	Inventaris Wob-verzoek W17-12								
		wordt verstrekt				**weigeringsgronden**			
nr.	**documenten NTS2016705**	**reeds openbaar**	**niet**	**geheel**	**deels**	**10.1.c**	**10.2.e**	**10.2.g**	**11.1**
1	Aanvraagformulier				x		x	x	
2	Projectvoorstel				x			x	
3	Niet-technische samenvatting	x							
4	Bijlage beschrijving dierproeven 1				x		x	x	
5	Bijlage beschrijving dierproeven 2				x		x	x	
6	Bijlage beschrijving dierproeven 3				x		x	x	
7	DEC-advies				x		x	x	
8	Ontvangstbevestiging				x		x	x	
9	Verzoek aanvulling aanvraag				x		x	x	
10	Reactie verzoek aanvulling				x		x	x	
11	Advies CCD		x						x
12	Beschikking en vergunning				x		x	x	

705

1.

Centrale Commissie Dierproeven

Aanvraag
Projectvergunning Dierproeven
Administratieve gegevens

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

1 Gegevens aanvrager

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

☐ Ja > *Vul uw deelnemernummer in*	11600
☐ Nee > *U kunt geen aanvraag doen*	

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

Naam instelling of organisatie	Academisch Ziekenhuis Leiden	
Naam van de portefeuillehouder of diens gemachtigde	Leids Universitair Medisch Centrum	
KvK-nummer	27366422	
Straat en huisnummer	Albinusdreef	2
Postbus	9600	
Postcode en plaats	2300 RC	Leiden
IBAN	NL11DEUT0451001400	
Tenaamstelling van het rekeningnummer	LUMC	

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

1.4 Vul de gegevens in van de verantwoordelijke onderzoeker.

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

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 - 7 - 2017
		Einddatum	30 - 6 - 2022
3.2	Wat is de titel van het project?	Investigating the dynamic role of the TGF-β pathway in liver metastases to come closer towards therapies.	
3.3	Wat is de titel van de niet-technische samenvatting?	Het onderzoeken van moleculaire en cellulaire aspecten van kanker uitzaaiingen.	
3.4	Wat is de naam van de Dierexperimentencommissie (DEC) aan wie de instellingsvergunninghouder doorgaans haar projecten ter toetsing voorlegt?	Naam DEC	DEC Leiden
		Postadres	[redacted] LUMC Postbus 9600 2300 RC Leiden
		E-mailadres	[redacted]

4 Betaalgegevens

4.1 Om welk type aanvraag gaat het?

☒ Nieuwe aanvraag Projectvergunning € 1541 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	Gemandateerd vergunninghouder
Plaats	Leiden
Datum	1 - 6 - 2017
Handtekening	[redacted]

Centrale Commissie Dierproeven

Form
Project proposal

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

1 General information

1.1	Provide the approval number of the 'Netherlands Food and Consumer Product Safety Authority'.	11600
1.2	Provide the name of the licenced establishment.	LUMC
1.3	Provide the title of the project.	Investigating the dynamic role of the TGF-β pathway in liver metastases to come closer towards therapies

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.

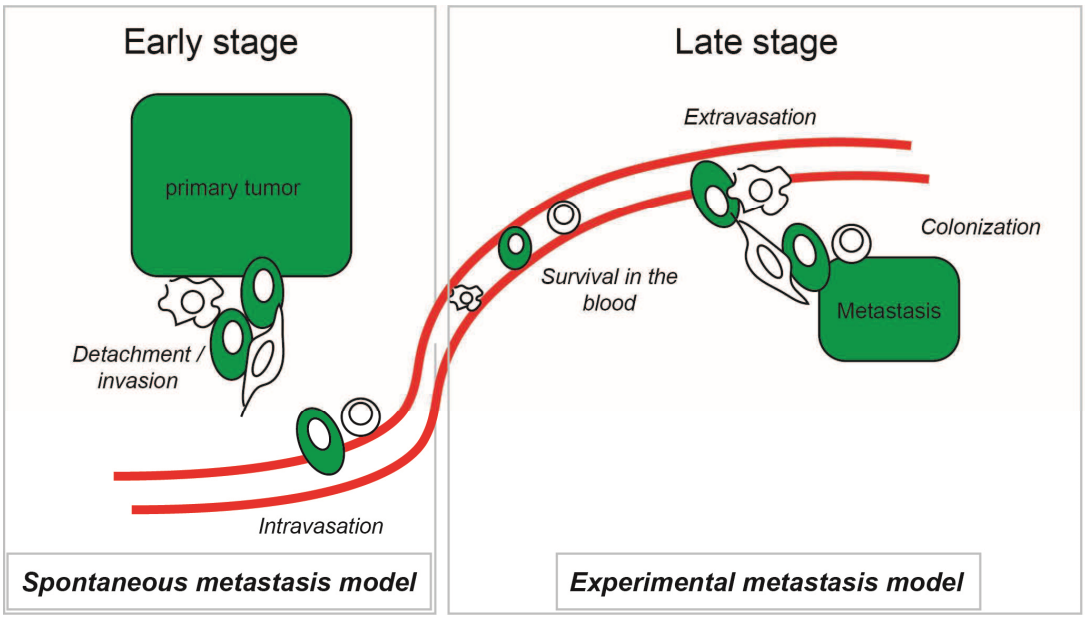
- For legally required animal procedures, indicate which statutory or regulatory requirements apply (with respect to the intended use and market authorisation).
- For routine production, describe what will be produced and for which uses.
- For higher education or training, explain why this project is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

Cancer is one of the deadliest diseases in the western world. About 40.000 patients die of cancer in the Netherlands on a yearly basis. Most of them do not die because of the primary tumor, but rather from metastases because they arise in hard to resect places. One of those places is the liver; if liver

metastases are observed, this usually results in a bad prognosis.

Breast and skin cancer patients that have liver metastases have a poor survival prognostic: on average 4 months which compares unfavourably to metastasis at other sites (Tas, J Oncol, 2012; Patanaphan et al, 1988; O'Reilly et al, 1990). Moreover, for both type of cancer patients we do not have good treatment options when liver metastases are present (Agarwala, Cancer, 2014). Therefore, we would like to better understand the regulation of metastasis colonization in the liver and how to target it with therapy.

The metastatic process is complex and can be divided in an early and late stage (see figure). In the early stage cells cells detach from the primary tumor, then invade the surrounding tissue and intravasate in the blood stream. In the late stage, cells survive in the blood, extravasate from the blood and colonize a distant tissue (Beerling, JCS, 2012). Sometimes, during colonization, tumor cells can enter into a dormancy state. This is a temporary phase in which the cell is not proliferating, and which can last many years to decades. It is not known how and why cancer cells switch to this state, however, once they escape from dormancy and start proliferating again, it leads to the formation of a metastasis and relapse of the patient (Sosa, NRC, 2014). For both immediate or delayed metastasis formation, the tumor microenvironment (TME) plays an important role. Factors known to play a major role in a variety of the metastatic cascade are fibroblasts, T cells, blood vessels and the extracellular matrix.



Despite all this knowledge, much of the details of this process are still unknown, which explains the lack of therapies to prevent and reduce metastases. Therefore, **a better understanding of the basic pathways that regulate this metastatic process is warranted.**

We are interested in studying the TGF-β pathway as it plays a role in metastasis, but its precise role in liver metastasis is unclear (Zhang, Trends Biochem Sci, 2013). Interestingly, depending on the cellular context, TGF-β can exert pro- and anti-tumorigenic effects (Massague, NRMCB, 2012). In cancer cells, TGF-β signalling often leads to increased migration and invasion of the tumor cells (Padua, Cell Res, 2009). Importantly, besides affecting the tumor cells themselves, TGF-β also affects the tumor microenvironment which will then affect metastasis (Pickup, NRC, 2013). For example, TGF-β activates fibroblasts which can increase tissue stiffness and tumor cell migration, and it inhibits T cell responses that would otherwise eliminate tumor cells. Recently, TGF-β components have been involved in the induction of dormancy pointing out its importance in both direct and delayed metastasis (Bragado, NCB, 2013; Ghajar, NCB, 2013; Gao, Cell, 2012). For all those reasons, TGF-β is a potentially interesting therapeutic to target the metastatic process and inhibitors are already available and used as monotherapy. However, these inhibitors showed mixed responses in humans, indicating that we lack a big part of the basic understanding of how the TGFβ pathway regulates metastasis.

Therefore, the aim of this project is to better understand the role of this pathway in liver metastasis of triple negative breast cancer and metastatic melanoma (skin cancer) patients. We hypothesize that TGF-β must be regulated in a dynamic manner in order to allow for successful metastasis formation. For example, TGF-β induced migration and invasion is thought to be beneficial for the early stage of metastasis (detachment, invasion and intravasation), but is likely to be hampering the later stage of metastasis formation (outgrowth of cells in the secondary site). In this example, TGF-β should first be upregulated and later be downregulated. Similar scenarios can be envisioned with regards to the tumor microenvironment. To better understand the dynamic regulation of TGF-β during the metastatic process, it is essential to study the individual steps of metastasis, and study them in the same animal over time. Conventional studies using ex vivo analyses like immunohistochemistry provide snapshots of the tissue, and cannot study invasion, or intravasation separately. Our lab is experienced with the intravital microscopy technique (IVM) in which single tumor cells can be visualized inside a living animal over multiple days. It is the *only* platform that allows us to study dynamic processes like tumor cell migration, intravasation, extravasation, but also the growth of a metastasis over time (colonization) in an animal. It is therefore the best platform to study the dynamic role of TGF-β in breast or melanoma liver metastases.

By comparing the role of the TGF-β pathway in two very different types of cancers (breast cancer and skin cancer), we hope to identify commonalities that may help to treat all cancer patients with liver metastases. However, we also expect to identify specific differences between the two types of cancers, which might lead to tumor-type specific treatments.

3.2 Purpose

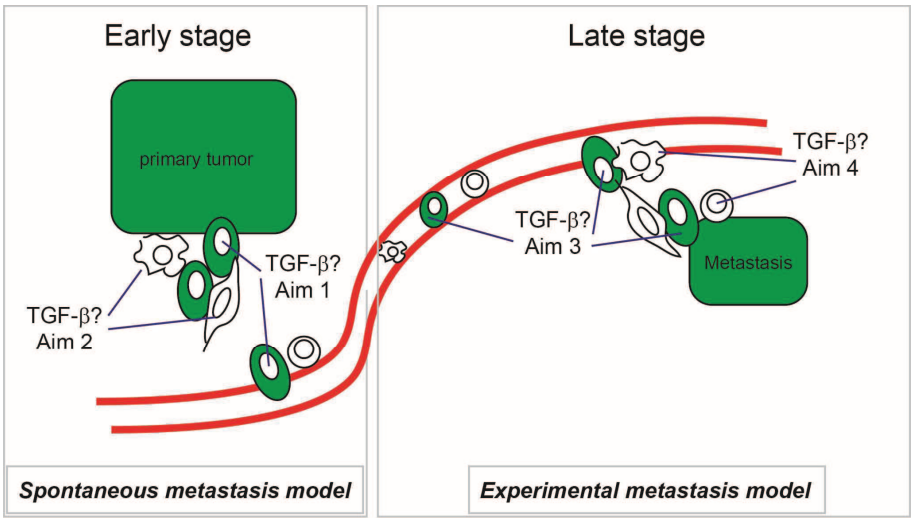
Describe the project's main objective and explain why this objective is achievable.

- 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?

TGF-β can be pro and anti-metastatic, and is known to be important during metastasis. In addition, TGF-β is known to play a major role in the tumor microenvironment. We hypothesize that TGF-β must be regulated in a dynamic manner in order to allow for successful metastasis formation. This relates to TGF-β regulation in the tumor cells ànd microenvironment. For example, TGF-β induced migration and invasion is thought to be beneficial for the initial stage of metastasis (invasion, intravasation and extravasation), but is likely to be hampering the later stage of metastasis formation (outgrowth of cells in the secondary site). Until now, no one has been able to study the dynamic regulation and expression of the TGF-β pathway in vivo. With IVM we are able to do this. **Thus, the goal of this project is to understand the dynamic role of the TGF-β pathway in breast and skin cancer liver metastases.**

We will achieve this goal by focussing on the following aims:

1. What is the role of TGF-β-related genes during the early stage of metastasis
2. What is the role of TGF-β in the tumor microenvironment during the early stage of metastasis
3. What is the role of TGF-β-related genes during the late stage of metastasis
4. What is the role of TGF-β in the tumor microenvironment during the late stage of metastasis



Why is our goal achievable:
The applicant has 7 years of experience working on breast cancer (metastasis), and 3 years working on melanoma (metastasis), clearly showing expertise in these areas. Moreover, the LUMC is specialized in breast and skin cancer (melanoma), so a lot of expertise in this area is present in house (e.g. via a collaboration with a melanoma clinical specialist at LUMC). Moreover, the applying group is embedded in a department [redacted] that has been studying metastasis for over a decade, and has close collaborations with the groups who are experts[redacted]. Regarding the study of the tumor microenvironment, the applicant has 7 years of experience in this field, and the group has connections with the immunology department and experts in stromal cell biology in the LUMC. In addition, the applicant has over 6 years of experience with the IVM technique, [redacted] The LUMC owns a multiphoton microscope that is already used for IVM experiments, allowing us to implement the IVM platform to study liver metastasis. A collaboration with [redacted] in the Netherlands strengthens this expertise even more. Finally, the group has received multiple sources of funding: [redacted]. Altogether, we believe that this lab is perfectly situated to conduct the proposed research.

3.3 Relevance

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

Metastasis is a deadly disease, as most cancer patients do not die from a primary tumor, but rather from secondary metastases. Breast cancer and melanoma patients with liver metastases have a very bad prognosis: A median survival of 1-14 months for breast cancer patients and 4 months for melanoma patients (Tas, J Oncology, 2012). For most patients, few drugs are available that completely eliminate these metastases (Agarwala, Cancer, 2014). Limited knowledge on which pathways regulate the formation of metastasis in the liver is to blame. Thus, it is of utmost importance to study the metastatic process in its greatest detail to ultimately find drugs that eliminate metastases or prevent the formation of (liver) metastases. By studying the dynamics of the metastatic process in using new in vivo methods (intravital microscopy), we expect to find new insights that can help understand the process of liver metastasis in more detail. To be more specific, we aim to investigate the role of specific genes from the TGF-β pathway during various steps of the metastatic process. **This research will be important for the scientific community as it will show that studying metastasis in vivo in a dynamic manner offers new insights into this intriguing process (scientific relevance). Ultimately, we expect that the knowledge of the role of specific TGF-β genes in metastasis might help formulate new therapies or treatment strategies (Societal relevance).**

3.4 Research strategy

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

The goal of this project is to understand the dynamic role of the TGF-β pathway in liver metastases.

This project will answer the following aims (see also figure in section 3.2):

1. What is the role of TGF-β-related genes during the initial stage of metastasis (Appendix 1 and 3).
2. What is the role of TGF-β in the tumor microenvironment during the initial stage of metastasis (Appendix 1 and 3).
3. What is the role of TGF-β-related genes during the later stage of metastasis (Appendix 2 and 3).
4. What is the role of TGF-β in the tumor microenvironment during the later stage of metastasis (Appendix 2 and 3)

- For each aim we will start by **characterizing** the metastatic potential of tumor cell lines.
- If after characterization no cell line is available that is completely dormant in the liver (meaning the cells are alive, but not forming metastases), we will generate such a **dormant cell line** ourselves (appendix 2, experiment 2).
- To find TGF-β related genes that are important for liver metastasis, we are performing multiple experiments among which an **in vitro screen** in which the TGF-β pathway is targeted in non-metastatic cells grown in 3D. These cells do not proliferate in 3D (they are in a non-metastatic dormant-like state), so we are screening for TGF-β-related targets that can increase proliferation and make these cells more metastatic. Hits will be verified using other in vitro assays like an apoptosis assay, invasion assay, 2D migration assay. Top hits will be verified in vivo.
- In addition to the in vitro screen, we will also perform **RNA-seq** on metastatic versus non-metastatic metastases to identify TGF-β-related genes that might be involved in metastasis formation (appendix 2, experiment 3). Top hits will be verified in vivo.
- Based on the in vitro results and/or RNA-seq results we will pick 3 genes for further in vivo validation. This number is based on the amount of time and people we have to perform the experiments. Moreover, experience has taught us that not every gene validates in vivo, but the chances are high at least one or two of three genes will validate. First, we will **validate the expression** of these genes in tumor or microenvironmental cells using immunohistochemistry or qPCR on tumor tissue from the characterization phase.
- Then, we will **assess the role of TGF-β-related genes** by manipulating these genes in tumor cells (aim 1 and 2) or tumor microenvironmental cells (aim 3 and 4). We will specifically assess the role of those genes during the initial or late stage of the metastatic process.

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

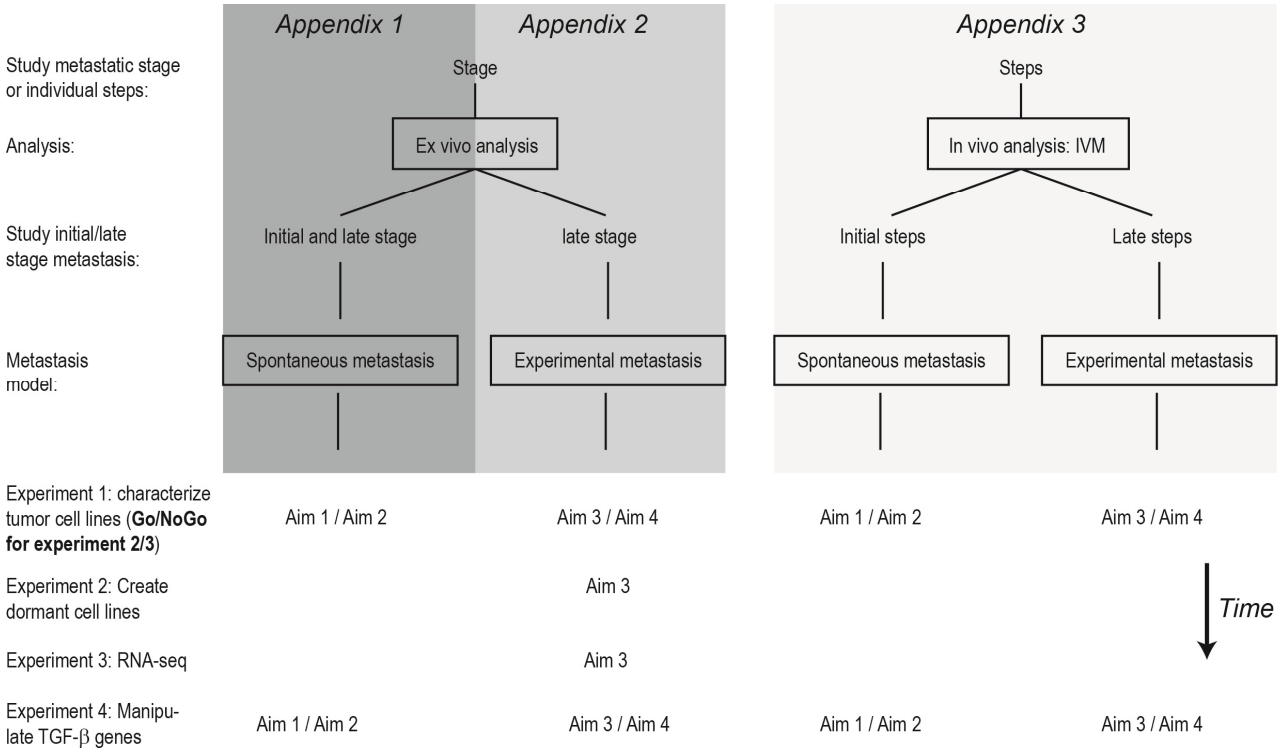
The metastastic process can be subdivided in two stages, each consisting of multiple steps: initial stage (tumor cell dissociation, migration, and intravation into the vasculature), and late stage (tumor cell survival in the blood, extravasation from the vasculature, and colonization of a distant organ). We will study the role of TGF-β-related genes during these metastatic steps using two distinct methods: ex vivo (appendix 1 and 2) and in vivo analyses (appendix 3).

Ex vivo analyses make use of immunohistochemistry on mouse tissue to study the initial or late *stage* of metastasis, as one cannot discriminate the various steps. For example, one can assess the number of metastases, size of metastases, presence of specific cell types or markers etc. Metastases will be studied using a *spontaneous model*, in which tumors are grown and one waits for the tumors to form metastases spontaneously. Ex vivo analyses on this model studies the role of TGF-β-related genes on the entire metastatic cascade, so it is not possible to pinpoint a role for a specific step (e.g. extravasation) (appendix 1). In a second model of metastasis, the *experimental metastasis model*, it is possible to study the role of TGF-β-related genes on the later stages of metastasis, as in this model the initial stage is skipped because cells are injected directly in the vasculature of mice to form metastases (appendix 2). Ex vivo analyses are important as they provide us with information on the ultimate effect TGF-β related genes have on metastasis, and it allows one to study the tissue more carefully for specific proteins or genes using for example immunohistochemistry.

In vivo analysis: A lot of the steps of metastasis are dynamic in nature. To study the role of TGF-β related genes in each *individual step*, intravital microscopy is necessary as it allows one to study these

individual steps over time in a living animal, and analyse the relationship between the various steps. Moreover, it is important to study those processes in vivo, as metastasis is highly dependent on the tumor microenvironment. Intravital microscopy is the *only* technique to study these processes *dynamically* at the cellular resolution in a living animal. Other imaging techniques like the often used bio-luminescence technique or PET scans do not suffice as the resolution is not adequate to image single cells or small micrometastases. Using the *spontaneous model*, in vivo microscopy allows one to study tumor cell detachment from the primary tumor, migration through the surrounding tissue and intravasation in a blood vessel all in one animal. By visualizing TGF-β expression in the same tumor cell during these various steps, it becomes possible to determine the specific role for TGF-β during these steps. Using the *experimental metastasis model* it is possible to study the later steps of metastasis such as extravasation, niche finding and colonization of a secondary organ. These analyses allow us to study the role of TGF-β related genes on specific steps of the metastastic process, and study the dynamics during those steps (Appendix 3). Both the spontaneous metastasis model and the experimental metastasis model will be used for in vivo analysis.

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



We will study the role of TGF-β related genes during the early or late *stage* of metastasis (using ex vivo analysis) or *steps* of metastasis (using in vivo analysis). Early metastasis (aim 1 and 2) can be studied using the spontaneous metastasis model and late metastasis (aim 3 and 4) can be studied using the experimental metastasis model. In all aims we will start with characterizing tumor cell lines for their metastatic potential. Then we will manipulate TGF-β-related genes in tumor cells (aim 1 and 3) or tumor microenvironmental cells (aim 2 and 4) to assess their role. In this way we will be able to address the role of TGF-β related genes in early and late stage metastasis, and their role during the various initial and late steps of metastasis. Ultimately, this leads to a better understanding of the dynamic role of the TGF-β pathway in liver metastasis.

- Note that the characterization of the tumor cell lines will always be performed before TGF-β-related gene manipulation.
- The characterization in experiment 1, are a go/no go for experiment 2 and 3 in appendix 2.
- Appendix 1 and 2 will be performed before appendix 3, because the choice of cell lines in appendix 3 is based on the cell lines used in appendix 1 and 2.

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	Study *spontaneous* metastases ex vivo
2	Study *experimental* metastases ex vivo
3	Study metastases in vivo
4	
5	
6	
7	
8	
9	
10	

Appendix
Description animal procedures

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

1 General information

1.1	Provide the approval number of the 'Netherlands Food and Consumer Product Safety Authority'.	11600
1.2	Provide the name of the licenced establishment.	LUMC
1.3	List the serial number and type of animal procedure. *Use the serial numbers provided in Section 3.4.4 of the Project Proposal form.*	Serial number: 1 — Type of animal procedure: Study spontaneous metastasis ex vivo

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.

We will study the entire metastatic process (initial and late stage) using ex vivo analyses on tissues obtained from mice injected with tumor cells. We will make use of the *spontaneous metastasis model*, meaning that we will inject mice with tumor cells, wait until a tumor has formed and metastases spontaneously arise. These metastases can arise in a variety of organs, which we will collect and analyse ex vivo (for example by immunohistochemistry, qPCR, westernblot or FACS sorting). These ex vivo analyses are important as they provide us with information on the effect TGF-β-related genes have on metastasis.
We will assess the following primary outcome parameter:
- The number of metastases,

And the following secondary outcome parameters:
- Size of metastases
- Presence of specific microenvironmental cells
- Presence/absence of specific genes/proteins

Who chose this method as it is a widely-used method in cancer research and allows one to study the metastatic cascade in its entirety, mimicking the human disease as closely as possible. The applicant has over 8 years of experience with this method.

We will perform the following experiments using the spontaneous metastasis model:
1. Characterization of tumor cell lines (aim 1 and 2):
   To characterize tumor cell lines, we will determine growth of the primary tumor and assess how fast and at which places metastases are forming. We will use this information to identify

timepoints at which the animals should be sacrificed for analysis in experiment 2. In addition, it allows us to determine the variation between mice to form metastases and estimate a coefficient of variation, which can be used to better estimate the number of animals required in experiment 2.
We will also use this experiment to pick the line with the best tumor/metastasis properties to study the TGF-β-related gene (for example, we might need a highly metastatic line to study a gene that reduces metastasis). We will study 3 TGF-β-related genes (see experiment 2, below) in both human and mouse lines, so we will characterize 2 mouse and 2 human lines per gene to pick the best line to study this gene. The lines will be picked based on literature, availability and our experience in working with the lines. Thus, a maximum of 12 breast cancer and 12 melanoma lines will be studied, of which half human and half mouse. This number will be less if a line will be used for more than one gene.
Why study both human and mouse lines? Lines from murine origin can be implanted in immunocompetent mice, providing data on the metastatic process in an isogenic background with an unperturbed immune system. However, contrary to mouse lines, human lines are considered to resemble disease in human patients more closely. But, as human lines cannot be studied in immunocompetent mice, but rather in immunodeficient mice that lac a proper immune system, both murine and human lines offer different insights into the biology and are therefore considered complementary. Hence, we deem it necessary to study both mouse and human lines.

2. Determine the role of TGF-β-related genes by gene manipulation in tumor cells (aim 1) or tumor microenvironmental cells (aim 2):

We will validate genes in the TGF-β pathway. Those genes have been selected for their ability to alter metastasis formation and growth in vitro. Examples are TGF-β2 and BMP7 that induce delayed metastasis formation in vivo (Bragado et al, NBC, 2013). We are currently running an in vitro screen to identify new genes of interest. We will assess the top 3 most likely candidates from these assays for further in vivo validation, in one mouse and one human line (picked based on experiment 1). Using this experiment, we can determine the role of a TGF-β-related gene in metastasis formation. In addition, we can use the tissues containing the metastases and compare the gene manipulated vs. the non-gene manipulated using for example immunohistochemistry or qPCR to obtain more information on the mechanism by which this gene might influence metastasis formation.

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

1. Characterization of tumor cell lines:
   Mice will be injected with (fluorescently labelled) tumor cells at orthotopic site (Breast cancer: mammary fat pad, melanoma: subcutaneous) in a max. volume of 200 ul. The growth of the tumor will be followed by taking measurements at least once a week by calliper (no anesthesia is required, this will cause more discomfort than quickly (<5 mins) measuring the tumor). Mice will be sacrificed at three different time points, but before tumor reaches >2 cm^3. This is important to establish a timeline for metastasis formation (how fast are the metastasis growing, how long does it take before tumor cells have spread to a certain organ, etc) which can be used to determine the best timepoint for analysis in experiment 2. In this experiment the organs will be harvested and used for ex vivo analyses.

2. Determine the role of TGF-β-related genes by gene manipulation in tumor cells (aim 1) or tumor microenvironmental cells (aim 2):
   Mice will be injected with (fluorescently labelled)(genetically manipulated) tumor cells (aim 1) and (Fluorescently labelled)(genetically manipulated) tumor microenvironmental cells (aim 2)) at orthotopic site (Breast cancer: mammary fat pad, melanoma: subcutaneous) in a max. volume of 200 ul. The growth of the tumor will be followed by taking measurements at least once a week by calliper (no anesthesia is required, this will cause more discomfort than quickly (<5 mins) measuring the tumor). Depending on the type of gene manipulation (see below), mice will be given doxycycline to drinking water, injected with tamoxifen i.p (max volume 150 ul), or injected with specific drugs that inhibit our TGF-β-related gene of interest (max volume 150 ul). Mice will be sacrificed at one time point based on experiment 1. The

organs will be harvested and used for ex vivo analyses.

Gene manipulation of tumor or microenvironmental cells:

- To study a gene of interest we will have to manipulate it, meaning overexpression, gene knockout (using e.g. CRISPR) or gene knock down (using e.g. siRNA). Depending on the process that's being studied, it might be necessary to perform inducible manipulation because the gene is essential for a certain process that is not of interest but which precedes the process of interest. Inducible manipulation during a later stage is then warranted. Inducible manipulation can be obtained using one of three systems: the Dox system, the CreERT2-lox system or using chemicals.
    - Using the Doxycycline inducible gene system, the gene of interest is under the control of a Doxycycline inducible promotor. By adding doxycycline to the water of the mouse, the gene or siRNA gets expressed. This does not lead to extra discomfort.
    - Using the CreERT2-lox inducible gene system (part of) the gene of interest is flanked by loxP sites. Upon i.p. injection of tamoxifen, CreERT2 gets activated and will recombine the loxP sites, resulting in gene expression or gene knockouts. The mouse will be i.p. injected with tamoxifen for a maximum of 2x (to get sufficient induction; max volume 150 µl). The discomfort is minimal but we still aim to give the injections simultaneously with tumor measurements (under anaesthesia) to reduce discomfort.
    - Gene manipulation can easily be obtained using drugs (chemicals) that are available from pharma companies. We will use drugs that specifically inhibit our gene of interest. To use drugs, they need to be administered s.c., i.p., i.m., or i.v. or using a slow release pump. We will opt for the least invasive method of injection if multiple drugs or routes of injection are at our disposal (slow release pump > s.c. > i.p. > i.m > i.v.). The animals will be injected for a maximum of 10 times in 1 months' time.
- Based on available plasmids for that gene we will chose for non-inducible gene manipulation, doxycycline inducible gene manipulation, CreERT2 inducible gene manipulation or drugs based inducible gene manipulation. If all methods can be used, we will opt for non-inducible gene manipulation or Dox inducible gene manipulation, because this does not lead to additional discomfort.

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

Minimise number of animals:
To minimise the number of animals used in experiment 2, we will establish a time line for tumor and metastasis growth in experiment 1 that can be used as a reference. Thus, by performing the experiment 1, we will reduce the number of animals needed in experiment 2. As experiment 2 will be performed for 3 different genes, this ultimately reduces the number of animals required.
In addition, we have the option to characterize multiple cell lines in experiment 1, so that the cell line that has the best properties to study a TGF-β-related gene can be picked in experiment 2. This ultimately reduces the number of animals.
TGF-β-related genes that will be studied in experiment 2 have already been shown to affect dormancy in vitro. Moreover, the presence of these genes in the metastatic cells or microenvironmental cells can be verified in tissues from experiment 1. Only the top 3 most promising candidates will be validated in vivo.

Statistics:
Experiment 1:
Based on experience we know that there is quite some variation (coefficient of variation approx. 25%) between animals with regards to metastases formation (Eckhardt, Mol Canc Res, 2005 / Johnstone, Dis Mod Mech, 2015). To obtain a reliable coefficient of variation in experiment 1 that can be used to calculate animal numbers for experiment 2, we will need at least 5 animals per experiment. This number of animals is based on similar experiments done by others: Eckhardt, Mol Canc Res, 2005 / Johnstone, Dis Mod Mech, 2015. However, if we obtain results that are highly variable (coefficient of variation >25%), it will increase the number of animals needed for further experiment 2, as they are based on this. Therefore, we deem it important to get a more reliable standard deviation by adding more mice.

Our lab has repeatedly performed gene manipulation experiments using the above described cell lines and often required 8 mice per experimental group [redacted] Thus, we will start by using 5 animals per experiment, but can increase this number to 8 if 5 has a standard deviation that is >25%.

Experiment 2:
Our primary outcome parameters will be "number of metastases". We will use the coefficient of variation calculated in experiment 1. We used data from a previous experiment that was comparable to what we are expecting to get now [redacted] in order to derive realistic values for the size of the variability in the data and the treatment/intervention effect. In particular, a linear mixed effects model have been fitted on the log-transformed sizes of tumors (R package lme4, Bates et al 2015). The transformation has been applied due to skewness in the raw data. Next we computed the sample size needed to detect a 82% relative decrease in the tumor size in the manipulated group vs the non-manipulated group with 80% power. The computation has been done via simulation and using the values from [redacted]. **We found that 10 mice per group needed in this case.**

B. The animals

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

Species: Mus musculus.

For BC mouse lines BALB/c: isogenic background.
For BC human lines: Immunodeficient mice (to enable xenografting).
For MM mouse lines C57/Bl6: isogenic background.
for MM human lines: Immunodeficient mice (to enable xenografting).

For all breast cancer studies we will make use of female mice exclusively, because breast cancer mainly occurs in females. Moreover, we focus on triple negative breast cancers, whereas males predominantly get ER^+ breast cancer, suggesting that our results would not be applicable to the male population.
In case of melanoma, males are more likely to cause infractions of the tumors (tumors become unusable) because they are more aggressive. Thus, using males would require us to house the mice in solitary, causing more discomfort, or use more mice to account for the loss of some tumors. As such, we have decided to use female mice for our melanoma research.

Origin: LUMC or external licenced breeders like Jackson labs and Charles River.

Life stages: Between 8-12 weeks old, this is based on literature. Also, at 8 weeks the mammary fat pads are fully grown and developed.

Estimated number for each experiment (calculations are done based on the maximum number of 8 mice, but are estimated to be performed with only 5 mice, see statistics. Moreover, calculations are based on experiments being performed for all cell lines, but are estimated to be performed for only half of them):

1. Breast cancer (BC): 8 mice x 3 time points x 12 tumor lines (6 mouse, 6 human, see part A) x 2 aims (1 and 2) = 576 mice. Melanoma skin cancer (MC): 8 mice x 3 time points x 12 tumor lines (6 mouse, 6 human, see part A) x 2 aims (1 and 2) = 576 mice. Total (BC and MC): 1152 mice.
2. BC: 10 mice x 2 experimental groups (manipulated vs not manipulated) x 2 tumor lines (1 mouse, 1 human) x 3 genes x 2 aims (1 and 2) = 240 mice. MC: 10 mice x 2 experimental groups (manipulated vs not manipulated) x 2 tumor lines (1 mouse, 1 human) x 3 genes x 2 aims (1 and 2) = 240 mice. Total (BC and MC): 480 mice.

Combined: 1152 + 480 = 1632 mice.

C. Re-use

Will the animals be re-used?

☒ No, continue with question D.

☐ Yes > Explain why re-use is considered acceptable for this animal procedure.

Are the previous or proposed animal procedures classified as 'severe'?

☐ No

☐ Yes> Provide specific justifications for the re-use of these animals during the procedures.

D. Replacement, reduction, refinement

Describe how the principles of replacement, reduction and refinement were included in the research strategy, e.g. the selection of the animals, the design of the procedures and the number of animals.

Replacement: We have replaced animal experiments by performing initial experiments in vitro. However, to study the different steps of metastasis we cannot use in vitro data because we need an intact animal with the various organs to be able to study the metastases in these organs.
Reduction: The experiments described in experiment 1 are condition finding. Hence, the data obtained in experiment 1will be used as a guide for experiment 2. For example, it will inform us on the coefficient of variation for the measured parameters, which will be helpful for a better estimation of the number of animals required for experiments 2, thereby reducing the number of animals.
Refinement: By making use of female mice, the animals can be housed in groups. In addition, when we will manipulate genes, we will opt for the least invasive method: slow release pump > s.c. > i.p. > i.m > i.v.. This reduces discomfort for the animals as much as possible.

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

1. Tumor measurements will be performed by experienced researchers without the need for anaesthesia, which would cause more discomfort (fear/stress).
2. There are no adverse effects on the environment; all animals will be housed under strict D1 conditions.

Repetition and duplication

E. Repetition

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

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 short-term anaesthesia (<5 mins) will be used during the injection of tumor cells in the mammary fat pad. It will be performed by trained researchers only.

I. Other aspects compromising the welfare of the animals

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

The animals may experience mild discomfort from the injection of tumor cells and therapeutic compounds, tumor measurements and anaesthesia.

Explain why these effects may emerge.

The injection or anaesthesia induction can cause mild discomfort to the animal.

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

The anaesthesia will be performed as short as possible by an experienced researcher. For measuring tumor growth and injection of cells in the fat pad < 5 mins.

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.

In all cases we will use criteria according to the code of practice Animals in Cancer Research. In case of tumor/metastases growth and/or window implantation, these are: severe loss of body weight (monitoring every other day based on body conditioning score), severe circulation or breathing problems, changes in behavior (posture, general signals of severe sickness or discomfort), severe clinical appearance of tumor (ulceration, growth hampering mobility, swollen abdomen), a total tumor mass that is too big (>2 cm^3).

Indicate the likely incidence.

< 1%

K. Classification of severity of procedures

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

The mice will experience short-term discomfort from injections or tumor measurements. All these procedures are assigned to the category: Mild

Description	Animal species/strain and sex	Number of animals	Discomfort (expected cumulative discomfort)	Discomfort is a sum of following procedures
Experiment 1/2	-Mus musculus. -BALB/c, C57/Bl6, immunodeficient mice. -Female.	1632	Mild 100%	Tumor cell injections, tumor/metastasis growth, tumor measurements by calliper, if necessary injections for drugs to manipulate genes
			Moderate 0%	
			Severe 0%	
		Total	100%	

End of experiment

L. Method of killing

Will the animals be killed during or after the procedures?

☐ No

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

The animals will have tumors and metastases. If the experiment is finished the animals will have to be sacrificed because otherwise the tumors/metastases will keep growing. Moreover, the organs will be used for further analyses using immunohistochemistry to determine cellular and molecular aspects of tumor/metastasis growth.

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'.	11600
1.2	Provide the name of the licenced establishment.	LUMC
1.3	List the serial number and type of animal procedure. *Use the serial numbers provided in Section 3.4.4 of the Project Proposal form.*	Serial number: 2 Type of animal procedure: Study experimental metastasis ex vivo

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.

We will study the late stage of the metastatic process using ex vivo analyses on tissues obtained from mice injected with tumor cells. We will make use of the *experimental metastasis model*, meaning that we will inject mice with melanoma or breast cancer cells directly in mesenteric vein or spleen. Cells will then rapidly reach the liver, extravasate and form metastases. No primary tumor formation is involved in this assay, and therefore we will only study the later stage of metastasis. The livers will be collected and analysed ex vivo (for example by immunohistochemistry, qPCR, westernblot or FACS sorting). These ex vivo analyses are important as they provide us with information on the effect TGF-β-related genes have on metastasis.
We will assess the following primary outcome parameter:
- Hepatic replacement area (the total area of liver that is replaced by metastases)

And the following secondary outcome parameters:
- The number of metastases,
- Size of metastases,
- Presence of specific microenvironmental cells
- Presence/absence of specific genes/proteins

We chose this method as it is a widely-used method in cancer research and allows us to study the late stage of the metastatic cascade. As such, it becomes easier to attribute a role for genes in this part of metastatic process. Moreover, the use of this model is essential in metastasis research because often gene manipulation will result in differences in primary tumor growth, making it impossible to study the effect of this gene on metastasis in the spontaneous tumor model; the experimental metastasis model offers a solution because the tumor cells are injected into the vasculature so there is no primary tumor.

We have over 8 years of experience with this method.

We will perform the following experiments using the experimental metastasis model:

1. Characterization of tumor cell lines (aim 3 and 4):
   To characterize tumor cell lines, we will assess how fast and how many metastases are forming. We will use this information to identify time points at which the animals should be sacrificed for analysis in experiment 2. In addition, it allows us to determine the variation between mice to form metastases and estimate a coefficient of variation, which can be used to better estimate the number of animals required in experiment 2.
   Similar to appendix 1, we will study a maximum of 12 breast cancer and 12 melanoma lines, of which half human and half mouse. The experiment will be performed for aim 3 and aim 4. For more information on the numbers see appendix 1.

2. Create a new dormant cell line (aim 3):
   Here we have the option of generating a new dormant non-metastatic cell line. This is necessary to study the process of tumor cells awakening from dormancy and forming metastases (by characterizing this cell line (experiment 1), and later by using it to study the effect of gene manipulation on the escape of tumor cells from dormancy (experiment 4)). The currently available cell lines have been shown to have dormant characteristics in some reports, but not in others. Thus, if the cells are not completely dormant and non-metastatic, we will generate dormant non-metastatic lines derived from the parental line by repeatedly isolating the dormant cells from the liver using FACS sorting and reinjecting them in donor mice. This method selects for cells with dormant properties and has been shown to work (de Cock, Cancer Research, 2016). Moreover, we have experience with isolating live cells from organs. This experiment will only be performed if the results in experiment 1 show that the cells are not completely dormant (go/no go). In case of melanoma there are no dormant lines, so we will have to generate them anyway. It will be performed only for 1 human and 1 mouse cell line, as we will continue with just 1 human and 1 mouse cell line in the next experiments.

3. Isolate cells for RNA-seq (aim 3):
   To identify TGF-β related genes that are important for metastasis in an unbiased manner we will use the experimental metastasis model to create liver metastases which we will isolate using FACS sorting and process for RNA-seq. We will compare cells from metastases that were growing rapidly (highly metastatic) versus cells from metastases that were not growing rapidly (non-metastatic) using a method that has been described in de Cock, Cancer Research, 2016. This experiment will only be performed if the results in experiment 1 show that there are cell lines that show huge differences in metastatic outgrowth of tumor cells within a single animal (go/no go). It will be performed in one mouse and one human line, for both Breast cancer and melanoma.

4. Determine the role of TGF-β-related genes by gene manipulation in tumor cells (aim 3) or tumor microenvironmental cells (aim 4):
   Using this experiment, we can determine the role of a TGF-β-related gene in the *late* stage of metastasis formation. In addition, we can use the tissues containing the metastases and compare the gene manipulated vs. the non-gene manipulated using for example immunohistochemistry or qPCR to obtain more information on the mechanism by which this gene might influence metastasis formation. The experiment will be performed twice (for aim 3, manipulating TGF-β related genes in the tumor cells, and for aim 4, manipulating TGF-β related genes in the tumor microenvironmental cells). We will validate genes in the TGF-β pathway (see appendix 1 for an explanation on how the genes were selected). We will assess the top 3 most likely candidates from these assays for further in vivo validation, in one mouse and one human line (picked based on experiment 1).

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

1. Characterization of tumor cell lines:
   Mice will be injected with (fluorescently labelled) tumor cells in the mesenteric vein or spleen in a max. volume of 100 ul. In this way, the liver is the first capillary network the tumor cells encounter where they get stuck and grow out to form liver metastases. Mice will be sacrificed at three different time points to establish a timeline for metastasis formation (how fast are the metastasis growing, what is the take rate of cells that grow out into a metastasis etc) which can be used to determine the best timepoint for analysis in experiments 2-4. In this experiment the organs will be harvested and used for ex vivo analyses.

2. Create a new dormant cell line:
   Similar to experiment 1, but mice will only be sacrificed at one timepoint when dormant cells are still present (based on experiment 1). The liver will be harvested, dissociated, and dormant tumor cells will be isolated and injected into a new donor mouse (again intrasplenically or intramesenterically). By repeating this process 10 times a dormant cell line can be generated (de Cock, Cancer Research, 2016).

3. Isolate cells for RNA-seq:
   Similar to experiment 1, but mice will only be sacrificed at one timepoint when big differences between metastastic outgrowth are still present (based on experiment 1). The liver will be harvested, dissociated, and fluorescent tumor cells will be isolated using FACS sorting (de Cock, Cancer Research, 2016). Then, RNA will be extracted for sequencing.

4. Determine the role of TGF-β-related genes by gene manipulation in tumor cells (aim 3) or tumor microenvironmental cells (aim 4):
   Mice will be injected with (fluorescently labelled)(genetically manipulated) tumor cells (aim 3) and (Fluorescently labelled)(genetically manipulated) tumor microenvironmental cells (aim 4)) in the mesenteric vein or spleen in a max. volume of 200 ul. Depending on the type of gene manipulation (see appendix 1), mice will be given doxycycline to drinking water, injected with tamoxifen i.p (max volume 150 ul), or injected with specific drugs that inhibit our TGF-β-related gene of interest (max volume 150 ul). Mice will be sacrificed at one time point based on experiment 1. The organs will be harvested and used for ex vivo analyses like immunohistochemistry and qPRC.

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

Minimise number of animals:
To minimise the number of animals used in experiment 2-4, we will establish a time line for metastasis growth in experiment 1 that can be used as a reference. Thus, by performing experiment 1, we will reduce the number of animals needed in experiment 2 because we will only need to sacrifice the animals at 1 timepoint. As experiment 4 will be performed for 3 different genes, this ultimately reduces the number of animals required.
The same argument can be made for the characterization of multiple cell lines in experiment 1. By doing this, the cell line that has the best properties to study a TGF-β-related gene can be picked in experiment 2-4. This ultimately reduces the number of animals.

Statistics:
Experiment 1:
See appendix 1. We need a maximum of **8 mice** per cell line.

Experiment 2:
To generate a dormant cell line, cells have to be injected in 10 successive mice (see protocol de Cock, Cancer Research, 2016). So, we need **10 mice** per cell line.

Experiment 3:
To obtain enough cells from the non-metastatic clone to perform RNA-seq, we will need to pool the cells

from 3 mice. Moreover, for RNA-seq, at least 2 biological repeats should be performed. Hence, we need **6 mice** per cell line.

Experiment 4:
Our primary outcome parameter is hepatic replacement area. We will use the coefficient of variation calculated in experiment 1. We used data from a previous experiment that was comparable to what we are expecting to get now [redacted] Sci transl Med, 2012) in order to derive realistic values for the size of the variability in the data and the treatment/intervention? effect. In particular, we computed the sample size needed to detect an effect size of 2 between the manipulated and the non-manipulated groups with 80% power using the function pwr.t.test in the R package pwr (Champely, 2016). **We found that 5 mice per group are needed in this case.**

B. The animals

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

Species: Mus musculus.

For BC mouse lines BALB/c: isogenic background.
For BC human lines: Immunodeficient mice (to enable xenografting).
For MM mouse lines C57/Bl6: isogenic background.
for MM human lines: Immunodeficient mice (to enable xenografting).

For all breast cancer studies we will make use of female mice exclusively, because breast cancer mainly occurs in females. Moreover, we focus on triple negative breast cancers, whereas males predominantly get ER^+ breast cancer, suggesting that our results would not be applicable to the male population.
In case of melanoma, males are more likely to cause infractions of the tumors (tumors become unusable) because they are more aggressive. Thus, using males would require us to house the mice in solitary, causing more discomfort, or use more mice to account for the loss of some tumors. As such, we have decided to use female mice for our melanoma research.
Origin: LUMC or external licenced breeders like Jackson labs and Charles River.

Life stages: Between 8-12 weeks old, this is based on literature. Also, at 8 weeks the mammary fat pads are fully grown and developed.

Estimated number for each experiment:

1. Breast cancer (BC): 8 mice x 3 time points x 12 tumor lines (6 mouse, 6 human, see part A) x 2 aims (aim 3 and 4) = 576 mice. Melanoma skin cancer (MC): 8 mice x 3 time points x 12 tumor lines (6 mouse, 6 human, see part A) x 2 aims (aim 3 and 4) = 576 mice. Total (BC and MC): 1152 mice. * Less mice might be used if similar cell lines are used to study multiple genes.
2. BC: 10 mice x 2 tumor lines (1 mouse, 1 human) = 20 mice. MC: 10 mice x 2 tumor lines (1 mouse, 1 human) = 20 mice. Total (BC and MC) = 40 mice.
3. BC: 6 mice x 2 tumor lines (1 mouse, 1 human) = 12 mice. MC: 6 mice x 2 tumor lines (1 mouse, 1 human) = 12 mice. Total (BC and MC): 24 mice.
4. BC: 5 mice x 2 experimental groups (manipulated vs not manipulated) x 2 tumor lines (1 mouse, 1 human) x 3 genes x 2 aims (3 and 4) = 120 mice. MC: 5 mice x 2 experimental groups (manipulated vs not manipulated) x 2 tumor lines (1 mouse, 1 human) x 3 genes x 2 aims (3 and 43) = 120 mice. Total (BC and MC): 240 mice.

Combined: 1152 + 40 + 24 + 240 = 1456 mice.

C. Re-use

Will the animals be re-used?

☒ No, continue with question D.

☐ Yes > Explain why re-use is considered acceptable for this animal procedure.

Are the previous or proposed animal procedures classified as 'severe'?

☐ No

☐ Yes> Provide specific justifications for the re-use of these animals during the procedures.

D. Replacement, reduction, refinement

Describe how the principles of replacement, reduction and refinement were included in the research strategy, e.g. the selection of the animals, the design of the procedures and the number of animals.

Replacement: We have replaced animal experiments by performing initial experiments in vitro. However, to study the different steps of metastasis we cannot use in vitro data because we need an intact animal with the various organs to be able to study the metastases in these organs.
Reduction: The experiments described in experiment 1 are condition finding. Hence, the data obtained in experiment 1 will be used as a guide for experiment 2-4. For example, it will inform us on the coefficient of variation for the measured parameters, which will be helpful for a better estimation of the number of animals required for experiments 2-4, thereby reducing the number of animals.
In addition, experiment 2 and 3 are go/no go and will only be performed if experiment 1 shows that it is necessary or possible to perform those experiments.
Refinement: By making use of female mice, the animals can be housed in groups. In addition, when we will manipulate genes, we will opt for the least invasive method: slow release pump > s.c. > i.p. > i.m > i.v.. This reduces discomfort for the animals as much as possible.

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

1. Animals will be monitored intensively after receiving surgery to inject the tumor cells. Before start of the surgery the animals will receive pain killers. Moreover, animals will be monitored on a daily basis by the animal caretakers and at least once a week by the researcher to look for signs of distress caused by tumor growth.
2. There are no adverse effects on the environment; all animals will be housed under strict D1 conditions.

Repetition and duplication

E. Repetition

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

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 anaesthesia will be used during the injection of tumor cells in the mesenteric vein or spleen. The animals will be a pain killer before surgery.

I. Other aspects compromising the welfare of the animals

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

The animals may experience mild discomfort from the injection of therapeutic compounds.

Explain why these effects may emerge.

The injection can cause mild discomfort to the animal.

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

The injection of the compound will be performed by an experience researcher, without anaesthesia, as this will cause more distress than a short injection.

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.

In all cases we will use criteria according to the code of practice Animals in Cancer Research. In case of tumor/metastases growth these are: severe loss of body weight (monitoring every other day based on body conditioning score), severe circulation or breathing problems, changes in behavior (posture, general signals of severe sickness or discomfort), severe clinical appearance of tumor (ulceration, growth hampering mobility, swollen abdomen), a total tumor mass that is too big (>2 cm^3).

Indicate the likely incidence.

< 1%

K. Classification of severity of procedures

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

The injection of tumor cells in the mesenteric vein or spleen is assigned to the category: Moderate.

Description	Animal species/strain and sex	Number of animals	Discomfort (expected cumulative discomfort)	Discomfort is a sum of following procedures
			Mild 0%	
Experiment 1/2/3/4	-Mus musculus. -BALB/c, C57/Bl6, immunodeficient mice. -Female.	1456	Moderate 100%	Tumor cell injections in spleen or mesenteric vein, metastasis growth, if necessary injections for drugs to manipulate genes.
			Severe 0%	
		Total	100%	

End of experiment

L. Method of killing

Will the animals be killed during or after the procedures?

☐ No

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

The animals will have metastases. If the experiment is finished the animals will have to be sacrificed because otherwise the metastases will keep growing. Moreover, the organs will be used for further analyses using immunohistochemistry to determine cellular and molecular aspects of metastasis growth.

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'.	11600
1.2	Provide the name of the licenced establishment.	LUMC
1.3	List the serial number and type of animal procedure. *Use the serial numbers provided in Section 3.4.4 of the Project Proposal form.*	Serial number: 3 Type of animal procedure: Study metastasis in vivo

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.

We will study individual steps of the entire metastatic process (early and late stage) using *in vivo* analyses on tissues obtained from mice injected with tumor cells. It is important to study the individual steps of metastasis because we expect that TGF-β-related proteins might be differentially regulated in each of the steps. The only method to study the individual steps of metastasis in vivo is using intravital microscopy.
We will make use of the *spontaneous metastasis model in combination with intravital microscopy* to study the early steps of metastasis (detachment, invasion, intravasation) by imaging individual tumor cells over time. This allows us to measure parameters like cell speed, plasticity, directionality etc, which are all important for metastasis.
We will also make use of *the experimental metastasis model in combination with intravital microscopy* to study the late steps of metastasis (Survival in the blood, extravasation, colonization). By imaging individual tumor cells over time, we can measure cell migration, but also cell proliferation/cell doubling, which is important for colonization.

We chose this method as it is the *only* method to study cells at the single level inside a living animal, and as such it is the *only* method to study the individual steps of the metastatic cascade. The applicant has over 8 years of experience with this method.

We will perform the following experiments using the spontaneous metastasis and the experimental metastasis model for aim 1 - 4:

1. Characterization of tumor cell lines (aim 1-4):
   To characterize tumor cell lines, we will assess tumor cell migration and intravasation in the

primary tumor (using the spontaneous model and intravital microscopy) and survival in the blood, extravasation and colonization (using the experimental model and intravital microscopy). This allows us to determine the variation between mice and estimate a coefficient of variation, which can be used to better estimate the number of animals required in experiment 2.
We will study 3 TGF-β-related genes (see experiment 2, below) in both human and mouse lines, so we will characterize 2 mouse and 2 human lines per gene to pick the best line to study this gene. The lines will be picked based on experiments performed in appendix 1 and 2. Thus, a maximum of 2 breast cancer and 2 melanoma lines will be studied, of which half human and half mouse.
Why study both human and mouse lines? See appendix 1

2. Determine the role of TGF-β-related genes by gene manipulation in tumor cells (aim 1/3) or tumor microenvironmental cells (aim 1-4):
We will validate genes in the TGF-β pathway (see appendix 1 for an explanation on how the genes were selected). We will assess the top 3 most likely candidates from these assays for further in vivo validation, in one mouse and one human line (picked based on experiment 1). Using this experiment, we can determine the role of a TGF-β-related gene in *each individual step* of the metastasis process. The spontaneous assay and the experimental assay will be performed twice (manipulating TGF-β related genes in the tumor cells, and manipulating TGF-β related genes in the tumor microenvironmental cells).

3. Training:
The success of this project is for a large part dependent on the successful execution of intravital microscopy (IVM). Surgery comprises an important part of IVM, and proper execution of this surgery is vital for the experiment and determines for a large part the discomfort of the animal. Therefore, we deem it necessary to practise these procedures with new labmembers before allowing them to perform these types of experiments. It is essential that all researchers who will perform IVM will learn the techniques because it is essential surgeries are done at a certain stage or time. Hence, it is not possible to have a single dedicated member doing all the procedures. Each new lab member will learn intrasplenic/intramesenteric injections, short-term IVM, long-term IVM. Depending on the experience of the researcher with microsurgeries, more or less animals will be used; however, for each trainee a maximum number of animals is given that should not be exceeded. A training will be finished if the procedure can be performed successfully and within a certain time limit without the help of an experienced supervisor. This will be assessed by an independent researcher.

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

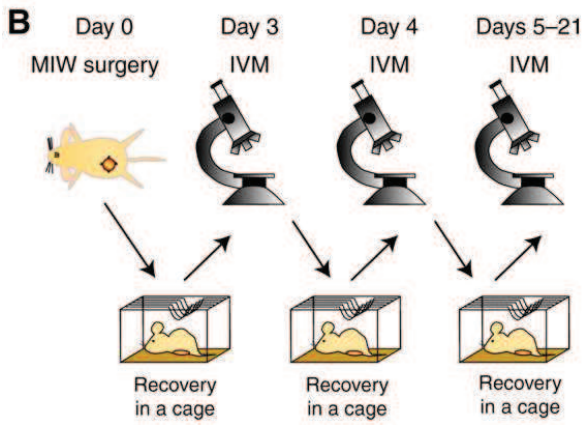
1. Characterization of tumor cell lines:
For this experiment, we will only use cell lines that have been selected for use in appendix 1 and 2. Hence, it reduces the number of cell lines to characterize to 1 human and 1 mouse line for both breast cancer and melanoma.
Spontaneous metastasis model. Mice will be injected with fluorescently labelled tumor cells at orthotopic site (Breast cancer: mammary fat pad, melanoma: subcutaneous) in a max. volume of 200 ul. The growth of the tumor will be followed by taking measurements at least once a week by calliper (no anesthesia is required, this will cause more discomfort than quickly (<5 mins) measuring the tumor). Short and long-term intravital microscopy will be performed (see explanation below) on the primary tumor before tumor reaches >2 cm^3 (timepoint based on appendix 2, experiment 1). This is important to determine which cell line can best be used for analysis in in experiment 2. After intravital microscopy the organs will be harvested and used for ex vivo analyses.
Experimental metastasis model. Mice will be injected with fluorescently labelled tumor cells in the mesenteric vein or spleen in a max. volume of 100 ul. Short and long-term intravital microscopy will be performed (see explanation below) on the liver metastases (timepoint based on appendix 2, experiment 1). This is important to determine which cell line can best

be used for analysis in in experiment 2. After intravital microscopy the organs will be harvested and used for ex vivo analyses.

2. Determine the role of TGF-β-related genes by gene manipulation in tumor cells (aim 1/3) or tumor microenvironmental cells (aim 2/4):
   Spontaneous metastasis model. Mice will be injected with fluorescently labelled (genetically manipulated) tumor cells (aim 1/3) and (Fluorescently labelled)(genetically manipulated) tumor microenvironmental cells (aim 2/4)) at orthotopic site (Breast cancer: mammary fat pad, melanoma: subcutaneous) in a max. volume of 200 ul. The growth of the tumor will be followed by taking measurements at least once a week by calliper (no anesthesia is required, this will cause more discomfort than quickly (<5 mins) measuring the tumor). Depending on the type of gene manipulation (see appendix 1), mice will be given doxycycline to drinking water, injected with tamoxifen i.p (max volume 150 ul), or injected with specific drugs that inhibit our TGF-β-related gene of interest (max volume 150 ul). Short and long-term intravital microscopy will be performed (see explanation below) on the primary tumor before tumor reaches >2 cm^3. The organs will be harvested and used for ex vivo analyses. This experiment allows us to assess the role of TGF-β related genes in specific steps of the early metastatic cascade.
   Experimental metastasis model. Same as for spontaneous model, except that cells will be injected in the mesenteric vein or spleen, and imaging will be performed on the liver. This experiment allows us to assess the role of TGF-β related genes in specific steps of the latey metastatic cascade.

3. Training animals:
   Procedures are similar to experiment 1.

- *Short and long-term intravital microscopy*
  Intravital microscopy, meaning visualization of single fluorescent cells in living animals, will be used to study the individual steps of the metastatic cascade in vivo. Intravital microscopy can be performed short-term or long-term.
  Short-term (< 24 hours): Mice are anesthetized, the organ/tumor gets exposed and imaged on the microscope. This is a terminal procedure: after imaging the mouse is sacrificed. This procedure is used for assessing for example tumor cell migration or extravasation.
  Long-term (max 28 days): Mice are anesthetized and an imaging window will be implanted on top of the tumor/organ. The tumor cells can be visualized on the microscope through the imaging window. After imaging the mouse will recover in its cage. By repeating this process over multiple days, processes that take more than one day can be visualized (see figure below). For example, long-term migration, proliferation, and formation of micrometastases. Both methods are required to be able to study all of the different steps of metastasis. We have a lot of expertise in both intravital microscopy methods (> 6 years) and have developed various methods to perfrom intravital microscopy [redacted]



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

Minimise number of animals:
By performing characterization in experiment 1, we can determine a more reliable coefficient of variation to make a better estimate of the required number of animals to get statistically significant data in experiment 2.
In this appendix, we will only use cell lines that have been selected for use in appendix 1 and 2. Hence, it reduces the number of cell lines to characterize, and therefore it also reduces the number of animals.
Furthermore, by performing long-term IVM, we can study the same process over time in the same animal. Ultimately, this reduces the number of animals required for these kind experiments, because we don't have to sacrifice multiple animals at various time points.
In the training animals, we chose to combine the teaching of intrasplenic/intramesenteric vein injections and intravital microscopy in the same animal, as it will reduce the number of animals required for teaching purposes. Moreover, a competent supervisor will ensure proper training of the new labmember; if the new labmember is deemed sufficiently trained by the supervisor with fewer animals than the maximum requested, the other animals will not be used.

Statistics:
Experiment 1:
See appendix 1. We need a maximum of **8 mice** per cell line.

Experiment 2:
We used data from a previous experiment that was comparable to what we are expecting to get now [redacted] Sci Transl Med, 2012) in order to derive realistic values for the size of the variability in the data and the treatment/intervention effect. In particular, a linear mixed effects model have been fitted on the log-transformed migration speed (R package lme4, Bates et al 2015). The transformation has been applied due to skewness in the raw data. Next we computed the sample size needed to detect a 55% relative decrease in the migration speed in the manipulated group vs the non-manipulated group with 80% power. The computation has been done via simulation and using the values from the experiment of [redacted], Sci Transl Med, 2012. **We found that 6 mice per group needed in this case.**

Experiment 3:
From experience, we know that ~10 animals are required to learn intrasplenic/intramesenteric injections, ~5 animals are required to learn the skinflap procedure (short-terim IVM), and ~8 animals are required to learn the window procedure (long-term IVM). So, 10 animals for short term-IVM and 10 animals for long-term IVM should be sufficient. If the labmembers are not deemed sufficiently trained after exceeding this number of animals, the members will not be allowed to perform the surgery.
We estimate that we will have to train 5 new labmembers. If this number is not met, the other animals will not be used.

B. The animals

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

Species: Mus musculus.

For BC mouse lines BALB/c: isogenic background.
For BC human lines: Immunodeficient mice (to enable xenografting).
For MM mouse lines C57/Bl6: isogenic background.
for MM human lines: Immunodeficient mice (to enable xenografting).

For all breast cancer studies we will make use of female mice exclusively, because breast cancer mainly occurs in females. Moreover, we focus on triple negative breast cancers, whereas males predominantly get ER^+ breast cancer, suggesting that our results would not be applicable to the male population.
In case of melanoma, males are more likely to cause infractions of the tumors (tumors become unusable) because they are more aggressive. Thus, using males would require us to house the mice in solitary, causing more discomfort, or use more mice to account for the loss of some tumors. As such, we have decided to use female mice for our melanoma research.
Origin: LUMC or external licenced breeders like Jackson labs and Charles River.

Life stages: Between 8-12 weeks old, this is based on literature. Also, at 8 weeks the mammary fat pads are fully grown and developed.

Estimated number for each experiment:

1. Breast cancer (BC): 8 mice x 2 tumor lines (1 mouse, 1 human, see part A) x 2 methods (spontaneous and experimental) x 2 IVM (short and long-term) x 4 aims = 256 mice. Melanoma skin cancer (MC): 8 mice x 2 tumor lines (1 mouse, 1 human, see part A) x 2 methods (spontaneous and experimental) x 2 IVM (short and long-term) x 4 aims = 256 mice. Total (BC and MC): 512 mice.
2. BC: 6 mice x 2 experimental groups (manipulated vs not manipulated) x 2 tumor lines (1 mouse, 1 human) x 3 genes x 2 methods (spontaneous and experimental) x 4 aims = 576 mice. MC: 6 mice x 2 experimental groups (manipulated vs not manipulated) x 2 tumor lines (1 mouse, 1 human) x 3 genes x 2 methods (spontaneous and experimental) x 4 aims = 576 mice. Total (BC and MC): 1152 mice.
3. 10 mice x 2 IVM (short and long-term) x 5 labmembers = 100 mice

Combined: 512 + 1152 + 100 = 1764 mice.

C. Re-use

Will the animals be re-used?

☒ No, continue with question D.

☐ Yes > Explain why re-use is considered acceptable for this animal procedure.

Are the previous or proposed animal procedures classified as 'severe'?

☐ No

☐ Yes> Provide specific justifications for the re-use of these animals during the procedures.

D. Replacement, reduction, refinement

Describe how the principles of replacement, reduction and refinement were included in the research strategy, e.g. the selection of the animals, the design of the procedures and the number of animals.

Replacement: We have replaced animal experiments by performing initial experiments in vitro. However, to study the different steps of metastasis we cannot use in vitro data because we need an intact animal with the various organs to be able to study the metastases in these organs.

Reduction: The experiments described in experiment 1 are condition finding. Hence, the data obtained in experiment 1will be used as a guide for experiment 2. For example, it will inform us on the coefficient of variation for the measured parameters, which will be helpful for a better estimation of the number of animals required for experiments 2, thereby reducing the number of animals.
By making use of multi-day IVM experiments we will also reduce the number of animals because we can use a single animal for multiple time points.
Refinement: We refine the experiments by first performing ex vivo experiments and based on those experiments we decide which cell lines to use in vivo. Because the ex vivo experiments cause less discomfort compared to the in vivo experiments we reduce the amount of discomfort for a lot of mice. In addition, when we will manipulate genes, we will opt for the least invasive method: slow release pump > s.c. > i.p. > i.m > i.v.. This reduces discomfort for the animals as much as possible.

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

1. We will perform surgeries under adequate anaesthesia. Analgesia will be provided prior and post-surgery to reduce the pain. We also reduce animal suffering by sacrificing the animal immediately after the last IVM sessions, so while the animal is still under anaesthesia. Tumor measurements will be performed by experienced researchers without the need for anaesthesia, which would cause more discomfort.
2. There are no adverse effects on the environment; all animals will be housed under strict D1 conditions.

Repetition and duplication

E. Repetition

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

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.

1. Adequate analgesia will be given before surgery (to make sure the analgesia is effective). Surgery will be performed by trained researchers only.
2. Adequate short-term anaesthesia (<5 mins) will be used during the injection of tumor cells in the mammary fat pad. It will be performed by trained researchers only.
3. Adequate anaesthesia will also be used during the IVM sessions to ensure the animal is not moving and not stressed by the fixation that is required for proper imaging.

I. Other aspects compromising the welfare of the animals

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

1. The animals that receive an imaging window will be housed solitary for 1 day to prevent other mice from biting the wound and hampering wound healing.
2. The animals may experience mild discomfort from the injection of tumor cells, tumor measurements and anaesthesia for IVM.
3. The animals will experience moderate discomfort after imaging window surgery because of the surgery itself, and because of the presence of the window.

Explain why these effects may emerge.

1. Mice are social animals and don't like to be housed in solitary.
2. The injection or anaesthesia induction can cause mild discomfort to the animal.
3. The surgical procedure will cause the animal moderate discomfort.

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

1. Only animals that have received an imaging window will be housed in solitary. The other animals described in this appendix will not be housed in solitary. The animals will be housed in solitary only for 1 day during which they show reduced mobility as the result of surgery.
2. The anaesthesia will be performed as short as possible. For measuring tumor growth and injection of cells in the fat pad < 5 mins.
3. The surgery will be performed by experienced researchers and the animal will be monitored closely after surgery. The first two days after surgery daily by the researcher, and if fine, daily by animal caretakers.

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.

In all cases we will use criteria according to the code of practice Animals in Cancer Research. In case of tumor/metastases growth and/or window implantation, these are: severe loss of body weight (monitoring every other day based on body conditioning score), severe circulation or breathing problems, changes in behavior (posture, general signals of severe sickness or discomfort), severe clinical appearance of tumor (ulceration, growth hampering mobility, swollen abdomen), a total tumor mass that is too big (>2 cm^3).
In case of window implantation, we will also sacrifice the animal if the tissue underneath the window gets infected or infested (milky white), or if the skin surrounding the window gets infected (red and

swollen).
In case of window implantation, we will also sacrifice the animal if the tissue underneath the window gets infected or infested (milky white), or if the skin surrounding the window gets infected (red and swollen).

Indicate the likely incidence.

< 1%

K. Classification of severity of procedures

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

The injection of tumor cells in the mesenteric vein or spleen, and intravital microscopy is defined as moderate. The housing in solitary for 1 day is defined as mild.

Description	Animal species/strain and sex	Number of animals	Discomfort (expected cumulative discomfort)	Discomfort is a sum of following procedures
			Mild 0%	
Experiment 1/2/3	-Mus musculus. -BALB/c, C57/Bl6, immunodeficient mice. -Female.	1764	Moderate 100%	Tumor cell injections (in the skin, spleen or mesenteric vein), tumor/metastasis growth, if applicable tumor measurements by calliper, if applicable injections for drugs to manipulate genes, Intravital microscopy.
			Severe 0%	
		Total	100%	

End of experiment

L. Method of killing

Will the animals be killed during or after the procedures?

☐ No

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

The animals will have tumors and metastases. If the experiment is finished the animals will have to be sacrificed because otherwise the tumors/metastases will keep growing. Moreover, the organs will be used for further analyses using immunohistochemistry to determine cellular and molecular aspects of tumor/metastasis growth.

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

DEC-advies

A. Algemene gegevens over de procedure

1. Aanvraagnummer: AVD116002016705

2. Titel van het project: Investigating the dynamic role of the TGF-β pathway in liver metastases to come closer towards therapies.

3. Titel van de NTS: Het onderzoeken van moleculaire en cellulaire aspecten van kanker uitzaaiingen.

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

5. Contactgegevens DEC:
   - naam DEC: DEC Leiden
   - telefoonnummer contactpersoon: [redacted]
   - e-mailadres contactpersoon: [redacted]

6. Adviestraject (data dd-mm-jjjj):
   - ✓ ontvangen door DEC: 31-01-2017
   - ✓ aanvraag compleet: 31-01-2017
   - ✓ in vergadering besproken: 09-02-2017 & 09-03-2017
   - ✓ anderszins behandeld: via emailronde
   - ✓ termijnonderbreking(en) van 14-02-2017 t/m 24-02-2017 & 21-03-2017 t/m/ 22-05-2017
   - ☐ besluit van CCD tot verlenging van de totale adviestermijn met maximaal 15 werkdagen
   - ✓ aanpassing aanvraag: 24-02-2017 & 22-05-2017
   - ✓ advies aan CCD: 02-06-2017

7. De IvD geeft aan dat de aanvrager de aanvraag met de IvD heeft afgestemd en dat deze de instemming heeft van de IvD

8. Eventueel horen van aanvrager
   N.v.t.

9. Correspondentie met de aanvrager
   - Datum: 14-02-2017
   - Strekking van de gestelde vragen:
     De DEC heeft bij de aanvrager aangegeven dat zij vanwege de enorme omvang en complexiteit moeite had met de leesbaarheid en navolgbaarheid van het projectvoorstel. Zij heeft tevens aanvullende informatie ingewonnen met betrekking tot de achtergrond, het gebruikte figuur, de onderzoeksvragen, de haalbaarheid & samenwerking met andere afdelingen, gebruikte referenties, de strategie, gebruikte milestones & beslismomenten en de verfijning.
   - Naar aanleiding van deze vragen is het projectvoorstel inclusief bijlages herschreven.
   - Datum: 21-03-2017
   - Strekking van de gestelde vragen:

De DEC heeft naar aanleiding van de herschreven aanvraag bij de aanvrager aanvullende informatie ingewonnen met betrekking tot de strategie, de motivatie voor het gebruik van de IVM, de statistiek, keuze voor het geslacht, verfijning, de individuele huisvesting, training personeel en het ongerief.
- Naar aanleiding van deze vragen is het projectvoorstel inclusief bijlages en de NTS naar tevredenheid door de aanvrager aangepast.

10. Eventuele adviezen door experts (niet lid van de DEC)
N.v.t.

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 over deze projectaanvraag te adviseren. De benodigde expertise op dit wetenschappelijke terrein is aanwezig binnen de DEC.

4. Geen van de DEC leden is betrokken bij het betreffende project.

C. Beoordeling (inhoud)

1. Deze aanvraag heeft een concrete doelstelling en kan getypeerd worden als een project. De aanvraag komt overeen met voorbeeld 1 en 4B uit de handreiking 'Wat is een project': De verschillende subdoelen zijn uitkomstafhankelijk van elkaar of worden parallel uitgevoerd. Deze subdoelen zijn allemaal noodzakelijk om de doelstelling te behalen. Het is helder welke handelingen individuele dieren zullen ondergaan. Hierdoor is ook duidelijk welk ongerief individuele dieren zullen ondergaan. De aanvrager heeft duidelijk beschreven op basis van welke criteria deze zal besluiten het project wel of niet te continueren. De DEC is er daardoor van overtuigd dat de aanvrager gedurende het project op zorgvuldige wijze besluiten zal nemen over de voortgang van het project en er niet onnodig dieren gebruikt zullen worden. Gezien bovenstaande is de DEC van mening dat de aanvraag toetsbaar is en voldoende samenhang heeft.

2. Voor zover de DEC kan beoordelen is er geen sprake van tegenstrijdige wetgeving die het uitvoeren van de proef in de weg zou kunnen staan.

3. De in de aanvraag aangekruiste doelcategorie is in overeenstemming met de hoofddoelstelling.

Belangen en waarden

4. Het directe doel van dit project het verkrijgen van inzicht in de dynamische rol van de TGF-β pathway in lever metastasen bij borst- en huidkanker. Het uiteindelijke doel is het identificeren van overeenkomsten en verschillen tussen de rol van de TGF-β pathway in huid- en borstkanker, die kunnen helpen bij de behandeling van kankerpatiënten met metastasen en die kunnen leiden tot tumor-type specifieke behandeling. De DEC is van mening dat er een duidelijke relatie is tussen het directe en uiteindelijk doel. De aanvrager heeft helder gemaakt wat de status is van het onderzoeksveld en wat de bijdrage van dit project aan het onderzoeksveld zal zijn. Ondanks dat er al veel bekend is over het metastatisch proces zijn er ook nog veel details onbekend. Door gebrek aan kennis over welke pathways de vorming van levermetastasen reguleren zijn er geen therapieën beschikbaar die lever metastasen

voorkomen of verminderen. De DEC is van mening dat het directe doel gerechtvaardigd is binnen de context van het onderzoeksveld.

5. De belangrijkste belanghebbenden in dit project dat gericht is op het verkrijgen van kennis over de regulatie van tumor metastasen in de lever zijn de proefdieren, de onderzoekers en de patiënt.
   Waarden die voor proefdieren in het geding zijn: De integriteit van de dieren zal worden aangetast, de dieren zullen beperkt worden in hun natuurlijke gedrag en gedurende de proeven zullen de dieren stress ondervinden en pijn ondergaan.
   Waarden die voor de onderzoekers bevorderd worden: De wetenschappers zullen kennis verkrijgen. Ook zullen de carrièremogelijkheden van de wetenschappers verbeteren door publicaties.
   Waarden die voor de patiënten bevorderd worden: Meer kennis over het metastatische proces kan mogelijk lijden tot het vinden van een therapie voor de behandeling van lever metastasen.

6. Voor zover de DEC kan beoordelen is er geen sprake van substantiële milieueffecten.

   Proefopzet en haalbaarheid

7. Naar de overtuiging van de DEC beschikt de aanvrager over voldoende expertise en voorzieningen om de projectdoelstelling met de gekozen strategie binnen de gevraagde termijn te realiseren. Het project bouwt verder op langlopend onderzoek dat wordt uitgevoerd door de onderzoeksgroep in samenwerking met nationale onderzoeksgroepen. De onderzoeksgroep heeft veel expertise op het gebied van dierexperimenteel onderzoek met betrekking tot borst- en huidkankeronderzoek en het gebruik van IVM om levermetastasen te kunnen bestuderen in muizen. In de afgelopen jaren zijn volgens vergelijkbare strategieën en aanpak belangrijke wetenschappelijke resultaten behaald resulterend in een aantal publicaties in internationaal gerenommeerde wetenschappelijke tijdschriften. Daarnaast zijn er belangrijke subsidies voor dit onderzoek binnen gehaald.

8. De DEC is er van overtuigd dat het projectvoorstel aansluit bij recente wetenschappelijke inzichten en geen hiaten bevat die de bruikbaarheid van de resultaten in de weg zullen staan. De voorgestelde experimentele opzet en uitkomstparameters zijn logisch en helder gekozen en sluiten aan bij de aangegeven doelstellingen en de gekozen strategie en experimentele aanpak kunnen naar de mening van de DEC leiden tot het behalen van de doelstelling binnen de looptijd van het project.

   Welzijn dieren

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

10. De DEC is ervan overtuigd dat de dieren gehuisvest en verzorgd worden op een wijze die voldoet aan de eisen die zijn opgenomen in bijlage III van de richtlijn. Het proefdiercentrum van het LUMC beschikt over uitstekende faciliteiten en uitsluitend bevoegd en competent personeel zal zorg dragen voor de verzorging van de dieren en de uitvoering van de dierproeven.

11. De DEC heeft zich ervan verzekerd dat de aanvrager al het mogelijke heeft gedaan om het eventuele ongerief voor de proefdieren te identificeren, te verminderen en

waar mogelijk te voorkomen. De DEC schat dat het merendeel van de dieren cumulatief maximaal matig ongerief zullen ondervinden als gevolg van de orthotope tumorcel injectie en de IVM. De overige muizen zullen maximaal mild ongerief ondervinden. Deze inschatting is in overeenstemming met het niveau van het cumulatief ongerief ingeschat door de onderzoekers.

12. De integriteit van dieren wordt fysiek aangetast doordat de dieren tumoren en metastasen ontwikkelen. De integriteit zal ook gedragsmatig worden aangetast. Gedurende het project worden de dieren namelijk beperkt in hun bewegingsvrijheid. Hierdoor zullen de dieren minder natuurlijk gedrag kunnen vertonen.

13. Naar mening van de DEC zijn de humane eindpunten zorgvuldig beschreven en is de inschatting van de incidentie met betrekking tot het bereiken van een humaan eindpunt eveneens zorgvuldig beschreven in de projectaanvraag.

3V's

14. In het project wordt de keuze voor de diermodellen duidelijk onderbouwd. De betrokken dieren en het gekozen diermodel zijn het meest geschikt voor deze studieopzet. De desbetreffende dierproef berokkent de dieren het minste pijn, lijden, angst of blijvende schade. Met behulp van het spontane model wordt het mogelijk om de specifieke rol voor TGF-β tijdens deze stappen te bepalen. Terwijl met behulp van het experimentele metastasemodel het mogelijk is om de latere stappen van metastase te bestuderen. De DEC is ervan overtuigd dat er geen alternatieven beschikbaar zijn voor het voorgestelde gebruik van intacte dieren om de doelstelling van dit project te realiseren.

15. In het project wordt optimaal tegemoet gekomen aan de vereisten van vermindering van dierproeven. Zo maakt in vivo microscopie het mogelijk om tumorcelafscheiding van de primaire tumor, migratie door het omliggende weefsel en intravasatie in een bloedvat te onderzoeken in één dier. Naar inzien van de DEC zijn de beschreven go/no-go momenten realistisch, helder en eenduidig omschreven, waardoor er geen onnodig onderzoek zal worden uitgevoerd. De DEC is ervan overtuigd dat het onderzoek ethisch verantwoord zal worden uitgevoerd. De DEC acht het maximale aantal te gebruiken dieren realistisch geschat.

16. De uitvoering van het project is in overeenstemming met de vereisten van verfijning van dierproeven en is zo opgezet dat de dierproeven met zo min mogelijk ongerief worden uitgevoerd. Bij de opzet van dit onderzoek wordt rekening gehouden met dierenwelzijn door eerst *ex vivo* te onderzoeken welke cellijnen er het beste gebruikt kunnen worden voor het *in vivo* onderzoek. De DEC is ervan overtuigd dat de beschreven dierproeven zo humaan mogelijk zullen worden uitgevoerd.

17. Het betreft hier geen wettelijk vereist onderzoek.

Dieren in voorraad gedood en bestemming dieren na afloop proef

18. Deze aanvraag is gericht op drievoudig negatieve borstkanker, terwijl mannen overwegend ER+ borstkanker krijgen, dit betekent dat de resultaten niet van toepassing zijn op de mannelijke bevolking. De aanvrager zal in het project daarom alleen vrouwelijke dieren gebruiken. Tevens is bij het gebruik van mannelijke muizen de kans groter dat de tumor beschadigd door vechten, waardoor de uitval hoger zou worden. De onderzoeker heeft dit naar mening van de DEC voldoende onderbouwd in de projectaanvraag.

19. De dieren worden in het kader van het project gedood. De organen zullen worden uitgenomen en gebruikt worden voor verdere analyses met behulp van immuno-histochemie om cellulaire en moleculaire aspecten van tumor / metastase groei te bepalen. Het doden van de dieren gebeurd volgens een voor de diersoort passende dodingsmethode die vermeld staat in bijlage IV van richtlijn 2010/63/EU.

20. Er worden voor dit projectvoorstel geen niet-humane primaten, honden, katten of landbouwhuisdieren gebruikt.

    NTS

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

D. Ethische afweging

1. Rechtvaardigt onderzoek doen naar de dynamische rol van de TGF-β pathway in lever metastasen bij borst- en huidkanker met als uiteindelijke doel het identificeren van overeenkomsten en verschillen tussen de rol van de TGF-β pathway in huid- en borstkanker, die kunnen helpen bij de behandeling van kankerpatiënten met metastasen en die kunnen leiden tot tumor-type specifieke behandeling het ongerief dat de dieren wordt aangedaan?

2. Project gericht op het verkrijgen van kennis over de regulatie van metastasen in de lever en hoe dit getarget kan worden met therapie.
   Waarden die voor proefdieren in het geding zijn: matig nadeel.
   Waarden die voor onderzoekers bevorderd worden: gering voordeel.
   Waarden die voor de patiënten (incl. de samenleving) bevorderd worden: groot voordeel.
   De DEC is van mening dat de belangen van de samenleving in het algemeen en de patiënten in het bijzonder in dit project zwaarder wegen dan de belangen/waarden van de proefdieren.
   Kanker is één van de dodelijkste ziektes ter wereld. In Nederland sterven jaarlijks 40.000 patiënten aan kanker. De meeste hiervan sterven niet als gevolg van de primaire tumor, maar vanwege de metastasen op moeilijk te reseceren plekken zoals de lever. De prognose voor borst- of huidkankerpatiënten met levermetastasen is gemiddeld slechts 4 maanden. Ondanks dat er al veel bekend is over het metastatisch proces zijn er ook nog veel details onbekend. Door gebrek aan kennis over welke pathways de vorming van levermetastasen reguleren zijn er geen therapieën beschikbaar die lever metastasen voorkomen of verminderen. De DEC acht het bestuderen van de individuele stappen van metastase in de loop der tijd van essentieel belang om de dynamische regulering van TGF-β beter te begrijpen tijdens het metastatische proces. Het is aannemelijk dat de doelstelling behaald zal worden. Hiertoe zullen dieren worden gebruikt. De onderzoekers doen er echter alles aan om het lijden van de dieren te beperken, waardoor het ongerief van de dieren zo veel mogelijk beperkt blijft.

3. De DEC is overtuigd van het belang van de doelstelling van dit project. De DEC is van mening dat de waarden die voor de doelgroep bevorderd kunnen worden zwaarder wegen dan de waarden die voor de proefdieren in het geding zijn. Het project is goed opgezet. De DEC is bovendien van mening dat de voorgestelde experimentele opzet en uitkomstparameters logisch en helder aansluiten bij de aangegeven doelstelling en dat de gekozen strategie en experimentele aanpak kunnen leiden tot het behalen van de doelstelling binnen het kader van het project. De DEC is er verder van overtuigd

dat de onderzoeksgroep voldoende ervaring heeft met de gekozen onderzoeksstrategie en met de voorgestelde dierproeven om de doelstelling te behalen en dat de aanvrager voldoende maatregelen treft om zowel het ongerief van de dieren alsmede het aantal benodigde dieren tot een minimum te beperken. De DEC onderschrijft dat de doelstelling niet zonder het gebruik van proefdieren behaald kunnen worden en acht het gebruik van het aantal dieren en het daarmee samenhangende ongerief bij de dieren 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
     - ☐ Op grond van het wettelijk vereiste dient de projectleider bij beëindiging van het project een beoordeling achteraf aan te leveren die is afgestemd met de IvD.
     - ☐ Voor de uitvoering van dit project is tevens ministeriële ontheffing vereist
     - ☐ Overige door de DEC aan de uitvoering verbonden voorwaarden, te weten...
   - ☐ De DEC adviseert de vergunning niet te verlenen vanwege:
     - ☐ De vaststelling dat het project niet vergunning plichtig is om de volgende redenen:…
     - ☐ De volgende doorslaggevende ethische bezwaren:…
     - ☐ De volgende tekortkomingen in de aanvraag:…

2. Het uitgebrachte advies is gebaseerd op consensus.

3. Er zijn tijdens de beoordeling van dit projectvoorstel geen echte knelpunten en of duidelijke dillema’s naar voren gekomen.

8.

Centrale Commissie Dierproeven

> Retouradres Postbus 20401 2500 EK Den Haag

Academisch Ziekenhuis Leiden h.o.d.n. LUMC
Mevr. Leids Universitair Medisch Centrum
Postbus 9600
2300 RC LEIDEN

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

Onze referentie
Aanvraagnummer
AVD116002016705

Bijlagen
2

Datum 22 juni 2017
Betreft Ontvangstbevestiging aanvraag projectvergunning Dierproeven

Geachte mevrouw Leids Universitair Medisch Centrum,

Wij hebben uw aanvraag voor een projectvergunning dierproeven ontvangen op 20 juni 2017. Het gaat om uw project "Investigating the dynamic role of the TGF-R pathway in liver metastases to come closer towards therapies". Het aanvraagnummer dat wij aan deze aanvraag hebben toegekend is AVD116002016705. 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:
22 juni 2017
Aanvraagnummer:
AVD116002016705

Met vriendelijke groet,

Centrale Commissie Dierproeven

Deze brief is automatisch aangemaakt en daarom niet ondertekend.

Bijlagen:
- Gegevens aanvraagformulier
- Factuur

Datum:
22 juni 2017
Aanvraagnummer:
AVD116002016705

Gegevens aanvrager

Uw gegevens	
Deelnemersnummer NVWA:	11600
Naam instelling of organisatie:	Academisch Ziekenhuis Leiden h.o.d.n. LUMC
Naam portefeuillehouder of diens gemachtigde:	Mevr. Leids Universitair Medisch Centrum
KvK-nummer:	27366422
Straat en huisnummer:	Albinusdreef 2
Postbus:	9600
Postcode en plaats:	2300 RC LEIDEN
IBAN:	NL11DEUT0451001400
Tenaamstelling van het rekeningnummer:	LUMC

Gegevens verantwoordelijke onderzoeker

Naam:
Functie:
Afdeling:
Telefoonnummer:
E-mailadres:

Datum:
22 juni 2017
Aanvraagnummer:
AVD116002016705

Gegevens plaatsvervangende verantwoordelijke onderzoeker

Naam: [redacted]
Functie:
Afdeling:
Telefoonnummer:
E-mailadres:

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 juni 2017
Geplande einddatum:	31 mei 2022
Titel project:	Investigating the dynamic role of the TGF-R pathway in liver metastases to come closer towards therapies
Titel niet-technische samenvatting:	Het onderzoeken van moleculaire en cellulaire aspecten van kanker uitzaaiingen.
Naam DEC:	DEC Leiden
Postadres DEC:	[redacted] LUMC Postbus 9600 ': 2300 RC Leiden
E-mailadres DEC:	[redacted]

Betaalgegevens

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

Checklist bijlagen

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

Overige bijlagen: [x] DEC-advies

Datum:
22 juni 2017
Aanvraagnummer:
AVD116002016705

Ondertekening

Naam:	[redacted]
Functie:	Gemandateerd functiehouder
Plaats:	Leiden
Datum:	1 juni 2017

Centrale Commissie Dierproeven

> Retouradres Postbus 20401 2500 EK Den Haag

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Onze referentie
Aanvraagnummer
AVD116002016705

Bijlagen
2

Datum 22 juni 2017
Betreft Factuur aanvraag projectvergunning Dierproeven

Factuur
Factuurdatum: 22 juni 2017
Vervaldatum: 22 juli 2017
Factuurnummer: 170705

Omschrijving	**Bedrag**
Betaling leges projectvergunning dierproeven Betreft aanvraag AVD116002016705	€ 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.

9.

[redacted]

Van: Info-zbo
Verzonden: dinsdag 22 augustus 2017 16:56
Aan: Wob CCD
Onderwerp: FW: Aanvraag AVD116002016705

Zie onder

Van: Info-zbo
Verzonden: maandag 17 juli 2017 15:40
Aan: [redacted]
CC: [redacted]
Onderwerp: Aanvraag AVD116002016705

Geachte [redacted]
Op 20 juni 2017 hebben wij uw projectaanvraag "Investigating the dynamic role of the TGF-R pathway in liver metastases to come closer towards therapies" met aanvraagnummer AVD116002016705 ontvangen.
Wij hebben nog aanvullende informatie van u nodig.

- Voor alle drie de bijlagen dierproeven is het ons onder vraag D niet duidelijk wat u in vitro onderzocht heeft en op basis waarvan u besloten heeft om dierproeven te gaan doen. Kunt u ons inzicht geven in hoe de in vitro resultaten de in vivo experimenten sturen?

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

Stuur de ontbrekende informatie binnen veertien dagen na de datum van deze brief op. U kunt dit aanleveren via NetFTP. Stuurt u het per post op, gebruik dan het formulier dat u bij deze brief krijgt.

De behandeling van uw aanvraag wordt opgeschort tot het moment dat wij de aanvullende informatie hebben ontvangen.

Met vriendelijke groet,

[redacted]

Medewerker behandelen en ontwikkelen
Centrale Commissie Dierproeven www.centralecommissiedierproeven.nl

Postbus 20401 | 2500 EK | Den Haag

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

Leids Universitair Medisch Centrum

afdeling [redacted]
postzone
afzender [redacted]
bezoekadres [redacted]
telefoon [redacted]
fax
e-mail [redacted]
onze referentie
uw referentie [redacted]
datum 20 juli 2017
onderwerp Aanvraag AVD116002016705
aantal pagina's 2

aan Centrale Commissie Dierproeven
Postbus 20401
2500 EK
Den Haag

Geachte [redacted]

Afgelopen maandag 17 juli 2017 heeft u ons een email gestuurd met daarin het verzoek tot aanvullende informatie betrekkende mijn CCD aanvraag “Investigating the dynamic role of the TGF-b pathway in liver metastases to come closer towards therapies” met aanvraagnummer AVD116002016705.
Uw vraag luidde als volgt:

- Voor alle drie de bijlagen dierproeven is het ons onder vraag D niet duidelijk wat u in vitro onderzocht heeft en op basis waarvan u besloten heeft om dierproeven te gaan doen. Kunt u ons inzicht geven in hoe de in vitro resultaten de in vivo experimenten sturen?

Ons antwoord daarop met de aanvullende informatie is als volgt:
2D in vitro culture models can be used to study tumor growth and metastasis, but only to a certain extend. We have setup a 3D in vitro model that recapitulates in vivo tumor/metastasis growth more closely compared to the 2D in vitro models. This is evident by the fact that some cell lines that proliferate in 2D do not proliferate in 3D. Similarly, in literature it was shown that these cell lines do not form metastases in vivo. As such, the 3D situation mimics the in vivo situation more closely.
We have used this 3D in vitro assay to study the proliferation of the cell lines we want to test in vivo. Indeed, some lines are hardly growing in the 3D assay whereas other lines are growing fine. In order to identify genes that are important for metastatic growth we have performed RNA-sequencing of the lines grown in the 3D assay and compared them. The sequencing is performed as we speak, so after we obtain the list with genes that are differentially regulated, we will make a top 10 list of interesting genes to study (related to the TGF-b pathway). These genes will be up or downregulated experimentally (siRNA, overexpression) in the cells, and we will then study their effect on proliferation, apoptosis and cell cycle using the 3D in vitro model. The top 3 genes that perform best in these assays will be used to guide the in vivo experiments.

afdeling [redacted]

onze referentie

datum 20 juli 2017

onderwerp Aanvraag AVD116002016705

aantal pagina's 2 van 2

aan Centrale Commissie Dierproeven

Dit antwoord geldt voor alle drie de bijlagen.

Ik hoop u hiermee voldoende te hebben geïnformeerd, maar mocht u nog meer vragen hebben dan hoor ik dat graag.

Met vriendelijke groet,

[redacted]

Centrale Commissie Dierproeven

> Retouradres Postbus 20401 2500 EK Den Haag

Academisch Ziekenhuis Leiden h.o.d.n. LUMC
t.a.v. [redacted]
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Onze referentie
Aanvraagnummer
AVD116002016705
Bijlagen
1

Datum 21 juli 2017
Betreft Beslissing aanvraag projectvergunning Dierproeven

Geachte mevrouw [redacted]

Op 20 juni 2017 hebben wij uw aanvraag voor een projectvergunning dierproeven ontvangen. Het gaat om uw project "Investigating the dynamic role of the TGF-R pathway in liver metastases to come closer towards therapies" met aanvraagnummer AVD116002016705. Wij hebben uw aanvraag beoordeeld.

Op 20 juli 2017 heeft u uw aanvraag aangevuld. Op ons verzoek is het in vitro werk dat voorafgaat aan het onderliggende project nader uitgelegd.

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 "Investigating the dynamic role of the TGF-R pathway in liver metastases to come closer towards therapies" starten. De vergunning wordt afgegeven van 21 juli 2017 tot en met 30 juni 2022. Deze termijn is anders dan in uw aanvraag, omdat de door u aangevraagde startdatum in het verleden ligt.

Overige wettelijke bepalingen blijven van kracht.

Procedure
Bij uw aanvraag heeft u een advies van de Dierexperimentencommissie DEC Leiden gevoegd. Dit advies is opgesteld op 2 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).

Datum:
21 juli 2017
Aanvraagnummer:
AVD116002016705

Centrale Commissie Dierproeven
namens deze:

[redacted]

ir. G.
Algemeen Secretaris

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

Projectvergunning

gelet op artikel 10a van de Wet op de Dierproeven

Verleent de Centrale Commissie Dierproeven aan

Naam:	Academisch Ziekenhuis Leiden h.o.d.n. LUMC
Adres:	Postbus 9600
Postcode en plaats:	2300 RC LEIDEN
Deelnemersnummer:	11600

deze projectvergunning voor het tijdvak 21 juli 2017 tot en met 30 juni 2022, voor het project "Investigating the dynamic role of the TGF-R pathway in liver metastases to come closer towards therapies" met aanvraagnummer AVD116002016705, volgens advies van Dierexperimentencommissie DEC Leiden. 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 20 juni 2017
2 de bij het aanvraagformulier behorende bijlagen:
a Projectvoorstel, zoals ontvangen per digitale indiening op 20 juni 2017;
b Niet-technische Samenvatting van het project, zoals ontvangen per digitale indiening op 20 juni 2017;
c Advies van dierexperimentencommissie d.d. 2 juni 2017, ontvangen op 20 juni 2017.
d De aanvullingen op uw aanvraag, ontvangen op 20 juli 2017

Naam proef	Diersoort/ Stam	Aantal dieren	Ernst	Opmerkingen
3.4.4.1 Study spontaneous metastasis ex vivo				
	Muizen (Mus musculus) /	1.632	Licht	
3.4.4.2 Study experimental metastasis ex vivo				
	Muizen (Mus musculus) /	1.456	Matig	
3.4.4.3 Study metastasis in vivo				
	Muizen (Mus musculus) /	1.764	Matig	

Voorwaarden

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

Aanvraagnummer:
AVD116002016705

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

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

Aanvraagnummer:
AVD116002016705

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.