		Inventaris Wob-verzoek W17-12								
			wordt verstrekt				**weigeringsgronden**			
nr.		**document NTS 20172485**	**reeds openbaar**	**niet**	**geheel**	**deels**	**10.1.c**	**10.2.e**	**10.2.g**	**11.1**
1		Origineel aanvraagformulier				x		x	x	
2		NTS	x							
3		Projectvoorstel				x		x	x	
4		Bijlage beschrijving dierproeven 1				x		x	x	
5		Bijlage beschrijving dierproeven 2				x		x	x	
6		Bijlage beschrijving dierproeven 3			x					
7		Referentielijst			x					
8		Factuur				x		x	x	
9		DEC-advies				x		x	x	
10		Adviesnota CCD		x						x
11		Beschikking en vergunning				x		x	x	

Centrale Commissie Dierproeven

1.

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*	11500
☐ Nee > *U kunt geen aanvraag doen*	

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

Naam instelling of organisatie	UMC Utrecht
Naam van de portefeuillehouder of diens gemachtigde	[redacted]
KvK-nummer	30244197

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	Instantie voor Dierenwelzijn Utrecht
Postbus	12007
Postcode en plaats	3501AA Utrecht
IBAN	NL27INGB0000425267
Tenaamstelling van het rekeningnummer	Universiteit Utrecht

1.4 Vul de gegevens in van de verantwoordelijke onderzoeker.

(Titel) Naam en voorletters	[redacted]	☐ Dhr. ☒ Mw.
Functie	[redacted]	
Afdeling	[redacted]	
Telefoonnummer	[redacted]	
E-mailadres	[redacted]	

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

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

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

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

1.7 Is er voor deze projectaanvraag een gemachtigde?

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

☐ Nee

2 Over uw aanvraag

2.1 Wat voor aanvraag doet u?

☒ Nieuwe aanvraag > Ga verder met vraag 3

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

Vul uw vergunde projectnummer in en ga verder met vraag 2.2

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

Vul uw vergunde projectnummer in en ga verder met vraag 2.3

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

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

☐ Nee > Ga verder met vraag 3

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

☐ Nee > Ga verder met vraag 3

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

3 Over uw project

3.1 Wat is de geplande start- en einddatum van het project?

Startdatum	1 - 9 - 2017
Einddatum	1 - 9 - 2022

3.2 Wat is de titel van het project?

Strategies for vascularized bone regeneration in maxillofacial surgical interventions

3.3 Wat is de titel van de niet-technische samenvatting?

Strategieën voor gevascullariseerde botregeneratie in kaak- en aangezichtschirurgische ingrepen

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

Naam DEC	DEC Utrecht
Postadres	Postbus 85500, 3508 GA Utrecht
E-mailadres	dec-utrecht@umcutrecht.nl

4 Betaalgegevens

4.1 Om welk type aanvraag gaat het?

☒ Nieuwe aanvraag Projectvergunning € 1.541 Lege

☐ Wijziging € Lege

4.2 Op welke wijze wilt u dit bedrag aan de CCD voldoen.

Bij een eenmalige incasso geeft u toestemming aan de CCD om eenmalig het bij 4.1 genoemde bedrag af te schrijven van het bij 1.2 opgegeven rekeningnummer.

☐ Via een eenmalige incasso

☒ Na ontvangst van de factuur

5 Checklist bijlagen

5.1 Welke bijlagen stuurt u mee?

Verplicht

☒ Projectvoorstel

☒ Niet-technische samenvatting

Overige bijlagen, indien van toepassing

☐ Melding Machtiging

☐

6 Ondertekening

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

Centrale Commissie Dierproeven
Postbus 20401
2500 EK Den Haag

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

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

Naam [redacted]

Functie

Plaats Utrecht

Datum 30 [redacted]

Handtekening

Centrale Commissie Dierproeven

Form
Project proposal

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

1 General information

1.1	Provide the approval number of the 'Netherlands Food and Consumer Product Safety Authority'.	11500
1.2	Provide the name of the licenced establishment.	UMC Utrecht
1.3	Provide the title of the project.	Strategies for vascularized bone regeneration in maxillofacial surgical interventions

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.

3.1.1 Motivation and background

Bone is the second most commonly transplanted tissue (1), as bone grafting is required in several orthopaedic, neurosurgical and dental procedures such as craniomaxillofacial reconstructions, spinal fusion techniques or

delayed fusion of bone defects (delayed unions and non-unions) (2, 3). Autografts, which represent the current golden standard, have an excellent success rate and a complete immunocompatibility. However, due to several drawbacks, including donor-site morbidity, limited tissue availability and surgical complications, this procedure is suboptimal or not always an option (4). Allografts are considered the surgeon's second best option. However, the percentage of successful graft incorporation is lower and the procedure is not completely free from the risk of immune rejection and pathogen transmission (4, 5). To overcome these limitations, a bone substitute is required, preferably as an off-the-shelf product. Currently, next to tissue transplantation, several non-regenerative treatment options are available such as polymer or titanium prostheses that do not adapt or grow. These prosthetic solutions have a limited lifespan in the patient, most commonly due to infections.
As mentioned, substitutes could be engineered via regenerative medicine strategies. These can be based on the use of (stem) cells and/or biomaterials. The latter is less complex and has found its way into the clinic. Various calcium phosphate-based materials (representing the inorganic component of bone tissue) are applied in the clinic when no tissue can be transplanted. However, the clinical outcomes with these materials are inferior to the gold standard transplantation treatment options. This warrants the exploration of creating bone substitutes based on both cells and materials.

3.1.2 State of the art

So far, several strategies for bone regeneration have been explored in order to combine the most effective cell population with the ideal carrier material and the optimal stimulation factors, but no fully satisfactory and clinically relevant results have been obtained yet (6). The main challenges are related to the scale-up of tissue size to a clinically relevant dimension. Initial studies showed that large engineered living tissue constructs cannot survive the implantation when exceeding a size of 400µm. Therefore, the current focus in the field is to work around this limitation by 1) introducing vascular structures in the constructs, or by 2) investigating the alternative route to bone formation via a cartilage intermediate, the endochondral route to bone formation. The present proposal will focus on the first approach.

Introduction of vascular structures in engineered tissues has focused on the establishment of capillary networks and the development of engineered blood vessels or endothelial-lined channels. [redacted] started working on generating capillary networks in engineered bone tissue in [redacted]. Typically, [redacted] a co-culture in hydrogels of bone progenitor cells (MSCs) and endothelial progenitor cells (ECFCs) to promote the formation of prevascular structures in bone-like tissue [7]. [redacted] the functionality of these structures as they were capable to connect to a host's vasculature upon subcutaneous implantation [redacted] Also, osteogenic differentiation was observed in these constructs in vitro. More recently, [redacted] the development of engineered blood vessels and endothelial-lined channels. The combination of both is the next step in this field. The interaction of vessels and capillaries and their effects on the surrounding osteogenic tissue can be studied in AV loop models.

3.1.3 Research lines

[redacted], bone regenerative research lines are inspired by the two natural processes of bone formation in humans: these are the (vascularized) intramembranous and the endochondral routes that contribute to developmental bone formation and to bone healing (7). By mimicking these mechanisms - using various combinations of cells, signals and materials - [redacted] to develop bone regenerative strategies for maxillofacial applications.

The flowchart below illustrates the interaction between the intramembranous and the endochondral research lines. It further highlights the translational approach from the more fundamental in vitro evaluations to large animal models. The present project proposal will focus on the part shown in the flowchart within the intramembranous research line (outlined by red box) with experiments that will be performed in small and large animal models following 'go/no go' evaluation (*e.g.* confirmation of osteogenic and vasculogenic potential) in [redacted] established *in vitro* models.

Comprehensive flow chart showing interconnection of aims/strategies for bone regeneration at various experimental levels, ranging from a more fundamental 'in vitro' level up to a 'large animal model'. Also, the go/no go indicators are included at the arrows.

Intramembranous route in the flowchart For engineering of (pre)vascularized tissues, we can for example culture (stem) cells in hydrogels. Once non-toxicity, osteogenesis and vasculogenesis are confirmed *in vitro*, constructs can be evaluated in small animal models (rat). The sc rat model can give a first impression of potential for osteogenesis, anastomosis and functional perfusion of the prevascular network. It is also suitable as a screening model to establish optimal construct composition as multiple samples of different groups can be implanted in one animal. The AV loop model in the rat can provide insight in bone forming potential and anastomosis to a larger vessel with functional perfusion of a larger construct. Once optimal construct composition is established and bone formation and vascular perfusion are confirmed in the rat AV loop model, construct performance can be evaluated in the goat AV loop model. This model can provide insight in construct performance for tissue formation of clinically relevant size.

An example illustrating the different research levels
An example of one of the strategies for vascularized bone regeneration, , is shown below. On the far left of the figure, the classical cell culture on a flat surface can be seen. Our work focuses on the other four steps to clinical implementation:

1. Culture of cells in a threedimensional (3D) matrix made from a natural material (e.g. decellularized matrix) or from a (semi)synthetic biomaterial (e.g. functionalized hydrogel).
2. of complex geometries with embedded cells.
3. Maturation of a construct *in vitro*, e.g. by growth factor stimulation or
4. *In vivo* testing and translation of tissue engineering protocols to clinically applicable versions.

Figure from [redacted] *showing the evolutionary stages from 2D cell culture to the development of 3D tissue analogs.*

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 aim of this proposal is to create vascularized constructs for bone regeneration, with a strong translational focus on [redacted] clinical applications:

1. we aim to fabricate an extensive perfusable microvascular network, which can connect to the host vasculature *in vivo* to supply the engineered bone tissue with blood and which also supports bone formation,
2. we aim to create vessels (mm-scale diameter) that can sprout to form and/or connect to a microvascular network to support and perfuse an engineered bone constructs.

***Aim 1*: To fabricate an extensive microvascular perfusable network in an osteogenic construct**
- Select the most suitable material and fabrication technique to obtain high biocompatibility and osteoinductivity and an extensive perfusable microvascular network
- Evaluate early and late *in vivo* vascular and bone tissue formation following implantation of prevascularized constructs
- To obtain functional anastomosis of the construct's prevascular network and the host's circulation

***Aim 2:* To create a mm-scale vessel to sprout and support engineered bone**
- Determine effective construct vascularization in a construct with an inserted small diameter blood vessel (*i.e.* sprouting and connection of the vessel into the surrounding construct; AV loop model)
- To obtain functional anastomosis of the construct's prevascular network and the host's circulation
- Upscale the dimension of living constructs to a clinically relevant size (i.e. cm scale)
- Increase our understanding of the interplay of vascularization and intramembranous bone tissue regeneration

The aims are achievable in 5 years:
- At present, connection of microvascular structures to a host vasculature upon subcutaneous implantation is achievable. [redacted] also demonstrated this before [redacted] However, simultaneous bone formation is challenging. To solve this, we will adapt [redacted] approach by [redacted]. This can be done by including [redacted] biomaterials or growth factors, such as [redacted]. Also, [redacted] developed new biomaterials that better mimic the natural environment of [redacted] to stimulate the [redacted] process.

- The AV loop models have been developed and used by others (8-11).
- In addition to the establishment of *in vitro* models and *in vivo* models, the [redacted] animal models for bone regeneration (12-14). [redacted] on cartilage and bone regenerative strategies, both *in vitro* and *in vivo,* resulting in [redacted] (12-18). Since [redacted]. So far, [redacted] published [redacted] articles and her current h-index [redacted]
- The [redacted] longstanding expertise in bone regeneration research and all equipment and infrastructure required for this project are already available (the [redacted] in the new [redacted], where regenerative facilities and expertise are clustered). Further, the [redacted] surgeons are dedicated to contribute to research where applicable and will be involved in the surgical aspects of studies.
- [redacted] with other [redacted] groups are essential to this project, including the [redacted]. Further, the group also collaborates with [redacted] labs, including [redacted] will work on the project, ensuring sufficient manpower to perform the required studies. Continuous application for research funding results in regular grants enabling sustenance of our research and manpower. In total, [redacted] has secured over [redacted] of funding, of which [redacted] Part of the proposed studies [redacted] and as such accepted by international reviewers as feasible and realistic studies that can be performed within the project's time frame within the next five years.
- We have published a paper describing [redacted]

3.3 Relevance

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

Scientific relevance:
[redacted] basic and translational research to induce effective vascularized bone restoration. This approach allows [redacted] face the challenges that have so far hampered the clinical translation of the engineered cell-constructs, while collecting precious information about the complex mechanisms involved in bone tissue regeneration.
(aim 1&2) Developing for example, an in vitro vascularized tissue construct, not only provides information about the interplay between the angiogenic (vessels) and osteogenic (bone) systems, but can also play a role in improving construct survival. The fabrication of such a prevascularized construct could also impact the upscaling of any other type of engineered tissue (e.g. heart, kidney, liver) to a clinically relevant size. Further, improved understanding of sprouting behavior and vascular anastomoses can be obtained based on the proposed studies.
Taken together, these results will generate knowledge that is essential to bring vascularized bone tissue regeneration closer to clinical translation. All studies will be compiled into conference abstracts and papers to be published in respected journals in the field.

Social relevance:
From the perspective of the patient, a lot can potentially be gained by the proposed studies. It has been estimated that every year 2.2 million bone grafts are implanted worldwide, with a related cost of $2.5 billion (20). Currently available treatment strategies for non-healing bone defects include autograft and allograft transplantation. Autografts represent the best alternative because of their complete immunocompatibility and their osteoconductive and osteoinductive properties. However, this solution is far from optimal. Among its drawbacks are the limited bone availability, the need of an additional surgical procedure, which is closely related to donor site morbidity and the difficulties in modelling the graft into the desired shape (4). Likewise, allograft procedures are associated with limited bone supply. In addition, these grafts are not completely immunocompatible, pose possibility of disease transmission, and the grafting materials need to undergo chemical or physical treatment, which often undermine their osteoinductive properties (4, 5).
To overcome these limitations, a bone substitute is required. Biomaterials have been developed to successfully

restore bone defects in patients. However, biomaterials cover only an estimated 20% of the market as they cannot be used to restore large, challenging bone defects. Therefore, we aim to develop bone regenerative strategies.
The proposed research can impact current patient care standards. Few cell-based regenerative constructs are nowadays translated to clinical application. Here, we can advance the field by designing the construct in such a way that it is more tailored towards clinical application. For example, if it would be possible to engineer vascularized large tissue constructs for implantation in humans, that could impact tissue and organ transplantations in the long term. With the well-known shortage of donor organs, regenerative medicine based solutions offering completely vascularized tissues that could be implanted in a patient would open up novel treatment avenues.

3.4 Research strategy

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

The overall design of the project and strategies are presented in the flowchart included in section 3.1.3. In vitro evaluated constructs that show confirmed osteogenesis, vasculogenesis and are non-toxic can progress into the subcutaneous or AV loop model in the rats. Depending on previous experience with the materials/cells either the subcutaneous model (e.g. to screen new materials or cell types for their in vivo performance for osteogenesis and vasculogenesis) or to the AV loop model for the rat (e.g. to answer specific questions regarding sprouting from an existing vessel). Constructs that have been evaluated in the subcutaneous rat model and perform well for osteogenesis and vascularization can also be included in the AV loop model for the rat. Only constructs that have been evaluated in the rat AV loop model and have shown osteogenesis and formation of vasculature can proceed to the goat AV loop model. Finally, constructs that show confirmed osteogenesis and/or vasculogenesis can proceed from the subcutaneous rat model to the intramuscular goat or os ilium model, where the aspect of scale-up of construct size can be assessed.
Outside of the present project, cartilage-based constructs (from [redacted] project 'Cartilage-based strategies for bone regeneration in maxillofacial surgical interventions') resulting in bone regeneration in the endochondral rat models (subcutaneous and femur) can proceed to large animal models, such as the goat where an ectopic intramuscular implantation or an orthotopic os ilium implantation can be used to assess bone regeneration in large defects.

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.

General outline
To investigate the aims, different types of animal models are required. Overall, subcutaneous rodent models are required to screen for optimal construct composition for tissue regeneration. The subcutaneous models offer the opportunity to reduce the overall number of animals required, as multiple samples (up to 6) can be simultaneously implanted in one animal. Next, the optimized constructs can be evaluated in an AV loop model (typically only 1 defect per animal possible). In this AV loop model, the construct should still be effective in anastomosis/induction of the vascular network, while supporting bone formation. However, scale-up of construct size must be assessed in even larger animals, such as goat that can accommodate large, cm-scale constructs.

Screening for optimal parameters at subcutaneous locations [redacted] *in flowchart of 3.1.3)*
Subcutaneous implantation in rodents is a suitable model to study anastomosis and intramembranous bone regeneration. Due to the relatively high number of samples that can be implanted at this location, the subcutaneous model is attractive for screening of the most promising construct compositions resulting from the *in vitro* experiments. For example, it can be used for comparing anastomosis, vasculogenesis and bone regeneration for different biomaterials, after various predifferentiation periods, early and late time points, or with different cell types (12, 13).
More specifically, under general anaesthesia, up to six subcutaneous dorsal pouches will be created via 7-10 mm incisions. By limiting the total number of pouches to 6, the risk of pouch-to-pouch connection is also limited. Prevascularized samples will be inserted into the pouches that are then intracutaneously closed by sutures. For studies involving implantation of human cells, the rat species can be changed to immunodeficient animals. To monitor bone formation, the rats will be included in the experiment for up to 12 weeks. Similarly, in the large animal models, subcutaneous and intramuscular pouches could be used to optimize various construct parameters specifically for larger samples for up to 24 weeks.

AV loop models [redacted] *in flowchart of 3.1.3)*
Vascular invasion models (to study an *in vivo* vascularization strategy) in mammals can entail relaying or transplanting an existing millimeter scale vessel of the host through an (osteogenic) construct. Sprouting and vascular migration of the vessel into the construct and subsequent bone regeneration can be studied in this model. An example is the arteriovenous loop (AV loop) model that can be inserted in a large animal model (8). Recently, this model was also extended to reconstruct goat mandibles (9). Additionally, anastomosis of capillary-like vasculature can be studied following subcutaneous implantation, as already mentioned under the subcutaneous models.
The AV-loop model is an intrinsic prevascularization method, in which an arteriovenous loop is used to improve initial vascularization of a (bone) construct. In more detail, an artery and vein are anastomosed in a loop to increase blood perfusion in the vicinity of the loop. The loop can be constructed by re-routing a vessel from the animal through the construct or by harvesting a vessel from another location and transplanting to the site where the construct is implanted. For example, in rats the AV-loop can be constructed from the femoral artery and vein with an interposed femoral vein graft, harvested from the contralateral side. A first screening of various construct compositions could be performed in this small animal model for up to 12 weeks. Once the most promising conditions are defined (*i.e.* combination of cells, materials, and/or growth factors), a goat model will be used to study feasibility of regeneration by larger constructs for up to 24 weeks.
This model could be combined with the intramuscular and the iliac crest model [redacted] in flowchart in 3.1.3) where required.

Readouts
The *in vivo* responses in terms of bone formation can be monitored by micro-CT imaging in the rat models. For rats and larger animals, fluorochrome injections can be an additional means to assess bone formation at various intervals during the experimental period without euthanizing animal at every time point (21). All other readouts (including end point micro-CT, visualization of vascularization by MICROFIL®, immunohistochemistry of tissue development and gene expression analysis) are obtained post-mortem.

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 coherence has been detailed in section 3.1.3 beneath the flowchart. Briefly, all questions related to vascularization of engineered bone constructs and anastomosis will be addressed in the subcutaneous, intramuscular, orthotopic or AV loop model in the rat. Once novel cell and/or material combinations have resulted in successful creation of prevascular microstructures and osteogenic differentiation in our established *in vitro* models, validation of the potential of a microvascular network to connect to the host's vasculature and to be perfused functionally upon implantation can be performed in the subcutaneous rat model. These microvascular samples could be introduced in the AV loop model where connection to a larger vessel can be established. Yet, even larger samples could be generated and evaluated in the large animal models in an orthotopic location (iliac crest), intramuscular (ectopic) or in combination with an AV loop.

The overall project includes a first screening step in the rat models. This screening step will ensure that only the most promising approaches will be further developed and optimized for studies in larger animal models.

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	Subcutaneous construct implantation in rats
2	AV loop model in rats
3	Intramuscular+ orthotopic+ AV loop model in goat
4	
5	
6	
7	
8	

9	
10	

Centrale Commissie Dierproeven

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'.	11500
1.2	Provide the name of the licenced establishment.	UMC Utrecht
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: 3.4.4.1 Type of animal procedure: Subcutaneous construct implantation in rats

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.

Experimental approach:
The aim of this project is to develop strategies to improve the characteristics of the currently available bone substitutes. Since different cell types, scaffold materials and growth factors will be optimized and combined in different ways, a screening of the most promising conditions needs to be performed. Subcutaneous pouches are the ideal model as they have been reported to be an adequate model to evaluate ectopic bone formation [1,2]. Besides, the model allows for the screening of several conditions at the same time, reducing the required amount of animals to a minimum. Further, the independence of the individual pouches will be maximized by limiting the total number of pouches to be created to six and by making one incision to form each pouch.

During the surgery, a maximum of six subcutaneous dorsal pouches will be created per rat. This model will be used for the first aim of the project:

- To fabricate an extensive microvascular perfusable network in an osteogenic construct

The criteria that need to be met *in vitro* before moving to this *in vivo* model are described in section 3.1.3 of the project proposal. The animal type (immunocompetent or immunocompromised) will be chosen according to the specific research question, *e.g.* implantation of [redacted] in immunocompromised animals.

Outcomes:
Primary outcome measures

- The evaluation of ectopic bone formation *in vivo* will be the primary outcome. It will be assessed by microCT analysis, both at early and late timepoints.
- A second primary outcome measure could be included; the assessment of vascularization by

MICROFIL®.

Secondary outcome measures

- Bone formation: Fluorochrome labels might be administered at different timepoints after the implantation to establish the onset of bone formation. After the explantation, *ex vivo* analysis will be performed to complement the information obtained with the microCT. In particular, bone content and remodelling will be evaluated via histological and immunohistochemical analysis (*e.g*. H&E, safranin-O, TRAP staining, collagen type I).
- Vascularization: After explantation of the constructs, histological and immunohistochemical analysis will be performed to evaluate blood vessels network growth and sprouting (*e.g.* H&E, CD34).

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

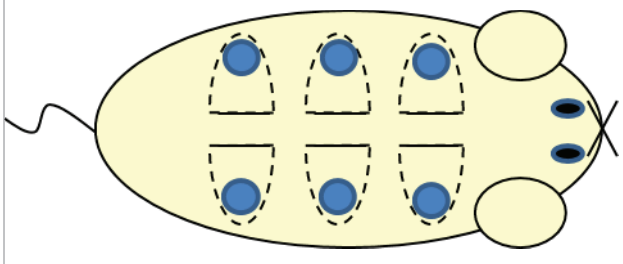
Animal procedures (12,13)
The animals are allowed at least one week of acclimatization before the start of the experiment. Open or filter top (in case of immunocompromised rat strain) cages will be selected according to the rat strain that is used and will be specified in the work protocol. The rats will always be housed in pairs, except for up to 3 days after the operation to allow for wound healing. The animals will be maintained on rodent chow and water *ad libitum*.

Anaesthesia protocol
All operations will be performed under adequate anaesthesia.

Pain management
Analgesia is provided by a subcutaneous injection of pain medication, before surgery and daily until 3 days after surgery.

Antiseptic techniques
All operations will be performed under aseptic conditions. The surgeon will wear scrubs, gown, surgical mask, head cover and gloves. After shaving, the skin will be disinfected with ethanol and the surgical site will be draped. To reduce perioperative infection risk, all rats receive a dose of antibiotics before surgery. All the surgical equipment will be sterilize before the surgery.

Surgical technique
As shown in the figure below, a maximum of 6 dorsal pockets (dotted lines) are created, each by blunt dissection through one skin incision (from 7 to 10 mm, solid black lines at pocket) and filled with one implant (blue circles). The skin will be closed transcutaneously with resorbable sutures.
Total operation time per animal: 30 minutes



Postoperative care
The animals are postoperatively treated with the analgesic as previously described. In case of failing sutures in unclosed wet wounds, the wound will be cleaned, debrided and re-sutured under adequate anaesthesia. The wounds will be examined daily for three days following surgery, thereafter weekly.

Bone regeneration measurements
Where applicable, fluorochromes will be administered by subcutaneous injection. For the *in vivo* bone formation analysis by microCT, rats are sedated using proper anaesthesia.
Total handling time per animal: 15 minutes

Euthanasia protocols
The rats will be euthanized according one of the methods listed in the appendix IV of directive 2010/63/EU. Methods will be selected based on their influence on vascular interference and compatibility with MICROFIL injection. The implants will be retrieved afterwards.

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

The required amount of animals has been selected based on previously published studies (12,13).

- To fabricate an extensive microvascular perfusable network in an osteogenic construct

  The criteria required to start an *in vivo* experiment using this model are descibed in section 3.1.3 of the project proposal. Here a schematic representation of the variables that are taken into account in this aim.

Variables		
1) Biomaterials (e.g. hydrogels, synthetic polymers, osteoinductive materials)	2) Progenitor cells (e.g. osteoprogenitor, endothelial progenitor cells)	3) Growth factors (e.g. chemoattractants, VEGF, BMPs)

  In a typical experiment, these three variables are combined. For example, in an experiment based on the 3 listed variables a maximum of four different groups, including the controls conditions can be made (biomaterial only, biomaterial + progenitor cells, biomaterial + growth factor, biomaterial + progenitor cells+ growth factor). Two additional groups can be added in case sub-variables are included (e.g. two different progenitor cells type or two different growth factor concentration). Therefore, in each experiment, 4 to 6 different experimental conditions and controls can be included.
  Based on previously published studies (12,13) the power analysis has been performed using an online tool (http://homepage.stat.uiowa.edu/~rlenth/Power/). For the statistical analysis a one-way anova with Tukey/HSD post hoc correction has been performed. A sample size of maximum 6 implants per condition was defined to be able to detect a contrast of 30% with a power of 82%, a standard deviation of 0.13 and α=0.05.

  Based on this sample size, a maximum amount of constructs per experiment can be determined: 4 to 6 conditions * 6 (n)= 24 to 36 implants in total per experiment.
  Number of animals required: 24 to 36 implants/ 6 implants per rat= 4-6 rats per experiment, considering a drop out of 5% of the implants (based on previous experience), 2 to 3 extra pockets might be required. For this reason a maximum of 5 to 7 animals are required per experiment.

  For a complete screening it is expected that several combinations of the three variables have to be compared as new biomaterials, growth factors and cell types are continuously developed and detected. The basic format of one experiment as outlined above will be repeated for different biomaterials, cell types or stimuli. As part of our ongoing research we expect to discover 1-2 promising new variables each year that will have completed the *in vitro* evaluations and have passed the go/no go criteria. So in total, for a five year period we would need to perform a maximum of 10 experiments, involving 5-7 rats each. This would amount to a total number of 70 rats. Furthermore, the exact amount of animals per experiment will be defined consulting a statistician and the animal welfare body and it will be specified in the work protocol.

Total number of animals required for this appendix (including the drop out rate): 70

B. The animals

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

A total of 70 animals is required. The appropriate animal strain will be chosen according to the specific type of graft that is implanted during the experiment (e.g. biomaterial only or non-autologous cell seeded constructs) and they will be purchased from a registered breeder (e.g. Charles River). To have sufficient space between the

pouches and avoid potential interference, skeletally mature rats are required. For this reason, only rats older than 12 weeks at the time of implantation will be used. Further, male and female rats show differences in bone metabolism (22,23,27,28), the use of a mixed population will cause a stronger increase in the observed variance. Since estrogen levels in female rats depend on stress and age (24) and can affect bone regeneration (25,26), male rats have been identified as the most suitable model.

- Male rats older than 12 weeks selected from an outbred colony (e.g. Wistar) will be used when a broad screening is required. These rats are a suitable screening model to test tissue regeneration in a cell-free approach.
- Male rats older than 12 weeks selected from an inbred strain (e.g Fischer 344, Brown Norway or Lewis) will be used when syngeneic transplantation of bone marrow derived stem cells needs to be performed. Further, less variation can be expected when this animal model is used, compared to the outbred colony rats. For this reason, valuable information can be collected using an inbred animal model during the screening phase.
- Male rats older than 12 weeks selected from an inbred nude strain (RH-Foxn1rnu rats) will be used when immunodeficient animals are required, [redacted] will be implanted (e.g. human cells)

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

No *in vitro* options are currently available to fully recapitulate the complexity of new bone formation or the combination of all the players involved in new tissue vascularization. Further, connection to the blood supply and invasion of (stem) cells from the host can only be mimicked in an animal model. For these reasons, the use of an animal model is a necessity in order to test the regenerative potential of the developed constructs.

Reduction

The available *in vitro* methods to pre-screen cell behavior and cytocompatibility of the constructs will be part of the evaluations before progressing to animal studies, as shown in the flow chart in the appendix. This will ensure that only a promising selection of constructs will progress to the stage of implantation, thus reducing the number of animals used. By performing the first screening in a subcutaneous model, fewer animals are required during the optimization process. Furthermore, 6 different conditions can be tested in one animal. In this way, only the promising substitutes will be tested in an orthotopic defect model (one per animal).

Refinement

- Subcutaneous model has been selected as an ideal model for this first screening phase because it allows to evaluate the regenerative potential of the implants (evaluating the ectopic bone formation). At the same time, it allows to compare multiple conditions within an animal, reducing the total amount of rats required.
- *In vivo* bone formation analysis will be performed using a microCT under adequate anaesthesia.
- Follow-up will be daily for one week after surgery, after that a minimum of one time weekly.
- The researches will be trained in order to perform the implantation as atraumatic as possible.

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

on the environment.

To reduce animal fear, the researcher will get the animals used to their presence, practicing the handling procedures before the surgery. During surgery, respiration will be observed continuously and both before and after surgery the animals will be placed on a heating mat. The rats will be returned to routine housing after they have recovered from anaesthesia and paired once the wounds are closed. Standard housing conditions will be allowed post-op.

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.

Analgesia is provided by a subcutaneous injection of pain medication, before surgery and twice per day until 3 days after surgery. Where applicable, additional local analgesics might be used. Adequate anaesthesia will be provided during the surgery and every time it will be needed (*e.g.* during microCT scan).

I. Other aspects compromising the welfare of the animals

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

Adverse events:
1) Skin irritation due to subcutaneous injections: mild (expected occurrence based on previous experients: 5-10%)
2) Rats might bite and remove the stitches before the wounds are completely closed: mild, might cause irritation and a delay in wound closure (expected occurrence based on previous experiences: 5%)
3) Infection might occur at the surgical site: mild to moderate according to the infection (expected occurrence based on previous experiences: 1%)

Explain why these effects may emerge.

It has been observed that the animals might show some irritation due to the subcutaneous injections, especially when fluorochromes are used to assess bone formation. If this is the case, a different location will be used for the following injections. Rats might also bite and remove the sutures before the wounds are completely closed. This might cause a delay in wound closure and/or infection. In case of failing sutures in unclosed wet wounds, the wound will be cleaned, debrided and re-sutured under adequate anaesthesia. Only in case of falling out of the implant outside the pocket, the sample will be excluded. The other pockets from the same animals will still be included, unless the human end-point has been reached.

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

Animal's breathing will be regularly checked during and after surgery and every time anaesthesia is induced. The adequate depth of narcosis will be monitored by observing the animal's respiration and testing whether the animal is still awake before starting any procedure. Animals will be housed singularly for up to 3 days after the surgery to allow full wound closure. Tools used during the surgery are sterile, to prevent an infection to occur.

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.

Rats will be closely monitored on general well being. If abnormalities are observed, the veterinarian will be consulted, and based on the severity of discomfort the animal will be euthanized.

Complications that have been observed with this model in the past:
- Rarely, after implantation, a local infection occurs.
- Also, irritation after injections may occur.
- Rats might chew on sutures and/or implanted samples

The animal will be euthanized if scoring ≥2 in the following list: infection of surgical sites (2), visible spine or ribcage (2), panting (1), salivation (1), immobility (2), persistent tremors (2), persistent convulsions (2), self-mutilation (2), pilo erection (2), abnormal posture (1).

Furthermore, the weight of the animals will be closely monitored and will be used as an additional indicator of animal (dis)comfort. A relevant unacceptable weight loss within a few days in comparison to its peers will be considered a humane endpoint.

Indicate the likely incidence.

5%

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').

Expected discomfort:
1) Animal discomfort due to the eventual presence of filter top cages: mild
2) Animal discomfort due to the handling: mild
3) Animal discomfort due to the subcutaneous injections: mild
4) Animal discomfort due to the surgery: moderate
5) Animal discomfort due to the anaesthetic induction: mild
6) Animal discomfort and pain postoperatively due to the bone substitute implantation with adequate pain

medication: mild

7) Animal discomfort due to the euthanasia under anaesthesia: mild

Cumulative discomfort:
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 implanted constructs need to be explanted for further analysis.

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

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

☒ Yes

Centrale Commissie Dierproeven

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'.	11500
1.2	Provide the name of the licenced establishment.	UMC Utrecht
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: 3.4.4.2 Type of animal procedure: AV loop model in rats

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.

Experimental approach:
The creation of an arteriovenous (AV) loop model for bone engineering is thought to be suitable for direct anastomosis and improvement of blood circulation in the implanted osteogenic construct, immediately after implantation. In this way, sprouting and blood vessel invasion is known to be faster, reducing the formation of a necrotic core in the implant. This model will be used as a screening step to identify which combination of scaffold, cell types and growth factors will most benefit vasculogenesis/angiogenesis and bone formation inside implanted constructs. The criteria that need to be met *in vitro* before moving to this *in vivo* model are described in section 3.1.3 of the project proposal.

The AV-loop model will be used for the second aim of the project: to create a mm-scale vessel to sprout and support engineered bone. The rat type [redacted] will be chosen depending on the required implantation [redacted] on the specific research question.

Outcomes:
Two main outcome parameters will be evaluated in this model: blood vessel sprouting and new bone formation. Both parameters will be evaluated at early and late time points.

Vessel outcomes:
- Primary outcome: The vascular structures will be evaluated with microCT using MICROFIL® after the end of the experiment.
- Secondary outcome: The organization and quality of the vascular network will be investigated by (immuno)histochemical staining techniques for several markers involved in vascularization.

Bone outcomes:

- Primary outcome: *In vivo* bone formation will be assessed by microCT analysis.
- Secondary outcome: Fluorochrome labelling will be administered at various time points after the implantation to monitor the progression of bone formation.
- Secondary outcome: Histology of the explants will confirm the nature of mineralized tissue as detected by micro-CT. Paraffin and/or MMA-embedded tissue sections will be stained with (immuno)histological stainings to identify the bone tissue and associated cells. After the explantation, *ex vivo* analysis will be performed to complement the information obtained with the microCT. In particular, bone content and remodelling will be evaluated via histological and immunohistochemical analysis.

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

Animal procedures (29)
The rats are allowed at least one week of acclimatization before the start of the experiment. Open or filter top cages will be selected according to the rat strain that it is used and will be specified in the work protocol (e.g. filter top cages will be used in case of immunocompromised animals). They will be housed in pairs, except for 2-3 days following surgery. Rats will be maintained on rodent chow and water ad libitum.

Anesthesia protocol
All operations will be performed under adequate anaesthesia specific for the animal species and the procedure.

Pain management
Adequate analgesia is provided to the animals before induction of the surgery and afterwards to minimize the discomfort of the animals.

Antiseptic techniques
All operations will be performed under aseptical conditions. The surgeon will wear scrubs, gown, mask and gloves. After shaving of the animal, the skin will be disinfected with ethanol and the surgical site will be draped. To reduce perioperative infection risk, all rats receive a single dose of antibiotics before surgery. All surgical equipment will be sterilized before the surgery.

Surgical techniques
AV loop model: This procedure has already been performed and described by [redacted] groups (29,30).
Researchers carrying out the procedure in our group will be trained in microsurgery.

A femoral vein graft (approximately 10 mm) will be harvested from the groin and flushed with heparinized saline solution. This graft can be used to create an AV-loop; an analogous procedure is performed to expose the inguinal ligament and the femoral vessel on the contralateral limb. Using microsurgery tools, the femoral artery and vein are cut transversely and the blood is flushed out. The previously prepared vein graft is anastomosed to the recipient femoral vein and artery. A synthetic chamber *can* be introduced to keep the AV-loop in place and to create an niche in which hydrogels, cells and/or growth factors can be loaded. The AV-loop will then be positioned in the tissue chamber. The chamber will be secured on the underlying muscular fascia with sutures. After insertion of the implant into the defect, the fascia and skin are sutured on both sides in layers (30).
Future tissue engineering perspectives are on the generation of vascularized bone tissue by incorporation of a synthethic vascular graft. As such, no autologous vessels from the other limb are necessary anymore for the creation of an AV-loop in an osteogenic construct. Therefore, also synthethic vascular grafts will be used for the creation of an AV-loop in a defined chamber or used as a graft for end-end artery-artery or vein-vein anastomosis, *eg.* in the groin or abdominal aorta. The dissection method will be the same as the one stated above; only, the use of the femoral vein from the donor leg will not be necessary anymore as it will be replaced by the synthethic vascular graft. Alternatively, this model with a straight vascular supply might be used instead of the AV-loop model for investigating optimal construct compositions.
Estimated time of surgery per animal: 1.5 hours.

Postoperative care
The rats are postoperatively treated with the analgesic as previously described. They will be housed separately

until wounds are closed (typically 1-3 days). After, they will be housed in pairs, keeping the same pairs before and after surgery. In case of failing sutures, the wound will be cleaned, trimmed and re-sutured under anesthesia. The weight of the rats will be examined closely after surgery.

Bone regeneration measurements
For the *in vivo* bone formation analysis by microCT, the rats are sedated (approximate handling time: 15 minutes). Furthermore, depending on the specific research question, they might receive fluorochrome marker injection at varying time points, postoperatively.

Euthanasia protocols
The rats will be euthanized according to one of the methods listed in the appendix IV of directive 2010/63/EU. Methods will be selected based on their influence on vascular interference and compatibility with Microfill injection. The implants will be retrieved afterwards.

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

A description of the different phases in this project is described below. Groups will be formed with variations in hydrogel, progenitor cells and/or growth factors and synthetic grafts, and inserted in the chambers. In the table, a schematic representation of a possible combination of the variables is displayed:

Variables		
1) Biomaterials (e.g. hydrogels, synthetic polymers, osteoinductive materials, synthetic vascular graft)	2) Progenitor cells (e.g. osteoprogenitor, endothelial progenitor cells)	3) Growth factors (e.g. VEGF, BMPs)

An example of a power analysis on a possible experiment is made for phase 2, based on vascular outcomes [34]. For example, 4 groups can be made, based on the 3 variables that can be combined (hydrogel (control); hydrogel + cells; hydrogel + growth factor; hydrogel + growth factor + cells) with an additional 2 groups if we have additional sub-variables (eg. extra cell types or synthetic vessel graft); this thus ends up to a maximal of 6 groups. A power analysis (one way anova; Tukey) was performed based on the vasculature outcome parameter at the end of the follow up period. The power analysis was performed according to the Lenth, R. V. (2006-9. Java Applets for Power and Sample Size [Computer software]; Retrieved March 22nd, 2017 from http://www.stat.uiowa.edu/~rlenth/Power). A detectable contrast of 30% is set, with a standard deviation of 0.17. The number of comparisons is thus set at 6. A total amount of 12 rats per experiment is calculated, with an additional drop-out rate of 2 animal already included (based on both bone and vascular outcome parameters (31). Calculations and drop-out rates were made, based on publications of the group of Andreas Arkudas, M.D., and Ulrich Kneser M.D (for example: (31)).
All calculations above were based on this paper and made for both the osteogenic and vasculogenic primary outcome parameters: they did display the same n of animals. The amount of animals per comparison typically used in the described experiments of Arkudas *et al* range from 6 to 19.

The highest number of animals for this experiment then includes 12 * 6 = 72 rats. Both phase 2 and phase 3 are expected to comprise 2 (independent) experiments. The estimated maximum amount of animals for all phases (n=12) is expected to be 4 experiments * (12 (n) * 6) = 288 rats. Additionally, to perfect surgical skills the pilot phase (phase 1), 2 (researchers with microsurgical course) *4 (animals to practice) = 8 practice rats will add this amount up to 296 rats. The exact amount of animals and groups of each experiment will be defined consulting a statistician and the animal welfare body and it will be specified in the work protocol.

- **Pre-experimental phase**
  The researchers carrying out this procedure will be trained in microsurgery for a week. Moreover, pre-experimental practicing will be carried out on cadavers (4 animals; surplus rats from other groups). Only cadavers with hind legs and blood vessels that are not affected in the previous handlings will be included. Handlings that will be practiced here involve isolation of the femoral vein, connection to femoral artery and suturing techniques or anastomosis with a synthetic graft. Also, the placement and attachment of the chamber will be will be optimized in this step. This extra step will contribute to

refinement of the procedures and of the experiments.

- **First phase – Pilot**
  The pilot phase is divided in 2 parts with both its own group of rats (4 rats per group).
  First, the procedure of anastomosis (autologous or synthetic graft) will be optimized in living animals. Possible problems with blood flow and vessel leaking, such as thrombosis, will be identified in this phase. No chamber (previously described) will be used in this group. Animals will be observed over a short time period.
  After success in the first group, a second group will be operated in which the procedure will be elaborated by means of addition of a sample chamber (as described in 'surgical techniques'), with or without hydrogel addition. Possible problems with irritation of the chamber on the hind leg will be observed in this phase. Animals will be observed over a short time period.

- **Second phase**
  The different experiments will not be carried out at the same time in the animals, but instead, independent experiments will be performed over time. The different experiments will contain different variables and sub-variables (eg. other progenitor cells and/or growth factors). In this long-term phase of the study, the experiments will be used to examine the influence of growth factor (involved in vasculogenesis/angiogenesis) and/or cells in the chamber on vascular sprouting. Therefore, in this phase groups can be formed based on defined combinations of biomaterials, endothelial progenitor cells, growth factors and/or synthetic vascular grafts.

- **Third phase**
  Also here, the different conditions will not be carried out at the same time in the animals, but instead, several independent experiments will be performed over time with different variables. In the long-term third phase of the experimental set up, the influence of growth factor (involved in osteogenesis) and/or cells in the chamber on vascular sprouting and osteogenic differentiation will be evaluated. We will continue with the optimal condition for vascular sprouting found in phase 2. Therefore, the final combinations of the groups to be assessed, should be determined after the results of phase 2. Groups can for example be formed based on combinations of hydrogels, (osteogenic) progenitor cells, growth factors, synthetic vascular grafts and/or calciumphosphates.

B. The animals

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

A maximum of 296 rats will be included in this appendix.

- Skeletally mature rats are needed for this model, therefore 12 week old male rats will be selected from an inbred rat strain (e.g. Fischer 344, Brown Norway or Lewis) and will be purchased from a registered breeder (eg. Charles river). The male rats have been identified as a suitable, reliable and reproducible model when immunocompetent animals are needed, as stated in references (29, 30,32). Only male rats will be selected as there needs to be sufficient room for the placing of the chamber in the groin; the smaller skeletal size of female rats will not provide this needed space. Furthermore, male and female rats show a different bone metabolism (22,23, 27,28). When using a mixed sex population, this will be likely to cause a strong increase in the observed variance. We therefore aim to use male rats only.
- 12 week old male rats from an inbred nude strain (RH-Foxn1rnu rats) will be used when immunodeficient animals are required. This model is the best option if the influence of the immune response on bone tissue regeneration is not the main research question and when xenogeneic or allogeneic cells or tissues are implanted.

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
Extensive *in vitro* research has been carried out on the subject of osteogenic differentiation in combination with vasculogenesis/angiogenesis (3D hydrogel co-cultures) and bloodvessel engineering in our lab. No *in vitro* options are currently available to fully recapitulate the complexity of new bone formation and remodelling, to model the complex interactions of the regenerative process with the immune system, or to evaluate the integration of the capillaries to the host circulation. Therefore, the use of an animal model is fundamental.

Reduction
The number of rats cannot be reduced more than has been done already, as only 1 construct per rat is feasible. Inclusion of more chambers per rat will lead to elevated discomfort for the animal. Moreover, only 1 donor vessel can be harvested per animal in the groups without the synthethic graft. Reduction of the amount of animals is achieved by having the *in vitro* 3D hydrogel model in our lab, for testing of progenitor cell combinations in combination with growth factors. With this pre-selection method, only promising combinations will be used in the transition from *in vitro* to *in vivo.*

Refinement
This model has already been described in papers of other groups; their experience will help us with refining the operation and we can build on their experience to determine the most innovative groups, type of implants and defining the outcome parameters. Also, the project has been built up in different phases. Only the best results from phase 2 will be used in phase 3, leading to refinement of the project and reduction of the number of animals. Moreover, considering the complexity of the surgery, the researchers will be extensively trained in order to reduce the implant failure to the minimum. During the follow up time, the animals will undergo microCT (under adequate anasthesia), therefore data will be collected on bone formation at various time points without the need for additional animals.

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

The rats will have at least 1 week to acclimatize to the new environment before surgery. To reduce animal fear, the researcher will get the animals used to their presence, practicing the handling procedures before the surgery. During surgery, respiration will be observed continuously and both during and after surgery the animals will be placed on a heating mat. The rats will be returned to routine housing after they have recovered from anaesthesia and will be paired once the wounds are closed. They will be maintained on rodent chow and water ad libitum. Cage enrichments and bedding materials will be provided. For specific information see refinement.

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.

Adequate analgesia is provided before and after the surgery. Further, adaquate anaesthesia is provided during surgery and when required (*e.g.* during microCT)

I. Other aspects compromising the welfare of the animals

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

Adverse events that might occur:

1) Skin irritation due to subcutaneous injections: mild
2) Rats might bite and remove the stitches before the wounds are completely closed: mild, might cause irritation and a delay in wounds closure
3) AV loop occlusion or failure (20% rates have been reported previously): moderate pain, might reduce the mobility of the animal (29,32)
4) discomfort when walking: mild
5) Infection might occur at the surgical site: mild to moderate according to the infection

Explain why these effects may emerge.

1) It has been reported before that subcutaneous injections might cause skin irritation, specifically for fluorochromes. If this is the case, a different location will be used for the following injections.
2) Rats might also bite and remove the sutures before the wounds are completely closed. This might cause a delay in wound closure.
3) It has been reported before that the creation of an AV loop can lead to thrombosis and occlusion of the vessels due to the handling of the blood vessels and the disturbance of the blood flow (29,32).
4) discomfort with walking can be caused by the placement of the chamber in the groin
5) Infection at the surgical site can occur due to unsterile handling of due to biting on the wound.

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

Animal's breathing will be regularly checked both during and after the surgery and every time anaesthesia is induced. The adequate depth of narcosis will be monitored by observing the animal respiration and testing

whether the animal is still awake before starting any procedure.
2) Animals will be housed singularly to prevent biting on each other wounds, and will be housed together as soon as the wounds are healed.
3) To prevent vessel occlusion or the formation of thrombi, microsurgery tools will be used. Further, researchers will follow an adequate microsurgery training, to handle the vessels in the least traumatic way possible.
4) The circulation of the lower limb will be closely monitored by gross inspection, in particular signs of swelling or oedema will be examined. If an unexpected adverse event will occur, the adequate actions will be taken, in consultation with the veterinary where required (see the section below).
5) Tools used during the surgery are sterile, to prevent infections.

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.

Rats will be closely monitored on general wellbeing. Also, weight loss will be monitored as an indication when assessing the humane endpoint. If abnormalities are observed, the veterinarian will be consulted, and based on the severity of discomfort the animal will be euthanized.

Local infection or failure of the vessel anastomosis might occur. The animal will be euthanized if scoring ≥2 in the following list: infection of surgical sites that can not be treated with antibiotics (2), weight loss during the entire length of the experiment (visible spine or ribcage (2)), panting (1), salivation (1), immobility (2), persistent tremors (2), persistent convulsions (2), self-mutilation (2), pilo erection (2), abnormal posture (1), oedema and swelling (1-2 according to the severity).

Indicate the likely incidence.

<20% (32,34,35)

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').

Expected discomfort:

1) Animal discomfort due to the eventual presence of filter top cages: mild
2) Animal discomfort due to the handling: mild
3) Animal discomfort due to the subcutaneous injections: mild
4) Animal discomfort due to the surgery: moderate
5) Animal discomfort due to the anaesthetic induction with isoflurane: mild
6) Animal discomfort and pain postoperatively due to the AV loop formation and vein graft harvest with adequate pain medication: moderate
7) Animal discomfort due to microCT analysis: mild
8) Animal discomfort due to the euthanasia under anaesthesia: mild

Cumulative discomfort:
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 euthanised at the endpoint as it is necessary to explant the construct to perform histological analysis

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

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

choice.

☒ Yes

Appendix
Description animal procedures

- This appendix should be enclosed with the project proposal for animal procedures.
- A different appendix 'description animal procedures' should be enclosed for each type of animal procedure.
- For more information, see our website (www.centralecommissiedierproeven.nl).
- Or contact us by phone (0900-2800028).

1 General information

1.1	Provide the approval number of the 'Netherlands Food and Consumer Product Safety Authority'.	11500
1.2	Provide the name of the licenced establishment.	UMC Utrecht
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: 3.4.4.3 Type of animal procedure: Intramuscular+orthotopic+AV loop model in goat

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.

One goat can accommodate three different types of defect and/or surgical procedures:

1) arteriovenous (AV) loop in a bone defect (orthotopic)
2) bilateral 17 millimetre diameter critical size os ilium defect (orthotopic)
3) a maximum of four implants positioned in the paraspinal muscles (ectopic)

The three defect types will be seen as independent experiments

The use of a large animal model is required in order to assess the feasibility of scaling up the size of a bone regenerative construct to clinically relevant dimensions (e.g. at least 1cm^3). Studies involving a rat AV loop model (described in 3.4.4.2) will precede the goat studies described here. Overall, the size of the defects in the goat model and their rate of bone formation comparable to humans, make this an attractive bone regenerative model. Large samples can be implanted to investigate if inclusion of an AV loop or other construct properties can enhance bone regeneration in large samples. Typically, implantation of small samples in small animal models cannot answer these dimensional questions.
The combination of implantation at orthotopic and ectopic locations allows the comparison of implant performance in an osteoconductive and a non-osteoconductive environment.
The three surgical procedures will be performed during the same surgical session, obviating an additional anaesthetic procedure. This will reduce the discomfort of the involved animals.

The above porcedures will be used for the second aim of the project, to create a mm-scale vessel to sprout and support engineered bone

Outcomes:
The main goal of this step is to evaluate the regenerative potential of the developed constructs when focusing on upscaling of implantation size. The primary outcome that will be evaluated is new bone formation. To establish the onset of bone formation, fluorochrome labels (secondary) might be administered at different time points after implantation. Additionally, when an AV loop is included in the study, sprouting and new vessels formation can be evaluated using MICROFIL®. After the explantation, *ex vivo* analysis will be performed to complement the information obtained via X-ray. In particular, bone content and remodelling will be evaluated via histological and immunohistochemical analysis.

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

Animal procedures (33,34)
The animals will be housed together at the Central Laboratory Animal Institute (Utrecht University) for the entire duration of the study, in accordance with Annex III of the Directive 2010/63/EU, in an establishment that is licenced by the Dutch authorities (NVWA). One week is given as acclimatization period before the start of the experiment. The goat will be kept in social housing with enrichment (e.g. toys and bedding material) and sufficient freedom of movement will be guaranteed. Each animal will be identified with an ear tag and unique code. Food and water are provided ad libitum besides up to 24h prior to operation, to limit regurgitation (10).

Anesthesia protocol
All operations will be performed under adequate anaesthesia specified for the animal and the procedure. Their vital parameters will be monitored constantly.

Pain management
Adequate analgesia is provided to the animals before induction of the surgery and afterwards to minimize the discomfort of the animals.

Antiseptic techniques
The surgery procedure will be performed under aseptical conditions. The surgeon will wear sterile scrubs, gloves, mask and gown. After shaving, the skin will be disinfected with iodine and the surgical site will be draped. To reduce perioperative infection risk, animals will be treated with prophylactic antibiotics once before surger. In addition, antibiotics will be administrated.

AV loop surgical technique, Os ilium defect and intramuscular implantation.
AV loop model: This model has been adapted from previously described procedures (8,9). A long skin incision is made in the left submandibular region. After identification of the facial vessels, a critical size defect is created using a custom-made drill. The implant (content is based on findings described in the procedures for the rat AV-loop model and previous described findings (8,9)) will be fixed to the mandible with screws and, using a surgical microscope, the facial vessels will be anastomosed in order to create an AV fistula around the implant or within a special groove. The wound will be closed in layers. The same procedure can also be performed in the contralateral hemimandible (10).

Os ilium defect: The surgical procedure has been already performed successfully and described in detail previously (34). Two central skin incisions of the dorsal lumbar area are made to expose the iliac crest, it is cleared of muscle tissue and the os ilium is exposed by elevation of the periosteum. The intended defect location is on the dorsal and lateral side. A critical size defect is performed using a custom-made drill. During the drilling, a constant cooling with sterile saline will be performed. The explanted bone might be used as autograft control, whereas the implant is press fit into the defect. The periosteum will be sutured back to its original position to keep the samples in place. The muscles, the fat tissue and the skin over the defect are going to be sutured using resorbable sutures.
Intramuscular defect: In the shaved lumbar area, skin incisions are performed to create pockets. The muscle fascia is exposed and cut. Using blunt dissection, an intramuscular pocket is created. After filling the pockets, the fascia and the skin will be closed with resorbable suture(33).

Estimated operation time: 4 hours (if receiving all three defect types)

Postoperative care
The goats are postoperatively treated with analgesics and antibiotics. The animals will be housed separately postoperatively for approximately 1 day, after which they will be housed together. In collaboration with the Central Laboratory Animal Institute staff members, animals will be closely monitored on general wellbeing after surgery. Additionally, their weight will be monitored weekly throughout the study. When surgical complications are encountered in one or more animals, risk of severe discomfort will be discussed with the veterinarian. In case of AV loop implantation in the mandible, only oral fluids will be allowed during the first postoperative day, with gradual return to normal diet. Unrestricted weight bearing and activity will be allowed post-op.

Bone regeneration measurements
According to the specific research question, goat might receive fluorochrome markers at different time points.

Euthanasia protocols
After sedation, animals will be euthanized according one of the methods listed in the appendix IV of directive 2010/63/EU. Methods will be selected based on their influence on vascular interference and compatibility with Microfill injection. Subsequently, the tissues of interest will be retrieved and their analyses will be performed post-mortem.

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

An example of a power analysis on a possible experiment is made, the total amount of animals is based on this estimation. Where possible, the three different models (i.e. AV loop, iliac crest, intramuscular) will be combined in the same animals to reduce the total number of animals involved in the study. The three different models will be seen as independent experiments, and thus not compared to each other; comparisons will be made within one model/experiment-type. Variables which can be included in the constructs are displayed in the table below. Since several screening and optimization steps will be performed in rodents (appendix 3.4.4.1&2), only the most promising conditions will be tested in this large animal setting.

Variables		
1) Biomaterials (e.g. hydrogels, synthetic polymers, osteoinductive materials, synthetic vascular graft)	2) Progenitor cells (e.g. osteoprogenitor, endothelial progenitor cells)	3) Growth factors (e.g. VEGF, BMPs)

To determine the required amount of animals, a power analysis based on a previously published study (bone outcome parameters) was performed (34) (with online tool at http://homepage.stat.uiowa.edu/~rlenth/Power/). The sample size has been calculated considering the AV loop model as the limiting element, since it is possible to implant 1 AV loop per animal but up to 2 os ilium defects and 4 intramuscular pockets. Here we assume to find 3 optimal combinations of variables after the first screening step (AV-loop rat model 3.4.4.2) to be evaluated in the mandibular AV loop model. For example, material X with cells, material X with growth factor A, and materials X with cells and growth factor A. with Further, a suitable control condition is included in the experimental set up (*e.g.* the material only). A contrast of 30.5% can be detected with a power of 80%, a standard deviation of 0.17 and α=0.05, based on previous findings from Eweida *et al., 2014* (9,11). This gives an n=8 animals per comparison. The AV loop model is considered the most critical part of the surgery and previous research has shown a 15% drop out due to occlusion of the loop using a similar method (10). Therefore a total number of 2 animals per comparison is required when this drop-out rate is taken into account: n=10. This means that a total amount of goats is estimated at 4* 10 (3 experimental conditions+ an adequate control condition) = 40 animals in total.
The exact amount of animals and groups for each experiment will be defined consulting a statistician and the animal welfare body and it will be specified in the work protocol.

B. The animals

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

A maximum of 40 animals will be used. Adult male goats will be used in this study, as it allows the implantation of bone substitutes of clinically relevant size and has been described to be a sufficient animal model for the experimental outcomes we are interested in (9,11,34). The goats need to be approximately 3 years old as after

only 32 months all permanent molars and incisors are erupted and all bones are fused, and not interfering with the placing of the AV loop in the mandible and/or growth (35). As it has been reported that female hormones can have a negative influence on bone formation, the use of female goat has been precluded for this bone study (22,23,27,28). Animals will be purchased from a Dutch farmer via GDL contact.

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
Extensive *in vitro* research has been carried out on the subject of osteogenic differentiation in combination with vasculogenesis/angiogenesis (3D hydrogel co-cultures) and bloodvessel engineering. Unfortunately, no *in vitro* options are currently available to fully recapitulate the complexity of new bone formation and remodelling, to model the complex interactions of the regenerative process with the immune system, or to evaluate the integration of the capillaries to the host circulation. Therefore, the use of an animal model is fundamental. The decision for a large animal made, is based on answering the research aim to mimic a clinically relevant size and situation, and is needed in order to assess the feasibility of scaling up the size of a bone regenerative construct to clinically relevant dimensions (e.g. at least $1cm^3$). For these reasons, the use of an large animal model is required in order to truly determine the clinical potential of the developed constructs (project proposal 3.4).

Reduction
By combining the three different types of defect (optimized in a rat model), a relatively small group of animals will be required, as several research questions can be tested at the same time.

Refinement
- The goat will receive adequate anaesthetics to prevent harm during surgery.
- Adequate analgesia medication will be administered after surgery to prevent harm post-operatively.
- Only oral fluids were allowed during the first postoperative day, with gradual return to normal diet within 3 days.
- In case of abnormalities, adverse events or unnecessary discomfort, a veretinarian will be consulted.
- To prevent the formation of thrombi, before opening of the anastomosis and post-operatively, anticoagulant drugs will be administered (e.g. heparin).

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

The goat will have at least 1 week to acclimatize to the new environment before surgery. Cage enrichments and bedding materials will be provided. During and right after surgery, respiration will be monitored continuously. Afterwards, animals will be returned to routine housing. To assess their well being, animals will be weighed weekly throughout the study. Cage-side observations will include, but not be limited to, changes in skin, eyes and mucous membranes and behaviour pattern. For specific information see refinement.

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.

To reduce to the minimum the pain, goat will receive before the surgery adequate analgesic. Post operatively, animals will be treated with adequate analgesics daily for 5 days post-surgery. Adequate anaesthesia will be provided.

I. Other aspects compromising the welfare of the animals

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

Adverse events:
1) Infections due to the surgical procedure (<5%): mild
2) Removal of the stiches before the wounds are completely closed: mild, might cause irritation and a delay in wounds closure
3) Thrombi in case AV loop model is included: moderate, imbalance in the blood circulation will be closely evaluated.
4) Occlusions in case AV loop model is included (15%): moderate, imbalance in the blood circulation will be closely evaluated

Explain why these effects may emerge.

The surgery can take up to 4 hours. This might increase the chances of infections. To prevent it, surgeons will

wear sterile scrubs, gloves and gown. Further, after shaving, the skin will be disinfected, the surgical site will be draped and the animals will be treated before and after the surgery with prophylactic antibiotics. To prevent the formation of thrombi or occlusions, anticoagulant will be administered before and after the surgery. Animal circulation will be closely monitored after the surgery. For specific information see the surgery section

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

Animals will be taken care of by trained personnel and their wellbeing will be monitored daily during the entire study. If it is noticed that one or more of the animals is in severe distress, we will consult the veterinarian.

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.

Goat will be closely monitored on general well being. If abnormalities are observed, the veterinarian will be consulted, and based on the severity of discomfort the animal will be euthanized.

Severe wound/implant infections, poor mobility, severe weight loss based on start weight, and impaired wound healing will be considered severe discomfort, in consultation with the responsible staff.

Indicate the likely incidence.

Very unlikely (<10%) to unlikely (15%) if the AV loop model is included

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').

Expected discomfort:
1) Animal discomfort due to the analgesia and antibiotics administration: mild
3) Animal discomfort due to the surgery: moderate
4) Animal discomfort and pain postoperatively due to the bone substitute implantation with adequate pain medication: moderate
5) Animal discomfort and pain postoperatively due to the intramuscular implantation with adequate pain medication: mild
6) Animal discomfort and pain postoperatively due to the AV loop model with adequate pain medication: moderate
7) Animal discomfort due to the euthanasia under anaesthesia: non recovery

Cumulative discomfort: 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 euthanised after the long-term follow-up experiment, as it is necessary to explant the construct to perform histological analysis

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

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

☒ Yes

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8.

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Aanvraagnummer
AVD1150020172485

Bijlagen
2

Datum 4 juli 2017
Betreft Ontvangstbevestiging aanvraag projectvergunning Dierproeven

Geachte [redacted]

Wij hebben uw aanvraag voor een projectvergunning dierproeven ontvangen op 30 juni 2017. Het gaat om uw project "Strategies for vascularized bone regeneration in maxillofacial surgical interventions". Het aanvraagnummer dat wij aan deze aanvraag hebben toegekend is AVD1150020172485. Gebruik dit nummer wanneer u contact met de CCD opneemt.

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

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

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

Datum:
4 juli 2017
Aanvraagnummer:
AVD1150020172485

Met vriendelijke groet,

Centrale Commissie Dierproeven

Deze brief is automatisch aangemaakt en daarom niet ondertekend.

Bijlagen:
- Gegevens aanvraagformulier
- Factuur

Datum:
4 juli 2017
Aanvraagnummer:
AVD1150020172485

Gegevens aanvrager

Uw gegevens	
Deelnemersnummer NVWA:	11500
Naam instelling of organisatie:	UMC Utrecht / Instantie voor Dierenwelzijn Utrecht
Naam portefeuillehouder of diens gemachtigde:	[redacted]
KvK-nummer:	30244197
Postbus:	12007
Postcode en plaats:	3501 AA UTRECHT
IBAN:	NL27INGB0000425267
Tenaamstelling van het rekeningnummer:	Universiteit Utrecht

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

Datum:
4 juli 2017
Aanvraagnummer:
AVD1150020172485

Over uw aanvraag

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

Over uw project

Geplande startdatum:	1 september 2017
Geplande einddatum:	1 september 2022
Titel project:	Strategies for vascularized bone regeneration in maxillofacial surgical interventions
Titel niet-technische samenvatting:	Strategieën voor gevascullariseerde botregeneratie in kaak- en aangezichtschirurgische
Naam DEC:	DEC-RUG
Postadres DEC:	Postbus 85500 3508 GA Utrecht
E-mailadres DEC:	dec-utrecht@umcutrecht.nl

Betaalgegevens

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

Checklist bijlagen

Verplichte bijlagen:	[x] Projectvoorstel [x] Beschrijving Dierproeven [x] Niet-technische samenvatting

Ondertekening

Naam:	[redacted]
Functie:	[redacted]
Plaats:	Utrecht
Datum:	29 juni 2017

Centrale Commissie Dierproeven

> Retouradres Postbus 20401 2500 EK Den Haag

UU-ASC
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Onze referentie
Aanvraagnummer
AVD1150020172485

Bijlagen
2

Datum 4 juli 2017
Betreft Factuur aanvraag projectvergunning Dierproeven

Factuur
Factuurdatum: 4 juli 2017
Vervaldatum: 3 augustus 2017
Factuurnummer: 172485
Ordernummer: CB. 841910.3.01.011

Omschrijving	**Bedrag**
Betaling leges projectvergunning dierproeven Betreft aanvraag AVD1150020172485	€ 1.541,00

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

DEC UTRECHT

A. Algemene gegevens over de procedure

1. Aanvraagnummer : 2017.I.543.008
2. Titel van het project : Strategies for vascularized bone regeneration in maxillofacial surgical interventions
3. Titel van de NTS : Strategieën voor gevascularisseerde botregeneratie in kaak- en aangezichtschirurgische ingrepen

4. Type aanvraag:
   - ☒ nieuwe aanvraag projectvergunning
   - ☐ wijziging van vergunning met nummer :

5. Contactgegevens DEC

Naam DEC	: DEC Utrecht
Telefoonnummer contactpersoon	: 088 – 75 59 247
Emailadres contactpersoon	: dec-utrecht@umcutrecht.nl

6. Adviestraject (data dd-mm-jjjj):
   - ☒ ontvangen door DEC: 13-04-2017
   - ☐ aanvraag compleet:
   - ☒ in vergadering besproken: 03-05-2017 en 31-05-2017
   - ☐ anderszins behandeld:
   - ☒ termijnonderbreking(en) van / tot : 08-05-2017/22-05-2017 en 06-06-2017/07-06-2017
   - ☐ besluit van CCD tot verlenging van de totale adviestermijn met max. 15 werkdagen:
   - ☐ aanpassing aanvraag:
   - ☒ advies aan CCD: 26-06-2017

7. De aanvraag is afgestemd met de IvD en deze is hiermee akkoord.

8. Eventueel horen van aanvrager
   - Datum:
   - Plaats:
   - Aantal aanwezige DEC-leden:
   - Aanwezige (namens) aanvrager:
   - Gestelde vragen en verstrekte antwoorden:
   - Het horen van de aanvrager heeft geleid tot aanpassing van de aanvraag.

9. Correspondentie met de aanvrager
   - Datum vragen: 08-05-2017
   - Datum antwoord: 22-05-2017
   - Gestelde vragen en antwoorden:

Projectvoorstel

- 3.1 Achtergrond: Er is uitvoerig gesproken of deze aanvraag wel één project is, meerdere projecten, of een programma. De onderzoekers presenteren de aanvraag als één project over botregeneratie. Botregeneratie op zichzelf is echter dusdanig algemeen en breed, dat dit wordt gezien als een programma in plaats van een project. De DEC meent dat het minimaal om twee projecten gaat en mogelijk zelfs drie: één project betreft het 'intramembranous' onderdeel, en één project betreft het 'endochondral[redacted] onderdeel, waarbij het gedeelte over de interactie tussen het implantaat en het [redacted], zeker als men wil gaan onderzoek hoe de reactie van het [redacted], ook nog als een apart project kan worden opgevat. Deze onderdelen zijn onderzoekslijnen die interessante inzichten in botregeneratie kunnen opleveren, maar staan in feite los van elkaar. De DEC verwacht dat de CCD dit zal zien als losse projecten en de onderzoekers zal vragen om de aanvraag op te splitsen. Daarom zou de DEC de onderzoekers adviseren om ofwel losse projecten in te dienen, of duidelijk aan te geven hoe de onderdelen verband houden met elkaar. Dat het subcutane model met de pockets in de rat voor beide onderdelen als model worden gebruikt, is op zich geen argument om het geheel als één project te beschouwen. Daar zijn inhoudelijke argumenten voor nodig. Als de DEC het goed heeft begrepen worden de beschreven onderdelen niet na elkaar uitgevoerd, maar naast elkaar en tegelijk. Wanneer de aanvrager de huidige aanvraag als één project zou willen indienen, dan dient er onderlinge afhankelijkheid tussen de beschreven onderdelen te zijn. Het is de DEC wel duidelijk dat zonder vascularisatie de cellen en constructen niet overleven; wellicht kunnen de onderzoekers hierop inhaken en op die manier duidelijk maken dat de twee onderdelen sterk samenhangen. De DEC zou de onderzoekers willen verwijzen naar het document 'Handreiking Invulling Definitie Project' op de CCD website onder het kopje onderwerpen, of de link: https://www.centralecommissiedierproeven.nl/onderwerpen/handreiking-invulling-definitie-project. In dit document staan een aantal schematisch uitgewerkte voorbeelden. De DEC is van mening dat de aanvraag in zijn huidige format valt onder voorbeeld 4A. Indien de onderzoekers de samenhang kunnen aantonen en beschrijven op het niveau van de onderdelen 'intramembranous' en 'endochondral-[redacted]' (o.a. in de flowchart) kan het project wellicht vallen onder voorbeeld 4B. Het verdere verloop van de aanvraag waarbij de in vitro modellen worden beschreven, waarna wordt overgegaan op ratten en vervolgens geiten is helder en komt overeen met voorbeeld 1 van de 'Handreiking Invulling Definitie Project'.
  Er is voor gekozen om de aanvraag op te splitsen in twee projecten: de endochondrale botregeneratie en gevasculariseerde constructen voor botvorming. Voor vereenvoudiging is verder het [redacted] aspect bij de endochondrale aanpak minder benadrukt omdat het inderdaad gaat om een secundaire uitkomstparameter. Hierna zullen de vragen die betrekking hebben op het gevascullariseerde deel beantwoord worden. Dit deel is als een nieuwe aanvraag aangeboden.

- 3.1 Achtergrond: De DEC adviseert om de bijlage met de flowchart, de tekst en het voorbeeld figuur op te nemen in de aanvraag zelf onder 3.1.3. Dit maakt de structuur van de aanvraag meteen duidelijk, waardoor ook de leesbaarheid van het vervolg toeneemt.
  De flowchart en delen van de tekst zijn ingevoegd in 3.1.3. De toevoeging van de tekst is verdeeld over de twee nieuwe aanvragen.
- 3.2 Doel: Het gaat om langlopend onderzoek. De DEC zou graag willen weten wat de onderzoekers na 5 jaar onderzoek willen hebben bereikt. Zonder concrete doelstellingen en milestones voor de duur van dit project kan de haalbaarheid van dit project niet goed worden beoordeeld.
  Onder 3.2 is een extra punt opgenomen onder 'The aims are achievable in 5 years', waarin voor het gevasculariseerd bot deel staat: 'At present, connection of microvascular structures to a host vasculature upon subcutaneous implantation is achievable. [redacted] *also demonstrated this before* [redacted] *However, simultaneous bone formation is challenging. To solve this, we will adapt our previous approach by* [redacted] *. This can be done by including* [redacted] *biomaterials or growth factors, such as* [redacted] *. Also, we have developed new biomaterials that better mimic the natural environment of bone cells to stimulate the osteogenic process.*
  The AV loop models have been developed and used by others.
  Ook in 3.3 is voor elk (sub-)aim een zin opgenomen die aangeeft wat het project kan opleveren.
- 3.4 Onderzoeksstrategie: De DEC raadt de onderzoekers aan om in de tekst onder 3.4.2. te verwijzen naar de bijlages; dit werkt verhelderend en zo wordt de lezer beter verwezen naar wat hij/zij waar kan vinden.
  De lezer wordt in 3.4.2 nu verwezen naar de box in het figuur in de flowchart in 3.1.3 waarover in elke paragraaf over gesproken wordt.
- 3.4 Onderzoeksstrategie: Er wordt nergens in het project vermeld hoe lang de dieren in experiment blijven. De DEC ziet graag dat U deze informatie toevoegt en beargumenteert.
  De duur van experimenten is in 3.4.2 van beide voorstellen toegevoegd. Voor de ratten is de duur maximaal 12 weken en voor de geiten is dit maximaal 24 weken.

Bijlage 1:

- H. Pijn en pijnbestrijding. De DEC merkt op dat u verzuimt hier de anesthesie te noemen. Dit geldt voor alle bijlagen.
  De anesthesie is in alle bijlagen toegevoegd onder H.
- K. Classificatie van ongerief. De DEC is van mening dat deze dierproeven matig ongerief veroorzaken. De IvD, aanwezig bij de vergadering deelde, na enige discussie, deze mening. Volgens de DEC betreffen chirurgische ingrepen en het bijkomen uit de anaesthesie vrijwel altijd matig ongerief ongeacht het type operatie. Graag veranderen.
  De mate van ongerief voor de chirurgische interventie is aangepast naar matig.

- K. Classificatie van ongerief. De DEC vraagt zich af of de naakte, immunodeficiënte dieren ongerief ervaren als gevolg van hun genetische mutatie? Kunt u de aspecten van het fenotype beschrijven die volgens u tot ongerief leiden?
  Er werd verondersteld dat alle genetisch gemuteerde dieren enig ongerief zouden ervaren. Dit is onjuist. Punt 1 is daarom geheel verwijderd.

Bijlage 3 (let op: dit is nu bijlage 2):

- B. De dieren: De onderzoekers geven aan ratten te gebruiken van 7-10 weken oud terwijl in bijlage 1 en 2 ratten worden gebruikt van tenminste 12 weken. De DEC is van mening dat ratten van 7-10 weken nog niet geheel volgroeid zijn. Wat is de argumentatie voor deze leeftijd?
  Dit is inderdaad niet correct. De leeftijd is aangepast naar de volgroeide leeftijd.
- B. De dieren: Benoem ook hier het aantal dieren wat wordt aangevraagd.
  Het aantal dieren is toegevoegd.

Bijlage 4 (let op: dit is nu bijlage 3):

- Experimentele aanpak en primaire uitkomstparameters: A.3: De DEC adviseert om wellicht meer dieren aan te vragen, aangezien de berekening van de statisticus hoger uitvalt. Dit zal U dan moeten onderbouwen.
  Waarschijnlijk was de toelichting van de berekening onduidelijk. Het gaat in totaal om vier groepen, waaronder een controle. De tekst is verduidelijkt om dit beter weer te geven. De berekening blijft hetzelfde. Indien het met de nieuwe uitleg nog steeds niet klopt, zouden wij graag meer details over de berekening van de statisticus ontvangen om te begrijpen waar het verschil in zit en het aantal dieren te kunnen aanpassen.
- B. De dieren: De DEC adviseert u om het aantal dieren hier ook te noemen.
  Het aantal dieren is toegevoegd.
- E. Herhaling: N.v.t. invullen. Deze tekst hoort hier niet.
  De tekst is verwijderd.

- De antwoorden hebben geleid tot aanpassing van de aanvraag.

- Datum vragen: 06-06-2017
- Datum antwoord: 07-06-2017
- Gestelde vragen en antwoorden:
  Bijlage 2
  - K. Classificatie van ongerief: immunodeficiënte dieren (1) kunnen hier verwijderd worden omdat ze geen ongerief ervaren.
    Dit is aangepast.

Bijlage 3

- Experimentele aanpak en primaire uitkomstparameters: In de zin: "*Studies involving a rat AV loop model (described in 3.4.4.2) will proceed the goat studies described here*", moet proceed vermoedelijk zijn precede.
  Dit is aangepast.

- De antwoorden hebben geleid tot aanpassing van de aanvraag.

10. Eventuele adviezen door experts (niet lid van de DEC)
    - Aard expertise:
    - Deskundigheid expert:
    - Datum verzoek:
    - Strekking van het verzoek:
    - Datum expert advies:
    - Advies expert:

B. Beoordeling (adviesvraag en behandeling)

1. Het project is vergunningplichtig (dierproeven in de zin der wet).
2. De aanvraag betreft een nieuwe aanvraag.
3. De DEC is competent om hierover te adviseren.
4. Er zijn geen DEC-leden betrokken bij het betreffende project.

C. Beoordeling (inhoud):

1. Door trauma, aangeboren afwijkingen of het verwijderen van tumoren, kunnen patiënten botweefsel missen. Momenteel wordt dit botweefsel vervangen door bot elders uit het lichaam, maar hierdoor ontstaat er een extra defect en bovendien vergt het een langdurige operatie. Een mogelijke oplossing hiervoor is het maken van botweefsel in het laboratorium. Tot nu toe zijn er verschillende strategieën voor botregeneratie onderzocht, waarbij geprobeerd is om de meest effectieve celpopulatie te combineren met het ideale dragermateriaal en de optimale stimulatiefactoren. De belangrijkste uitdaging daarbij is het opschalen van weefselgrootte naar een klinisch relevante dimensie (in de cm range), omdat gebleken is dat weefselconstructen de implantatie niet kunnen 'overleven' wanneer zij groter zijn dan 400µm. Onderzoekers proberen dit nu op te lossen door: 1) vasculaire structuren in de constructies te introduceren, of door 2) botvorming vanuit kraakbeen te stimuleren, de endochondrale route naar botvorming. Het onderhavige projectvoorstel richt zich op de eerste aanpak.
   Er zal worden gestart met een *in vitro* studie (geen onderdeel van het projectvoorstel), waarbij de constructen getest worden op non-toxiciteit, osteogenese en vasculogenese. Wanneer dit is bevestigd, worden de constructen geëvalueerd in het subcutane rattenmodel. Dit model geeft een eerste indruk van het potentieel voor osteogenese, anastomose en functionele perfusie van het pre-vasculaire netwerk. Het is ook geschikt als screeningsmodel om de optimale constructsamenstelling te bepalen aangezien meerdere samples van verschillende groepen geïmplanteerd kunnen worden in één dier. Alleen de meest geschikte constructen zullen

vervolgens in de volgende stap worden toegepast: het AV loop model in de rat. Dit model wordt gebruikt om te bepalen welke combinatie van scaffolds, celtypen en groeifactoren leidt tot de meeste vasculogenese/angiogenese en botvorming in de geïmplanteerde constructen. De meest optimale constructen worden geëvalueerd in het rat AV-loop model en vervolgens in het geit AV-loopmodel. Het laatste model kan tevens inzicht geven in of het construct zorgt voor weefselvorming op klinisch relevante grootte.
De aanvraag komt qua structuur overeen met voorbeeld 1 uit de "Handreiking Invulling Definitie Project". De DEC is derhalve van mening dat de aanvraag toetsbaar is en voldoende samenhang heeft.

2. Voor zover de DEC bekend, is er geen mogelijk tegenstrijdige wetgeving die het uitvoeren van de dierexperimenten in de weg zou kunnen staan.

3. De in de aanvraag aangekruiste doelcategorieën sluiten aan bij de hoofddoelstelling(en).

Belangen en waarden

4. Het directe doel van het project is het creëren van een gevascullariseerd construct voor botregeneratie, met de focus op [redacted] klinische toepassing. Hiertoe wordt er geprobeerd om 1) een uitgebreid perfuseerbaar microvasculair netwerk op te zetten, dat verbonden kan worden met de bloedvoorziening na implantatie, en 2) een groter vat te creëren waarop dit netwerk kan worden aangesloten en dat stevig genoeg is om te kunnen vasthechten aan een bestaand vat (AV-loop model). Het uiteindelijke doel van het project is het vervangen van botweefsel in de kaak en het aangezicht door middel van botweefsel uit het laboratorium. De DEC is van mening dat er in voldoende mate een relatie is tussen het directe doel en het uiteindelijke doel.

5. De belangrijkste belanghebbenden in dit onderzoeksproject zijn: de proefdieren, de doelgroep/patiënt en de onderzoekers. Voor de proefdieren geldt dat hun welzijn en integriteit worden aangetast. De dieren zullen beperkt worden in hun natuurlijke gedrag en gedurende de proeven zullen de dieren stress ondervinden en pijn ervaren. De dieren zullen in het kader van het onderzoek gedood worden.
Zoals bij C1 vermeld, is het vervangen van botweefsel door bot elders uit het lichaam een ingreep die vaak gepaard gaat met letsel op de plek waar het bot vandaan wordt gehaald. Door het ontwikkelen van botweefsel in het laboratorium, dat ook bij een grootte >400µm kan overleven, kunnen patiënten in de toekomst eenvoudiger, zonder extra chirurgische ingreep en zonder bijkomende defecten als gevolg van het verwijderen van botweefsel, behandeld worden. Voor de onderzoekers geldt dat dit project kan bijdragen aan een goede wetenschappelijke reputatie en kan leiden tot nieuwe wetenschappelijke inzichten. Wetenschappelijke reputatie kan door de onderzoeker van belang geacht worden, maar dient naar de mening van de DEC geen rol te spelen in de ethische afweging over de toelaatbaarheid van het gebruik van proefdieren.

6. De aanvrager geeft niet aan nadelige effecten op het milieu te verwachten. De DEC ziet geen aanleiding om aan te nemen dat zich toch nadelige effecten zullen voordoen.

Proefopzet en haalbaarheid

7. De onderzoeksgroep heeft veel ervaring met het doen van onderzoek naar botregeneratie en de PI specifiek met diermodellen voor onderzoek naar botregeneratie. Daarnaast is er, zowel binnen als buiten de instelling, samenwerking met groepen die zich eveneens bezighouden met botregeneratie. De DEC is er daarom van overtuigd dat de aanvrager over voldoende expertise en voorzieningen beschikt om de projectdoelstelling met de gekozen strategie/aanpak binnen de gevraagde termijn te realiseren. De DEC is er bovendien van overtuigd dat de aanvrager gedurende het project zal kunnen voldoen aan de 3V-beginselen om te voorkomen dat teveel proefdieren zullen worden ingezet en dat ze onnodig nadeel zullen ondervinden van de experimenten.

8. Het project is goed opgezet, de voorgestelde experimentele opzet en uitkomstparameters sluiten logisch en helder aan bij de aangegeven doelstellingen en de gekozen strategie en experimentele aanpak kan leiden tot het behalen van de doelstelling binnen het kader van het project (zie hiervoor ook de tekst bij C1).

Welzijn dieren

9. Er is geen sprake van de volgende bijzonderheden op het gebied van categorieën van dieren, omstandigheden of behandeling van de dieren:
   - ☐ Bedreigde diersoort(en) (10e lid 4)
   - ☐ Niet-menselijke primaten (10e)
   - ☐ Dieren in/uit het wild (10f)
   - ☐ Niet gefokt voor dierproeven (11, bijlage I EU richtlijn)
   - ☐ Zwerfdieren (10h)
   - ☐ Hergebruik (1e lid 2)
   - ☐ Locatie: buiten instelling vergunninghouder (10g)
   - ☐ Geen toepassing verdoving/pijnbestrijding (13)
   - ☐ Dodingsmethode niet volgens bijlage IV EU richtlijn (13c lid 3)

10. De dieren worden gehuisvest en verzorgd op een wijze die voldoet aan de eisen die zijn opgenomen in bijlage III van de EU richtlijn. Alleen na de operatie worden de dieren enkele dagen solitair gehuisvest totdat de wond geheeld is.

11. Het cumulatieve ongerief als gevolg van de dierproeven is realistisch ingeschat en geclassificeerd. De experimentele handelingen aan de dieren zullen (gezien hun aard) licht tot matig ongerief veroorzaken. Cumulatief is voor alle bijlagen het ongerief ingeschat als matig.

12. De integriteit van de dieren wordt fysiek aangetast door het implanteren van de scaffolds.

13. De humane eindpunten zijn in de bijlage dierproeven goed gedefinieerd en het percentage dieren dat naar verwachting een humaan eindpunt bereikt is goed ingeschat. In bijlage 1 wordt verwacht dat maximaal dan 5% van de dieren het humane eindpunt bereikt. Voor bijlage 2 is dat minder dan 20%. De ratten in deze bijlagen zullen nauwlettend gemonitord worden op algeheel welzijn, waarbij met name wordt gelet op een infectie van de chirurgische wond(en), hijgen, speekselvloed, immobiliteit, aanhoudend beven, aanhoudende krampen, zelfmutilatie, pilo erectie en abnormale houding en onacceptabel/langdurig gewichtsverlies (zichtbare wervelkolom of rib). In bijlage 2 wordt tevens gelet op een infectie of defect van de anastomose, oedeem en zwelling. In bijlage 3 wordt verwacht dat minder dan 15% van de dieren (afhankelijk van of het AV loop model wordt toegepast) het humane eindpunt bereikt. De geiten in deze bijlage zullen eveneens nauwlettend gemotord worden op het algeheel welzijn, waarbij met name gelet wordt op ernstige wond-/implantaat-infecties, slechte mobiliteit, ernstig gewichtsverlies t.o.v. het start gewicht en langzame wondgenezing.

3V's

14. De aanvrager heeft voldoende aannemelijk gemaakt dat er geen geschikte vervangingsalternatieven zijn. Voor aanvang van de *in vivo* studies wordt *in vitro* onderzoek verricht, waarbij de constructen getest worden op non-toxiciteit, osteogenese en vasculogene. Omdat de systemische effecten van de implantaten, zoals de invloed op de aantrekking van stamcellen uit de bloedstroom en het beenmerg, en de ingroei en binding van het implantaat met het omliggende weefsel, alsook de snelheid van omgroei naar nieuw bot, waarbij cellen nodig zijn vanuit het lichaam, niet *in vitro* na te bootsen zijn is het gebruik van diermodellen noodzakelijk. Ook de aansluiting van een vaatnetwerk of klein vat op de bloedsomloop kan niet volledig worden nagebootst in het lab. Om op termijn de translatiestap naar de mens te maken is het noodzakelijk om de klinische situatie zo goed mogelijk na te bootsen in een groot dier.

15. Het aantal te gebruiken dieren is realistisch ingeschat en er is een heldere strategie om ervoor te zorgen dat tijdens het project met het kleinst mogelijke aantal dieren wordt gewerkt waarmee nog een betrouwbaar resultaat kan worden verkregen. Voor het berekenen van het aantal benodigde dieren worden statistische methoden toegepast. Daarnaast wordt onnodig proefdiergebruik voorkomen met behulp van heldere go/no-go momenten waarbij alleen de best werkende combinatie gebruikt wordt bij de volgende fase. De dierproeven zijn zodanig opgezet dat er onderhuids meerdere combinaties (van scaffolds, celtypen en groeifactoren) in één dier geplaatst kunnen worden. Dit vermindert het aantal benodigde dieren.

16. Het project is in overeenstemming met de vereiste van verfijning van dierproeven en het project is zodanig opgezet dat de dierproeven zo humaan mogelijk kunnen worden uitgevoerd. De dieren worden goed gemonitord om ongerief tijdig te kunnen vaststellen en onnodig lijden te voorkomen en zullen worden geëuthanaseerd zodra ze één van de vooraf nauwkeurig gedefinieerde humane eindpunten bereiken. De onderzoekers zullen een specifieke training in

microchirurgie volgen en er wordt uitgebreid op kadavermateriaal geoefend voordat de experimentele fase gestart wordt. In bijlage 1 wordt het s.c. rattenmodel gebruikt omdat het een goede screening van de implantaten toestaat en er meerdere verschillende combinaties van implantaten in één dier geplaatst kunnen worden. Voor het AV- loop rattenmodel is gekozen omdat het reeds beschreven in is in de literatuur door andere groepen. Deze ervaring kan de onderzoeksgroep helpen om de operatie te verfijnen, de meest innovatieve groepen en type implantaten te bepalen en de uitkomstparameters vast te stellen. Een groot diermodel in de geit wordt gebruikt om de situatie van de mens zo goed mogelijk na te bootsen.

17. Er is geen sprake van wettelijk vereist onderzoek.

Dieren in voorraad gedood en bestemming dieren na afloop proef

18. Omdat mannelijke en vrouwelijke ratten een ander bot metabolisme hebben, wordt de voorkeur gegeven aan het gebruik van één sekse. Het gebruik van beide seksen zal leiden tot een grotere variatie en het gebruik van meer dieren. Aangezien stress en leeftijd invloed hebben op het oestrogeen niveau en oestrogenen van invloed zijn op bot regeneratie, gaat in bijlage 1 de voorkeur uit naar het gebruik van mannelijke muizen. In bijlage 2 gaat eveneens de voorkeur naar het gebruik van mannelijke ratten omdat het skelet van de vrouwelijke rat niet groot genoeg is om de scaffolds in de lies te plaatsen. In bijlage 3 worden mannelijke geiten aangevraagd ook vanwege de vrouwelijke hormonale invloed op de botvorming. De DEC is er van overtuigd dat de aanvrager in voldoende mate wetenschappelijk heeft onderbouwd dat het, om de doelstellingen te bereiken, noodzakelijk is om de proeven met alleen mannelijke dieren uit te voeren.

19. De dieren worden in het kader van het project gedood, voor histologisch onderzoek van de constructen. De dieren worden volgens een, bijlage IV van de EU richtlijn, passende methode gedood.

20 De vraag over hergebruik is niet van toepassing omdat de dieren gedood worden in het kader van het experiment.

NTS

21. De niet-technische samenvatting is een evenwichtige weergave van het project en begrijpelijk geformuleerd.

D. Ethische afweging

1. De morele vraag die de DEC dient te beantwoorden is of het belang van dit onderzoek, namelijk het creëren van een gevascullariseerd construct voor botregeneratie, voor maxillofaciale klinische toepassing, de onvermijdelijke aantasting van het welzijn en de integriteit van de gebruikte proefdieren kan rechtvaardigen.

2. Er vindt een aanzienlijke aantasting van welzijn en integriteit van de proefdieren plaats, met matig ongerief.
   Echter, indien de hierboven genoemde doelstellingen behaald worden, dan zal dit project er toe bijdragen dat patiënten die botweefsel missen in de kaak of het aangezicht, een transplantatie kunnen ondergaan van, in het laboratorium ontwikkeld, gevasculariseerd bot, zonder de nadelen die een bottransplantatie uit een ander deel van het lichaam van de patiënt met zich meebrengt. De DEC kent daar veel gewicht aan toe. Wanneer het onderzoekers bovendien lukt om dit gevascularisseerde botweefsel te ontwikkelen in het lab, kan in de toekomst wellicht ook de vertaalslag worden gemaakt naar andere weefsels en organen.
   Het is aannemelijk dat de fundamentele en translationele doelstellingen behaald zullen worden. Daarvoor is de inzet van proefdieren noodzakelijk, maar de onderzoekers doen al het mogelijke om het ongerief voor de dieren en het aantal dieren tot een minimum te beperken.

3. Op grond van het bovenstaande is de DEC van oordeel dat, hoewel de experimenten leiden tot een aanzienlijke aantasting van het welzijn en de integriteit van de proefdieren, het in het laboratorium ontwikkelen van gevascularisseerd botweefsel, ter vervanging van een bottransplantatie elders uit het lichaam, een substantieel belang vertegenwoordigt dat opweegt tegen de aantasting van het welzijn en de integriteit van de proefdieren. Het gebruik van de proefdieren zoals beschreven in de aanvraag is daarmee gerechtvaardigd.

E. Advies

1. Advies aan de CCD
   ☒ De DEC adviseert de vergunning te verlenen.

   ☐ De DEC adviseert de vergunning te verlenen onder de volgende voorwaarden.

   ☐ De DEC adviseert de vergunning niet te verlenen vanwege:

2. Het uitgebrachte advies is gebaseerd op consensus.

3. Er zijn geen knelpunten/dilemma's naar voren gekomen tijdens het beoordelen van de aanvraag en het opstellen van het advies.

11.

Centrale Commissie Dierproeven

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Onze referentie
Aanvraagnummer
AVD1150020172485
Bijlagen
1

Datum 20 juli 2017
Betreft Beslissing aanvraag projectvergunning Dierproeven

Geachte [redacted]

Op 30 juni 2017 hebben wij uw aanvraag voor een projectvergunning dierproeven ontvangen. Het gaat om uw project "Strategies for vascularized bone regeneration in maxillofacial surgical interventions" met aanvraagnummer AVD1150020172485. Wij hebben uw aanvraag beoordeeld.

Beslissing
Wij keuren uw aanvraag goed op grond van artikel 10a van de Wet op de Dierproeven (hierna: de wet). Hierbij gelden de voorwaarden zoals genoemd in de vergunning.
De algemene voorwaarde(n) zijn opgenomen op grond van artikel 1d lid 4, artikel 10a1 lid 2, artikel 10 lid 2 en/of artikel 10a3 van de wet.

U kunt met uw project "Strategies for vascularized bone regeneration in maxillofacial surgical interventions" starten. De vergunning wordt afgegeven van 1 september 2017 tot en met 31 augustus 2022. Deze termijn is anders dan in uw aanvraag, omdat een vergunning een looptijd van maximaal 5 jaar kan hebben.

Overige wettelijke bepalingen blijven van kracht.

Procedure
Wij hebben advies gevraagd bij de Dierexperimentencommissie DEC Utrecht. Dit advies is opgesteld op 26 juni 2017. Bij de beoordeling van uw aanvraag is dit advies betrokken overeenkomstig artikel 10a, lid 3 van de wet.
Wij kunnen ons vinden in de inhoud van het advies van de Dierexperimentencommissie. Dit advies van de commissie nemen wij over,

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

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

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

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

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

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

Centrale Commissie Dierproeven
namens deze:

[redacted]

Algemeen Secretaris

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

Centrale Commissie Dierproeven

Projectvergunning

gelet op artikel 10a van de Wet op de Dierproeven

Verleent de Centrale Commissie Dierproeven aan

Naam:	UMC Utrecht / Instantie voor Dierenwelzijn Utrecht
Adres:	Postbus 12007
Postcode en plaats:	3501 AA UTRECHT
Deelnemersnummer:	11500

deze projectvergunning voor het tijdvak 1 september 2017 tot en met 31 augustus 2022, voor het project "Strategies for vascularized bone regeneration in maxillofacial surgical interventions" met aanvraagnummer AVD1150020172485, volgens advies van Dierexperimentencommissie DEC Utrecht. Er worden aanvullende algemene voorwaarde(n) gesteld.
De functie van de verantwoordelijk onderzoeker is [redacted]
De aanvraag omvat de volgende bescheiden:

1 een aanvraagformulier projectvergunning dierproeven, ontvangen op 30 juni 2017
2 de bij het aanvraagformulier behorende bijlagen:
a Projectvoorstel, zoals ontvangen per digitale indiening op 30 juni 2017;
b Niet-technische Samenvatting van het project, zoals ontvangen per digitale indiening op 30 juni 2017;
c Advies van dierexperimentencommissie d.d. 26 juni 2017, ontvangen op 30 juni 2017.

Naam proef	Diersoort/ Stam	Aantal dieren	Ernst	Opmerkingen
3.4.4.1 Subcutaneous construct implantation in rats				
	Ratten (Rattus norvegicus) /	70	Matig	
3.4.4.2 AV loop model in rats				
	Ratten (Rattus norvegicus) /	296	Matig	
3.4.4.3 Intramuscular+orthotopic+AV loop model in goat				
	Geiten (Capra aegagrus hircus) /	40	Matig	

Voorwaarden

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

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

Aanvraagnummer:
AVD1150020172485

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

Aanvraagnummer:
AVD1150020172485

Weergave wet- en regelgeving

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

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

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

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

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