CCD, d.d. 14-10-2020_met opmerkingen				x		x		x	x
18	Adviesnota aan CCD, d.d. 19-10-2020				x		x			
19	E-mail CCD aan vergunninghouder over aanvraag, d.d. 19-10-2020				x		x		x	
20	Reactie na vragen CCD			x						
21	NTS na CCD vragen en definitieve versie			x						
22	Adviesnota aan CCD, d.d. 05-11-2020				x		x		x	
23	Beschikking, d.d. 06-11-2020				x		x		x	
24	E-mail CCD aan DEC over aanvraag projectvergunning, d.d. 09-11-2020				x		x		x	

Centrale Commissie Dierproeven

9866

23 OKT 2020

Aanvraag
Projectvergunning Dierproeven
Administratieve gegevens

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

1 Gegevens aanvrager

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

☒ Ja > *Vul uw deelnemernummer in* 5.1 lid2h

☐ Nee > *U kunt geen aanvraag doen*

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

Naam instelling of organisatie	5.1 lid2h
Naam van de portefeuillehouder of diens gemachtigde	5.1 lid2e
KvK-nummer	5.1 lid2h

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	5.1 lid2h
Postbus	
Postcode en plaats	
IBAN	
Tenaamstelling van het rekeningnummer	

1.4 Vul de gegevens in van de verantwoordelijke onderzoeker.

(Titel) Naam en voorletters	5.1 lid2e	☒ Dhr. ☐ Mw.
Functie	5.1 lid2e	
Afdeling	5.1 lid2e	
Telefoonnummer	5.1 lid2e	
E-mailadres	5.1 lid2e	

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

(Titel) Naam en voorletters	5.1 lid2e	☐ Dhr. ☒ Mw.
Functie	5.1 lid2e	
Afdeling	5.1 lid2h	
Telefoonnummer	5.1 lid2e	
E-mailadres	5.1 lid2e	

1.6	*(Optioneel)* Vul hier de gegevens in van de persoon die er verantwoordelijk voor is dat de uitvoering van het project in overeenstemming is met de projectvergunning.	(Titel) Naam en voorletters	☐ Dhr. ☐ Mw.
		Functie	
		Afdeling	
		Telefoonnummer	
		E-mailadres	
1.7	Is er voor deze projectaanvraag een gemachtigde?	☒ Ja > *Stuur dan het ingevulde formulier Melding Machtiging mee met deze aanvraag*	
		☐ Nee	

2 Over uw aanvraag

2.1	Wat voor aanvraag doet u?	☒ Nieuwe aanvraag > Ga verder met vraag 3
		☐ Wijziging op (verleende) vergunning die negatieve gevolgen kan hebben voor het dierenwelzijn
		Vul uw vergunde projectnummer in en ga verder met vraag 2.2
		☐ Melding op (verleende) vergunning die geen negatieve gevolgen kan hebben voor het dierenwelzijn
		Vul uw vergunde projectnummer in en ga verder met vraag 2.3
2.2	Is dit een *wijziging* voor een project of dierproef waar al een vergunning voor verleend is?	☐ Ja > Beantwoord dan in het projectplan en de niet-technische samenvatting alleen de vragen waarop de wijziging betrekking heeft en onderteken het aanvraagformulier
		☐ Nee > Ga verder met vraag 3
2.3	Is dit een *melding* voor een project of dierproef waar al een vergunning voor is verleend?	☐ Nee > Ga verder met vraag 3
		☐ Ja > Geef hier onder een toelichting en ga verder met vraag 6

3 Over uw project

3.1	Wat is de geplande start- en einddatum van het project?	Startdatum	1 september 2020
		Einddatum	31 augustus 2025
3.2	Wat is de titel van het project?	Towards a novel treatment of pulmonary arterial hypertension; preclinical testing of novel interventions targeting both right ventricular pressure overload and pulmonary vascular remodelling.	
3.3	Wat is de titel van de niet-technische samenvatting?	Verbeterde behandeling van longziekte: het testen van kandidaat-behandelmethoden in diermodellen.	
3.4	Wat is de naam van de Dierexperimentencommissie (DEC) aan wie de instellingsvergunninghouder doorgaans haar projecten ter toetsing voorlegt?	Naam DEC	5.1 lid2h
		Postadres	
		E-mailadres	5.1 lid2e

4 Betaalgegevens

4.1 Om welk type aanvraag gaat het?

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.

☒ Nieuwe aanvraag Projectvergunning € Lege
☐ Wijziging € Lege
☐ Via een eenmalige incasso
☒ Na ontvangst van de factuur*

23 OKT 2020

* Wanneer de factuur direct naar de financiële afdeling van 5.1 lid2h dient te gaan moet hier een inkooporder nummer en factuuradres worden toegevoegd door de onderzoekers graag van te voren afstemmen met de financiële afdeling.

Inkooporder nummer: 5.1 lid2h
Factuur e-mailadres: 5.1 lid2h

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

5 Checklist bijlagen

5.1 Welke bijlagen stuurt u mee?

Verplicht
☒ Projectvoorstel
☒ Niet-technische samenvatting

Overige bijlagen, indien van toepassing
☒ Melding Machtiging
☐

6 Ondertekening

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

Centrale Commissie
Dierproeven
Postbus 20401
2500 EK Den Haag

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

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

Naam	5.1 lid2e
Functie	5.1 lid2e
Plaats	5.1 lid2h
Datum	07 - 05 - 2020
Handtekening	5.1 lid2e

15-10-2020

5.1 lid2e

Centrale Commissie Dierproeven

Aanvraag
Projectvergunning Dierproeven
Administratieve gegevens

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

1 Gegevens aanvrager

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

☒ Ja > *Vul uw deelnemernummer in* 5.1 lid2h
☐ Nee > *U kunt geen aanvraag doen*

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

Naam instelling of organisatie	5.1 lid2h
Naam van de portefeuillehouder of diens gemachtigde	5.1 lid2e
KvK-nummer	5.1 lid2h
Straat en huisnummer	5.1 lid2h
Postbus	
Postcode en plaats	
IBAN	
Tenaamstelling van het rekeningnummer	

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

1.4 Vul de gegevens in van de verantwoordelijke onderzoeker.

(Titel) Naam en voorletters	5.1 lid2e	☒ Dhr. ☐ Mw.
Functie	5.1 lid2e	
Afdeling	5.1 lid2h	
Telefoonnummer	5.1 lid2e	
E-mailadres	5.1 lid2e	

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

(Titel) Naam en voorletters	5.1 lid2e	☐ Dhr. ☒ Mw.
Functie	5.1 lid2e	
Afdeling	5.1 lid2h	
Telefoonnummer	5.1 lid2e	
E-mailadres	5.1 lid2e	

1.6	*(Optioneel)* Vul hier de gegevens in van de persoon die er verantwoordelijk voor is dat de uitvoering van het project in overeenstemming is met de projectvergunning.	(Titel) Naam en voorletters		☐ Dhr. ☐ Mw.
		Functie		
		Afdeling		
		Telefoonnummer		
		E-mailadres		
1.7	Is er voor deze projectaanvraag een gemachtigde?	☒ Ja > *Stuur dan het ingevulde formulier Melding Machtiging mee met deze aanvraag*		
		☐ Nee		

2 Over uw aanvraag

2.1	Wat voor aanvraag doet u?	☒ Nieuwe aanvraag > Ga verder met vraag 3	
		☐ Wijziging op (verleende) vergunning die negatieve gevolgen kan hebben voor het dierenwelzijn	
		Vul uw vergunde projectnummer in en ga verder met vraag 2.2	
		☐ Melding op (verleende) vergunning die geen negatieve gevolgen kan hebben voor het dierenwelzijn	
		Vul uw vergunde projectnummer in en ga verder met vraag 2.3	
2.2	Is dit een *wijziging* voor een project of dierproef waar al een vergunning voor verleend is?	☐ Ja > Beantwoord dan in het projectplan en de niet-technische samenvatting alleen de vragen waarop de wijziging betrekking heeft en onderteken het aanvraagformulier	
		☐ Nee > Ga verder met vraag 3	
2.3	Is dit een *melding* voor een project of dierproef waar al een vergunning voor is verleend?	☐ Nee > Ga verder met vraag 3	
		☐ Ja > Geef hier onder een toelichting en ga verder met vraag 6	

3 Over uw project

3.1	Wat is de geplande start- en einddatum van het project?	Startdatum	1 september 2020
		Einddatum	31 augustus 2025
3.2	Wat is de titel van het project?	Towards a novel treatment of pulmonary arterial hypertension; preclinical testing of novel interventions targeting both right ventricular pressure overload and pulmonary vascular remodelling.	
3.3	Wat is de titel van de niet-technische samenvatting?	Verbeterde behandeling van longziekte: het testen van kandidaat-behandelmethoden in diermodellen.	
3.4	Wat is de naam van de Dierexperimentencommissie (DEC) aan wie de instellingsvergunninghouder doorgaans haar projecten ter toetsing voorlegt?	Naam DEC	5.1 lid2h
		Postadres	
		E-mailadres	5.1 lid2e

4 Betaalgegevens

4.1 Om welk type aanvraag gaat het?

☒ Nieuwe aanvraag Projectvergunning € 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*

* Wanneer de factuur direct naar de financiële afdeling van 5.1 lid2h dient te gaan moet hier een inkooporder nummer en factuuradres worden toegevoegd door de onderzoekers, graag van te voren afstemmen met de financiële afdeling.

Inkooporder nummer: 5.1 lid2h
Factuur e-mailadres: 5.1 lid2h

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

5 Checklist bijlagen

5.1 Welke bijlagen stuurt u mee?

Verplicht

☒ Projectvoorstel

☒ Niet-technische samenvatting

Overige bijlagen, indien van toepassing

☒ Melding Machtiging

☐

6 Ondertekening

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

Centrale Commissie
Dierproeven
Postbus 20401
2500 EK Den Haag

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

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

Naam	5.1 lid2e
Functie	5.1 lid2e
Plaats	5.1 lid2h
Datum	07 - 05 - 2020
Handtekening	5.1 lid2e

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'.	5.1 lid2h
1.2	Provide the name of the licenced establishment.	5.1 lid2h
1.3	Provide the title of the project.	Towards a novel treatment of pulmonary arterial hypertension; preclinical testing of novel interventions targeting both right ventricular pressure overload and pulmonary vascular remodelling

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.

This project proposal describes testing of novel therapeutic interventions in laboratory animals (rats and mice) in the field of cardiopulmonary medicine, in particular for pulmonary arterial hypertension (PAH). The included studies comprise both fundamental research (studying the underlying pathology of PAH) and translational research, including the testing of novel (combinations of) therapeutic interventions in mice and rat models for PAH.

Cardiopulmonary medicine focuses on a range of disorders that affect the heart ("cardio-") as well as the lungs ("-pulmonary"). The two organ systems work closely together to make sure the body has the oxygen-rich blood it needs to function. Lung and cardiovascular (heart) disease are increasingly recognized to occur in the same patient populations [1]. Whether these diseases develop as a result of unique mechanisms or shared pathways remains uncertain, but growing evidence indicates that they may share common origins [2]. Example of such a disease is pulmonary arterial hypertension, a chronic severe disease with a poor prognosis. Although the origin of the disease is located in the lungs, the majority of patients die from right heart failure.

HEALTH CARE PROBLEM: PULMONARY ARTERIAL HYPERTENSION
Pulmonary arterial hypertension is a progressive and fatal disease [3]. Its prevalence in the Netherlands is around 16-29 patients per million inhabitants, equal to around 300-500 patients [4]. New cases are estimated to occur in 2.2 individuals per million each year in the Netherlands, i.e. around 37 new patients annually [4]. Due to progressive nature of the disease, a patient may experience only mild symptoms at first, but will eventually require treatment and increasing medical care to maintain a reasonable quality of life. Apart from lung transplantation, no curative treatment for PAH is available. The average life expectancy is currently only 3-5 years. While PAH is rare, other types of pulmonary hypertension (PH) are much more prevalent and carry significant morbidity and mortality. Many of the pathophysiological and pathobiological changes that are seen in the lungs and hearts of patients with PAH are also found in patients with other types of PH.

PREVIOUS RESEARCH IN PAH PATHOBIOLOGY
The pathology in PAH can be categorized by abnormal remodelling of pulmonary vessels (causing the arteries in the lungs to become narrowed, thickened or stiff), leading to a progressive increase in pulmonary artery pressure and increased right ventricular (RV) afterload (Figure 1). Effectively, the RV must work harder to push blood through the narrowed pulmonary arteries. The RV adapts to this increased load via several compensatory mechanisms. Although RV adaption mechanisms initially suffice, the progressive increases in pressure-overload leads almost inevitably to RV dysfunction and right heart failure, which is the predominant cause of death in PAH. Hence, PAH is a complex and multi-factorial disease, involving the two organ systems (lung, heart) concurrently.

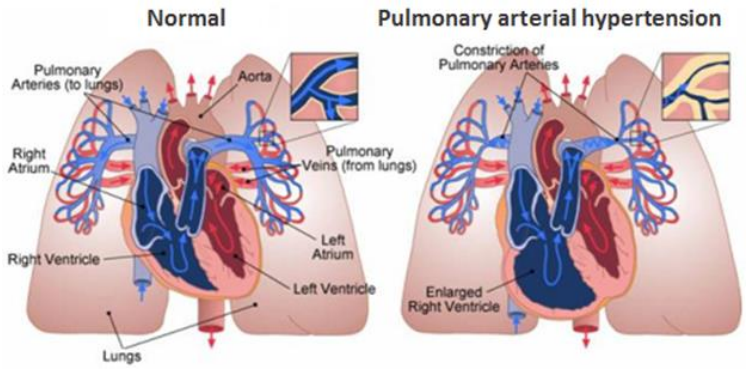

Figure 1: Illustration of pathology occurring in pulmonary arterial hypertension (PAH, right panel) compared to the normal condition (left). PAH is categorized by abnormal remodelling of pulmonary vessels (causing construction of the pulmonary arteries), leading to a progressive increase in pulmonary

artery pressure and increased right ventricular afterload. The right ventricle adapts to this increased load via several compensatory mechanisms (incl. increase in size). The progressive increases in pressure-overload leads almost inevitably to right ventricle dysfunction and right heart failure, which is the predominant cause of death in PAH. (Source: https://www.labroots.com/trending/cardiology/3394/bad-pulmonary-arterial-hypertension)

Due to its complex nature, the pathobiology of PAH remains incompletely understood. Research has focused for many years on the pathophysiology that is occurring in the lung as starting point for PAH. It is believed that changes in the layer of cells (endothelial cells) that line the small arteries of the lung ('abnormal vascular remodelling'), either causing or being linked to changes in the smooth muscle cells in the vessel wall, initiates the narrowing of the pulmonary arteries. Several studies (including of our research group) have shown that the abnormal vascular remodelling in the lung is associated with endothelial cell dysfunction, increased proliferation of smooth muscle cells, loss of pre-capillary arteries, inflammation and impaired bone morphogenetic protein (BMP) signalling [5,6]. However, far less is known about the events leading to right heart failure in PAH. Although it is known for some years that RV adaptation is of clinical importance, it has just recently become clear that RV diastolic stiffness increases and may contribute to disease progression in PAH. We and others have recently shown that although increased RV afterload is the initial trigger for PAH-induced RV dysfunction, the degree of pressure overload does not predict the development of RV failure. This suggest that the response of the RV to pressure overload, rather than the degree of pressure overload, determines the fate of the right ventricle in PAH patients. Hence, further studies are warranted in order to increase our understanding of PAH pathogenesis, including the processes resulting in right heart failure (RV dysfunction). Gaining insights in the pathology occurring in the lungs - as well as the heart - is pivotal in order to develop new therapies for PAH.

CURRENT STATE-OF-THE-ART IN THERAPY DEVELOPMENT

Over the past three decades, based on the advances in our understanding of the pathobiology of PAH, new targeted therapies have been developed, resulting also in improved patient outcomes [5,7,8]. Current drug therapies used in the clinic for PAH are particularly focused on the molecular and cellular pathways underlying pulmonary vascular remodelling (in particular endothelial dysfunction [9]), vasoconstriction, inflammation and thrombosis [8,9]. Novel potential targets in PAH drug development include vascular inflammation, metabolic derangements and aberrant BMPR2 signalling [10]. Despite these advances, PAH remains an incurable disease as mortality rates are high and prognosis of patients remains poor. There is still an unmet medical need for new PAH therapies, possibly targeting alternative pathways. Moreover, the current therapies are selectively focused on exerting an effect on the pulmonary system. Little is known about their effects on right heart function. In addition, there has been little consideration in the field for the possibility that PAH patients may benefit from a cardiac-specific treatment, which has no direct effect on the pulmonary vasculature, but presents or reverses right heart dysfunction.

One of the missions is to ultimately improve diagnosis and treatment of PAH. Fundamental, translational and clinical research to expand the understanding of PAH RV overload are all essential to achieve this. Our research group has a unique focus on the integrated pathophysiological consequences of PAH and right heart failure and is not only working to restore the PAH lung vasculature, but also to develop PAH-treatments that are cardiac-specific with no direct effects on the pulmonary vasculature. Through our research we have gained in-depth information and new insights into potential new druggable targets in PAH and right heart failure.

Testing of novel therapeutic interventions in preclinical models, before the start of clinical studies, is an essential component of our research programme. It is ethically unacceptable and practically impossible to test all (combinations of) treatments immediately in patients. Therefore, preclinical studies in relevant disease models (cellular/animal models for PAH) are required to provide information on the soundness of the strategy, the best candidate agents and the most optimal combinations of treatments. As such,

animal studies form an indispensable part of our research and PAH treatment development. Translational studies have resulted in the identification of specific (cell-type dependent) blockers for TGFβ and/or enhancers of BMP signalling to restore the TGFβ/BMP imbalance in endothelial cells and the validation of these compounds in experimental PAH models (to ultimately develop a drug with beneficial effects in the lungs and the heart). Successful preclinical studies have allowed us to select one compound for testing in a Phase IIb clinical trial for the treatment of PAH patients. Through the studies included in the current project proposal, these and other research lines will be further expanded with the ultimate goal to improve the treatment and quality of life of PAH patients. The interventions act on 7 different established targets for PAH, namely BMPR2 signalling, vascular tone, RV diastolic stiffness, inflammation, coagulation, integrin and VHL gene expression (Figure 2).

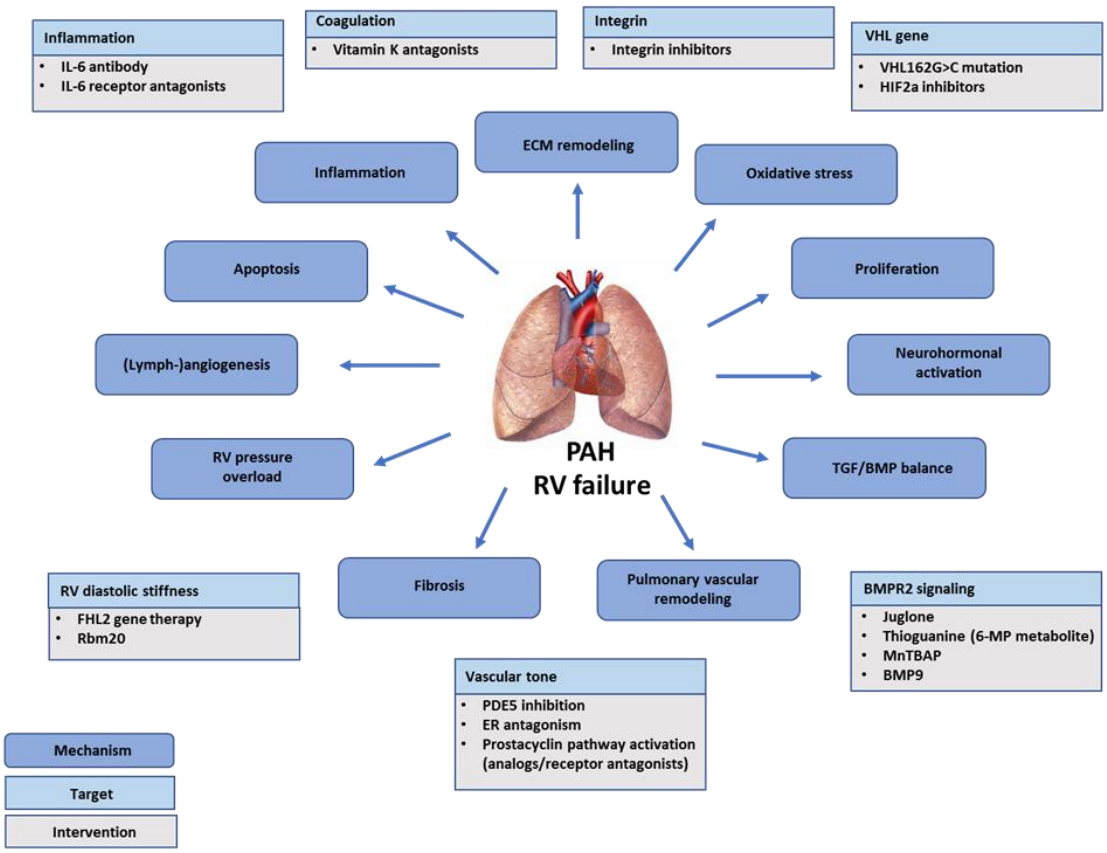

Figure 2: Illustration of the included interventions in the project and the targets upon which they act. Should during the course of this project our fundamental/clinical research (not included in this project), result in the identification of new interventions addressing these targets, these interventions will be added to the study.

References used in this section:

[1] P. Carter et al., "Association of Cardiovascular Disease with Respiratory Disease," Journal of the American College of Cardiology, vol. 73, no. 17, pp. 2166–2177, May 2019, doi: 10.1016/j.jacc.2018.11.063.
[2] A. Morris, "Heart–Lung Interaction via Infection," Ann Am Thorac Soc, vol. 11, no. Suppl 1, pp. S52–S56, Jan. 2014, doi: 10.1513/AnnalsATS.201306-157MG.
[3] G. Simonneau et al., "Updated clinical classification of pulmonary hypertension," J. Am. Coll. Cardiol., vol. 54, no. 1 Suppl, pp. S43-54, Jun. 2009, doi: 10.1016/j.jacc.2009.04.012.
[4] Long Alliantie Nederland, Longziekten feiten en cijfers 2013. Amersfoort, Nederland: Long Alliantie Nederland, 2013.
[5] M. Humbert et al., "Pathology and pathobiology of pulmonary hypertension: state of the art and research perspectives," Eur. Respir. J., vol. 53, no. 1, Jan. 2019, doi: 10.1183/13993003.01887-2018.

[6] A. R. Hemnes and M. Humbert, "Pathobiology of pulmonary arterial hypertension: understanding the roads less travelled," Eur Respir Rev, vol. 26, no. 146, Dec. 2017, doi: 10.1183/16000617.0093-2017.
[7] E. M. T. Lau, E. Giannoulatou, D. S. Celermajer, and M. Humbert, "Epidemiology and treatment of pulmonary arterial hypertension," Nat Rev Cardiol, vol. 14, no. 10, pp. 603–614, Oct. 2017, doi: 10.1038/nrcardio.2017.84.
[8] M. Humbert and H.-A. Ghofrani, "The molecular targets of approved treatments for pulmonary arterial hypertension," Thorax, vol. 71, no. 1, pp. 73–83, Jan. 2016, doi: 10.1136/thoraxjnl-2015-207170.
[9] R. Nogueira-Ferreira, R. Ferreira, and T. Henriques-Coelho, "Cellular interplay in pulmonary arterial hypertension: Implications for new therapies," Biochimica et Biophysica Acta (BBA) - Molecular Cell Research, vol. 1843, no. 5, pp. 885–893, May 2014, doi: 10.1016/j.bbamcr.2014.01.030.
[10] S. Bonnet et al., "Translating Research into Improved Patient Care in Pulmonary Arterial Hypertension," Am J Respir Crit Care Med, vol. 195, no. 5, pp. 583–595, Mar. 2017, doi: 10.1164/rccm.201607-1515PP.
[11] K. R. Stenmark, B. Meyrick, N. Galie, W. J. Mooi, and I. F. McMurtry, "Animal models of pulmonary arterial hypertension: the hope for etiological discovery and pharmacological cure," Am. J. Physiol. Lung Cell Mol. Physiol., vol. 297, no. 6, pp. L1013-1032, Dec. 2009, doi: 10.1152/ajplung.00217.2009.
[12] S. Andersen et al., "A Pulmonary Trunk Banding Model of Pressure Overload Induced Right Ventricular Hypertrophy and Failure," J Vis Exp, no. 141, 29 2018, doi: 10.3791/58050.

3.2 Purpose

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

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

The main objectives of the research included in this project are to:

- Evaluate novel (combinations of) therapeutic interventions in mice and rat models for PAH, targeting both pulmonary vascular remodelling and RV pressure overload;
- Improve fundamental understanding of PAH and RV failure and their underlying pathologies;
- Discover novel therapeutic targets for PAH.

The results of the studies will render pivotal information on the usefulness of the therapeutic interventions in subsequent clinical trials and will support the use of relevant PAH disease models. As such, they will contribute to improve the treatment of PAH.

The research group has available a wide range of relevant animal models for PAH and assays to measure cardiopulmonary function in rodents (see Section 3.4.1-2 for details) and all the expertise to conduct the studies described in this project documented by >40 papers published by the PI and his group in international journals on PAH in the last 11 years. The close relationship between the preclinical and clinical research additionally facilitates both clinical translation of research results and as well as feedback from the clinic to guide new research directions.

The infrastructure required for the studies (animal facility, surgery facility and wet-lab for e.g. tissue analyses) is available and the department has an active collaboration with other research groups. Standard operating procedures (SOPs) for animal handling/experimentation and extensive expertise on animal handling are available. In the project, 2 PIs, 1 technician, 2 postdocs and 3 PhD students will be involved. The studies will be performed and supervised by dedicated and well-trained staff. The studies will be financed through grant funding, which has been guaranteed. From experience we know that the studies take around 3 months (12 weeks) to complete. Based on the number of interventions we would like to test (see Section 3.4), we estimate that the research included in this project will take 5 years.

3.3 Relevance

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

SCIENTIFIC RELEVANCE
Not only will the proposed studies allow us to identify therapeutic interventions for PAH with the highest efficacy likelihood and the lowest toxicity potential before starting clinical trials, they will also increase our understanding of the processes underlying abnormal pulmonary vascularisation and that controlling the transition of RV adaptation towards right heart failure. Our research group is one of the few who takes a combined approach by studying the pathological effects of PAH in the lungs and the right heart concurrently. This will allow us to investigate the relatively new concept that PAH patients may benefit from a cardiac-specific treatment, which has no direct effect on the pulmonary vasculature, but presents or reverses right heart dysfunction. Moreover, novel therapeutic targets for future clinical research may be identified.
Although this research proposal is focused on PAH, right heart failure is also the main cause of death in several other conditions such as left heart failure and critical illness. We have for example shown that not only the RV but also the LV is affected in PAH-patients. We believe that RV remodelling observed in PAH patients shares important pathophysiological mechanisms with the cardiac remodelling observed in left heart failure patients. As such, the findings of this proposal may also advance research in left heart failure. The scientific relevance of our findings is therefore not limited to PAH-induced right heart failure.

SOCIETAL RELEVANCE
PAH remains an incurable debilitating disease, with high mortality rates and poor prognosis for patients. Besides the enormous impact of the disease on the quality of life of PAH patients, the disease also carries considerable economic consequences because patients and/or care-givers drop out of the work force and patients require expensive medical treatments, including lung transplantation. New PAH therapies, also targeting alternative pathways are urgently needed. In this project, we address apart from established PAH-targets (e.g. BMPR2 signalling), also relatively new ones, such as RV diastolic stiffness. Up till now, there has been little consideration in the field of the notion that PAH patients could benefit from a cardiac-specific treatment, which has no direct effect on the pulmonary vasculature. With the results of this project, we will be able to select those interventions with promising effectiveness in PAH animal models for further clinical testing. This will bring us hopefully a step closer to the development of an effective PAH treatment. In the future, patients at risk of developing right heart failure (PAH) may benefit from these new treatment options. Although PAH is rare, other types of pulmonary hypertension (PH) are much more prevalent and carry significant morbidity and mortality. Moreover, right heart failure is becoming a great clinical problem as leading cause of death in several diseases such as left heart failure, and the critical ill at the intensive care. Currently, no therapeutic strategies are available to improve RV function or prevent right heart failure. As such, the societal relevance of our studies extends far beyond PAH alone.

3.4 Research strategy

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

Each animal study included in the proposal will be initiated based on identified PAH targets or candidate targets obtained by fundamental research (e.g. genetical, molecular, cellular, histopathological studies on tissue of animal models) or clinical research (e.g. genetic studies on human samples or other observations, such as fMRI) as illustrated in Figure 3. In all cases, a clear rationale for the animal experiments will be provided based on in vitro data or other considerations (e.g. clinical data). Ultimate end goal of all studies is to identify those therapeutic interventions that can proceed to clinical testing. Main selection criteria for interventions to progress to the clinical phase is a demonstrated in vivo efficacy in reducing abnormal pulmonary vascular remodelling and/or improving RV adaptation.

All studies will be grouped based on similarities in study goals and protocols. The procedures are introduced in Section 3.4.2 and described in detail in Appendix 1-4. Selection criteria for and connection between procedures are described below and in Section 3.4.3.

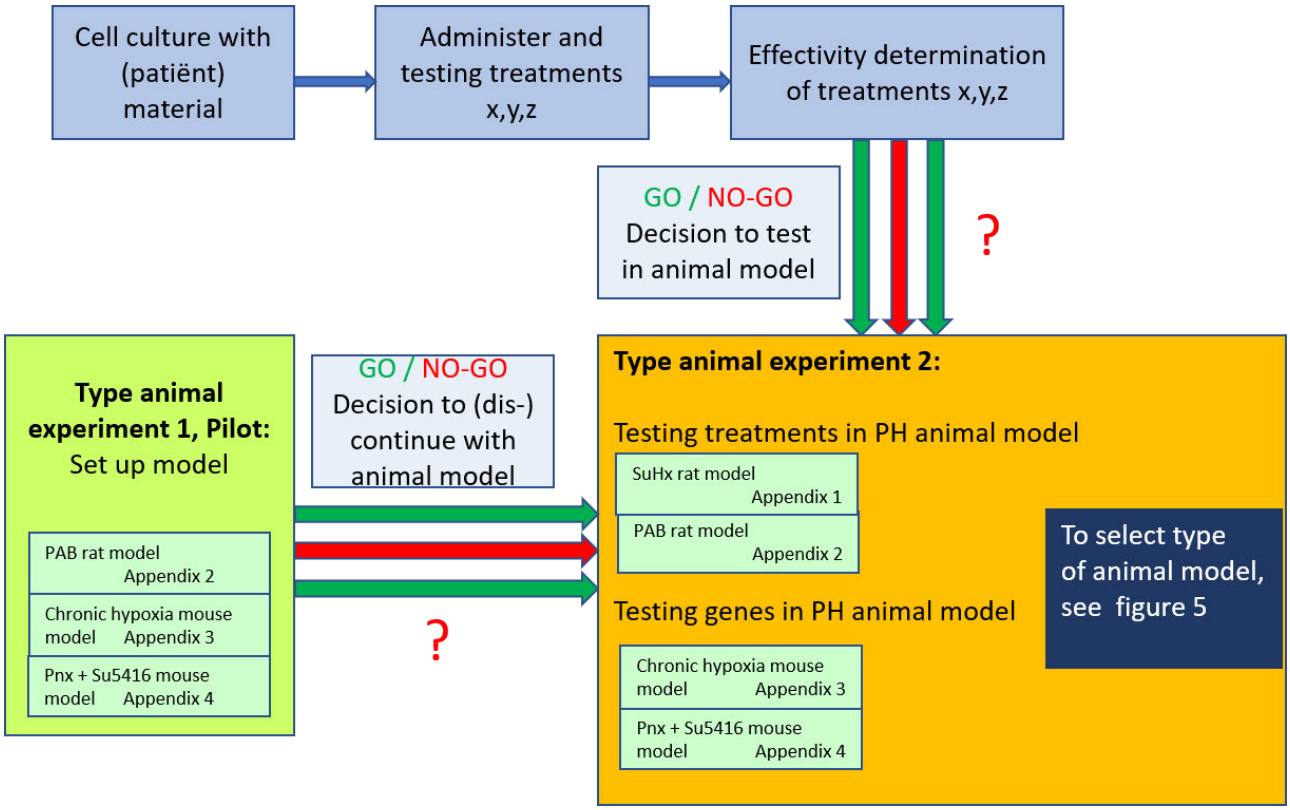

Figure 3: Position of this project within the overall strategy to develop a new therapeutic intervention for PAH. In case a candidate treatment has positive results without severe side-effects, the treatment will be selected for clinical testing.

SELECTION CRITERIA FOR THE ANIMAL MODELS TO BE USED

At present, there is no animal model available that fully recapitulates human PAH [10,11]. To maximize the chances of a successful outcome and to be able to compare the results with those of previous animal studies (AVD5.1 lid2h), we will evaluate the novel (combinations of) therapeutic interventions in four animal models for PAH. These include:

5.1 lid2h

In the 5.1 lid2h pulmonary vascular remodelling and right heart failure are induced by the combined exposure to the vascular endothelial growth factor receptor (VEGFR) inhibitor SU5416 and hypoxia. A single administration of SU5416 is followed by 3-4 weeks transient exposure to hypoxia and 2 weeks normoxic re-exposure. Extensive pulmonary vascular remodelling and first signs of right ventricle dysfunction is observed after 5-6 weeks. After an additional 4 weeks, the rats can develop right heart failure.

Pulmonary artery banding (PAB) model (rat model).

The pulmonary artery banding rat model for PAH is a surgical model. A titanium clip is compressed around the pulmonary artery with a modified ligating clip applier. When subjecting rats to pulmonary artery banding for 6-8 weeks [12, and own published data], they developed cardiac phenotypes with RV hypertrophy and dysfunction.

Chronic hypoxia model (mouse model)

In the chronic hypoxia model, mice are exposed to chronic hypoxia (10% O2, for 3 weeks). Pulmonary vascular remodelling and a mild increase in right ventricular pressures are observed after three weeks. To test the involvement of targets genes in pulmonary vascular or right ventricular remodelling (as identified above) that are promising novel therapeutic interventions for PAH, knock-in and knock-out mice will be tested in this model. As an example, this model includes EpoR-null mutant mice expressing

erythropoietin receptor (EpoR) exclusively in the erythroid lineage (EpoR-/- rescued mice). Because systemic deletion of EpoR is embryo-lethal, mice are rescued with EpoR that is exclusively expressed in erythroid progenitor cells under the regulatory domain of globin transcription factor 1 (GATA-1) (EpoR-/- rescued mice). Mice (EpoR-/- rescued and wild-type controls) are exposed to hypoxia (10% O2, chronically) for 3 weeks. The development of pulmonary vascular remodelling is accelerated in EpoR-/- rescued mice compared with wild-type mice.

Pneumonectomy+SU5416 model (mouse model)
The pneumonectomy (PNX)+SU5416 mouse model will be used to validate genetic targets that are promising as novel therapeutic intervention for PAH. The target genes include identified PAH targets or candidate targets obtained by fundamental research (e.g. genetical, molecular, cellular, histopathological studies on tissue of animal models) or clinical research (e.g. genetic studies on human samples or other observations, such as fMRI) – see also Figure 3. In the pneumonectomy (Pnx) +SU5416 mouse model, the left lung will be removed which results in increased pulmonary blood flow and vascular remodelling. A single administration of SU5416 one week after the surgery will result in severe PAH and RV dysfunction. The effect of the genetic interventions will be tested when PAH has developed in the mice subjected to the PNX+SU5416 model.

These animal models are selected because they all have a close resemblance to the clinical symptomology observed in PAH patients. The four animal models also supplement each other. The PAB model is included as direct effects of interventions on RV adaptation can be studied. Interventions from which we expect it will have an effect in the heart, will be first tested in this model. Furthermore, the model will be used to validate results obtained with the SuHx model. When not using this approach, it is possible that specific RV toxicity may be overlooked, because that intervention has such a profound effect on the lung. If such an intervention would be used in human, it is possible that the effect in the lung is not as large in patients as it is in animals, while cardiotoxicity is the same. This would result in severe side-effects of the treatment intervention in patients, which was not anticipated from animal studies. With the SuHx model more mechanistic information can be obtained and interventions from which we expect it will have an effect in the lung, will be first tested in this model. Since genetic modification is more efficient in mice than in rat, the addition of the chronic hypoxia mouse model allows for testing of genetic interventions (knock-out and knock-in) in the genes identified in human genetic studies.

SELECTION CRITERIA FOR THE INTERVENTIONS
Only interventions in this project are included that act on an identified PAH target (or candidate target) as demonstrated by previous fundamental research or clinical research. Based on this premise, we have selected at least 15 interventions to be included in our studies. The interventions act on 7 different established targets for PAH, namely BMPR2 signalling, vascular tone, RV diastolic stiffness, inflammation, coagulation, integrin and VHL gene expression. Should new interventions be identified during the course of this project addressing these targets, they will be added to the study.

EXPERIMENTAL READOUTS
The interventions will be tested when PAH has developed in the four animal models (therapeutics vs preventive), as this study design has the highest clinical relevance. We will particularly focus in these studies on assessing the impact of the intervention on RV overload and pulmonary vascular remodelling, as both are clinically relevant end-points for PAH. Moreover, we have shown in the past that interventions targeted at the lungs, also had an effect on cardiac function and vice versa. This clearly illustrates that the two organ systems are interconnected.

As the focus of this project is on the evaluation of the efficacy of novel (combinations of) therapeutic PAH-interventions, we have chosen for clinically relevant end-points as primary outcome measures. These will be combined with advanced blood serum and tissue analyses to study the mechanisms

underlying the chosen interventions and to possibly identify new targets (in untreated experimental animals). The outcomes measures include:

- Time-to-right-heart failure: the time (in days) from PAH induction to clinical manifestation of right heart failure;
- Lung and right heart (RV) function (cardiac output, RV hypertrophy and dilatation, RV functional measurements through echocardiography and pressure volume loop analysis);
- Measurements of vascular leakage;
- Systemic blood pressure;
- Imaging (MRI myocardial tagging, Diffusion Tensor Imaging-MRI quantification of the helical muscle fibre architecture in the RV)
- Blood and lung, heart, and muscle tissue analysis (to assess degree of pulmonary vascular remodelling).

References used in this section:

[10] S. Bonnet et al., "Translating Research into Improved Patient Care in Pulmonary Arterial Hypertension," Am J Respir Crit Care Med, vol. 195, no. 5, pp. 583–595, Mar. 2017, doi: 10.1164/rccm.201607-1515PP.
[11] K. R. Stenmark, B. Meyrick, N. Galie, W. J. Mooi, and I. F. McMurtry, "Animal models of pulmonary arterial hypertension: the hope for etiological discovery and pharmacological cure," Am. J. Physiol. Lung Cell Mol. Physiol., vol. 297, no. 6, pp. L1013-1032, Dec. 2009, doi: 10.1152/ajplung.00217.2009.
[12] S. Andersen et al., "A Pulmonary Trunk Banding Model of Pressure Overload Induced Right Ventricular Hypertrophy and Failure," J Vis Exp, no. 141, 29 2018, doi: 10.3791/58050.

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 studies in this project have been grouped into four different procedures:

PROCEDURE 1: INTERVENTION STUDIES USING THE 5.1 lid2h
The SuHx model in Sprague-Dawley rats is well-established (in use >7 years) in our department. The rats receive a single subcutaneous (s.c.) injection of SU5416, followed by 3-4 weeks transient exposure to 10% hypoxia and 2 weeks normoxic re-exposure. In the study a control group is included that receives a vehicle-injection and no hypoxia. Following confirmation of PAH induction with echocardiography, the animals are randomly divided into two groups: the intervention group and an vehicle-control group (Figure 4). After ~2-6 weeks of treatment (time-point will vary dependent on type of intervention), the experimental readouts as defined in Section 3.4.1 will be assessed by means of echocardiography of the heart (under anaesthesia), hemodynamic assessments via a catheter (under anaesthesia) and in a subset of the animals by MRI-imaging (MRI myocardial tagging, under anaesthesia). At the end of these assessments (Figure 4), the animals are sacrificed and blood and lung/cardiac tissues is collected for further analysis (histology, RNA analyses and protein analyses). The discomfort level of the SuHx model is considered to be moderate, to maximal 10% of the animals severe.

PROCEDURE 2: INTERVENTION STUDIES USING THE PULMONARY ARTERY BANDING (PAB) MODEL
Rats are subjected to pulmonary artery banding for 6-8 weeks. A sham-operated control group is included in the study that will not receive the pulmonary artery banding (Figure 4). Upon verification of PH induction in the animals that have received the pulmonary artery banding compared to controls (via echocardiography of the heart), the intervention will be started (Figure 4). The PH-rats will be randomly allocated to the intervention group or the placebo-control (vehicle-treated) group. After ~6 weeks of treatment (time-point will vary dependent on type of intervention), the experimental readouts as defined in Section 3.4.1 will be assessed by means of echocardiography of the heart (e.g. measurements of the RV wall thickness, under anaesthesia), hemodynamic assessments of the heart/lung (e.g. RV pressure-volume loops) via a catheter (under anaesthesia). A subset of the treated animals is subjected to MRI myocardial tagging and/or DT-MRI quantification of the helical muscle fiber architecture in the RV. A DT-MRI quantification of the helical muscle fiber architecture in the RV will be performed *ex vivo*, since bulk cardiac motion *in vivo* may lead to image artefacts. Hearts will be perfused and fixated to keep the RV

open, and the diffusion-weighted images will be acquired. In this way, we will get information on the fiber orientation of the RV. At the end of these experiments, all animals are sacrificed and blood and tissue (heart and lung) is collected for subsequent analysis (histology, RNA analyses and protein analyses). The discomfort level of the PAB model is considered to be moderate to severe, depending on the inner diameter of the clip around the pulmonary artery.

PROCEDURE 3: INTERVENTION STUDIES USING THE CHRONIC HYPOXIA MOUSE MODEL
The wildtype versus knock-in/knock-out mice will be exposed to hypoxia (10% O_2, chronically) for 3 weeks. After that, the animals are randomly divided into two groups: the intervention group and an vehicle-control group (Figure 4). The effect of the genetic interventions will be tested when PAH has developed in the mice subjected to the chronic hypoxia model. The mice are anesthetized and hemodynamic measurements are performed (among others right ventricular pressure with a Millar catheter) and vascular leakage is measured - see also Section 3.4.1. Following exsanguination, serum and plasma samples are taken, and heart and lung tissues are collected for tissue, protein and RNA analyses. The discomfort level of the chronic hypoxia mouse model is considered mild to moderate.

PROCEDURE 4: INTERVENTION STUDIES USING THE PNEUMONECTOMY+SU5146 MOUSE MODEL
The wildtype versus knock-in/knock-out mice will be exposed to the pneumonectomy (PNX) +SU5416 model. In this model, the left lung will be surgically removed (pneumonectomy), which results in increased pulmonary blood flow and vascular remodelling. A single administration of SU5416 one week after the surgery will result in severe PAH and RV dysfunction. The effect of the genetic interventions will be tested when PAH has developed in the mice subjected to the PNX+SU5416 model. The mice are anesthetized and hemodynamic measurements are performed. Following exsanguination, serum and plasma samples are taken, and heart and lung tissues are collected for tissue, protein and RNA analyses.

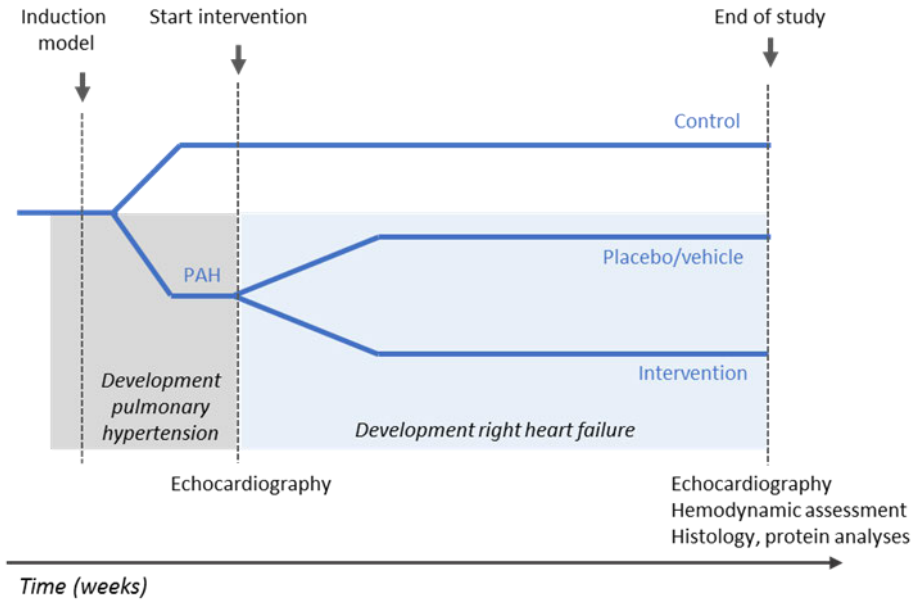

Figure 4: Graphical illustration of the approach that will be taken to test the therapeutic efficacy of the selected interventions in the animal models for PAH. The intervention will be administered after pulmonary hypertension has developed. Development of PAH will be confirmed compared to a control group, in which no PAH will be induced. The animals with confirmed PAH will be randomly allocated to the group that receives the intervention, or the placebo/vehicle-treated group.

ESTIMATED NUMBER OF ANIMALS/DISCOMFORT LEVEL

The estimated number of animals needed for each type of procedures and an indication of discomfort levels per procedure is provided in Table 1. Further details on animal numbers and discomfort levels will be provided in the corresponding Appendices.

Table 1: Estimated animal numbers and discomfort levels per procedure.

Procedure	Goal of study	# Mice	Discomfort level	% at risk*	# Rats	Discomfort level	% at risk*
1.	Intervention studies using SuHx model (rat)	n/a	Mild	n/a	300	Mild	20
			Moderate	n/a		Moderate	78
			Severe	n/a		Severe	2
2.	Intervention studies using PAB model (rat)	n/a	Mild	n/a	300	Mild	20
			Moderate	n/a		Moderate	70
			Severe	n/a		Severe	10
3.	Intervention studies using chronic hypoxia model (mouse)	320	Mild	15	n/a	Mild	n/a
			Moderate	80		Moderate	n/a
			Severe	5		Severe	n/a
4.	Intervention studies using pneumonectomy (Pnx) + SU5146 mouse model	336	Mild	15	n/a	Mild	n/a
			Moderate	80		Moderate	n/a
			Severe	5		Severe	n/a

***Expected risk of premature death due to PAH is max. 10%.**

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.

The main aim of the studies in this proposal is to examine the effect of novel (combinations of) interventions for PAH. To this end, four different animal models are used (Appendices 1-4). Each study in the project will start with selection of an appropriate model to test the intervention. Firstly, the animal model should contain the appropriate target (see Figure 3). Secondly, the level of discomfort should be considered. If no previous *in vivo* data are present on the new intervention, the relevant model with the lowest possible level of discomfort will be selected for initial *in vivo* studies. If good quality data are already available from previous (own or published) data, more stringent and complex models may be selected from the outset.

In general, a first *in vivo* intervention study will be performed using the SuHx or PAB model (Appendix 1, 2). If these initial *in vivo* tests are unsuccessful, the investigational intervention will be discontinued (GO/NO GO DECISION). If initial *in vivo* tests are successful, it will in general be necessary to perform confirmatory studies in the other models (Appendix 1-4). Confirmatory studies may also be performed on models lacking the appropriate target, as a negative control to understand or confirm the mechanism of action.

Depending on the type of intervention, preliminary studies may also be necessary, as outlined in Figure 5. Prior to using interventions that have never previously been tested *in vivo*, particularly for new pharmacotherapeutic interventions, a pilot pharmacokinetics study will be performed to determine the most optimal dose, route and schedule. If the study aims to test a new combination of interventions, a pharmacokinetics study will determine potential drug-drug interactions. When no data is available on previous dose-finding of pharmacological interventions, tolerability studies and/or dose-finding studies will also be performed to determine the maximum tolerated dose. If the highest safe dose is not expected to provide sufficient systemic exposure for efficacy, further *in vivo* studies will be halted (GO/NO GO DECISION).

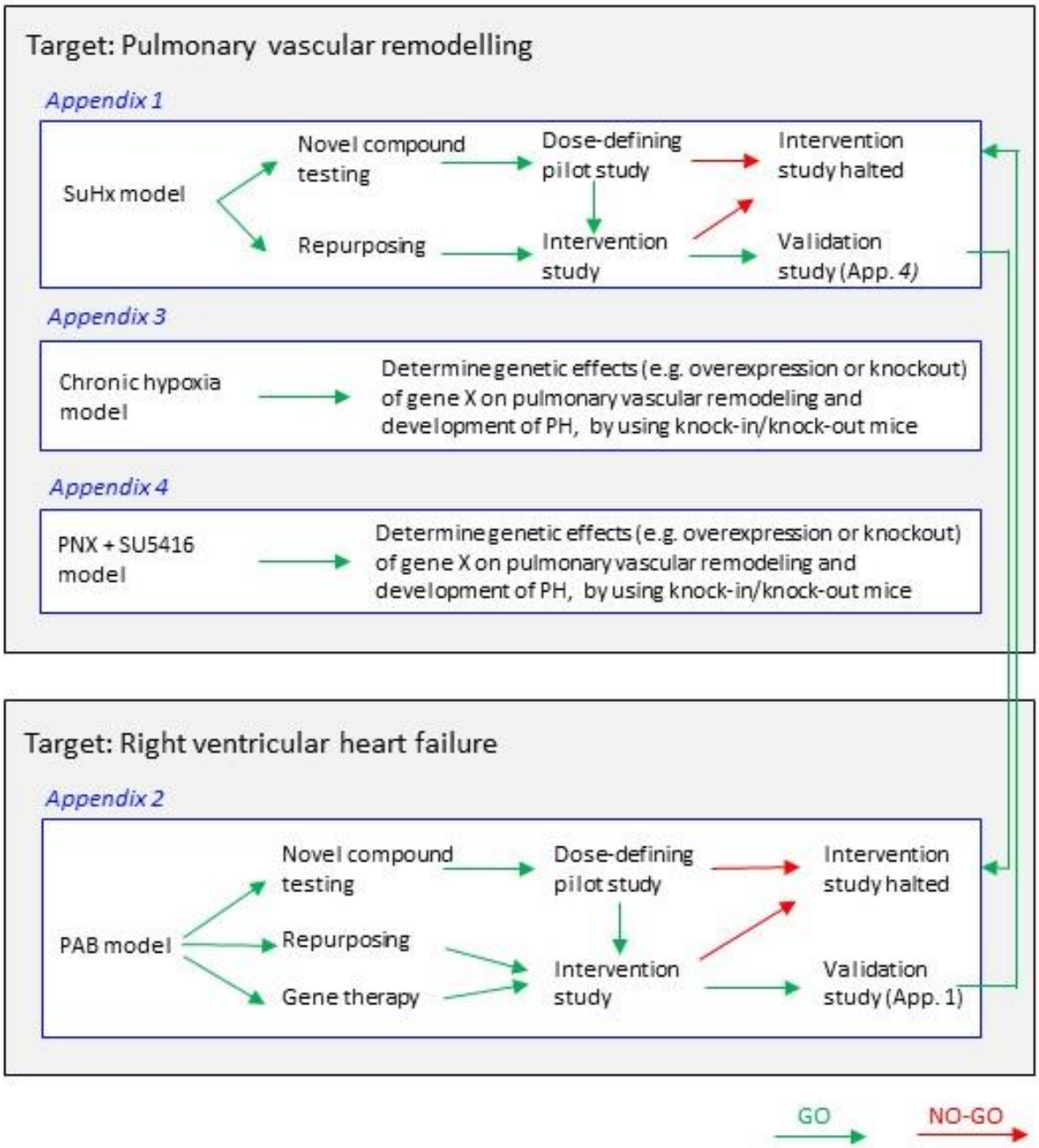

Figure 5: Flow charts summarizing the general research strategy and the key decision/selection points. The SuHx model will be used to test compounds (either novel compounds or compounds that are already used for other purposes ("repurposing")) that target the pulmonary vascular remodelling. When the compound shows positive effects, the PAB model will be used to validate these results.
The mouse models (Chronix hypoxia and PNX+SU5416) will be used to determine genetic effects by using knock-in/knock-out mice. No compounds will be tested in these models. Because both mouse models are new to our department, it is not decided when to use the Chronic hypoxia model or the PNX+SU5416 model. This will depend on the Pilot Set-up Model experiments, which are followed by a GO / NO-GO decision (fig. 3).
The PAB model will be used to test compounds that target right ventricular heart failure. When the compound shows positive effects, the SuHx model will be used to validate these results.
SuHx: SU5416 & Hypoxia, PNX: pneumonectomy, PH: Pulmonary Hypertension, PAB: Pulmonary Artery Banding.

Thus, subsequent research steps will be determined based on study outcome:

- If the study has been conducted technically satisfactory and the outcome is conclusive and negative, the study will be halted.
- If the study results are not conclusive, but warrant further exploration, the experiment will be redesigned and repeated, provided that improvements are feasible. If not, the study will be halted.
- If the study has been conducted technically satisfactory and the outcome is positive:
  i. the study may be considered completed,
     or
  ii. efficacy may need to be confirmed in an additional, usually more stringent, model,
     or
  iii. further testing may be needed to support translation to clinical studies, such as testing at adapted dose levels or with different dosing schedules.

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	Intervention studies using SuHx model
2	Intervention studies using PAB model
3	Intervention studies using Chronic hypoxia mouse model
4	Intervention studies using Pneumonectomy (Pnx)-Su5416 mouse model
5	
6	
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'.	5.1 lid2h
1.2	Provide the name of the licenced establishment.	5.1 lid2h
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: 1 — Type of animal procedure: Intervention studies using SuHx model

2 Description of animal procedures

A. Experimental approach and primary outcome parameters

Describe the general design of the animal procedures in relation to the primary outcome parameters. Justify the choice of these parameters.

This Appendix describes the use of the 5.1 lid2h to validate novel therapeutic interventions for PAH. In the 5.1 lid2h pulmonary vascular remodelling and right heart failure are induced by the combined exposure to the vascular endothelial growth factor receptor (VEGFR) inhibitor SU5416 and hypoxia. A single administration of SU5416 is followed by 3-4 weeks transient exposure to hypoxia and 2 weeks normoxic re-exposure. Extensive pulmonary vascular remodelling and first signs of right ventricle dysfunction is observed after 5-6 weeks. Upon verification of PAH induction in the animals (via echocardiography of the heart), typically 5-6 weeks after induction of the model, the intervention will be started. Only interventions in this project are included that act on an identified PAH target (or candidate target) as demonstrated by previous fundamental research or clinical research – see project proposal Figure 2). The interventions act on 7 different established targets for PAH, namely BMPR2 signalling, vascular tone, RV diastolic stiffness, inflammation, coagulation, integrin and VHL gene expression. Should new interventions be identified during the course of this project addressing these targets, they will be added to the study. The interventions will be tested when PAH has developed in the SuHx model (therapeutics vs preventive), as this study design has the highest clinical relevance. We will particularly focus in these studies on assessing the impact of the intervention on RV overload and pulmonary vascular remodelling, as both are clinical relevant end-points for PAH. The primary outcomes measures include the time (in days) from PAH induction to clinical manifestation of right heart failure; lung and right heart (RV) function and structure (assessed via imaging and tissue/protein analysis). The advanced blood serum and tissue analyses are also included to study the mechanisms underlying the chosen genetic interventions and to possibly identify new targets (in control animals).

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

Induction SuHx model
Sprague-Dawley rats receive a single subcutaneous (s.c.) injection of the vascular endothelial growth factor receptor (VEGFR) inhibitor SU5416 (25 mg/kg in CMC), followed by 3-4 weeks transient exposure to 10% hypoxia in a hypoxia chamber and 2 weeks normoxic re-exposure). In the study, a control group is included that receives a vehicle-injection and no hypoxia.

Follow-up development and progression
Pulmonary vascular remodelling and a mild increase in right ventricular pressures are observed after 5-6 weeks. To verify PAH induction, the rats are subjected to echocardiography of the heart. The right ventricular end-diastolic diameter (RVEDD) and tricuspid annular plane systolic excursion (TAPSE), are measured according to standard protocols at our Department. Following confirmation of PAH induction with echocardiography, the SuHx-animals are randomly divided into two groups: an intervention group and a vehicle-control (for illustration of experimental design see Figure 1 below).

Interventions
Interventions include treatment by any kind of agent (e.g. dietary, chemical, biological, genetic, radiopharmaceutical), or combination of these agents. All agents will be administered by the appropriate route, time of day, duration and frequency as required. Examples include oral gavage, bolus injections (i.v., i.p., s.c.), continuous infusion in cannulated animals, minipumps and slow release pellets. Procedures requiring surgery (cannulation, implantation of minipumps) will be performed under general anaesthesia and analgesia. The selection of agent(s), dose, time of day, and route of application depends on the target of the intervention and the details of the treatment of each study will be discussed with the IvD.

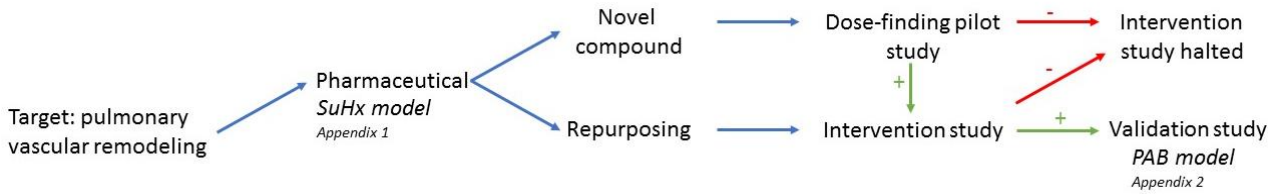

Figure 1. Study design SuHx studies, with GO / NO-GO decisions.

After ~2-6 weeks of treatment (time-point will vary dependent on type of intervention), the experimental readouts as defined in Section 3.4.1 of the project proposal will be assessed by means of echocardiography of the heart and hemodynamic assessments via a catheter (under anaesthesia).

Echocardiography
After treatment, all animals will be subjected to echocardiographic assessments (see above) under anaesthesia, to measure RV wall thickness (RVWT), RV end diastolic diameter (RVEDD), tricuspid annular plane systolic excursion (TAPSE), stroke volume (SV), heart rate (HR), cardiac output (CO), pulmonary artery acceleration time (PAAT).

Haemodynamic measurements
After the treatment period, rats are anaesthetized for hemodynamic assessment via open-chest RV catheterization. RV systolic pressure (RVSP) will be determined from steady state measurement, as well as RV afterload (Ea-Arterial elastance). Pressure-volume loops after vena-cava occlusion will be obtained and used to derive end-systolic elastance (Ees), and end-diastolic elastance (Eed). Arterial ventricular coupling will be calculated as Ees/Ea. After the haemodynamic measurements, the animals are sacrificed and blood and tissue (heart and lung) is collected for subsequent analysis.

Termination
At the end of the study, all animals are killed by an approved method (e.g. removal of blood and organs under anaesthesia or CO2 asphyxiation,) and blood and lung/cardiac tissues are collected for further analysis (histology, RNA analyses and protein analyses). The animals are maximally for 12 weeks in experiment.

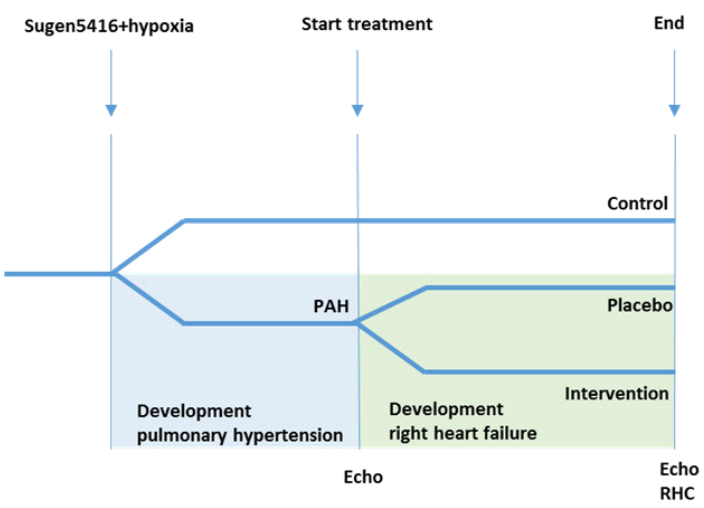

Figure 2. Experimental design SuHx studies. After two weeks of acclimatization we start with a single injection of Sugen5416 and start the 10% hypoxia period of 3-4 weeks (not the control group). At start treatment we perform an echocardiography and at the end experiments an echocardiography and right heart catheterization is performed. The animals are maximally for 12 weeks in experiment.

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

To demonstrate a 50% improvement in terms of pulmonary vascular remodeling between two groups (treatment vs control) with an overall variability of ~30%, a group size of 7 evaluable animals per group is needed (power > 0.8 with α = 0.05, two sided). Because our study design will be comprised of 3 groups (control, SuHx, SuHx+intervention) and 2 comparisons will be performed, an α of 0.025 (0.05/2) is defined as statistically significant. Therefore, an estimated group size of 10 evaluable animals will be needed to perform a 3-arm study. We typically use n=12 in model/intervention groups, n=6 in control group. This includes potential losses due to human end points and losses because of animals not developing pulmonary hypertension. The appropriateness of the chosen group sizes has been confirmed in previous studies conducted with the SuHx model at our Department and described in the literature.

B. The animals

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

Species: *Rattus norvegicus*, wild-type

Origin: Commercial breeder

Sex: Both male and female animals (equally divided) will be used throughout the project.

Justification: Sprague-Dawley rats (150-250 gr) will be used for the experiments. They will be 5-6 weeks of age at the start of the experiments and 12-16 weeks at sacrifice. The SuHx model is well-validated in this rat strain and for these stages of life, it is in use >7 years at our department.

Estimated numbers:
The SuHx model will be performed in 10 intervention studies during the project, with an average of ~30 rats per experiment (see above), resulting in an estimated total of 10 x 30 = 300 rats in total (in 5 years). The appropriateness of the chosen group sizes has been confirmed in previous studies conducted with the SuHx model at our Department and described in the literature.

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.

Replacement
All proposed interventions that will be tested throughout this project will be assessed first in other, non-animal, models, such as cell culture experiments. Only if these experiments yield sufficiently promising results, in vivo tests will be undertaken. In vivo experiments are a necessary step in development of interventions that may one day be used in the clinic, since currently no other methods allow the evaluation of the effect of an intervention on the organism as a whole. Cell culture, organoids, lab-on-a-chip or computer models are not (yet) sophisticated enough to assess the interaction between the PAH pathology and host environmental factors such as the oxygen supply, the immune system and metabolism.

Reduction
The proposed number of evaluable animals per study arm (n=12 in model/intervention groups, n=6 in control group) is calculated as described above, and is in line with generally accepted protocols in scientific literature and our previous studies with the SuHx model at our Department. Further reduction of the number of animals per group would decrease statistical power, and might thus disqualify the experiment.

Refinement
State-of-the-art methods and equipment for assessing pulmonary vascular remodelling and RV overload, in particular advanced imaging techniques, will be used to refine protocols and minimize discomfort to the animals especially in support of the application of humane endpoints. International guidelines will be applied to ensure that best practice is followed.

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

The procedures conducted under this protocol will inevitably cause discomfort for the animals in the studies. In order to minimize suffering, all studies will adhere to the national and internationally accepted rules for handling of lab animals [1], [2]. Under these rules, the animals will be humanely killed when a humane endpoint (see below) is reached. Within the institute, standard operating procedures (SOP) for animal handling are in place. These also include dedicated anaesthesia and analgesia (A&A) SOPs that will accompany invasive procedures to minimize pain and discomfort.
To minimize animal suffering, pain and fear the following measures will be taken. The animals will be allowed to acclimatize to their new environment for two weeks upon arrival at the animal facility. Animals are housed in groups (socially housed) at all times (also during hypoxia. Except during surgery, animals are returned to the group directly after surgery) and environmental enrichment strategies are applied in the cages to improve animal welfare.
In case the therapeutic interventions require surgical procedures (e.g. cannula/mini-pump implantation), this will be done under general anaesthesia in combination with pain treatment. During haemodynamic measurements, the animals will be kept under general anaesthesia in a temperature-controlled environment. During imaging procedures, animals will be kept under general anaesthesia in a temperature-controlled environment.
No adverse effects on the environment are expected because animals are kept and procedures are performed in a controlled environment, all waste will be safely discarded.

References used in this section:
[1] Zutphen, L. Van, Handboek proefdierkunde: proefdieren, dierproeven, alternatieven en ethiek. .
[2] J. Guillen, "FELASA Guidelines and Recommendations," J Am Assoc Lab Anim Sci, vol. 51, no. 3, pp. 311–321, May 2012.

Repetition and duplication

E. Repetition

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

N.A.

Accommodation and care

F. Accommodation and care

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

☐ No

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

The animals will be socially housed in standard conditions (food and water available ad libitum) and environmental enrichment strategies are applied in the cages to improve animal welfare conform the Directive 2010/63/EU. However, as part of the experimental model, the animals will also be housed under low oxygen conditions (10% O2) for 3-4 weeks (the chronic hypoxia period).

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.

In case the therapeutic interventions require surgical procedures (e.g. cannula/mini-pump implantation), this will be done under general anaesthesia in combination with perioperative pain treatment. General anaesthesia will also be applied in order to perform the haemodynamic measurements (via open-chest RV catheterization). For some procedures (e.g. echocardiography) anaesthesia will be applied in the absence of any risk for pain.
Within the institute, standard operating procedures (SOP) for animal handling are in place. These also include dedicated anaesthesia and analgesia (A&A) SOPs that will accompany invasive procedures to minimize pain and discomfort. These A&A SOPs describe the best practice methods for anaesthesia and

analgesia for each (surgical) procedure and are regularly checked as new concepts or procedures become available. All experiments performed within this project will conform to these A&A SOPs.

I. Other aspects compromising the welfare of the animals

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

Exposure to hypoxia (10% O2) will result in a temporary increase in respiration and pulse. Following a number of days in the hypoxia conditions, the respiration level and heartbeat will begin to acclimatize. It is our experience with hypoxia-induced animals that their growth in body weight falls ~10% behind compared to control animals. Apart from this observation, we have not observed any other physical or behavioural changes in the hypoxia-treated animals. Due to the SuHx model (and consequently, the induced right heart failure), the animals may lose weight, experience shortness in breath and become lethargic. Apart from discomfort directly caused by the procedures as described above, animals may develop complications due to the therapeutic interventions (e.g. toxic side-effects), which in some cases may result in adverse effects on the animals' welfare. These side-effects are still unknown as well as the discomfort the animal can experience. The maximum tolerated level of discomfort is moderate. When this is exceeded, an HEP will be applied.

Explain why these effects may emerge.

These effects are a consequence of the induction of PAH and right heart failure due to the SuHx model and the applied interventions respectively.

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

In general, adverse effects on the animals' welfare caused by induction of pulmonary vascular remodeling and RV pressure overload cannot be completely prevented. In order to minimize adverse effects, the animals will be monitored at a frequency that is dictated by the model (2 times per week) and timely killed when a humane endpoint (see below) is met. When profound weight drop occurs, daily monitoring will be applied. The CO2 level, humidity and temperature in the hypoxia chamber are kept constant and will not deviate from the by law defined norms.
Should unforeseen complications due to the interventions or procedures occur, either the effect of these complications will be minimized by adjusted procedures, such as providing easy access to food (mush-feeding), or if this is not possible, the humane endpoints as defined below will be taking into account.

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.

The most important humane endpoints applicable to all studies are:

- Weight loss $\geq$20% of maximum body weight in adult animals, measured from the start of the treatment
- Weight loss $\geq$10% of body weight during 24h, in combination with:
    - Sustained abnormal breathing, dyspnoea (symptom PAH/right heart failure)
    - Sustained lethargy (symptom PAH/right heart failure)
- Sustained abnormal behaviour
- Complications of interventions
- Other procedure-specific endpoints

Indicate the likely incidence.

It is our experience that the end stadium of PAH will be achieved in the SuHx model at maximally day 70 after induction of the model. At day 70, around 85-90% of the induced animals do not experience symptoms of right heart failure yet (sustained periods of shortness of breath, dyspnoea, lethargy). Thus, humane endpoints are expected to occur in <10% of all cases.

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').

Discomfort levels categorized according to the SuHx model are as follows:

- Animals that are subjected to the SuHx model, injection + chronic hypoxia, experience mild discomfort.
- Control animals, experience mild discomfort.

Discomfort levels categorized according to the interventions are as follows:

- Animals that receive therapeutic interventions during the study period, can experience moderate discomfort.
- Control animals (vehicle treated) during the study period, can experience moderate discomfort.

Discomfort levels categorized according to the procedures used for assessment of pulmonary vascular remodelling and right ventricular pressures (echocardiography, haemodynamic measurements) are as follows:

- Echocardiography: mild discomfort.
- Haemodynamic measurements, using general anaesthesia: non-recovery.

Other procedures that will be used, which are not expected to alter the total level of discomfort experienced:

- Simple well tolerated interventions (e.g. drug treatment): mild discomfort.
- Simple but frequent handling procedures (e.g. weighing): mild discomfort.
- Minimally invasive procedures and those requiring anaesthesia (e.g. non-invasive imaging): mild discomfort.

Table 1: Procedures and discomfort classification.

Procedures	**Category**	**Expected percentage (%) of animals**	**Frequency and duration of the procedure**
1. Obtaining rats: Transport to animal facility	mild	100%	1x
2. Induction SuHx model: a. Injection b. Induction hypoxia	 mild moderate	 100% 80%	 1x 1x 3-4 weeks
3. Echocardiography (pre-treatment)	mild	100%	1x ~10min
4. Applying therapeutic interventions a. Frequent handling procedures b. Bolus injections (i.v., i.p., s.c, oral gavage) c. Procedures requiring surgery (cannulation, implantation of minipumps) under brief adequate anaesthesia and postoperative analgesia d. Local infusion, either acute or chronic through a cannula	 mild moderate moderate moderate	 100% 80% 25% 25%	 Max 4 weeks Max 4 weeks 1x ~30-60 min Max 4 weeks
5. Potential adverse effects of treatments*	max. moderate	40%	Max 4 weeks
6. Heart failure	severe	40%	Max 2 days
6. Echocardiography (post-treatment)	non-recovery	100%	1x ~10 min

7.Heamodynamic measurements (under general anaesthesia and analgesia)	non-recovery	100%	1x ~30 min
9. Blood sampling	mild	25%	Max 2x per week <2 min
10. Sacrifice	non-recovery	100%	1x <1 min

* This is based on previous experience, the adverse effects may vary between light and moderate discomfort. When the discomfort exceeds moderate an HEP will be applied.

Based on this table, we expect that cumulative discomfort for the SuHx groups (80%) will be moderate to severe. For the control groups (20%) this will be mild.

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 animals will be humanely killed at the end of the procedure, to collect large blood samples and tissues for further analysis. Also, animals will also be humanely killed in the case when one of the humane end-points will be reached.

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'.	5.1 lid2h
1.2	Provide the name of the licenced establishment.	5.1 lid2h
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: 2 Type of animal procedure: Intervention studies using PAB model

2 Description of animal procedures

A. Experimental approach and primary outcome parameters

Describe the general design of the animal procedures in relation to the primary outcome parameters. Justify the choice of these parameters.

This Appendix describes the use of the pulmonary artery banding (PAB) model in rats to validate novel therapeutic interventions for PAH. This model has to be implemented and optimized at our department. The PAB rat model is a surgical model mimicking the increased afterload on the right heart as occurs in PAH as well as in other forms of pulmonary hypertension. Rats are subjected to pulmonary artery banding for 6-8 weeks (a sham-operated control group is included). Upon verification of pressure overload in the animals that have received the pulmonary artery banding compared to controls (via echocardiography of the heart), the intervention will be started. Only interventions in this project are included that act on an identified PAH target (or candidate target) as demonstrated by previous fundamental research or clinical research – see project proposal Figure 2. The interventions act on 7 different established targets for PAH, namely BMPR2 signaling, vascular tone, RV diastolic stiffness, inflammation, coagulation, integrin and VHL gene expression. Should new interventions be identified during the course of this project addressing these targets, they will be added to the study. The interventions will be tested when pressure overload has developed in the PAB model (therapeutics vs preventive), as this study design has the highest clinical relevance. We will particularly focus in these studies on assessing the impact of the intervention on RV overload. The primary outcomes measures include the time (in days) from afterload induction to clinical manifestation of right heart failure; lung and right heart (RV) function and structure (assessed via imaging and tissue/protein analysis). The advanced blood serum and tissue analyses are also included to study the mechanisms underlying the chosen genetic interventions and to possibly identify new targets (in control animals).

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

Induction PAB model
The pulmonary artery banding rat model is a surgical model. The rats are put under general anaesthesia and analgesia, the chest is opened and a titanium clip is compressed around the pulmonary artery with a modified ligating clip applier. By using different sizes of pulmonary artery constriction, a phenotype of moderate RV dysfunction and severe RV failure can be generated. Following the surgical procedure, the rats are daily monitored and receive pain medication for at least two days. Rats are subjected to pulmonary artery banding for 6-8 weeks [1]. A sham-operated control group is included in the study that will not receive the titanium clip

Follow-up development and progression
After the surgery, growth of the animal ensures a progressive increase in right ventricular pressure. To verify induction of pressure overload, the rats are subjected to echocardiography of the heart. Pulmonary artery acceleration time (PAAT/cl, estimate of RV pressure), right ventricular end-diastolic diameter (RVEDD, dilatation) and tricuspid annular plane systolic excursion (TAPSE, RV dysfunction), are measured according to standard protocols at our Department. Following confirmation of pressure overload with echocardiography, the animals are randomly divided into two groups: the intervention group and a vehicle-control group (for illustration of experimental design see Figure 1 below).

Interventions
Interventions include treatment by any kind of agent (e.g. dietary, chemical, biological, genetic, radiopharmaceutical), or combination of these agents. All agents will be administered by the appropriate route, time of day, duration and frequency as required. Examples include oral gavage, bolus injections (i.v., i.p., s.c.), continuous infusion in cannulated animals, minipumps and slow release pellets. Procedures requiring surgery (cannulation, implantation of minipumps) will be performed under general anaesthesia and analgesia. The selection of agent(s), dose, time of day, and route of application depends on the target of the intervention and the details of the treatment of each study will be discussed with the IvD.

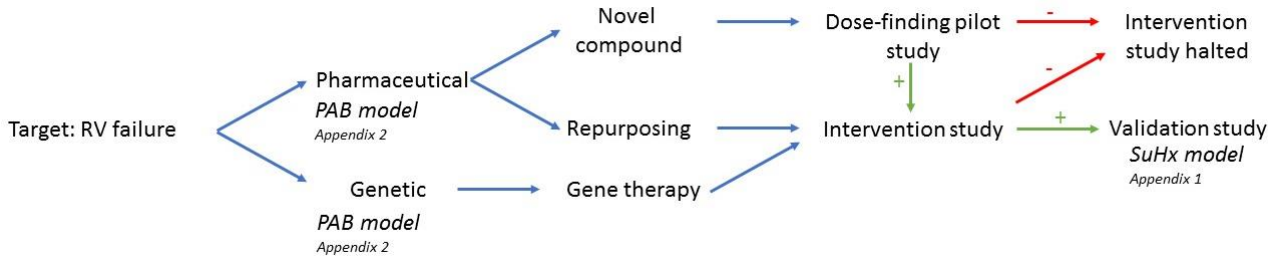

Figure 1. Study design PAB studies with GO / NO-GO decisions.

After ~2-6 weeks of treatment (time-point will vary dependent on type of intervention), the experimental readouts as defined in Section 3.4.1 of the project proposal will be assessed by means of echocardiography of the heart, hemodynamic assessments via a catheter (under anaesthesia) and in a subset of the animals by MRI-imaging (MRI myocardial tagging or DT-MRI, under anaesthesia).

Echocardiography
After treatment, all animals will be subjected to echocardiographic assessments (see above) under anaesthesia, to measure RV wall thickness (RVWT), RV end diastolic diameter (RVEDD), tricuspid annular plane systolic excursion (TAPSE), stroke volume (SV), heart rate (HR), cardiac output (CO) and pulmonary artery acceleration time (PAAT).

Hemodynamic measurements
After echocardiography, hemodynamic assessments are done via open-chest RV catheterization. RV systolic pressure (RVSP) will be determined from steady state measurement, as well as RV afterload (Ea-Arterial elastance). Pressure-volume loops after vena-cava occlusion will be obtained and used to derive end-systolic elastance (Ees), and end-diastolic elastance (Eed). Arterial ventricular coupling will be calculated as Ees/Ea. After the haemodynamic measurements, the animals are sacrificed and blood and tissue (heart and lung) is collected for subsequent analysis.

Imaging
A subset of the treated animals is subjected to MRI-imaging (MRI myocardial tagging, under anaesthesia) and/or DT-MRI quantification of the helical muscle fiber architecture in the RV (Figure 1

below). A DT-MRI quantification of the helical muscle fiber architecture in the RV will be performed ex vivo, since bulk cardiac motion in vivo may lead to image artefacts. Hearts will be perfused and fixated to keep the RV open, and the diffusion-weighted images will be acquired. In this way, we will get information on the fiber orientation of the RV.

Termination
At the end of the study, all animals are killed by an approved method (e.g. removal of blood and organs under anaesthesia or CO2 asphyxiation,) and blood and lung/cardiac tissues are collected for further analysis (histology, RNA analyses and protein analyses). The animals are maximally for 12 weeks in the experiment.

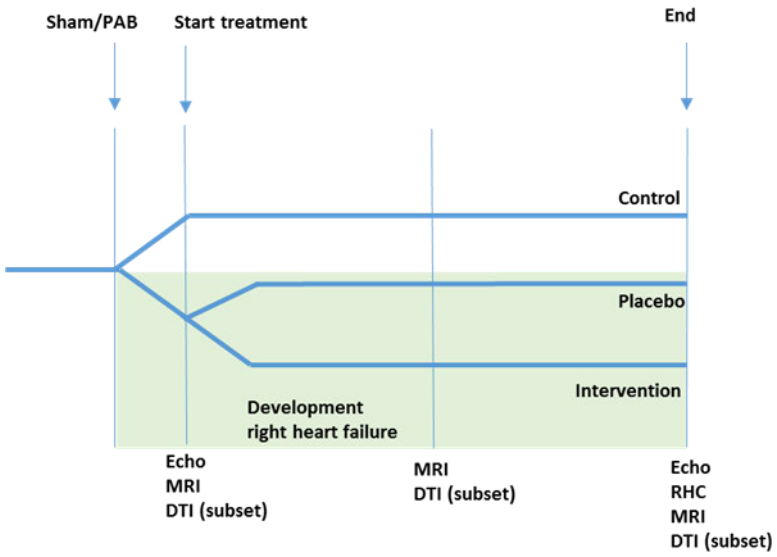

Figure 2. Experimental design PAB studies. After two weeks of acclimatization, animals undergo PAB or sham operation. At start treatment (1-2 weeks after PAB/sham surgery) we perform an echocardiography and at the end experiments an echocardiography and right heart catheterization is performed. The animals are maximally for 12 weeks in the experiment.

References used in this section:
[1] Andersen S, et al. Pulmonary Trunk Banding Model of Pressure Overload Induced Right Ventricular Hypertrophy and Failure. J Vis Exp. 2018 Nov 29;(141). doi: 10.3791/58050. PMID: 30582605.

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

To demonstrate a 50% improvement in terms of RV function between two groups (treatment vs control) with an overall variability of ~30%, a group size of 7 evaluable animals per group is needed (power > 0.8 with α = 0.05, two sided). Because our study design will be comprised of 3 groups (sham, PAB, PAB+intervention) and 2 comparisons will be performed, an α of 0.025 (0.05/2) is defined as statistically significant. Therefore, an estimated group size of 10 evaluable animals will be needed to perform a 3-arm study. We typically use n=12 in model/intervention groups, n=6 in control group. This includes potential losses due to human end points. The appropriateness of the chosen group sizes has been confirmed in previous studies conducted with the PAB model at our Department and described in the literature.

B. The animals

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

Species: *Rattus norvegicus*, wild-type

Origin: Own breeding or university or commercial breeder.

Sex: Both male and female animals (equally divided) will be used throughout the project.

Justification: Wistar rats will be used for the experiments. They will be 4 to 6 weeks of age at the start of the experiments and ~14 weeks at sacrifice.

Estimated numbers:
The PAB model will be performed in 10 intervention studies during the project, with an average of ~30 rats per experiment (see above), resulting in an estimated total of 10 x 30 = 300 rats in total (in 5 years). The appropriateness of the chosen group sizes has been confirmed in previous studies conducted with the PAB model at our Department and described in the literature.

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.

Replacement
All proposed interventions that will be tested throughout this project will be assessed first in other, non-animal, models, such as cell culture experiments. Only if these experiments yield sufficiently promising results, in vivo tests will be undertaken. In vivo experiments are a necessary step in development of interventions that may one day be used in the clinic, since currently no other methods allow the evaluation of the effect of an intervention on the organism as a whole. Cell culture, organoids, lab-on-a-chip or computer models are not (yet) sophisticated enough to assess the interaction between the PAH pathology and host environmental factors such as the oxygen supply, the immune system and metabolism.

Reduction
The proposed number of evaluable animals per study arm (n=12 in model/intervention groups, n=6 in control group) is calculated as described above, and is in line with generally accepted protocols in scientific literature and our previous studies with the PAB model. Further reduction of the number of animals per group would decrease statistical power, and might thus disqualify the experiment.

Refinement
State-of-the-art methods and equipment for assessing pulmonary vascular remodelling and RV overload, in particular advanced imaging techniques, will be used to refine protocols and minimize discomfort to the animals especially in support of the application of humane endpoints. International guidelines will be applied to ensure that best practice is followed.

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

The procedures conducted under this protocol will inevitably cause discomfort for the animals in the studies. In order to minimize suffering, all studies will adhere to the national and internationally accepted rules for handling of lab animals [2, 3]. Under these rules, the animals will be humanely killed when a humane endpoint (see below) is reached. Within the institute, standard operating procedures (SOP) for

animal handling are in place. These also include dedicated anaesthesia and analgesia (A&A) SOPs that will accompany invasive procedures to minimize pain and discomfort.
To minimize animal suffering, pain and fear the following measures will be taken. The animals will be allowed to acclimatize to their new environment for two weeks upon arrival at the animal facility. Animals are housed in groups (socially housed) at all times (except during surgery, animals are returned to the group directly after surgery) and environmental enrichment strategies are applied in the cages to improve animal welfare.
The PAB surgical model is performed under general anaesthesia and analgesia (pre- and post-surgery). During haemodynamic measurements, animals will be kept under anaesthesia in a temperature-controlled environment. During imaging procedures, animals will be kept under anaesthesia in a temperature-controlled environment.
No adverse effects on the environment are expected because animals are kept and procedures are performed in a controlled environment, all waste will be safely discarded.

References used in this section:
[2] Zutphen, L. Van, Handboek proefdierkunde: proefdieren, dierproeven, alternatieven en ethiek.
[3] J. Guillen, "FELASA Guidelines and Recommendations," J Am Assoc Lab Anim Sci, vol. 51, no. 3, pp. 311–321, May 2012.

Repetition and duplication

E. Repetition

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

N.A.

Accommodation and care

F. Accommodation and care

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

☒ No

☐ 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.

The PAB surgical model is performed under general anaesthesia and analgesia (pre- and post-surgery). In case the therapeutic interventions require surgical procedures (e.g. cannula/mini-pump implantation), this will be done under general anaesthesia in combination with perioperative pain treatment. General anaesthesia will also be applied in order to perform the haemodynamic measurements (via open-chest RV catheterization). For some procedures (e.g. imaging) anaesthesia will be applied in the absence of any risk for pain.
Within the institute, SOPs for animal handling are in place. These also include dedicated A&A SOPs that will accompany invasive procedures to minimize pain and discomfort. These A&A SOPs describe the best practice methods for anaesthesia and analgesia for each (surgical) procedure and are regularly checked as new concepts or procedures become available. All experiments performed within this project will conform to these A&A SOPs.

I. Other aspects compromising the welfare of the animals

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

Due to the PAB model (and consequently, the induced right heart failure), the animals may lose weight, experience shortness in breath (dyspnea) and can become lethargic. Apart from discomfort directly caused by the procedures as described above, animals may develop complications due to the therapeutic interventions (e.g. toxic side-effects), which in some cases may result in adverse effects on the animals' welfare. These side-effects are still unknown as well as the discomfort the animal can experience. The maximum tolerated level of discomfort is moderate. When this is exceeded, an HEP will be applied.

Explain why these effects may emerge.

These effects are a consequence of the induction of pressure overload (right heart failure) due to the PAB model and the applied interventions respectively.

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

In general, adverse effects on the animals' welfare caused by induction of RV pressure overload cannot be completely prevented. In order to minimize adverse effects, the animals will be monitored at a frequency that is dictated by the model (2 times per week) and timely killed when a humane endpoint (see below) is met. When profound weight drop occurs, daily monitoring will be applied. Should unforeseen complications due to the interventions or procedures occur, either the effect of these complications will be minimized by adjusted procedures, such as providing easy access to food (mush-feeding), or if this is not possible, the humane endpoints as defined below will be taking into account.

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.

The most important humane endpoints applicable to all studies are:

- Permanent weight loss $\geq$20% of initial body weight in adult animals, measured from the start of the treatment
- Weight loss $\geq$10% of body weight during 24h, in combination with:
  - Sustained abnormal breathing, dyspnea (symptom PAH/right heart failure)
  - Sustained lethargy (symptom PAH/right heart failure)
- Sustained abnormal behavior
- Complications of interventions

- Other procedure-specific endpoints

Indicate the likely incidence.

Humane endpoints expected to occur in <10% of all cases.

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').

Discomfort levels categorized according to the PAB model are as follows:

- Animals that are subjected to the PAB surgical model, under general anaesthesia and analgesia, experience moderate discomfort.
- Animals that are sham-operated, under general anaesthesia and analgesia, experience moderate discomfort.

Discomfort levels categorized according to the interventions are as follows:

- Animals that receive therapeutic interventions during the study period, can experience moderate discomfort.
- Control animals (vehicle-treated) during the study period, can experience moderate discomfort.

Discomfort levels categorized according to the procedures used for assessment of pulmonary vascular remodelling and right ventricular pressures (echocardiography, haemodynamic measurements, imaging) are as follows:

- Echocardiography: mild discomfort.
- Haemodynamic measurements, using general anaesthesia: non-recovery.
- More intensive imaging procedures and those requiring prolonged anaesthesia (e.g. MRI, DTI-MRI quantification): moderate.

Other procedures that will be used, which are not expected to alter the total level of discomfort experienced:

- Simple well tolerated interventions (e.g. drug treatment): mild discomfort.
- Simple but frequent handling procedures (e.g. weighing): mild discomfort.
- Minimally invasive procedures and those requiring anaesthesia (e.g. non-invasive imaging): mild discomfort.

Table 1: Procedures and discomfort classification.

Procedures	**Category**	**Expected percentage (%) of animals**	**Frequency and duration of the procedure**
1. Obtaining rats: Transport to animal facility	mild	100%	1x
2. Induction PAB model: a. Surgical procedure (under general anaesthesia and analgesia)	moderate	80%	1x ~90 min
3. Echocardiography (pre-treatment)	mild	100%	1x ~10 min

4. Applying therapeutic interventions a. Frequent handling procedures b. Bolus injections (i.v., i.p., s.c, oral gavage) c. Procedures requiring surgery (cannulation, implantation of minipumps) under brief adequate anaesthesia and postoperative analgesia d. Local infusion, either acute or chronic through a cannula	 mild moderate moderate moderate	 100% 80% 25% 25%	 Max 4 weeks Max 4 weeks 1x ~30-60 min Max 4 weeks
5. Potential adverse effects of treatments*	max. moderate	40%	Max 4 weeks
6. Heart failure	severe	40%	Max 2 days
7. Echocardiography (post-treatment)	mild	100%	1x ~10 min
8. Haemodynamic measurements (under general anaesthesia and analgesia)	non-recovery	100%	1x ~30 min
9. MRI	moderate	25%	Max. 3x ~2.5 hours
10. Blood sampling	mild	25%	Max 2 per week <2 min
11. Sacrifice	non-recovery	100%	1x <1 min

* This is based on previous experience, the adverse effects may vary between light and moderate discomfort. When the discomfort exceeds moderate an HEP will be applied.

Based on this table, we expect that cumulative discomfort for the PAB groups (80%) will be moderate to severe. For the control groups (20%) this will be mild to 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.

The animals will be humanely killed at the end of the procedure, to collect large blood samples and tissues for further analysis. Also, animals will also be humanely killed in the case when one of the humane end-points will be reached.

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'.	5.1 lid2h
1.2	Provide the name of the licenced establishment.	5.1 lid2h
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: 3 Type of animal procedure: Intervention studies using Chronic hypoxia mouse model

2 Description of animal procedures

A. Experimental approach and primary outcome parameters

Describe the general design of the animal procedures in relation to the primary outcome parameters. Justify the choice of these parameters.

This Appendix describes the use of chronic hypoxia mouse model to validate genetic targets that are promising as novel therapeutic intervention for PAH. This model has to be implemented and optimized at our department. To test the involvement of target genes in pulmonary vascular or right ventricular (RV) remodelling, knock-in and knock-out mice will be tested in this model. The target genes include identified PAH targets or candidate targets obtained by fundamental research (e.g. genetical, molecular, cellular, histopathological studies on tissue of animal models) or clinical research (e.g. genetic studies on human samples or other observations, such as fMRI) such as, BMPR2 signalling, vascular tone, RV diastolic stiffness, inflammation, coagulation, integrin and VHL gene expression. Should new targets be identified during the course of this project, they will be added to the study. The effect of the genetic interventions will be tested when PAH has developed in the mice subjected to the chronic hypoxia model. We will particularly focus in these studies on assessing the impact of the intervention on RV overload and pulmonary vascular remodelling, as both are clinical relevant end-points for PAH. The primary outcomes measures include the time (in days) from PAH induction to clinical manifestation of right heart failure; lung and right heart (RV) function and structure (assessed via imaging and tissue/protein analysis). The advanced blood serum and tissue analyses are also included to study the mechanisms underlying the chosen genetic interventions and to possibly identify new targets (in wild-type experimental animals).

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

Genetic interventions

Knock-in or knock-out mice will be obtained from commercial suppliers or from collaborating institutions. The current proposal does not involve the generation of new genetic models. After obtaining knock-in or knock-out models from institutions abroad, genetic models will be imported in our animal facility via embryo transfer to guarantee that animals are free from pathogenic micro-organisms. A number of genes of interest (GOI) that will be studied can only be knocked-out at an adult age to prevent developmental problems. Examples include VEGFR2/KDR and SOX17. To achieve selective, inducible gene knockout, we will breed mice carrying loxP-flanked GOI with mice carrying tamoxifen-inducible Cre recombinase under control of the tissue of interest (e.g. VE-cadherin). Induction of Cre-recombinase will be performed by a 5-day course of tamoxifen-supplemented non-pelleted dry feed.

Induction chronic hypoxia model

The wildtype versus knock-in/knock-out mice will be exposed to chronic hypoxia (10% O_2) by placing them for 2 weeks in a hypoxia chamber. In the study, a control group is included that receives no hypoxia (for illustration of experimental design see Figure 1 below).

Echocardiography

To verify development of pulmonary vascular remodelling and a mild increase in right ventricular pressures, the mice are subjected echocardiography of the heart according to standard protocols at our Department.

Haemodynamic measurements

Mice are anesthetized for hemodynamic measurements via open-chest RV catheterization. RV systolic pressure (RVSP) will be determined from steady state measurement. After the haemodynamic measurements, the animals are sacrificed and blood and tissue (heart and lung) is collected for subsequent analysis.

Measurements of vascular leakage

After induction of anaesthesia, mice will receive a tail vein injection with 150µL 1% Evans Blue/phosphate buffered saline, which will be left circulating for one hour (under continuous anaesthesia). After 1 hour a catheter will be placed in the right ventricle, and the mice will be sacrificed by perfusion with phosphate buffered saline. Subsequently, organs will be harvested and processed for measurement of Evans Blue in the organs.

Termination

At the end of the study, animals will be killed by an approved method (e.g. Removal of blood and organs under anaesthesia or CO_2 asphyxiation, cervical dislocation, terminal anaesthesia). Following exsanguination, serum and plasma samples are taken, and heart and lung tissues are collected for tissue, protein and RNA analyses. The animals are maximally for 12 weeks in experiment.

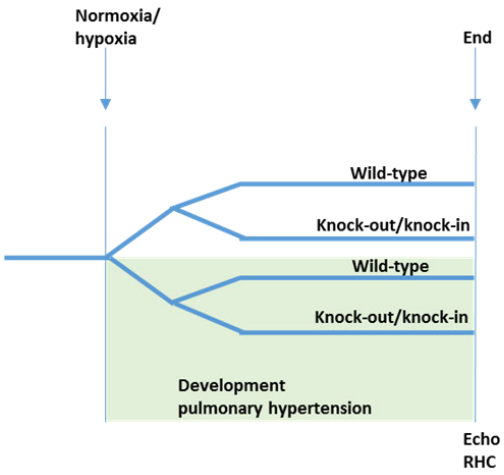

Figure 1. Experimental design studies with chronic hypoxia mouse model. After 2 weeks of acclimatization we start with the 10% hypoxia period of 2 weeks (not the control group). At the end experiments an echocardiography and right heart catheterization is performed. The animals are maximally for 12 weeks in experiment.

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

A typical intervention study will comprise several study arms, including control (no chronic hypoxia) and experimental (chronic hypoxia), genetically modified (knock-in/knock-out) and wildtype groups. To demonstrate a 50% change in terms of pulmonary vascular remodelling between two groups (genetic intervention vs control) with an overall variability of ~30, a group size of 10 evaluable animals per group is needed (power > 0.9 with α 0.05, two sided). In case of the multiple treatment groups here [1) No hypoxia – wildtype, 2) no hypoxia - knock-in/knock-out, 3) chronic hypoxia – wildtype, 4) chronic hypoxia - knock-in/knock-out], it will be necessary to increase the group size, to adjust for multiple comparisons, e.g. α of 0.05 will be divided by the number of treatment groups minus 1. A 4-arm study therefore, assuming 4 treatment groups, will have α = 0.05/3 = 0.013. Overall, an estimated group size of 10 evaluable animals will be needed to perform a 4-arm study. This includes potential losses due to human end points.

B. The animals

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

Species: *Mus musculus*, wild-type, knock-in, knock-out

Origin: Own breeding, university or commercial breeder.

Sex: Both male and female animals (equally divided) will be used throughout the project

Justification: Mice, being mammals, share many organ structures and similarities in genetic composition with humans. Other advantages include the short generation time and options to obtain genetically altered animals. Since genetic modification is more efficient in mice than in rat, the addition of the chronic hypoxia mouse model allows for testing of genetic interventions (knock-out and knock-in) in the genes identified in human genetic studies to be involved in PAH.

Estimated numbers: Chronic hypoxia mouse model will be performed in 8 intervention studies during the project, with an average of ~40 mice per experiment (see above), resulting in an estimated total of 8 x 40 = 320 mice in total (in 5 years).

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.

Replacement
In vivo experiments are a necessary step in development of interventions that may one day be used in the clinic, since currently no other methods allow the evaluation of the effect of an intervention on the

organism as a whole. Cell culture, organoids, lab-on-a-chip or computer models are not (yet) sophisticated enough to assess the interaction between the PAH pathology and host environmental factors such as the oxygen supply, the immune system and metabolism.

Reduction
The proposed number of evaluable animals per study arm (n=10) is calculated as described above, and is in line with generally accepted protocols in scientific literature. Further reduction of the number of animals per group would decrease statistical power, and might thus disqualify the experiment.

Refinement
State-of-the-art methods and equipment for assessing pulmonary vascular remodelling and RV overload, in particular advanced imaging techniques, will be used to refine protocols and minimize discomfort to the animals especially in support of the application of humane endpoints. International guidelines will be applied to ensure that best practice is followed.

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

The procedures conducted under this protocol will inevitably cause discomfort for the animals in the studies. In order to minimize suffering, all studies will adhere to the national and internationally accepted rules for handling of lab animals [1], [2]. Under these rules, the animals will be humanely killed when a humane endpoint (see below) is reached. Within the institute, standard operating procedures (SOP) for animal handling are in place. These also include dedicated anaesthesia and analgesia (A&A) SOPs that will accompany invasive procedures to minimize pain and discomfort.
To minimize animal suffering, pain and fear the following measures will be taken. The animals will be allowed to acclimatize to their new environment for two weeks upon arrival at the animal facility. Animals are housed in groups (socially housed) at all times (also during hypoxia) and environmental enrichment strategies are applied in the cages to improve animal welfare. During haemodynamic measurements, animals will be kept under anaesthesia in a temperature-controlled environment. Measurements of vascular leakage will be performed under continuous anaesthesia.
No adverse effects on the environment are expected because animals are kept and procedures are performed in a controlled environment, all waste will be safely discarded.

References used in this section:
[1] Zutphen, L. Van, Handboek proefdierkunde: proefdieren, dierproeven, alternatieven en ethiek. .
[2] J. Guillen, "FELASA Guidelines and Recommendations," J Am Assoc Lab Anim Sci, vol. 51, no. 3, pp. 311–321, May 2012.

Repetition and duplication

E. Repetition

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

N.A.

Accommodation and care

F. Accommodation and care

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

☐ No

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

The animals will be socially housed in standard conditions (food and water available ad libitum) and environmental enrichment strategies are applied in the cages to improve animal welfare conform the

Directive 2010/63/EU. However, as part of the experimental model, the animals will also be housed under low oxygen conditions (10% O2) for 2 weeks (the chronic hypoxia period).

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.

General anaesthesia, in combination with pain treatment, will also be applied in order to perform the haemodynamic measurements (via open-chest RV catheterization). Measurements of vascular leakage will be performed under continuous anaesthesia.
Within the institute, standard operating procedures (SOP) for animal handling are in place. These also include dedicated anaesthesia and analgesia (A&A) SOPs that will accompany invasive procedures to minimize pain and discomfort. These A&A SOPs describe the best practice methods for anaesthesia and analgesia for each (surgical) procedure and are regularly checked as new concepts or procedures become available. All experiments performed within this project will conform to these A&A SOPs.

I. Other aspects compromising the welfare of the animals

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

Exposure to hypoxia (10% O2) will result in a temporary increase in respiration and pulse. Following a number of days in the hypoxia conditions, the respiration level and heartbeat will begin to acclimatize. Hypoxia-induced animals may lose weight compared to control animals. Apart from this observation, we have not observed any other physical or behavioural changes in the hypoxia-treated animals.
The induction of the inducible knockout models requires a 5-day course of tamoxifen-supplemented non-pelleted dry feed, which is not expected to compromise animal welfare. Due to the genetic modifications that target genes in pulmonary vascular or right ventricular (RV) remodelling, the animals may lose weight, experience shortness in breath and become lethargic. Apart from discomfort directly caused by the procedures as described above, animals carrying genetic modifications are not expected to suffer from the genetic modulation itself, based on previous observations, which in some cases may result in adverse effects on the animals' welfare.

Explain why these effects may emerge.

These effects are a consequence of the induction of PAH (right heart failure) due to the applied genetic interventions respectively.

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

In general, adverse effects on the animals' welfare caused by induction of pulmonary vascular remodelling and RV pressure overload cannot be completely prevented. In order to minimize adverse effects, the animals will be monitored at a frequency that is dictated by the model (2 times per week) and timely killed when a humane endpoint (see below) is met. The CO2 level, humidity and temperature in the hypoxia chamber are kept constant and will not deviate from the by law defined norms.
Should unforeseen complications due to the interventions or procedures occur, either the effect of these complications will be minimized by adjusted procedures, such as providing easy access to food (mush-feeding), or if this is not possible, the humane endpoints as defined below will be taking into account.

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.

The most important humane endpoints applicable to all studies are:

- Weight loss ≥20% of maximum body weight in adult animals, measured from the start of the treatment
- Weight loss ≥15% of body weight during 24h, in combination with:
  - Sustained abnormal breathing, dyspnoea (symptom PAH/right heart failure)
  - Sustained lethargy (symptom PAH/right heart failure)
- Sustained abnormal behaviour
- Complications of interventions (<1%): No interventions are planned.

Indicate the likely incidence.

Humane endpoints expected to occur in <10% of all cases.

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').

Discomfort levels categorized according to the genetic interventions are as follows:

- Animals that carry genetic interventions during the study period, can experience mild discomfort.
- Animals that carry no genetic interventions (wild-type) during the study period, can experience mild discomfort.

Discomfort levels categorized according to the chronic hypoxia model are as follows:

- Animals that are subjected to the chronic hypoxia model experience mild discomfort.
- Animals that are not subjected to the chronic hypoxia model experience mild discomfort.

Discomfort levels categorized according to the procedures used for assessment of pulmonary vascular remodelling and right ventricular pressures (echocardiography, haemodynamic measurements, imaging) are as follows:

- Echocardiography: non-recovery.
- Haemodynamic measurements, using general anaesthesia and analgesia: non-recovery.
- Tail vein injection with Evans Blue: non-recovery.

All techniques mentioned above will be performed after induction of anaesthesia and analgesia and are performed as final experiments, indicating that animals will only experience the discomfort resulting from the induction of anaesthesia.

Other procedures that will be used, which are not expected to alter the total level of discomfort experienced:

- Simple but frequent handling procedures (e.g. weighing): mild discomfort.
- Minimally invasive procedures and those requiring anaesthesia (e.g. non-invasive imaging): mild discomfort.
- More intensive imaging procedures and those requiring prolonged anaesthesia (e.g. image guided radiotherapy): moderate discomfort

Table 1: Procedures and discomfort classification.

Procedures	Category	Expected percentage (%) of animals	Frequency and duration of the procedure
1. Obtaining mice: Transport to animal facility	mild	100%	1x
2. Potential adverse effects genetic interventions	mild	50%	~4-12 weeks
3. Induction hypoxia (chronic hypoxia model)	moderate	50%	2 weeks
4. Echocardiography (under general anaesthesia)	non-recovery	60%	1x ~10 min
5. Haemodynamic measurements (under general anaesthesia and analgesia)	non-recovery	60%	1x ~30 min
6. Tail vein injection Evans Blue (under general anaesthesia)	non-recovery	20%	1x ~10 min
7. Sacrifice	non-recovery	100%	1x <1 min

Based on this table, we expect that cumulative discomfort for the hypoxia groups (50%) will be moderate. For the control groups (50%) this will be mild.

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 animals will be humanely killed at the end of the procedure, to collect large blood samples and tissues for further analysis. Also, animals will also be humanely killed in the case when one of the humane end-points will be reached.

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'.	5.1 lid2h
1.2	Provide the name of the licenced establishment.	5.1 lid2h
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: 4 Type of animal procedure: Intervention studies using Pneumonectomy (PNX)-Su5416 mouse model

2 Description of animal procedures

A. Experimental approach and primary outcome parameters

Describe the general design of the animal procedures in relation to the primary outcome parameters. Justify the choice of these parameters.

This Appendix describes the use of the pneumonectomy (PNX)+SU5416 mouse model to validate genetic targets that are promising as novel therapeutic intervention for PAH. This model has to be implemented and optimized at our department. In the PNX+SU5416 mouse model, the left lung will be removed which results in increased pulmonary blood flow and vascular remodelling. A single administration of SU5416 one week after the surgery will result in severe PAH and RV dysfunction. To test the involvement of target genes in pulmonary vascular or right ventricular (RV) remodelling, knock-in and knock-out mice will be tested in this model. The target genes include identified PAH targets or candidate targets obtained by fundamental research (e.g. genetical, molecular, cellular, histopathological studies on tissue of animal models) or clinical research (e.g. genetic studies on human samples or other observations, such as fMRI) such as, BMPR2 signalling, vascular tone, RV diastolic stiffness, inflammation, coagulation, integrin and VHL gene expression. Should new targets be identified during the course of this project, they will be added to the study. The effect of the genetic interventions will be tested when PAH has developed in the mice subjected to the PNX+SU5416 model. We will particularly focus in these studies on assessing the impact of the intervention on RV overload and pulmonary vascular remodelling, as both are clinical relevant end-points for PAH. The primary outcomes measures include the time (in days) from PAH induction to clinical manifestation of right heart failure; ; lung and right heart (RV) function and structure (assessed via imaging and tissue/protein analysis). The advanced blood serum and tissue analyses are also included to study the mechanisms underlying the chosen genetic interventions and to possibly identify new targets (in wild-type experimental animals).

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

Genetic interventions

Knock-in or knock-out mice will be obtained from commercial suppliers or from collaborating institutions. The current proposal does not involve the generation of new genetic models. After obtaining knock-in or knock-out models from institutions abroad, genetic models will be imported in our animal facility via embryo transfer to guarantee that animals are free from pathogenic micro-organisms. A number of genes of interest (GOI) that will be studied can only be knocked-out at an adult age to prevent developmental problems. To achieve selective, inducible gene knockout, we will breed mice carrying loxP-flanked GOI with mice carrying tamoxifen-inducible Cre recombinase under control of the tissue of interest (e.g. VE-cadherin). Induction of Cre-recombinase will be performed by a 5-day course of tamoxifen-supplemented non-pelleted dry feed.

Induction pneumonectomy+SU5416 mouse model

The PNX+SU5416 mouse model is a surgical model. Mice (Knock-in/knock-out mice and wildtype) are put under general anaesthesia and analgesia, the thorax is opened and the left lung is removed. One week after surgery, mice receive a single subcutaneous (s.c.) injection of the vascular endothelial growth factor receptor (VEGFR) inhibitor SU5416 (20 mg/kg in CMC). A sham-operated control group will be included that will not undergo PNX and receive a vehicle-injection (for illustration of experimental design see Figure 1 below).

Echocardiography

To verify development of pulmonary vascular remodelling and a mild increase in right ventricular pressures, the mice are subjected echocardiography of the heart according to standard protocols at our Department.

Haemodynamic measurements

Mice are anesthetized for hemodynamic measurements via open-chest RV catheterization. RV systolic pressure (RVSP) will be determined from steady state measurement, After the haemodynamic measurements, the animals are sacrificed and blood and tissue (heart and lung) are collected for subsequent analysis.

Termination

At the end of the study, animals will be killed by an approved method (e.g. removal of blood and organs under anaesthesia or CO2 asphyxiation, cervical dislocation, terminal anaesthesia). Following exsanguination, serum and plasma samples are taken, and heart and lung tissues are collected for tissue, protein and RNA analyses. The animals are maximally for 12 weeks in experiment.

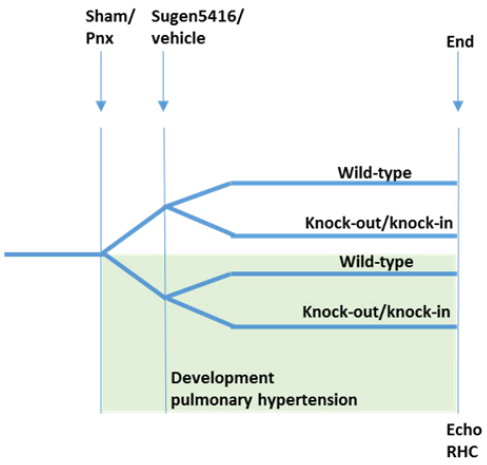

Figure 1. Experimental design studies with pneumonectomy (PNX)+SU5416 mouse model. After 2 weeks of acclimatization, pneumonectomy will be performed, followed by single injection of Sugen5416 one

week later. At the end experiments an echocardiography and right heart catheterization is performed. The animals are maximally for 12 weeks in experiment.

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

A typical intervention study will comprise several study arms, including control (no PNX+SU5416) and experimental (PNX+SU5416), genetically modified (knock-in/knock-out) and wildtype groups. To demonstrate a 50% improvement in terms of pulmonary vascular remodelling between two groups (genetic intervention vs control) with an overall variability of ~30, a group size of 10 evaluable animals per group is needed (power > 0.9 with α 0.05, two sided). In case of the multiple treatment groups here [1) no PNX+SU5416–wildtype, 2) no PNX+SU5416–knock-in/knock-out, 3) PNX+SU5416–wildtype, 4) PNX+SU541–knock-in], it will be necessary to increase the group size to adjust for multiple comparisons, e.g. α of 0.05 will be divided by the number of treatment groups minus 1. A 4-arm study therefore will have α = 0.05/3 = 0.013. Overall, an estimated group size of 10 evaluable animals will be needed. To accommodate for drop-out of animals due to the surgery, we use n=12 in the model/intervention groups, n=6 in the control group. This includes potential losses due to human end points.

B. The animals

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

Species: *Mus musculus*, wild-type, knock-in, knock-out

Origin: Own breeding or university or commercial breeder.

Sex: Both male and female animals (equally divided) will be used throughout the project

Justification: Mice, being mammals, share many organ structures and similarities in genetic composition with humans. Other advantages include the short generation time and options to obtain genetically altered animals. Since genetic modification is more efficient in mice than in rat, the addition of the PNX-SU5416 mouse model allows for testing of genetic interventions (knock-out and knock-in) in the genes identified in human genetic studies to be involved in PAH.

Estimated numbers: PNX+SU5416 mouse model will be performed in 8 intervention studies during the project, with an average of ~42 mice per experiment (see above), resulting in an estimated total of 8 x 42 = 336 mice in total (in 5 years).

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.

Replacement
In vivo experiments are a necessary step in development of interventions that may one day be used in the clinic, since currently no other methods allow the evaluation of the effect of an intervention on the organism as a whole. Cell culture, organoids, lab-on-a-chip or computer models are not (yet)

sophisticated enough to assess the interaction between the PAH pathology and host environmental factors such as the oxygen supply, the immune system and metabolism.

Reduction
The proposed number of evaluable animals per study arm (n=12 in model/intervention groups, n=6 in control group) is calculated as described above, and is in line with generally accepted protocols in scientific literature. Further reduction of the number of animals per group would decrease statistical power, and might thus disqualify the experiment.

Refinement
State-of-the-art methods and equipment for assessing pulmonary vascular remodelling and RV overload, in particular advanced imaging techniques, will be used to refine protocols and minimize discomfort to the animals especially in support of the application of humane endpoints. International guidelines will be applied to ensure that best practice is followed.

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

The procedures conducted under this protocol will inevitably cause discomfort for the animals in the studies. In order to minimize suffering, all studies will adhere to the national and internationally accepted rules for handling of lab animals [1], [2]. Under these rules, the animals will be humanely killed when a humane endpoint (see below) is reached. Within the institute, standard operating procedures (SOP) for animal handling are in place. These also include dedicated anaesthesia and analgesia (A&A) SOPs that will accompany invasive procedures to minimize pain and discomfort. In case the pulmonary hypertension is too severe, we may choose to omit the SU5416 injection.
To minimize animal suffering, pain and fear the following measures will be taken. The animals will be allowed to acclimatize to their new environment for two weeks upon arrival at the animal facility. Animals are housed in groups (socially housed) at all times (except during surgery, animals are returned to the group directly after surgery) and environmental enrichment strategies are applied in the cages to improve animal welfare. The PNX surgical model is performed under general anaesthesia and analgesia (pre- and post-surgery). During haemodynamic measurements, animals will be kept under anaesthesia in a temperature-controlled environment.
No adverse effects on the environment are expected because animals are kept and procedures are performed in a controlled environment, all waste will be safely discarded.

References used in this section:
[1] Zutphen, L. Van, Handboek proefdierkunde: proefdieren, dierproeven, alternatieven en ethiek.
[2] J. Guillen, "FELASA Guidelines and Recommendations," J Am Assoc Lab Anim Sci, vol. 51, no. 3, pp. 311–321, May 2012.

Repetition and duplication

E. Repetition

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

N.A.

Accommodation and care

F. Accommodation and care

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

☒ No

☐ 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.

The PNX will be performed under general anaesthesia and analgesia (pre- and post-surgery). General anaesthesia, in combination with perioperative pain treatment, will also be applied in order to perform the haemodynamic measurements (via open-chest RV catheterization).
Within the institute, standard operating procedures (SOP) for animal handling are in place. These also include dedicated anaesthesia and analgesia (A&A) SOPs that will accompany invasive procedures to minimize pain and discomfort. These A&A SOPs describe the best practice methods for anaesthesia and analgesia for each (surgical) procedure and are regularly checked as new concepts or procedures become available. All experiments performed within this project will conform to these A&A SOPs.

I. Other aspects compromising the welfare of the animals

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

The induction of the inducible knockout models requires a 5-day course of tamoxifen-supplemented non-pelleted dry feed, which is not expected to compromise animal welfare. Due to the genetic modifications and PNX+SU5416 model (and consequently, the induced right heart failure), that target genes in pulmonary vascular or right ventricular (RV) remodelling, the animals may lose weight, experience shortness in breath and become lethargic. Apart from discomfort directly caused by the procedures as described above, animals may develop complications due to the therapeutic interventions (e.g. toxic side-effects), which in some cases may result in adverse effects on the animals' welfare.

Explain why these effects may emerge.

These effects are a consequence of the induction of PAH and right heart failure due to the PNX+SU5416 model and the applied genetic interventions respectively.

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

In general, adverse effects on the animals' welfare caused by induction of pulmonary vascular remodelling and RV pressure overload cannot be completely prevented. In order to minimize adverse effects, the animals will be monitored at a frequency that is dictated by the model (2 times per week) and timely killed when a humane endpoint (see below) is met. When profound weight drop occurs, daily monitoring will be applied. Should unforeseen complications due to the interventions or procedures occur, either the effect of these complications will be minimized by adjusted procedures, such as providing easy access to food (mush-feeding), or if this is not possible, the humane endpoints as defined below will be taking into account.

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.

The most important humane endpoints applicable to all studies are:

- Weight loss ≥20% of maximum body weight in adult animals, measured from the start of the treatment
- Weight loss ≥15% of body weight during 24h, in combination with:
    - Sustained abnormal breathing, dyspnoea (symptom PAH/right heart failure)
    - Sustained lethargy (symptom PAH/right heart failure)
- Sustained abnormal behaviour
- Complications of interventions
    - • Other procedure-specific endpoints
    -

Indicate the likely incidence.

Humane endpoints expected to occur in <10% of all cases.

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').

Discomfort levels categorized according to the genetic interventions are as follows:

- Animals that carry genetic interventions during the study period, can experience mild discomfort.
- Animals that carry no genetic interventions (wild-type) during the study period, can experience mild discomfort.

Discomfort levels categorized according to the PNX-SU5416 model are as follows:

- Animals that are subjected to the PNX-SU5416 model experience moderate discomfort.
- Animals that are not subjected to the PNX-SU5416 model experience mild discomfort.

Discomfort levels categorized according to the procedures used for assessment of pulmonary vascular remodelling and right ventricular pressures (echocardiography, haemodynamic measurements, imaging) are as follows:

- Echocardiography: non-recovery.
- Haemodynamic measurements, using general anaesthesia and analgesia: non-recovery.
- More intensive imaging procedures and those requiring prolonged anaesthesia: non-recovery.

Other procedures that will be used, which are not expected to alter the total level of discomfort experienced:

- Simple but frequent handling procedures (e.g. weighing): mild discomfort.
- Minimally invasive procedures and those requiring anaesthesia (e.g. non-invasive imaging): mild discomfort.
- More intensive imaging procedures and those requiring prolonged anaesthesia (e.g. image guided radiotherapy): moderate discomfort.
-

Table 1: Procedures and discomfort classification.

Procedures	**Category**	**Expected percentage (%) of animals**	**Frequency and duration of the procedure**
1. Obtaining mice: Transport to animal facility	mild	100%	1x
2. Potential adverse effects genetic interventions	mild	50%	~4-12 weeks

3. Induction PNX-SU5416 model: a. PNX surgery (under general anaesthesia and analgesia) b. Injection with SU5416	moderate mild	58% 58%	1x ~90 min 1x <1 min
4. Echocardiography (under general anaesthesia)	non-recovery	100%	1x ~10 min
5. Haemodynamic measurements (under general anaesthesia and analgesia)	non-recovery	100%	1x ~30 min
6. Sacrifice	non-recovery	100%	1x <1 min

Based on this table, we expect that cumulative discomfort for the PNX-SU5416 groups (58%) will be moderate. For the control groups (42%) this will be mild.

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 animals will be humanely killed at the end of the procedure, to collect large blood samples and tissues for further analysis. Also, animals will also be humanely killed in the case when one of the humane end-points will be reached.

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
Niet-technische samenvatting

- Dit format gebruikt u om uw niet-technische samenvatting te schrijven
- Meer informatie over de niet-technische samenvatting vindt u op de website www.centralecommissiedierproeven.nl.
- Of neem telefonisch contact op. (0900-2800028).

1 Algemene gegevens

1.1	Titel van het project	Verbeterde behandeling van longziekte: het testen van kandidaat-behandelmethoden in diermodellen
1.2	Looptijd van het project	5 jaar
1.3	Trefwoorden (maximaal 5)	Longziekte, rechter hartfalen, behandeling, muismodellen, ratmodellen

2 Categorie van het project

2.1 In welke categorie valt het project.

U kunt meerdere mogelijkheden kiezen.

- ☒ Fundamenteel onderzoek
- ☒ Translationeel of toegepast onderzoek
- ☐ Wettelijk vereist onderzoek of routinematige productie
- ☐ Onderzoek ter bescherming van het milieu in het belang van de gezondheid
- ☐ Onderzoek gericht op het behoud van de diersoort
- ☐ Hoger onderwijs of opleiding
- ☐ Forensisch onderzoek
- ☐ Instandhouding van kolonies van genetisch gemodificeerde dieren, niet gebruikt in andere dierproeven

3 Projectbeschrijving

3.1	Beschrijf de doelstellingen van het project (bv de wetenschappelijke vraagstelling of het wetenschappelijk en/of maatschappelijke belang)	Pulmonale arteriële hypertensie (PAH) is een zeldzame maar dodelijke longziekte. Bij patiënten met PAH leidt een belemmerde bloeddoorstroming door het longvaatbed tot een verhoogde bloeddruk in de longen en een overbelast hart. De overbelasting van het hart leidt tot hartfalen en uiteindelijk de dood. Omdat genezing van deze ziekte meestal niet mogelijk is, hebben patiënten een sterk verminderde kwaliteit van leven en een beperkte levensverwachting. De levensverwachting van patiënten met deze ziekte is slechts 3 tot 5 jaar (bij een relatief jonge patiëntengroep van 50 jaar of jonger). De huidige behandeling bestaat uit een combinatie van bloedvat verwijdende medicijnen die selectief op de longbloedvaten werken. Deze huidige behandeling is echter niet voldoende om het hartfalen te stoppen of te voorkomen. De enige manier om het hart te ontlasten en te

		laten herstellen in deze ziekte, is een longtransplantatie. Door schaarste in het aantal beschikbare organen en voortschrijding van de ziekte komt deze optie te laat voor de meeste patiënten. Binnen dit project zullen we werken aan de ontwikkeling van nieuwe kandidaat-behandelingen voor deze longziekte. Deze kandidaat-behandelingen en bijbehorende methodes zullen worden getest en verfijnd in muizen of ratten, zodat ze vervolgens kunnen worden onderzocht in patiënten en bij positief resultaat op de langere termijn kunnen worden ontwikkeld tot effectieve behandelingsmethode voor deze longziekte.
3.2	Welke opbrengsten worden van dit project verwacht en hoe dragen deze bij aan het wetenschappelijke en/of maatschappelijke belang?	De opbrengsten uit dit project zijn enerzijds in diermodellen geteste kandidaat-behandelingen voor PAH en anderzijds nieuwe kennis en inzichten rond de ziekteprocessen onderliggend aan dit ziektebeeld. Wetenschappelijk belang: de door het uitvoeren van interventiestudies in muizen en ratten zullen de onderzoekers een beter inzicht krijgen in de factoren die het succes van een kandidaat-behandeling bepalen. Dit is belangrijk omdat in het wetenschappelijk onderzoek de rol van een aantal van deze factoren (bijv. bepaalde genen, eiwitten) en de mogelijke interacties tussen factoren tot nu toe nog niet voldoende aandacht heeft gekregen. Daarnaast zal de nieuw verkregen kennis bijdragen aan nieuwe inzichten in de ziekteprocessen onderliggend aan de ziekte en ander gerelateerde ziektebeelden, bijvoorbeeld betreffende de rol van de linkerhartkamer. Maatschappelijk belang: de kandidaat-behandelingen kunnen op basis van de uitkomsten uit deze studies verder ontwikkeld worden op de langere termijn tot een behandeling die veilig en effectief is voor gebruik in patiënten. Dit onderzoek is van groot maatschappelijk belang omdat deze ziekte een zeer ernstige conditie is waarvoor tot op heden geen goede behandeling beschikbaar is.
3.3	Welke diersoorten en geschatte aantallen zullen worden gebruikt?	In dit project zullen experimenten worden uitgevoerd op muizen en ratten. Wij verwachten voor dit onderzoek maximaal 656 muizen en 600 ratten nodig te hebben in 5 jaar. De totale som van het aantal proefdieren is 1256.
3.4	Wat zijn bij dit project de verwachte negatieve gevolgen voor het welzijn van de proefdieren?	Om PAH in een diermodel te kunnen nabootsen ontstaan bij de dieren ook negatieve gevolgen door de symptomen van deze ziekte. Dit is helaas een belangrijk onderdeel van dit onderzoek en niet te voorkomen. Bij hartfalen is een afname van lichaamsgewicht te zien, de dieren worden dagelijks gecontroleerd en gewogen en indien nodig worden ze voortijdig uit de proef genomen (een humaan eindpunt toegepast). Daarnaast zullen negatieve gevolgen voor de proefdieren voortkomen uit de behandelingen en het monitoren van de effecten.
3.5	Hoe worden de dierproeven in het project ingedeeld naar de verwachte ernst?	Licht ongerief: maximaal 10% van de proefdieren Matig ongerief: maximaal 80% van de proefdieren Ernstig ongerief: maximaal 10% van de proefdieren Hartfalen is helaas een belangrijk onderdeel van dit onderzoek en is niet te voorkomen, hierbij kan bij een deel van de dieren ernstig ongerief optreden.
3.6	Wat is de bestemming van de dieren na afloop?	Alle dieren worden na afloop van de experimenten gedood, waarna weefsel wordt gebruikt voor verder onderzoek.

4 Drie V's

4.1 **Vervanging** Geef aan waarom het gebruik van dieren nodig is voor de beschreven doelstelling en waarom proefdiervrije alternatieven niet gebruikt kunnen worden.	Alle behandelingen die in dit project getest worden in dieren, zijn eerst uitgebreid getest in relevante andere systemen, zoals gekweekte cellen of op eerder verzameld patiënt weefselmateriaal. De experimenten in proefdieren zijn erop gericht informatie te verkrijgen over complexe processen die met alternatieve methodes niet getest kunnen worden. Het gaat hierbij bijvoorbeeld over de verdeling van een (experimenteel) geneesmiddel in het hele lichaam, interactie met het immuunsysteem, en de interactie tussen hartfunctie en longbloedvat-afwijkingen. Dit kan vooralsnog niet nagebootst worden in celkweek of andere modelsystemen. Als zodanig heeft onderzoek in proefdieren geeft belangrijke informatie over veiligheid en effectiviteit van een kandidaat-behandeling, die van groot belang is, voor de toepassing van de nieuwe behandeling in patiënten.
4.2 **Vermindering** Leg uit hoe kan worden verzekerd dat een zo gering mogelijk aantal dieren wordt gebruikt.	Ter vermindering van het aantal dieren, zullen de volgende overwegingen gebruikt worden bij ieder experiment: - De groepsgrootte benodigd voor het verkrijgen van goed onderbouwde resultaten zal door middel van statistische methodes bepaald worden. Hierbij zal gestreefd worden naar het minimaliseren van het aantal gebruikte controle dieren. - Het onderzoek wordt uitgevoerd met behulp van standaard procedures en metingen om variatie tussen individuele experimenten te voorkomen. - Er wordt gebruik gemaakt van niet-invasieve beeldvorming (echo van het hart) om long- en hartfunctie gedurende een langere periode in een dier te kunnen volgen. Voor het project als geheel geldt dat de studies worden uitgevoerd in een gefaseerde opzet waardoor gebruik van het optimale aantal dieren wordt gewaarborgd.
4.3 **Verfijning** Verklaar de keuze voor de diersoort(en). Verklaar waarom de gekozen diermodel(len) de meest verfijnde zijn, gelet op de doelstellingen van het project.	De experimenten in dit project worden uitgevoerd in ratten of muizen. Muizen en ratten vertonen qua orgaanstructuur en genetische opbouw grote overeenkomsten met de mens. Daarnaast zijn in muizen veel genetische technieken mogelijk. Gedurende de afgelopen decennia is veel ervaring opgedaan met het onderzoek in muizen, waardoor veel vergelijkingsmateriaal, verschillende muizenstammen en modellen beschikbaar zijn. Ook zijn muizen goed te houden en te hanteren, wat het onderzoek vergemakkelijkt. Voor ratten gelden grotendeels dezelfde argumenten voor gebruik, behalve genetische modellen. Daarnaast zijn ratten veel groter dan muizen, waardoor bepaalde procedures die in muizen niet uitgevoerd kunnen worden, in ratten wel getest kunnen worden. We hebben gekozen voor vier diermodellen welke wetenschappelijk erkent zijn als op dit moment het beste diermodel voor pulmonale arteriële hypertensie. We hebben veel ervaring met de toegepaste onderzoekstechnieken, hiermee wordt onnodig lijden bij de dieren voorkomen.
Vermeld welke algemene maatregelen genomen worden om de negatieve	De proefdieren zullen gezamenlijk worden gehuisvest in een omgeving met kooiverrijking. Bij de operaties en andere invasieve behandelingen worden algehele narcose en effectieve pijnstilling toegepast. In geval van ernstig

(schadelijke) gevolgen voor het welzijn van de proefdieren zo beperkt mogelijk te houden.	onverwacht ongerief worden de humane eindpunten toegepast. Al het onderzoek in dit project zal door gekwalificeerd personeel worden uitgevoerd in een gespecialiseerde proefdierfaciliteit. Daarnaast zal ervaren personeel zorgdragen voor de controle van het welzijn van de dieren. Er zijn protocollen aanwezig waarin procedures voor het hanteren van dieren, alsmede richtlijnen voor narcose en pijnstilling, zijn vastgelegd.

5 In te vullen door de CCD

Publicatie datum	
Beoordeling achteraf	
Andere opmerkingen	

Van: info@zbo-ccd.nl
Verzonden: vrijdag 8 mei 2020 10:51
Aan: 5.1 lid2e
Onderwerp: Verzoek om advies over projectvergunningsaanvraag AVD5.1 lid2h20209866
Bijlagen: Appendix_2_behandeling_PAH.pdf; aanvraag_5.1 lid2e_PAH_final.pdf; Appendix_1_behandeling_PAH.pdf; NTS.pdf; Appendix_3_behandeling_PAH.pdf; Appendix_4_behandeling_PAH.pdf; Project_proposal.pdf

Geachte leden van 5.1 lid2h

De Centrale Commissie Dierproeven (hierna: CCD) verzoekt u in het kader van vergunningverlening (of wijziging van een vergunning) advies te geven over het project met als titel: "Towards a novel treatment of pulmonary arterial hypertension; preclinical testing of novel interventions targeting both right ventricular pressure overload and pulmonary vascular remodelling." en aanvraagnummer: AVD5.1 lid2h20209866.

Uw commissie wordt verzocht op grond van artikel 10.a.2 van de Wet op de dierproeven de aanvraag te beoordelen en een ethische toetsing uit te voeren waarbij wordt afgewogen of de doelstelling van het project, de verwachte voordelen voor mens, dier of milieu en de haalbaarheid van de doelstellingen, het gebruik van dieren en de schade die zal worden toegebracht aan de dieren in de vorm van lijden, pijn en angst kan rechtvaardigen.

Graag ontvangen wij van u bericht dat deze e-mail goed is ontvangen en wanneer u dit advies in de vergadering gaat bespreken.

Voor het in te dienen advies dient de DEC gebruik te maken van de meest actuele versie van het op de website van de CCD gepubliceerde Format DEC-advies en de toelichting daarbij. U dient deze aanvraag vertrouwelijk te behandelen. Voor de communicatie met de CCD dient u gebruik te maken van FileSecure.

De CCD verzoekt u uiterlijk binnen 20 werkdagen, na 08-05-2020, uw advies bij de CCD in te dienen. Indien de aanvraag door uw commissie niet in behandeling kan worden genomen, dient u dit per ommegaande per e-mail aan de CCD te melden.

Ingeval uw commissie tussentijds aanvullende informatie wil inwinnen bij de aanvrager wordt de termijn opgeschort en geeft u in uw advies aan wanneer dit is geweest. Opschorting van de adviestermijn vindt niet plaats ingeval u ten behoeve van uw advies een onafhankelijk extern expert raadpleegt. Mocht u verwachten door een andere reden dan opschorting uw advies later dan 20 werkdagen na 08-05-2020 bij de CCD in te dienen, dan verzoeken wij u dit direct aan de CCD te melden.

Mocht u vragen hebben, dan kunt u uiteraard contact met ons opnemen.

Met vriendelijke groet,
Centrale Commissie Dierproeven

www.centralecommissiedierproeven.nl

.....
Postbus 93118 | 2509 AC | Den Haag
.....

T: 0900 2800028
E: info@zbo-ccd.nl

Format DEC-advies

A. Algemene gegevens over de procedure

1. Aanvraagnummer:
Het NVWA nummer is 5.1 lid2h *het aanvraagnummer is AVD*5.1 lid2h*20209866.*

2. Titel van het project:
Towards a novel treatment of pulmonary arterial hypertension; preclinical testing of novel interventions targeting both right ventricular pressure overload and pulmonary vascular remodelling

3. Titel van de NTS:
Verbeterde behandeling van longziekte: het testen van kandidaat-behandelmethoden in diermodellen

4. Type aanvraag:
Nieuwe aanvraag projectvergunning

5. Contactgegevens DEC:
   - naam DEC: 5.1 lid2h
   - telefoonnummer contactpersoon:5.1 lid2e
   - e-mailadres contactpersoon:5.1 lid2e

6. Adviestraject (data dd-mm-jjjj):
   - ontvangen door DEC: *08-05-2020*
   - aanvraag compleet: *08-05-2020*
   - in vergadering besproken: *12-05-2020*
   - anderszins behandeld: *n.v.t*
   - termijnonderbreking(en) van / tot: 20-5-2020 tot *03-08-2020 en van 04-09-2020 tot 25-09-2020*
   - besluit van CCD tot verlenging van de totale adviestermijn met maximaal 15 werkdagen: *n.v.t.*
   - aanpassing aanvraag: *11-09-2020*
   - advies aan CCD: *30-09-2020*

7. Geef aan of de aanvraag is afgestemd met de IvD en deze de instemming heeft van de IvD.
-Datum advies IvD: *08-05-2020*
-Strekking advies IvD: *De IvD geeft aan dat de aanvrager het project met de IvD heeft afgestemd en dat deze de instemming heeft van de IvD.*

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

9. Correspondentie met de aanvrager

Vraagronde 1
- Datum: *20-05-2020*
- Strekking gestelde vragen*: **1. NTS:** Onderdeel 3.4: Geef hier meer uitleg over hoe dit ongerief kan ontstaat. Hoe zit het precies met het ernstig ongerief? Wanneer stop je de experimenten (HEP)? Waarom is ernstig ongerief niet te vermijden? Graag ziet de DEC dat hier meer uitleg wordt*

toegevoegd. NTS Onderdeel 3.5: Hier staat ernstig ongerief max 10%, dit lijkt niet hetzelfde als in het projectvoorstel? Graag checken. Geef onder 3.5 ook de maximale duur van het ernstige ongerief weer (in het projectvoorstel staat 2 dagen hartfalen?!). ***2. Projectvoorstel****: Onderdeel 3.3: De DEC ziet graag dat ook de bredere belangen worden vermeld i.p.v. alleen de behandeling van de PAH. Bovendien ziet de DEC graag dat er getallen en literatuur verwijzingen aan dit onderdeel worden toegevoegd. Onderdeel 3.4.1 Figuur 3: de pilot (experiment 1) die hier wordt benoemd is moeilijk terug te vinden in de tekst en de rest van het project, graag ook laten terugkomen in de rest van de strategie. Wat zijn precies de GO en NO GO momenten bij de pilotstudie? Gaat men een pilot doen met alle diermodellen? Er moet hier meer informatie over worden gegeven. Onderdeel 3.4.1 en 3.4.2 Wat voor soort interventies ga je doen? (er staat alleen 15 interventions to be included in our studies). 3.4.2 tabel 1. De tekst onder Procedure 1 (10% severe discomfort) lijkt niet overeen te komen met de tekst in tabel 1? Ook bij Procedure 3 komt het discomfort niet overeen met tabel 1? Graag de tekst aanpassen zodat alles klopt. 3.4.3 see figure 3, Moet dit niet een verwijzing zijn naar figuur2? Graag checken. Wat is het target precies? Graag checken en aanpassen. 3.4.3 De DEC snapt waarom men naar de muis wil, maar graag krijgt de DEC meer uitleg over wat de meerwaarde is om beide muis modellen te gebruiken.* ***3. Bijlage 1****: Onderdeel A: Bij statistische methodes staat dat men wil kijken naar "50% improvement in terms of PV (?) remodeling". Wat is een verbetering? en hoe gaat men dat meten? Wat voor soort interventies ga je doen? Noem dit ook al eerder in de proefopzet (projectvoorstel) en onderbouw dit! Onderdeel B: Hoeveel dieren gaat men in totaal gebruiken? graag meer uitleg in de tabel. Onderdeel D: Wat is het nut en hoe kan men ''severe'' ongerief voorkomen? Hoe staan de experimenten/dit project in verhouding tot het ernstige ongerief? Waarom is dit niet te voorkomen? Is het mogelijk het gebruik van het aantal dieren met ernstig ongerief te verminderen? Benoem hier ook jullie ervaring uit het verleden. Onderdeel I: Dit gaat over de therapeutische interventies. Waarom gaat men geen dosis proeven doen, Is dit wel maximaal moderate? En alleen bij de medicatie of ook bij andere onderdelen? Graag kloppend maken. Onderdeel K (<40%) komt niet overeen met tekst onder J (<10%)? Laatste zin onder K klopt ook niet? Graag kloppend maken. Geef echte aantallen! Hoeveel dieren ondergaan moderate ongerief? Hoeveel mild ongerief en hoeveel severe ongerief? Waarom is severe ongerief bij deze experimenten niet te vermijden?* ***4. Bijlage 2,*** *zie de vragen bij bijlage 1, die gelden ook voor bijlage 2. Onderdeel A figuur 1 niet helder, figuur 2: Er wordt 1 model met de PAB gebruikt in figuur 2 en niet twee modellen? De figuren lijken niet te kloppen, graag checken en aanpassen.* ***5. Bijlage 3****, zie de vragen bij bijlage 1, die gelden ook voor bijlage 3. Onderdeel A: bij de Evans blue meeting (blz 2). Staat ''Measurements vasclulair …'' Waarom gaat men bij de dieren een katheter plaatsen in plaats van gewoon inspuiten met vloeistof? Dit tweede zou minder ongerief geven, graag onderbouwen waarom voor de katheter is gekozen.* ***6. Bijlage 4****, zie de vragen bij bijlage 1, die gelden ook voor bijlage 4. Geen verdere opmerkingen.*

- Datum antwoord: *03-08-2020*
- Strekking van de antwoord(en): *Naar aanleiding van de vragen van de DEC is de aanvraag op veel punten aangepast, hieronder de strekking van de antwoorden.* ***1. De NTS*** *is aangepast, de meeste dieren zullen tot het eind van het experiment geen hartfalen ontwikkelen, hierbij zal het ongerief niet boven matig uitkomen. Een deel van de dieren kan wel hartfalen ontwikkelen met ernstig ongerief. De eerste dag van hartfalen, is moeilijk te herkennen, omdat dit zowel een dagelijkse schommeling van het lichaamsgewicht kan zijn, als het eerste signaal van hartfalen. Bij een tweede dag van afvallen kan vrijwel altijd met zekerheid gezegd worden dat een dier hartfalen heeft, bij 10% afname van het lichaamsgewicht bij ratten en 15% bij muizen.* ***2. Het projectvoorstel*** *is op veel punten aangepast. Dit project zal niet alleen leiden tot nieuwe informatie over PH en rechter hartfalen, maar zal ook nieuwe*

inzichten geven in cardiale en endotheel fysiologie. Dit kan nuttig zijn voor andere longziekten en linker hartfalen. Het PAB model en beide muismodellen zijn nieuwe modellen in onze groep en moeten nog opgezet en gevalideerd worden. Met pilotstudie bedoelen we hier het opzetten en valideren van deze modellen, en niet iets wat in deze modellen getest gaat worden. Wanneer er dieren in een model succesvol PH ontwikkelen betekent dit een GO, anders een NO-GO. Een aantal van de interventies die we gaan doen zijn al bekend. Er is een tabel toegevoegd op pagina 9 van het projectvoorstel waar deze interventies beschreven worden. We verwachten echter ook dat we de komende vijf jaar nieuwe targets zullen identificeren. Het klopt inderdaad dat de getallen niet correct waren. In 3.4.2 van het projectvoorstel op pagina 10 en 11 zijn alle vermeldingen van het ongerief verwijderd en tabel 1 op pagina 12 is eruit gehaald. Bij alle documenten zijn de ongerief waardes nogmaals tegen het licht gehouden en aangepast waar nodig. Op dit moment is het chronische hypoxia muismodel de gouden standaard om PH in muizen te bestuderen. De PH die deze muizen ontwikkelen is echter niet heel ernstig en de dieren ontwikkelen geen hartfalen. Hierdoor zijn ze minder representatief voor de patiënt. Dat is de reden waarom we een nieuw model willen ontwikkelen, maar dit nieuwe model zullen we wel eerst uit moeten zetten tegen het bestaande muismodel. ***3-4. Bijlage 1 en 2*** *zijn aangepast. Bij de vier appendices is het kopje "Total" toegevoegd, waarbij de aantallen zijn weergegeven. Ernstig ongerief is helaas niet te vermijden. Patiënten komen vaak ook binnen in de kliniek met tekenen van rechter hartfalen. Daarom is rechter hartfalen ook onderdeel van onze uitkomstmaat. Dit is nodig om deze dieren te kunnen vergelijken met onze patiënten. In de tekst stond inderdaad niet vermeld dat we ook dosis proeven doen indien de dosering niet bekend is en de interventie nieuw is. Bij appendices 1 en 2 op pagina 6 is het is veranderd. Figuur 1 was inderdaad onjuist en is aangepast. Nu komt duidelijk uit het model naar voren dat er één PAB model is waarin we zowel medicatie als gentherapie gaan testen.* ***5-6. Bijlage 3 en 4*** *zijn aangepast. De vloeistof wordt ingespoten. De katheter die hier genoemd wordt betreft de katheter voor de PV loops. Tekst in bijlage is aangepast om het duidelijker te maken. Bij appendices 3 en 4 wordt geen severe ongerief verwacht omdat muizen vrijwel nooit hartfalen ontwikkelen bij PH. Bij appendices 3 en 4 worden geen therapeutische interventies toegepast.*

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

Vraagronde 2

- Datum: 04-09-2020

*- Strekking gestelde vragen: 1. Jullie hebben in de Appendices extra tekst toegevoegd en ook een soort diagram (dosis defining pilot studies). De DEC ziet bij de dieren onderdeel B echter geen extra dieren terug voor deze pilots? klopt dit? Graag ziet de DEC dat de aantallen dieren voor de pilot studies apart worden vermeld en berekend. 2. In Appendix 2 lijkt de tabel en uitleg (zie **) niet hetzelfde te zijn als in Appendix 1 terwijl er toch wel ongeveer dezelfde handelingen plaatsvinden? Graag deze tabel nog nakijken en gelijk maken aan Appendix 1 voor zover deze overeenkomen. 3. In Appendix 1 is de kans op een HEP minder of gelijk aan 15%, bij Appendix 2 is dit 10%, klopt dit wel? omdat de dieren in Appendix 2 ook ernstig ongerief kunnen ondervinden en zelfs vaker dan in Appendix 1. Graag checken.*

- Datum antwoord: *11-09-2020 en 25-09-2020*
- Strekking van de antwoord(en): *Naar aanleiding van de vragen van de DEC is de aanvraag op veel punten aangepast, hieronder de strekking van de antwoorden. 1. Dit klopt, de aantallen voor deze pilot studies zijn toegevoegd. Ook zijn er dieren toegevoegd voor het opzetten van de modellen die nog niet op de afdeling lopen. De getallen en percentages zijn ook aangepast in de NTS. 2. De tabel in Appendix 2 is gelijk gemaakt aan de tabel in Appendix 1. 3. 10% was inderdaad niet correct, dit moet 32% zijn. Dit is aangepast op pagina 7 van Appendix 2.*

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

B. Beoordeling (adviesvraag en behandeling)

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

C. Beoordeling (inhoud)

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

   Deze aanvraag heeft een concrete doelstelling en kan getypeerd worden als een project. Het doel van dit project is de ontwikkeling van nieuwe kandidaat-behandelingen voor de longziekte Pulmonale arteriële hypertensie (PAH). Bij patiënten met PAH leidt een belemmerde bloeddoorstroming door het longvaatbed tot een verhoogde bloeddruk in de longen en een overbelast hart. De overbelasting van het hart leidt tot hartfalen en uiteindelijk de dood. De veiligheid en effectiviteit van de nieuwe kandidaat-behandelingen zullen worden getest in verschillende diermodellen voor PAH, hiervoor zullen zowel muizen als ratten worden gebruikt.

   Na het stellen van vragen en het aanpassen van de aanvraag is het voor de DEC helder geworden welke handelingen de individuele dieren zullen ondergaan. Na navraag is het ook duidelijk welk ongerief individuele dieren zullen ondergaan. De aanvrager heeft na de vragenrondes duidelijk de go/no go momenten beschreven. De DEC is er daardoor van overtuigd dat de aanvrager gedurende het project op zorgvuldige wijze besluiten zal nemen over de voortgang van het project en er niet onnodig dieren gebruikt zullen worden. Gezien het bovenstaande is de DEC van mening dat de aanvraag toetsbaar is en voldoende samenhang heeft.

2. Signaleer of er mogelijk tegenstrijdige wetgeving is die het uitvoeren van de proef in de weg zou kunnen staan. Het gaat hier om wetgeving die gericht is op de gezondheid en welzijn van het dier of het voortbestaan van de soort (bijvoorbeeld Wet dieren en Wet Natuurbescherming).: *n.v.t.*

3. *De in de aanvraag aangekruiste doelcategorieën fundamenteel en translationeel onderzoek zijn in overeenstemming met de hoofddoelstelling. De doelstelling is helder omschreven. De opbrengsten uit dit project zijn enerzijds in diermodellen geteste kandidaat-behandelingen voor PAH en anderzijds nieuwe kennis en inzichten rond de ziekteprocessen onderliggend aan dit ziektebeeld.*

   Belangen en waarden

4. Benoem zowel het directe doel als het uiteindelijke doel en geef aan of er een directe en reële relatie is tussen beide doelstellingen. Beoordeel of het directe doel gerechtvaardigd is binnen de context van het onderzoeksveld.

   Pulmonale arteriële hypertensie (PAH) is een zeldzame maar dodelijke longziekte. De levensverwachting van patiënten met deze ziekte is slechts 3 tot 5 jaar (bij een relatief jonge patiëntengroep van 50 jaar of jonger). De huidige behandeling bestaat uit een combinatie van bloedvat verwijdende medicijnen die selectief op de longbloedvaten werken. Deze huidige behandeling is echter niet voldoende om het hartfalen te stoppen of te voorkomen. De enige manier

om het hart te ontlasten en te laten herstellen in deze ziekte, is een longtransplantatie. Door schaarste in het aantal beschikbare organen en voortschrijding van de ziekte komt deze optie te laat voor de meeste patiënten.

Het directe doel van deze studie is om een beter inzicht te krijgen in de factoren die het succes van een kandidaat-behandeling bepalen voor de behandeling van PAH. De kandidaat-behandelingen kunnen op basis van de uitkomsten uit deze studies verder ontwikkeld worden en op de langere termijn tot een veilige en effectieve behandeling zorgen voor patiënten met PAH. Het uiteindelijke doel van de studie is om het verloop van de ziekte PAH te verbeteren. Er is een reële relatie tussen deze beide doelstellingen. Het directe doel is nodig om het uiteindelijke doel te bereiken.

5. Benoem de belanghebbenden in het project en beschrijf voor elk van de belanghebbenden welke morele waarden in het geding zijn of bevorderd worden *(Zie Praktische handreiking ETK: Stap 2.B en tabel 1; zie bijlage I voor voorbeeld)*

De belangrijkste belanghebbenden in dit project zijn: de proefdieren, de onderzoekers en de patiënten. De waarden die voor proefdieren in het geding zijn: De integriteit van de dieren zal worden aangetast omdat de dieren ingrepen ondergaan en omdat de dieren worden gedood. De waarde van deze proef voor onderzoekers is: Het vergroten van de wetenschappelijke kennis. Waarden die voor patiënten bevorderd worden: De kennis van dit onderzoek zal bijdragen aan het ontwikkelen van betere behandelingsopties voor patiënten met PAH.

6. Is er aanleiding voor de DEC om de in de aanvraag beschreven effecten op het milieu in twijfel te trekken?: *n.v.t*

Proefopzet en haalbaarheid

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

De DEC heeft veel vragen gesteld bij deze aanvraag, zie de vragenrondes bovenaan dit document. De DEC is van mening dat naast aanwezige apparatuur, kennis, personeel en financiering ook het goed opzetten van experimenten (en daarbij het inschatten van het aantal dieren) de haalbaarheid van een projectvoorstel bepaalt en ziet graag dat de onderzoekers bij de uitvoering goed voor ogen houden wat ze precies willen doen en dat goed inplannen om fouten te voorkomen. Alle technische voorzieningen die benodigd zijn voor uitvoering van het project zijn voorhanden, evenals voldoende deskundigheid en financiering om het project succesvol uit te voeren. Ervaring binnen het onderzoeksinstituut met vergelijkbaar onderzoek waarborgt het technisch succesvol uitvoeren van de dierexperimenten. Na navraag is de DEC ervan overtuigd dat de projectdoelstelling met de gekozen strategie/aanpak binnen de gevraagde termijn is te realiseren.

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

Na de aanpassingen heeft het project een navolgbare opbouw en strategie. De voorgestelde experimentele opzet en uitkomstparameters zijn logisch en helder, en sluiten aan bij de aangegeven doelstellingen. Het project bestaat uit 4 type dierproeven met elk hun eigen diermodel: In Appendix 1 gebruikt men het SuHx ratmodel, in Appendix 2 het PAB ratmodel, in Appendix 3 het chronische hypoxia muismodel en in Appendix 4 het Pnx +Su muismodel. Deze diermodellen zijn geselecteerd omdat ze allemaal de klinische symptomen van PAH-patiënten nabootsen. Met het

SuHx-model kan meer mechanistische informatie worden verkregen en zullen interventies die effect kunnen hebben in de longen worden getest. Ingrepen die een effect kunnen hebben in het hart zullen eerst worden getest in het PAB ratmodel. Het chronische hypoxia muismodel maakt het testen van genetische interventies (knock-out en knock-in) in de genen die geïdentificeerd zijn in genetische studies bij mensen mogelijk. Het Pnx +Su muismodel zal worden gebruikt om genetische doelwitten te valideren die veelbelovend zijn als nieuwe therapeutische interventie voor PAH.

De interventies zullen worden getest wanneer PAH zich heeft ontwikkeld in de vier diermodellen. Interventies omvatten behandeling met elk soort middel (bijv. dieet, chemisch, biologisch, genetisch, radio farmaceutisch) of een combinatie van deze middelen. Sommige van deze interventies zijn al bekend, waaronder FHL2, RBM20 en integrin (zie tabel 1 van het voorstel). Alle middelen zullen worden toegediend via de juiste route, het tijdstip, de duur en de frequentie zoals vereist, de details van de behandeling van elk experiment zullen met de IvD worden besproken. Indien er in de loop van dit project nieuwe interventies worden geïdentificeerd die deze doelen aanpakken, dan zullen deze aan de studie worden toegevoegd. Over het algemeen zal een eerste in vivo interventiestudie worden uitgevoerd met behulp van het SuHx- of PAB-model (Appendix 1, 2 GO / NO GO moment). Als de eerste in-vivo-tests succesvol zijn, zullen bevestigende onderzoeken worden uitgevoerd in de andere modellen.

De DEC acht het reëel om te veronderstellen dat op basis van de resultaten van de voorgenomen reeks experimenten beschreven in het project, nieuwe en/of aanvullende kennis zal worden verkregen. De nieuw verkregen inzichten kunnen bijdragen aan het beschikbaar komen van betere behandelingsopties voor patiënten met PAH. De gevraagde looptijd van 5 jaar acht de DEC reëel gezien de opbouw en de financiële ondersteuning.

Welzijn dieren

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

De locatie van de dierproef is binnen de instelling van de vergunninghouder. Er is geen sprake van bedreigde diersoorten, niet-menselijke primaten, zwerfdieren en/of dieren in/uit het wild. De dieren krijgen adequate verdoving.

10. Geef aan of de dieren gehuisvest en verzorgd worden op een wijze die voldoet aan de eisen die zijn opgenomen in bijlage III van richtlijn 2010/63/EU.

De dieren worden gehuisvest en verzorgd op een wijze die voldoet aan de eisen die zijn opgenomen in bijlage III van de richtlijn. De dieren in Appendix 1 (SuHx model) en 3 (Chronic hypoxia mouse model) worden voor maximaal 4 weken gehuisvest in een hypoxische omgeving met 10% zuurstof, deze hypoxie leidt tot pulmonale hypertensie.

11. Beoordeel of het cumulatieve ongerief als gevolg van de dierproeven voor elk dier realistisch is ingeschat en geclassificeerd. Licht uw beoordeling toe.

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

De dieren zullen licht tot ernstig ongerief ondervinden. Maximaal 34% van de proefdieren ondervindt licht ongerief, 54% matig ongerief en 12% ernstig ongerief.

De dieren ondervinden licht ongerief als gevolg van: transport, injecties, inductie hypoxie,

echocardiografie, handling, bloedafname en het doden van de dieren onder anesthesie. In Appendix 3 en 4 kunnen de dieren ook licht ongerief ondervinden vanwege de mogelijke nadelige effecten van de genetische interventies. Alle dieren in Appendix 3 (100%) ondervinden maximaal licht ongerief. In Appendix 4 is dat 41% van de dieren.

De dieren ondervinden matig ongerief als gevolg van: bolus injecties, operaties onder anesthesie met pijnbestrijding en lokale infusie. In Appendix 4 zorgt dit voor matig ongerief bij 59% van de dieren. In Appendix 1 en 2 kunnen de dieren ook matig ongerief ondervinden vanwege de mogelijke negatieve bijwerkingen van de behandeling. In Appendix 1 ondervindt 85% van de dieren matig ongerief, in Appendix 2 is dit 68%.

Een deel van de dieren (ratten) in Appendix 1 (15%) en 2 (32%) kunnen ernstig ongerief ervaren als gevolg van hartfalen. Hartfalen is helaas een belangrijk onderdeel van dit onderzoek en is niet te voorkomen, hierbij kan bij een deel van de dieren ernstig ongerief optreden. De meeste dieren zullen tot het eind van het experiment geen hartfalen ontwikkelen, hierbij zal het ongerief niet boven de matig uitkomen. Maximaal 12% van de dieren kan wel hartfalen ontwikkelen met ernstig ongerief (voor maximaal 2 dagen). Bij hartfalen is een afname van lichaamsgewicht te zien, de dieren worden daarom dagelijks gecontroleerd en gewogen. De DEC is van mening dat het ongerieflevel aan de onderkant van ernstig ligt, zoals dat door de EU is gedefinieerd, de dieren hebben waarschijnlijk geen pijn maar missen energie. De DEC is akkoord met deze inschatting van het ongerieflevel.

12. Het uitvoeren van dierproeven zal naast het ongerief vaak gepaard gaan met aantasting van de integriteit van het dier. Beschrijf op welke wijze er sprake is van aantasting van integriteit.

De integriteit van de dieren zal worden aangetast door de injecties, de hypoxie, echocardiografie, handling, bloedafname, bijwerkingen van de interventies, operaties, infusie en het doden. Daarnaast zal een deel van de dieren hartfalen ontwikkelen. Helaas is het ontwikkelen hiervan essentieel, vanwege het translationele karakter van deze dierstudie. Het ernstig ongerief wordt beperkt door dagelijks te monitoren op basis van vooraf gestelde criteria (humane eindpunten).

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

De criteria voor de humane eindpunten zijn goed gedefinieerd.

Het humane eindpunt is, in lijn met eerdere protocollen als volgt gedefinieerd: "Het moment waarop, in de periode dat het begin van hartfalen wordt verwacht, voor het eerst een duidelijke gewichtsafname waarneembaar is, die niet past binnen de normale dagelijkse schommelingen." In de praktijk komt dat neer op het vaststellen van een duidelijke gewichtsafname ≥10% van gewicht binnen 24 uur (in combinatie met symptomen van PAH/hartfalen) of een opvallend grote gewichtsafname ≥20% van het maximale lichaamsgewicht, gemeten vanaf de start van de behandeling.

Daarnaast zal expliciet gelet worden op andere klinische tekenen van hartfalen: zoals cyanose, dyspneu, lethargie, afwijkend gedrag, complicaties van interventies en een slechte verzorging. Door dagelijks te observeren en te wegen kan het eindpunt goed worden bepaald, en kan onnodig lijden worden voorkomen. Gebaseerd op eerdere experimenten wordt er rekening gehouden 15% kans op een humaan eindpunt voor de dieren in Appendix 1, bij Appendix 2 is de kans 32% en de kans in Appendix 3 en 4 is 10%.

3V's

14. Beoordeel of de aanvrager voldoende aannemelijk heeft gemaakt dat er geen geschikte vervangingsalternatieven zijn. Licht uw beoordeling toe.

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

Voordat de behandelingen worden getest in dieren zijn deze eerst uitgebreid getest in celkweek of patiënt weefselmateriaal. Het gaat bij dit onderzoek om complexe processen, zoals de verdeling van een (experimenteel) geneesmiddel in het hele lichaam, interactie met het immuunsysteem, en de interactie tussen hartfunctie en longbloedvat-afwijkingen, dit kan vooralsnog niet nagebootst worden in celkweek of andere modelsystemen. Het project geeft belangrijke informatie over veiligheid en effectiviteit van een kandidaat-behandeling, die van groot belang is, voor de toepassing van de nieuwe behandeling in patiënten.

De keuze voor het gebruik van muizen en ratten is naar het oordeel van de DEC gerechtvaardigd. Muizen en ratten hebben een orgaanstructuur en genetische opbouw die grote overeenkomsten vertoont met de mens. Daarnaast zijn in muizen veel genetische technieken mogelijk, waardoor veel vergelijkingsmateriaal, verschillende muizenstammen en modellen beschikbaar zijn. Voor ratten is gekozen omdat deze veel groter zijn dan muizen, waardoor bepaalde procedures die in muizen niet uitgevoerd kunnen worden, in ratten wel getest kunnen worden.

15. Beoordeel of het aantal te gebruiken dieren realistisch is ingeschat en of er een heldere strategie is om ervoor te zorgen dat tijdens het project met zo min mogelijk dieren wordt gewerkt waarmee een betrouwbaar resultaat kan worden verkregen. Licht uw beoordeling toe.

In het project wordt optimaal tegemoetgekomen aan de vereiste van de vermindering van dierproeven.

Door het gefaseerd uitvoeren van de experimenten wordt voorkomen dat er teveel of te weinig dieren worden gebruikt. De groepsgrootte zal door middel van statistische methodes bepaald worden, hierbij zal gestreefd worden naar het minimaliseren van het aantal gebruikte controle dieren. Er wordt gebruik gemaakt van niet-invasieve beeldvorming (echo) om long- en hartfunctie gedurende een langere periode in een dier te kunnen volgen. Onnodige duplicatie van experimenten wordt voorkomen doordat de onderzoekers goed bekend zijn met het onderzoeksveld en samenwerken met de andere onderzoeksgroepen die vergelijkbaar onderzoek verrichten.

Het maximale aantal proefdieren is proportioneel ten opzichte van de gekozen strategie en de looptijd. De DEC onderschrijft dat het project kan worden uitgevoerd met maximaal 776 muizen en 840 ratten en acht dit aantal realistisch onderbouwd.

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

Het project is in overeenstemming met de vereiste van de verfijning van dierproeven en het project is zo opgezet dat de dierproeven met zo min mogelijk ongerief worden uitgevoerd.

Passende anesthesie en pijnstilling zal de gevolgen van de ingrepen minimaliseren. Alle procedures zullen uitgevoerd worden door ervaren en bekwaam personeel. De dieren zullen gezamenlijk worden gehuisvest. Daarnaast zal ervaren personeel zorgdragen voor de controle van het welzijn van de dieren. Als de dieren te veel afvallen of anderszins lijden worden de humane eindpunten toegepast.

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

Dieren in voorraad gedood en bestemming dieren na afloop proef

18. Geef aan of dieren van beide geslachten in gelijke mate ingezet zullen worden. Indien alleen dieren van één geslacht gebruikt worden, beoordeel of de aanvrager dat in voldoende mate wetenschappelijk heeft onderbouwd.

Tijdens de experimenten zal men gebruik maken van zowel mannelijke als vrouwelijke dieren.

19. Geef aan of dieren gedood worden in kader van het project (tijdens of na afloop van de dierproef). Indien dieren gedood worden, geef aan of en waarom dit noodzakelijk is voor het behalen van de doelstellingen van het project. Indien dieren gedood worden, geef aan of er een voor de diersoort passende dodingsmethode gebruikt wordt die vermeld staat in bijlage IV van richtlijn 2010/63/EU. Zo niet, beoordeel of dit in voldoende mate is onderbouwd. Licht uw beoordeling toe. Indien van toepassing, geeft ook aan of er door de aanvrager ontheffing is aangevraagd.

De dieren zullen worden gedood om grote bloedvolumes en weefsel te verzamelen voor analyses. De experimenten kunnen niet uitgevoerd worden zonder dat de dieren gedood worden. Er wordt een dodingsmethode uit bijlage IV van richtlijn 2010/63/EU gebruikt.

20. Indien niet-humane primaten, honden, katten of landbouwhuisdieren worden gedood om niet-wetenschappelijke redenen, is herplaatsing of hergebruik overwogen? Licht toe waarom dit wel/niet mogelijk is: *n.v.t.*

NTS

21. Is de niet-technische samenvatting een evenwichtige weergave van het project en begrijpelijk geformuleerd?

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

D. Ethische afweging

1. Benoem de centrale morele vraag

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

Rechtvaardigt de ontwikkeling van nieuwe kandidaat-behandelingen voor de longziekte Pulmonale arteriële hypertensie (PAH), om hiermee het verloop van de ziekte te verbeteren, het gebruik van maximaal 776 muizen en 840 ratten die daarvan licht tot ernstig ongerief ondervinden?

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

De waarden die voor de proefdieren in het geding zijn: De integriteit van de proefdieren wordt aangetast en de dieren ondervinden licht tot ernstig ongerief. Dat leidt tot veel nadeel voor deze proefdieren. De waarden voor de onderzoekers: voordeel vanwege de kennisontwikkeling over het verloop van PAH. De waarden die voor de patiënten bevorderd worden: Mogelijk veel voordeel wanneer de dierproef bijdraagt aan het ter beschikking komen van betere behandelopties voor hartfalen bij patiënten met pulmonale hypertensie.

De DEC is van mening dat de belangen van de patiënten in dit project zwaarder wegen dan de belangen van de 776 muizen en 840 ratten, die hiervoor als proefdieren gebruikt worden. Voor het verkrijgen van meer kennis over pulmonale hypertensie en hartfalen is onderzoek in diermodellen noodzakelijk. Er zijn op dit moment geen alternatieven voor deze dierproeven beschikbaar waarmee men de doelstellingen kan bereiken.

3. Beantwoord de centrale morele vraag. Maak voor het beantwoorden van deze vraag gebruik van bovenstaande afweging van morele waarden.

Volgens de DEC rechtvaardigen de doeleinden van dit project het voorgestelde gebruik van dieren. Het directe doel van deze studie is de ontwikkeling van nieuwe kandidaat-behandelingen voor de longziekte Pulmonale arteriële hypertensie (PAH). Het verwachte resultaat, in het kader van het beschikbaar komen van betere behandelingsopties voor patiënten met hartfalen, is afgewogen tegen het licht tot ernstig geschatte ongerief en de aantasting van integriteit, inclusief het doden van de dieren in de proef.

De DEC onderschrijft dat de doelstellingen niet zonder het gebruik van proefdieren kunnen worden behaald en acht het gebruik van 776 muizen en 840 ratten, en de daarmee samenhangende schade aan deze dieren gerechtvaardigd. Bij het uitvoeren van de dierproeven wordt een adequate invulling gegeven aan de vereisten op het gebied van de vervanging, vermindering en verfijning van de dierproeven. Het project is (1) van substantieel belang en (2) van goede kwaliteit.

(1) Het maatschappelijk belang en wetenschappelijk belang zijn beide substantieel. De resultaten van dit onderzoek zullen bijdragen aan meer kennis over pulmonale hypertensie en het beschikbaar komen van betere behandelingsopties voor patiënten met hartfalen.

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

Samenvattend kan worden gesteld dat het als substantieel te kwalificeren maatschappelijk en wetenschappelijk belang van het onderzoek naar het oordeel van de DEC opweegt tegen het gebruik van maximaal 776 muizen en 840 ratten en het daarbij verwachte lichte tot ernstige ongerief.

E. Advies

1. Advies aan de CCD

De DEC adviseert de vergunning te verlenen.

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

Het uitgebrachte advies is gebaseerd op meerderheid.

Er is een lid dat niet meegaat met het positieve advies, omdat het vertrouwen in de haalbaarheid van dit project voor dit lid gecompromitteerd is: het goed opzetten van dierexperimenten bepaalt mede de haalbaarheid van een projectvoorstel. Het inschatten van het aantal dieren is hierbij een voorwaarde. Gezien het twee vragenrondes duurde voordat het juiste aantal dieren in het protocol opgenomen waren hebben de aanvragers het in de ogen van het lid nagelaten om goed over de experimenten na te denken.

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

Er is geen dilemma geconstateerd.

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'.	5.1 lid2h
1.2	Provide the name of the licenced establishment.	5.1 lid2h
1.3	Provide the title of the project.	Towards a novel treatment of pulmonary arterial hypertension; preclinical testing of novel interventions targeting both right ventricular pressure overload and pulmonary vascular remodelling

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.

This project proposal describes testing of novel therapeutic interventions in laboratory animals (rats and mice) in the field of cardiopulmonary medicine, in particular for pulmonary arterial hypertension (PAH). The included studies comprise both fundamental research (studying the underlying pathology of PAH) and translational research, including the testing of novel (combinations of) therapeutic interventions in mice and rat models for PAH.

Cardiopulmonary medicine focuses on a range of disorders that affect the heart ("cardio-") as well as the lungs ("-pulmonary"). The two organ systems work closely together to make sure the body has the oxygen-rich blood it needs to function. Lung and cardiovascular (heart) disease are increasingly recognized to occur in the same patient populations [1]. Whether these diseases develop as a result of unique mechanisms or shared pathways remains uncertain, but growing evidence indicates that they may share common origins [2]. Example of such a disease is pulmonary arterial hypertension, a chronic severe disease with a poor prognosis. Although the origin of the disease is located in the lungs, the majority of patients die from right heart failure.

HEALTH CARE PROBLEM: PULMONARY ARTERIAL HYPERTENSION
Pulmonary arterial hypertension is a progressive and fatal disease [3]. Its prevalence in the Netherlands is around 16-29 patients per million inhabitants, equal to around 300-500 patients [4]. New cases are estimated to occur in 2.2 individuals per million each year in the Netherlands, i.e. around 37 new patients annually [4]. Due to progressive nature of the disease, a patient may experience only mild symptoms at first, but will eventually require treatment and increasing medical care to maintain a reasonable quality of life. Apart from lung transplantation, no curative treatment for PAH is available. The average life expectancy is currently only 3-5 years. While PAH is rare, other types of pulmonary hypertension (PH) are much more prevalent and carry significant morbidity and mortality. Many of the pathophysiological and pathobiological changes that are seen in the lungs and hearts of patients with PAH are also found in patients with other types of PH.

PREVIOUS RESEARCH IN PAH PATHOBIOLOGY
The pathology in PAH can be categorized by abnormal remodelling of pulmonary vessels (causing the arteries in the lungs to become narrowed, thickened or stiff), leading to a progressive increase in pulmonary artery pressure and increased right ventricular (RV) afterload (Figure 1). Effectively, the RV must work harder to push blood through the narrowed pulmonary arteries. The RV adapts to this increased load via several compensatory mechanisms. Although RV adaption mechanisms initially suffice, the progressive increases in pressure-overload leads almost inevitably to RV dysfunction and right heart failure, which is the predominant cause of death in PAH. Hence, PAH is a complex and multi-factorial disease, involving the two organ systems (lung, heart) concurrently.

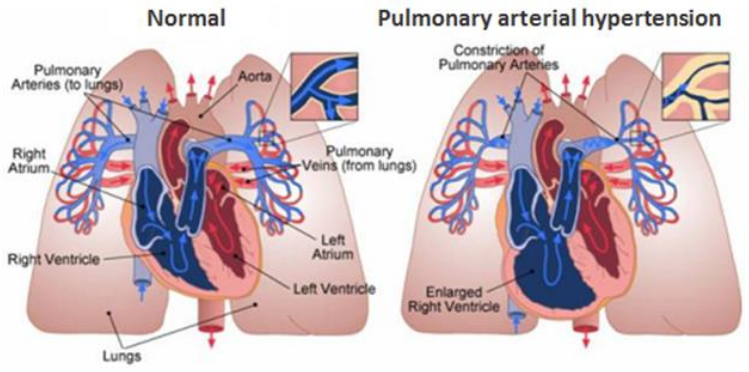

Figure 1: Illustration of pathology occurring in pulmonary arterial hypertension (PAH, right panel) compared to the normal condition (left). PAH is categorized by abnormal remodelling of pulmonary vessels (causing construction of the pulmonary arteries), leading to a progressive increase in pulmonary

artery pressure and increased right ventricular afterload. The right ventricle adapts to this increased load via several compensatory mechanisms (incl. increase in size). The progressive increases in pressure-overload leads almost inevitably to right ventricle dysfunction and right heart failure, which is the predominant cause of death in PAH. (Source: https://www.labroots.com/trending/cardiology/3394/bad-pulmonary-arterial-hypertension)

Due to its complex nature, the pathobiology of PAH remains incompletely understood. Research has focused for many years on the pathophysiology that is occurring in the lung as starting point for PAH. It is believed that changes in the layer of cells (endothelial cells) that line the small arteries of the lung ('abnormal vascular remodelling'), either causing or being linked to changes in the smooth muscle cells in the vessel wall, initiates the narrowing of the pulmonary arteries. Several studies (including of our research group) have shown that the abnormal vascular remodelling in the lung is associated with endothelial cell dysfunction, increased proliferation of smooth muscle cells, loss of pre-capillary arteries, inflammation and impaired bone morphogenetic protein (BMP) signalling [5,6]. However, far less is known about the events leading to right heart failure in PAH. Although it is known for some years that RV adaptation is of clinical importance, it has just recently become clear that RV diastolic stiffness increases and may contribute to disease progression in PAH. We and others have recently shown that although increased RV afterload is the initial trigger for PAH-induced RV dysfunction, the degree of pressure overload does not predict the development of RV failure. This suggest that the response of the RV to pressure overload, rather than the degree of pressure overload, determines the fate of the right ventricle in PAH patients. Hence, further studies are warranted in order to increase our understanding of PAH pathogenesis, including the processes resulting in right heart failure (RV dysfunction). Gaining insights in the pathology occurring in the lungs - as well as the heart - is pivotal in order to develop new therapies for PAH.

CURRENT STATE-OF-THE-ART IN THERAPY DEVELOPMENT

Over the past three decades, based on the advances in our understanding of the pathobiology of PAH, new targeted therapies have been developed, resulting also in improved patient outcomes [5,7,8]. Current drug therapies used in the clinic for PAH are particularly focused on the molecular and cellular pathways underlying pulmonary vascular remodelling (in particular endothelial dysfunction [9]), vasoconstriction, inflammation and thrombosis [8,9]. Novel potential targets in PAH drug development include vascular inflammation, metabolic derangements and aberrant BMPR2 signalling [10]. Despite these advances, PAH remains an incurable disease as mortality rates are high and prognosis of patients remains poor. There is still an unmet medical need for new PAH therapies, possibly targeting alternative pathways. Moreover, the current therapies are selectively focused on exerting an effect on the pulmonary system. Little is known about their effects on right heart function. In addition, there has been little consideration in the field for the possibility that PAH patients may benefit from a cardiac-specific treatment, which has no direct effect on the pulmonary vasculature, but presents or reverses right heart dysfunction.

One of the missions is to ultimately improve diagnosis and treatment of PAH. Fundamental, translational and clinical research to expand the understanding of PAH RV overload are all essential to achieve this. Our research group has a unique focus on the integrated pathophysiological consequences of PAH and right heart failure and is not only working to restore the PAH lung vasculature, but also to develop PAH-treatments that are cardiac-specific with no direct effects on the pulmonary vasculature. Through our research we have gained in-depth information and new insights into potential new druggable targets in PAH and right heart failure.

Testing of novel therapeutic interventions in preclinical models, before the start of clinical studies, is an essential component of our research programme. It is ethically unacceptable and practically impossible to test all (combinations of) treatments immediately in patients. Therefore, preclinical studies in relevant disease models (cellular/animal models for PAH) are required to provide information on the soundness of the strategy, the best candidate agents and the most optimal combinations of treatments. As such,

animal studies form an indispensable part of our research and PAH treatment development. Translational studies have resulted in the identification of specific (cell-type dependent) blockers for TGFβ and/or enhancers of BMP signalling to restore the TGFβ/BMP imbalance in endothelial cells and the validation of these compounds in experimental PAH models (to ultimately develop a drug with beneficial effects in the lungs and the heart). Successful preclinical studies have allowed us to select one compound for testing in a Phase IIb clinical trial for the treatment of PAH patients. Through the studies included in the current project proposal, these and other research lines will be further expanded with the ultimate goal to improve the treatment and quality of life of PAH patients. The interventions act on 7 different established targets for PAH, namely BMPR2 signalling, vascular tone, RV diastolic stiffness, inflammation, coagulation, integrin and VHL gene expression (Figure 2).

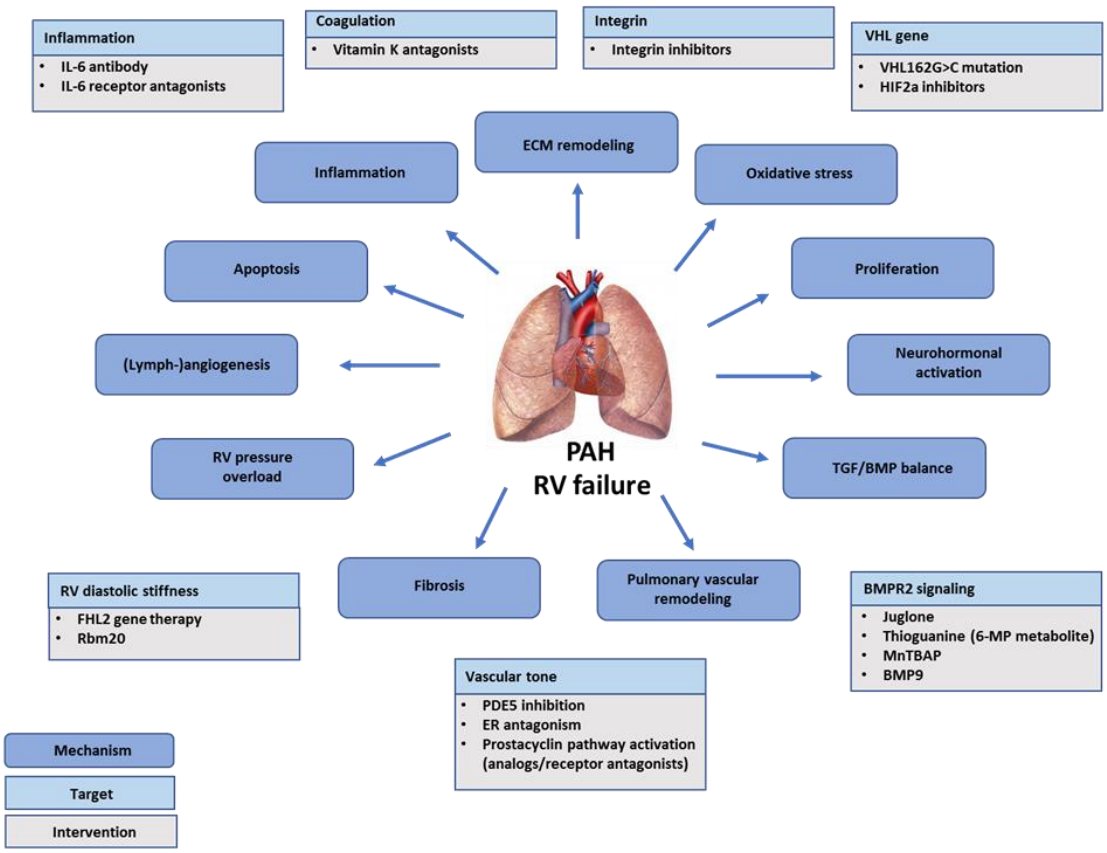

Figure 2: Illustration of the included interventions in the project and the targets upon which they act. Should during the course of this project our fundamental/clinical research (not included in this project), result in the identification of new interventions addressing these targets, these interventions will be added to the study.

References used in this section:

[1] P. Carter et al., "Association of Cardiovascular Disease with Respiratory Disease," Journal of the American College of Cardiology, vol. 73, no. 17, pp. 2166–2177, May 2019, doi: 10.1016/j.jacc.2018.11.063.
[2] A. Morris, "Heart–Lung Interaction via Infection," Ann Am Thorac Soc, vol. 11, no. Suppl 1, pp. S52–S56, Jan. 2014, doi: 10.1513/AnnalsATS.201306-157MG.
[3] G. Simonneau et al., "Updated clinical classification of pulmonary hypertension," J. Am. Coll. Cardiol., vol. 54, no. 1 Suppl, pp. S43-54, Jun. 2009, doi: 10.1016/j.jacc.2009.04.012.
[4] Long Alliantie Nederland, Longziekten feiten en cijfers 2013. Amersfoort, Nederland: Long Alliantie Nederland, 2013.
[5] M. Humbert et al., "Pathology and pathobiology of pulmonary hypertension: state of the art and research perspectives," Eur. Respir. J., vol. 53, no. 1, Jan. 2019, doi: 10.1183/13993003.01887-2018.

[6] A. R. Hemnes and M. Humbert, "Pathobiology of pulmonary arterial hypertension: understanding the roads less travelled," Eur Respir Rev, vol. 26, no. 146, Dec. 2017, doi: 10.1183/16000617.0093-2017.
[7] E. M. T. Lau, E. Giannoulatou, D. S. Celermajer, and M. Humbert, "Epidemiology and treatment of pulmonary arterial hypertension," Nat Rev Cardiol, vol. 14, no. 10, pp. 603–614, Oct. 2017, doi: 10.1038/nrcardio.2017.84.
[8] M. Humbert and H.-A. Ghofrani, "The molecular targets of approved treatments for pulmonary arterial hypertension," Thorax, vol. 71, no. 1, pp. 73–83, Jan. 2016, doi: 10.1136/thoraxjnl-2015-207170.
[9] R. Nogueira-Ferreira, R. Ferreira, and T. Henriques-Coelho, "Cellular interplay in pulmonary arterial hypertension: Implications for new therapies," Biochimica et Biophysica Acta (BBA) - Molecular Cell Research, vol. 1843, no. 5, pp. 885–893, May 2014, doi: 10.1016/j.bbamcr.2014.01.030.
[10] S. Bonnet et al., "Translating Research into Improved Patient Care in Pulmonary Arterial Hypertension," Am J Respir Crit Care Med, vol. 195, no. 5, pp. 583–595, Mar. 2017, doi: 10.1164/rccm.201607-1515PP.
[11] K. R. Stenmark, B. Meyrick, N. Galie, W. J. Mooi, and I. F. McMurtry, "Animal models of pulmonary arterial hypertension: the hope for etiological discovery and pharmacological cure," Am. J. Physiol. Lung Cell Mol. Physiol., vol. 297, no. 6, pp. L1013-1032, Dec. 2009, doi: 10.1152/ajplung.00217.2009.
[12] S. Andersen et al., "A Pulmonary Trunk Banding Model of Pressure Overload Induced Right Ventricular Hypertrophy and Failure," J Vis Exp, no. 141, 29 2018, doi: 10.3791/58050.

3.2 Purpose

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

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

The main objectives of the research included in this project are to:

- Evaluate novel (combinations of) therapeutic interventions in mice and rat models for PAH, targeting both pulmonary vascular remodelling and RV pressure overload;
- Improve fundamental understanding of PAH and RV failure and their underlying pathologies;
- Discover novel therapeutic targets for PAH.

The results of the studies will render pivotal information on the usefulness of the therapeutic interventions in subsequent clinical trials and will support the use of relevant PAH disease models. As such, they will contribute to improve the treatment of PAH.

The research group has available a wide range of relevant animal models for PAH and assays to measure cardiopulmonary function in rodents (see Section 3.4.1-2 for details) and all the expertise to conduct the studies described in this project documented by >40 papers published by the PI and his group in international journals on PAH in the last 11 years. The close relationship between the preclinical and clinical research additionally facilitates both clinical translation of research results and as well as feedback from the clinic to guide new research directions.

The infrastructure required for the studies (animal facility, surgery facility and wet-lab for e.g. tissue analyses) is available and the department has an active collaboration with other research groups. Standard operating procedures (SOPs) for animal handling/experimentation and extensive expertise on animal handling are available. In the project, 2 PIs, 1 technician, 2 postdocs and 3 PhD students will be involved. The studies will be performed and supervised by dedicated and well-trained staff. The studies will be financed through grant funding, which has been guaranteed. From experience we know that the studies take around 3 months (12 weeks) to complete. Based on the number of interventions we would like to test (see Section 3.4), we estimate that the research included in this project will take 5 years.

3.3 Relevance

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

SCIENTIFIC RELEVANCE
Not only will the proposed studies allow us to identify therapeutic interventions for PAH with the highest efficacy likelihood and the lowest toxicity potential before starting clinical trials, they will also increase our understanding of the processes underlying abnormal pulmonary vascularisation and that controlling the transition of RV adaptation towards right heart failure. Our research group is one of the few who takes a combined approach by studying the pathological effects of PAH in the lungs and the right heart concurrently. This will allow us to investigate the relatively new concept that PAH patients may benefit from a cardiac-specific treatment, which has no direct effect on the pulmonary vasculature, but presents or reverses right heart dysfunction. Moreover, novel therapeutic targets for future clinical research may be identified.
Although this research proposal is focused on PAH, right heart failure is also the main cause of death in several other conditions such as left heart failure and critical illness. We have for example shown that not only the RV but also the LV is affected in PAH-patients. We believe that RV remodelling observed in PAH patients shares important pathophysiological mechanisms with the cardiac remodelling observed in left heart failure patients. As such, the findings of this proposal may also advance research in left heart failure. The scientific relevance of our findings is therefore not limited to PAH-induced right heart failure.

SOCIETAL RELEVANCE
PAH remains an incurable debilitating disease, with high mortality rates and poor prognosis for patients. Although the incidence is low (2.2 per million), the current life expectancy is only 3-5 years [4]. Besides the enormous impact of the disease on the quality of life of PAH patients, the disease also carries considerable economic consequences because patients and/or care-givers drop out of the work force and patients require expensive medical treatments, including lung transplantation. New PAH therapies, also targeting alternative pathways are urgently needed. In this project, we address apart from established PAH-targets (e.g. BMPR2 signalling) [10], also relatively new ones, such as RV diastolic stiffness. Up till now, there has been little consideration in the field of the notion that PAH patients could benefit from a cardiac-specific treatment, which has no direct effect on the pulmonary vasculature. With the results of this project, we will be able to select those interventions with promising effectiveness in PAH animal models for further clinical testing. This will bring us hopefully a step closer to the development of an effective PAH treatment. In the future, patients at risk of developing right heart failure (PAH) may benefit from these new treatment options. Although PAH is rare, other types of pulmonary hypertension (PH) are much more prevalent and carry significant morbidity and mortality. Moreover, right heart failure is becoming a great clinical problem as leading cause of death in several diseases such as left heart failure, and the critical ill at the intensive care. Currently, no therapeutic strategies are available to improve RV function or prevent right heart failure. Beside knowledge on PH and right heart failure, this project will also provide new insight in cardiac and endothelial physiology, which will be useful in other lung diseases and left heart failure. As such, the societal relevance of our studies extends far beyond PAH alone.

References used in this section:
[4] Long Alliantie Nederland, Longziekten feiten en cijfers 2013. Amersfoort, Nederland: Long Alliantie Nederland, 2013.
[10] S. Bonnet et al., "Translating Research into Improved Patient Care in Pulmonary Arterial Hypertension," Am J Respir Crit Care Med, vol. 195, no. 5, pp. 583–595, Mar. 2017, doi: 10.1164/rccm.201607-1515PP.

3.4 Research strategy

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

Each animal study included in the proposal will be initiated based on identified PAH targets or candidate targets obtained by fundamental research (e.g. genetical, molecular, cellular, histopathological studies on tissue of animal models) or clinical research (e.g. genetic studies on human samples or other observations, such as fMRI) as illustrated in Figure 3. In all cases, a clear rationale for the animal experiments will be provided based on in vitro data or other considerations (e.g. clinical data). Ultimate

end goal of all studies is to identify those therapeutic interventions that can proceed to clinical testing. Main selection criteria for interventions to progress to the clinical phase is a demonstrated in vivo efficacy in reducing abnormal pulmonary vascular remodelling and/or improving RV adaptation.

All studies will be grouped based on similarities in study goals and protocols. The procedures are introduced in Section 3.4.2 and described in detail in Appendix 1-4. Selection criteria for and connection between procedures are described below and in Section 3.4.3.

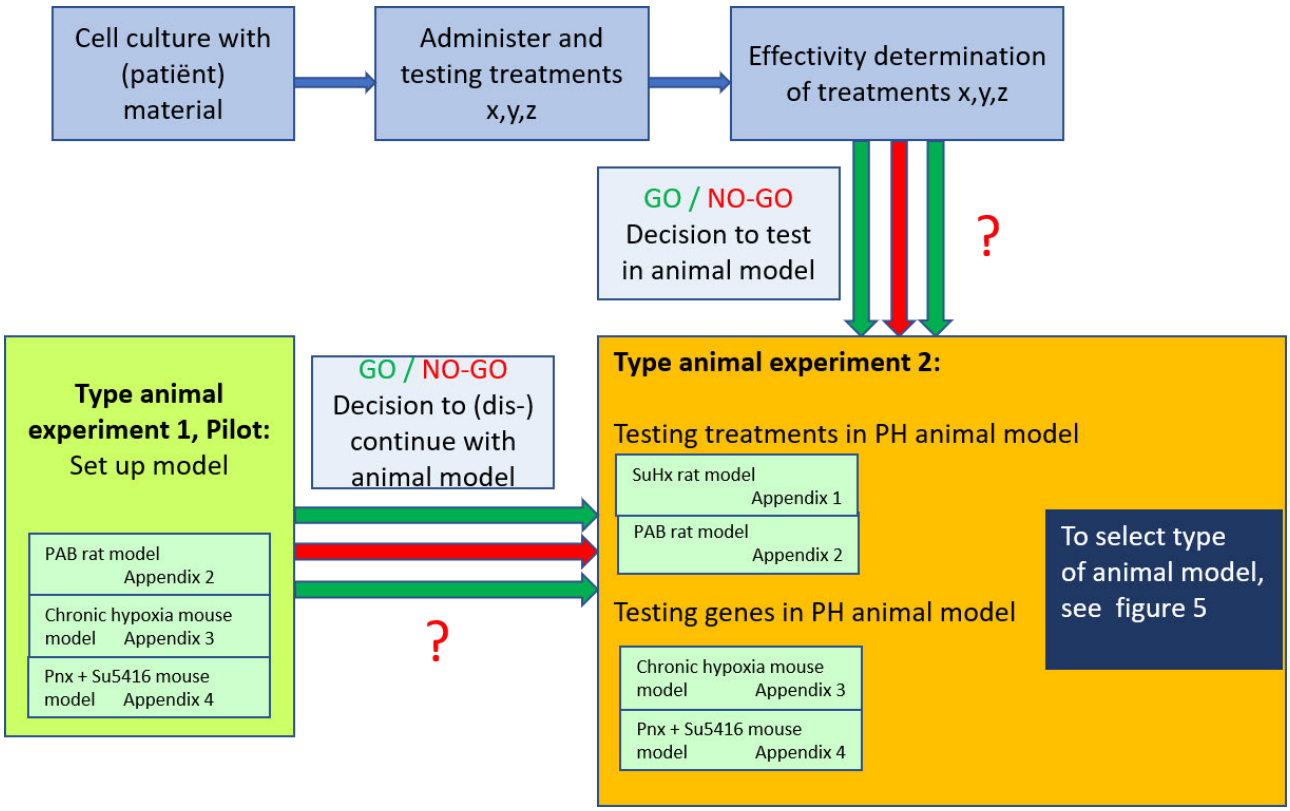

Figure 3: Position of this project within the overall strategy to develop a new therapeutic intervention for PAH. In case a candidate treatment has positive results without severe side-effects, the treatment will be selected for clinical testing. Since the PAB rat model and both mouse models are not established models at out department yet, we will first set up these models. If animals successfully develop PH (rats: RVSP > 40 mmHg; mice: RVSP > 30 mmHg), we will continue with the model. Both the PAB model and chronic hypoxia mouse model are validated models used by several other research groups. Therefore, we expect that we will successfully set up these models.

SELECTION CRITERIA FOR THE ANIMAL MODELS TO BE USED

At present, there is no animal model available that fully recapitulates human PAH [10,11]. To maximize the chances of a successful outcome and to be able to compare the results with those of previous animal studies (AVD5.1 lid2h), we will evaluate the novel (combinations of) therapeutic interventions in four animal models for PAH. These include:

5.1 lid2h

In the 5.1 lid2h pulmonary vascular remodelling and right heart failure are induced by the combined exposure to the vascular endothelial growth factor receptor (VEGFR) inhibitor SU5416 and hypoxia. A single administration of SU5416 is followed by 3-4 weeks transient exposure to hypoxia and 2 weeks normoxic re-exposure. Extensive pulmonary vascular remodelling and first signs of right ventricle dysfunction is observed after 5-6 weeks. After an additional 4 weeks, the rats can develop right heart failure.

Pulmonary artery banding (PAB) model (rat model).

Since the PAB rat is not an established model at out department yet, we will first set up this model. If animals successfully develop PH, we will continue with the model (GO/NO-GO). The PAB model is a validated model used by several other research groups. Therefore, we expect that we will successfully set up these model.

The pulmonary artery banding rat model for PAH is a surgical model. A titanium clip is compressed around the pulmonary artery with a modified ligating clip applier. When subjecting rats to pulmonary artery banding for 6-8 weeks [12, and own published data], they developed cardiac phenotypes with RV hypertrophy and dysfunction.

Chronic hypoxia model (mouse model)

Since the Chronic hypoxia mouse is not an established model at out department yet, we will first set up this model. If animals successfully develop PH, we will continue with the model (GO/NO-GO). The chronic hypoxia mouse model is a validated model used by several other research groups. Therefore, we expect that we will successfully set up this model.

In the chronic hypoxia model, mice are exposed to chronic hypoxia (10% O2, for 3 weeks). Pulmonary vascular remodelling and a mild increase in right ventricular pressures are observed after three weeks. To test the involvement of targets genes in pulmonary vascular or right ventricular remodelling (as identified above) that are promising novel therapeutic interventions for PAH, knock-in and knock-out mice will be tested in this model. As an example, this model includes EpoR-null mutant mice expressing erythropoietin receptor (EpoR) exclusively in the erythroid lineage (EpoR-/- rescued mice). Because systemic deletion of EpoR is embryo-lethal, mice are rescued with EpoR that is exclusively expressed in erythroid progenitor cells under the regulatory domain of globin transcription factor 1 (GATA-1) (EpoR-/- rescued mice). Mice (EpoR-/- rescued and wild-type controls) are exposed to hypoxia (10% O2, chronically) for 3 weeks. The development of pulmonary vascular remodelling is accelerated in EpoR-/- rescued mice compared with wild-type mice.

Pneumonectomy+SU5416 model (mouse model)

Since the Pneumonectomy+SU5416 mouse is not an established model at out department yet, we will first set up this model. If animals successfully develop PH, we will continue with the model (GO/NO-GO).

The pneumonectomy (PNX)+SU5416 mouse model will be used to validate genetic targets that are promising as novel therapeutic intervention for PAH. The target genes include identified PAH targets or candidate targets obtained by fundamental research (e.g. genetical, molecular, cellular, histopathological studies on tissue of animal models) or clinical research (e.g. genetic studies on human samples or other observations, such as fMRI) – see also Figure 3. In the pneumonectomy (Pnx) +SU5416 mouse model, the left lung will be removed which results in increased pulmonary blood flow and vascular remodelling. A single administration of SU5416 one week after the surgery will result in severe PAH and RV dysfunction. The effect of the genetic interventions will be tested when PAH has developed in the mice subjected to the PNX+SU5416 model.

These animal models are selected because they all have a close resemblance to the clinical symptomology observed in PAH patients. The four animal models also supplement each other. The PAB model is included as direct effects of interventions on RV adaptation can be studied. Interventions from which we expect it will have an effect in the heart, will be first tested in this model. Furthermore, the model will be used to validate results obtained with the SuHx model. When not using this approach, it is possible that specific RV toxicity may be overlooked, because that intervention has such a profound effect on the lung. If such an intervention would be used in human, it is possible that the effect in the lung is not as large in patients as it is in animals, while cardiotoxicity is the same. This would result in severe side-effects of the treatment intervention in patients, which was not anticipated from animal studies. With the SuHx model more mechanistic information can be obtained and interventions from which we expect it will have an effect in the lung, will be first tested in this model. Since genetic modification is more efficient in mice than in rat, the addition of the chronic hypoxia mouse model allows

for testing of genetic interventions (knock-out and knock-in) in the genes identified in human genetic studies.

SELECTION CRITERIA FOR THE INTERVENTIONS

Only interventions in this project are included that act on an identified PAH target (or candidate target) as demonstrated by previous fundamental research or clinical research. Based on this premise, we have selected several interventions to be included in our studies (Table 1). The interventions act on 7 different established targets for PAH, namely BMPR2 signalling, vascular tone, RV diastolic stiffness, inflammation, coagulation, integrin and VHL gene expression. Other pathways we would like to target are the TGFβ/BMP pathway (using nanobodies/small molecules), GDF15 (using nanobodies/peptide inhibitors), Sox9, FHL2 and several microRNAs. Should new interventions be identified during the course of this project addressing these targets, they will be added to the study.

Table 1. Overview interventions

Target	Intervention	Type of intervention	Hypothesis	Reference
RV diastolic stiffness	FHL2	Gene therapy	Increased FHL2 expression will restore RV function	Hojayev B et al., Mol Cell Biol, 2012; Granzier HL et al., Proc Nat Acad Sci USA, 2014
RV diastolic stiffness	RBM20	Genetic	Compliant titin due to mutations in RBM20 will prevent RV failure	Methawasin M et al., Circulation, 2016
Integrins	cilengitide	Drug	Inhibition of integrins will reverse pulmonary vascular remodeling	Cowan et al., JCI, 2000
Integrins	PLN-74809	Drug	Inhibition of integrins will reverse pulmonary vascular remodeling	Cowan et al., JCI, 2000
Integrins	ATN-161	Drug	Inhibition of integrins will reverse pulmonary vascular remodeling	Cowan et al., JCI, 2000
Endothelin receptor	Macitentan	Drug	Endothelin receptor antagonist will reverse pulmonary vascular remodeling	Pulido et al., NEJM, 2013
Prostacyclin receptor	Selexipag	Drug	Prostacyclin receptor agonist will reverse pulmonary vascular remodeling	Sitbon et al., NEJM, 2015
PDE5	Sildenafil	Drug	PDE5 inhibition will reverse pulmonary vascular remodeling	Galie et al., NEJM, 2005

EXPERIMENTAL READOUTS

The interventions will be tested when PAH has developed in the four animal models (therapeutics vs preventive), as this study design has the highest clinical relevance. We will particularly focus in these studies on assessing the impact of the intervention on RV overload and pulmonary vascular remodelling, as both are clinically relevant end-points for PAH. Moreover, we have shown in the past that interventions targeted at the lungs, also had an effect on cardiac function and vice versa. This clearly illustrates that the two organ systems are interconnected.

As the focus of this project is on the evaluation of the efficacy of novel (combinations of) therapeutic PAH-interventions, we have chosen for clinically relevant end-points as primary outcome measures. These will be combined with advanced blood serum and tissue analyses to study the mechanisms underlying the chosen interventions and to possibly identify new targets (in untreated experimental animals). The outcomes measures include:

- Time-to-right-heart failure: the time (in days) from PAH induction to clinical manifestation of right heart failure;
- Lung and right heart (RV) function (cardiac output, RV hypertrophy and dilatation, RV functional measurements through echocardiography and pressure volume loop analysis);
- Measurements of vascular leakage;
- Systemic blood pressure;
- Imaging (MRI myocardial tagging, Diffusion Tensor Imaging-MRI quantification of the helical muscle fibre architecture in the RV)
- Blood and lung, heart, and muscle tissue analysis (to assess degree of pulmonary vascular remodelling).

References used in this section:

[10] S. Bonnet et al., "Translating Research into Improved Patient Care in Pulmonary Arterial Hypertension," Am J Respir Crit Care Med, vol. 195, no. 5, pp. 583–595, Mar. 2017, doi: 10.1164/rccm.201607-1515PP.
[11] K. R. Stenmark, B. Meyrick, N. Galie, W. J. Mooi, and I. F. McMurtry, "Animal models of pulmonary arterial hypertension: the hope for etiological discovery and pharmacological cure," Am. J. Physiol. Lung Cell Mol. Physiol., vol. 297, no. 6, pp. L1013-1032, Dec. 2009, doi: 10.1152/ajplung.00217.2009.
[12] S. Andersen et al., "A Pulmonary Trunk Banding Model of Pressure Overload Induced Right Ventricular Hypertrophy and Failure," J Vis Exp, no. 141, 29 2018, doi: 10.3791/58050.

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 studies in this project have been grouped into four different procedures:

PROCEDURE 1: INTERVENTION STUDIES USING THE 5.1 lid2h

The SuHx model in Sprague-Dawley rats is well-established (in use >7 years) in our department. The rats receive a single subcutaneous (s.c.) injection of SU5416, followed by 3-4 weeks transient exposure to 10% hypoxia and 2 weeks normoxic re-exposure. In the study a control group is included that receives a vehicle-injection and no hypoxia. Following confirmation of PAH induction with echocardiography, the animals are randomly divided into two groups: the intervention group and an vehicle-control group (Figure 4). After ~2-6 weeks of treatment (time-point will vary dependent on type of intervention), the experimental readouts as defined in Section 3.4.1 will be assessed by means of echocardiography of the heart (under anaesthesia), hemodynamic assessments via a catheter (under anaesthesia) and in a subset of the animals by MRI-imaging (MRI myocardial tagging, under anaesthesia). At the end of these assessments (Figure 4), the animals are sacrificed and blood and lung/cardiac tissues is collected for further analysis (histology, RNA analyses and protein analyses).

PROCEDURE 2: INTERVENTION STUDIES USING THE PULMONARY ARTERY BANDING (PAB) MODEL

Rats are subjected to pulmonary artery banding for 6-8 weeks. A sham-operated control group is included in the study that will not receive the pulmonary artery banding (Figure 4). Upon verification of PH induction in the animals that have received the pulmonary artery banding compared to controls (via echocardiography of the heart), the intervention will be started (Figure 4). The PH-rats will be randomly allocated to the intervention group or the placebo-control (vehicle-treated) group. After ~6 weeks of treatment (time-point will vary dependent on type of intervention), the experimental readouts as defined in Section 3.4.1 will be assessed by means of echocardiography of the heart (e.g. measurements of the RV wall thickness, under anaesthesia), hemodynamic assessments of the heart/lung (e.g. RV pressure-volume loops) via a catheter (under anaesthesia). A subset of the treated animals is subjected to MRI myocardial tagging and/or DT-MRI quantification of the helical muscle fiber architecture in the RV. A DT-

MRI quantification of the helical muscle fiber architecture in the RV will be performed *ex vivo*, since bulk cardiac motion *in vivo* may lead to image artefacts. Hearts will be perfused and fixated to keep the RV open, and the diffusion-weighted images will be acquired. In this way, we will get information on the fiber orientation of the RV. At the end of these experiments, all animals are sacrificed and blood and tissue (heart and lung) is collected for subsequent analysis (histology, RNA analyses and protein analyses).

PROCEDURE 3: INTERVENTION STUDIES USING THE CHRONIC HYPOXIA MOUSE MODEL
The wildtype versus knock-in/knock-out mice will be exposed to hypoxia (10% O_2, chronically) for 3 weeks. After that, the animals are randomly divided into two groups: the intervention group and an vehicle-control group (Figure 4). The effect of the genetic interventions will be tested when PAH has developed in the mice subjected to the chronic hypoxia model. The mice are anesthetized and hemodynamic measurements are performed (among others right ventricular pressure with a Millar catheter) and vascular leakage is measured - see also Section 3.4.1. Following exsanguination, serum and plasma samples are taken, and heart and lung tissues are collected for tissue, protein and RNA analyses.

PROCEDURE 4: INTERVENTION STUDIES USING THE PNEUMONECTOMY+SU5146 MOUSE MODEL
The wildtype versus knock-in/knock-out mice will be exposed to the pneumonectomy (PNX) +SU5416 model. In this model, the left lung will be surgically removed (pneumonectomy), which results in increased pulmonary blood flow and vascular remodelling. A single administration of SU5416 one week after the surgery will result in severe PAH and RV dysfunction. The effect of the genetic interventions will be tested when PAH has developed in the mice subjected to the PNX+SU5416 model. The mice are anesthetized and hemodynamic measurements are performed. Following exsanguination, serum and plasma samples are taken, and heart and lung tissues are collected for tissue, protein and RNA analyses.

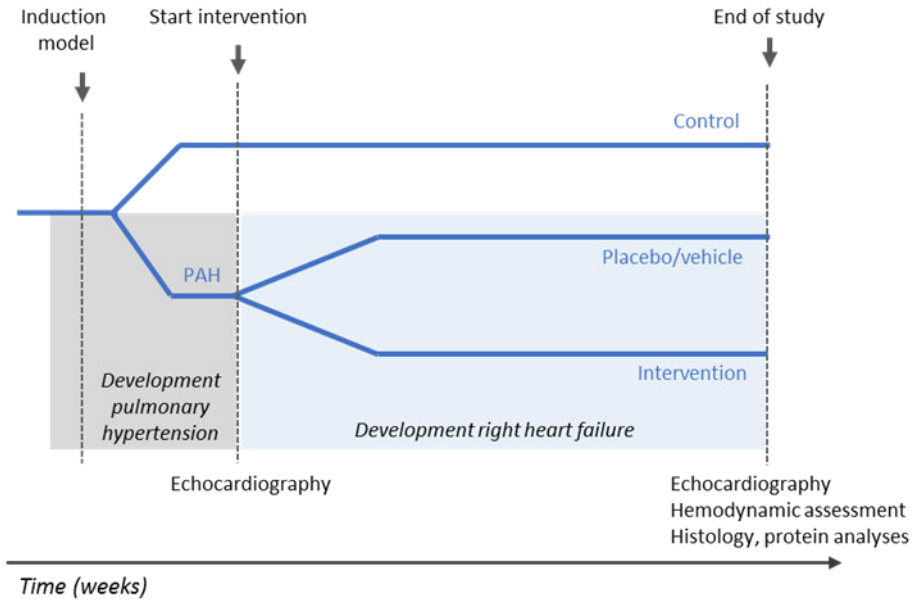

Figure 4: Graphical illustration of the approach that will be taken to test the therapeutic efficacy of the selected interventions in the animal models for PAH. The intervention will be administered after pulmonary hypertension has developed. Development of PAH will be confirmed compared to a control group, in which no PAH will be induced. The animals with confirmed PAH will be randomly allocated to the group that receives the intervention, or the placebo/vehicle-treated group.

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.

The main aim of the studies in this proposal is to examine the effect of novel (combinations of) interventions for PAH. To this end, four different animal models are used (Appendices 1-4). Each study in the project will start with selection of an appropriate model to test the intervention. Firstly, the animal model should contain the appropriate target (if the target is pulmonary vascular remodelling we will first use the SuHx model, and if the target is right ventricular heart failure we will first use the PAB model) (see Figure 5). Secondly, the level of discomfort should be considered. If no previous *in vivo* data are present on the new intervention, the relevant model with the lowest possible level of discomfort will be selected for initial *in vivo* studies (which is the SuHx model). If good quality data are already available from previous (own or published) data, more stringent and complex models may be selected from the outset.

In general, a first *in vivo* intervention study will be performed using the SuHx or PAB model (Appendix 1, 2). If these initial *in vivo* tests are unsuccessful, the investigational intervention will be discontinued (GO/NO GO DECISION). If initial *in vivo* tests are successful, it will in general be necessary to perform confirmatory studies in the other models (Appendix 1-4). Confirmatory studies may also be performed on models lacking the appropriate target, as a negative control to understand or confirm the mechanism of action.

Depending on the type of intervention, preliminary studies may also be necessary, as outlined in Figure 5. Prior to using interventions that have never previously been tested *in vivo*, particularly for new pharmacotherapeutic interventions, a pilot pharmacokinetics study will be performed to determine the most optimal dose, route and schedule. If the study aims to test a new combination of interventions, a pharmacokinetics study will determine potential drug-drug interactions. When no data is available on previous dose-finding of pharmacological interventions, tolerability studies and/or dose-finding studies will also be performed to determine the maximum tolerated dose. If the highest safe dose is not expected to provide sufficient systemic exposure for efficacy, further *in vivo* studies will be halted (GO/NO GO DECISION).

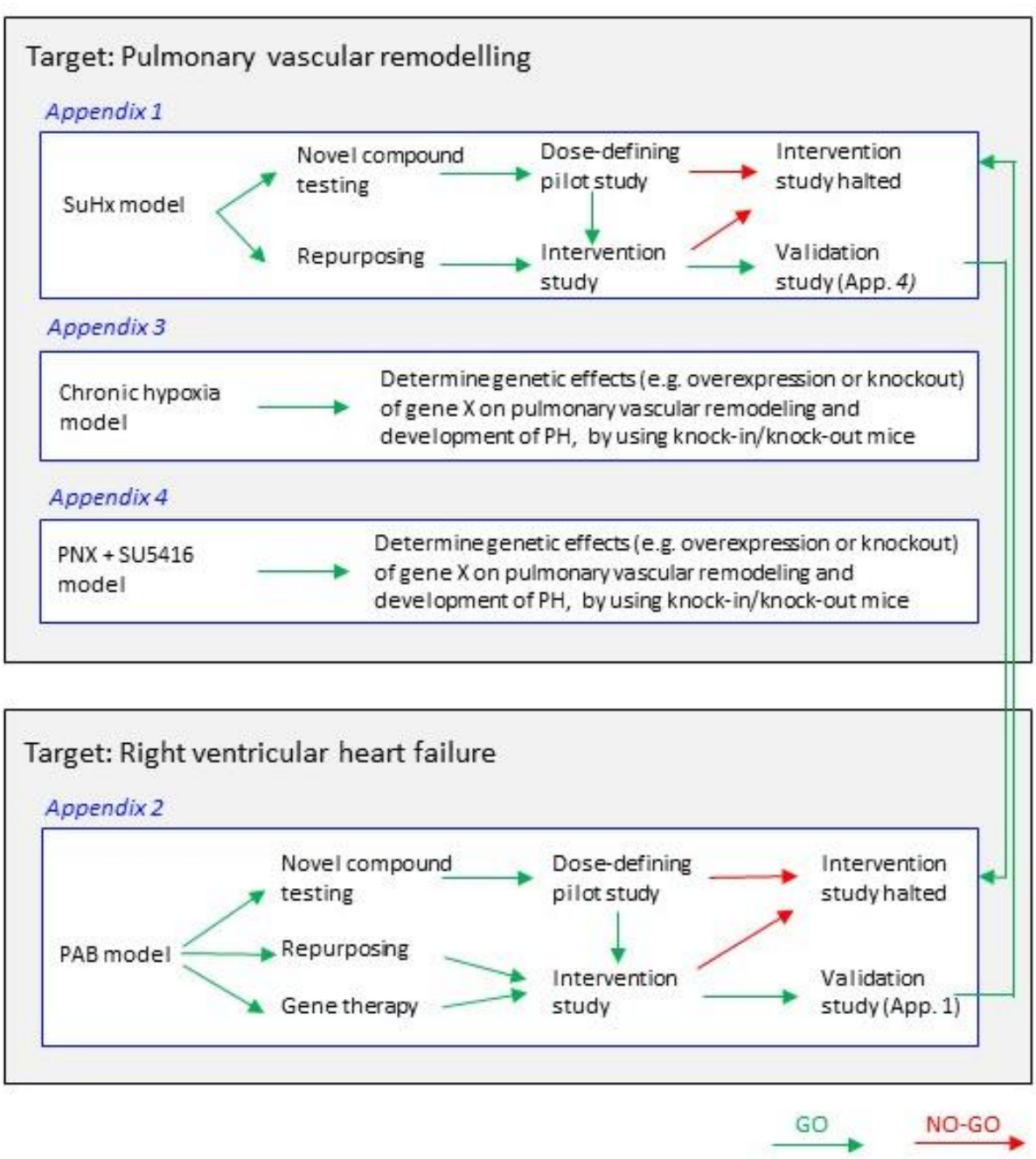

Figure 5: Flow charts summarizing the general research strategy and the key decision/selection points. The SuHx model will be used to test compounds (either novel compounds or compounds that are already used for other purposes ("repurposing")) that target the pulmonary vascular remodelling. When the compound shows positive effects, the PAB model will be used to validate these results.
The mouse models (Chronix hypoxia and PNX+SU5416) will be used to determine genetic effects by using knock-in/knock-out mice. No compounds will be tested in these models. Because both mouse models are new to our department, it is not decided when to use the Chronic hypoxia model or the PNX+SU5416 model. This will depend on the Pilot Set-up Model experiments, which are followed by a GO / NO-GO decision (fig. 3). Based on how successfully animals will develop PH, we will decide which model we will use in the future. At the moment, the chronic hypoxia model is the golden standard to study PH in mice, although these animals do not develop severe PH and right ventricle failure. This makes the model less representative for our patients. Therefore, we want to set up the PNX+SU5416 model, but we do have to compare this model with the existing model. The PAB model will be used to test compounds

that target right ventricular heart failure. When the compound shows positive effects, the SuHx model will be used to validate these results.
SuHx: SU5416 & Hypoxia, PNX: pneumonectomy, PH: Pulmonary Hypertension, PAB: Pulmonary Artery Banding.

Thus, subsequent research steps will be determined based on study outcome:

- If the study has been conducted technically satisfactory and the outcome is conclusive and negative, the study will be halted.
- If the study results are not conclusive, but warrant further exploration, the experiment will be redesigned and repeated, provided that improvements are feasible. If not, the study will be halted.
- If the study has been conducted technically satisfactory and the outcome is positive:
    i. the study may be considered completed,
    or
    ii. efficacy may need to be confirmed in an additional, usually more stringent, model,
    or
    iii. further testing may be needed to support translation to clinical studies, such as testing at adapted dose levels or with different dosing schedules.

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	Intervention studies using SuHx model
2	Intervention studies using PAB model
3	Intervention studies using Chronic hypoxia mouse model
4	Intervention studies using Pneumonectomy (Pnx)-Su5416 mouse model
5	
6	
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'.	5.1 lid2h
1.2	Provide the name of the licenced establishment.	5.1 lid2h
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: 1 — Type of animal procedure: Intervention studies using SuHx model

2 Description of animal procedures

A. Experimental approach and primary outcome parameters

Describe the general design of the animal procedures in relation to the primary outcome parameters. Justify the choice of these parameters.

This Appendix describes the use of the 5.1 lid2h to validate novel therapeutic interventions for PAH. In the 5.1 lid2h pulmonary vascular remodelling and right heart failure are induced by the combined exposure to the vascular endothelial growth factor receptor (VEGFR) inhibitor SU5416 and hypoxia. A single administration of SU5416 is followed by 3-4 weeks transient exposure to hypoxia and 2 weeks normoxic re-exposure. Extensive pulmonary vascular remodelling and first signs of right ventricle dysfunction is observed after 5-6 weeks. Upon verification of PAH induction in the animals (via echocardiography of the heart), typically 5-6 weeks after induction of the model, the intervention will be started. Only interventions in this project are included that act on an identified PAH target (or candidate target) as demonstrated by previous fundamental research or clinical research – see project proposal Figure 2). The interventions act on 7 different established targets for PAH, namely BMPR2 signalling, vascular tone, RV diastolic stiffness, inflammation, coagulation, integrin and VHL gene expression. Should new interventions be identified during the course of this project addressing these targets, they will be added to the study. The interventions will be tested when PAH has developed in the SuHx model (therapeutics vs preventive), as this study design has the highest clinical relevance. We will particularly focus in these studies on assessing the impact of the intervention on RV overload and pulmonary vascular remodelling, as both are clinical relevant end-points for PAH. The primary outcomes measures include the time (in days) from PAH induction to clinical manifestation of right heart failure; lung and right heart (RV) function and structure (assessed via imaging and tissue/protein analysis). The advanced blood serum and tissue analyses are also included to study the mechanisms underlying the chosen genetic interventions and to possibly identify new targets (in control animals).

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

Induction SuHx model
Sprague-Dawley rats receive a single subcutaneous (s.c.) injection of the vascular endothelial growth factor receptor (VEGFR) inhibitor SU5416 (25 mg/kg in CMC), followed by 3-4 weeks transient exposure to 10% hypoxia in a hypoxia chamber and 2 weeks normoxic re-exposure). In the study, a control group is included that receives a vehicle-injection and no hypoxia.

Follow-up development and progression
Pulmonary vascular remodelling and a mild increase in right ventricular pressures are observed after 5-6 weeks. To verify PAH induction, the rats are subjected to echocardiography of the heart. The right ventricular end-diastolic diameter (RVEDD) and tricuspid annular plane systolic excursion (TAPSE), are measured according to standard protocols at our Department. Following confirmation of PAH induction with echocardiography, the SuHx-animals are randomly divided into two groups: an intervention group and a vehicle-control (for illustration of experimental design see Figure 1 below).

Interventions
Interventions include treatment by any kind of agent (e.g. dietary, chemical, biological, genetic, radiopharmaceutical), or combination of these agents. Some of these interventions are already known, including FHL2, RBM20 and integrin (see table 1 of the proposal). Should new interventions be identified during the course of this project addressing these targets, they will be added to the study. All agents will be administered by the appropriate route, time of day, duration and frequency as required. Examples include oral gavage, bolus injections (i.v., i.p., s.c.), continuous infusion in cannulated animals, minipumps and slow release pellets. Procedures requiring surgery (cannulation, implantation of minipumps) will be performed under general anaesthesia and analgesia. The selection of agent(s), dose, time of day, and route of application depends on the target of the intervention and the details of the treatment of each study will be discussed with the IvD.

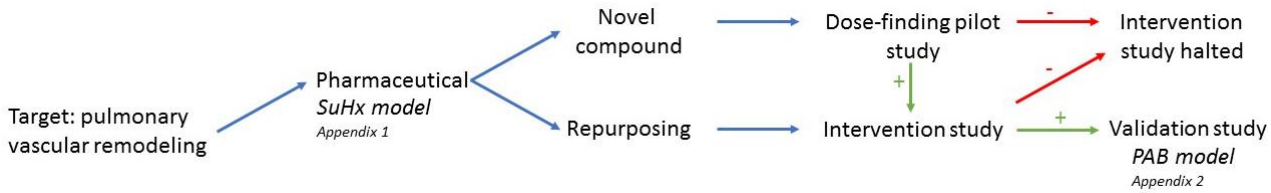

Figure 1. Study design SuHx studies, with GO / NO-GO decisions.

After ~2-6 weeks of treatment (time-point will vary dependent on type of intervention), the experimental readouts as defined in Section 3.4.1 of the project proposal will be assessed by means of echocardiography of the heart and hemodynamic assessments via a catheter (under anaesthesia).

Echocardiography
After treatment, all animals will be subjected to echocardiographic assessments (see above) under anaesthesia, to measure RV wall thickness (RVWT), RV end diastolic diameter (RVEDD), tricuspid annular plane systolic excursion (TAPSE), stroke volume (SV), heart rate (HR), cardiac output (CO), pulmonary artery acceleration time (PAAT).

Haemodynamic measurements
After the treatment period, rats are anaesthetized for hemodynamic assessment via open-chest RV catheterization. RV systolic pressure (RVSP) will be determined from steady state measurement, as well as RV afterload (Ea-Arterial elastance). Pressure-volume loops after vena-cava occlusion will be obtained and used to derive end-systolic elastance (Ees), and end-diastolic elastance (Eed). Arterial ventricular coupling will be calculated as Ees/Ea. After the haemodynamic measurements, the animals are sacrificed and blood and tissue (heart and lung) is collected for subsequent analysis.

Termination
At the end of the study, all animals are killed by an approved method (e.g. removal of blood and organs under anaesthesia or CO2 asphyxiation,) and blood and lung/cardiac tissues are collected for further

analysis (histology, RNA analyses and protein analyses). The animals are maximally for 12 weeks in experiment.

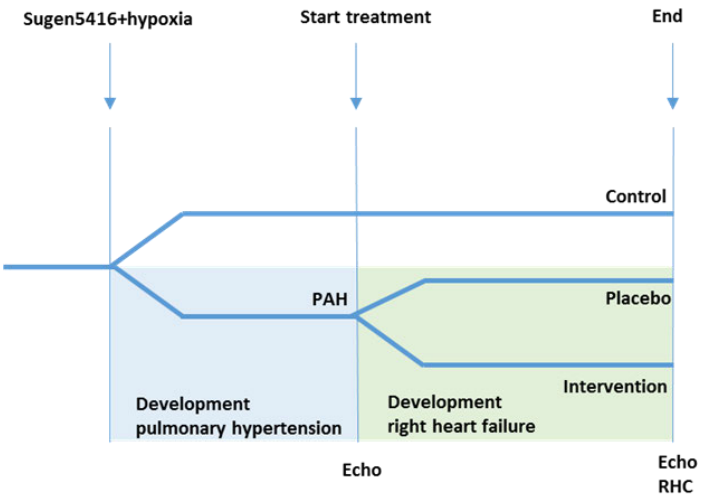

Figure 2. Experimental design SuHx studies. After two weeks of acclimatization we start with a single injection of Sugen5416 and start the 10% hypoxia period of 3-4 weeks (not the control group). At start treatment we perform an echocardiography and at the end experiments an echocardiography and right heart catheterization is performed. The animals are maximally for 12 weeks in experiment.

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

To demonstrate a 50% improvement in terms of pulmonary vascular remodelling, (measuring the diameter of the media and intima wall thickness of pulmonary arterioles in lung cryosections), between two groups (treatment vs control) with an overall variability of ~30%, a group size of 7 evaluable animals per group is needed (power > 0.8 with α = 0.05, two sided). Because our study design will be comprised of 3 groups (control, SuHx, SuHx+intervention) and 2 comparisons will be performed, an α of 0.025 (0.05/2) is defined as statistically significant. Therefore, an estimated group size of 10 evaluable animals will be needed to perform a 3-arm study. We typically use n=12 in model/intervention groups, n=6 in control group. This includes potential losses due to human end points and losses because of animals not developing pulmonary hypertension. The appropriateness of the chosen group sizes has been confirmed in previous studies conducted with the SuHx model at our Department and described in the literature.

B. The animals

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

Species: *Rattus norvegicus*, Sprague Dawley rat, wild-type.

Origin: Commercial breeder.

Sex: Both male and female animals (equally divided) will be used throughout the project.

Total: 400 rats, 60 control rats and 340 SuHx rats.

Justification: Sprague-Dawley rats (150-250 gr) will be used for the experiments. They will be 5-6 weeks of age at the start of the experiments and 12-16 weeks at sacrifice. The SuHx model is well-validated in this rat strain and for these stages of life, it is in use >7 years at our department.

Estimated numbers:
The SuHx model will be performed in 10 intervention studies during the project, with an average of ~30 rats per experiment, resulting in an estimated total of 10 x 30 = 300 rats. We typically use n=12 in

model/intervention groups, n=6 in control group. This includes potential losses due to human end points and losses because of animals not developing pulmonary hypertension. The appropriateness of the chosen group sizes has been confirmed in previous studies conducted with the SuHx model at our Department and described in the literature. For dose defining pilot studies we included 100 animals (10 animals per compound, maximally 10 interventions). This number is based on our previous experience with these pilot studies. The total amount of rats is 400 for a period of 5 years.

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.

Replacement
All proposed interventions that will be tested throughout this project will be assessed first in other, non-animal, models, such as cell culture experiments. Only if these experiments yield sufficiently promising results, in vivo tests will be undertaken. In vivo experiments are a necessary step in development of interventions that may one day be used in the clinic, since currently no other methods allow the evaluation of the effect of an intervention on the organism as a whole. Cell culture, organoids, lab-on-a-chip or computer models are not (yet) sophisticated enough to assess the interaction between the PAH pathology and host environmental factors such as the oxygen supply, the immune system and metabolism.

Reduction
The proposed number of evaluable animals per study arm (n=12 in model/intervention groups, n=6 in control group) is calculated as described above, and is in line with generally accepted protocols in scientific literature and our previous studies with the SuHx model at our Department. Further reduction of the number of animals per group would decrease statistical power, and might thus disqualify the experiment.

Refinement
State-of-the-art methods and equipment for assessing pulmonary vascular remodelling and RV overload, in particular advanced imaging techniques, will be used to refine protocols and minimize discomfort to the animals especially in support of the application of humane endpoints. International guidelines will be applied to ensure that best practice is followed.

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

The procedures conducted under this protocol will inevitably cause discomfort for the animals in the studies. In order to minimize suffering, all studies will adhere to the national and internationally accepted rules for handling of lab animals [1], [2]. Under these rules, the animals will be humanely killed when a humane endpoint (see below) is reached. Within the institute, standard operating procedures (SOP) for animal handling are in place. These also include dedicated anaesthesia and analgesia (A&A) SOPs that will accompany invasive procedures to minimize pain and discomfort.
To minimize animal suffering, pain and fear the following measures will be taken. The animals will be allowed to acclimatize to their new environment for two weeks upon arrival at the animal facility. Animals are housed in groups (socially housed) at all times (also during hypoxia. Except during surgery, animals are returned to the group directly after surgery) and environmental enrichment strategies are applied in the cages to improve animal welfare.

In case the therapeutic interventions require surgical procedures (e.g. cannula/mini-pump implantation), this will be done under general anaesthesia in combination with pain treatment. During haemodynamic measurements, the animals will be kept under general anaesthesia in a temperature-controlled environment. During imaging procedures, animals will be kept under general anaesthesia in a temperature-controlled environment.
No adverse effects on the environment are expected because animals are kept and procedures are performed in a controlled environment, all waste will be safely discarded.
Right ventricular (RV) failure is the predominant cause of death in patients with pulmonary hypertension. Most rats will not suffer from heart failure until the end of the experiment, and their discomfort will not exceed moderate. Maximally 15% (based on previous experiments) of the rats can develop heart failure with severe discomfort. It is very difficult to see if a rat is having heart failure (which can occur in the weeks after hypoxia). A decrease in bodyweight is the first sign of heart failure which is then already occurring for one day. It is normal to have a decreasing bodyweight of the rat during the day, because of sleeping, less drinking and eating during the day (daily fluctuations). To be sure it is heart failure (and not daily fluctuations of bodyweight), a bodyweight decrease of 10% is being established. Also cyanosis, dyspnea, lethargy and poor grooming can be observed. This is usually on the second day. Then an HEP will be applied.
Severe discomfort is unfortunately unavoidable. Patients usually present at the stage of heart failure. Right heart failure is an important outcome measure of our research. Heart failure is necessary to compare the rats with patients.

References used in this section:
[1] Zutphen, L. Van, Handboek proefdierkunde: proefdieren, dierproeven, alternatieven en ethiek. .
[2] J. Guillen, "FELASA Guidelines and Recommendations," J Am Assoc Lab Anim Sci, vol. 51, no. 3, pp. 311–321, May 2012.

Repetition and duplication

E. Repetition

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

N.A.

Accommodation and care

F. Accommodation and care

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

☐ No

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

The animals will be socially housed in standard conditions (food and water available ad libitum) and environmental enrichment strategies are applied in the cages to improve animal welfare conform the Directive 2010/63/EU. However, as part of the experimental model, the animals will also be housed under low oxygen conditions (10% O2) for 3-4 weeks (the chronic hypoxia period).

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.

In case the therapeutic interventions require surgical procedures (e.g. cannula/mini-pump implantation), this will be done under general anaesthesia in combination with perioperative pain treatment. General anaesthesia will also be applied in order to perform the haemodynamic measurements (via open-chest RV catheterization). For some procedures (e.g. echocardiography) anaesthesia will be applied in the absence of any risk for pain.
Within the institute, standard operating procedures (SOP) for animal handling are in place. These also include dedicated anaesthesia and analgesia (A&A) SOPs that will accompany invasive procedures to minimize pain and discomfort. These A&A SOPs describe the best practice methods for anaesthesia and analgesia for each (surgical) procedure and are regularly checked as new concepts or procedures become available. All experiments performed within this project will conform to these A&A SOPs.

I. Other aspects compromising the welfare of the animals

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

Exposure to hypoxia (10% O2) will result in a temporary increase in respiration and pulse. Following a number of days in the hypoxia conditions, the respiration level and heartbeat will begin to acclimatize. It is our experience with hypoxia-induced animals that their growth in body weight falls ~10% behind compared to control animals. Apart from this observation, we have not observed any other physical or behavioural changes in the hypoxia-treated animals. Due to the SuHx model (and consequently, the induced right heart failure), the animals may lose weight, experience shortness in breath and become lethargic. Apart from discomfort directly caused by the procedures as described above, animals may develop complications due to the therapeutic interventions (e.g. toxic side-effects), which in some cases may result in adverse effects on the animals' welfare. These side-effects are still unknown as well as the discomfort the animal can experience. The maximum tolerated level of discomfort is moderate. When this is exceeded, an HEP will be applied.
Applying therapeutic interventions, i.e. Bolus injections or procedures requiring surgery or local infusion, can also lead to moderate discomfort.
Prior to using interventions that have never previously been tested *in vivo*, particularly for new pharmacotherapeutic interventions, a pilot pharmacokinetics study will be performed to determine the most optimal dose, route and schedule. If the study aims to test a new combination of interventions, a pharmacokinetics study will determine potential drug-drug interactions. When no data is available on previous dose-finding of pharmacological interventions, tolerability studies and/or dose-finding studies will also be performed to determine the maximum tolerated dose. If the highest safe dose is not expected to provide sufficient systemic exposure for efficacy, further *in vivo* studies will be halted (GO/NO GO DECISION).

Explain why these effects may emerge.

These effects are a consequence of the induction of PAH and right heart failure due to the SuHx model and the applied interventions respectively.

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

In general, adverse effects on the animals' welfare caused by induction of pulmonary vascular remodeling and RV pressure overload cannot be completely prevented. In order to minimize adverse effects, the animals will be monitored at a frequency that is dictated by the model and timely killed when a humane endpoint (see below) is met. When profound weight drop occurs, daily monitoring will be

applied. The CO2 level, humidity and temperature in the hypoxia chamber are kept constant and will not deviate from the by law defined norms.
Should unforeseen complications due to the interventions or procedures occur, either the effect of these complications will be minimized by adjusted procedures, such as providing easy access to food (mush-feeding), or if this is not possible, the humane endpoints as defined below will be taking into account.

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.

The most important humane endpoints applicable to all studies are:

- Weight loss ≥20% of maximum body weight in adult animals, measured from the start of the treatment
- Weight loss ≥10% of body weight during 24h, in combination with:
  - Sustained abnormal breathing, dyspnoea (symptom PAH/right heart failure)
  - Sustained lethargy (symptom PAH/right heart failure)
- Sustained abnormal behaviour
- Complications of interventions
- Other procedure-specific endpoints

Indicate the likely incidence.

It is our experience that the end stadium of PAH will be achieved in the SuHx model at maximally day 70 after induction of the model. At day 70, around 85% of the induced animals do not experience symptoms of right heart failure yet (sustained periods of shortness of breath, dyspnoea, lethargy). Thus, humane endpoints are expected to occur in <15% of all cases.

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').

Discomfort levels categorized according to the SuHx model are as follows:

- Animals that are subjected to the SuHx model, injection + chronic hypoxia, experience mild discomfort.
- Control animals, experience mild discomfort.

Discomfort levels categorized according to the interventions are as follows:

- Animals that receive therapeutic interventions during the study period, can experience moderate discomfort.
- Control animals (vehicle treated) during the study period, can experience moderate discomfort.

Discomfort levels categorized according to the procedures used for assessment of pulmonary vascular remodelling and right ventricular pressures (echocardiography, haemodynamic measurements) are as follows:

- Echocardiography: mild discomfort.
- Haemodynamic measurements, using general anaesthesia: non-recovery.

Other procedures that will be used, which are not expected to alter the total level of discomfort experienced:

- Simple well tolerated interventions (e.g. drug treatment): mild discomfort.
- Simple but frequent handling procedures (e.g. weighing): mild discomfort.
- Minimally invasive procedures and those requiring anaesthesia (e.g. non-invasive imaging): mild discomfort.

Table 1: Procedures and discomfort classification.

Procedures	Category	Expected percentage (%) of animals	Frequency and duration of the procedure
1. Obtaining rats: Transport to animal facility	mild	100%	1x
2. Induction SuHx model: a. Injection b. Induction hypoxia	 mild mild	 100% 85%	 1x 1x 3-4 weeks
3. Echocardiography (pre-treatment)	mild	100%	1x ~10min
4. Applying therapeutic interventions* a. Frequent handling procedures b. Bolus injections (i.v., i.p., s.c, oral gavage) c. Procedures requiring surgery (cannulation, implantation of minipumps) under brief adequate anaesthesia and postoperative analgesia d. Local infusion, either acute or chronic through a cannula	 mild moderate moderate moderate	 100% 85% 25% 25%	 Max 4 weeks Max 4 weeks 1x ~30-60 min Max 4 weeks
5. Potential adverse effects of treatments**	max. moderate	40%	Max 4 weeks
6. Heart failure***	Max. severe	15%	Max 2 days
7. Echocardiography (post-treatment)	non-recovery	100%	1x ~10 min
8.Heamodynamic measurements (under general anaesthesia and analgesia)	non-recovery	100%	1x ~30 min
9. Blood sampling	mild	25%	Max 2x per week <2 min
10. Sacrifice	non-recovery	100%	1x <1 min

*The therapeutic intervention is unknown yet. The interventions act on 7 different established targets for PAH, namely BMPR2 signalling, vascular tone, RV diastolic stiffness, inflammation, coagulation, integrin and VHL gene expression. Should new interventions be identified during the course of this project addressing these targets, they will be added to the study.

** This is based on previous experience, the adverse effects may vary between light and moderate discomfort. When the discomfort exceeds moderate an HEP will be applied.

***Right ventricular (RV) failure is the predominant cause of death in patients with pulmonary hypertension. Most rats will not suffer from heart failure until the end of the experiment, and their discomfort will not exceed moderate. Maximally 15% (based on previous experiments) of the rats can develop heart failure with severe discomfort. It is very difficult to see if a rat is having heart failure (which can occur in the weeks after hypoxia). A decrease in bodyweight is the first sign of heart failure which is then already occurring for one day. To be sure it's heart failure, (and not daily fluctuations of bodyweight), a bodyweight decrease of 10% is being established. This is usually at the second day and an HEP will be applied.

Based on this table, we expect that cumulative discomfort for 340 rats (85%) will be moderate, and maximum 60 rats (15%) severe discomfort.

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 animals will be killed at the end of the procedure, to collect large blood samples and tissues for further analysis. Also, animals will also be killed in the case when one of the humane end-points will be reached.

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'.	5.1 lid2h
1.2	Provide the name of the licenced establishment.	5.1 lid2h
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: 2 Type of animal procedure: Intervention studies using PAB model

2 Description of animal procedures

A. Experimental approach and primary outcome parameters

Describe the general design of the animal procedures in relation to the primary outcome parameters. Justify the choice of these parameters.

This Appendix describes the use of the pulmonary artery banding (PAB) model in rats to validate novel therapeutic interventions for PAH. This model has to be implemented and optimized at our department. The PAB rat model is a surgical model mimicking the increased afterload on the right heart as occurs in PAH as well as in other forms of pulmonary hypertension. Rats are subjected to pulmonary artery banding for 6-8 weeks (a sham-operated control group is included). Upon verification of pressure overload in the animals that have received the pulmonary artery banding compared to controls (via echocardiography of the heart), the intervention will be started. Only interventions in this project are included that act on an identified PAH target (or candidate target) as demonstrated by previous fundamental research or clinical research – see project proposal Figure 2. The interventions act on 7 different established targets for PAH, namely BMPR2 signalling, vascular tone, RV diastolic stiffness, inflammation, coagulation, integrin and VHL gene expression. Should new interventions be identified during the course of this project addressing these targets, they will be added to the study. The interventions will be tested when pressure overload has developed in the PAB model (therapeutics vs preventive), as this study design has the highest clinical relevance. We will particularly focus in these studies on assessing the impact of the intervention on RV overload. The primary outcomes measures include the time (in days) from afterload induction to clinical manifestation of right heart failure; lung and right heart (RV) function and structure (assessed via imaging and tissue/protein analysis). The advanced blood serum and tissue analyses are also included to study the mechanisms underlying the chosen genetic interventions and to possibly identify new targets (in control animals).

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

Induction PAB model
The pulmonary artery banding rat model is a surgical model. The rats are put under general anaesthesia and analgesia, the chest is opened and a titanium clip is compressed around the pulmonary artery with a modified ligating clip applier. By using different sizes of pulmonary artery constriction, a phenotype of moderate RV dysfunction and severe RV failure can be generated. Following the surgical procedure, the rats are daily monitored and receive pain medication for at least two days. Rats are subjected to pulmonary artery banding for 6-8 weeks [1]. A sham-operated control group is included in the study that will not receive the titanium clip.

Follow-up development and progression
After the surgery, growth of the animal ensures a progressive increase in right ventricular pressure. To verify induction of pressure overload, the rats are subjected to echocardiography of the heart. Pulmonary artery acceleration time (PAAT/cl, estimate of RV pressure), right ventricular end-diastolic diameter (RVEDD, dilatation) and tricuspid annular plane systolic excursion (TAPSE, RV dysfunction), are measured according to standard protocols at our Department. Following confirmation of pressure overload with echocardiography, the animals are randomly divided into two groups: the intervention group and a vehicle-control group (for illustration of experimental design see Figure 1 below).

Interventions
Interventions include treatment by any kind of agent (e.g. dietary, chemical, biological, genetic, radiopharmaceutical), or combination of these agents. Some of these interventions are already known, including FHL2, RBM20 and integrin (see table 1 of the proposal). Should new interventions be identified during the course of this project addressing these targets, they will be added to the study. All agents will be administered by the appropriate route, time of day, duration and frequency as required. Examples include oral gavage, bolus injections (i.v., i.p., s.c.), continuous infusion in cannulated animals, minipumps and slow release pellets. Procedures requiring surgery (cannulation, implantation of minipumps) will be performed under general anaesthesia and analgesia. The selection of agent(s), dose, time of day, and route of application depends on the target of the intervention and the details of the treatment of each study will be discussed with the IvD.

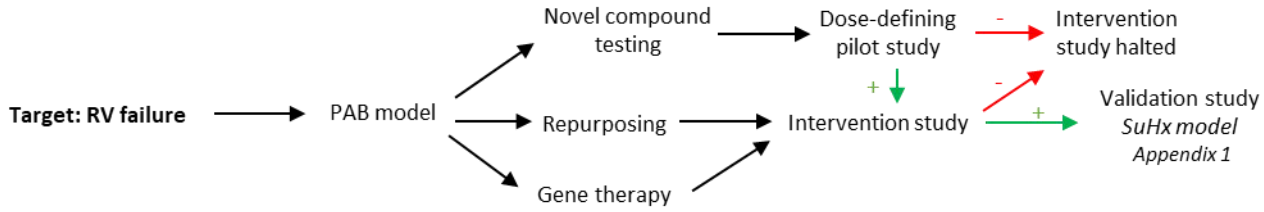

Figure 1. Study design PAB studies with GO / NO-GO decisions.

After ~2-6 weeks of treatment (time-point will vary dependent on type of intervention), the experimental readouts as defined in Section 3.4.1 of the project proposal will be assessed by means of echocardiography of the heart, hemodynamic assessments via a catheter (under anaesthesia) and in a subset of the animals by MRI-imaging (MRI myocardial tagging or DT-MRI, under anaesthesia).

Echocardiography
After treatment, all animals will be subjected to echocardiographic assessments (see above) under anaesthesia, to measure RV wall thickness (RVWT), RV end diastolic diameter (RVEDD), tricuspid annular plane systolic excursion (TAPSE), stroke volume (SV), heart rate (HR), cardiac output (CO) and pulmonary artery acceleration time (PAAT).

Hemodynamic measurements
After echocardiography, hemodynamic assessments are done via open-chest RV catheterization. RV systolic pressure (RVSP) will be determined from steady state measurement, as well as RV afterload (Ea-Arterial elastance). Pressure-volume loops after vena-cava occlusion will be obtained and used to derive end-systolic elastance (Ees), and end-diastolic elastance (Eed). Arterial ventricular coupling will be calculated as Ees/Ea. After the haemodynamic measurements, the animals are sacrificed and blood and tissue (heart and lung) is collected for subsequent analysis.

Imaging

A subset of the treated animals is subjected to MRI-imaging (MRI myocardial tagging, under anaesthesia) and/or DT-MRI quantification of the helical muscle fiber architecture in the RV (Figure 1 below). A DT-MRI quantification of the helical muscle fiber architecture in the RV will be performed ex vivo, since bulk cardiac motion in vivo may lead to image artefacts. Hearts will be perfused and fixated to keep the RV open, and the diffusion-weighted images will be acquired. In this way, we will get information on the fiber orientation of the RV.

Termination

At the end of the study, all animals are killed by an approved method (e.g. removal of blood and organs under anaesthesia or CO2 asphyxiation,) and blood and lung/cardiac tissues are collected for further analysis (histology, RNA analyses and protein analyses). The animals are maximally for 12 weeks in the experiment.

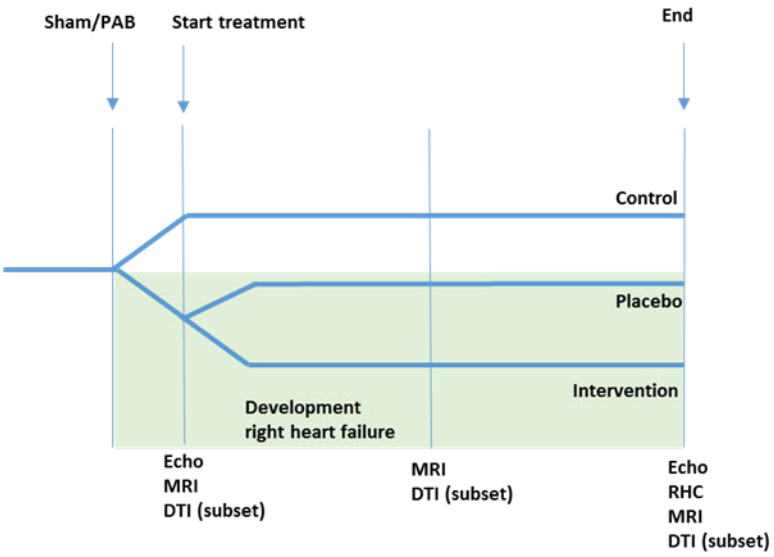

Figure 2. Experimental design PAB studies. After two weeks of acclimatization, animals undergo PAB or sham operation. At start treatment (1-2 weeks after PAB/sham surgery) we perform an echocardiography and at the end experiments an echocardiography and right heart catheterization is performed. The animals are maximally for 12 weeks in the experiment.

References used in this section:

[1] Andersen S, et al. Pulmonary Trunk Banding Model of Pressure Overload Induced Right Ventricular Hypertrophy and Failure. J Vis Exp. 2018 Nov 29;(141). doi: 10.3791/58050. PMID: 30582605.

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

To demonstrate a 50% improvement in terms of RV function between two groups (measured with RV pressure and echocardiography CO, to estimate the pulmonary resistance, treatment vs control) with an overall variability of ~30%, a group size of 7 evaluable animals per group is needed (power > 0.8 with α = 0.05, two sided). Because our study design will be comprised of 3 groups (sham, PAB, PAB+intervention) and 2 comparisons will be performed, an α of 0.025 (0.05/2) is defined as statistically significant. Therefore, an estimated group size of 10 evaluable animals will be needed to perform a 3-arm study. We typically use n=12 in model/intervention groups, n=6 in control group. This includes potential losses due to human end points. The appropriateness of the chosen group sizes has been confirmed in previous studies conducted with the PAB model at our Department and described in the literature.

B. The animals

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

Species: *Rattus norvegicus*, Wistar, wild-type

Origin: Own breeding or university or commercial breeder.

Sex: Both male and female animals (equally divided) will be used throughout the project.

Justification: Wistar rats will be used for the experiments. They will be 4 to 6 weeks of age at the start of the experiments and ~14 weeks at sacrifice.

Total: 440 rats, 60 control rats and 380 PAB rats.

Estimated numbers:
The PAB model will be performed in 10 intervention studies during the project, with an average of ~30 rats per experiment (see above), resulting in an estimated total of 10 x 30 = 300 rats in this study. We typically use n=12 in model/intervention groups, n=6 in control group. This includes potential losses due to human end points. The appropriateness of the chosen group sizes has been confirmed in previous studies conducted with the PAB model at our Department and described in the literature. For dose defining pilot studies we included 100 animals (10 animals per compound, maximally 10 interventions). This number is based on our previous experience with these pilot studies. Since this concerns a new model at our department, we also included 40 animals to set up this model. The total amount of rats is 440 for a period of 5 years.

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.

Replacement
All proposed interventions that will be tested throughout this project will be assessed first in other, non-animal, models, such as cell culture experiments. Only if these experiments yield sufficiently promising results, in vivo tests will be undertaken. In vivo experiments are a necessary step in development of interventions that may one day be used in the clinic, since currently no other methods allow the evaluation of the effect of an intervention on the organism as a whole. Cell culture, organoids, lab-on-a-chip or computer models are not (yet) sophisticated enough to assess the interaction between the PAH pathology and host environmental factors such as the oxygen supply, the immune system and metabolism.

Reduction
The proposed number of evaluable animals per study arm (n=12 in model/intervention groups, n=6 in control group) is calculated as described above, and is in line with generally accepted protocols in scientific literature and our previous studies with the PAB model. Further reduction of the number of animals per group would decrease statistical power, and might thus disqualify the experiment.

Refinement
State-of-the-art methods and equipment for assessing pulmonary vascular remodelling and RV overload, in particular advanced imaging techniques, will be used to refine protocols and minimize discomfort to the animals especially in support of the application of humane endpoints. International guidelines will be applied to ensure that best practice is followed.

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

The procedures conducted under this protocol will inevitably cause discomfort for the animals in the studies. In order to minimize suffering, all studies will adhere to the national and internationally accepted rules for handling of lab animals [2, 3]. Under these rules, the animals will be humanely killed when a humane endpoint (see below) is reached. Within the institute, standard operating procedures (SOP) for animal handling are in place. These also include dedicated anaesthesia and analgesia (A&A) SOPs that will accompany invasive procedures to minimize pain and discomfort.
To minimize animal suffering, pain and fear the following measures will be taken. The animals will be allowed to acclimatize to their new environment for two weeks upon arrival at the animal facility. Animals are housed in groups (socially housed) at all times (except during surgery, animals are returned to the group directly after surgery) and environmental enrichment strategies are applied in the cages to improve animal welfare.
The PAB surgical model is performed under general anaesthesia and analgesia (pre- and post-surgery). During haemodynamic measurements, animals will be kept under anaesthesia in a temperature-controlled environment. During imaging procedures, animals will be kept under anaesthesia in a temperature-controlled environment.
No adverse effects on the environment are expected because animals are kept and procedures are performed in a controlled environment, all waste will be safely discarded.
Right ventricular (RV) failure is the predominant cause of death in patients with pulmonary hypertension. Maximally 32% of the rats can develop heart failure with severe discomfort. It is due to the diameter of the band around the pulmonary artery. A decrease in bodyweight is the first sign of heart failure which is then already occurring for one day. It is normal to have a decreasing bodyweight of the rat during the day, because of sleeping, less drinking and eating during the day (daily fluctuations). To be sure it is heart failure (and not daily fluctuations of bodyweight), a bodyweight decrease of 10% is being established. Also cyanosis, dyspnea, lethargy and poor grooming can be observed. This is usually at the second day and an HEP will be applied. Based on experience with this model in Denmark: Seven weeks survival rate was 80% for rats subjected to severe banding and close to 100% in rats subjected to mild or moderate banding or sham surgery[1].
Severe discomfort is unfortunately unavoidable. Patients usually present at the stage of heart failure. Right heart failure is an important outcome measure of our research. Heart failure is necessary to compare the rats with patients.

References used in this section:
[1] Andersen S, et al. Pulmonary Trunk Banding Model of Pressure Overload Induced Right Ventricular Hypertrophy and Failure. J Vis Exp. 2018 Nov 29;(141). doi: 10.3791/58050. PMID: 30582605.
[2] Zutphen, L. Van, Handboek proefdierkunde: proefdieren, dierproeven, alternatieven en ethiek.
[3] J. Guillen, "FELASA Guidelines and Recommendations," J Am Assoc Lab Anim Sci, vol. 51, no. 3, pp. 311–321, May 2012.

Repetition and duplication

E. Repetition

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

N.A.

Accommodation and care

F. Accommodation and care

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

☒ No

☐ 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.

The PAB surgical model is performed under general anaesthesia and analgesia (pre- and post-surgery). In case the therapeutic interventions require surgical procedures (e.g. cannula/mini-pump implantation), this will be done under general anaesthesia in combination with perioperative pain treatment. General anaesthesia will also be applied in order to perform the haemodynamic measurements (via open-chest RV catheterization). For some procedures (e.g. imaging) anaesthesia will be applied in the absence of any risk for pain.
Within the institute, SOPs for animal handling are in place. These also include dedicated A&A SOPs that will accompany invasive procedures to minimize pain and discomfort. These A&A SOPs describe the best practice methods for anaesthesia and analgesia for each (surgical) procedure and are regularly checked as new concepts or procedures become available. All experiments performed within this project will conform to these A&A SOPs.

I. Other aspects compromising the welfare of the animals

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

Due to the PAB model (and consequently, the induced right heart failure), the animals may lose weight, experience shortness in breath (dyspnea) and can become lethargic. Apart from discomfort directly caused by the procedures as described above, animals may develop complications due to the therapeutic interventions (e.g. toxic side-effects), which in some cases may result in adverse effects on the animals' welfare. These side-effects are still unknown as well as the discomfort the animal can experience. The maximum tolerated level of discomfort is moderate. When this is exceeded, an HEP will be applied.
Applying therapeutic interventions, i.e. Bolus injections or procedures requiring surgery or local infusion, can also lead to moderate discomfort.
Prior to using interventions that have never previously been tested *in vivo*, particularly for new pharmacotherapeutic interventions, a pilot pharmacokinetics study will be performed to determine the most optimal dose, route and schedule. If the study aims to test a new combination of interventions, a pharmacokinetics study will determine potential drug-drug interactions. When no data is available on previous dose-finding of pharmacological interventions, tolerability studies and/or dose-finding studies

will also be performed to determine the maximum tolerated dose. If the highest safe dose is not expected to provide sufficient systemic exposure for efficacy, further *in vivo* studies will be halted (GO/NO GO DECISION).

Explain why these effects may emerge.

These effects are a consequence of the induction of pressure overload (right heart failure) due to the PAB model and the applied interventions respectively.
Unfortunately severe discomfort is unavoidable. Patients are presented in the clinic when they have heart failure. Right heart failure is a measure of outcome in our study. This is necessary to compare the animals with PAH patients. Maximum 32% of rats can develop heart failure with severe discomfort. The reason is the small diameter of the PAB, resulting in severe PAH. The second group of rats receives a slightly larger diameter PAB, resulting in a milder PAH. In this way we can compare these two groups with the two patients groups, mild and sever PAH.

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

In general, adverse effects on the animals' welfare caused by induction of RV pressure overload cannot be completely prevented. In order to minimize adverse effects, the animals will be monitored at a frequency that is dictated by the model and timely killed when a humane endpoint (see below) is met. When profound weight drop occurs, daily monitoring will be applied. Should unforeseen complications due to the interventions or procedures occur, either the effect of these complications will be minimized by adjusted procedures, such as providing easy access to food (mush-feeding), or if this is not possible, the humane endpoints as defined below will be taking into account.

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.

The most important humane endpoints applicable to all studies are:

- Permanent weight loss ≥20% of initial body weight in adult animals, measured from the start of the treatment
- Weight loss ≥10% of body weight during 24h, in combination with:
  - Sustained abnormal breathing, dyspnea (symptom PAH/right heart failure)
  - Sustained lethargy (symptom PAH/right heart failure)
- Sustained abnormal behavior
- Complications of interventions
- Other procedure-specific endpoints

Indicate the likely incidence.

Humane endpoints expected to occur in <32% of all cases.

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').

Discomfort levels categorized according to the PAB model are as follows:

- Animals that are subjected to the PAB surgical model, under general anaesthesia and analgesia, experience moderate discomfort.
- Animals that are sham-operated, under general anaesthesia and analgesia, experience moderate discomfort.

Discomfort levels categorized according to the interventions are as follows:

- Animals that receive therapeutic interventions during the study period, can experience moderate discomfort.
- Control animals (vehicle-treated) during the study period, can experience moderate discomfort.

Discomfort levels categorized according to the procedures used for assessment of pulmonary vascular remodelling and right ventricular pressures (echocardiography, haemodynamic measurements, imaging) are as follows:

- Echocardiography: mild discomfort.
- Haemodynamic measurements, using general anaesthesia: non-recovery.
- More intensive imaging procedures and those requiring prolonged anaesthesia (e.g. MRI, DTI-MRI quantification): moderate.

Other procedures that will be used, which are not expected to alter the total level of discomfort experienced:

- Simple well tolerated interventions (e.g. drug treatment): mild discomfort.
- Simple but frequent handling procedures (e.g. weighing): mild discomfort.
- Minimally invasive procedures and those requiring anaesthesia (e.g. non-invasive imaging): mild discomfort.

Table 1: Procedures and discomfort classification.

Procedures	**Category**	**Expected percentage (%) of animals**	**Frequency and duration of the procedure**
1. Obtaining rats: Transport to animal facility	mild	100%	1x
2. Induction PAB model: a. Surgical procedure (under general anaesthesia and analgesia)	moderate	86%	1x ~90 min
3. Echocardiography (pre-treatment)	mild	100%	1x ~10 min
4. Applying therapeutic interventions* a. Frequent handling procedures b. Bolus injections (i.v., i.p., s.c, oral gavage) c. Procedures requiring surgery (cannulation, implantation of minipumps) under brief adequate anaesthesia and postoperative analgesia d. Local infusion, either acute or chronic through a cannula	 mild moderate moderate moderate	 100% 86% 25% 25%	 Max 4 weeks Max 4 weeks 1x ~30-60 min Max 4 weeks
5. Potential adverse effects of treatments**	max. moderate	40%	Max 4 weeks
6. Heart failure***	Max. severe	32%	Max 2 days
7. Echocardiography (post-treatment)	mild	100%	1x ~10 min
8. Haemodynamic measurements (under general anaesthesia and analgesia)	non-recovery	100%	1x ~30 min
9. MRI	moderate	25%	Max. 3x ~2.5 hours

10. Blood sampling	mild	25%	Max 2 per week <2 min
11. Sacrifice	non-recovery	100%	1x <1 min

*The therapeutic intervention is unknown yet. The interventions act on 7 different established targets for PAH, namely BMPR2 signalling, vascular tone, RV diastolic stiffness, inflammation, coagulation, integrin and VHL gene expression. Should new interventions be identified during the course of this project addressing these targets, they will be added to the study.

**This is based on previous experience, the adverse effects may vary between light and moderate discomfort. When the discomfort exceeds moderate an HEP will be applied.

***Right ventricular (RV) failure is the predominant cause of death in patients with pulmonary hypertension. Maximally 32% of the rats can develop heart failure with severe discomfort. It is due to the diameter of the band around the pulmonary artery. A decrease in bodyweight is the first sign of heart failure which is then already occurring for one day. To be sure it's heart failure, (and not daily fluctuations of bodyweight), a bodyweight decrease of 10% is being established. This is usually at the second day and an HEP will be applied.

Based on this table, we expect that cumulative discomfort for 300 rats (68%) will be moderate, and 140 rats (32%) severe discomfort.

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 animals will be killed at the end of the procedure, to collect large blood samples and tissues for further analysis. Also, animals will also be killed in the case when one of the humane end-points will be reached.

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'.	5.1 lid2h
1.2	Provide the name of the licenced establishment.	5.1 lid2h
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: 3 Type of animal procedure: Intervention studies using Chronic hypoxia mouse model

2 Description of animal procedures

A. Experimental approach and primary outcome parameters

Describe the general design of the animal procedures in relation to the primary outcome parameters. Justify the choice of these parameters.

This Appendix describes the use of chronic hypoxia mouse model to validate genetic targets that are promising as novel therapeutic intervention for PAH. This model has to be implemented and optimized at our department. To test the involvement of target genes in pulmonary vascular or right ventricular (RV) remodelling, knock-in and knock-out mice will be tested in this model. The target genes include identified PAH targets or candidate targets obtained by fundamental research (e.g. genetical, molecular, cellular, histopathological studies on tissue of animal models) or clinical research (e.g. genetic studies on human samples or other observations, such as fMRI) such as BMPR2, integrin and VHL signalling, as well as regulators of vascular tone, RV diastolic stiffness, inflammation and coagulation. Should new targets be identified during the course of this project, they will be added to the study. The effect of the genetic interventions will be tested when PAH has developed in the mice subjected to the chronic hypoxia model. We will particularly focus in these studies on assessing the impact of the intervention on RV overload and pulmonary vascular remodelling, as both are clinical relevant end-points for PAH. The primary outcomes measures include the time (in days) from PAH induction to clinical manifestation of right heart failure; lung and right heart (RV) function and structure (assessed via imaging and tissue/protein analysis). The advanced blood serum and tissue analyses are also included to study the mechanisms underlying the chosen genetic interventions and to possibly identify new targets (in wild-type experimental animals).

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

Genetic interventions

Knock-in or knock-out mice will be obtained from commercial suppliers or from collaborating institutions. The current proposal does not involve the generation of new genetic models. After obtaining knock-in or knock-out models from institutions abroad, genetic models will be imported in our animal facility via embryo transfer to guarantee that animals are free from pathogenic micro-organisms. A number of genes of interest (GOI) that will be studied can only be knocked-out at an adult age to prevent developmental problems. Examples include VEGFR2/KDR and SOX17. To achieve selective, inducible gene knockout, we will breed mice carrying loxP-flanked GOI with mice carrying tamoxifen-inducible Cre-recombinase under control of the tissue of interest (e.g. VE-cadherin). Induction of Cre-recombinase will be performed by a 5-day course of tamoxifen-supplemented non-pelleted dry feed.

Induction chronic hypoxia model

The wildtype versus knock-in/knock-out mice will be exposed to chronic hypoxia (10% O_2) by placing them for 2 weeks in a hypoxia chamber. In the study, a control group is included that receives no hypoxia (for illustration of experimental design see Figure 1 below).

Echocardiography

To verify development of pulmonary vascular remodelling and a mild increase in right ventricular pressures, the mice are subjected echocardiography of the heart according to standard protocols at our Department.

Haemodynamic measurements

Mice are anesthetized for hemodynamic measurements via open-chest RV catheterization. RV systolic pressure (RVSP) will be determined from steady state measurement. After the haemodynamic measurements, the animals are sacrificed and blood and tissue (heart and lung) is collected for subsequent analysis.

Measurements of vascular leakage

After induction of anaesthesia, mice will receive a tail vein injection with 150µL 1% Evans Blue/phosphate buffered saline, which will be left circulating for one hour (under continuous anaesthesia). After 1 hour a pressure catheter will be placed in the right ventricle to perform haemodynamic measurements. The mice will be sacrificed by perfusion with phosphate buffered saline. Subsequently, organs will be harvested and processed for measurement of Evans Blue in the organs.

Termination

At the end of the study, animals will be killed by an approved method (e.g. Removal of blood and organs under anaesthesia or CO_2 asphyxiation, cervical dislocation, terminal anaesthesia). Following exsanguination, serum and plasma samples are taken, and heart and lung tissues are collected for tissue, protein and RNA analyses. The animals are maximally for 52 weeks in experiment. Age is an important factor involved in stability and loss of small vessels. Effects of the genes we want to study can be more efficient at a higher age, therefore we will keep the animals for 52 weeks (aging mouse) in experiment.

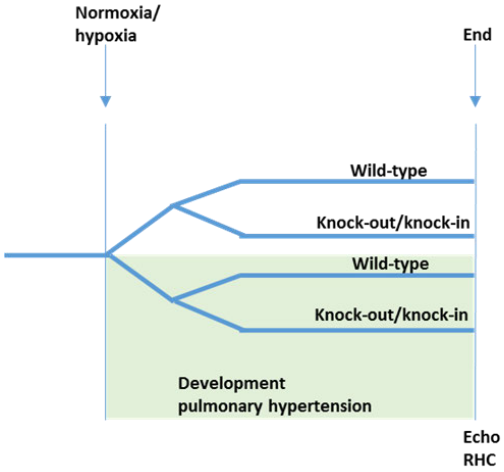

Figure 1. Experimental design studies with chronic hypoxia mouse model. After 2 weeks of acclimatization we start with the 10% hypoxia period of 2 weeks (not the control group). At the end experiments an echocardiography and right heart catheterization is performed. The animals are maximally for 52 weeks in experiment.

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

A typical intervention study will comprise several study arms, including control (no chronic hypoxia) and experimental (chronic hypoxia), genetically modified (knock-in/knock-out) and wildtype groups. To demonstrate a 50% change in terms of pulmonary vascular remodelling (as measured by right ventricular systolic pressure) between two groups (genetic intervention vs control) with an overall variability of ~30, a group size of 10 evaluable animals per group is needed (power > 0.9 with α 0.05, two sided). In case of the multiple treatment groups here [1) No hypoxia – wildtype, 2) no hypoxia - knock-in/knock-out, 3) chronic hypoxia – wildtype, 4) chronic hypoxia - knock-in/knock-out], it will be necessary to increase the group size, to adjust for multiple comparisons, e.g. α of 0.05 will be divided by the number of treatment groups minus 1. A 4-arm study therefore, assuming 4 treatment groups, will have α = 0.05/3 = 0.013. Overall, an estimated group size of 10 evaluable animals will be needed to perform a 4-arm study. This includes potential losses due to human end points.

B. The animals

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

Species: *Mus musculus*, wild-type, knock-in, knock-out.

Origin: Own breeding, university or commercial breeder.

Sex: Both male and female animals (equally divided) will be used throughout the project.

Total: 400 mice, 100 mice No hypoxia–wildtype, 100 mice No hypoxia-knock-in/out, 100 mice Chronic hypoxia–wildtype, 100 mice Chronic hypoxia-knock-in/out.

Justification: Mice, being mammals, share many organ structures and similarities in genetic composition with humans. Other advantages include the short generation time and options to obtain genetically altered animals. Since genetic modification is more efficient in mice than in rat, the addition of the chronic hypoxia mouse model allows for testing of genetic interventions (knock-out and knock-in) in the genes identified in human genetic studies to be involved in PAH.

Estimated numbers: Chronic hypoxia mouse model will be performed in 8 intervention studies during the project, with an average of ~40 mice per experiment (see above), resulting in an estimated total of 8 x 40 = 320 mice in this study. Since this concerns a new model at our department, we also included 80 animals to set up this model. The total amount of mice is 400 for a period of 5 years.

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.

Replacement
In vivo experiments are a necessary step in development of interventions that may one day be used in the clinic, since currently no other methods allow the evaluation of the effect of an intervention on the organism as a whole. Cell culture, organoids, lab-on-a-chip or computer models are not (yet) sophisticated enough to assess the interaction between the PAH pathology and host environmental factors such as the oxygen supply, the immune system and metabolism.

Reduction
The proposed number of evaluable animals per study arm (n=10) is calculated as described above, and is in line with generally accepted protocols in scientific literature. Further reduction of the number of animals per group would decrease statistical power, and might thus disqualify the experiment.

Refinement
State-of-the-art methods and equipment for assessing pulmonary vascular remodelling and RV overload, in particular advanced imaging techniques, will be used to refine protocols and minimize discomfort to the animals especially in support of the application of humane endpoints. International guidelines will be applied to ensure that best practice is followed.

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

The procedures conducted under this protocol will inevitably cause discomfort for the animals in the studies. In order to minimize suffering, all studies will adhere to the national and internationally accepted rules for handling of lab animals [1], [2]. Under these rules, the animals will be humanely killed when a humane endpoint (see below) is reached. Within the institute, standard operating procedures (SOP) for animal handling are in place. These also include dedicated anaesthesia and analgesia (A&A) SOPs that will accompany invasive procedures to minimize pain and discomfort.
To minimize animal suffering, pain and fear the following measures will be taken. The animals will be allowed to acclimatize to their new environment for two weeks upon arrival at the animal facility. Animals are housed in groups (socially housed) at all times (also during hypoxia) and environmental enrichment strategies are applied in the cages to improve animal welfare. During haemodynamic measurements, animals will be kept under anaesthesia in a temperature-controlled environment. Measurements of vascular leakage will be performed under continuous anaesthesia.
No adverse effects on the environment are expected because animals are kept and procedures are performed in a controlled environment, all waste will be safely discarded.
In these experiments in mice with PAH there is no heart failure expected, and the discomfort will not transcend mild.

References used in this section:
[1] Zutphen, L. Van, Handboek proefdierkunde: proefdieren, dierproeven, alternatieven en ethiek. .
[2] J. Guillen, "FELASA Guidelines and Recommendations," J Am Assoc Lab Anim Sci, vol. 51, no. 3, pp. 311–321, May 2012.

Repetition and duplication

E. Repetition

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

N.A.

Accommodation and care

F. Accommodation and care

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

☐ No

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

The animals will be socially housed in standard conditions (food and water available ad libitum) and environmental enrichment strategies are applied in the cages to improve animal welfare conform the Directive 2010/63/EU. However, as part of the experimental model, the animals will also be housed under low oxygen conditions (10% O2) for 2 weeks (the chronic hypoxia period).

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.

General anaesthesia, in combination with pain treatment, will also be applied in order to perform the haemodynamic measurements (via open-chest RV catheterization). Measurements of vascular leakage will be performed under continuous anaesthesia.
Within the institute, standard operating procedures (SOP) for animal handling are in place. These also include dedicated anaesthesia and analgesia (A&A) SOPs that will accompany invasive procedures to minimize pain and discomfort. These A&A SOPs describe the best practice methods for anaesthesia and analgesia for each (surgical) procedure and are regularly checked as new concepts or procedures become available. All experiments performed within this project will conform to these A&A SOPs.

I. Other aspects compromising the welfare of the animals

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

Exposure to hypoxia (10% O2) will result in a temporary increase in respiration and pulse. Following a number of days in the hypoxia conditions, the respiration level and heartbeat will begin to acclimatize. Hypoxia-induced animals may lose weight compared to control animals. Apart from this observation, we have not observed any other physical or behavioural changes in the hypoxia-treated animals.
The induction of the inducible knockout models requires a 5-day course of tamoxifen-supplemented non-pelleted dry feed, which is not expected to compromise animal welfare. Due to the genetic modifications that target genes in pulmonary vascular or right ventricular (RV) remodelling, the animals may lose weight, experience shortness in breath and become lethargic. Apart from discomfort directly caused by the procedures as described above, animals carrying genetic modifications are not expected to suffer from the genetic modulation itself, based on previous observations, which in some cases may result in adverse effects on the animals' welfare.

Explain why these effects may emerge.

These effects are a consequence of the induction of PAH (right heart failure) due to the applied genetic interventions respectively.

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

In general, adverse effects on the animals' welfare caused by induction of pulmonary vascular remodelling and RV pressure overload cannot be completely prevented. In order to minimize adverse effects, the animals will be monitored at a frequency that is dictated by the model and timely killed when a humane endpoint (see below) is met. The CO2 level, humidity and temperature in the hypoxia chamber are kept constant and will not deviate from the by law defined norms.
Should unforeseen complications due to the interventions or procedures occur, either the effect of these complications will be minimized by adjusted procedures, such as providing easy access to food (mush-feeding), or if this is not possible, the humane endpoints as defined below will be taking into account.

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.

The most important humane endpoints applicable to all studies are:

- Weight loss $\geq$20% of maximum body weight in adult animals, measured from the start of the treatment
- Weight loss $\geq$15% of body weight during 24h, in combination with:
  - Sustained abnormal breathing, dyspnoea (symptom PAH/right heart failure)
  - Sustained lethargy (symptom PAH/right heart failure)
- Sustained abnormal behaviour
- Complications of interventions (<1%): No interventions are planned.

Indicate the likely incidence.

Humane endpoints expected to occur in <10% of all cases.

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').

Discomfort levels categorized according to the genetic interventions are as follows:

- Animals that carry genetic interventions during the study period, can experience mild discomfort.
- Animals that carry no genetic interventions (wild-type) during the study period, can experience mild discomfort.

Discomfort levels categorized according to the chronic hypoxia model are as follows:

- Animals that are subjected to the chronic hypoxia model experience mild discomfort.
- Animals that are not subjected to the chronic hypoxia model experience mild discomfort.

Discomfort levels categorized according to the procedures used for assessment of pulmonary vascular remodelling and right ventricular pressures (echocardiography, haemodynamic measurements, imaging) are as follows:

- Echocardiography: non-recovery.
- Haemodynamic measurements, using general anaesthesia and analgesia: non-recovery.
- Tail vein injection with Evans Blue: non-recovery.

All techniques mentioned above will be performed after induction of anaesthesia and analgesia and are performed as final experiments, indicating that animals will only experience the discomfort resulting from the induction of anaesthesia.

Other procedures that will be used, which are not expected to alter the total level of discomfort experienced:

- Simple but frequent handling procedures (e.g. weighing): mild discomfort.
- Minimally invasive procedures and those requiring anaesthesia (e.g. non-invasive imaging): mild discomfort.
- More intensive imaging procedures and those requiring prolonged anaesthesia (e.g. image guided radiotherapy): moderate discomfort

Table 1: Procedures and discomfort classification.

Procedures	**Category**	**Expected percentage (%) of animals**	**Frequency and duration of the procedure**
1. Obtaining mice: Transport to animal facility	mild	100%	1x
2. Potential adverse effects genetic interventions	mild	50%	~4-52 weeks
3. Induction hypoxia (chronic hypoxia model)	mild	50%	2 weeks
4. Echocardiography (under general anaesthesia)	non-recovery	60%	1x ~10 min
5. Haemodynamic measurements (under general anaesthesia and analgesia)	non-recovery	60%	1x ~30 min
6. Tail vein injection Evans Blue (under general anaesthesia)	non-recovery	20%	1x ~10 min
7. Sacrifice	non-recovery	100%	1x <1 min

Based on this table, we expect that cumulative discomfort will be mild.

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 animals will be killed using transcardiac perfusion at the end of the procedure, to collect large blood samples and tissues for further analysis. Also, animals will also be killed in the case when one of the humane end-points will be reached.

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'.	5.1 lid2h
1.2	Provide the name of the licenced establishment.	5.1 lid2h
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: 4 Type of animal procedure: Intervention studies using Pneumonectomy (PNX)-Su5416 mouse model

2 Description of animal procedures

A. Experimental approach and primary outcome parameters

Describe the general design of the animal procedures in relation to the primary outcome parameters. Justify the choice of these parameters.

This Appendix describes the use of the pneumonectomy (PNX)+SU5416 mouse model to validate genetic targets that are promising as novel therapeutic intervention for PAH. This model has to be implemented and optimized at our department. In the PNX+SU5416 mouse model, the left lung will be removed which results in increased pulmonary blood flow and vascular remodelling. A single administration of SU5416 one week after the surgery will result in severe PAH and RV dysfunction. To test the involvement of target genes in pulmonary vascular or right ventricular (RV) remodelling, knock-in and knock-out mice will be tested in this model. The target genes include identified PAH targets or candidate targets obtained by fundamental research (e.g. genetical, molecular, cellular, histopathological studies on tissue of animal models) or clinical research (e.g. genetic studies on human samples or other observations, such as fMRI) such as, BMPR2 signalling, vascular tone, RV diastolic stiffness, inflammation, coagulation, integrin and VHL gene expression. Should new targets be identified during the course of this project, they will be added to the study. The effect of the genetic interventions will be tested when PAH has developed in the mice subjected to the PNX+SU5416 model. We will particularly focus in these studies on assessing the impact of the intervention on RV overload and pulmonary vascular remodelling, as both are clinical relevant end-points for PAH. The primary outcomes measures include the time (in days) from PAH induction to clinical manifestation of right heart failure; ; lung and right heart (RV) function and structure (assessed via imaging and tissue/protein analysis). The advanced blood serum and tissue analyses are also included to study the mechanisms underlying the chosen genetic interventions and to possibly identify new targets (in wild-type experimental animals).

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

Genetic interventions

Knock-in or knock-out mice will be obtained from commercial suppliers or from collaborating institutions. The current proposal does not involve the generation of new genetic models. After obtaining knock-in or knock-out models from institutions abroad, genetic models will be imported in our animal facility via embryo transfer to guarantee that animals are free from pathogenic micro-organisms. A number of genes of interest (GOI) that will be studied can only be knocked-out at an adult age to prevent developmental problems. To achieve selective, inducible gene knockout, we will breed mice carrying loxP-flanked GOI with mice carrying tamoxifen-inducible Cre-recombinase under control of the tissue of interest (e.g. VE-cadherin). Induction of Cre-recombinase will be performed by a 5-day course of tamoxifen-supplemented non-pelleted dry feed.

Induction pneumonectomy+SU5416 mouse model

The PNX+SU5416 mouse model is a surgical model. Mice (Knock-in/knock-out mice and wildtype) are put under general anaesthesia and analgesia, the thorax is opened and the left lung is removed. One week after surgery, mice receive a single subcutaneous (s.c.) injection of the vascular endothelial growth factor receptor (VEGFR) inhibitor SU5416 (20 mg/kg in CMC). A sham-operated control group will be included that will not undergo PNX and receive a vehicle-injection (for illustration of experimental design see Figure 1 below).

Echocardiography

To verify development of pulmonary vascular remodelling and a mild increase in right ventricular pressures, the mice are subjected echocardiography of the heart according to standard protocols at our Department.

Haemodynamic measurements

Mice are anesthetized for hemodynamic measurements via open-chest RV catheterization. RV systolic pressure (RVSP) will be determined from steady state measurement, After the haemodynamic measurements, the animals are sacrificed and blood and tissue (heart and lung) are collected for subsequent analysis.

Termination

At the end of the study, animals will be killed by an approved method (e.g. removal of blood and organs under anaesthesia or CO2 asphyxiation, cervical dislocation, terminal anaesthesia). Following exsanguination, serum and plasma samples are taken, and heart and lung tissues are collected for tissue, protein and RNA analyses. The animals are maximally for 12 weeks in experiment.

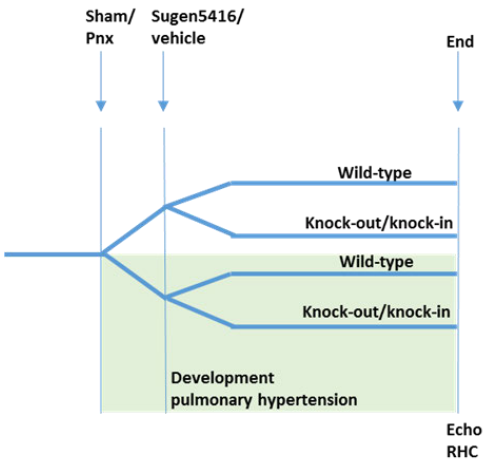

Figure 1. Experimental design studies with pneumonectomy (PNX)+SU5416 mouse model. After 2 weeks of acclimatization, pneumonectomy will be performed, followed by single injection of Sugen5416 one

week later. At the end experiments an echocardiography and right heart catheterization is performed. The animals are maximally for 12 weeks in experiment.

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

A typical intervention study will comprise several study arms, including control (no PNX+SU5416) and experimental (PNX+SU5416), genetically modified (knock-in/knock-out) and wildtype groups. To demonstrate a 50% improvement in terms of pulmonary vascular remodelling (measuring the diameter of the media and intima wall thickness of pulmonary arterioles in lung cryosections), between two groups (genetic intervention vs control) with an overall variability of ~30, a group size of 10 evaluable animals per group is needed (power > 0.9 with α 0.05, two sided). In case of the multiple treatment groups here [1) no PNX+SU5416–wildtype, 2) no PNX+SU5416–knock-in/knock-out, 3) PNX+SU5416–wildtype, 4) PNX+SU541–knock-in], it will be necessary to increase the group size to adjust for multiple comparisons, e.g. α of 0.05 will be divided by the number of treatment groups minus 1. A 4-arm study therefore will have α = 0.05/3 = 0.013. Overall, an estimated group size of 10 evaluable animals will be needed. To accommodate for drop-out of animals due to the surgery, we use n=12 in the model/intervention groups, n=6 in the control group. This includes potential losses due to human end points.

B. The animals

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

Species: *Mus musculus*, wild-type, knock-in, knock-out

Origin: Own breeding or university or commercial breeder.

Sex: Both male and female animals (equally divided) will be used throughout the project.

Total: 376 mice, 58 control mice No PNX+SU5416–wildtype, and 96 mice No PNX+SU5416–knock-in/out, and 126 mice PNX+SU5416–wildtype, and 96 mice PNX+SU541–knock-in/out.

Justification: Mice, being mammals, share many organ structures and similarities in genetic composition with humans. Other advantages include the short generation time and options to obtain genetically altered animals. Since genetic modification is more efficient in mice than in rat, the addition of the PNX-SU5416 mouse model allows for testing of genetic interventions (knock-out and knock-in) in the genes identified in human genetic studies to be involved in PAH.

Estimated numbers: PNX+SU5416 mouse model will be performed in 8 intervention studies during the project, with an average of ~42 mice per experiment (see above), resulting in an estimated total of 8 x 42 = 336 mice in this study. Since this concerns a new model at our department, we also included 40 animals to set up this model. The total amount of mice is 376 for a period of 5 years.

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.

Replacement

In vivo experiments are a necessary step in development of interventions that may one day be used in the clinic, since currently no other methods allow the evaluation of the effect of an intervention on the organism as a whole. Cell culture, organoids, lab-on-a-chip or computer models are not (yet) sophisticated enough to assess the interaction between the PAH pathology and host environmental factors such as the oxygen supply, the immune system and metabolism.

Reduction
The proposed number of evaluable animals per study arm (n=12 in model/intervention groups, n=6 in control group) is calculated as described above, and is in line with generally accepted protocols in scientific literature. Further reduction of the number of animals per group would decrease statistical power, and might thus disqualify the experiment.

Refinement
State-of-the-art methods and equipment for assessing pulmonary vascular remodelling and RV overload, in particular advanced imaging techniques, will be used to refine protocols and minimize discomfort to the animals especially in support of the application of humane endpoints. International guidelines will be applied to ensure that best practice is followed.

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

The procedures conducted under this protocol will inevitably cause discomfort for the animals in the studies. In order to minimize suffering, all studies will adhere to the national and internationally accepted rules for handling of lab animals [1], [2]. Under these rules, the animals will be humanely killed when a humane endpoint (see below) is reached. Within the institute, standard operating procedures (SOP) for animal handling are in place. These also include dedicated anaesthesia and analgesia (A&A) SOPs that will accompany invasive procedures to minimize pain and discomfort. In case the pulmonary hypertension is too severe, we may choose to omit the SU5416 injection.
To minimize animal suffering, pain and fear the following measures will be taken. The animals will be allowed to acclimatize to their new environment for two weeks upon arrival at the animal facility. Animals are housed in groups (socially housed) at all times (except during surgery, animals are returned to the group directly after surgery) and environmental enrichment strategies are applied in the cages to improve animal welfare. The PNX surgical model is performed under general anaesthesia and analgesia (pre- and post-surgery). During haemodynamic measurements, animals will be kept under anaesthesia in a temperature-controlled environment.
No adverse effects on the environment are expected because animals are kept and procedures are performed in a controlled environment, all waste will be safely discarded.
In these experiments in mice with PAH there is no heart failure expected, and the discomfort will not transcend moderate.

References used in this section:
[1] Zutphen, L. Van, Handboek proefdierkunde: proefdieren, dierproeven, alternatieven en ethiek.
[2] J. Guillen, "FELASA Guidelines and Recommendations," J Am Assoc Lab Anim Sci, vol. 51, no. 3, pp. 311–321, May 2012.

Repetition and duplication

E. Repetition

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

N.A.

Accommodation and care

F. Accommodation and care

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

☒ No

☐ 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.

The PNX will be performed under general anaesthesia and analgesia (pre- and post-surgery). General anaesthesia, in combination with perioperative pain treatment, will also be applied in order to perform the haemodynamic measurements (via open-chest RV catheterization).
Within the institute, standard operating procedures (SOP) for animal handling are in place. These also include dedicated anaesthesia and analgesia (A&A) SOPs that will accompany invasive procedures to minimize pain and discomfort. These A&A SOPs describe the best practice methods for anaesthesia and analgesia for each (surgical) procedure and are regularly checked as new concepts or procedures become available. All experiments performed within this project will conform to these A&A SOPs.

I. Other aspects compromising the welfare of the animals

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

The induction of the inducible knockout models requires a 5-day course of tamoxifen-supplemented non-pelleted dry feed, which is not expected to compromise animal welfare. Due to the genetic modifications and PNX+SU5416 model (and consequently, the induced right heart failure), that target genes in pulmonary vascular or right ventricular (RV) remodelling, the animals may lose weight, experience shortness in breath and become lethargic. Apart from discomfort directly caused by the procedures as described above, animals may develop complications due to the therapeutic interventions (e.g. toxic side-effects), which in some cases may result in adverse effects on the animals' welfare.

Explain why these effects may emerge.

These effects are a consequence of the induction of PAH and right heart failure due to the PNX+SU5416 model and the applied genetic interventions respectively.

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

In general, adverse effects on the animals' welfare caused by induction of pulmonary vascular remodelling and RV pressure overload cannot be completely prevented. In order to minimize adverse effects, the animals will be monitored at a frequency that is dictated by the model and timely killed when a humane endpoint (see below) is met. When profound weight drop occurs, daily monitoring will be applied. Should

unforeseen complications due to the interventions or procedures occur, either the effect of these complications will be minimized by adjusted procedures, such as providing easy access to food (mush-feeding), or if this is not possible, the humane endpoints as defined below will be taking into account.

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.

The most important humane endpoints applicable to all studies are:

- Weight loss ≥20% of maximum body weight in adult animals, measured from the start of the treatment
- Weight loss ≥15% of body weight during 24h, in combination with:
    - Sustained abnormal breathing, dyspnoea (symptom PAH/right heart failure)
    - Sustained lethargy (symptom PAH/right heart failure)
- Sustained abnormal behaviour
- Complications of interventions
    - • Other procedure-specific endpoints
    -

Indicate the likely incidence.

Humane endpoints expected to occur in <10% of all cases.

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').

Discomfort levels categorized according to the genetic interventions are as follows:

- Animals that carry genetic interventions during the study period, can experience mild discomfort.
- Animals that carry no genetic interventions (wild-type) during the study period, can experience mild discomfort.

Discomfort levels categorized according to the PNX-SU5416 model are as follows:

- Animals that are subjected to the PNX-SU5416 model experience moderate discomfort.
- Animals that are not subjected to the PNX-SU5416 model experience mild discomfort.

Discomfort levels categorized according to the procedures used for assessment of pulmonary vascular remodelling and right ventricular pressures (echocardiography, haemodynamic measurements, imaging) are as follows:

- Echocardiography: non-recovery.
- Haemodynamic measurements, using general anaesthesia and analgesia: non-recovery.
- More intensive imaging procedures and those requiring prolonged anaesthesia: non-recovery.

Other procedures that will be used, which are not expected to alter the total level of discomfort experienced:

- Simple but frequent handling procedures (e.g. weighing): mild discomfort.
- Minimally invasive procedures and those requiring anaesthesia (e.g. non-invasive imaging): mild discomfort.
- More intensive imaging procedures and those requiring prolonged anaesthesia (e.g. image guided radiotherapy): moderate discomfort.
-

Table 1: Procedures and discomfort classification.

Procedures	Category	Expected percentage (%) of animals	Frequency and duration of the procedure
1. Obtaining mice: Transport to animal facility	mild	100%	1x
2. Potential adverse effects genetic interventions	mild	50%	~4-12 weeks
3. Induction PNX-SU5416 model: a. PNX surgery (under general anaesthesia and analgesia) b. Injection with SU5416	moderate mild	59% 59%	1x ~90 min 1x <1 min
4. Echocardiography (under general anaesthesia)	non-recovery	100%	1x ~10 min
5. Haemodynamic measurements (under general anaesthesia and analgesia)	non-recovery	100%	1x ~30 min
6. Sacrifice	non-recovery	100%	1x <1 min

Based on this table, we expect that cumulative discomfort for the PNX-SU5416 groups, 222 mice (59%) will be moderate. For the control groups, 154 mice (41%) this will be mild.

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 animals will be killed at the end of the procedure, to collect large blood samples and tissues for further analysis. Also, animals will also be killed in the case when one of the humane end-points will be reached.

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
Niet-technische samenvatting

- Dit format gebruikt u om uw niet-technische samenvatting te schrijven
- Meer informatie over de niet-technische samenvatting vindt u op de website www.centralecommissiedierproeven.nl.
- Of neem telefonisch contact op. (0900-2800028).

1 Algemene gegevens

1.1	Titel van het project	Verbeterde behandeling van longziekte: het testen van kandidaat-behandelmethoden in diermodellen
1.2	Looptijd van het project	5 jaar
1.3	Trefwoorden (maximaal 5)	Longziekte, rechter hartfalen, behandeling, muismodellen, ratmodellen

2 Categorie van het project

2.1 In welke categorie valt het project.

U kunt meerdere mogelijkheden kiezen.

- ☒ Fundamenteel onderzoek
- ☒ Translationeel of toegepast onderzoek
- ☐ Wettelijk vereist onderzoek of routinematige productie
- ☐ Onderzoek ter bescherming van het milieu in het belang van de gezondheid
- ☐ Onderzoek gericht op het behoud van de diersoort
- ☐ Hoger onderwijs of opleiding
- ☐ Forensisch onderzoek
- ☐ Instandhouding van kolonies van genetisch gemodificeerde dieren, niet gebruikt in andere dierproeven

3 Projectbeschrijving

3.1	Beschrijf de doelstellingen van het project (bv de wetenschappelijke vraagstelling of het wetenschappelijk en/of maatschappelijke belang)	Pulmonale arteriële hypertensie (PAH) is een zeldzame maar dodelijke longziekte. Bij patiënten met PAH leidt een belemmerde bloeddoorstroming door het longvaatbed tot een verhoogde bloeddruk in de longen en een overbelast hart. De overbelasting van het hart leidt tot hartfalen en uiteindelijk de dood. Omdat genezing van deze ziekte meestal niet mogelijk is, hebben patiënten een sterk verminderde kwaliteit van leven en een beperkte levensverwachting. De levensverwachting van patiënten met deze ziekte is slechts 3 tot 5 jaar (bij een relatief jonge patiëntengroep van 50 jaar of jonger). De huidige behandeling bestaat uit een combinatie van bloedvat verwijdende medicijnen die selectief op de longbloedvaten werken. Deze huidige behandeling is echter niet voldoende om het hartfalen te stoppen of te voorkomen. De enige manier om het hart te ontlasten en te

		laten herstellen in deze ziekte, is een longtransplantatie. Door schaarste in het aantal beschikbare organen en voortschrijding van de ziekte komt deze optie te laat voor de meeste patiënten. Binnen dit project zullen we werken aan de ontwikkeling van nieuwe kandidaat-behandelingen voor deze longziekte. Deze kandidaat-behandelingen en bijbehorende methodes zullen worden getest en verfijnd in muizen of ratten, zodat ze vervolgens kunnen worden onderzocht in patiënten en bij positief resultaat op de langere termijn kunnen worden ontwikkeld tot effectieve behandelingsmethode voor deze longziekte.
3.2	Welke opbrengsten worden van dit project verwacht en hoe dragen deze bij aan het wetenschappelijke en/of maatschappelijke belang?	De opbrengsten uit dit project zijn enerzijds in diermodellen geteste kandidaat-behandelingen voor PAH en anderzijds nieuwe kennis en inzichten rond de ziekteprocessen onderliggend aan dit ziektebeeld. Wetenschappelijk belang: de door het uitvoeren van interventiestudies in muizen en ratten zullen de onderzoekers een beter inzicht krijgen in de factoren die het succes van een kandidaat-behandeling bepalen. Dit is belangrijk omdat in het wetenschappelijk onderzoek de rol van een aantal van deze factoren (bijv. bepaalde genen, eiwitten) en de mogelijke interacties tussen factoren tot nu toe nog niet voldoende aandacht heeft gekregen. Daarnaast zal de nieuw verkregen kennis bijdragen aan nieuwe inzichten in de ziekteprocessen onderliggend aan de ziekte en ander gerelateerde ziektebeelden, bijvoorbeeld betreffende de rol van de linkerhartkamer. Maatschappelijk belang: de kandidaat-behandelingen kunnen op basis van de uitkomsten uit deze studies verder ontwikkeld worden op de langere termijn tot een behandeling die veilig en effectief is voor gebruik in patiënten. Dit onderzoek is van groot maatschappelijk belang omdat deze ziekte een zeer ernstige conditie is waarvoor tot op heden geen goede behandeling beschikbaar is.
3.3	Welke diersoorten en geschatte aantallen zullen worden gebruikt?	In dit project zullen experimenten worden uitgevoerd op muizen en ratten. Wij verwachten voor dit onderzoek maximaal 776 muizen en 840 ratten nodig te hebben in 5 jaar. De totale som van het aantal proefdieren is 1616.
3.4	Wat zijn bij dit project de verwachte negatieve gevolgen voor het welzijn van de proefdieren?	Om PAH in een diermodel te kunnen nabootsen ontstaan bij de dieren ook negatieve gevolgen door de symptomen van deze ziekte. Dit is helaas een belangrijk onderdeel van dit onderzoek en niet te voorkomen. Bij hartfalen is een afname van lichaamsgewicht te zien, de dieren worden dagelijks gecontroleerd en gewogen en indien nodig worden ze voortijdig uit de proef genomen (een humaan eindpunt toegepast). De meeste dieren zullen tot het eind van het experiment geen hartfalen ontwikkelen, hierbij zal het ongerief niet boven de matig uitkomen. Maximaal 12% van de dieren (alleen de ratten uit appendix 1 en 2) kan wel hartfalen ontwikkelen met ernstig ongerief. Ernstig ongerief is helaas niet te vermijden. Patiënten komen vaak ook binnen in de kliniek met tekenen van rechter hartfalen. Daarom is rechter hartfalen ook onderdeel van onze uitkomstmaat. Dit is nodig om deze dieren te kunnen vergelijken met onze patiënten, zodat de uitkomsten van onze experimenten representatief zijn. Daarnaast zullen negatieve gevolgen voor de proefdieren voortkomen uit de behandelingen en het monitoren van de effecten.
3.5	Hoe worden de dierproeven in het project ingedeeld naar de verwachte ernst?	Licht ongerief: maximaal 34% van de proefdieren Matig ongerief: maximaal 54% van de proefdieren Ernstig ongerief: maximaal 12% van de proefdieren, max 2 dagen. Alleen bij ratten, in appendix 1 en 2.

		Hartfalen is helaas een belangrijk onderdeel van dit onderzoek en is niet te voorkomen, hierbij kan bij een deel van de dieren ernstig ongerief optreden. Het is erg moeilijk om hartfalen te kunnen zien bij knaagdieren. De lichaamsgewichten van deze dieren schommelen enkele grammen gedurende de dag. Overdag slapen deze dieren en zullen ze weinig eten en drinken, maar wel ontlasting produceren. De eerste dag van hartfalen, is moeilijk te herkennen, omdat dit zowel een dagelijkse schommeling van het lichaamsgewicht kan zijn, als het eerste signaal van hartfalen. Bij een tweede dag van afvallen kan vrijwel altijd met zekerheid gezegd worden dat een dier hartfalen heeft.
3.6	Wat is de bestemming van de dieren na afloop?	Alle dieren worden na afloop van de experimenten gedood, waarna weefsel wordt gebruikt voor verder onderzoek.

4 Drie V's

4.1	**Vervanging** Geef aan waarom het gebruik van dieren nodig is voor de beschreven doelstelling en waarom proefdiervrije alternatieven niet gebruikt kunnen worden.	Alle behandelingen die in dit project getest worden in dieren, zijn eerst uitgebreid getest in relevante andere systemen, zoals gekweekte cellen of op eerder verzameld patiënt weefselmateriaal. De experimenten in proefdieren zijn erop gericht informatie te verkrijgen over complexe processen die met alternatieve methodes niet getest kunnen worden. Het gaat hierbij bijvoorbeeld over de verdeling van een (experimenteel) geneesmiddel in het hele lichaam, interactie met het immuunsysteem, en de interactie tussen hartfunctie en longbloedvat-afwijkingen. Dit kan vooralsnog niet nagebootst worden in celkweek of andere modelsystemen. Als zodanig heeft onderzoek in proefdieren geeft belangrijke informatie over veiligheid en effectiviteit van een kandidaat-behandeling, die van groot belang is, voor de toepassing van de nieuwe behandeling in patiënten.
4.2	**Vermindering** Leg uit hoe kan worden verzekerd dat een zo gering mogelijk aantal dieren wordt gebruikt.	Ter vermindering van het aantal dieren, zullen de volgende overwegingen gebruikt worden bij ieder experiment: - De groepsgrootte benodigd voor het verkrijgen van goed onderbouwde resultaten zal door middel van statistische methodes bepaald worden. Hierbij zal gestreefd worden naar het minimaliseren van het aantal gebruikte controle dieren. - Het onderzoek wordt uitgevoerd met behulp van standaard procedures en metingen om variatie tussen individuele experimenten te voorkomen. - Er wordt gebruik gemaakt van niet-invasieve beeldvorming (echo van het hart) om long- en hartfunctie gedurende een langere periode in een dier te kunnen volgen. Voor het project als geheel geldt dat de studies worden uitgevoerd in een gefaseerde opzet waardoor gebruik van het optimale aantal dieren wordt gewaarborgd.
4.3	**Verfijning** Verklaar de keuze voor de diersoort(en). Verklaar waarom de gekozen diermodel(len) de meest verfijnde zijn, gelet op de	De experimenten in dit project worden uitgevoerd in ratten of muizen. Muizen en ratten vertonen qua orgaanstructuur en genetische opbouw grote overeenkomsten met de mens. Daarnaast zijn in muizen veel genetische technieken mogelijk. Gedurende de afgelopen decennia is veel ervaring opgedaan met het onderzoek in muizen, waardoor veel vergelijkingsmateriaal, verschillende muizenstammen en modellen

doelstellingen van het project.	beschikbaar zijn. Ook zijn muizen goed te houden en te hanteren, wat het onderzoek vergemakkelijkt. Voor ratten gelden grotendeels dezelfde argumenten voor gebruik, behalve genetische modellen. Daarnaast zijn ratten veel groter dan muizen, waardoor bepaalde procedures die in muizen niet uitgevoerd kunnen worden, in ratten wel getest kunnen worden. We hebben gekozen voor vier diermodellen welke wetenschappelijk erkent zijn als op dit moment het beste diermodel voor pulmonale arteriële hypertensie. We hebben veel ervaring met de toegepaste onderzoekstechnieken, hiermee wordt onnodig lijden bij de dieren voorkomen.
Vermeld welke algemene maatregelen genomen worden om de negatieve (schadelijke) gevolgen voor het welzijn van de proefdieren zo beperkt mogelijk te houden.	De proefdieren zullen gezamenlijk worden gehuisvest in een omgeving met kooiverrijking. Bij de operaties en andere invasieve behandelingen worden algehele narcose en effectieve pijnstilling toegepast. In geval van ernstig onverwacht ongerief worden de humane eindpunten toegepast. Al het onderzoek in dit project zal door gekwalificeerd personeel worden uitgevoerd in een gespecialiseerde proefdierfaciliteit. Daarnaast zal ervaren personeel zorgdragen voor de controle van het welzijn van de dieren. Er zijn protocollen aanwezig waarin procedures voor het hanteren van dieren, alsmede richtlijnen voor narcose en pijnstilling, zijn vastgelegd.

5 In te vullen door de CCD

Publicatie datum	
Beoordeling achteraf	
Andere opmerkingen	

Centrale Commissie Dierproeven

Advies aan CCD

Datum 14 oktober 2020
Betreft Advies Secretariaat over Aanvraag projectvergunning Dierproeven AVD20209866

Instelling:	5.1 lid2h
Onderzoeker:	5.1 lid2e
Project:	Towards a novel treatment of pulmonary arterial hypertension; preclinical testing of novel interventions targeting both right ventricular pressure overload and pulmonary vascular remodelling.
Aanvraagnummer:	AVD20209866
Betreft:	Nieuwe aanvraag
Categorieën:	Fundamenteel onderzoek Translationeel of toegepast onderzoek

1 Inzicht in aanvraag en de eventuele knelpunten en risico's

Proces	De DEC heeft 41 werkdagen gedaan over het opstellen van het advies. De volgende vragen zijn nog gesteld aan de aanvrager: In sectie 3.4 van de NTS schrijft u dat dieren'vroegtijdig uit de proef genomen'worden. Dit kan als verhullend taalgebruik worden gezien. Graag dit aanpassen naar 'gedood'. In secties 3.4 en 3.5 van de NTS verwijst u naar appendix 1 en 2. De NTS dient zelfstandig leesbaar te zijn. Graag de verwijzingen naar de appendices verwijderen. U wordt verzocht in sectie 3.5 de ongeriefsclassificaties weer te geven voor ratten en muizen afzonderlijk.

Overzicht van opmerkingen bij AdviesNotaCCD_5.1 lid2e.pdf

Pagina: 1

Nummer: 1 Auteur: 5.1 lid2e Onderwerp: Notitie Datum: 14-10-2020 12:20:49
Eens met de vragen over de NTS.

Nummer: 2 Auteur: 5.1 lid2e Onderwerp: Notitie Datum: 14-10-2020 12:04:13
Ik begrijp je twijfel over 'sustained' bij de HEPs. Ik zou aan ze vragen vanaf wanneer ze die afwijkende ademhaling, kortademigheid, lethargie en afwijkend gedrag gaan meten. Is dit meteen vanaf het eerste moment dat ze de hypertensie induceren? Is dat vanaf het moment van therapie? Als ze deze tekenen vertonen na hoe lang haal je ze dan uit de proef? Zijn hele praktische vragen maar aan de hand van ons NVWA overleg denk wel goed om in de bijlagen dierproeven te hebben staan,

Naam proef	Diersoort	Stam	Aantal dieren	Herkomst
3.4.4.1 Intervention studies using SuHx model				
	Ratten (Rattus norvegicus)	Sprague Dawley	400	Dieren die voor onderzoek gefokt zijn
3.4.4.2 Intervention studies using PAB model				
	Ratten (Rattus norvegicus)	Wistar	440	Dieren die voor onderzoek gefokt zijn
3.4.4.3 Intervention studies using Chronic hypoxia mouse model				
	Muizen (Mus musculus)	WT, knock-in, knock-out	400	Dieren die voor onderzoek gefokt zijn
3.4.4.4 Intervention studies using Pneumonectomy (PNX)-Su5416 mouse model				
	Muizen (Mus musculus)	WT, knock-in, knock-out	376	Dieren die voor onderzoek gefokt zijn

Huisvesting en verzorging anders dan Bijlage III Richtlijn

3.4.4.1 Intervention studies using SuHx model

Citaat: ... However, as part of the experimental model, the animals will also be housed under low oxygen conditions (10% O2) for 3-4 weeks (the chronic hypoxia period).

3.4.4.3 Intervention studies using Chronic hypoxia mouse model

Citaat: ...However, as part of the experimental model, the animals will also be housed under low oxygen conditions (10% O2) for 2 weeks (the chronic hypoxia period).

Gebruik van mannelijke en vrouwelijke dieren

3.4.4.1 Intervention studies using SuHx model

Ratten (Rattus norvegicus) Er worden zowel mannelijke als vrouwelijke dieren gebruikt.

3.4.4.2 Intervention studies using PAB model

Ratten (Rattus norvegicus) Er worden zowel mannelijke als vrouwelijke dieren gebruikt.

3.4.4.3 Intervention studies using Chronic hypoxia mouse model

Muizen (Mus musculus) Er worden zowel mannelijke als vrouwelijke dieren gebruikt.

3.4.4.4 Intervention studies using Pneumonectomy (PNX)-Su5416 mouse model

Muizen (Mus musculus) Er worden zowel mannelijke als vrouwelijke dieren gebruikt.

Een deel van de dieren in bijlage 3.4.4.1 en 3.4.4.3 zal tijdelijk worden gehuisvest onder lage zuurstofcondities. Dit is nodig voor het opwekken van het ziektebeeld.

Locatie uitvoering experimenten	- Alle proeven vinden plaats in een instelling van een vergunninghouder. - Er zijn geen problemen bekend met de vergunninghouder.

2 DEC advies

DEC-advies	De DEC heeft in 2 vragenrondes vragen gesteld aan de aanvrager. In vraagronde 1 werden vragen gesteld over: de NTS, belangen en onderbouwing van het belang, opzet van de de pilot, go-no go momenten, soorten interventies, percentage dieren dat ernstig ongerief zal ondervinden, benoemen van het target, inconsistenties in de aanvraag, onderbouwing gekozen diermodellen, beschrijving van de te verwachten verbeteringen door de interventies, onderbouwing dieraantallen, voorkomen van ernstig ongerief mogelijk?, waarom geen dosis proeven, ongerief door interventies, onderbouwing gebruik katheters, In vraagronde 2 zijn vragen gesteld over o.a.: verheldering dieraantallen pilotstudies, verheldering tabellen, inschatting aantal dieren dat HEP bereikt, Citaten: C10 (huisvesting): De dieren worden gehuisvest en verzorgd op een wijze die voldoet aan de eisen die zijn opgenomen in bijlage III van de richtlijn. De dieren in Appendix 1 (SuHx model) en 3 (Chronic hypoxia mouse model) worden voor maximaal 4 weken gehuisvest in een hypoxische omgeving met 10% zuurstof, deze hypoxie leidt tot pulmonale hypertensie. C11 (ongerief): (...) Hartfalen is helaas een belangrijk onderdeel van dit onderzoek en is niet te voorkomen, hierbij kan bij een deel van de dieren ernstig ongerief optreden. De meeste dieren zullen tot het eind van het experiment geen hartfalen ontwikkelen, hierbij zal het ongerief niet boven de matig uitkomen. Maximaal 12% van de dieren kan wel hartfalen ontwikkelen met ernstig ongerief (voor maximaal 2 dagen). Bij hartfalen is een afname van lichaamsgewicht te zien, de dieren worden daarom dagelijks gecontroleerd en gewogen. De DEC is van mening dat het ongerieflevel aan de onderkant van ernstig ligt, zoals dat door de EU is gedefinieerd, de dieren hebben waarschijnlijk geen pijn maar missen energie. De DEC is akkoord met deze inschatting van het ongerieflevel. C13 (Humane eindpunten): De criteria voor de humane eindpunten zijn goed gedefinieerd. Het humane eindpunt is, in lijn met eerdere protocollen als volgt gedefinieerd: "Het moment waarop, in de periode dat het begin van hartfalen wordt verwacht, voor het eerst een duidelijke gewichtsafname

Nummer: 1 Auteur: 5.1 lid2e Onderwerp: Notitie Datum: 14-10-2020 11:39:04

Zet je dit citaat erin omdat een deel ernstig ongerief ondervindt? 5.2 lid1

5.2 lid1

waarneembaar is, die niet past binnen de normale dagelijkse schommelingen." In de praktijk komt dat neer op het vaststellen van een duidelijke gewichtsafname ≥10% van gewicht binnen 24 uur (in combinatie met symptomen van PAH/hartfalen) of een opvallend grote gewichtsafname ≥20% van het maximale lichaamsgewicht, gemeten vanaf de start van de behandeling.
Daarnaast zal expliciet gelet worden op andere klinische tekenen van hartfalen: zoals cyanose, dyspneu, lethargie, afwijkend gedrag, complicaties van interventies en een slechte verzorging. Door dagelijks te observeren en te wegen kan het eindpunt goed worden bepaald, en kan onnodig lijden worden voorkomen. Gebaseerd op eerdere experimenten wordt er rekening gehouden 15% kans op een humaan eindpunt voor de dieren in Appendix 1, bij Appendix 2 is de kans 32% en de kans in Appendix 3 en 4 is 10%.

Ethische afweging van de DEC:
Citaat: Rechtvaardigen de doeleinden van dit project het voorgestelde gebruik van de dieren? Rechtvaardigt de ontwikkeling van nieuwe kandidaat-behandelingen voor de longziekte Pulmonale arteriële hypertensie (PAH), om hiermee het verloop van de ziekte te verbeteren, het gebruik van maximaal 776 muizen en 840 ratten die daarvan licht tot ernstig ongerief ondervinden?

2. De waarden die voor de proefdieren in het geding zijn: De integriteit van de proefdieren wordt aangetast en de dieren ondervinden licht tot ernstig ongerief. Dat leidt tot veel nadeel voor deze proefdieren. De waarden voor de onderzoekers: voordeel vanwege de kennisontwikkeling over het verloop van PAH. De waarden die voor de patiënten bevorderd worden: Mogelijk veel voordeel wanneer de dierproef bijdraagt aan het ter beschikking komen van betere behandelopties voor hartfalen bij patiënten met pulmonale hypertensie.
De DEC is van mening dat de belangen van de patiënten in dit project zwaarder wegen dan de belangen van de 776 muizen en 840 ratten, die hiervoor als proefdieren gebruikt worden. Voor het verkrijgen van meer kennis over pulmonale hypertensie en hartfalen is onderzoek in diermodellen noodzakelijk. Er zijn op dit moment geen alternatieven voor deze dierproeven beschikbaar waarmee men de doelstellingen kan bereiken.

3. Volgens de DEC rechtvaardigen de doeleinden van dit project het

	voorgestelde gebruik van dieren. Het directe doel van deze studie is de ontwikkeling van nieuwe kandidaat-behandelingen voor de longziekte Pulmonale arteriële hypertensie (PAH). Het verwachte resultaat, in het kader van het beschikbaar komen van betere behandelingsopties voor patiënten met hartfalen, is afgewogen tegen het licht tot ernstig geschatte ongerief en de aantasting van integriteit, inclusief het doden van de dieren in de proef. De DEC onderschrijft dat de doelstellingen niet zonder het gebruik van proefdieren kunnen worden behaald en acht het gebruik van 776 muizen en 840 ratten, en de daarmee samenhangende schade aan deze dieren gerechtvaardigd. Bij het uitvoeren van de dierproeven wordt een adequate invulling gegeven aan de vereisten op het gebied van de vervanging, vermindering en verfijning van de dierproeven. Het project is (1) van substantieel belang en (2) van goede kwaliteit. (1) Het maatschappelijk belang en wetenschappelijk belang zijn beide substantieel. De resultaten van dit onderzoek zullen bijdragen aan meer kennis over pulmonale hypertensie en het beschikbaar komen van betere behandelingsopties voor patiënten met hartfalen. (2) De DEC is van mening dat dit project verantwoord is vanuit wetenschappelijk oogpunt en acht het waarschijnlijk dat op basis van de resultaten van de voorgenomen reeks experimenten beschreven in het project, nieuwe en/of aanvullende kennis zal worden verkregen. De onderzoekers beschikken over ruime ervaring en kennis op het gebied van de te gebruiken methoden en werken nauw samen met andere onderzoeksgroepen. Dit in combinatie met de beschikbare faciliteiten en infrastructuur betekent dat de onderzoekers goed gekwalificeerd en geoutilleerd zijn voor het uitvoeren van het in dit project beschreven onderzoek. Samenvattend kan worden gesteld dat het als substantieel te kwalificeren maatschappelijk en wetenschappelijk belang van het onderzoek naar het oordeel van de DEC opweegt tegen het gebruik van maximaal 776 muizen en 840 ratten en het daarbij verwachte lichte tot ernstige ongerief. De DEC heeft extern advies ingewonnen bij - de aanvrager is om aanvullingen gevraagd Het DEC advies is Positief

	Het uitgebrachte advies is niet gebaseerd op consensus. Het uitgebrachte advies is gebaseerd op meerderheid. Er is een lid dat niet meegaat met het positieve advies, omdat het vertrouwen in de haalbaarheid van dit project voor dit lid gecompromitteerd is: het goed opzetten van dierexperimenten bepaalt mede de haalbaarheid van een projectvoorstel. Het inschatten van het aantal dieren is hierbij een voorwaarde. Gezien het twee vragenrondes duurde voordat het juiste aantal dieren in het protocol opgenomen waren hebben de aanvragers het in de ogen van het lid nagelaten om goed over de experimenten na te denken.

3 Kwaliteit DEC advies

Kwaliteit DEC-advies	
Het DEC advies is helder en navolgbaar. In het DEC advies is op heldere wijze inzicht gegeven in de vragen die aan de aanvrager zijn gesteld. Bij de beantwoording van de beoordelingsvragen verstrekt u over het algemeen een heldere onderbouwing. De ethische afweging volgt op logische wijze uit de beantwoording van de C vragen. 1 Bij vraag C10 benoemt u alleen dat de dieren tijdelijk onder lage O2-condities gehuisvest worden, maar u geeft hierbij niet de mening van de DEC weer. U heeft de noodzaak van het opleggen van een beoordeling achteraf aan deze vergunning niet benoemd. Uw advies is tot stand gekomen op basis van een meerderheidsstandpunt. Het minderheidsstandpunt is duidelijk weergegeven en u maakt onder de vragen C7 en C8 duidelijk waarom de meerderheid van de DEC een andere afweging maakt. Wij volgen het meerderheidsstandpunt van de DEC, omdat wij, gezien de vele publicaties van deze onderzoeksgroep, en gezien het nu voorliggende project 2rstel voldoende vertrouwen hebben in de haalbaarheid van de doelstellingen van dit project. De behandeltijd van deze aanvraag bij uw DEC heeft meer dan 20 werkdagen in beslag genomen. Het valt ons op dat er een lange periode tussen indienen van de gewijzigde versie door de aanvrager en de bespreking in de DEC zat.	

Pagina: 6

Nummer: 1 Auteur: 5.1 lid2e Onderwerp: Notitie Datum: 14-10-2020 11:41:40

Je kan nog iets zeggen dat ze veel vragen hebben gesteld, maar dat de CCD ziet dat dit was om helder te hebben hoeveel beesten wat ondergaan, zodat tot een ethische afweging kon worden gekomen, en nietomdat de de DEC wilde meeschrijven. Iets in die richting.

Nummer: 2 Auteur: 5.1 lid2e Onderwerp: Notitie Datum: 14-10-2020 11:40:16

Mooi

4 Inhoudelijke beoordeling

Doelstelling Doelstelling	Citaat: The main objectives of the research included in this project are to: • Evaluate novel (combinations of) therapeutic interventions in mice and rat models for PAH, targeting both pulmonary vascular remodelling and RV pressure overload; • Improve fundamental understanding of PAH and RV failure and their underlying pathologies; • Discover novel therapeutic targets for PAH. The results of the studies will render pivotal information on the usefulness of the therapeutic interventions in subsequent clinical trials and will support the use of relevant PAH disease models. As such, they will contribute to improve the treatment of PAH.
Wetenschappelijk en maatschappelijk belang	Citaat: SCIENTIFIC RELEVANCE Not only will the proposed studies allow us to identify therapeutic interventions for PAH with the highest efficacy likelihood and the lowest toxicity potential before starting clinical trials, they will also increase our understanding of the processes underlying abnormal pulmonary vascularisation and that controlling the transition of RV adaptation towards right heart failure. Our research group is one of the few who takes a combined approach by studying the pathological effects of PAH in the lungs and the right heart concurrently. This will allow us to investigate the relatively new concept that PAH patients may benefit from a cardiac-specific treatment, which has no direct effect on the pulmonary vasculature, but presents or reverses right heart dysfunction. Moreover, novel therapeutic targets for future clinical research may be identified. Although this research proposal is focused on PAH, right heart failure is also the main cause of death in several other conditions such as left heart failure and critical illness. We have for example shown that not only the RV but also the LV is affected in PAH-patients. We believe that RV remodelling observed in PAH patients shares important pathophysiological mechanisms with the cardiac remodelling observed in left heart failure patients. As such, the findings of this proposal may also advance research in left heart failure. The scientific relevance of our findings is therefore not limited to PAH-induced right heart failure. SOCIETAL RELEVANCE PAH remains an incurable debilitating disease, with high mortality rates and poor prognosis for patients. Although the incidence is low (2.2 per million), the current life expectancy is only 3-5 years [4]. Besides the enormous impact of the disease on the quality of life of PAH patients, the disease also carries considerable economic consequences because patients and/or care-givers drop out of the work force and patients

	require expensive medical treatments, including lung transplantation. New PAH therapies, also targeting alternative pathways are urgently needed. In this project, we address apart from established PAH-targets (e.g. BMPR2 signalling) [10], also relatively new ones, such as RV diastolic stiffness. Up till now, there has been little consideration in the field of the notion that PAH patients could benefit from a cardiac-specific treatment, which has no direct effect on the pulmonary vasculature. With the results of this project, we will be able to select those interventions with promising effectiveness in PAH animal models for further clinical testing. This will bring us hopefully a step closer to the development of an effective PAH treatment. In the future, patients at risk of developing right heart failure (PAH) may benefit from these new treatment options. Although PAH is rare, other types of pulmonary hypertension (PH) are much more prevalent and carry significant morbidity and mortality. Moreover, right heart failure is becoming a great clinical problem as leading cause of death in several diseases such as left heart failure, and the critical ill at the intensive care. Currently, no therapeutic strategies are available to improve RV function or prevent right heart failure. Beside knowledge on PH and right heart failure, this project will also provide new insight in cardiac and endothelial physiology, which will be useful in other lung diseases and left heart failure. As such, the societal relevance of our studies extends far beyond PAH alone.
Onderbouwing wetenschappelijk en maatschappelijk belang	Voldoende beschreven.

Wetenschappelijke kwaliteit Kwaliteit aanvrager/ onderzoeksgroep en onderzoek	Citaat uit DEC advies C7: De DEC heeft veel vragen gesteld bij deze aanvraag, zie de vragenrondes bovenaan dit document. De DEC is van mening dat naast aanwezige apparatuur, kennis, personeel en financiering ook het goed opzetten van experimenten (en daarbij het inschatten van het aantal dieren) de haalbaarheid van een projectvoorstel bepaalt en ziet graag dat de onderzoekers bij de uitvoering goed voor ogen houden wat ze precies willen doen en dat goed inplannen om fouten te voorkomen. Alle technische voorzieningen die benodigd zijn voor uitvoering van het project zijn voorhanden, evenals voldoende deskundigheid en financiering om het project succesvol uit te voeren. Ervaring binnen het onderzoeksinstituut met vergelijkbaar onderzoek waarborgt het technisch succesvol uitvoeren van de dierexperimenten. Na navraag is de DEC ervan overtuigd dat de projectdoelstelling met de gekozen strategie/aanpak binnen de gevraagde termijn is te realiseren. Eén van de DEC leden is van mening dat "het vertrouwen in de haalbaarheid van dit project voor dit lid gecompromitteerd is: het goed opzetten van dierexperimenten bepaalt mede de haalbaarheid van een projectvoorstel. Het inschatten van het aantal dieren is hierbij een voorwaarde. Gezien het twee vragenrondes duurde voordat het juiste aantal dieren in het protocol opgenomen waren hebben de aanvragers het in de ogen van het lid nagelaten om goed over de experimenten na te denken."

Pagina: 9

Nummer: 1 Auteur: 5.1 lid2e Onderwerp: Notitie Datum: 14-10-2020 11:42:51

5.2 lid1 Kun je copy pasten uit je terugkoppeling aan de DEC.

3V's

Vervanging	
	3.4.4.1 Intervention studies using SuHx model: Citaat: All proposed interventions that will be tested throughout this project will be assessed first in other, non-animal, models, such as cell culture experiments. Only if these experiments yield sufficiently promising results, in vivo tests will be undertaken. In vivo experiments are a necessary step in development of interventions that may one day be used in the clinic, since currently no other methods allow the evaluation of the effect of an intervention on the organism as a whole. Cell culture, organoids, lab-on-a-chip or computer models are not (yet) sophisticated enough to assess the interaction between the PAH pathology and host environmental factors such as the oxygen supply, the immune system and metabolism.
	3.4.4.2 Intervention studies using PAB model: Citaat: All proposed interventions that will be tested throughout this project will be assessed first in other, non-animal, models, such as cell culture experiments. Only if these experiments yield sufficiently promising results, in vivo tests will be undertaken. In vivo experiments are a necessary step in development of interventions that may one day be used in the clinic, since currently no other methods allow the evaluation of the effect of an intervention on the organism as a whole. Cell culture, organoids, lab-on-a-chip or computer models are not (yet) sophisticated enough to assess the interaction between the PAH pathology and host environmental factors such as the oxygen supply, the immune system and metabolism.
	3.4.4.3 Intervention studies using Chronic hypoxia mouse model: Citaat: In vivo experiments are a necessary step in development of interventions that may one day be used in the clinic, since currently no other methods allow the evaluation of the effect of an intervention on the organism as a whole. Cell culture, organoids, lab-on-a-chip or computer models are not (yet) sophisticated enough to assess the interaction between the PAH pathology and host environmental factors such as the oxygen supply, the immune system and metabolism.
	3.4.4.4 Intervention studies using Pneumonectomy (PNX)-Su5416 mouse model: Citaat: In vivo experiments are a necessary step in development of interventions that may one day be used in the clinic, since currently no other methods allow the evaluation of the effect of an intervention on the organism as a whole. Cell culture, organoids, lab-on-a-chip or computer models are not (yet) sophisticated enough to assess the interaction between the PAH pathology and host environmental factors such as the oxygen supply, the immune system and metabolism.

Pagina: 10

Nummer: 1 Auteur: 5.1 lid2e Onderwerp: Notitie Datum: 14-10-2020 11:45:08
Zie bijlage 3.4.4.1

Nummer: 2 Auteur: 5.1 lid2e Onderwerp: Markering Datum: 14-10-2020 11:44:52

Nummer: 3 Auteur: 5.1 lid2e Onderwerp: Notitie Datum: 14-10-2020 11:45:57
Zie bijlage 3.4.4.3

Nummer: 4 Auteur: 5.1 lid2e Onderwerp: Markering Datum: 14-10-2020 11:45:45

Verminderen	
	3.4.4.1 Intervention studies using SuHx model: Citaat: The proposed number of evaluable animals per study arm (n=12 in model/intervention groups, n=6 in control group) is calculated as described above, and is in line with generally accepted protocols in scientific literature and our previous studies with the SuHx model at our Department. Further reduction of the number of animals per group would decrease statistical power, and might thus disqualify the experiment.
	3.4.4.2 Intervention studies using PAB model: Citaat: The proposed number of evaluable animals per study arm (n=12 in model/intervention groups, n=6 in control group) is calculated as described above, and is in line with generally accepted protocols in scientific literature and our previous studies with the PAB model. Further reduction of the number of animals per group would decrease statistical power, and might thus disqualify the experiment.
	3.4.4.3 Intervention studies using Chronic hypoxia mouse model: Citaat: The proposed number of evaluable animals per study arm (n=10) is calculated as described above, and is in line with generally accepted protocols in scientific literature. Further reduction of the number of animals per group would decrease statistical power, and might thus disqualify the experiment.
	3.4.4.4 Intervention studies using Pneumonectomy (PNX)-Su5416 mouse model: Citaat: The proposed number of evaluable animals per study arm (n=12 in model/intervention groups, n=6 in control group) is calculated as described above, and is in line with generally accepted protocols in scientific literature. Further reduction of the number of animals per group would decrease statistical power, and might thus disqualify the experiment.
Verfijnen	
	3.4.4.1 Intervention studies using SuHx model: Citaat: State-of-the-art methods and equipment for assessing pulmonary vascular remodelling and RV overload, in particular advanced imaging techniques, will be used to refine protocols and minimize discomfort to the animals especially in support of the application of humane endpoints. International guidelines will be applied to ensure that best practice is followed. Citaat: The procedures conducted under this protocol will inevitably cause discomfort for the animals in the studies. In order to minimize suffering, all studies will adhere to the national and internationally accepted rules for handling of lab animals [1], [2]. Under these rules, the animals will be

	humanely killed when a humane endpoint (see below) is reached. Within the institute, standard operating procedures (SOP) for animal handling are in place. These also include dedicated anaesthesia and analgesia (A&A) SOPs that will accompany invasive procedures to minimize pain and discomfort. To minimize animal suffering, pain and fear the following measures will be taken. The animals will be allowed to acclimatize to their new environment for two weeks upon arrival at the animal facility. Animals are housed in groups (socially housed) at all times (also during hypoxia. Except during surgery, animals are returned to the group directly after surgery) and environmental enrichment strategies are applied in the cages to improve animal welfare. In case the therapeutic interventions require surgical procedures (e.g. cannula/mini-pump implantation), this will be done under general anaesthesia in combination with pain treatment. During haemodynamic measurements, the animals will be kept under general anaesthesia in a temperature-controlled environment. During imaging procedures, animals will be kept under general anaesthesia in a temperature-controlled environment. (...) Most rats will not suffer from heart failure until the end of the experiment, and their discomfort will not exceed moderate. Maximally 15% (based on previous experiments) of the rats can develop heart failure with severe discomfort. It is very difficult to see if a rat is having heart failure (which can occur in the weeks after hypoxia). A decrease in bodyweight is the first sign of heart failure which is then already occurring for one day. It is normal to have a decreasing bodyweight of the rat during the day, because of sleeping, less drinking and eating during the day (daily fluctuations). To be sure it is heart failure (and not daily fluctuations of bodyweight), a bodyweight decrease of 10% is being established. Also cyanosis, dyspnea, lethargy and poor grooming can be observed. This is usually on the second day. Then an HEP will be applied. Severe discomfort is unfortunately unavoidable. Patients usually present at the stage of heart failure. Right heart failure is an important outcome measure of our research. Heart failure is necessary to compare the rats with patients.
	3.4.4.2 Intervention studies using PAB model: Citaat: State-of-the-art methods and equipment for assessing pulmonary vascular remodelling and RV overload, in particular advanced imaging techniques, will be used to refine protocols and minimize discomfort to the animals especially in support of the application of humane endpoints. International guidelines will be applied to ensure that best practice is followed. Citaat: The procedures conducted under this protocol will inevitably cause

	discomfort for the animals in the studies. In order to minimize suffering, all studies will adhere to the national and internationally accepted rules for handling of lab animals [2, 3]. Under these rules, the animals will be humanely killed when a humane endpoint (see below) is reached. Within the institute, standard operating procedures (SOP) for animal handling are in place. These also include dedicated anaesthesia and analgesia (A&A) SOPs that will accompany invasive procedures to minimize pain and discomfort. To minimize animal suffering, pain and fear the following measures will be taken. The animals will be allowed to acclimatize to their new environment for two weeks upon arrival at the animal facility. Animals are housed in groups (socially housed) at all times (except during surgery, animals are returned to the group directly after surgery) and environmental enrichment strategies are applied in the cages to improve animal welfare. The PAB surgical model is performed under general anaesthesia and analgesia (pre- and post-surgery). During haemodynamic measurements, animals will be kept under anaesthesia in a temperature-controlled environment. During imaging procedures, animals will be kept under anaesthesia in a temperature-controlled environment. (...) Right ventricular (RV) failure is the predominant cause of death in patients with pulmonary hypertension. Maximally 32% of the rats can develop heart failure with severe discomfort. It is due to the diameter of the band around the pulmonary artery. A decrease in bodyweight is the first sign of heart failure which is then already occurring for one day. It is normal to have a decreasing bodyweight of the rat during the day, because of sleeping, less drinking and eating during the day (daily fluctuations). To be sure it is heart failure (and not daily fluctuations of bodyweight), a bodyweight decrease of 10% is being established. Also cyanosis, dyspnea, lethargy and poor grooming can be observed. This is usually at the second day and an HEP will be applied. Based on experience with this model in Denmark: Seven weeks survival rate was 80% for rats subjected to severe banding and close to 100% in rats subjected to mild or moderate banding or sham surgery[1]. Severe discomfort is unfortunately unavoidable. Patients usually present at the stage of heart failure. Right heart failure is an important outcome measure of our research. Heart failure is necessary to compare the rats with patients.

	3.4.4.3 Intervention studies using Chronic hypoxia mouse model: Citaat: State-of-the-art methods and equipment for assessing pulmonary vascular remodelling and RV overload, in particular advanced imaging techniques, will be used to refine protocols and minimize discomfort to the animals especially in support of the application of humane endpoints. International guidelines will be applied to ensure that best practice is followed. Explain what measures will be taken to minimise 1) animal suffering, pain or fear and 2) adverse effects on the environment. The procedures conducted under this protocol will inevitably cause discomfort for the animals in the studies. In order to minimize suffering, all studies will adhere to the national and internationally accepted rules for handling of lab animals [1], [2]. Under these rules, the animals will be humanely killed when a humane endpoint (see below) is reached. Within the institute, standard operating procedures (SOP) for animal handling are in place. These also include dedicated anaesthesia and analgesia (A&A) SOPs that will accompany invasive procedures to minimize pain and discomfort. To minimize animal suffering, pain and fear the following measures will be taken. The animals will be allowed to acclimatize to their new environment for two weeks upon arrival at the animal facility. Animals are housed in groups (socially housed) at all times (also during hypoxia) and environmental enrichment strategies are applied in the cages to improve animal welfare. During haemodynamic measurements, animals will be kept under anaesthesia in a temperature-controlled environment. Measurements of vascular leakage will be performed under continuous anaesthesia. (...) In these experiments in mice with PAH there is no heart failure expected, and the discomfort will not transcend mild.

	3.4.4.4 Intervention studies using Pneumonectomy (PNX)-Su5416 mouse model: Citaat: State-of-the-art methods and equipment for assessing pulmonary vascular remodelling and RV overload, in particular advanced imaging techniques, will be used to refine protocols and minimize discomfort to the animals especially in support of the application of humane endpoints. International guidelines will be applied to ensure that best practice is followed. Explain what measures will be taken to minimise 1) animal suffering, pain or fear and 2) adverse effects on the environment. The procedures conducted under this protocol will inevitably cause discomfort for the animals in the studies. In order to minimize suffering, all studies will adhere to the national and internationally accepted rules for handling of lab animals [1], [2]. Under these rules, the animals will be humanely killed when a humane endpoint (see below) is reached. Within the institute, standard operating procedures (SOP) for animal handling are in place. These also include dedicated anaesthesia and analgesia (A&A) SOPs that will accompany invasive procedures to minimize pain and discomfort. In case the pulmonary hypertension is too severe, we may choose to omit the SU5416 injection. To minimize animal suffering, pain and fear the following measures will be taken. The animals will be allowed to acclimatize to their new environment for two weeks upon arrival at the animal facility. Animals are housed in groups (socially housed) at all times (except during surgery, animals are returned to the group directly after surgery) and environmental enrichment strategies are applied in the cages to improve animal welfare. The PNX surgical model is performed under general anaesthesia and analgesia (pre- and post-surgery). During haemodynamic measurements, animals will be kept under anaesthesia in a temperature-controlled environment. (...) In these experiments in mice with PAH there is no heart failure expected, and the discomfort will not transcend moderate.
3.4.4.1 Intervention studies using SuHx model: Voldoende beschreven.	
3.4.4.2 Intervention studies using PAB model: Voldoende beschreven.	
3.4.4.3 Intervention studies using Chronic hypoxia mouse model: Voldoende beschreven.	
3.4.4.4 Intervention studies using Pneumonectomy (PNX)-Su5416 mouse model: Voldoende beschreven.	

Hergebruik	Er is geen sprake van hergebruik van dieren.

Naam proef	Worden de dieren gedood?	Doden volgens richtlijn?
3.4.4.1 Intervention studies using SuHx model	Ja	volgens de richtlijn.
3.4.4.2 Intervention studies using PAB model	Ja	volgens de richtlijn.
3.4.4.3 Intervention studies using Chronic hypoxia mouse model	Ja	volgens de richtlijn.
3.4.4.4 Intervention studies using Pneumonectomy (PNX)-Su5416 mouse model	Ja	volgens de richtlijn.

Naam proef		
3.4.4.1 Intervention studies using SuHx model	HEP: <15%	Citaat: The most important humane endpoints applicable to all studies are: • Weight loss >20% of maximum body weight in adult animals, measured from the start of the treatment • Weight loss >10% of body weight during 24h, in combination with: • Sustained abnormal breathing, dyspnoea (symptom PAH/right heart failure) • Sustained lethargy (symptom PAH/right heart failure) • Sustained abnormal behaviour • Complications of interventions • Other procedure-specific endpoints
Ratten (Rattus norvegicus)	Ongerief: 15,0% Ernstig 85,0% Matig	

3.4.4.2 Intervention studies using PAB model	HEP: <32%	The most important humane endpoints applicable to all studies are: • Permanent weight loss >20% of initial body weight in adult animals, measured from the start of the treatment • Weight loss >10% of body weight during 24h, in combination with: o Sustained abnormal breathing, dyspnea (symptom PAH/right heart failure) o Sustained lethargy (symptom PAH/right heart failure) • Sustained abnormal behavior • Complications of interventions • Other procedure-specific endpoints
Ratten (Rattus norvegicus)	Ongerief: 32,0% Ernstig 68,0% Matig	
3.4.4.3 Intervention studies using Chronic hypoxia mouse model	HEP: <10%	Citaat: The most important humane endpoints applicable to all studies are: • Weight loss >20% of maximum body weight in adult animals, measured from the start of the treatment • Weight loss >15% of body weight during 24h, in combination with: o Sustained abnormal breathing, dyspnoea (symptom PAH/right heart failure) o Sustained lethargy (symptom PAH/right heart failure) • Sustained abnormal behaviour • Complications of interventions (<1%): No interventions are planned.
Muizen (Mus musculus)	Ongerief: 100,0% Licht	

3.4.4.4 Intervention studies using Pneumonectomy (PNX)-Su5416 mouse model	HEP: <10%	Citaat: The most important humane endpoints applicable to all studies are: • Weight loss >20% of maximum body weight in adult animals, measured from the start of the treatment • Weight loss >15% of body weight during 24h, in combination with: • Sustained abnormal breathing, dyspnoea (symptom PAH/right heart failure) • Sustained lethargy (symptom PAH/right heart failure) • Sustained abnormal behaviour • Complications of interventions • • Other procedure-specific endpoints
Muizen (Mus musculus)	Ongerief: 59,0% Matig 41,0% Licht	

5 Samenvatting

5.2 lid1

Er worden enkele dieren extra aangevraagd voor het geval van uitval van dieren. Dit is voldoende onderbouwd.

Een deel van de dieren, in bijlagen 3.4.4.1 en 3.4.4.3 wordt gedurende enkele weken gehuisvest onder condities met lage zuurstofspanning. Dit is nodig om het ziektebeeld te induceren, en heeft dus een wetenschappelijke noodzaak.
5.2 lid1

Het DEC advies is gebaseerd op een meerderheidsstandpunt. (citaat): "Er is een lid dat niet meegaat met het positieve advies, omdat het vertrouwen in de haalbaarheid van dit project voor dit lid gecompromitteerd is: het goed opzetten van dierexperimenten bepaalt mede de haalbaarheid van een projectvoorstel. Het inschatten van het aantal dieren is hierbij een voorwaarde. Gezien het twee vragenrondes duurde voordat het juiste aantal dieren in het protocol opgenomen waren hebben de aanvragers het in de ogen van het lid nagelaten om goed over de experimenten na te denken." 5.2 lid1

5.2 lid1

Pagina: 18

Nummer: 1 Auteur: 5.1 lid2e Onderwerp: Notitie Datum: 14-10-2020 12:00:29

Eens.

Nummer: 2 Auteur: 5.1 lid2e Onderwerp: Notitie Datum: 14-10-2020 12:03:22

Deze zou ik er ook bij zetten omdat je dit in je samenvatting van de DEC ook aanhaalt:

Het ongerief in dit onderzoek is voor 12% van de ratten op ernstig ingeschat. De DEC zegt over deze inschatting, citaat: *De DEC is van mening dat het ongerieflevel aan de onderkant van ernstig ligt, zoals dat door de EU is gedefinieerd, de dieren hebben waarschijnlijk geen pijn maar missen energie. De DEC is akkoord met deze inschatting van het ongerieflevel.* 5.2 lid1

6 Voorstel besluit incl. voorstel geldigheidsduur van de vergunning

5.2 lid1

Beoordeling achteraf [1]
In dit project worden dierproeven toegepast die vallen in de categorie ernstig volgens artikel 10b van de wet. Daarom bent u verplicht om na afloop van de vergunning in een Beoordeling achteraf over uw project te rapporteren. Deze beoordeling zal uiterlijk augustus 2026 plaatsvinden. Er zal dan conform artikel 10a2, derde lid van de wet, beoordeeld worden of de doelstellingen van het project werden bereikt.

De ingangsdatum van de vergunning kan niet voor de verzenddatum van de beschikking zijn en zal indien van toepassing aangepast worden. Dit is ook het geval bij een voorgenomen besluit.

7 Concept beschikking voor akkoord CCD

Nummer: 1 Auteur: 5.2 lid1 Onderwerp: Notitie Datum: 14-10-2020 12:20:18

Eens.

Centrale Commissie Dierproeven

Advies aan CCD

Datum 19 oktober 2020
Betreft Advies Secretariaat over Aanvraag projectvergunning Dierproeven AVD20209866

Instelling:	5.1 lid2h
Onderzoeker:	5.1 lid2e
Project:	Towards a novel treatment of pulmonary arterial hypertension; preclinical testing of novel interventions targeting both right ventricular pressure overload and pulmonary vascular remodelling.
Aanvraagnummer:	AVD20209866
Betreft:	Nieuwe aanvraag
Categorieën:	Fundamenteel onderzoek Translationeel of toegepast onderzoek

1 Inzicht in aanvraag en de eventuele knelpunten en risico's

Proces	De DEC heeft 41 werkdagen gedaan over het opstellen van het advies. De volgende vragen zijn nog gesteld aan de aanvrager: In sectie 3.4 van de NTS schrijft u dat dieren'vroegtijdig uit de proef genomen'worden. Dit kan als verhullend taalgebruik worden gezien. Graag dit aanpassen naar 'gedood'. In secties 3.4 en 3.5 van de NTS verwijst u naar appendix 1 en 2. De NTS dient zelfstandig leesbaar te zijn. Graag de verwijzingen naar de appendices verwijderen. U wordt verzocht in sectie 3.5 van de NTS de ongeriefsclassificaties weer te geven voor ratten en muizen afzonderlijk. U geeft bij de beschrijving van de humane eindpunten in de verschillende bijlagen dierproeven aan dat de dieren een humaan eindpunt bereiken als ze bepaalde symptomen "sustained" hebben. Kunt u verhelderen wat u met "sustained" bedoelt? Hoe lang moeten de dieren deze symptomen laten zien voordat het humane eindpunt wordt toegepast?

Naam proef	Diersoort	Stam	Aantal dieren	Herkomst
3.4.4.1 Intervention studies using SuHx model				
	Ratten (Rattus norvegicus)	Sprague Dawley	400	Dieren die voor onderzoek gefokt zijn
3.4.4.2 Intervention studies using PAB model				
	Ratten (Rattus norvegicus)	Wistar	440	Dieren die voor onderzoek gefokt zijn
3.4.4.3 Intervention studies using Chronic hypoxia mouse model				
	Muizen (Mus musculus)	WT, knock-in, knock-out	400	Dieren die voor onderzoek gefokt zijn
3.4.4.4 Intervention studies using Pneumonectomy (PNX)-Su5416 mouse model				
	Muizen (Mus musculus)	WT, knock-in, knock-out	376	Dieren die voor onderzoek gefokt zijn

Huisvesting en verzorging anders dan Bijlage III Richtlijn

3.4.4.1 Intervention studies using SuHx model

Citaat: ... However, as part of the experimental model, the animals will also be housed under low oxygen conditions (10% O2) for 3-4 weeks (the chronic hypoxia period).

3.4.4.3 Intervention studies using Chronic hypoxia mouse model

Citaat: ...However, as part of the experimental model, the animals will also be housed under low oxygen conditions (10% O2) for 2 weeks (the chronic hypoxia period).

Gebruik van mannelijke en vrouwelijke dieren

3.4.4.1 Intervention studies using SuHx model

Ratten (Rattus norvegicus) Er worden zowel mannelijke als vrouwelijke dieren gebruikt.

3.4.4.2 Intervention studies using PAB model

Ratten (Rattus norvegicus) Er worden zowel mannelijke als vrouwelijke dieren gebruikt.

3.4.4.3 Intervention studies using Chronic hypoxia mouse model

Muizen (Mus musculus) Er worden zowel mannelijke als vrouwelijke dieren gebruikt.

3.4.4.4 Intervention studies using Pneumonectomy (PNX)-Su5416 mouse model

Muizen (Mus musculus) Er worden zowel mannelijke als vrouwelijke dieren gebruikt.

Een deel van de dieren in bijlage 3.4.4.1 en 3.4.4.3 zal tijdelijk worden gehuisvest onder lage zuurstofcondities. Dit is nodig voor het opwekken van het ziektebeeld.

Locatie uitvoering experimenten	- Alle proeven vinden plaats in een instelling van een vergunninghouder. - Er zijn geen problemen bekend met de vergunninghouder.

2 DEC advies

DEC-advies	De DEC heeft in 2 vragenrondes vragen gesteld aan de aanvrager. In vraagronde 1 werden vragen gesteld over: de NTS, belangen en onderbouwing van het belang, opzet van de de pilot, go-no go momenten, soorten interventies, percentage dieren dat ernstig ongerief zal ondervinden, benoemen van het target, inconsistenties in de aanvraag, onderbouwing gekozen diermodellen, beschrijving van de te verwachten verbeteringen door de interventies, onderbouwing dieraantallen, voorkomen van ernstig ongerief mogelijk?, waarom geen dosis proeven, ongerief door interventies, onderbouwing gebruik katheters, In vraagronde 2 zijn vragen gesteld over o.a.: verheldering dieraantallen pilotstudies, verheldering tabellen, inschatting aantal dieren dat HEP bereikt, Citaten: C10 (huisvesting): De dieren worden gehuisvest en verzorgd op een wijze die voldoet aan de eisen die zijn opgenomen in bijlage III van de richtlijn. De dieren in Appendix 1 (SuHx model) en 3 (Chronic hypoxia mouse model) worden voor maximaal 4 weken gehuisvest in een hypoxische omgeving met 10% zuurstof, deze hypoxie leidt tot pulmonale hypertensie. C11 (ongerief): (...) Hartfalen is helaas een belangrijk onderdeel van dit onderzoek en is niet te voorkomen, hierbij kan bij een deel van de dieren ernstig ongerief optreden. De meeste dieren zullen tot het eind van het experiment geen hartfalen ontwikkelen, hierbij zal het ongerief niet boven de matig uitkomen. Maximaal 12% van de dieren kan wel hartfalen ontwikkelen met ernstig ongerief (voor maximaal 2 dagen). Bij hartfalen is een afname van lichaamsgewicht te zien, de dieren worden daarom dagelijks gecontroleerd en gewogen. De DEC is van mening dat het ongerieflevel aan de onderkant van ernstig ligt, zoals dat door de EU is gedefinieerd, de dieren hebben waarschijnlijk geen pijn maar missen energie. De DEC is akkoord met deze inschatting van het ongerieflevel. C13 (Humane eindpunten): De criteria voor de humane eindpunten zijn goed gedefinieerd. (...) Door dagelijks te observeren en te wegen kan het eindpunt goed worden bepaald, en kan onnodig lijden worden voorkomen. (...)

Ethische afweging van de DEC:
Citaat: Rechtvaardigen de doeleinden van dit project het voorgestelde gebruik van de dieren? Rechtvaardigt de ontwikkeling van nieuwe kandidaat-behandelingen voor de longziekte Pulmonale arteriële hypertensie (PAH), om hiermee het verloop van de ziekte te verbeteren, het gebruik van maximaal 776 muizen en 840 ratten die daarvan licht tot ernstig ongerief ondervinden?

2. De waarden die voor de proefdieren in het geding zijn: De integriteit van de proefdieren wordt aangetast en de dieren ondervinden licht tot ernstig ongerief. Dat leidt tot veel nadeel voor deze proefdieren. De waarden voor de onderzoekers: voordeel vanwege de kennisontwikkeling over het verloop van PAH. De waarden die voor de patiënten bevorderd worden: Mogelijk veel voordeel wanneer de dierproef bijdraagt aan het ter beschikking komen van betere behandelopties voor hartfalen bij patiënten met pulmonale hypertensie.
De DEC is van mening dat de belangen van de patiënten in dit project zwaarder wegen dan de belangen van de 776 muizen en 840 ratten, die hiervoor als proefdieren gebruikt worden. Voor het verkrijgen van meer kennis over pulmonale hypertensie en hartfalen is onderzoek in diermodellen noodzakelijk. Er zijn op dit moment geen alternatieven voor deze dierproeven beschikbaar waarmee men de doelstellingen kan bereiken.

3. Volgens de DEC rechtvaardigen de doeleinden van dit project het voorgestelde gebruik van dieren. Het directe doel van deze studie is de ontwikkeling van nieuwe kandidaat-behandelingen voor de longziekte Pulmonale arteriële hypertensie (PAH). Het verwachte resultaat, in het kader van het beschikbaar komen van betere behandelingsopties voor patiënten met hartfalen, is afgewogen tegen het licht tot ernstig geschatte ongerief en de aantasting van integriteit, inclusief het doden van de dieren in de proef.
De DEC onderschrijft dat de doelstellingen niet zonder het gebruik van proefdieren kunnen worden behaald en acht het gebruik van 776 muizen en 840 ratten, en de daarmee samenhangende schade aan deze dieren gerechtvaardigd. Bij het uitvoeren van de dierproeven wordt een adequate invulling gegeven aan de vereisten op het gebied van de vervanging, vermindering en verfijning van de dierproeven. Het project is (1) van substantieel belang en (2) van goede kwaliteit.
(1) Het maatschappelijk belang en wetenschappelijk belang zijn beide

	substantieel. De resultaten van dit onderzoek zullen bijdragen aan meer kennis over pulmonale hypertensie en het beschikbaar komen van betere behandelingsopties voor patiënten met hartfalen. (2) De DEC is van mening dat dit project verantwoord is vanuit wetenschappelijk oogpunt en acht het waarschijnlijk dat op basis van de resultaten van de voorgenomen reeks experimenten beschreven in het project, nieuwe en/of aanvullende kennis zal worden verkregen. De onderzoekers beschikken over ruime ervaring en kennis op het gebied van de te gebruiken methoden en werken nauw samen met andere onderzoeksgroepen. Dit in combinatie met de beschikbare faciliteiten en infrastructuur betekent dat de onderzoekers goed gekwalificeerd en geoutilleerd zijn voor het uitvoeren van het in dit project beschreven onderzoek. Samenvattend kan worden gesteld dat het als substantieel te kwalificeren maatschappelijk en wetenschappelijk belang van het onderzoek naar het oordeel van de DEC opweegt tegen het gebruik van maximaal 776 muizen en 840 ratten en het daarbij verwachte lichte tot ernstige ongerief. De DEC heeft extern advies ingewonnen bij - de aanvrager is om aanvullingen gevraagd Het DEC advies is Positief Het uitgebrachte advies is niet gebaseerd op consensus. Het uitgebrachte advies is gebaseerd op meerderheid. Er is een lid dat niet meegaat met het positieve advies, omdat het vertrouwen in de haalbaarheid van dit project voor dit lid gecompromitteerd is: het goed opzetten van dierexperimenten bepaalt mede de haalbaarheid van een projectvoorstel. Het inschatten van het aantal dieren is hierbij een voorwaarde. Gezien het twee vragenrondes duurde voordat het juiste aantal dieren in het protocol opgenomen waren hebben de aanvragers het in de ogen van het lid nagelaten om goed over de experimenten na te denken.

3 Kwaliteit DEC advies

Kwaliteit DEC-advies	
Het DEC advies is helder en navolgbaar. In het DEC advies is op heldere wijze inzicht gegeven in de vragen die aan de aanvrager zijn gesteld. Wij zien dat u veel vragen heeft gesteld, wij begrijpen dat dit nodig was om een helder beeld te krijgen hoeveel dieren welke handelingen en bijbehorend ongerief ondervinden, nodig voor het kunnen maken van een ethische afweging. Bij de beantwoording van de beoordelingsvragen verstrekt u over het algemeen een heldere onderbouwing. De ethische afweging volgt op logische wijze uit de beantwoording van de C vragen. Bij vraag C10 benoemt u alleen dat de dieren tijdelijk onder lage O2-condities gehuisvest worden, maar u geeft hierbij niet de mening van de DEC weer. U heeft de noodzaak van het opleggen van een beoordeling achteraf aan deze vergunning niet benoemd. Uw advies is tot stand gekomen op basis van een meerderheidsstandpunt. Het minderheidsstandpunt is duidelijk weergegeven en u maakt onder de vragen C7 en C8 duidelijk waarom de meerderheid van de DEC een andere afweging maakt. Wij volgen het meerderheidsstandpunt van de DEC, omdat wij, gezien de vele publicaties van deze onderzoeksgroep, en gezien het nu voorliggende projectvoorstel voldoende vertrouwen hebben in de haalbaarheid van de doelstellingen van dit project. De behandeltijd van deze aanvraag bij uw DEC heeft meer dan 20 werkdagen in beslag genomen. Het valt ons op dat er een lange periode tussen indienen van de gewijzigde versie door de aanvrager en de bespreking in de DEC zat.	

4 Inhoudelijke beoordeling

Doelstelling Doelstelling	Citaat: The main objectives of the research included in this project are to: • Evaluate novel (combinations of) therapeutic interventions in mice and rat models for PAH, targeting both pulmonary vascular remodelling and RV pressure overload; • Improve fundamental understanding of PAH and RV failure and their underlying pathologies; • Discover novel therapeutic targets for PAH. The results of the studies will render pivotal information on the usefulness of the therapeutic interventions in subsequent clinical trials and will support the use of relevant PAH disease models. As such, they will contribute to improve the treatment of PAH.
Wetenschappelijk en maatschappelijk belang	Citaat: SCIENTIFIC RELEVANCE Not only will the proposed studies allow us to identify therapeutic interventions for PAH with the highest efficacy likelihood and the lowest

toxicity potential before starting clinical trials, they will also increase our understanding of the processes underlying abnormal pulmonary vascularisation and that controlling the transition of RV adaptation towards right heart failure. Our research group is one of the few who takes a combined approach by studying the pathological effects of PAH in the lungs and the right heart concurrently. This will allow us to investigate the relatively new concept that PAH patients may benefit from a cardiac-specific treatment, which has no direct effect on the pulmonary vasculature, but presents or reverses right heart dysfunction. Moreover, novel therapeutic targets for future clinical research may be identified. Although this research proposal is focused on PAH, right heart failure is also the main cause of death in several other conditions such as left heart failure and critical illness. We have for example shown that not only the RV but also the LV is affected in PAH-patients. We believe that RV remodelling observed in PAH patients shares important pathophysiological mechanisms with the cardiac remodelling observed in left heart failure patients. As such, the findings of this proposal may also advance research in left heart failure. The scientific relevance of our findings is therefore not limited to PAH-induced right heart failure.

SOCIETAL RELEVANCE
PAH remains an incurable debilitating disease, with high mortality rates and poor prognosis for patients. Although the incidence is low (2.2 per million), the current life expectancy is only 3-5 years [4]. Besides the enormous impact of the disease on the quality of life of PAH patients, the disease also carries considerable economic consequences because patients and/or care-givers drop out of the work force and patients require expensive medical treatments, including lung transplantation. New PAH therapies, also targeting alternative pathways are urgently needed. In this project, we address apart from established PAH-targets (e.g. BMPR2 signalling) [10], also relatively new ones, such as RV diastolic stiffness. Up till now, there has been little consideration in the field of the notion that PAH patients could benefit from a cardiac-specific treatment, which has no direct effect on the pulmonary vasculature. With the results of this project, we will be able to select those interventions with promising effectiveness in PAH animal models for further clinical testing. This will bring us hopefully a step closer to the development of an effective PAH treatment. In the future, patients at risk of developing right heart failure (PAH) may benefit from these new treatment options. Although PAH is rare, other types of pulmonary hypertension (PH) are much more prevalent and carry significant morbidity and mortality. Moreover, right heart failure is becoming a great clinical problem as leading cause of death in several diseases such as left heart failure, and the critical ill at the intensive care. Currently, no therapeutic strategies

	are available to improve RV function or prevent right heart failure. Beside knowledge on PH and right heart failure, this project will also provide new insight in cardiac and endothelial physiology, which will be useful in other lung diseases and left heart failure. As such, the societal relevance of our studies extends far beyond PAH alone.
Onderbouwing wetenschappelijk en maatschappelijk belang	Voldoende beschreven.
Wetenschappelijke kwaliteit Kwaliteit aanvrager/ onderzoeksgroep en onderzoek	Citaat uit DEC advies C7: De DEC heeft veel vragen gesteld bij deze aanvraag, zie de vragenrondes bovenaan dit document. De DEC is van mening dat naast aanwezige apparatuur, kennis, personeel en financiering ook het goed opzetten van experimenten (en daarbij het inschatten van het aantal dieren) de haalbaarheid van een projectvoorstel bepaalt en ziet graag dat de onderzoekers bij de uitvoering goed voor ogen houden wat ze precies willen doen en dat goed inplannen om fouten te voorkomen. Alle technische voorzieningen die benodigd zijn voor uitvoering van het project zijn voorhanden, evenals voldoende deskundigheid en financiering om het project succesvol uit te voeren. Ervaring binnen het onderzoeksinstituut met vergelijkbaar onderzoek waarborgt het technisch succesvol uitvoeren van de dierexperimenten. Na navraag is de DEC ervan overtuigd dat de projectdoelstelling met de gekozen strategie/aanpak binnen de gevraagde termijn is te realiseren. Eén van de DEC leden is van mening dat "het vertrouwen in de haalbaarheid van dit project voor dit lid gecompromitteerd is: het goed opzetten van dierexperimenten bepaalt mede de haalbaarheid van een projectvoorstel. Het inschatten van het aantal dieren is hierbij een voorwaarde. Gezien het twee vragenrondes duurde voordat het juiste aantal dieren in het protocol opgenomen waren hebben de aanvragers het in de ogen van het lid nagelaten om goed over de experimenten na te denken." Het Secretariaat volgt het meerderheidsstandpunt van de DEC, en heeft, gezien de vele publicaties van deze onderzoeksgroep, en gezien het nu voorliggende projectvoorstel voldoende vertrouwen in de haalbaarheid van de doelstellingen van dit project.

3V's

Vervanging	
	3.4.4.1 Intervention studies using SuHx model: Citaat: All proposed interventions that will be tested throughout this project will be assessed first in other, non-animal, models, such as cell culture experiments. Only if these experiments yield sufficiently promising results, in vivo tests will be undertaken. In vivo experiments are a necessary step in development of interventions that may one day be used in the clinic, since currently no other methods allow the evaluation of the effect of an intervention on the organism as a whole. Cell culture, organoids, lab-on-a-chip or computer models are not (yet) sophisticated enough to assess the interaction between the PAH pathology and host environmental factors such as the oxygen supply, the immune system and metabolism.
	3.4.4.2 Intervention studies using PAB model: Zie bijlage 3.4.4.1.
	3.4.4.3 Intervention studies using Chronic hypoxia mouse model: Citaat: In vivo experiments are a necessary step in development of interventions that may one day be used in the clinic, since currently no other methods allow the evaluation of the effect of an intervention on the organism as a whole. Cell culture, organoids, lab-on-a-chip or computer models are not (yet) sophisticated enough to assess the interaction between the PAH pathology and host environmental factors such as the oxygen supply, the immune system and metabolism.
	3.4.4.4 Intervention studies using Pneumonectomy (PNX)-Su5416 mouse model: Zie bijlage 3.4.4.3.

Verminderen	
	3.4.4.1 Intervention studies using SuHx model: Citaat: The proposed number of evaluable animals per study arm (n=12 in model/intervention groups, n=6 in control group) is calculated as described above, and is in line with generally accepted protocols in scientific literature and our previous studies with the SuHx model at our Department. Further reduction of the number of animals per group would decrease statistical power, and might thus disqualify the experiment.
	3.4.4.2 Intervention studies using PAB model: Citaat: The proposed number of evaluable animals per study arm (n=12 in model/intervention groups, n=6 in control group) is calculated as described above, and is in line with generally accepted protocols in scientific literature and our previous studies with the PAB model. Further reduction of the number of animals per group would decrease statistical power, and might thus disqualify the experiment.
	3.4.4.3 Intervention studies using Chronic hypoxia mouse model: Citaat: The proposed number of evaluable animals per study arm (n=10) is calculated as described above, and is in line with generally accepted protocols in scientific literature. Further reduction of the number of animals per group would decrease statistical power, and might thus disqualify the experiment.
	3.4.4.4 Intervention studies using Pneumonectomy (PNX)-Su5416 mouse model: Citaat: The proposed number of evaluable animals per study arm (n=12 in model/intervention groups, n=6 in control group) is calculated as described above, and is in line with generally accepted protocols in scientific literature. Further reduction of the number of animals per group would decrease statistical power, and might thus disqualify the experiment.
Verfijnen	
	3.4.4.1 Intervention studies using SuHx model: Citaat: State-of-the-art methods and equipment for assessing pulmonary vascular remodelling and RV overload, in particular advanced imaging techniques, will be used to refine protocols and minimize discomfort to the animals especially in support of the application of humane endpoints. International guidelines will be applied to ensure that best practice is followed. Citaat: The procedures conducted under this protocol will inevitably cause discomfort for the animals in the studies. In order to minimize suffering, all studies will adhere to the national and internationally accepted rules for handling of lab animals [1], [2]. Under these rules, the animals will be

	humanely killed when a humane endpoint (see below) is reached. Within the institute, standard operating procedures (SOP) for animal handling are in place. These also include dedicated anaesthesia and analgesia (A&A) SOPs that will accompany invasive procedures to minimize pain and discomfort. To minimize animal suffering, pain and fear the following measures will be taken. The animals will be allowed to acclimatize to their new environment for two weeks upon arrival at the animal facility. Animals are housed in groups (socially housed) at all times (also during hypoxia. Except during surgery, animals are returned to the group directly after surgery) and environmental enrichment strategies are applied in the cages to improve animal welfare. In case the therapeutic interventions require surgical procedures (e.g. cannula/mini-pump implantation), this will be done under general anaesthesia in combination with pain treatment. During haemodynamic measurements, the animals will be kept under general anaesthesia in a temperature-controlled environment. During imaging procedures, animals will be kept under general anaesthesia in a temperature-controlled environment. (...) Most rats will not suffer from heart failure until the end of the experiment, and their discomfort will not exceed moderate. Maximally 15% (based on previous experiments) of the rats can develop heart failure with severe discomfort. It is very difficult to see if a rat is having heart failure (which can occur in the weeks after hypoxia). A decrease in bodyweight is the first sign of heart failure which is then already occurring for one day. It is normal to have a decreasing bodyweight of the rat during the day, because of sleeping, less drinking and eating during the day (daily fluctuations). To be sure it is heart failure (and not daily fluctuations of bodyweight), a bodyweight decrease of 10% is being established. Also cyanosis, dyspnea, lethargy and poor grooming can be observed. This is usually on the second day. Then an HEP will be applied. Severe discomfort is unfortunately unavoidable. Patients usually present at the stage of heart failure. Right heart failure is an important outcome measure of our research. Heart failure is necessary to compare the rats with patients.
	3.4.4.2 Intervention studies using PAB model: Citaat: State-of-the-art methods and equipment for assessing pulmonary vascular remodelling and RV overload, in particular advanced imaging techniques, will be used to refine protocols and minimize discomfort to the animals especially in support of the application of humane endpoints. International guidelines will be applied to ensure that best practice is followed. Citaat: The procedures conducted under this protocol will inevitably cause

	discomfort for the animals in the studies. In order to minimize suffering, all studies will adhere to the national and internationally accepted rules for handling of lab animals [2, 3]. Under these rules, the animals will be humanely killed when a humane endpoint (see below) is reached. Within the institute, standard operating procedures (SOP) for animal handling are in place. These also include dedicated anaesthesia and analgesia (A&A) SOPs that will accompany invasive procedures to minimize pain and discomfort. To minimize animal suffering, pain and fear the following measures will be taken. The animals will be allowed to acclimatize to their new environment for two weeks upon arrival at the animal facility. Animals are housed in groups (socially housed) at all times (except during surgery, animals are returned to the group directly after surgery) and environmental enrichment strategies are applied in the cages to improve animal welfare. The PAB surgical model is performed under general anaesthesia and analgesia (pre- and post-surgery). During haemodynamic measurements, animals will be kept under anaesthesia in a temperature-controlled environment. During imaging procedures, animals will be kept under anaesthesia in a temperature-controlled environment. (...) Right ventricular (RV) failure is the predominant cause of death in patients with pulmonary hypertension. Maximally 32% of the rats can develop heart failure with severe discomfort. It is due to the diameter of the band around the pulmonary artery. A decrease in bodyweight is the first sign of heart failure which is then already occurring for one day. It is normal to have a decreasing bodyweight of the rat during the day, because of sleeping, less drinking and eating during the day (daily fluctuations). To be sure it is heart failure (and not daily fluctuations of bodyweight), a bodyweight decrease of 10% is being established. Also cyanosis, dyspnea, lethargy and poor grooming can be observed. This is usually at the second day and an HEP will be applied. Based on experience with this model in Denmark: Seven weeks survival rate was 80% for rats subjected to severe banding and close to 100% in rats subjected to mild or moderate banding or sham surgery[1]. Severe discomfort is unfortunately unavoidable. Patients usually present at the stage of heart failure. Right heart failure is an important outcome measure of our research. Heart failure is necessary to compare the rats with patients.

	3.4.4.3 Intervention studies using Chronic hypoxia mouse model: Citaat: State-of-the-art methods and equipment for assessing pulmonary vascular remodelling and RV overload, in particular advanced imaging techniques, will be used to refine protocols and minimize discomfort to the animals especially in support of the application of humane endpoints. International guidelines will be applied to ensure that best practice is followed. Explain what measures will be taken to minimise 1) animal suffering, pain or fear and 2) adverse effects on the environment. The procedures conducted under this protocol will inevitably cause discomfort for the animals in the studies. In order to minimize suffering, all studies will adhere to the national and internationally accepted rules for handling of lab animals [1], [2]. Under these rules, the animals will be humanely killed when a humane endpoint (see below) is reached. Within the institute, standard operating procedures (SOP) for animal handling are in place. These also include dedicated anaesthesia and analgesia (A&A) SOPs that will accompany invasive procedures to minimize pain and discomfort. To minimize animal suffering, pain and fear the following measures will be taken. The animals will be allowed to acclimatize to their new environment for two weeks upon arrival at the animal facility. Animals are housed in groups (socially housed) at all times (also during hypoxia) and environmental enrichment strategies are applied in the cages to improve animal welfare. During haemodynamic measurements, animals will be kept under anaesthesia in a temperature-controlled environment. Measurements of vascular leakage will be performed under continuous anaesthesia. (...) In these experiments in mice with PAH there is no heart failure expected, and the discomfort will not transcend mild.

	3.4.4.4 Intervention studies using Pneumonectomy (PNX)-Su5416 mouse model: Citaat: State-of-the-art methods and equipment for assessing pulmonary vascular remodelling and RV overload, in particular advanced imaging techniques, will be used to refine protocols and minimize discomfort to the animals especially in support of the application of humane endpoints. International guidelines will be applied to ensure that best practice is followed. Explain what measures will be taken to minimise 1) animal suffering, pain or fear and 2) adverse effects on the environment. The procedures conducted under this protocol will inevitably cause discomfort for the animals in the studies. In order to minimize suffering, all studies will adhere to the national and internationally accepted rules for handling of lab animals [1], [2]. Under these rules, the animals will be humanely killed when a humane endpoint (see below) is reached. Within the institute, standard operating procedures (SOP) for animal handling are in place. These also include dedicated anaesthesia and analgesia (A&A) SOPs that will accompany invasive procedures to minimize pain and discomfort. In case the pulmonary hypertension is too severe, we may choose to omit the SU5416 injection. To minimize animal suffering, pain and fear the following measures will be taken. The animals will be allowed to acclimatize to their new environment for two weeks upon arrival at the animal facility. Animals are housed in groups (socially housed) at all times (except during surgery, animals are returned to the group directly after surgery) and environmental enrichment strategies are applied in the cages to improve animal welfare. The PNX surgical model is performed under general anaesthesia and analgesia (pre- and post-surgery). During haemodynamic measurements, animals will be kept under anaesthesia in a temperature-controlled environment. (...) In these experiments in mice with PAH there is no heart failure expected, and the discomfort will not transcend moderate.
3.4.4.1 Intervention studies using SuHx model: Voldoende beschreven.	
3.4.4.2 Intervention studies using PAB model: Voldoende beschreven.	
3.4.4.3 Intervention studies using Chronic hypoxia mouse model: Voldoende beschreven.	
3.4.4.4 Intervention studies using Pneumonectomy (PNX)-Su5416 mouse model: Voldoende beschreven.	

Hergebruik	Er is geen sprake van hergebruik van dieren.

Naam proef	Worden de dieren gedood?	Doden volgens richtlijn?
3.4.4.1 Intervention studies using SuHx model	Ja	volgens de richtlijn.
3.4.4.2 Intervention studies using PAB model	Ja	volgens de richtlijn.
3.4.4.3 Intervention studies using Chronic hypoxia mouse model	Ja	volgens de richtlijn.
3.4.4.4 Intervention studies using Pneumonectomy (PNX)-Su5416 mouse model	Ja	volgens de richtlijn.

Naam proef		
3.4.4.1 Intervention studies using SuHx model	HEP: <15%	Citaat: The most important humane endpoints applicable to all studies are: • Weight loss >20% of maximum body weight in adult animals, measured from the start of the treatment • Weight loss >10% of body weight during 24h, in combination with: • Sustained abnormal breathing, dyspnoea (symptom PAH/right heart failure) • Sustained lethargy (symptom PAH/right heart failure) • Sustained abnormal behaviour • Complications of interventions • Other procedure-specific endpoints
Ratten (Rattus norvegicus)	Ongerief: 15,0% Ernstig 85,0% Matig	

3.4.4.2 Intervention studies using PAB model	HEP: <32%	The most important humane endpoints applicable to all studies are: • Permanent weight loss >20% of initial body weight in adult animals, measured from the start of the treatment • Weight loss >10% of body weight during 24h, in combination with: o Sustained abnormal breathing, dyspnea (symptom PAH/right heart failure) o Sustained lethargy (symptom PAH/right heart failure) • Sustained abnormal behavior • Complications of interventions • Other procedure-specific endpoints
Ratten (Rattus norvegicus)	Ongerief: 32,0% Ernstig 68,0% Matig	
3.4.4.3 Intervention studies using Chronic hypoxia mouse model	HEP: <10%	Citaat: The most important humane endpoints applicable to all studies are: • Weight loss >20% of maximum body weight in adult animals, measured from the start of the treatment • Weight loss >15% of body weight during 24h, in combination with: o Sustained abnormal breathing, dyspnoea (symptom PAH/right heart failure) o Sustained lethargy (symptom PAH/right heart failure) • Sustained abnormal behaviour • Complications of interventions (<1%): No interventions are planned.
Muizen (Mus musculus)	Ongerief: 100,0% Licht	

3.4.4.4 Intervention studies using Pneumonectomy (PNX)-Su5416 mouse model	HEP: <10%	Citaat: The most important humane endpoints applicable to all studies are: • Weight loss >20% of maximum body weight in adult animals, measured from the start of the treatment • Weight loss >15% of body weight during 24h, in combination with: • Sustained abnormal breathing, dyspnoea (symptom PAH/right heart failure) • Sustained lethargy (symptom PAH/right heart failure) • Sustained abnormal behaviour • Complications of interventions • • Other procedure-specific endpoints
Muizen (Mus musculus)	Ongerief: 59,0% Matig 41,0% Licht	

5 Samenvatting

5.2 lid1 [redacted]

Er worden enkele dieren extra aangevraagd voor het geval van uitval van dieren. Dit is voldoende onderbouwd.

Een deel van de dieren, in bijlagen 3.4.4.1 en 3.4.4.3 wordt gedurende enkele weken gehuisvest onder condities met lage zuurstofspanning. Dit is nodig om het ziektebeeld te induceren, en heeft dus een wetenschappelijke noodzaak.

5.2 lid1 [redacted]

Het ongerief in dit onderzoek is voor 12% van de ratten op ernstig ingeschat. De DEC zegt over deze inschatting: "De DEC is van mening dat het ongerieflevel aan de onderkant van ernstig ligt, zoals dat door de EU is gedefinieerd, de dieren hebben waarschijnlijk geen pijn maar missen energie. De DEC is akkoord met deze inschatting van het ongerieflevel." Het

5.2 lid1 [redacted]

Het DEC advies is gebaseerd op een meerderheidsstandpunt. (citaat): "Er is een lid dat niet meegaat met het positieve advies, omdat het vertrouwen in de haalbaarheid van dit project voor dit lid gecompromitteerd is: het goed opzetten van dierexperimenten bepaalt mede de haalbaarheid van een projectvoorstel. Het inschatten van het aantal dieren is hierbij een voorwaarde. Gezien het twee vragenrondes duurde voordat het juiste aantal

dieren in het protocol opgenomen waren hebben de aanvragers het in de ogen van het lid nagelaten om goed over de experimenten na te denken." 5.2 lid1

De aanvrager is nog gevraagd bij de beschrijving van de humane eindpunten te verhelderen wat met "sustained" bedoeld wordt. Het is onduidelijk hoe lang de dieren deze symptomen moeten laten zien voordat de dieren het humane eindpunt hebben bereikt.

6 Voorstel besluit incl. voorstel geldigheidsduur van de vergunning

5.2 lid1

Beoordeling achteraf
In dit project worden dierproeven toegepast die vallen in de categorie ernstig volgens artikel 10b van de wet. Daarom bent u verplicht om na afloop van de vergunning in een Beoordeling achteraf over uw project te rapporteren. Deze beoordeling zal uiterlijk augustus 2026 plaatsvinden. Er zal dan conform artikel 10a2, derde lid van de wet, beoordeeld worden of de doelstellingen van het project werden bereikt.

De ingangsdatum van de vergunning kan niet voor de verzenddatum van de beschikking zijn en zal indien van toepassing aangepast worden. Dit is ook het geval bij een voorgenomen besluit.

7 Concept beschikking voor akkoord CCD

Van: info@zbo-ccd.nl
Verzonden: maandag 19 oktober 2020 17:45
Aan: 5.1 lid2e
CC: 5.1 lid2e
Onderwerp: Aanhouden AVD5.1 lid2h20209866

Categorieën: Dossier: 5.1 lid2e

Geachte 5.1 lid2e ,

Op 08-05-2020 hebben wij uw aanvraag voor een projectvergunning dierproeven ontvangen. Het gaat om uw project "Towards a novel treatment of pulmonary arterial hypertension; preclinical testing of novel interventions targeting both right ventricular pressure overload and pulmonary vascular remodelling." met aanvraagnummer AVD5.1 lid2h20209866. In uw aanvraag zitten voor ons nog enkele onduidelijkheden. In dit bericht leest u wat wij nog nodig hebben en wanneer u een beslissing kunt verwachten.

Welke informatie nog nodig
Wij hebben de volgende informatie van u nodig om uw aanvraag verder te kunnen beoordelen:

Niet technische samenvatting

Onduidelijkheden
In sectie 3.4 van de NTS schrijft u dat dieren'vroegtijdig uit de proef genomen'worden. Dit kan als verhullend taalgebruik worden gezien. Graag dit aanpassen naar 'gedood'.

In secties 3.4 en 3.5 van de NTS verwijst u naar appendix 1 en 2. De NTS dient zelfstandig leesbaar te zijn. Graag de verwijzingen naar de appendices verwijderen.

U wordt verzocht in sectie 3.5 van de NTS de ongeriefsclassificaties weer te geven voor ratten en muizen afzonderlijk.

U geeft bij de beschrijving van de humane eindpunten in de verschillende bijlagen dierproeven aan dat de dieren een humaan eindpunt bereiken als ze bepaalde symptomen "sustained" hebben. Kunt u verhelderen wat u met "sustained" bedoelt? Hoe lang moeten de dieren deze symptomen laten zien voordat het humane eindpunt wordt toegepast?

Zonder deze aanvullende informatie kan de beslissing nadelig voor u uitvallen omdat de gegevens onvolledig of onduidelijk zijn.

Opsturen binnen veertien dagen
Stuur de ontbrekende informatie binnen veertien dagen na de datum van dit bericht op. U kunt dit aanleveren via NetFTP.

Uw aanvraag wordt besproken

Wanneer een beslissing
De behandeling van uw aanvraag wordt opgeschort tot het moment dat wij de aanvullende informatie hebben

ontvangen. Als u goedkeuring krijgt op uw aanvraag, kunt u daarna beginnen met het project.

Mocht u vragen hebben, dan kunt u uiteraard contact met ons opnemen.

Met vriendelijke groet,
Namens de Centrale Commissie Dierproeven

www.centralecommissiedierproeven.nl

.....
Postbus 93118 | 2509 AC | Den Haag
.....
T: 0900 2800028
E: info@zbo-ccd.nl

Vragen NTS project 9866

1. In sectie 3.4 van de NTS schrijft u dat dieren 'vroegtijdig uit de proef genomen' worden. Dit kan als verhullend taalgebruik worden gezien. Graag dit aanpassen naar 'gedood'.
   Dit is aangepast, 'vroegtijdig uit de proef genomen' is veranderd in 'vroegtijdig gedood'.

2. In secties 3.4 en 3.5 van de NTS verwijst u naar appendix 1 en 2. De NTS dient zelfstandig leesbaar te zijn. Graag de verwijzingen naar de appendices verwijderen.
   De verwijzingen naar de appendices in sectie 3.4 en 3.5 zijn verwijderd.

3. U wordt verzocht in sectie 3.5 van de NTS de ongeriefsclassificaties weer te geven voor ratten en muizen afzonderlijk.
   Dit is aangepast en wordt nu als volgt weergegeven in sectie 3.5:
   Licht ongerief: maximaal 71% van de muizen, maximaal 0% van de ratten
   Matig ongerief: maximaal 29% van de muizen, maximaal 76% van de ratten
   Ernstig ongerief: maximaal 0% van de muizen, maximaal 24% van de ratten (max 2 dagen).

4. U geeft bij de beschrijving van de humane eindpunten in de verschillende bijlagen dierproeven aan dat de dieren een humaan eindpunt bereiken als ze bepaalde symptomen "sustained" hebben. Kunt u verhelderen wat u met "sustained" bedoelt? Hoe lang moeten de dieren deze symptomen laten zien voordat het humane eindpunt wordt toegepast?
   Met "sustained" bedoelen we dat de dieren constante ademhalingsklachten, lethargie of afwijkend gedrag vertonen. Wanneer deze klachten gedurende een periode van 48 uur ononderbroken aanhouden, zal het humane eindpunt worden toegepast.

Format
Niet-technische samenvatting

- Dit format gebruikt u om uw niet-technische samenvatting te schrijven
- Meer informatie over de niet-technische samenvatting vindt u op de website www.centralecommissiedierproeven.nl.
- Of neem telefonisch contact op. (0900-2800028).

1 Algemene gegevens

1.1	Titel van het project	Verbeterde behandeling van longziekte: het testen van kandidaat-behandelmethoden in diermodellen
1.2	Looptijd van het project	5 jaar
1.3	Trefwoorden (maximaal 5)	Longziekte, rechter hartfalen, behandeling, muismodellen, ratmodellen

2 Categorie van het project

2.1 In welke categorie valt het project.

U kunt meerdere mogelijkheden kiezen.

- ☒ Fundamenteel onderzoek
- ☒ Translationeel of toegepast onderzoek
- ☐ Wettelijk vereist onderzoek of routinematige productie
- ☐ Onderzoek ter bescherming van het milieu in het belang van de gezondheid
- ☐ Onderzoek gericht op het behoud van de diersoort
- ☐ Hoger onderwijs of opleiding
- ☐ Forensisch onderzoek
- ☐ Instandhouding van kolonies van genetisch gemodificeerde dieren, niet gebruikt in andere dierproeven

3 Projectbeschrijving

3.1	Beschrijf de doelstellingen van het project (bv de wetenschappelijke vraagstelling of het wetenschappelijk en/of maatschappelijke belang)	Pulmonale arteriële hypertensie (PAH) is een zeldzame maar dodelijke longziekte. Bij patiënten met PAH leidt een belemmerde bloeddoorstroming door het longvaatbed tot een verhoogde bloeddruk in de longen en een overbelast hart. De overbelasting van het hart leidt tot hartfalen en uiteindelijk de dood. Omdat genezing van deze ziekte meestal niet mogelijk is, hebben patiënten een sterk verminderde kwaliteit van leven en een beperkte levensverwachting. De levensverwachting van patiënten met deze ziekte is slechts 3 tot 5 jaar (bij een relatief jonge patiëntengroep van 50 jaar of jonger). De huidige behandeling bestaat uit een combinatie van bloedvat verwijdende medicijnen die selectief op de longbloedvaten werken. Deze huidige behandeling is echter niet voldoende om het hartfalen te stoppen of te voorkomen. De enige manier om het hart te ontlasten en te

		laten herstellen in deze ziekte, is een longtransplantatie. Door schaarste in het aantal beschikbare organen en voortschrijding van de ziekte komt deze optie te laat voor de meeste patiënten. Binnen dit project zullen we werken aan de ontwikkeling van nieuwe kandidaat-behandelingen voor deze longziekte. Deze kandidaat-behandelingen en bijbehorende methodes zullen worden getest en verfijnd in muizen of ratten, zodat ze vervolgens kunnen worden onderzocht in patiënten en bij positief resultaat op de langere termijn kunnen worden ontwikkeld tot effectieve behandelingsmethode voor deze longziekte.
3.2	Welke opbrengsten worden van dit project verwacht en hoe dragen deze bij aan het wetenschappelijke en/of maatschappelijke belang?	De opbrengsten uit dit project zijn enerzijds in diermodellen geteste kandidaat-behandelingen voor PAH en anderzijds nieuwe kennis en inzichten rond de ziekteprocessen onderliggend aan dit ziektebeeld. Wetenschappelijk belang: de door het uitvoeren van interventiestudies in muizen en ratten zullen de onderzoekers een beter inzicht krijgen in de factoren die het succes van een kandidaat-behandeling bepalen. Dit is belangrijk omdat in het wetenschappelijk onderzoek de rol van een aantal van deze factoren (bijv. bepaalde genen, eiwitten) en de mogelijke interacties tussen factoren tot nu toe nog niet voldoende aandacht heeft gekregen. Daarnaast zal de nieuw verkregen kennis bijdragen aan nieuwe inzichten in de ziekteprocessen onderliggend aan de ziekte en ander gerelateerde ziektebeelden, bijvoorbeeld betreffende de rol van de linkerhartkamer. Maatschappelijk belang: de kandidaat-behandelingen kunnen op basis van de uitkomsten uit deze studies verder ontwikkeld worden op de langere termijn tot een behandeling die veilig en effectief is voor gebruik in patiënten. Dit onderzoek is van groot maatschappelijk belang omdat deze ziekte een zeer ernstige conditie is waarvoor tot op heden geen goede behandeling beschikbaar is.
3.3	Welke diersoorten en geschatte aantallen zullen worden gebruikt?	In dit project zullen experimenten worden uitgevoerd op muizen en ratten. Wij verwachten voor dit onderzoek maximaal 776 muizen en 840 ratten nodig te hebben in 5 jaar. De totale som van het aantal proefdieren is 1616.
3.4	Wat zijn bij dit project de verwachte negatieve gevolgen voor het welzijn van de proefdieren?	Om PAH in een diermodel te kunnen nabootsen ontstaan bij de dieren ook negatieve gevolgen door de symptomen van deze ziekte. Dit is helaas een belangrijk onderdeel van dit onderzoek en niet te voorkomen. Bij hartfalen is een afname van lichaamsgewicht te zien, de dieren worden dagelijks gecontroleerd en gewogen en indien nodig worden ze voortijdig gedood (een humaan eindpunt toegepast). De meeste dieren zullen tot het eind van het experiment geen hartfalen ontwikkelen, hierbij zal het ongerief niet boven de matig uitkomen. Maximaal 12% van de dieren (alleen de ratten) kan wel hartfalen ontwikkelen met ernstig ongerief. Ernstig ongerief is helaas niet te vermijden. Patiënten komen vaak ook binnen in de kliniek met tekenen van rechter hartfalen. Daarom is rechter hartfalen ook onderdeel van onze uitkomstmaat. Dit is nodig om deze dieren te kunnen vergelijken met onze patiënten, zodat de uitkomsten van onze experimenten representatief zijn. Daarnaast zullen negatieve gevolgen voor de proefdieren voortkomen uit de behandelingen en het monitoren van de effecten.
3.5	Hoe worden de dierproeven in het project ingedeeld naar de verwachte ernst?	Licht ongerief: maximaal 71% van de muizen, maximaal 0% van de ratten Matig ongerief: maximaal 29% van de muizen, maximaal 76% van de ratten Ernstig ongerief: maximaal 0% van de muizen, maximaal 24% van de ratten (max 2 dagen).

		Hartfalen is helaas een belangrijk onderdeel van dit onderzoek en is niet te voorkomen, hierbij kan bij een deel van de dieren ernstig ongerief optreden. Het is erg moeilijk om hartfalen te kunnen zien bij knaagdieren. De lichaamsgewichten van deze dieren schommelen enkele grammen gedurende de dag. Overdag slapen deze dieren en zullen ze weinig eten en drinken, maar wel ontlasting produceren. De eerste dag van hartfalen, is moeilijk te herkennen, omdat dit zowel een dagelijkse schommeling van het lichaamsgewicht kan zijn, als het eerste signaal van hartfalen. Bij een tweede dag van afvallen kan vrijwel altijd met zekerheid gezegd worden dat een dier hartfalen heeft.
3.6	Wat is de bestemming van de dieren na afloop?	Alle dieren worden na afloop van de experimenten gedood, waarna weefsel wordt gebruikt voor verder onderzoek.

4 Drie V's

4.1	**Vervanging** Geef aan waarom het gebruik van dieren nodig is voor de beschreven doelstelling en waarom proefdiervrije alternatieven niet gebruikt kunnen worden.	Alle behandelingen die in dit project getest worden in dieren, zijn eerst uitgebreid getest in relevante andere systemen, zoals gekweekte cellen of op eerder verzameld patiënt weefselmateriaal. De experimenten in proefdieren zijn erop gericht informatie te verkrijgen over complexe processen die met alternatieve methodes niet getest kunnen worden. Het gaat hierbij bijvoorbeeld over de verdeling van een (experimenteel) geneesmiddel in het hele lichaam, interactie met het immuunsysteem, en de interactie tussen hartfunctie en longbloedvat-afwijkingen. Dit kan vooralsnog niet nagebootst worden in celkweek of andere modelsystemen. Als zodanig heeft onderzoek in proefdieren geeft belangrijke informatie over veiligheid en effectiviteit van een kandidaat-behandeling, die van groot belang is, voor de toepassing van de nieuwe behandeling in patiënten.
4.2	**Vermindering** Leg uit hoe kan worden verzekerd dat een zo gering mogelijk aantal dieren wordt gebruikt.	Ter vermindering van het aantal dieren, zullen de volgende overwegingen gebruikt worden bij ieder experiment: - De groepsgrootte benodigd voor het verkrijgen van goed onderbouwde resultaten zal door middel van statistische methodes bepaald worden. Hierbij zal gestreefd worden naar het minimaliseren van het aantal gebruikte controle dieren. - Het onderzoek wordt uitgevoerd met behulp van standaard procedures en metingen om variatie tussen individuele experimenten te voorkomen. - Er wordt gebruik gemaakt van niet-invasieve beeldvorming (echo van het hart) om long- en hartfunctie gedurende een langere periode in een dier te kunnen volgen. Voor het project als geheel geldt dat de studies worden uitgevoerd in een gefaseerde opzet waardoor gebruik van het optimale aantal dieren wordt gewaarborgd.
4.3	**Verfijning** Verklaar de keuze voor de diersoort(en). Verklaar waarom de gekozen diermodel(len) de meest verfijnde zijn, gelet op de	De experimenten in dit project worden uitgevoerd in ratten of muizen. Muizen en ratten vertonen qua orgaanstructuur en genetische opbouw grote overeenkomsten met de mens. Daarnaast zijn in muizen veel genetische technieken mogelijk. Gedurende de afgelopen decennia is veel ervaring opgedaan met het onderzoek in muizen, waardoor veel vergelijkingsmateriaal, verschillende muizenstammen en modellen

doelstellingen van het project.	beschikbaar zijn. Ook zijn muizen goed te houden en te hanteren, wat het onderzoek vergemakkelijkt. Voor ratten gelden grotendeels dezelfde argumenten voor gebruik, behalve genetische modellen. Daarnaast zijn ratten veel groter dan muizen, waardoor bepaalde procedures die in muizen niet uitgevoerd kunnen worden, in ratten wel getest kunnen worden. We hebben gekozen voor vier diermodellen welke wetenschappelijk erkent zijn als op dit moment het beste diermodel voor pulmonale arteriële hypertensie. We hebben veel ervaring met de toegepaste onderzoekstechnieken, hiermee wordt onnodig lijden bij de dieren voorkomen.
Vermeld welke algemene maatregelen genomen worden om de negatieve (schadelijke) gevolgen voor het welzijn van de proefdieren zo beperkt mogelijk te houden.	De proefdieren zullen gezamenlijk worden gehuisvest in een omgeving met kooiverrijking. Bij de operaties en andere invasieve behandelingen worden algehele narcose en effectieve pijnstilling toegepast. In geval van ernstig onverwacht ongerief worden de humane eindpunten toegepast. Al het onderzoek in dit project zal door gekwalificeerd personeel worden uitgevoerd in een gespecialiseerde proefdierfaciliteit. Daarnaast zal ervaren personeel zorgdragen voor de controle van het welzijn van de dieren. Er zijn protocollen aanwezig waarin procedures voor het hanteren van dieren, alsmede richtlijnen voor narcose en pijnstilling, zijn vastgelegd.

5 In te vullen door de CCD

Publicatie datum	
Beoordeling achteraf	
Andere opmerkingen	

Centrale Commissie Dierproeven

Advies aan CCD

Datum 05 november 2020
Betreft Advies Secretariaat over Aanvraag projectvergunning Dierproeven AVD20209866

Instelling:	5.1 lid2h
Onderzoeker:	5.1 lid2e
Project:	Towards a novel treatment of pulmonary arterial hypertension; preclinical testing of novel interventions targeting both right ventricular pressure overload and pulmonary vascular remodelling.
Aanvraagnummer:	AVD20209866
Betreft:	Nieuwe aanvraag
Categorieën:	Fundamenteel onderzoek Translationeel of toegepast onderzoek

1 Inzicht in aanvraag en de eventuele knelpunten en risico's

Proces	De DEC heeft 41 werkdagen gedaan over het opstellen van het advies. De volgende vragen zijn nog gesteld aan de aanvrager: - In sectie 3.4 van de NTS schrijft u dat dieren'vroegtijdig uit de proef genomen'worden. Dit kan als verhullend taalgebruik worden gezien. Graag dit aanpassen naar 'gedood'. - In secties 3.4 en 3.5 van de NTS verwijst u naar appendix 1 en 2. De NTS dient zelfstandig leesbaar te zijn. Graag de verwijzingen naar de appendices verwijderen. - U wordt verzocht in sectie 3.5 van de NTS de ongeriefsclassificaties weer te geven voor ratten en muizen afzonderlijk. - U geeft bij de beschrijving van de humane eindpunten in de verschillende bijlagen dierproeven aan dat de dieren een humaan eindpunt bereiken als ze bepaalde symptomen "sustained" hebben. Kunt u verhelderen wat u met "sustained" bedoelt? Hoe lang moeten de dieren deze symptomen laten zien voordat het humane eindpunt wordt toegepast? De aanvrager heeft de NTS aangepast, deze aangepaste versie is de versie die u voor zich heeft.

Naam proef	Diersoort	Stam	Aantal dieren	Herkomst
3.4.4.1 Intervention studies using SuHx model				
	Ratten (Rattus norvegicus)	Sprague Dawley	400	Dieren die voor onderzoek gefokt zijn
3.4.4.2 Intervention studies using PAB model				
	Ratten (Rattus norvegicus)	Wistar	440	Dieren die voor onderzoek gefokt zijn
3.4.4.3 Intervention studies using Chronic hypoxia mouse model				
	Muizen (Mus musculus)	WT, knock-in, knock-out	400	Dieren die voor onderzoek gefokt zijn
3.4.4.4 Intervention studies using Pneumonectomy (PNX)-Su5416 mouse model				
	Muizen (Mus musculus)	WT, knock-in, knock-out	376	Dieren die voor onderzoek gefokt zijn

Huisvesting en verzorging anders dan Bijlage III Richtlijn

3.4.4.1 Intervention studies using SuHx model

Citaat: ... However, as part of the experimental model, the animals will also be housed under low oxygen conditions (10% O2) for 3-4 weeks (the chronic hypoxia period).

3.4.4.3 Intervention studies using Chronic hypoxia mouse model

Citaat: ...However, as part of the experimental model, the animals will also be housed under low oxygen conditions (10% O2) for 2 weeks (the chronic hypoxia period).

Gebruik van mannelijke en vrouwelijke dieren

3.4.4.1 Intervention studies using SuHx model

Ratten (Rattus norvegicus) Er worden zowel mannelijke als vrouwelijke dieren gebruikt.

3.4.4.2 Intervention studies using PAB model

Ratten (Rattus norvegicus) Er worden zowel mannelijke als vrouwelijke dieren gebruikt.

3.4.4.3 Intervention studies using Chronic hypoxia mouse model

Muizen (Mus musculus) Er worden zowel mannelijke als vrouwelijke dieren gebruikt.

3.4.4.4 Intervention studies using Pneumonectomy (PNX)-Su5416 mouse model

Muizen (Mus musculus) Er worden zowel mannelijke als vrouwelijke dieren gebruikt.

Een deel van de dieren in bijlage 3.4.4.1 en 3.4.4.3 zal tijdelijk worden gehuisvest onder lage zuurstofcondities. Dit is nodig voor het opwekken van het ziektebeeld.

Locatie uitvoering experimenten	- Alle proeven vinden plaats in een instelling van een vergunninghouder. - Er zijn geen problemen bekend met de vergunninghouder.

2 DEC advies

DEC-advies	De DEC heeft in 2 vragenrondes vragen gesteld aan de aanvrager. In vraagronde 1 werden vragen gesteld over: de NTS, belangen en onderbouwing van het belang, opzet van de de pilot, go-no go momenten, soorten interventies, percentage dieren dat ernstig ongerief zal ondervinden, benoemen van het target, inconsistenties in de aanvraag, onderbouwing gekozen diermodellen, beschrijving van de te verwachten verbeteringen door de interventies, onderbouwing dieraantallen, voorkomen van ernstig ongerief mogelijk?, waarom geen dosis proeven, ongerief door interventies, onderbouwing gebruik katheters, In vraagronde 2 zijn vragen gesteld over o.a.: verheldering dieraantallen pilotstudies, verheldering tabellen, inschatting aantal dieren dat HEP bereikt, Citaten: C10 (huisvesting): De dieren worden gehuisvest en verzorgd op een wijze die voldoet aan de eisen die zijn opgenomen in bijlage III van de richtlijn. De dieren in Appendix 1 (SuHx model) en 3 (Chronic hypoxia mouse model) worden voor maximaal 4 weken gehuisvest in een hypoxische omgeving met 10% zuurstof, deze hypoxie leidt tot pulmonale hypertensie. C11 (ongerief): (...) Hartfalen is helaas een belangrijk onderdeel van dit onderzoek en is niet te voorkomen, hierbij kan bij een deel van de dieren ernstig ongerief optreden. De meeste dieren zullen tot het eind van het experiment geen hartfalen ontwikkelen, hierbij zal het ongerief niet boven de matig uitkomen. Maximaal 12% van de dieren kan wel hartfalen ontwikkelen met ernstig ongerief (voor maximaal 2 dagen). Bij hartfalen is een afname van lichaamsgewicht te zien, de dieren worden daarom dagelijks gecontroleerd en gewogen. De DEC is van mening dat het ongerieflevel aan de onderkant van ernstig ligt, zoals dat door de EU is gedefinieerd, de dieren hebben waarschijnlijk geen pijn maar missen energie. De DEC is akkoord met deze inschatting van het ongerieflevel. C13 (Humane eindpunten): De criteria voor de humane eindpunten zijn goed gedefinieerd. (...) Door dagelijks te observeren en te wegen kan het eindpunt goed worden bepaald, en kan onnodig lijden worden voorkomen. (...)

Ethische afweging van de DEC:
Citaat: Rechtvaardigen de doeleinden van dit project het voorgestelde gebruik van de dieren? Rechtvaardigt de ontwikkeling van nieuwe kandidaat-behandelingen voor de longziekte Pulmonale arteriële hypertensie (PAH), om hiermee het verloop van de ziekte te verbeteren, het gebruik van maximaal 776 muizen en 840 ratten die daarvan licht tot ernstig ongerief ondervinden?

2. De waarden die voor de proefdieren in het geding zijn: De integriteit van de proefdieren wordt aangetast en de dieren ondervinden licht tot ernstig ongerief. Dat leidt tot veel nadeel voor deze proefdieren. De waarden voor de onderzoekers: voordeel vanwege de kennisontwikkeling over het verloop van PAH. De waarden die voor de patiënten bevorderd worden: Mogelijk veel voordeel wanneer de dierproef bijdraagt aan het ter beschikking komen van betere behandelopties voor hartfalen bij patiënten met pulmonale hypertensie.
De DEC is van mening dat de belangen van de patiënten in dit project zwaarder wegen dan de belangen van de 776 muizen en 840 ratten, die hiervoor als proefdieren gebruikt worden. Voor het verkrijgen van meer kennis over pulmonale hypertensie en hartfalen is onderzoek in diermodellen noodzakelijk. Er zijn op dit moment geen alternatieven voor deze dierproeven beschikbaar waarmee men de doelstellingen kan bereiken.

3. Volgens de DEC rechtvaardigen de doeleinden van dit project het voorgestelde gebruik van dieren. Het directe doel van deze studie is de ontwikkeling van nieuwe kandidaat-behandelingen voor de longziekte Pulmonale arteriële hypertensie (PAH). Het verwachte resultaat, in het kader van het beschikbaar komen van betere behandelingsopties voor patiënten met hartfalen, is afgewogen tegen het licht tot ernstig geschatte ongerief en de aantasting van integriteit, inclusief het doden van de dieren in de proef.
De DEC onderschrijft dat de doelstellingen niet zonder het gebruik van proefdieren kunnen worden behaald en acht het gebruik van 776 muizen en 840 ratten, en de daarmee samenhangende schade aan deze dieren gerechtvaardigd. Bij het uitvoeren van de dierproeven wordt een adequate invulling gegeven aan de vereisten op het gebied van de vervanging, vermindering en verfijning van de dierproeven. Het project is (1) van substantieel belang en (2) van goede kwaliteit.
(1) Het maatschappelijk belang en wetenschappelijk belang zijn beide

	substantieel. De resultaten van dit onderzoek zullen bijdragen aan meer kennis over pulmonale hypertensie en het beschikbaar komen van betere behandelingsopties voor patiënten met hartfalen. (2) De DEC is van mening dat dit project verantwoord is vanuit wetenschappelijk oogpunt en acht het waarschijnlijk dat op basis van de resultaten van de voorgenomen reeks experimenten beschreven in het project, nieuwe en/of aanvullende kennis zal worden verkregen. De onderzoekers beschikken over ruime ervaring en kennis op het gebied van de te gebruiken methoden en werken nauw samen met andere onderzoeksgroepen. Dit in combinatie met de beschikbare faciliteiten en infrastructuur betekent dat de onderzoekers goed gekwalificeerd en geoutilleerd zijn voor het uitvoeren van het in dit project beschreven onderzoek. Samenvattend kan worden gesteld dat het als substantieel te kwalificeren maatschappelijk en wetenschappelijk belang van het onderzoek naar het oordeel van de DEC opweegt tegen het gebruik van maximaal 776 muizen en 840 ratten en het daarbij verwachte lichte tot ernstige ongerief. De DEC heeft extern advies ingewonnen bij - de aanvrager is om aanvullingen gevraagd Het DEC advies is Positief Het uitgebrachte advies is niet gebaseerd op consensus. Het uitgebrachte advies is gebaseerd op meerderheid. Er is een lid dat niet meegaat met het positieve advies, omdat het vertrouwen in de haalbaarheid van dit project voor dit lid gecompromitteerd is: het goed opzetten van dierexperimenten bepaalt mede de haalbaarheid van een projectvoorstel. Het inschatten van het aantal dieren is hierbij een voorwaarde. Gezien het twee vragenrondes duurde voordat het juiste aantal dieren in het protocol opgenomen waren hebben de aanvragers het in de ogen van het lid nagelaten om goed over de experimenten na te denken.

3 Kwaliteit DEC advies

Kwaliteit DEC-advies	
Het DEC advies is helder en navolgbaar. In het DEC advies is op heldere wijze inzicht gegeven in de vragen die aan de aanvrager zijn gesteld. Wij zien dat u veel vragen heeft gesteld, wij begrijpen dat dit nodig was om een helder beeld te krijgen hoeveel dieren welke handelingen en bijbehorend ongerief ondervinden, nodig voor het kunnen maken van een ethische afweging. Bij de beantwoording van de beoordelingsvragen verstrekt u over het algemeen een heldere onderbouwing. De ethische afweging volgt op logische wijze uit de beantwoording van de C vragen. Bij vraag C10 benoemt u alleen dat de dieren tijdelijk onder lage O2-condities gehuisvest worden, maar u geeft hierbij niet de mening van de DEC weer. U heeft de noodzaak van het opleggen van een beoordeling achteraf aan deze vergunning niet benoemd. Uw advies is tot stand gekomen op basis van een meerderheidsstandpunt. Het minderheidsstandpunt is duidelijk weergegeven en u maakt onder de vragen C7 en C8 duidelijk waarom de meerderheid van de DEC een andere afweging maakt. Wij volgen het meerderheidsstandpunt van de DEC, omdat wij, gezien de vele publicaties van deze onderzoeksgroep, en gezien het nu voorliggende projectvoorstel voldoende vertrouwen hebben in de haalbaarheid van de doelstellingen van dit project. De behandeltijd van deze aanvraag bij uw DEC heeft meer dan 20 werkdagen in beslag genomen. Het valt ons op dat er een lange periode tussen indienen van de gewijzigde versie door de aanvrager en de bespreking in de DEC zat.	

4 Inhoudelijke beoordeling

Doelstelling Doelstelling	Citaat: The main objectives of the research included in this project are to: • Evaluate novel (combinations of) therapeutic interventions in mice and rat models for PAH, targeting both pulmonary vascular remodelling and RV pressure overload; • Improve fundamental understanding of PAH and RV failure and their underlying pathologies; • Discover novel therapeutic targets for PAH. The results of the studies will render pivotal information on the usefulness of the therapeutic interventions in subsequent clinical trials and will support the use of relevant PAH disease models. As such, they will contribute to improve the treatment of PAH.
Wetenschappelijk en maatschappelijk belang	Citaat: SCIENTIFIC RELEVANCE Not only will the proposed studies allow us to identify therapeutic interventions for PAH with the highest efficacy likelihood and the lowest

toxicity potential before starting clinical trials, they will also increase our understanding of the processes underlying abnormal pulmonary vascularisation and that controlling the transition of RV adaptation towards right heart failure. Our research group is one of the few who takes a combined approach by studying the pathological effects of PAH in the lungs and the right heart concurrently. This will allow us to investigate the relatively new concept that PAH patients may benefit from a cardiac-specific treatment, which has no direct effect on the pulmonary vasculature, but presents or reverses right heart dysfunction. Moreover, novel therapeutic targets for future clinical research may be identified. Although this research proposal is focused on PAH, right heart failure is also the main cause of death in several other conditions such as left heart failure and critical illness. We have for example shown that not only the RV but also the LV is affected in PAH-patients. We believe that RV remodelling observed in PAH patients shares important pathophysiological mechanisms with the cardiac remodelling observed in left heart failure patients. As such, the findings of this proposal may also advance research in left heart failure. The scientific relevance of our findings is therefore not limited to PAH-induced right heart failure.

SOCIETAL RELEVANCE
PAH remains an incurable debilitating disease, with high mortality rates and poor prognosis for patients. Although the incidence is low (2.2 per million), the current life expectancy is only 3-5 years [4]. Besides the enormous impact of the disease on the quality of life of PAH patients, the disease also carries considerable economic consequences because patients and/or care-givers drop out of the work force and patients require expensive medical treatments, including lung transplantation. New PAH therapies, also targeting alternative pathways are urgently needed. In this project, we address apart from established PAH-targets (e.g. BMPR2 signalling) [10], also relatively new ones, such as RV diastolic stiffness. Up till now, there has been little consideration in the field of the notion that PAH patients could benefit from a cardiac-specific treatment, which has no direct effect on the pulmonary vasculature. With the results of this project, we will be able to select those interventions with promising effectiveness in PAH animal models for further clinical testing. This will bring us hopefully a step closer to the development of an effective PAH treatment. In the future, patients at risk of developing right heart failure (PAH) may benefit from these new treatment options. Although PAH is rare, other types of pulmonary hypertension (PH) are much more prevalent and carry significant morbidity and mortality. Moreover, right heart failure is becoming a great clinical problem as leading cause of death in several diseases such as left heart failure, and the critical ill at the intensive care. Currently, no therapeutic strategies

	are available to improve RV function or prevent right heart failure. Beside knowledge on PH and right heart failure, this project will also provide new insight in cardiac and endothelial physiology, which will be useful in other lung diseases and left heart failure. As such, the societal relevance of our studies extends far beyond PAH alone.
Onderbouwing wetenschappelijk en maatschappelijk belang	Voldoende beschreven.
Wetenschappelijke kwaliteit Kwaliteit aanvrager/ onderzoeksgroep en onderzoek	Citaat uit DEC advies C7: De DEC heeft veel vragen gesteld bij deze aanvraag, zie de vragenrondes bovenaan dit document. De DEC is van mening dat naast aanwezige apparatuur, kennis, personeel en financiering ook het goed opzetten van experimenten (en daarbij het inschatten van het aantal dieren) de haalbaarheid van een projectvoorstel bepaalt en ziet graag dat de onderzoekers bij de uitvoering goed voor ogen houden wat ze precies willen doen en dat goed inplannen om fouten te voorkomen. Alle technische voorzieningen die benodigd zijn voor uitvoering van het project zijn voorhanden, evenals voldoende deskundigheid en financiering om het project succesvol uit te voeren. Ervaring binnen het onderzoeksinstituut met vergelijkbaar onderzoek waarborgt het technisch succesvol uitvoeren van de dierexperimenten. Na navraag is de DEC ervan overtuigd dat de projectdoelstelling met de gekozen strategie/aanpak binnen de gevraagde termijn is te realiseren. Eén van de DEC leden is van mening dat "het vertrouwen in de haalbaarheid van dit project voor dit lid gecompromitteerd is: het goed opzetten van dierexperimenten bepaalt mede de haalbaarheid van een projectvoorstel. Het inschatten van het aantal dieren is hierbij een voorwaarde. Gezien het twee vragenrondes duurde voordat het juiste aantal dieren in het protocol opgenomen waren hebben de aanvragers het in de ogen van het lid nagelaten om goed over de experimenten na te denken." Het Secretariaat volgt het meerderheidsstandpunt van de DEC, en heeft, gezien de vele publicaties van deze onderzoeksgroep, en gezien het nu voorliggende projectvoorstel voldoende vertrouwen in de haalbaarheid van de doelstellingen van dit project.

3V's

Vervanging	
	3.4.4.1 Intervention studies using SuHx model: Citaat: All proposed interventions that will be tested throughout this project will be assessed first in other, non-animal, models, such as cell culture experiments. Only if these experiments yield sufficiently promising results, in vivo tests will be undertaken. In vivo experiments are a necessary step in development of interventions that may one day be used in the clinic, since currently no other methods allow the evaluation of the effect of an intervention on the organism as a whole. Cell culture, organoids, lab-on-a-chip or computer models are not (yet) sophisticated enough to assess the interaction between the PAH pathology and host environmental factors such as the oxygen supply, the immune system and metabolism.
	3.4.4.2 Intervention studies using PAB model: Zie bijlage 3.4.4.1.
	3.4.4.3 Intervention studies using Chronic hypoxia mouse model: Citaat: In vivo experiments are a necessary step in development of interventions that may one day be used in the clinic, since currently no other methods allow the evaluation of the effect of an intervention on the organism as a whole. Cell culture, organoids, lab-on-a-chip or computer models are not (yet) sophisticated enough to assess the interaction between the PAH pathology and host environmental factors such as the oxygen supply, the immune system and metabolism.
	3.4.4.4 Intervention studies using Pneumonectomy (PNX)-Su5416 mouse model: Zie bijlage 3.4.4.3.

Verminderen	
	3.4.4.1 Intervention studies using SuHx model: Citaat: The proposed number of evaluable animals per study arm (n=12 in model/intervention groups, n=6 in control group) is calculated as described above, and is in line with generally accepted protocols in scientific literature and our previous studies with the SuHx model at our Department. Further reduction of the number of animals per group would decrease statistical power, and might thus disqualify the experiment.
	3.4.4.2 Intervention studies using PAB model: Citaat: The proposed number of evaluable animals per study arm (n=12 in model/intervention groups, n=6 in control group) is calculated as described above, and is in line with generally accepted protocols in scientific literature and our previous studies with the PAB model. Further reduction of the number of animals per group would decrease statistical power, and might thus disqualify the experiment.
	3.4.4.3 Intervention studies using Chronic hypoxia mouse model: Citaat: The proposed number of evaluable animals per study arm (n=10) is calculated as described above, and is in line with generally accepted protocols in scientific literature. Further reduction of the number of animals per group would decrease statistical power, and might thus disqualify the experiment.
	3.4.4.4 Intervention studies using Pneumonectomy (PNX)-Su5416 mouse model: Citaat: The proposed number of evaluable animals per study arm (n=12 in model/intervention groups, n=6 in control group) is calculated as described above, and is in line with generally accepted protocols in scientific literature. Further reduction of the number of animals per group would decrease statistical power, and might thus disqualify the experiment.
Verfijnen	
	3.4.4.1 Intervention studies using SuHx model: Citaat: State-of-the-art methods and equipment for assessing pulmonary vascular remodelling and RV overload, in particular advanced imaging techniques, will be used to refine protocols and minimize discomfort to the animals especially in support of the application of humane endpoints. International guidelines will be applied to ensure that best practice is followed. Citaat: The procedures conducted under this protocol will inevitably cause discomfort for the animals in the studies. In order to minimize suffering, all studies will adhere to the national and internationally accepted rules for handling of lab animals [1], [2]. Under these rules, the animals will be

	humanely killed when a humane endpoint (see below) is reached. Within the institute, standard operating procedures (SOP) for animal handling are in place. These also include dedicated anaesthesia and analgesia (A&A) SOPs that will accompany invasive procedures to minimize pain and discomfort. To minimize animal suffering, pain and fear the following measures will be taken. The animals will be allowed to acclimatize to their new environment for two weeks upon arrival at the animal facility. Animals are housed in groups (socially housed) at all times (also during hypoxia. Except during surgery, animals are returned to the group directly after surgery) and environmental enrichment strategies are applied in the cages to improve animal welfare. In case the therapeutic interventions require surgical procedures (e.g. cannula/mini-pump implantation), this will be done under general anaesthesia in combination with pain treatment. During haemodynamic measurements, the animals will be kept under general anaesthesia in a temperature-controlled environment. During imaging procedures, animals will be kept under general anaesthesia in a temperature-controlled environment. (...) Most rats will not suffer from heart failure until the end of the experiment, and their discomfort will not exceed moderate. Maximally 15% (based on previous experiments) of the rats can develop heart failure with severe discomfort. It is very difficult to see if a rat is having heart failure (which can occur in the weeks after hypoxia). A decrease in bodyweight is the first sign of heart failure which is then already occurring for one day. It is normal to have a decreasing bodyweight of the rat during the day, because of sleeping, less drinking and eating during the day (daily fluctuations). To be sure it is heart failure (and not daily fluctuations of bodyweight), a bodyweight decrease of 10% is being established. Also cyanosis, dyspnea, lethargy and poor grooming can be observed. This is usually on the second day. Then an HEP will be applied. Severe discomfort is unfortunately unavoidable. Patients usually present at the stage of heart failure. Right heart failure is an important outcome measure of our research. Heart failure is necessary to compare the rats with patients.
	3.4.4.2 Intervention studies using PAB model: Citaat: State-of-the-art methods and equipment for assessing pulmonary vascular remodelling and RV overload, in particular advanced imaging techniques, will be used to refine protocols and minimize discomfort to the animals especially in support of the application of humane endpoints. International guidelines will be applied to ensure that best practice is followed. Citaat: The procedures conducted under this protocol will inevitably cause

	discomfort for the animals in the studies. In order to minimize suffering, all studies will adhere to the national and internationally accepted rules for handling of lab animals [2, 3]. Under these rules, the animals will be humanely killed when a humane endpoint (see below) is reached. Within the institute, standard operating procedures (SOP) for animal handling are in place. These also include dedicated anaesthesia and analgesia (A&A) SOPs that will accompany invasive procedures to minimize pain and discomfort. To minimize animal suffering, pain and fear the following measures will be taken. The animals will be allowed to acclimatize to their new environment for two weeks upon arrival at the animal facility. Animals are housed in groups (socially housed) at all times (except during surgery, animals are returned to the group directly after surgery) and environmental enrichment strategies are applied in the cages to improve animal welfare. The PAB surgical model is performed under general anaesthesia and analgesia (pre- and post-surgery). During haemodynamic measurements, animals will be kept under anaesthesia in a temperature-controlled environment. During imaging procedures, animals will be kept under anaesthesia in a temperature-controlled environment. (...) Right ventricular (RV) failure is the predominant cause of death in patients with pulmonary hypertension. Maximally 32% of the rats can develop heart failure with severe discomfort. It is due to the diameter of the band around the pulmonary artery. A decrease in bodyweight is the first sign of heart failure which is then already occurring for one day. It is normal to have a decreasing bodyweight of the rat during the day, because of sleeping, less drinking and eating during the day (daily fluctuations). To be sure it is heart failure (and not daily fluctuations of bodyweight), a bodyweight decrease of 10% is being established. Also cyanosis, dyspnea, lethargy and poor grooming can be observed. This is usually at the second day and an HEP will be applied. Based on experience with this model in Denmark: Seven weeks survival rate was 80% for rats subjected to severe banding and close to 100% in rats subjected to mild or moderate banding or sham surgery[1]. Severe discomfort is unfortunately unavoidable. Patients usually present at the stage of heart failure. Right heart failure is an important outcome measure of our research. Heart failure is necessary to compare the rats with patients.

	3.4.4.3 Intervention studies using Chronic hypoxia mouse model: Citaat: State-of-the-art methods and equipment for assessing pulmonary vascular remodelling and RV overload, in particular advanced imaging techniques, will be used to refine protocols and minimize discomfort to the animals especially in support of the application of humane endpoints. International guidelines will be applied to ensure that best practice is followed. Explain what measures will be taken to minimise 1) animal suffering, pain or fear and 2) adverse effects on the environment. The procedures conducted under this protocol will inevitably cause discomfort for the animals in the studies. In order to minimize suffering, all studies will adhere to the national and internationally accepted rules for handling of lab animals [1], [2]. Under these rules, the animals will be humanely killed when a humane endpoint (see below) is reached. Within the institute, standard operating procedures (SOP) for animal handling are in place. These also include dedicated anaesthesia and analgesia (A&A) SOPs that will accompany invasive procedures to minimize pain and discomfort. To minimize animal suffering, pain and fear the following measures will be taken. The animals will be allowed to acclimatize to their new environment for two weeks upon arrival at the animal facility. Animals are housed in groups (socially housed) at all times (also during hypoxia) and environmental enrichment strategies are applied in the cages to improve animal welfare. During haemodynamic measurements, animals will be kept under anaesthesia in a temperature-controlled environment. Measurements of vascular leakage will be performed under continuous anaesthesia. (...) In these experiments in mice with PAH there is no heart failure expected, and the discomfort will not transcend mild.

	3.4.4.4 Intervention studies using Pneumonectomy (PNX)-Su5416 mouse model: Citaat: State-of-the-art methods and equipment for assessing pulmonary vascular remodelling and RV overload, in particular advanced imaging techniques, will be used to refine protocols and minimize discomfort to the animals especially in support of the application of humane endpoints. International guidelines will be applied to ensure that best practice is followed. Explain what measures will be taken to minimise 1) animal suffering, pain or fear and 2) adverse effects on the environment. The procedures conducted under this protocol will inevitably cause discomfort for the animals in the studies. In order to minimize suffering, all studies will adhere to the national and internationally accepted rules for handling of lab animals [1], [2]. Under these rules, the animals will be humanely killed when a humane endpoint (see below) is reached. Within the institute, standard operating procedures (SOP) for animal handling are in place. These also include dedicated anaesthesia and analgesia (A&A) SOPs that will accompany invasive procedures to minimize pain and discomfort. In case the pulmonary hypertension is too severe, we may choose to omit the SU5416 injection. To minimize animal suffering, pain and fear the following measures will be taken. The animals will be allowed to acclimatize to their new environment for two weeks upon arrival at the animal facility. Animals are housed in groups (socially housed) at all times (except during surgery, animals are returned to the group directly after surgery) and environmental enrichment strategies are applied in the cages to improve animal welfare. The PNX surgical model is performed under general anaesthesia and analgesia (pre- and post-surgery). During haemodynamic measurements, animals will be kept under anaesthesia in a temperature-controlled environment. (...) In these experiments in mice with PAH there is no heart failure expected, and the discomfort will not transcend moderate.
3.4.4.1 Intervention studies using SuHx model: Voldoende beschreven.	
3.4.4.2 Intervention studies using PAB model: Voldoende beschreven.	
3.4.4.3 Intervention studies using Chronic hypoxia mouse model: Voldoende beschreven.	
3.4.4.4 Intervention studies using Pneumonectomy (PNX)-Su5416 mouse model: Voldoende beschreven.	

Hergebruik	Er is geen sprake van hergebruik van dieren.

Naam proef	Worden de dieren gedood?	Doden volgens richtlijn?
3.4.4.1 Intervention studies using SuHx model	Ja	volgens de richtlijn.
3.4.4.2 Intervention studies using PAB model	Ja	volgens de richtlijn.
3.4.4.3 Intervention studies using Chronic hypoxia mouse model	Ja	volgens de richtlijn.
3.4.4.4 Intervention studies using Pneumonectomy (PNX)-Su5416 mouse model	Ja	volgens de richtlijn.

Naam proef		
3.4.4.1 Intervention studies using SuHx model	HEP: <15%	Citaat: The most important humane endpoints applicable to all studies are: • Weight loss >20% of maximum body weight in adult animals, measured from the start of the treatment • Weight loss >10% of body weight during 24h, in combination with: • Sustained abnormal breathing, dyspnoea (symptom PAH/right heart failure) • Sustained lethargy (symptom PAH/right heart failure) • Sustained abnormal behaviour • Complications of interventions • Other procedure-specific endpoints
Ratten (Rattus norvegicus)	Ongerief: 15,0% Ernstig 85,0% Matig	

3.4.4.2 Intervention studies using PAB model	HEP: <32%	The most important humane endpoints applicable to all studies are: • Permanent weight loss >20% of initial body weight in adult animals, measured from the start of the treatment • Weight loss >10% of body weight during 24h, in combination with: o Sustained abnormal breathing, dyspnea (symptom PAH/right heart failure) o Sustained lethargy (symptom PAH/right heart failure) • Sustained abnormal behavior • Complications of interventions • Other procedure-specific endpoints
Ratten (Rattus norvegicus)	Ongerief: 32,0% Ernstig 68,0% Matig	
3.4.4.3 Intervention studies using Chronic hypoxia mouse model	HEP: <10%	Citaat: The most important humane endpoints applicable to all studies are: • Weight loss >20% of maximum body weight in adult animals, measured from the start of the treatment • Weight loss >15% of body weight during 24h, in combination with: o Sustained abnormal breathing, dyspnoea (symptom PAH/right heart failure) o Sustained lethargy (symptom PAH/right heart failure) • Sustained abnormal behaviour • Complications of interventions (<1%): No interventions are planned.
Muizen (Mus musculus)	Ongerief: 100,0% Licht	

3.4.4.4 Intervention studies using Pneumonectomy (PNX)-Su5416 mouse model	HEP: <10%	Citaat: The most important humane endpoints applicable to all studies are: • Weight loss >20% of maximum body weight in adult animals, measured from the start of the treatment • Weight loss >15% of body weight during 24h, in combination with: • Sustained abnormal breathing, dyspnoea (symptom PAH/right heart failure) • Sustained lethargy (symptom PAH/right heart failure) • Sustained abnormal behaviour • Complications of interventions • • Other procedure-specific endpoints
Muizen (Mus musculus)	Ongerief: 59,0% Matig 41,0% Licht	

5 Samenvatting

5.2 lid1 [redacted]

Er worden enkele dieren extra aangevraagd voor het geval van uitval van dieren. Dit is voldoende onderbouwd.

Een deel van de dieren, in bijlagen 3.4.4.1 en 3.4.4.3 wordt gedurende enkele weken gehuisvest onder condities met lage zuurstofspanning. Dit is nodig om het ziektebeeld te induceren, en heeft dus een wetenschappelijke noodzaak.

5.2 lid1 [redacted]

Het ongerief in dit onderzoek is voor 12% van de ratten op ernstig ingeschat. De DEC zegt over deze inschatting: "De DEC is van mening dat het ongerieflevel aan de onderkant van ernstig ligt, zoals dat door de EU is gedefinieerd, de dieren hebben waarschijnlijk geen pijn maar missen energie. De DEC is akkoord met deze inschatting van het ongerieflevel." Het

5.2 lid1 [redacted]

Het DEC advies is gebaseerd op een meerderheidsstandpunt. (citaat): "Er is een lid dat niet meegaat met het positieve advies, omdat het vertrouwen in de haalbaarheid van dit project voor dit lid gecompromitteerd is: het goed opzetten van dierexperimenten bepaalt mede de haalbaarheid van een projectvoorstel. Het inschatten van het aantal dieren is hierbij een voorwaarde. Gezien het twee vragenrondes duurde voordat het juiste aantal

dieren in het protocol opgenomen waren hebben de aanvragers het in de ogen van het lid nagelaten om goed over de experimenten na te denken." 5.2 lid1

De aanvrager is nog gevraagd bij de beschrijving van de humane eindpunten te verhelderen wat met "sustained" bedoeld wordt. Het is onduidelijk hoe lang de dieren deze symptomen moeten laten zien voordat de dieren het humane eindpunt hebben bereikt. De aanvrager heeft hierop geantwoord: 'Met "sustained" bedoelen we dat de dieren constante ademhalingsklachten, lethargie of afwijkend gedrag vertonen. Wanneer deze klachten gedurende een periode van 48 uur ononderbroken aanhouden, zal het humane eindpunt worden toegepast.'

6 Voorstel besluit incl. voorstel geldigheidsduur van de vergunning

5.2 lid1

Beoordeling achteraf
In dit project worden dierproeven toegepast die vallen in de categorie ernstig volgens artikel 10b van de wet. Daarom bent u verplicht om na afloop van de vergunning in een Beoordeling achteraf over uw project te rapporteren. Deze beoordeling zal uiterlijk augustus 2026 plaatsvinden. Er zal dan conform artikel 10a2, derde lid van de wet, beoordeeld worden of de doelstellingen van het project werden bereikt.

De ingangsdatum van de vergunning kan niet voor de verzenddatum van de beschikking zijn en zal indien van toepassing aangepast worden. Dit is ook het geval bij een voorgenomen besluit.

7 Concept beschikking voor akkoord CCD

Centrale Commissie Dierproeven

23

> Retouradres Postbus 93118 2509 AC Den Haag

5.1 lid2h
5.1 lid2e
5.1 lid2h

Centrale Commissie Dierproeven
Postbus 93118
2509 AC Den Haag
centralecommissiedierproeven.nl
0900 28 000 28 (10 ct/min)
info@zbo-ccd.nl

Onze referentie
Aanvraagnummer
AVD5.1 lid2h20209866
Bijlagen
3

Datum 6 november 2020
Betreft Beslissing aanvraag projectvergunning Dierproeven

Geachte 5.1 lid2e ,

Op 8 mei 2020 hebben wij uw aanvraag voor een projectvergunning dierproeven ontvangen. Het gaat om uw project "Towards a novel treatment of pulmonary arterial hypertension; preclinical testing of novel interventions targeting both right ventricular pressure overload and pulmonary vascular remodelling." met aanvraagnummer AVD5.1 lid2h20209866. Wij hebben uw aanvraag beoordeeld.

Beslissing
Wij keuren uw aanvraag goed. Uit artikel 10a, eerste lid van de Wet op de dierproeven (hierna: de wet) volgt daarom dat het is toegestaan om uw project uit te voeren binnen de gestelde vergunningsperiode. Deze vergunning wordt afgegeven voor de periode van 6 november 2020 tot en met 31 augustus 2025.
Aan de vergunning hebben wij de volgende voorwaarde verbonden op grond van artikel 10a1, tweede lid van de wet.

Beoordeling achteraf
In dit project worden dierproeven toegepast die vallen in de categorie ernstig volgens artikel 10b van de wet. Daarom bent u verplicht om na afloop van de vergunning in een Beoordeling achteraf over uw project te rapporteren. Deze beoordeling zal uiterlijk augustus 2026 plaatsvinden. Er zal dan conform artikel 10a2, derde lid van de wet, beoordeeld worden of de doelstellingen van het project werden bereikt.

De onderbouwing van deze beslissing vindt u onder 'Overwegingen'.

Datum:
6 november 2020
Aanvraagnummer:
AVD5.1 lid2h 20209866

Procedure

Advies dierexperimentencommissie
Wij hebben advies gevraagd bij 5.1 lid2h
(hierna: DEC). Dit advies is ontvangen op 30 september 2020. Bij de beoordeling van uw aanvraag is dit advies betrokken overeenkomstig artikel 10a, derde lid van de wet.

Nadere vragen aanvrager
Op 19 oktober 2020 hebben wij u om aanvullingen gevraagd. U heeft tijdig antwoord gegeven. Het verzoek om aanvullingen had betrekking op aanpassingen in de NTS en verheldering van de humane eindpunten. U heeft aangegeven dat wanneer dieren constante ademhalingsklachten, lethargie of afwijkend gedrag vertonen en deze klachten gedurende een periode van 48 uur ononderbroken aanhouden, het humane eindpunt zal worden toegepast. Uw reactie is betrokken bij de behandeling van uw aanvraag.

Overwegingen

Wij kunnen ons vinden in de inhoud van het advies van de DEC, inclusief de daaraan ten grondslag liggende motivering.

Beoordeling achteraf
Na afloop van het project moet er een beoordeling plaatsvinden zoals bedoeld in artikel 10a1, eerste lid, onder d en artikel 10a1, derde lid van de wet. De reden van deze beoordeling achteraf is dat in dit project dieren ernstig ongerief ondergaan. Deze beoordeling zal uiterlijk augustus 2026 plaatsvinden. Meer informatie over de eisen die gesteld worden bij de beoordeling achteraf vindt u in de bijlage 'Weergave wet- en regelgeving'.

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 93118, 2509 AC 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. Nadat u een bezwaarschrift heeft ingediend kunt u een voorlopige voorziening vragen bij de voorzieningenrechter van de rechtbank in de vestigingsplaats van de

vergunninghouder. U moet dan wel kunnen aantonen dat er sprake is van een spoedeisende situatie.

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 vergunninghouder valt.

Meer informatie
Heeft u vragen, kijk dan op www.centralecommissiedierproeven.nl, stuur een e-mail naar info@zbo-ccd.nl of neem telefonisch contact met ons op: 0900 28 000 28 (10 ct/minuut).

Centrale Commissie Dierproeven
namens deze:

5.1 lid2h

drs. F. Braunstahl
Algemeen Secretaris

Bijlagen:
- Projectvergunning
- DEC-advies
- Weergave wet- en regelgeving

Centrale Commissie Dierproeven

Projectvergunning

gelet op artikel 10a van de Wet op de Dierproeven

Verleent de Centrale Commissie Dierproeven aan
Naam:
Adres:
Postcode en plaats:
Deelnemersnummer:

5.1 lid2h

deze projectvergunning voor het tijdvak 6 november 2020 tot en met 31 augustus 2025, voor het project "Towards a novel treatment of pulmonary arterial hypertension; preclinical testing of novel interventions targeting both right ventricular pressure overload and pulmonary vascular remodelling." met aanvraagnummer AVD5.1 lid2h20209866, na advies van 5.1 lid2h .
De functie van de verantwoordelijk onderzoeker is 5.1 lid2e

Het besluit is gebaseerd op de volgende (aangepaste) stukken:

1 een aanvraagformulier projectvergunning dierproeven, zoals ontvangen op 8 mei 2020
2 de bij het aanvraagformulier behorende bijlagen:
   a Projectvoorstel, zoals ontvangen op 30 september 2020;
   b Bijlagen dierproeven
      - 3.4.4.1 Intervention studies using SuHx model, zoals ontvangen op 30 september 2020;
      - 3.4.4.2 Intervention studies using PAB model, zoals ontvangen op 30 september 2020;
      - 3.4.4.3 Intervention studies using Chronic hypoxia mouse model, zoals ontvangen op 30 september 2020;
      - 3.4.4.4 Intervention studies using Pneumonectomy (PNX)-Su5416 mouse model, zoals ontvangen op 30 september 2020;
   c Niet-technische Samenvatting van het project, zoals ontvangen op 21 oktober 2020;
   d Advies van dierexperimentencommissie, zoals ontvangen op 30 september 2020
   e De aanvullingen op uw aanvraag, zoals ontvangen op 21 oktober 2020.

Aanvraagnummer:
AVD5.1 lid2h20209866

Naam proef	Diersoort/ Stam	Aantal dieren	Ongerief
3.4.4.1 Intervention studies using SuHx model			
	Ratten (Rattus norvegicus) / Sprague Dawley	400	15,0% Ernstig 85,0% Matig
3.4.4.2 Intervention studies using PAB model			
	Ratten (Rattus norvegicus) / Wistar	440	32,0% Ernstig 68,0% Matig
3.4.4.3 Intervention studies using Chronic hypoxia mouse model			
	Muizen (Mus musculus) / WT, knock-in, knock-out	400	100,0% Licht
3.4.4.4 Intervention studies using Pneumonectomy (PNX)-Su5416 mouse model			
	Muizen (Mus musculus) / WT, knock-in, knock-out	376	59,0% Matig 41,0% Licht

Voorwaarden

Beoordeling achteraf
In dit project worden dierproeven toegepast die vallen in de categorie ernstig volgens artikel 10b van de wet. Daarom bent u verplicht om na afloop van de vergunning in een Beoordeling achteraf over uw project te rapporteren. Deze beoordeling zal uiterlijk augustus 2026 plaatsvinden. Er zal dan conform artikel 10a2, derde lid van de wet, beoordeeld worden of de doelstellingen van het project werden bereikt.

Geldende voorschriften
Wij wijzen u op onderstaande geldende voorschriften, die volgen uit artikel 1d, vierde lid, artikel 10, eerste lid en/of artikel 10a3 van de wet.
- Go/ no go momenten worden voor aanvang van elk experiment afgestemd met de IvD.
- Het is verboden 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.
- Het is verboden dierproeven te verrichten voor een doel waarvan het belang niet opweegt tegen het ongerief dat aan het proefdier wordt berokkend.
- Overige wettelijke bepalingen blijven van kracht.

Centrale Commissie Dierproeven

Aanvraagnummer:
AVD5.1 lid2h20209866

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, derde lid van de wet. Uit artikel 10b, eerste lid van de wet 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, eerste lid van de wet de wijziging eerst voorgelegd worden en mag deze pas doorgevoerd worden na goedkeuren door de Centrale Commissie Dierproeven.
Artikel 10b, tweede en derde lid van de wet schrijven 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 van de wet 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 van de wet 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 13c van de wet 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 13d van de wet 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.

Beoordeling achteraf
Volgens artikel 10a1, eerste lid onder d en derde lid van de wet worden projecten waarbij niet-menselijke primaten worden gebruikt, projecten die als ernstig ingedeelde dierproeven omvatten of een dierproef die leidt tot ernstige mate van pijn, lijden, angst of blijvende schade die waarschijnlijk langdurig zal zijn en niet kan worden verzacht, achteraf beoordeeld.

Van: info@zbo-ccd.nl
Verzonden: maandag 9 november 2020 11:53
Aan: 5.1 lid2e
Onderwerp: Terugkoppeling over projectvergunningsaanvraag AVD5.1 lid2h20209866

Geachte 5.1 lid2h ,

Op 08-05-2020 hebben wij een aanvraag voor een projectvergunning dierproeven ontvangen waarover uw DEC advies heeft uitgebracht. Het gaat om het project 'Towards a novel treatment of pulmonary arterial hypertension; preclinical testing of novel interventions targeting both right ventricular pressure overload and pulmonary vascular remodelling.' met aanvraagnummer AVD5.1 lid2h20209866.

De CCD heeft de aanvrager aanvullende vragen gesteld. De aanvullingen hadden betrekking op aanpassingen in de NTS en verheldering van de humane eindpunten. De aanvrager heeft aangegeven dat wanneer dieren constante ademhalingsklachten, lethargie of afwijkend gedrag vertonen en deze klachten gedurende een periode van 48 uur ononderbroken aanhouden, het humane eindpunt zal worden toegepast.

De CCD heeft besloten de vergunning toe te wijzen. De aanvrager en verantwoordelijk onderzoeker zijn hierover ingelicht. De beschikking is verstuurd op 6-11-2020.

De vergunning wordt verleend onder de volgende voorwaarden:
Na afloop van het project zal er een beoordeling plaatsvinden, zoals bedoeld in artikel 10a1 lid 1 sub d en artikel 10a1 lid 3 van de wet. De reden van deze beoordeling achteraf is dat in dit project dieren ernstig ongerief ondergaan.

Het DEC advies is helder en navolgbaar. In het DEC advies is op heldere wijze inzicht gegeven in de vragen die aan de aanvrager zijn gesteld. Wij zien dat u veel vragen heeft gesteld. De CCD is van mening dat als zoveel vragen gesteld moeten worden, u de aanvraag als niet toetsbaar had moeten bestempelen, en de vragen moeten doorgeven aan de CCD. Bij de beantwoording van de beoordelingsvragen verstrekt u over het algemeen een heldere onderbouwing. De ethische afweging volgt op logische wijze uit de beantwoording van de C vragen. Bij vraag C10 benoemt u alleen dat de dieren tijdelijk onder lage O2-condities gehuisvest worden, maar u geeft hierbij niet de mening van de DEC weer. U heeft de noodzaak van het opleggen van een beoordeling achteraf aan deze vergunning niet benoemd. Uw advies is tot stand gekomen op basis van een meerderheidsstandpunt. Het minderheidsstandpunt is duidelijk weergegeven en u maakt onder de vragen C7 en C8 duidelijk waarom de meerderheid van de DEC een andere afweging maakt. Wij volgen het meerderheidsstandpunt van de DEC, omdat wij, gezien de vele publicaties van deze onderzoeksgroep, en gezien het nu voorliggende projectvoorstel voldoende vertrouwen hebben in de haalbaarheid van de doelstellingen van dit project. De behandeltijd van deze aanvraag bij uw DEC heeft meer dan 20 werkdagen in beslag genomen. Het valt ons op dat er een lange periode tussen indienen van de gewijzigde versie door de aanvrager en de bespreking in de DEC zat.

Mocht u vragen hebben over onze beslissing, dan kunt u uiteraard contact met ons opnemen.

Met vriendelijke groet,
Namens de Centrale Commissie Dierproeven

5.1 lid2e
www.centralecommissiedierproeven.nl

.....
Postbus 93118 | 2509 AC | Den Haag

………………………………………………………………
T: 0900 2800028
E: info@zbo-ccd.nl