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Establishment and MRI evaluation of two animal models of minimal residual disease (MRD) for studying new strategies of MRD detection.

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	Naam	Tel (+ Tel privé enkel VO, VVO en VM)	E-mailadres	Bevoegd-heid[5]	Cap. groep /afdeling
1. Verantwoordelijk onderzoeker (VO)				Art.9	
2. Vervanger VO (VVO)				Art.9	
3. Verantwoordelijk medewerker (VM) GGO[7]					
4. overige uitvoerenden				Art.12	
5. overige uitvoerenden				Art.9	

Diergroep	1A	1B	2A	2B	3A	4A	
ctrl/exp/sham	ctrl	exp	ctr	exp	exp	exp	
Diersoort	01	01	01	01	01	01	
Stam	NMRI nu/nu	NMRI nu/nu	NMRI nu/nu	NMRI nu/nu	NMRI nu/nu	NMRI nu/nu	
Construct / mutatie ?	-	-	-	-	-	-	
Herkomst (leverancier) *	02	02	02	02	02	02	
Aantal	**8**	**96**	**8**	**32**	**12**	**32**	
Geslacht	m/v	m/v	m/v	m/v	m/v	m/v	
Dieren immuuncompetent ?	nee	nee	nee	nee	nee	nee	
Leeftijd/gewicht	2m/28-30g	2m/28-30g	2m/28-30g	2m/28-30g	2m/28-30g	2m/28-30g	
Doel van de proef *	30	30	30	30	30	30	
Belang van de proef *	01	01	01	01	01	01	
Toxicologisch onderzoek *	01	01	01	01	01	01	
Bijzondere technieken *	01	01	01	01	01	01	
Anesthesie *	04	04	04	04	04	04	
Pijnbestrijding *	01	01	01	01	01	01	
Mate ongerief *	**05**	**05**	**05**	**05**	**05**	**05**	
Toestand dier einde exp*	01	01	01	01	01	01	

* VHI-coderingen zie bijlage

1 Verantwoording

Aanvraag dierproef DEC-UM **(kaders zijn licht flexibel, maar het geheel is max. 5 pag. versie 2006)**

Titel: *Establishment and MRI evaluation of two animal models of minimal residual disease (MRD) to study new strategies for MRD detection*

1. Doel van de proef.

Minimal residual disease (MRD) is a term used to describe any small population of malignant cells that cannot be detected in a routine medical examination. MRD is a serious problem observed in patients with colorectal cancer as well as other types of malignancies. A failure of its timely detection leads frequently to cancer recurrence and spread. In view of the current diagnostic incapability, the development of new strategies for non-invasive MRD assessment is a leading topic of this proposal.

First aim of this study is to establish two pre-clinical models of minimal residual disease (MRD), i.e., a and an MRD, which are required for development of new MRD detection strategies. Both models will be established in HT-29 colorectal human carcinoma-bearing nude mice. The MRD represents a clinically relevant model of the residual disease after (chemo)radiotherapy in patients with colorectal cancer. The second model of microscopic lesions, which is formed in the . can be related to the early stage of tumor development. Both types of MRD are important targets of oncologic diagnostics. Magnetic resonance imaging (MRI) will be used to evaluate the status of the disease and, thus, to characterise the MRD models. The application of this non-invasive diagnostic method, which is also applicable to humans, will assure more efficient translation of the preclinical findings to the clinical setting. The MRI results will be validated by histology.

Our second aim is to determine the feasibility of molecular imaging of angiogenesis for the MRD detection. To this aim, we will use a novel angiogenesis-specific contrast agent, . It will be tested in both aforementioned MRD models. The specificity and efficacy of contrast agent association with target cells will be assessed in vivo with MRI and ex vivo using optical methods. Moreover, a non-targeted contrast agent will be used as a control for the non-specific uptake in MRD.

To characterize the circulation kinetics and biodistribution of this novel contrast agent we will determine its circulation half-live and perform analysis of dissected organs. These properties will be also investigated for the non-targeted equivalent.

2. Maatschappelijke relevantie en/of wetenschappelijk belang

The detection of low-volume disease, which is present locally after partial response to (chemo)radiotherapy or originate from distant metastases is one of the biggest challenges of oncologic radiology. These microscopic cancer cell islets are a frequent source of tumor regrowth and spread, and, therefore, require timely detection and therapeutic intervention. Their successful visualization would enable better selection of patients for surgery after neoadjuvant therapy and early assessment of metastases. Unfortunately, the currently available MRI methods, including anatomical, morphological and functional imaging, are not capable of detecting these microscopic targets. Therefore, more sensitive approaches are needed.

The most promising appear to be the methods that exploit powerful MR contrast agents targeted to the specific molecules involved in cancerous processes. The evaluation of these novel imaging approaches requires, however, adequate pre-clinical models.

The proposed study integrates therefore both social and scientific objectives. The detection of premature disease would dramatically improve the prognosis of cancer patients. This early assessment remains, however, a major scientific challenge. The design of our study, including the use of colorectal tumor model and MRI as an evaluation method, is thought to speed up the translation of pre-clinical findings to the patients with colorectal cancer, who are a group of our particular interest.

3. Alternatieven

In vitro alternatives, such as isolated cell culture or co-culture of different cell types, do not reproduce the complexity of in vivo environment. This is particularly the case for the tumor microenvironment, which is a three-dimensional structure composed of neoplastic cells, blood vessels, immune cells and other supporting components. Additionally, the fibrotic tissue, which formation is triggered by (chemo)radiotherapy, is an important element of the post-therapeutic scenery. Furthermore, neither the circulation kinetics nor biodistribution, which co-contribute to the in vivo targeting properties of novel contrast agents, can be tested in vitro. Therefore, it is vital to evaluate novel imaging approaches in adequate animal models of the disease.

The use of humans as test subjects is impossible, as we would like to test novel contrast agents, thus, agents that are not approved by the Food and Drug Administration (FDA).

4. Ethische afweging

The topics of this study i.e., the detection of minimal residual disease and molecular imaging, are emerging areas of oncologic diagnostics. Investigators involved in this study are convinced about its importance for health and knowledge interests.

We believe that the gains from these experiments overweight animal suffering. This study does not include any procedures that cause serious suffering of animals. MRI allows for painless disease evaluation. The duration of MRI examination and interval between the subsequent measurements as well as all other experimental procedures are set in a way to minimize a degree of discomfort for animals.

In addition to in vivo results, the ex vivo evaluation of the experiments will be preformed. In this way, we will maximally the use of animals sacrificed for this study.

The number of animals required for this experiment is estimated on the basis of statistical analysis.

5. Wetenschappelijke onderbouwing

Minimal Residual Disease (MRD) is a term that refers to any small population of malignant cells that cannot be detected in a routine medical examination, using available imaging tools and routine laboratory analyses [1]. For some types of MRD, including the peripheral blood- and bone marrow-associated leukemic cells or disseminated cancer cells of different types, advanced immunocytochemical and molecular techniques have been developed. However, the detection of cancer residues at the site of primary tumor after incomplete response to (chemo)radiotherapy and organ-confined micrometastases remains a major diagnostic challenge. The most promising for this purpose appear to be the non-invasive imaging methods that enable characterization of the suspicious tissue on a cellular and a molecular level. They are, however, in the preliminary stage of development. The progress in MRD detection requires establishment of adequate pre-clinical models of MRD and novel imaging strategies.

The problem of MRD is frequently observed in patients with colorectal cancer [2]. It is particularly relevant for the evaluation of neoadjuvant (chemo)radiotherapy. There is a relatively large group of patients, who appear to be complete responders, based on routine pre- and post-therapeutic examinations. However, the histological analysis of primary tumor site reveals often the presence of small cancer cell islets, indicating that the response was partial. The assessment of larger lesions, in a range of several $mm,^3$ is also complicated due to the therapy-induced tissue changes, such as edema and fibrosis, which may mask the presence of tumor residues. Any remaining lesions are a potential source of cancer reoccurrence and spread. Therefore, their timely detection is of great importance. Another form of MRD, which is often associated with colorectal cancer, are metastases to lymph nodes and other organs.

A small size of MRD lesions is a major challenge for all leading imaging modality. Furthermore, the microenvironmental conditions, including edema, necrosis and fibrosis, co-contribute considerably to this state of MRD diagnostics. The development of new approaches for MRD detection is therefore of paramount importance. This requires, however, pre-clinical MRD models of colorectal cancer, when focusing on the aforementioned group of patients. A few examples of animal models of MRD have been described in the literature. These are either locally-induced microlesions [3,4] or micrometastases formed by highly metastatic tumors [5,6]. In this study, we aim to establish two models of primary microlesions i.e., the and n-induced model, which represent the post-therapeutic and premature stadium of the disease, respectively. These forms of MRD are associated with different microenvironmental components, which interfere in distinct ways with the lesion detection.

Magnetic Resonance Imaging (MRI) has become a first line technique for the diagnostics and therapy-monitoring of patients with colorectal cancer. Considering its wide clinical application, MRI-based MRD imaging strategies are the most desirable. Therefore, we focus our research on MRI as a primary evaluation method. MRI is a non-invasive imaging technique, which can provide valuable information on the anatomy, morphology and functional status of the tissue. Moreover, it is considered a promising technique for molecular imaging. The application of different MRI methods will enable us to characterize the lesions and to select the most reliable MRD model. The following MRI measurements will be performed: T_2-weighted, diffusion-weighted and dynamic contrast-enhanced T_1-weighted imaging (DCE-MRI). Both T_2-weighted and diffusion-weighted imaging are anatomical/morphological methods, which exploit the differences in the endogenous magnetic properties between the neoplastic and normal tissue. DCE-MRI will be used to visualize vascular formations at the site of interest, which may persist after incomplete tumor irradiation or can be

formed during tumor development.

It is crucial, however, to validate MRI results by histology, which is considered a gold standard for the detection of low-volume (microscopic) lesions. The histological analysis will include hematoxylin and eosin staining as well as staining for fibrin and immune components e.g. macrophages. This is important because we expect no obvious MRI contrast related to the presence of MRD and because the planed MRI measurements, although widely applied for imaging of large lesions, have rather unclear status for the assessment of small lesions. Therefore, this study is designed to enable the histological validation of each MRI examination. A combination of MRI and histology will enable comprehensive evaluation of the disease status and selection of the most optimal protocol for MRD induction.

Angiogenesis, which is the formation of new capillaries from pre-existing vessels, plays a crucial role in cancer development [7]. Similarly, cancer regrowth after incomplete response to chemoradiotherapy is dependent on the presence of functional vasculature. Since angiogenesis is an integral part of the tumor microenvironment and a key tumor growth propagator, we hypothesized that visualization of specific molecular markers of angiogenesis can serve as a method for MRD detection. The currently used vascular imaging methods trace the pharmacokinetics of non-specific contrast agents in the suspicious region of the body. This type of measurements will be used for the initial evaluation of the MRD models, as indicated above. However, considering the microscopic size of the lesions that we are aiming at, we think that more specific and sensitive imaging methods are required. The molecular visualization methods seem the most promising since they aim at molecular markers of angiogenesis, which expression proceeds the formation of the functional vascular network. The presence of specific molecular markers, which are virtually absent on normal endothelium, is one of the hallmarks of angiogenic vessels. Molecular MR imaging of angiogenesis requires, however, a powerful contrast agent that binds specifically and efficiently to the molecular epitope of interest. This can be achieved by functionalization of a conventional contrast agent with a ligand that binds with high affinity to the target receptor.

In the present study, we will evaluate the utility of for the MRD detection. $\alpha_v\beta_3$ integrin is a cell adhesion molecule and a widely investigated molecular marker of angiogenesis [8]. It is abundantly expressed by activated endothelial cells, whereas virtually absent on normal endothelium. $\alpha_v\beta_3$ integrin has been frequently used as a target for imaging and therapeutic purposes in solid tumors. However, it has never been evaluated as a MRD-relevant target. ; that will be used for $\alpha_v\beta_3$ integrin-targeting produce MRI contrast, which is powerful enough to enable single-cell imaging [9]. The combination of a potent contrast agent with a molecular-recognition motif creates therefore a promising molecular imaging agent, which can provide valuable prognostic measures on cancer reoccurrence.

6. Wetenschappelijke beoordeling

This project has been evaluated and approved by

5 Proefdier

7. Proefdier keuze

7a. Soort, stam / herkomst / eindbestemming

We would like to use NMRI-nu (nu/nu) mice, which are needed to establish human tumor xenografts. We chose the HT-29 colorectal human carcinoma since colorectal cancer is one of the leading topics of clinical research in our department. By using the preclinical model of this cancer type we will be able more efficiently translate the pre-clinical findings to the clinical setting.
The mice will be obtained from a registered breeder in .
At the end of the study, the animals will be sacrificed and histological analysis of muscles/tumors and normal organs will be performed.

7b. Sexe

The animal sex is not relevant in this study as growth of HT-29 colorectal human carcinoma is hormone-independent.

7.c. Aantallen

We set a power π = 80% as we expect considerable variability in the cancer cell growth in vivo and tumor response to radiotherapy. The significance level was set at α=0.05. To measure the group size we used the formula of L.Sachs :

$n = 2(z_{\alpha/2} - z_{\pi})^2 * (\sigma/\delta)^2$

$2(z_{\alpha/2} - z_{\pi})^2 = F.$

For $\alpha = 0.05$ and $\pi = 80\%$: F=15.7

Experiment 1. *Establishment and MRI evaluation of the l model of minimal residual disease (MRD).*

We estimated that the minimal effect considered meaningful (effect = MRI contrast change) around δ = 30% and standard deviation σ=20%.
Filling the formula of L. Sachs results: $n = 15.7 * (20/30)^2 = 6.9$ mice

Uitvalspercentage ***10% is related to difficulties with MRI measurements, such as technical failure or movement artifacts, which can affect the quality of data or make them even unusable.*** *The estimated percentage is based on the previous experience of the investigators. Great care will be taken to limit the technical-related drop out, however it is often operator-independent.*

$(a - 0.1a) = 4.5$ of $0.9a = 6.9$ or $6.9/0.9 = a$ $a = 7.666 = 8$ mice/group

Experiment 2. *Establishment and MRI evaluation of the minimal residual disease (MRD) model induced by*

We estimated that the minimal effect considered meaningful (effect = MRI contrast change) around δ = 30% and standard deviation σ=20%.
Filling the formula of L. Sachs results: $n = 15.7 * (20/30)^2 = 6.9$ mice

Uitvalspercentage ***10% is related to difficulties with MRI measurements, such as technical failure or movement artifacts, which can considerably affect the quality of data or make them even unusable.*** *The estimated percentage is based on the previous experience of the investigators. Great care will be taken to limit the technical-related drop out, however it is often operator-independent.*

(a – 0.1a) = 6.9 of 0.9a = 6.9 or 6.9/0.9 = a a = 7.666 = 8 mice/group

Experiment 3. *Determination of the circulation half-live of the integrin-targeted and non-targeted agent.*

We estimated that the minimal effect considered meaningful (effect = contrast agent concentration) around δ *= 75% and standard deviation of* σ*=30%.*
Filling the formula of L. Sachs results: $n = 15.7 * (30/75)^2 = 2.5 = 3$ mice

There is no drop out expected in this experiment.

Experiment 4*. In vivo MRI of MRD using angiogenesis-specific contrast agent.*

We estimated that the minimal effect considered meaningful (effect = MRI contrast change) around δ *= 30% and standard deviation* σ*=20%.*
Filling the formula of L. Sachs results: $n = 15.7 * (20/30)^2 = 6.9$ mice

Uitvalspercentage ***10% is related to difficulties with MRI measurements, such as technical failure or movement artifacts, which can considerably affect the quality of data or make them even unusable****. The estimated percentage is based on the previous experience of the investigators. Great care will be taken to limit the technical-related drop out, however it is often operator-independent.*

(a – 0.1a) =4.5 of 0.9a = 6.9 or 6.9/0.9 = a a = 7.666 = 8 mice/group

Exp1. *3 ; :s, 4 timepoints after y, 8 mice per group, + one non-treated group as a control (8 mice)* → *(3x4x8)+ 8 = 104*

Exp2. 8 mice per group, 4 timepoints after 1 + one group :2 as a control (8 mice) → *(4x8) + 8 = 40*

Exp3. *2 contrast agents, 2 MRD models, 3 mice per group* → *(2x2x3) = 12*

Exp4. *2 contrast agents, 2 MRD models, 8 mice per group* → *(2x2x8) = 32*

The total number of animals needed: 188

7 Dierproef

8. Experiment

All animal experiments and procedures within this research will be performed according to the code of practice related to animal research within the field of cancer (REF) [10]. All human endpoints are based on this document.

Experiment 1. *Establishment and MRI evaluation of the -induced model of minimal residual disease (MRD).*

Mice will be inoculated with tumor cells subcutaneously in lateral flank, according to SOP1. When tumors are established (tumor size of 150-250mm^3 measured with a Vernier calliper), mice will be anaesthetized and the infusion line for contrast agent injection will be placed in the tail vain (SOP2). The pre-treatment MRI (SOP3) will be performed, during which the contrast agent will be administered. The total time of measurements will be around 3 hours. The tumors will be on a subsequent day (SOP4). Mice will be assigned to one of the experimental groups, in which different treatment/readout protocols will be applied. The following parameters will be varied in the study groups:

1.
2. *time after (readout timepoint): 3, 6, 10 or 14 days after radiotherapy*

This experimental scheme is based on the previous experience with this tumor model, including its growth kinetics and response to The selected readout timepoints correspond to the early, intermediate and delayed effects of v. The post-treatment MRI (2nd MRI) will be done on either on the aforementioned timepoints after . Mice will be euthanized immediately thereafter. Tumors and normal tissues (liver, spleen, lung, muscle, kidney and heart) will be collected for immunohistochemical analyses.

Additionally, a group of mice that will not undergo treatment will be followed with MRI over time. In this group, first MRI examination will be performed on a day when tumors reach the size of 200-500mm^3. Subsequent MRI measurements will be performed 4, 7, 11 and 15 days after 1st MRI, which correspond to the readout timpoints after After these measurements mice will be sacrificed. Tumors and normal tissues (liver, spleen, lung, muscle, kidney and heart) will be collected for immunohistochemical analyses. The timelines of the ***Experiment 1*** *are presented in Figure 1.*

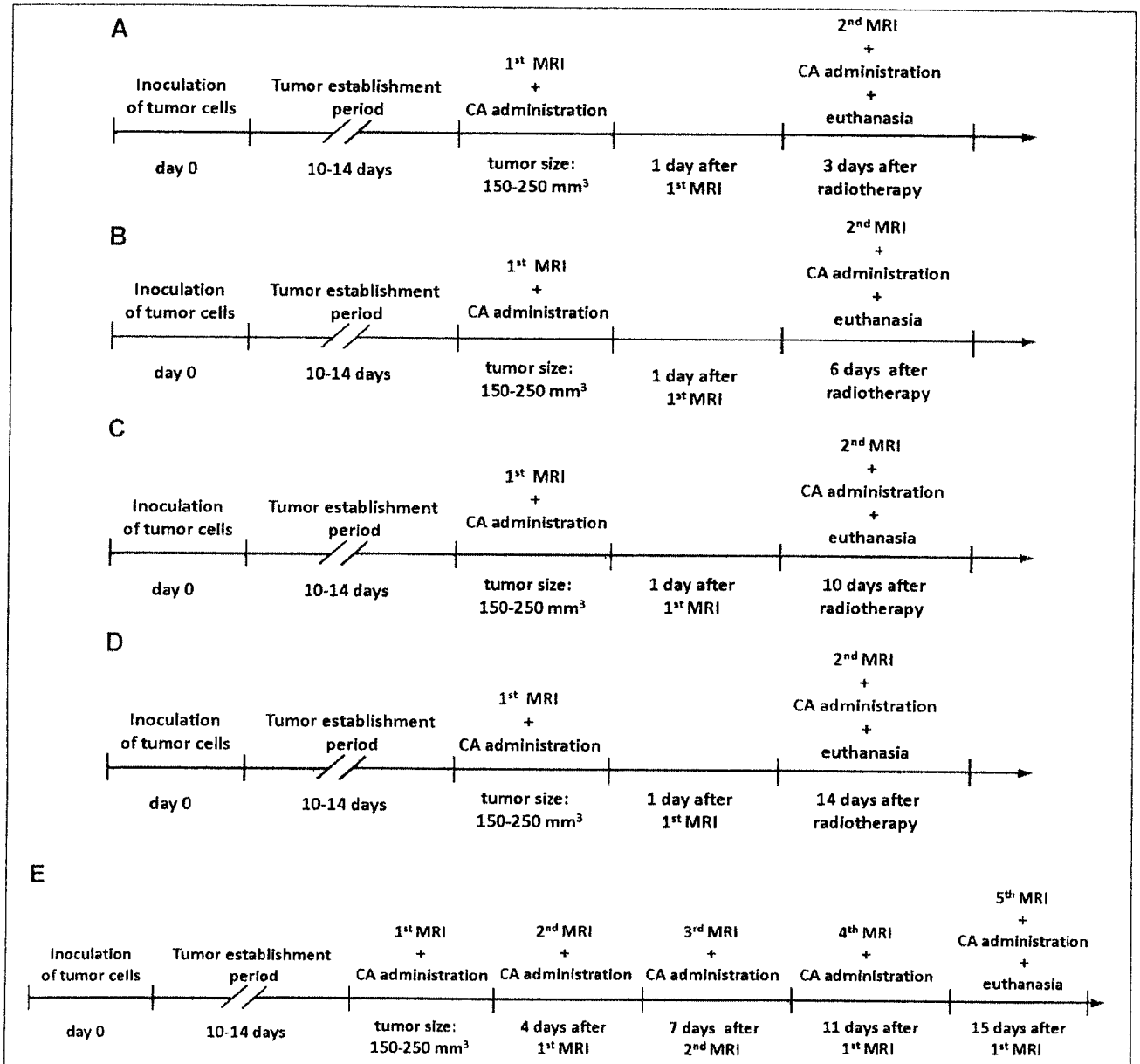

Figure 1: Timeline of the ***Experiment 1****.*

Experiment 2. *Establishment and MRI evaluation of the minimal residual disease (MRD) model induced by*

To characterize , which will be our reference point, mice will undergo MRI examination (1st MRI). One day after, cancer cells will be (SOP5), which was previously described by Oakley et al. [4]. Subsequently, the mice will be assigned to one of four experimental groups and scanned either 3, 6, 10 or 14 days after . These timepoints were based to the tumor growth kinetics, reported in the previous studies for the HT-29 colorectal human carcinoma in NMRI-nu (nu/nu) mice, and they represent different stages of tumor development. After the 2nd MRI examination, mice will be

euthanized. ; that were .... and normal tissues (liver, spleen, lung, muscle, kidney and heart) will be collected for immunohistochemical analyses.
Additionally, a group of mice that will be with saline ....s will be followed with MRI over time. The mice in this control group will be scanned (1st MRI) and with saline a day after. Subsequently, MRI measurements will be performed 3, 6, 10 and 14 days after saline
The timelines of this experiment are presented in Figure 2.

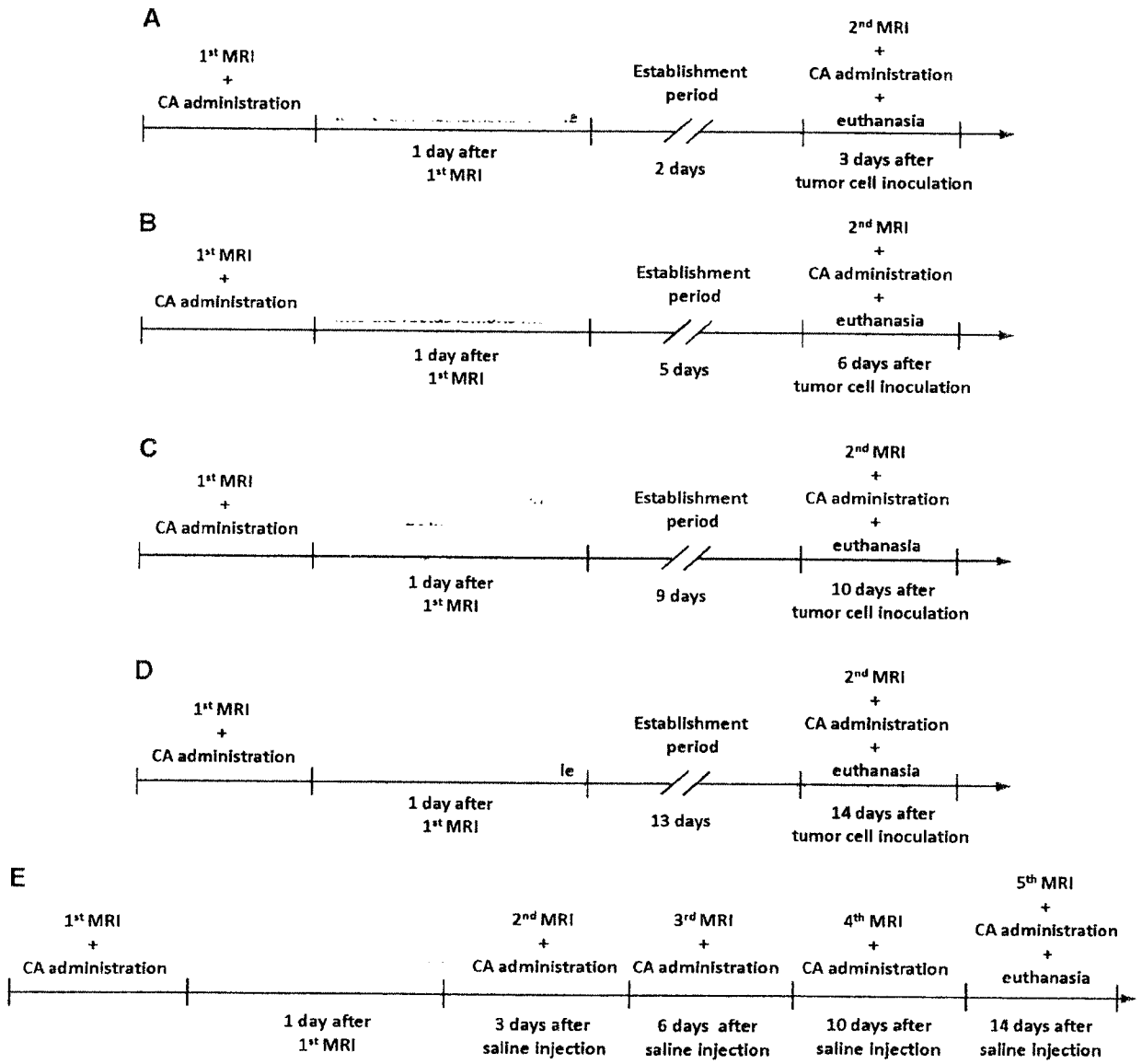

Figure 2: Timelines of the ***Experiment 2*** *for different experimental groups.*

The following experiments: ***Experiment 3*** *and* ***Experiment 4*** *will be performed after accomplished* ***Experiment 1*** *and* ***Experiment 2.***

***Experiment 1 and Experiment 2** aim to test the feasibility of conventional MRI to detect low-volume malignancy under different experimental conditions, resulting in different microenvironments. The ability to detect the minimal disease in one of the proposed models does not necessary mean that it will be possible in the other one. Therefore, **Experiment 1 and Experiment 2** are independent and, thus, have equal priority.*

***Experiment** 3. Determination of the circulation half-live and biodistribution of the integrin-targeted and non-targeted contrast agent.*

*First, either of MRD models will be induced in mice. The most optimal protocol will be used, as assessed from the **Experiment 1** and **Experiment 2**. At the timepoint when MRD is established, the mice will be anaesthetized and the infusion line filled with the contrast agent will be placed in the tail vain (SOP2). Before contrast agent injection 20µl of blood will be collected from the vena saphena (SOP6). Subsequently, the intravenous administration of a buffered contrast agent solution (maximal volume = 0.2ml) will be performed via catheter. 20 µl of blood will be collected from the vena saphena 2 min, 15 min, 30 min, 45 min, 1 hour after contrast agent administration. The animals will be recovered from the anesthesia. At 4, 8, 24, 48 and 72 hours after contrast agent injection, the animal will be fixed in a 50 ml falcon tube and 20 µl of blood will be sampled from the vena saphena. After the last blood sampling (72h after contrast agent injection) the animal will be anesthetized with isoflurane (induction: 2.5%) and the animal will be euthanatized by dislocation of cervical vertebrae. Tumors/muscles with MRD and other tissues (liver, spleen, lung, muscle, kidney and heart) will be collected for immunohistochemical analyses and Gadolinium (Gd) content determination. The Gd concentration in blood samples and organs will be assessed indirectly by MRI measurements and validated by Inductively Coupled Plasma-Mass Spectrometry (ICP-MS).*

***Experiment 4.** In vivo MRI of MRD using angiogenesis-specific contrast agent*

The following contrast agents will be evaluated:

1. *integrin-targeted contrast agent*
2. *non-targeted contrast agent*

*The MRD will be induced in mice using the most optimal protocol assessed in the **Experiment 1** and **Experiment 2**. Subsequently, MRI examination (SOP4) will be performed, during which one of the investigated contrast agents will be administered. The total time of measurements will be around 3 hours. A second MRI scan will be performed at the timepoint when the contrast agent is completely cleared from the blood to avoid the background signal. This timepoint will be determined from the circulation kinetics experiment (Experiment 3). We estimate that the contrast agent will be cleared from the blood around 24-48h after administration. The animals will be sacrificed immediately after the third MRI examination. Tumors/muscles with MRD and normal tissues (liver, spleen, lung, muscle, kidney and heart) will be collected for immunohistochemical analyses to validate MRI results. The timelines of the experiments on radiotherapy- and inoculation-induced MRD are presented in Figure 3 and Figure 4, respectively.*

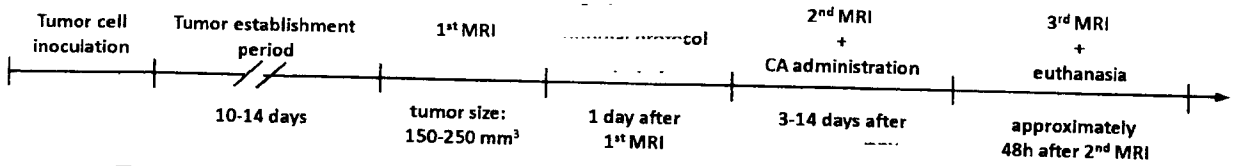

Figure 3: Timeline of the ***Experiment 4*** *using radiotherapy-induced MRD model.*

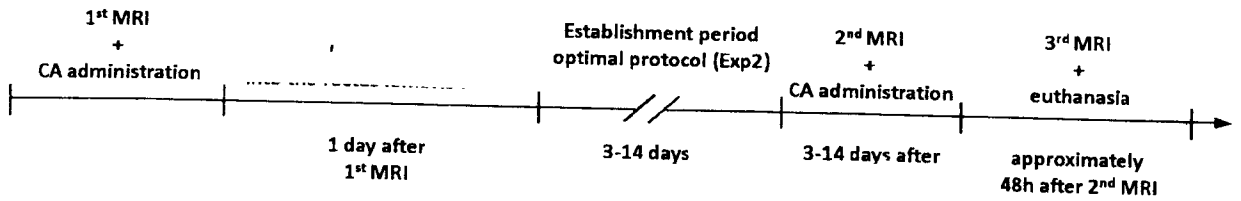

Figure 4: Timeline of the ***Experiment 4*** *using inoculation-induced MRD model.*

9. Experimentele condities

9a. Anesthesie

, placement of the infusion line in the tail vein of the mouse and MRI measurements will be performed under isoflurane anesthesia. The anaesthesia will be induced in the induction chamber with 2.5% of isoflurane and maintained with 1.5-2% of isoflurane delivered via a mouthcap. The medical air will be used as a carrier of isoflurane. The depth of anesthesia will be controlled with a calibrated vaporizer. During the MRI measurements the body temperature will be kept on the physiological level by the warming plate, on which the mouse is positioned. Respiration will be monitored with a balloon sensor connected to an ECG/respiratory unit.
During animals will be sedated with 30-100µl/kg of medetomidine administrated s.c.

9b. Pijnbestrijding

The subcutaneous injection (tumor cell inoculation) is expected to cause mild pain.
and will be performed under isoflurane anaesthesia. The intravenous injection will be performed under anaesthesia with isoflurane during MRI measurements. The will be done on animals sedated with medetomidine administered s.c. No post-treatment pain is expected, as only tumors will be exposed to the . Blood sampling from the vena saphena until one hour after contrast agent injection will be performed under anesthesia with isoflurane (anesthesia needed for i.v. injection of contrast agent), whereas in later timepoints without the use of anesthetic as it is expected to cause mild pain. Euthanasia by dislocation of cervical vertebrae will be performed while the animal is under anaesthesia. The mentioned above procedures do not require administration of any analgesics, except for the , which is performed in a form of the latter case, we will administer an analgesic (temgesic, 0.05mg/kg) 15 min before the . This dose will assure pain sufficient relief during and up to 8h after . This type of pain relief is preferred over NSAID agents because of the anti-inflammatory activity of the letter drugs, which might influence the tumor growth.

9c. Euthanasie en Humane eindpunten

The animals are euthanized under sedation at the end of the experiment by dislocation of the

cervical vertebrae. If during the experiment an animal becomes ill (body weight loss of 20%), or suffers heavily (infection, tumour volume>1500mm^3), then the animal will be euthanized. When there would exist a doubt over the seriousness of suffering or the possibility of treatment, then the CPV-veterinarian (art.14) will be consulted.

Zorg

10a. Ongerief

Gering = 01
Gering/matig = 02
Matig = 03
matig/ernstig = 04
Ernstig = 05
Zeer ernstig = 06

Subcutaneous tumor cell inoculation *(discomfort = 03, duration = 2 min)*
Restraining and subcutaneous injection

Tumor growth *(discomfort = 01, duration = approximately two weeks from a day of inoculation)*
There are no systemic side effects related to the tumor growth on the flank. The animal discomfort related to the presence of established tumor depends on the tumor size. Degree of discomfort can vary from none or minimal in the case of small tumors to moderate and serious when the tumors reach big size. When there will be threatening of serious discomfort (tumor volume>1500mm^3) the animal will be euthanized. However, we do not expect to find such an advance tumor stage during our experiments.

Tumor size monitoring *(discomfort = 03, duration = approximately two weeks from tumor establishment)*
Animal handling and restraining

discomfort = 04, duration = maximum 1h)
Subcutaneous administration of analgesic 15 min before the surgery, anesthesia with isoflurane, ... ı and . recovery from the anesthesia.

i.v. injection of the contrast agent *(discomfort = 04, duration = 15-45 min).*
Anesthesia with isoflurane, fixing an infusion line in the tail vein, injection of the contrast agent, recovery from the anesthesia. No side effects are expected to be induced by the contrast agent.

MRI examination *(discomfort = 04, duration ~3 hours, intervals of several days between the subsequent MRI experiments)*
Anesthesia with isoflurane, placement of an animal in a cradle, recovery from the anesthesia or euthanasia by cervical vertebrae dislocation.

. . (discomfort = 04, duration ~ 0.5 h).
Anesthesia (s.c. injection of medetomidine), n applied s, recovery from the anesthesia. Nno systemic effects are associated with the protocol.

Blood sampling *(discomfort = 04, duration = 72h with intervals)*
Anesthesia with isoflurane (first 1h), i.v. injection, puncture of the vena saphena in 15 min-long intervals, recovery from the anesthesia, animal restraining and puncture of the vena saphena at 4h, 8h, 24h, 48h and 72h after CA injection, euthanasia by cervical vertebrae dislocation.

Total discomfort per experimental group:

experimental group	experimental procedure	duration	discomfort	frequency	total discomfort
1A	Subcutaneous tumor cell inoculation	2 min	03	1	05
	Tumor growth	Max two weeks	01	continuous	
	Tumor size monitoring		03	multiple	
	MRI examination and i.v. CA injection	3 h	04	2	
1B	Subcutaneous tumor cell inoculation	2 min	03	1	05
	Tumor growth	Max two weeks	01	continuous	
	Tumor size monitoring		03	multiple	
	MRI examination and i.v. CA injection	3 h	04	2	
		0.5 h	04	1	
2A	MRI examination and i.v. CA injection	3 h	04	2	05
	ar saline	Max 1 h	04	1	
	Tumor growth	Max two weeks	01	continuous	
2B	MRI examination and i.v. CA injection	3 h	04	2	05
	r	Max 1 h	04	1	
	Tumor growth	Max two weeks	01	continuous	
3A		Max 1 h	04	1	05
	Tumor growth	Max two weeks	01	continuous	
	Or				
	Subcutaneous tumor cell inoculation	2 min	03	1	
	Tumor growth	Max two weeks	01	continuous	
	Tumor size monitoring		03	multiple	
		0.5 h	04	1	
	+				
	i.v. CA injection		04	1	
	Blood sampling		04	11 over 72h	
4A		Max 1 h	04	1	05
	n				
	Tumor growth	Max two weeks	01	continuous	
	Or				
	Subcutaneous tumor cell inoculation	2 min	03	1	
	Tumor growth	Max two weeks	01	continuous	
	Tumor size monitoring		03	multiple	
		0.5 h	04	1	
	+				
	MRI and i.v. CA injection	3 h	04	3	

10b. Welzijnsevaluatie

A logbook will be used where daily animals welfare will be evaluated. Tumor volume will be monitored regularly (two times a week or more).

11. Verzorging en huisvesting

Animals will be housed in animal housing facilities of CPV. Tumor cell inoculation as well as blood sampling will be performed in the animal laboratory of CPV. The MRI measurements will be done on whereas the euthanasia will be performed in the proefdierruimte located next to the . These facilities are located on the

During whole experiment animals will be housed socially (4-6 mice per cage). Mice undergoing the same treatment protocol will be housed in the same cage. Standard rodent chow and water will be available ad libitum throughout the study.

12. Deskundigheid

In the frame of this study the following experimental procedures are planned:
-Subcutaneous injection
-Induction and maintenance of the anaesthesia
-Intravenous injection
-Monitoring of the life parameters and adjustment of anaesthesia during MRI measurements.
-
-
-Blood sampling from vena saphena
-Euthanasia by dislocation of cervical vertebrae
They will be performed, as required, by a competent person (with art.9 qualifications). The people involved in this experiment have already experience in this type of investigations and they have the required licence.

13. Standard Operation Procedures (SOP)

***SOP1**: Subcutaneous inoculation of tumor cells.*

- *Restraining of the animal*
- *Subcutaneous injection of $1x10^6$ tumor cells (cell suspension in PBS or matrigel, volume of 100µl, needle 26G) in the right flank*
- *The inoculated mice will be marked on the tail with a markerpen*

***SOP2**: Placement of an infusion line in the tail vein*

- *Mice will be transported from CPV to facilities on in a closed cagekart*
- *Sedation of an animal with isoflurane*
  a) *Induction: 2.5% isoflurane in the induction chamber,*
  b) *Maintenance: the mouse is placed on the warming plate, 1.5% isoflurane delivered via a mouthcap with a flow of isoflurane with medical air as a gas carrier. The depth of anaesthesia will be controlled with a calibrated vaporizer.*
- *The tail will be warmed up with warm "gel dressing" in order to dilate the tail veins and make them more visible.*

- *The needle will be inserted in the vein lumen*
- *An infusion line (26G needle, catheter, and syringe) will be fixed in the tail vein with a glue and a tape. The infusion line will enable the intravenous injection during the MRI experiment (changes in the animal position are avoided).*

***SOP3**: MRI measurements*

- *Sedation of an animal with isoflurane (induction: 2.5% isoflurane in the induction chamber, maintenance: 1.5% isoflurane delivered via a mouthcap with a flow of isoflurane and medical air as a gas carrier. The depth of anaesthesia will be controlled with a calibrated vaporizer.*
- *An infusion line will placed and fixed in the tail vein (see SOP2) to enable the intravenous administration of the contrast agent during MRI measurements.*
- *Eye ointment will be administered on the mouse eyes to prevent from drying.*
- *The mouse is placed in a home built cradle, equipped with a mask for anaesthesia gas supply and a warm water pad. The cradle lies in a 3-cm quadrature-driven bridge coil in the MR scanner. Respiration is monitored with a balloon sensor connected to a respiratory unit and temperature with a temperature-sensitive rectal probe*
- *The total time of measurements will not exceed 3 hours. First T_2-weighted and diffusion-weighted imaging will be performed.*
- *T_1-weighted images will be generated before and after injection of a contrast agent .*
- *After MRI measurements the mouse will be allowed to recover marked with the marker and placed back in the cage.*
- *The second MRI examination in **Experiment 4** does not include the contrast agent injection.*
- *After the last planned MRI examination, the mouse will not recover after measurements, but will be euthanized, while still being under anesthesia, by cervical dislocation.*

***SOP4**: therapy protocol*

- *Mice will be transported from CPV facilities to in a closed cage*
- *Restraining of an animal*
- *Sedation with* medetomidine *(30-100 µl/kg; s.c.) administered 10 minutes before the treatment*
- *Mouse is placed on a warm water pad*
- *Eye ointment is applied on the mouse eyes to prevent from drying*
- *The treatment will be performed in a custom-made setup, in which only ..... is , whereas the rest of the mouse body is*
- *Depending on the experimental group, tumors wil'*
- *The total time of will be, depending on a dose, from*
- *After recovery from anaesthesia, mice will be transported back to the animal housing facility.*

***SOP5**:*

- *Subcutaneous injection of analgesic (temgesic, 0.05 mg/kg)*
- *Sedation of an animal with isoflurane*
  -Induction: 2.5% isoflurane in the induction chamber,
  -Maintenance: the mouse is placed on the warming plate, 1.5% isoflurane delivered via a mouthcap with a flow of isoflurane with medical air as a gas carrier. The depth of anaesthesia is controlled with a calibrated vaporizer and by testing the foot-reflex
- *Eye ointment will be administered on the mouse eyes to prevent from drying*
- *The incision site will be decontaminated with iodine solution*
- *The . . ! will be made to*
- *(1x10^{6}cells in PBS, 50µl, needle 26G) in . . s will be performed in the right leg*
- *In the control group saline will be injected*
- *The and the mouse will be recovered from the anesthesia*

***SOP6**: Blood collection from the vena saphena*

- *Sedation of an animal with isoflurane*
  -Induction: 2.5% isoflurane in the induction chamber,
  -Maintenance: the mouse is placed on the warming plate, 1.5% isoflurane delivered via a mouthcap with a flow of isoflurane with medical air as a gas carrier. The depth of anesthesia is controlled with a calibrated vaporizer and by testing the foot-reflex.
- *The tail will be warmed up with warm "gel dressing" in order to dilate the tail veins and make them more visible.*
- *The needle connected to the infusion line (26G needle, catheter, and syringe) will be inserted in the vein lumen and fixed on the tail with a glue and a tape.*
- *20ul of blood will be sampled from the vena saphena before contrast agent injection*
- *The contrast agent will be infused*
- *20 µl of blood will be collected from the vena saphena 2 min, 15 min, 30 min, 45 min, 1 h after contrast agent administration.*
- *The animal will recover from the anesthesia in the incubator.*
- *4, 24, 48 and 72 h after contrast agent injection the animal will be fixed in a 50 ml falcon tube and 20 µl of blood will be sampled from the vena saphena (blood collection from vena saphena will be performed according to the SOP: CPV-3-MR).*
- *After the last blood sampling (72h after contrast agent injection) the animal will be anesthetized (sedation with isoflurane (induction: 2.5%) carried by medical air).*
- *The animal will be euthanatized by dislocation of cervical vertebrae.*

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University Maastricht

Faculty of Health, Medicine and Life Sciences

Dierexperimenten Commissie

DEC

, voorzitter
p/a Secretariaat DEC-UM
Postbus 616
NL-6200 MD Maastricht
Telefoon:

Aan:

Uw referentie: Onze referentie Maastricht, 02-02-2011

Geachte Onderzoeker,

Uw projectaanvraag: "*Establishment and MRI evaluation of two animal models of minimal residual disease (MRD) for studying new strategies of MRD detection*", is op de DEC vergadering van 28 januari 2011 besproken.

De DEC heeft een aantal vragen en opmerkingen:

- Op dit protocol is GGO niet van toepassing en daarom kan de GGO medewerker van het voorblad verwijderd worden.
- De DEC verzoekt op het voorblad de duur van de proef aan te passen in 2 "maanden".
- De DEC suggereert eerst experiment 2 ( ) uit te voeren (om te kijken of die cellen detecteerbaar zijn), en pas na een positief resultaat het klinische model te gaan uitvoeren.
- Bij punt 6 verzoekt de DEC alleen aan te geven, "This project has been evaluated and approved by..."
- De DEC verzoekt bij punt 7c, na de uitvalberekening de groepsgrootte te bepalen (afronden) en daarna deze groepsgrootte te gebruiken voor de berekening van het totaal aantal dieren.
- De DEC vindt de uitvalpercentages erg hoog en wenst een betere motivering hiervoor. De DEC accepteert niet dat het uitvalpercentage zo hoog is door niet goed getrainde onderzoekers (bloedafname moet mogelijk zijn met nagenoeg geen uitval). Bij experiment 1 wordt als reden van uitval genoemd "de inter-tumor variability". Die zou opgenomen moeten zijn bij de berekening van de aantallen (variation parameter).
- Bij punt 8 (bladzijde 10) experiment 2, merkt de DEC op dat the "second" the "third" moet zijn.
- Bij punt 10a verzoekt de DEC in een tabel, de aard, ernst, duur en frequentie beter te definiëren per handeling en groep, en het totale ongerief per groep aan te geven. De DEC is van mening dat het totale ongerief code 05 is.

Conclusie:
Het project wordt aangehouden.

Gelieve eventuele vragen te beantwoorden in **een brief en indien noodzakelijk Uw project aan te passen en duidelijk de aanpassingen grijs te markeren.**
Uw project staat bij de DEC geregistreerd onder nummer **2011-006**, gelieve dit nummer in verdere correspondentie te vermelden.

Hoogachtend,

Voorzitter DEC-UM

07.02.2011

Dear DEC members,

Hereby, I address my responses to the comments of DEC on my proposal 2011-006 entitled: "*Establishment and MRI evaluation of two animal models of minimal residual disease (MRD) for studying new strategies of MRD detection*". The answers are given in **bold**.

De DEC heeft een aantal vragen en opmerkingen:

- Op dit protocol is GGO niet van toepassing en daarom kan de GGO medewerker van het voorblad verwijderd worden.

According to the DEC suggestion, the GGO responsible researcher has been removed from the list of involved investigators.

- De DEC verzoekt op het voorblad de duur van de proef aan te passen in 2 "maanden".

The word "manden" was corrected to "maanden".

- De DEC suggereert eerst experiment 2 (uit te voeren (om te kijken of die cellen detecteerbaar zijn), en pas na een positief resultaat het klinische model te gaan uitvoeren.

Experiment 1 and Experiment 2 aim to test the feasibility of conventional MRI to detect low-volume malignancy under different experimental conditions, which result in different microenvironments. The ability to detect the minimal disease in one of the proposed models does not necessary mean that it will be possible in the other one. Therefore, Experiment 1 and Experiment 2 are independent and, thus, have equal priority.

- Bij punt 6 verzoekt de DEC alleen aan te geven, "This project has been evaluated and approved by..."

Point 6 was adjusted as follows: This project has been evaluated and approved by from the Department and (and from of

- De DEC verzoekt bij punt 7c, na de uitvalberekening de groepsgrootte te bepalen (afronden) en daarna deze groepsgrootte te gebruiken voor de berekening van het totaal aantal dieren.

The point 7c was corrected. For the calculation of the total number of animals the rounded up number of animals was used.

- De DEC vindt de uitvalpercentages erg hoog en wenst een betere motivering hiervoor. De DEC accepteert niet dat het uitvalpercentage zo hoog is door niet goed getrainde onderzoekers (bloedafname moet mogelijk zijn met nagenoeg geen uitval). Bij experiment 1 wordt als reden van uitval genoemd "de inter-tumor variability". Die zou opgenomen moeten zijn bij de berekening van de aantallen (variation parameter).

The "uitvalpercentage" was decreased to 10% and in the case of blood sampling experiment to 0%. As a result, the number of requested animals decreased. The motivation for the remaining 10% is the following: Uitvalspercentage 10% is related to potential difficulties with MRI measurements, such as technical failure and movement artifacts, which can affect the quality of data considerably or make them even unusable. The estimated percentage is based on the previous experience of the investigators.

- Bij punt 8 (bladzijde 10) experiment 2, merkt de DEC op dat the "second" the "third" moet zijn.

The word "second" was corrected to "third".

- Bij punt 10a verzoekt de DEC in een tabel, de aard, ernst, duur en frequentie beter te definiëren per handeling en groep, en het totale ongerief per groep aan te geven. De DEC is van mening dat het totale ongerief code 05 is.

A table with detailed depiction of experimental procedures, their duration, discomfort and frequency per experimental group was added to the proposal on page 14. According to the suggestion of DEC, the total discomfort of animals was changed from 04 to 05.

I hope that the current version of my DEC proposal will be positively evaluated.

Kind regards,

Maastricht University

Faculty of Health, Medicine and Life Sciences

Aan:

Ons kenmerk	*Doorkiesnummer*	*Maastricht*
		07-03-2011

Project: *Establishment and MRI evaluation of two animal models of minimal residual disease (MRD) for studying new strategies of MRD detection.*

Verantwoordelijk onderzoeker (VO):

Hierbij delen wij U mede dat voornoemd project aan de ethische toetsingscriteria voor proefdiergebruik voldoet.
De DEC maakt geen bezwaar tegen uitvoering van dit project zoals aangevraagd en geeft een **positief advies**.

Projectnummer:	**2011-006**
Diersoort:	**muis**
Aantal dieren:	**188**
Einddatum:	**02-03-2015**

Uw project staat bij de DEC en CPV geregistreerd onder bovenstaand nummer. Gelieve dieren, die voor dit project bestemd zijn, ook onder dit nummer aan te vragen.

Voorzitter DEC-UM

Vice-Voorzitter DEC-UM

DEC-UM
Voorzitter DEC-UM

p/a secretariaat DEC-UM

Secretariaat DEC-UM

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