Begeleidingsformulier aanvraag dierproef DEC- UM

Versie 2006

Herziene versie

DECNR: 2011-129

Ontvangen: 11-11-2011

DEC datum goedkeuring#	Type aanvraag [2]
18-11-2011	Nieuw / ~~Herz.versie / Pilot~~

VROM/GGONR[3]
04-165 ($ApoE^{-/-}/TM^{pro/pro}$) 04-165 ($ApoE^{-/-}$) 04-165 ($LDLR^{-/-}$)

LNV/CBDNR[4]

Hoofdproject	CARIM	~~NUTRIM~~	~~Hersenen en gedrag~~	~~GROW~~	~~biomaterialen~~	~~Ander UM~~	~~Geen UM~~
Deelproject	3	~~1. 2. 3. 4.~~	~~1. 2. 3.~~	~~1. 2. 3.~~			

Financieel beheerder	

Budgetnummer	30981119B

Titel van het onderzoek:

The effect of thrombin inhibition on atherosclerotic plaque development in normal and pro-thrombotic mice

startdatum **October 2011** einddatum [9] **October 2015** *Duur van de proef [10]:* *14 weken*

	Naam	Tel (+ Tel privé enkel VO, VVO en VM)	E-mailadres	Bevoegdheid[5]	Cap. groep /afdeling
1.Verantwoordelijk onderzoeker (VO)				Art.9	
2. Vervanger VO (VVO)				Art.9	
3. Verantwoordelijk medewerker (VM) GGO[7]				Art.9	
4. overige uitvoerenden				Art.12 Art. 9	
5.PI/punt6					

Diergroep	1	2	3
ctrl/exp/sham	Exp 1	Exp 2	Exp 3
Diersoort	01, muis	01, muis	01, muis
Stam	C57/bl6	C57/bl6	C57/bl6
Construct / mutatie ?	$TM^{pro/pro}/ApoE^{-/-}$	$ApoE^{-/-}$	$LDLR^{-/-}$
Herkomst (leverancier) *	01, UM	01, UM	01, UM
Aantal	8(1a)+8(1b)+16(1c)+16(1d)=48	8(2a)+8(2b)+16(2c)+16(2d)=48	16(3a)+16(3b)+16(3c)+16(3d)=64
Geslacht	V	V	V
Dieren immuuncompetent ?	Ja/~~nee~~[8]	Ja/~~nee~~[8]	Ja/~~nee~~[8]
Leeftijd/gewicht	8-9 weeks	8-9 weeks	8-9 weeks
Doel van de proef *	31	31	31
Belang van de proef *	1	1	1
Toxicologisch onderzoek *	1	1	1
Bijzondere technieken *	1	1	1
Anesthesie *	4	4	4
Pijnbestrijding *	4	4	4
Mate ongerief *	3	3	3
Toestand dier einde exp*	1	1	1

* VHI-coderingen zie bijlage

1 Verantwoording

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

Titel: The effect of thrombin inhibition on atherosclerotic plaque development in normal and pro-thrombotic mice

1. Goal of the experiment

Atherosclerosis is the leading cause of mortality in the developed societies worldwide. The buildup of lipids, calcium, and cellular debris within the intima of the vessel wall results in plaque formation, vascular remodeling, acute and chronic luminal obstruction, abnormalities of blood flow and diminished oxygen supply to target organs. Hence, it results into the occurrence of cardiovascular incidents such as myocardial infarction or stroke. However, many studies demonstrate a cross-link between coagulation and inflammation in atherosclerosis, suggesting that hemostatic proteins might be involved in the modulation of the atherosclerotic progression. Based on previous experiments we performed, compliant with **DEC application 2004-151 and 2009-039**, we demonstrate that altered plasma thrombin levels might play an important role in development and progression of atherosclerosis.

Objective 1: Is the direct inhibition of thrombin causing a reduction in atherosclerotic plaque progression using a milder model of atherosclerosis?

Objective 2: Is it the higher thrombin generation that mediates the enhancement of atherosclerosis in $TM^{pro/pro}/ApoE^{-/-}$ mice?

For these purposes, we want to administer the direct thrombin inhibitor in a milder model of atherosclerosis, namely in $ApoE^{-/-}$ and $LDLR^{-/-}$ mice. By doing this, we want to investigate whether a direct thrombin inhibitor can also give a positive effect when milder plaque formation occurs. To reveal whether the observed effect is solely thrombin dependent, $ApoE^{-/-}$ and $LDLR^{-/-}$ mice with normal pro-thrombin levels will be used instead of mice with a mutation in the TM gene, which causes higher pro-thrombin levels. These experiments will help to reveal the effect of this drug on the plaque phenotypization and coagulation/lipid profile.

2. Social relevance and/or scientific significance

Cardiovascular diseases, respectively atherosclerosis, are the number one cause of morbidity and mortality for individuals in modern societies nowadays. Atherosclerosis in terms of its most common later complications – e.g. myocardial infarction or stroke, plays the most substantial and social relevant role for performing these experiments. Since atherosclerosis is a multifactorial disease, it is very hard for physicians to predict the incidence of thrombotic events. With the help of this project we would have the possibility to study different aspects of atherosclerotic development in relation to thrombosis. In case we prove that the altered levels of thrombin generation influence on the development and composition of the atherosclerotic plaque, this information would be exclusively valuable for the progress of medical behavior and drug intervention in terms of this nosological unit and would have a great social significance.

3. Alternatives

The most significant task in this project is to obtain information of drug intervention at different stages of the atherosclerotic plaque development in vivo. An alternative, as for instance cell culturing, is not appropriate in this case, since we will be studying a multifactor disease as atherosclerosis is. Therefore, there is no alternative method.

4. Ethical assessment

In case we prove that the altered levels of thrombin generation influence on the development and composition of the atherosclerotic plaque, this information would be of exclusively great social significance in terms of finding further understanding of this world-wide spreading systemic disease and eventually, improving the drug solutions for it.

5. Scientific substantiation

Little is known about the pathophysiological cross talk between coagulation proteins and inflammation in atherosclerosis. Thrombin is a coagulation protease with a central role in the coagulation cascade. Besides its well-known key role in thrombus formation, thrombin activation of protease activated receptors (PARs) may affect cellular processes, thus leading to inflammation, proliferation and apoptosis during development and progression of atherosclerosis. Using two transgenic mice models with altered thrombin generation capacity we demonstrated that atherosclerosis was decreased with a more smooth muscle cell rich plaque phenotype in animals with

, **(DEC 2004-151)**, which showed increased plaque formation compared to normal ApoE$^{-/-}$ mice. Conclusions from this study were that variations in the endogenous thrombin potential may be relevant to the risk of atherothrombosis. Either increased or normal endogenous thrombin potential or impaired APC production could possibly affect progression and thrombogenicity of the atherosclerotic lesions. When administering a direct thrombin inhibitor (**DEC 2009-039**) in the mice with a pro-thrombotic phenotype,

Therefore, we would like to implement 3 experimental setups:

- **Experiment 1: a cuff atherosclerotic model-TM $^{pro/pro}$/ApoE$^{-/-}$ administered with or without a direct thrombin inhibitor (Dabigatran)**
- **Experiment 2: a cuff atherosclerotic model-ApoE$^{-/-}$ administered with or without a direct thrombin inhibitor (Dabigatran)**
- **Experiment 3: a cuff atherosclerotic model-LDLR$^{-/-}$ administered with or without a direct thrombin inhibitor (Dabigatran)**

We expect that direct thrombin inhibitors might also show potency in decreasing the atherosclerotic burden in the ApoE$^{-/-}$ and LDLR$^{-/-}$mice by diminishing the production of thrombin, thereby leading to less thrombin-induced PAR-mediated pro-atherogenic effects.

For this purpose, we want to implement a control as well as a treatment group with either 8 or 14 weeks of plaque formation. From previous experiments (as described in DEC 2009-039), it is shown that these 2 time points are most optimal for detecting the effect of the treatment. Two different animal groups for the 2 time points have to be included as analysis of the arteries is necessary for determining the treatment effect. Unfortunately, this is not possible via a non-invasive procedure, so no single animal group can be used for monitoring the effects in time.

The control groups for the ApoE$^{-/-}$ and TM$^{pro/pro}$/ApoE$^{-/-}$ are equal to the control groups from the experiments as described in DEC 2009-039, therefore the number of animals in these groups will be halved and results from these previous experiments will be used.

6. Scientific appraisal

This DEC protocol is evaluated and has been approved by the

7. Selection of laboratory animals

7a. Sort/stam

- **ApoE$^{-/-}$** mice-C57/Bl 6 bred
  These mice are prone to develop spontaneous atherosclerosis and the give a good insight into the development of atherosclerosis in humans.
- **LDLR$^{-/-}$** mice-C57Bl 6
  These mice are prone to develop atherosclerosis, but to a lesser extent then ApoE$^{-/-}$ mice (which show higher cholesterol levels and more severe plaques).
- **ApoE$^{-/-}$/TM$^{pro/pro}$** mice-C57Bl 6 bred (88%), 12% 129 SV
  These mice have a mutation in the thrombin-binding domain, which results in the fact that protein C no longer can be activated to APC, resulting in higher thrombin levels. This in turn results in increased fibrin formation and clot formation. These mice are being used for both the simulation of elevated thrombin levels and of the atherosclerotic process.

ApoE$^{-/-}$ and ApoE$^{-/-}$/TM$^{pro/pro}$ mice are bred within our animal facility
LDLR$^{-/-}$ mice are from Jackson Laboratories

After the experiments, the mice will be sacrificed and blood and vessel material will be collected.

7b. Sex

Female mice will be utilized only because of a faster atherosclerotic plaque development and a lower variation of the stages of atherosclerotic plaque formation in comparison to the male mice.

7.c. Amount of laboratory animals

Student's t-test results with a confidence interval of 5% (α=0.05), and power capacity of 80% (π=0.8):

$Z_{\alpha/2}-Z_{\pi} = (n/2)^{½} * (\mu_1-\mu_2)/\sigma$

Variation coefficient is 25% and to detect a 25% difference in plaque area or in immunohistochemical quantification of cell-types, extracellular matrix content or necrotic core, this requires 16 animals for each group. Based on previous experiments, we don't expect loss of animals.

<u>Experiment – A cuff atherosclerotic model with direct thrombin inhibitor administration</u>

1. a) **TM$^{pro/pro}$/ApoE$^{-/-}$** mice for the cuff model administered without thrombin inhibitor (8 weeks) ⇨ n= 8
   b) **TM$^{pro/pro}$/ApoE$^{-/-}$** mice for the cuff model administered without thrombin inhibitor (14 weeks) ⇨ n= 8
   c) **TM$^{pro/pro}$/ApoE$^{-/-}$** mice for the cuff model administered with a direct thrombin inhibitor (8 weeks) ⇨ n= 16
   d) **TM$^{pro/pro}$/ApoE$^{-/-}$** mice for the cuff model administered with a direct thrombin inhibitor (14 weeks) ⇨ n= 16

2. a) **ApoE$^{-/-}$** mice for the cuff model administered without thrombin inhibitor (8 weeks) ⇨ n= 8
   b) **ApoE$^{-/-}$** mice for the cuff model administered without thrombin inhibitor (14 weeks) ⇨ n= 8
   c) **ApoE$^{-/-}$** mice for the cuff model administered with a direct thrombin inhibitor (8 weeks) ⇨ n= 16
   d) **ApoE$^{-/-}$** mice for the cuff model administered with a direct thrombin inhibitor (14 weeks) ⇨ n= 16

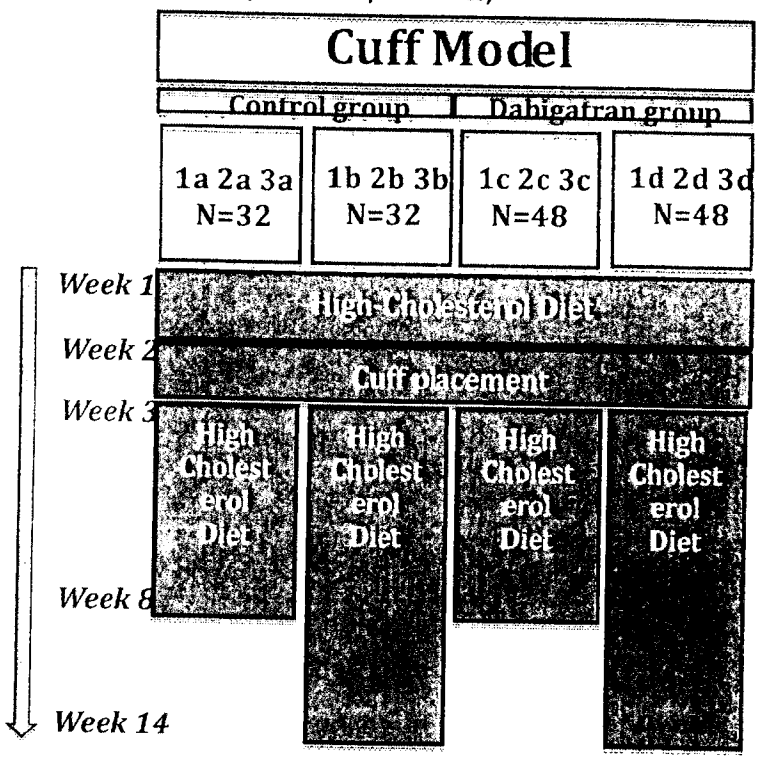
3. a) **LDLR$^{-/-}$** mice for the cuff model administered without thrombin inhibitor (8 weeks) ⇨ n= 16
   b) **LDLR$^{-/-}$** mice for the cuff model administered without thrombin inhibitor (14weeks) ⇨ n= 16
   c) **LDLR$^{-/-}$** mice for the cuff model with a direct thrombin inhibitor administered (8 weeks) ⇨ n= 16
   d) **LDLR$^{-/-}$** mice for the cuff model with a direct thrombin inhibitor administered (14 weeks) ⇨ n= 16

Total: n= 160

8. Experiments

Experiments 1, 2 and 3 will involve $TM^{pro/pro}/ApoE^{-/-}$, $ApoE^{-/-}$, and $LDLR^{-/-}$ female mice respectively, which have reached the age of 9 weeks. Then, the mice will be assigned on a Western high-cholesterol diet for 2 weeks prior to a collar (cuff) placement on both of the common carotid arteries. Hence, they will be left, for 6 more weeks (**group 1a+c, 2a+c, and 3a+c**) or 12 more weeks (**group 1b+d, 2b+d, and 3b+d**) on the same food regimen, thus leading to the development of atherosclerosis in the arteries, leading to a total duration of the experiment of 8 or 14 weeks. Experimental animals from **group 1c+d, 2c+d, and 3c+d** will receive their direct thrombin inhibitor medication per os, in powdered high fatty pellets, mixed up with dabigatran, in concentration of 7.5 mg/kg.

From the 3 animal groups, a control group will be included without Dabigatran administration. These animals will be placed under a western diet for a total 8 or 14 weeks. 2 weeks after the start of the diet, the cuff will be placed (**group 1a+b, 2a+b, and 3a+b**). At the end of the 8 or 14 weeks, all mice will be sacrificed, exsanguinated and both aortic trees and carotid arteries will be carefully prepared for further histological and morphometrical analysis. *(See protocol enclosed for more details on the experimental procedures)*



9. Experimental conditions

9a. Anaesthesia

Anaesthesia will be applied in all experiments and will be induced in an incubator using isoflurane, whereupon the anesthesia will be continued via a surgical mask with 3-4% isoflurane and will be maintained with 1.5-2.5% isofluran.

9b. Pain killing

0.1 mg/kg Temgesic (buprenorphine hydrochloride) will be administered subcutaneously prior to and after all surgical actions.

9c. Euthanasia en Human endpoints

Euthanasia is induced in all experiments by intraperitoneal application of pentobarbital (200 mg/kg). In case of complications during surgery, direct euthanasia will be performed. Euthanasia will be performed on all animals suffering from disease or pain (within the experimental procedure or during development of atherosclerosis). The art.12 will judge whether animals suffer from pain, based on experience and common sense. Discomfort during development of atherosclerosis will be judged by the experienced employees of the CPV. **Animals will be removed from the experiment when they suffer from wasting syndrome (weight loss, weakness, fur and nutritional problems).**

Zorg

10a. Level of discomfort

Procedures during cuff placement	Code (Level of Discomfort)	Duration/frequency
a) Preparation for the study	01	5 min/1
b) Cuff placement: operation under general anaesthesia	03	30 min/1
d) State of the animals at the end of the study	01	
e) During euthanasia	02	5 min/1
Total discomfort all animal groups	03	

10b. Welfare evaluation

From previous experiments, general discomfort of the animals is observed as described in the table.

11. Housing, provisions and care

A Western diet (High-cholesterol) and tap water ad libitum will be provided to the animals in **Experiments 1a+b, 2a+b and 3a+b** throughout the experimental period. **1c+d, 2c+d, and 3c+d** will be provided with a Western type of diet mixed up with Dabigatran (as stated above). Animals will be housed in group.

12. Expertise

Experiments will be performed by owns an article 12 status and is experienced in all experiments as described

13. Standard Operation Procedures (SOP)

Please find all necessary SOP protocols attached in the annex.

Relevant literature

University Maastricht

Faculty of Health, Medicine
and Life Sciences

Dierexperimenten Commissie

DEC

Aan:

voorzitter
p/a Secretariaat DEC-UM
Postbus 616
NL-6200 MD Maastricht
Telefoon: 043-

Uw referentie: Onze referentie Maastricht, 26-10-2011

Geachte Onderzoeker,

Uw projectaanvraag: "*The effect of thrombin inhibition on atherosclerotic plaque development in normal and pro-thrombotic mice*", is op de DEC vergadering van 21 oktober 2011 besproken.

De DEC heeft een aantal vragen en opmerkingen:

- De DEC verzoekt op het voorblad de GGO medewerker toe te voegen van nummer 02-141. Dit is van een andere onderzoeker.
- De duur van de proef op het voorblad is niet juist (dit is de langste periode binnen één project dat één dier in proef is). De DEC verzoekt dit aan te passen. *(14 wk)*
- Punt 5- De DEC verzoekt aan te geven of de experimenten uit het verleden op gelijke wijze zijn uitgevoerd (dit wil zeggen worden de experimenten in dit DEC protocol herhaald? Dit betreft de $ApoE^{-/-}//TM^{pro/pro}$ en de $ApoE^{-/-}$ controlegroep). Indien dit het geval is, vraagt de DEC zich af of de controlegroepen eventueel gehalveerd kunnen worden en (deels) gebruik kan worden gemaakt van historische controles.
- Punt 6- De DEC verzoekt aan te geven door wie of welke commissie **dit DEC protocol** wetenschappelijk is beoordeeld en goedgekeurd.
- Punt 7c- De DEC merkt op dat "N=17"- "N=16" moet zijn.
- Punt 7c- De DEC acht het hoogst onwaarschijnlijk dat de variatie coëfficiënt in alle genoemde parameters exact hetzelfde is (25%). De DEC wenst een specificatie op welke genoemde parameter de 25% variatie betrekking heeft en wenst een onderbouwing (historische gegevens en literatuur).
- Punt 7c De DEC vraagt of de genetische achtergrond van de $ApoE^{-/-}$ en de $TM^{pro/pro}/ApoE^{-/-}$ (gezien de Sv129 achtergrond) vergelijkbaar is?
- Punt 8- Vanwaar de grote spreiding in de te gebruiken doses van dabigatran? Gaarne toelichten.
- De DEC verzoekt bij punt 9a "in a box using a few drops of isoflurane" te verwijderen.
- Punt 9c- De DEC verzoekt de humane eindpunten te specificeren.

- Punt 10a- De DEC verzoekt het leven na de cuff placement en het 3x uit anesthesie bijkomen, ook te vermelden bij punt 10a. De DEC is van mening dat het totale ongerief code 03 is (ook het voorblad aanpassen).

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 **2011-129**, gelieve dit nummer in verdere correspondentie te vermelden.

Hoogachtend,

Voorzitter DEC-UM

Faculty of
Health, Medicine and Life Sciences

Voorzitter DEC-UMCPV,

Your reference	*Our reference*	*Direct line*	*Maastricht*
			10.11.2011

DEC 2011-129

Internal Medicine

Geachte leden van de DEC,

Met betrekking tot het commentaar op DEC-aanvraag 2011-129 : ' The effect of thrombin inhibition on atherosclerotic plaque development in normal and pro-thrombotic mice ' heeft de onderzoeker volgende antwoorden:

- Na telefonisch contact met , vallen alle muizen zoals beschreven in dit DEC protocol onder GGO nummer 04-165. Dit is ook aangepast op het voorblad.
- De duur van de proef op het voorblad is gewijzigd in 14 weken.
- De controlegroepen zijn inderdaad gelijk aan deze uit eerdere experimenten. De onderzoekers hebben dan ook de aantallen van de controlegroepen (voor de $ApoE^{-/-}/Tm^{Pro/Pro}$ en $ApoE^{-/-}$ muizen) gehalveerd naar 8. Voor de overige dieren zal gebruik gemaakt worden van historische controles.
- Dit DEC protocol is wetenschappelijk beoordeeld en goedgekeurd door de vakgroepvoorzitter . Dit is gewijzigd in de aanvraag en s om deze reden als PI toegevoegd op het voorblad
- De aantallen zijn gewijzigd zowel op het voorblad als in de aanvraag.
- De 25% variatie die we in deze DEC aanvraag gebruiken, duidt op de variatie in plaque grootte, die verkregen wordt met behandeling van de

T + 31
F + 31

Visiting address

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NL003475268B01

muizen door middel van anti-stolling. Deze 25% is gebaseerd op data verkregen uit de resultaten van de proeven zoals beschreven in DEC 2009-039.

- Gezien de genetische achtergrond van de $TM^{pro/pro}/ApoE^{-/-}$ voor 88% C57Bl6 is en gezien voorgaande positieve resultaten uit vorige experimenten, zijn de onderzoekers van mening dat beide muizenstammen vergelijkbaar zijn.
- De dosis Dabigatran die in de aanvraag vermeld stond was niet correct. De onderzoekers hebben de dosis van Dabigatran in punt 8 in de aanvraag gewijzigd in 7.5 mg/kg.
- Punt 9a is gewijzigd in de aanvraag.
- De humane eindpunten zoals beschreven in punt 9c van de aanvraag zijn gespecifieerd.
- De ongerief code is zowel in de aanvraag als op het voorblad aangepast naar 3. De onderzoekers zijn van mening dat het leven na cuff placement geen ongerief voor de dieren veroorzaakt en dit op basis van de welzijnsevaluaties van vorige DEC aanvragen. Verder willen de onderzoekers ook meedelen dat de dieren slechts 1x uit anesthesie ontwaken en dit na het plaatsen van de cuff.

Hopend hiermee naar tevredenheid antwoord te hebben gegeven,

Met vriendelijke groet,

Maastricht University

Faculty of Health, Medicine and Life Sciences

Aan:

Ons kenmerk *Doorkiesnummer* *Maastricht*
22-11-2011

Project: *The effect of thrombin inhibition on atherosclerotic plaque development in normal and pro-thrombotic mice.*

Verantwoordelijk onderzoeker (VO):

Namens de Vergunninghouder van de DEC-UM, 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:	**2011-129**
Diersoort:	**muis**
Aantal dieren:	**160**
Einddatum:	**18-11-2015**

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

Vicevoorzitter DEC-UM

DEC-UM
Voorzitter DEC-UM

p/a secretariaat DEC-UM

Secretariaat DEC-UM
T (043)

Bezoekadres

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