| | **Inventaris Wob-verzoek W17-11** | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | **wordt verstrekt** | | | | **weigeringsgronden** | | | |
| **nr.** | **documenten NTS2016607** | **reeds openbaar** | **niet** | **geheel** | **deels** | **10.1.c** | **10.2.e** | **10.2.g** | **11.1** |
| 1 | Aanvraagformulier | | | | x | | x | | |
| 2 | Projectvoorstel | | | | x | | x | | |
| 3 | Niet-technische samenvatting oud | | | x | | | | | |
| 4 | Bijlage beschrijving dierproeven 1 oud | | | | x | | x | | |
| 5 | Bijlage beschrijving dierproeven 2 oud | | | | x | | x | | |
| 6 | Bijlage beschrijving dierproeven 3 oud | | | | x | | x | | |
| 7 | Bijlage beschrijving dierproeven 4 oud | | | | x | | x | | |
| 8 | DEC-advies | | | | x | | x | | |
| 9 | Ontvangstbevestiging | | | | x | | x | | |
| 10 | Verzoek aanvulling aanvraag | | | | x | | x | | |
| 11 | Reactie verzoek aanvulling | | | x | | | | | |
| 12 | Projectvoorstel nieuw | | | | x | | x | | |
| 13 | Bijlage beschrijving dierproeven 1 nieuw | | | | x | | x | | |
| 14 | Bijlage beschrijving dierproeven 2 nieuw | | | | x | | x | | |
| 15 | Bijlage beschrijving dierproeven 3 nieuw | | | | x | | x | | |
| 16 | Bijlage beschrijving dierproeven 4 nieuw | | | | x | | x | | |
| 17 | Niet-technische samenvatting nieuw | x | | | | | | | |
| 18 | Bijlage beschrijving dierproeven 3 nieuwst | | | | x | | x | | |
| 19 | Bijlage beschrijving dierproeven 4 nieuwst | | | | x | | x | | |
| 20 | Advies CCD | | x | | | | | | x |
| 21 | Beschikking en vergunning | | | | x | | x | | |

15 MEI 2017

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] | |
| Functie | Associate Professor | |
| Afdeling | [redacted] | |
| Telefoonnummer | [redacted] | |
| E-mailadres | [redacted] | |

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

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

| | | | |
|---|---|---|---|
| 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<br>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<br>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 - 6 - 2017 |
| | | Einddatum | 1 - 6 - 2022 |
| 3.2 | Wat is de titel van het project? | The Role of the Solute Carrier Transporter 44A2 (SLC44A2) Gene in the Pathophysiology of Venous Thromboembolism | |
| 3.3 | Wat is de titel van de niet-technische samenvatting? | De Rol van de Erfelijke Factor 'Solute Carrier Transporter 44A2' (SLC44A2) in de Ontwikkeling van de Ziekte Veneuze Trombose | |
| 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] |
| | | E-mailadres | [redacted] |

# 4 Betaalgegevens

4.1 Om welk type aanvraag gaat het?

☒ Nieuwe aanvraag Projectvergunning € 1684 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 | 8 - 5 - 17 |
| Handtekening | [redacted] |

Leids Universitair
Medisch Centrum

**Begeleidend schrijven**


afdeling [redacted]
postzone
afzender
bezoekadres
telefoon
fax
e-mail [redacted]


onze referentie
uw referentie
datum 11-05-2017
onderwerp Administratieve gegevens
AVD116002016607
aantal pagina's 1

aan Centrale Commissie Dierproeven
Postbus 20401
2500 EK Den Haag

Geachte heer/mevrouw ,

Bijgaand doen wij u toekomen de administratieve gegevens van AVD116002016607: The Role of the Solute Carrier Transporter 44A2 (SLC44A2) Gene in the Pathophysiology of Venous Thromboembolism.

☐ naar aanleiding van uw telefonisch/schriftelijk verzoek d.d.

☐ op verzoek van

☐ volgens afspraak

☐ retour met dank voor inzage

☐ met verzoek om advies / bericht / nadere informatie

☑ met verzoek de (verdere) behandeling over te nemen

☐ ter kennisname / inzage

☐ ter doorzending aan

☐ gaarne retour

☐

Met vriendelijke groet,

[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. | [redacted] |
| 1.3 | Provide the title of the project. | The Role of the Solute Carrier Transporter 44A2 (SLC44A2) Gene in the Pathophysiology of Venous Thromboembolism |

## 2 Categories

2.1 Please tick each of the following boxes that applies to your project.

X 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.

Venous thromboembolism (VTE) is the third leading cause of cardiovascular mortality worldwide and is responsible for approximately half a million associated deaths in Western Europe each year (Cohen et al

*Thromb Haemost* 2007). In addition, adverse events linked to VTE, i.e. deep vein thrombosis (DVT) and pulmonary embolism (PE), are major contributing factors to the growing number of years lived with a disability and results in a marked reduction in the quality of life for millions of people every year. VTE is a complex thrombotic disorder influenced both by environmental aspects as well as in particular by genetic predisposition. As part of the [redacted], within the Leiden University Medical Center (LUMC), our research mainly focuses on the underlying mechanisms associated with VTE and places a strong emphasis on the role of genetics in this process. This has led to seminal contributions in the VTE field including the discovery of the factor V Leiden mutation in the mid-nineties and subsequently several other genetic variations which can predetermine thrombotic risk. Although there have been several advances in the field with the identification of many genetic variants linked to VTE, they still only explain a minor percentage of VTE risk in many cases.

Recently, as part of the international INVENT consortium, we undertook a meta-analysis of genome-wide association studies (GWAS) to identify additional VTE susceptibility genes (Germain et al. 2015 Am. J. Human Genetics). Twelve GWAS totalling 7,507 VTE cases and 52,632 controls formed our discovery stage where 6,751,884 single nucleotide polymorphisms (SNPs) were tested for association with VTE. Nine loci reached the genome-wide significance level including 6 already known to associate with VTE (ABO, F2, F5, F11, FGG and PROCR) and 3 unsuspected genes. SNPs mapping to the latter genes were selected for replication in 3 independent case-control studies totalling 3,009 VTE patients and 2,586 controls. This strategy lead to the identification of *SLC44A2* as a new risk locus ($p$ value = $2.75 \times 10^{-15}$). The lead SNP at the *SLC44A2* SNP locus is the non-synonymous rs2288904 previously shown to associate with transfusion related acute lung injury (TRALI). Importantly, a second independent GWAS study found that a locus also located within the *SLC44A2* gene is a genetic risk factor for both stroke and cardiovascular disease, further substantiating a possible role for SLC44A2 in VTE pathogenesis (Hinds et al. 2016 Hum Mol Genet.) SLC44A2 has not been described to associate with known haemostatic markers, thus interestingly, SLC44A2 does not belong to conventional pathways for thrombosis, nor has it been associated with other cardiovascular diseases or related quantitative biomarkers.

The VTE lead SNP, *SLC44A2* rs2288904 (A or G), coincides with an amino acid substitution in the extracellular domain of the Solute Carrier 44 A2 protein (SLC44A2 R154 or Q154, respectively) and is known to trigger antibody formation in carriers of the minor A allele, both during pregnancy and upon exposure to the major G allele variant. Upon transfusion, these antibodies induce neutrophil activation, sequestration, and finally, endothelial barrier damage which can result in possibly fatal transfusion related acute lung injury (TRALI). SLC44A2 is expressed on neutrophils, which is the postulated target cell for the anti-SLC44A2 antibodies in TRALI, although SLC44A2 is also expressed on vascular endothelial cells. Our collaborators at Giessen University, Germany, demonstrated *in vivo* that the anti-SLC44A2 antibodies induce loss of endothelial barrier integrity. In this model, neutrophils aggravated the destructive effects of anti-SLC44A2. Furthermore, they demonstrated *in vitro* that SLC44A2 can act as a receptor for von Willebrand Factor (vWF), a factor expressed on the endothelium and a key molecule important for normal haemostasis. In addition, it has become evident that two isoforms of SLC44A2 exist (i.e P1 and P2) with endothelial cells and neutrophils showing variable expression (P1 and P2 vs P1 only) possibly affecting SLC44A2 functionality.

Initial work characterizing the SLC44A2 global knockout mouse from a haemostasis perspective was performed in the laboratory of our collaborators at the University of Michigan in Ann Arbor, Michigan, USA. Preliminary testing found increased transcript levels of tissue plasminogen activator (tPA) in the lung of SLC44A2 knock out mice indicating that these mice may have a better capacity for clearing blood clots. Furthermore, upon laser induced injury of the cremaster muscle, SLC44A2 knockouts exhibited a reduction in fibrin formation, again suggesting that the mice have a faster clearance of clotting factors. These data point to a possible role for SLC44A2 in the process of haemostasis, which has not been previously described, however, the mechanisms behind these observations remain unclear.

Also of particular interest is the recent identification of autoantibodies against SLC44A2 in patients with autoimmune hearing loss (Kommareddi et al. 2009 Laryngoscope). The binding of these antibodies to SLC44A2 expressing cells in the inner ear leads to a detrimental effect on hair cell survival and hearing, again demonstrating that anti-SLC44A2 antibodies can have a disruptive effect on normal cellular

function. Several autoantibodies against haemostatic proteins such as prothrombin and thrombomodulin, in addition to antibodies targeting phospholipids, have been identified and linked to VTE, showing that autoantibodies can also contribute to the pathophysiology of VTE. We believe it is possible, as an alternative hypothesis, that a similar mechanism may also be occurring in the context of SLC44A2 and preliminary work by collaborators has even identified potential SLC44A2 autoantibodies in patients with VTE.

Altogether SLC44A2 appears to be relevant in both TRALI and VTE, two diseases where endothelial cells, neutrophils and their interactions are considered key to vascular pathophysiology. Importantly, antibodies can also play a destructive role in both of these conditions. Based on this knowledge, the current project proposal aims to further analyse the function of SLC44A2, including the interactions with autoantibodies, and unravel the mechanism underlying the association between SLC44A2 and VTE. We will focus in particular on the role of SLC44A2 in endothelial cells, neutrophils as well as with autoantibodies. We expect that the proposed project will provide novel therapeutic insight for the development of improved VTE treatments. Since all the current therapeutic strategies for VTE are directed at targeting coagulation factors and consequently have major bleeding as an unwanted side-effect and SLC44A2 has not been linked to traditional haemostasis, further understanding into its function may lead to the discovery of a unique treatment strategy without the adverse side effects of the current therapeutics.

**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?

SLC44A2 has been identified as a new VTE-associated locus, one with a strongly significant association with the disease ($p=2.75x10^{-15}$). However, at present, it is not understood how SLC44A2 and the allelic variation at amino acid 154 play a role in VTE pathophysiology. To further elucidate how SLC44A2 contributes to VTE pathogenesis, we formulated the following research questions: 1. What is the role of SLC44A2 during haemostasis? 2. How do autoantibodies targeting SLC44A2 affect haemostasis? 3. How does SLC44A2 contribute to VTE pathophysiology? 4. Do SLC44A2 targeting antibodies exacerbate the effects of VTE? 5. Are the contributions of SLC44A2 to VTE cell specific? Finding the answer to these questions will help us achieve our main objective which is to determine how SLC44A2 modulates the interactions between the blood and venous vessel wall, and thereby the initiation and/or propagation of venous thrombotic disease.

The current proposal is the result of a unique collaboration between the [redacted], LUMC, The Netherlands and the [redacted], Giessen, Germany. Thus, expertise from both institutes will join at the pre-clinical and basic science level to clarify the function of SLC44A2 and the relevance of SLC44A2 and of SLC44A2 antibodies in VTE. Recently, mice with a conditional floxed *Slc44a2* allele (with loxP sites flanking *Slc44a2* exon 3-10) were generated by a research group at [redacted] at the University of Michigan in Ann Arbor, Michigan, USA. We have established a collaboration with this group, and in March 2016 we received embryos of these conditional *Slc44a2* mice that will be used for the present project. Cryorecovery of the embryos was performed in April 2016 and the first offspring carrying the conditional allele became available in Leiden as of May 2016. As all parties involved have extensive experience with the experimental disease models included in this proposal, the principles of replacement, reduction and refinement will be wholly implemented in an effort to prevent as many negative consequences for the involved animals as possible.

Upon completion of this project, we expect to have a coherent answer to all of the above mentioned research questions and thus a much better understanding of the role of SLC44A2 not only in normal haemostasis, but in the pathological condition of VTE as well. With this knowledge, we can then begin to translate our observations into those from a clinical perspective with an aim to design better therapeutics

with less detrimental side effects for patients and ultimately improve the quality of life for those living with the symptoms associated with VTE.

### 3.3 Relevance

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

SLC44A2 is a novel susceptibility gene for VTE, however, very little is known about the protein and thus, at this juncture, it does not belong to any conventional pathways for VTE or have a role in haemostasis pathways. Therefore, the proposed objectives create an opportunity to elucidate the function of SLC44A2 in the context of physiological and pathological haemostasis. In regards to the impact this may have on the field as a whole, these studies may discover previously unknown mechanisms involved in the pathogenesis of VTE as well as establish a unique biomarker for the disease. Moreover, these findings may further prove significant in the clinical setting as targeting SLC44A2 might provide a more effective treatment strategy in VTE, as it is not linked to traditional haemostasis and would reduce the chance of adverse side effects such as bleeding, which accompanies all of the current therapeutics available.

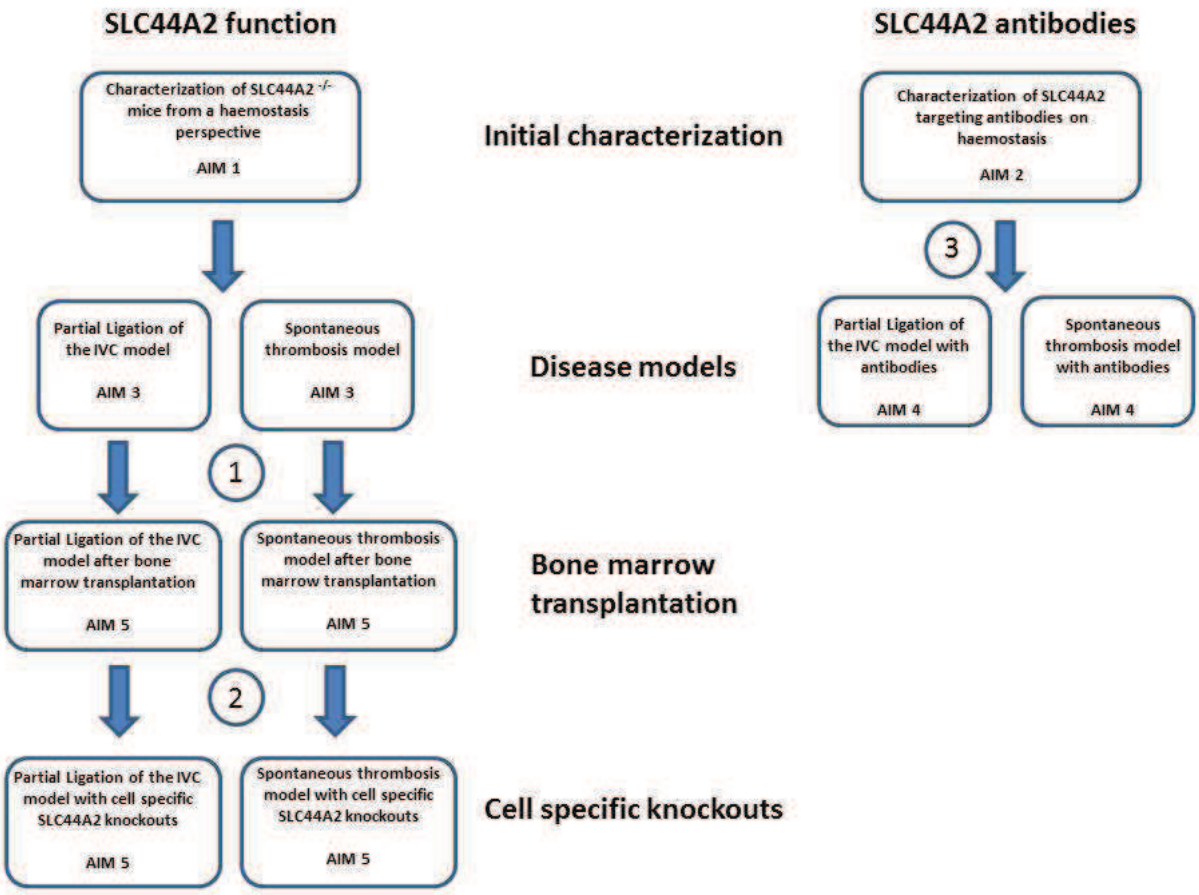
### 3.4 Research strategy

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

Haemostasis and subsequently VTE, are complex processes which involve various cell types and cell products from different biological systems including endothelial cells (circulatory), neutrophils (immune) and the production of coagulation factors by hepatocytes (digestive). There is also the added element of continuous blood flow, a physical factor that is difficult to replicate precisely *in vitro*, in addition to the possible changes in the immune profile. Therefore, it is only possible to truly define key interactions central to these processes using a model organism. In order to investigate the role of SLC44A2 in the pathogenesis of VTE we will make use of the recently generated *Slc44a2* exon 3-10 deficient knock out mice which allow for conditional deletions using the Cre-Lox recombination system. Our collaborator demonstrated that the *EIIa* Cre-mediated germ line inactivation of *Slc44a2* results in mice globally lacking *Slc44a2* (full knockouts) that appear viable and healthy with no spontaneous discomfort other than hearing loss upon a hearing challenge. This phenotype is only observed in the FVB genetic background and not the C57Black/6J background which we intend to use.

Using the mice in this manner we can then achieve the following aims:
1. Establish the role of SLC44A2 (if any) during traditional haemostasis
2. Determine if autoantibodies targeting SLC44A2 affect traditional haemostasis
3. Detect mechanisms which connect SLC44A2 to VTE pathophysiology
4. Test if SLC44A2 targeting antibodies exacerbate the effects of VTE
5. Dissect out the cell specific (i.e. endothelial cells or neutrophils) contributions of SLC44A2 to VTE

The following aims are summarized in the design overview below:




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.

In order to determine the importance of SLC44A2 function during normal haemostasis we will begin by characterizing the global *Slc44a2* knockout mice from a haemostasis perspective, focusing on the organs that are central to this process such as blood, liver and immune cells (AIM 1). It is at this point we will also determine whether or not it is necessary to include both male and female mice in the subsequent studies, as the response is well described to be bi-distributional and therefore the two genders cannot be combined. As obvious differences may not be detected in these mice under normal homeostatic conditions, regardless of the findings from this characterization, upon completion, we will move forward to the disease models (AIM 3). In this respect, we will employ two different models of venous thrombosis (VT), namely the partial ligation model and the spontaneous induced thrombosis model. With these two systems, we will cover all relevant aspects which are crucial to the induction of VT and overcome the limitations that each model presents such as retrograde thrombus formation in the ligation model and the lack of influence by neutrophils in the spontaneous model. The results from AIM 3 will then determine our first go/no go moment (arrow 1). If the disease models point to a clear effect of SLC44A2 in VT pathophysiology, we will then proceed to first part of AIM 5, which is the use of bone marrow transplantation in order to identify the cellular compartment expressing SLC44A2 that is contributing to the induction of VT, i.e. the stromal or hematopoietic compartment. The outcomes from these studies will then comprise our second go/no go moment (arrow 2). If the loss of SLC44A2 in either the stromal or hematopoietic compartment does not elicit differences in the propagation of VT, then we will conclude that ubiquitous expression of SLC44A2 is important in VT pathogenesis and no further study will be needed. However, if the findings point to a certain compartment as being the main contributor in the disease, then we will continue to pinpoint the specific cell type that is central to this process. To achieve this final objective of AIM 5, we will generate either endothelial cell (stromal) or neutrophil (hematopoietic) specific SLC44A2 knockout mice and then induce disease using the models described in

AIM 3.

In parallel to the above studies, we will also explore the contributions of SLC44A2 targeting antibodies in haemostasis and possibly VT. In order to do this, we must first find a working concentration of SLC44A2 antibody that can be tolerated by the mice without being cleared from the blood within a 72 hour time frame, specifically the duration of the disease model. In addition to titrating the amount of antibody that is suitable for later models, we will also determine the effect that these molecules have on haemostasis, These are the two main goals of AIM 2. The outcome of these studies will then form a go/no go moment for this portion of the project (arrow 3). If we can find an amount of SLC44A2 antibody that circulates freely within in the mouse without causing an acute reaction, we will proceed to use disease models in combination with autoantibodies (AIM4). However, if the mice have a severe response to the antibodies (which we do not expect) or if the antibodies do not remain in circulation long enough, then we will conclude this half of the project.

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.

Altogether the different components of this project culminate to form an explanation as to how SLC44A2 contributes to VTE. The initial study proposed in aim 1 will determine if SLC44A2 is involved in traditional haemostasis pathways through characterization of the *Slc44a2* global knockout mouse from a haemostasis perspective. We will evaluate whether the loss of SLC44A2 has any effect on the production of coagulation factors and subsequently blood clotting, which is the source of clinically manifested events associated with VTE. As the effects of the loss of SLC44A2 may not be evident under normal homeostatic conditions and only upon vascular injury, regardless of the outcome of this aim, we will proceed to the disease models described in aims 3-5 Additionally, in aim 2, we will also investigate the effect that SLC44A2 targeting antibodies have on haemostasis as these corresponding antibodies have already been implicated in vascular disease such as transfusion related acute lung injury (TRALI). Together these findings will form the first milestone of the project as such a detailed examination has yet to be described and is crucial before studying further aspects of SLC44A2 involvement in VTE. These results may also help us to hone our focus on certain mechanisms that may be amplified at later stages in the disease models included in aims 3-5. Moreover, the initial trials studying SLC44A2 targeting antibodies are of particular importance as these will establish the appropriate concentration of antibodies as well as administration schedule that will be used in aim 4. Upon completion of this initial characterization, we will move forward with aim 3 and determine the importance of SLC44A2 in the propagation of VTE using two independent disease models of thrombosis, namely the partial ligation and spontaneous model. It is vital to utilize both models of VTE as each has its disadvantages such as extensive surgical handling and retrograde thrombi formation in the ligation model and no role for neutrophils in the spontaneous model. Thus, using these systems in combination is essential and will ensure that we fully cover all aspects that are key to VTE pathophysiology. These data will then mark the second milestone of the project as it will become clear as to whether SLC44A2 is a central contributor in the induction of thrombosis. If mice lacking SLC44A2 are protected or exhibit a less severe thrombotic phenotype, we will conclude that this is directly linked to the loss of SLC44A2. However, if we do not observe such an effect, we will conclude that SLC44A2 does not play a role in VTE and will not proceed with experiments associated with aim 5. In addition, as detailed in aim 4, in order to distinguish the importance of SLC44A2 targeting antibodies in VTE pathogenesis we will include such antibodies, as defined by aim 2, in addition to the two thrombosis models and measure the additive effect that antibodies may have on vessel injury. The outcome from these studies will constitute another major milestone of the project as it would establish whether autoantibodies against SLC44A2 in particular can exacerbate the symptoms associated with VTE. This has yet to be described and may have strong implications for the clinical setting. Lastly, when the evidence from aim 3 supports further study of SLC44A2, we will continue to dissect out its importance by defining the cellular players that are contributing to disease induction, as described in aim 5. We will first utilize bone marrow isolated from WT and *Slc44a2* global knockout mice which is then transferred back to either WT or mice lacking *Slc44a* before the challenge of thrombosis. In this way we can begin to identify the cellular compartments (stromal or hematopoietic) that support the propagation of VTE as it relates to SLC44A2. Concurrently, we will also breed *Slc44a2* floxed mice with *Tie-2* Cre and *LysM* Cre in order to generate cell specific knockout mice, lacking SLC44A2 in either endothelial cells or neutrophils, respectively. Thrombosis will then be promoted in these mice and based on these results, we will be able to identify which of these cell types is driving the pathogenesis of VTE. This will be the final milestone of

the project as not only will we have determined the importance of SLC44A2 in VTE, but identified the principal cell types that are essential in SLC44A2 associated VTE pathogenesis.

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 | Haemostasis characterization of *Slc44a2* knockout mice |
| 2 | Effect of SLC44A2 targeting antibodies on haemostasis |
| 3 | Venous thrombosis through partial ligation of the caval vein |
| 4 | Spontaneous venous thrombosis through RNAi |
| 5 | |
| 6 | |
| 7 | |
| 8 | |
| 9 | |
| 10 | |

# Format
## Niet-technische samenvatting

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

## 1 Algemene gegevens

| | | |
|---|---|---|
| 1.1 | Titel van het project | De Rol van de Erfelijke Factor 'Solute Carrier Transporter 44A2' (SLC44A2) in de Ontwikkeling van de Ziekte Veneuze Trombose |
| 1.2 | Looptijd van het project | 5 jaar |
| 1.3 | Trefwoorden (maximaal 5) | SLC44A2; Veneuze Trombose |

## 2 Categorie van het project

2.1 In welke categorie valt het project.

*U kunt meerdere mogelijkheden kiezen.*

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

## 3 Projectbeschrijving

3.1 Beschrijf de doelstellingen van het project (bv de wetenschappelijke vraagstelling of het wetenschappelijk en/of maatschappelijke belang)

Erfelijke factoren bepalen voor een belangrijk deel wie veneuze trombose (een ziekte waarbij bloedstolsels in de aderen kunnen ontstaan) krijgt en wie niet, maar de erfelijke achtergrond van de ziekte is nog lang niet helemaal duidelijk. Onlangs ontdekten we dat het gen SLC44A2 (Solute Carrier Transporter 44A2) een rol zou kunnen spelen en we willen nu onderzoeken of dat klopt en wat die rol is. Het gen is actief in de witte afweercellen in ons bloed en in de cellen die onze bloedvaten bekleden.
Waar de andere bekende erfelijke factoren voor veneuze trombose ingrijpen op de cascade van reacties die tot

bloedstolling leidt, lijkt SLC44A2 op een andere manier van invloed te zijn. Kennis over de rol van dit gen kan dan ook nieuwe inzichten geven in het ontstaan van veneuze trombose en leiden tot nieuwe behandelingen. Bestaande medicijnen remmen de stollingscascade en verhogen daarmee het risico op spontane bloedingen.

Veneuze trombose raakt het leven van miljoenen mensen over de hele wereld. De ziekte kan fatale gevolgen hebben, maar ook de kwaliteit van leven van patiënten verminderen, onder meer doordat zij langdurig, soms levenslang, medicijnen moeten gebruiken.

3.2 Welke opbrengsten worden van dit project verwacht en hoe dragen deze bij aan het wetenschappelijke en/of maatschappelijke belang?

We hopen te ontrafelen hoe de nieuwe erfelijke factor bijdraagt aan de ontwikkeling van veneuze trombose, daardoor beter te begrijpen hoe de ziekte ontstaat en aanknopingspunten te vinden voor betere en veiliger behandelingsstrategieën.

Dit project heeft twee hoofdvragen:
1) Heeft SLC44A2 invloed op de normale functies van bloed en bloedvaatwand?
2) Wat is de rol van SLC44A2 in de ontwikkeling van veneuze trombose?
We zullen nagaan:

- of het ontbreken van SLC44A2 veranderingen in normale functies van bloed en de bloedvaatwand veroorzaakt;
- of andere factoren in bloed en de bloedvaatwand een interactie aangaan met SLC44A2;
- waarom en wanneer SLC44A2 belangrijk is in de ontwikkeling van veneuze trombose;
- in welke cellen SLC44A2 belangrijk is: de witte bloedcellen of cellen van de bloedvaatwand.

Onverwacht ontstond een goede mogelijkheid om dit onderzoek te doen: onlangs is in de Verenigde Staten een muis ontwikkeld waarin SLC44A2 ontbreekt, de zogenaamde SLC44A2 knock-out muis. Door deze muis te vergelijken met een normale muis kunnen we ontdekken of en hoe SLC44A2 betrokken is bij de ontwikkeling van veneuze trombose.

3.3 Welke diersoorten en geschatte aantallen zullen worden gebruikt?

We zullen maximaal 3770 muizen gebruiken.

3.4 Wat zijn bij dit project de verwachte negatieve gevolgen voor het welzijn van de proefdieren?

We zullen bij muizen veneuze trombose opwekken op twee manieren. Ten eerste vertragen we de bloedstroom in een bloedvat door een bloedvat tijdens een kleine operatie deels af te sluiten met een hechtdraad om dit vat; trombose treedt dan ter plekke op binnen twee dagen. Ten tweede injecteren we stoffen die productie van enkele stollingsfactoren remmen; ook

dan treedt trombose op binnen twee dagen. Om ongemak voor de muizen te voorkomen zullen we deze handelingen onder verdoving uitvoeren en pijnstilling geven.
Als zich trombose ontwikkelt zullen we de dieren doden, zodat we alle veranderingen die optreden in het lichaam kunnen onderzoeken. We volgen hierbij zeer strikte richtlijnen die beschrijven hoe euthanasie op de meest humane manier toegepast dient te worden.

3.5 Hoe worden de dierproeven in het project ingedeeld naar de verwachte ernst?

Voor de eerste hoofdvraag verzamelen we bloed en weefsels. We verwachten een gering ongemak voor de dieren vanwege injecties met een narcosemiddel voordat we bloed afnemen en voordat we ze, aan het eind van de proef, doden voor onderzoek.
Voor de tweede hoofdvraag wekken we bovendien trombose op. Hiervan verwachten wij een matig ongemak voor de dieren. De handelingen omvatten een kleine operatie in de buik of injecties met remmers van stollingsfactoren. Na injecties kunnen als gevolg van de trombose die ontstaat zwellingen bij de kop optreden. We zijn bekend met, en voorbereid op, deze symptomen. Om pijn te voorkomen behandelen we de muizen met geschikte pijnstillers. Om het ongemak door de zwellingen zo klein mogelijk te houden zullen we het gemakkelijker voor ze maken om te eten en te drinken.

3.6 Wat is de bestemming van de dieren na afloop?

Na alle experimenten analyseren we weefsels, bloed en eventuele stolsels. Daartoe wordt op alle dieren euthanasie toegepast.

## **4** Drie V's

4.1 **Vervanging**
Geef aan waarom het gebruik van dieren nodig is voor de beschreven doelstelling en waarom proefdiervrije alternatieven niet gebruikt kunnen worden.

Veneuze trombose is een zeer complexe ziekte waarbij onder meer bloedplasma, bloedcellen, bloedsomloop, immuun-systeem en lever betrokken zijn. Het proces van bloedstolling is niet buiten het lichaam na te bootsen, dus experimenten met intacte dieren zijn onvermijdelijk.

4.2 **Vermindering**
Leg uit hoe kan worden verzekerd dat een zo gering mogelijk aantal dieren wordt gebruikt.

Met statistische berekeningen hebben we bepaald hoeveel dieren nodig zijn om relevante effecten inderdaad te vinden als ze er zijn. Voor deze berekeningen hebben we gegevens gebruikt van andere onderzoekers uit recente internationale wetenschappelijke literatuur en eigen gegevens uit eerdere studies.

| | | |
|---|---|---|
| 4.3 | **Verfijning**<br>Verklaar de keuze voor de diersoort(en). Verklaar waarom de gekozen diermodel(len) de meest verfijnde zijn, gelet op de doelstellingen van het project. | Muizen zijn vergelijkbaar met de mens qua genetica, bloed, bloedsomloop, immuunsysteem en orgaansystemen. Daarnaast zijn muizen beschikbaar waar SLC44A2 in ontbreekt. Voor ons onderzoek zijn muizen dus de meest geschikte proefdieren. |
| | Vermeld welke algemene maatregelen genomen worden om de negatieve (schadelijke) gevolgen voor het welzijn van de proefdieren zo beperkt mogelijk te houden. | Een aantal muizen krijgt alleen een éénmalige onderhuidse injectie met narcosemiddel. Bloed wordt dan onder narcose afgenomen en hierna worden de muizen op humane wijze gedood voor analyse.<br><br>De experimenten waarbij we trombose opwekken kunnen tot ongerief leiden. Binnen de protocollen hebben we zorgvuldig momenten gezocht waar pijnstilling nodig is. Bovendien worden de muizen meerdere malen per dag gecontroleerd zodat we eventuele onverwachte veranderingen kunnen, registeren en zo nodig direct ingrijpen, bijvoorbeeld met voortijdige euthanasie als al pijnstilling toegepast is. |

## **5** In te vullen door de CCD

| | |
|---|---|
| Publicatie datum | |
| Beoordeling achteraf | |
| Andere opmerkingen | |

# 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. | [redacted] |

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 | Type of animal procedure |
|---|---|
| 3.4.4.1 | Haemostasis characterization of *Slc44a2* knockout mice |

## 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.

Blood and tissue samples from several organs central to the process of haemostasis will be collected from wild type and *Slc44a2* deficient animals, including 1) global knockout 2) cell specific knockout (endothelial or neutrophil) or 3) bone marrow transplanted animals which creates mice lacking SLC44A2 in either the stromal or hematopoietic compartment in addition to mice containing or lacking SLC44A2 in both compartments. This may include, but is not limited, to material such as blood, liver, lungs and venous tissue. Subsequently, these samples can be used to phenotype the *Slc44a2* knockout mouse (either global or cell/compartment specific) from a haemostasis perspective by measuring differences in the capacity of the blood to coagulate and related cells to produce coagulation factors, in addition to measuring changes in the immunological profile. In this way we can begin to answer how SLC44A2 affects both normal and pathological haemostasis. As it is difficult to isolate such material without causing injury to the animals, a total isolation of all tissues upon anesthetization, followed by euthanization, is the most humane procedure.

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

While under general anaesthesia, the abdomen of the mice will be opened and blood will be collected directly from the vena cava into a syringe containing sodium citrate so as to preserve the sample. Blood must be collected directly from the vein in order to avoid contamination with factors that activate coagulation (i.e. tissue factor) as it will be used for testing exactly that at a later time. The amount of blood retrieved will be the maximal amount possible, therefore following collection, the diaphragm of the

mouse will be punctured, euthanizing the mouse in a humane manner. At this point, several tissues will be collected and snap frozen. These can then be used to determine if *Slc44a2* mice exhibit any differences in haemostatic activity both at the mRNA and protein level.

For the mice undergoing bone marrow transplantation, donor mice will be euthanized using standard techniques and bone marrow cells isolated and prepared for injection into the recipient mice. Bone marrow transplant recipients will begin receiving acidified, antibiotic water one week prior to the irradiation procedure. At least six hours before receiving the transplantation, they will be sub lethally irradiated at a dose suitable for their background (C57BL/6 mice). Following radiation, mice will receive a tail vein injection of the donor mouse bone marrow cells and subsequently housed for 21 days until the repopulation of hematopoietic cells is complete.

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

Group size: For the calculation of determining how many animals are necessary per group to establish any meaningful differences, we use the parameter of thrombin generation as it has the largest standard deviation as compared to other readouts of haemostasis, such as gene expression by qPCR. After reviewing the most recent literature, we have determined that a reasonable mean for group 1, the wild type (WT) group in a C57BL/6 background, for endogenous thrombin potential (ETP) is approximately 220 nM*min and for group 2, a knockout mouse that is reasonably healthy as compared to its WT littermates, a value of 290 nM*min. We estimate the heterozygous mice to fall somewhere in between and therefore we use the mean value of 255 nM*min and an average standard deviation of 45. Because the readout will be comparing three groups, we will use a one way ANOVA test which will control for the type I error. Using the G*Power statistical software (http://www.gpower.hhu.de/) program to perform a POWER calculation with an alpha of 0.05 and a power of 0.80, we find the effect size f to be 0.63505 which would require a sample size of 30, signifying 10 animals per group.

Sex consideration: As it is unknown whether sex will influence the effect of the *SLC44A2* gene and its role in haemostasis, we will include both for this initial characterization study. If no significant differences are presented between genders, then we will continue only with females for the remainder of the project as there is generally less biological variation such as size/weight between age matched mice. Our rationale for starting with both sexes is due to the well described findings that hormones can play a role in haemostasis (i.e. contraception & thrombotic events), but because no differences in gender were described in the initial GWAS study linking *SLC44A2* to venous thrombosis, we do not expect sex to play a role. However, until we can rule this out we find it prudent to include both groups. In addition, as several studies report sex differences in biological response to murine models of VT (Alvarado et al Thromb Res 2011; Vanlangenvelde et al Comp Med 2005), we cannot justifiably group males and females together, as such bi-distributional readouts may obscure true differences which may be due to the gene alone.

**B. The animals**

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

The animals used in these studies are *Mus musculus,* from a C57BL/6J background, containing either a deleted region (exon 3-10) of the *Slc44a2* gene (KO) or a wild type (WT) allele. They originate from the lab of [redacted] at the University of Michigan but have been breed at the LUMC for many generations. In addition, final studies may also employ mice lacking the *Slc44a2* gene only in specific cell types. These will be generated in two distinct ways. First, we will use bone marrow transplants from WT or *Slc44a2* deficient mice to either WT or *Slc44a2* KO mice to create mice lacking SLC44A2 expression in either the stromal or hematopoietic compartments. Alternatively, we will also cross breed *Slc44a2* floxed mice, containing loxP sites on either side of exons 3-10, with mice expressing Cre-recombinase under the control of either the *Tie-2* or *LysM* promoter, resulting in mice lacking SLC44A2 in endothelial cells or neutrophils, respectively. To control for genetic variation as much as possible, we will make use of matched litter mates, using WT, HET and KO siblings, generated from parents who are heterozygous for the KO allele. The mice will be included between ages of 8-14 weeks, which is the same age that will be used in the experimental disease models described in appendices 3.4.4.3-4. We estimate that the characterization of these knockout animals from a haemostatic perspective will involve approximately 180-290 animals, based on the calculations below:

General characterization of global knockout animals:
3 genotypes (KO, HET, WT) x 10 (group size) x 2 sex (males and females) = approximately 60 animals

General characterization of cell specific knockout animals:
3 genotypes (KO, HET, WT) x 10 (group size) x 1 sex (female) = approximately 30 animals
If sex differences are found in the general characterization of global knockouts then we will also use approximately 30 males.

Training and set up of bone marrow transplantation procedure:
5 WT and 5 KO animals

General characterization of bone marrow transplant animals:
4 systems (WT, WT hemo/KO stromal, KO hemo/WT stromal, KO) x 2 transfer (donor, recipient) x 10 (group size) x 1 sex (female) = approximately 80 animals
If sex differences are found in the general characterization of global knockouts then we will also use approximately 80 males.

**C. Re-use**

Will the animals be re-used?

X 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'?

X 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:
Due to the complex nature of haemostasis, which involves several different biological systems including the circulatory, immune and digestive system, in addition to the physical interaction between flowing blood and the vessel wall, it cannot be accurately replicated *in vitro*. Thus an *in vivo* system, which includes interactions between all such factors, is essential in studying this process. In addition, the ability to target deletion in cellular compartments through bone marrow transplantation and cell specific knockout animals and continue to study the interaction between the different biological systems is a very unique tool that cannot be readily reproduced. However, once we have conducted the studies contained in this proposal, it is likely that we will be able to pinpoint the cellular players and signalling pathways that are central to this process, specifically in relation to SLC44A2, and we can then follow up using *in vitro* models with human tissues to confirm and therefore strengthen our conclusions.

Reduction:
In order to reduce the number of animals necessary, we have taken several steps and carefully considered all options. First, we are making use of littermates so as to reduce the chance of genetic variability. Second, we will also save most organs and materials from the mice and not only the tissues that are classically involved in haemostasis. In this way, if the data points to a novel finding and further analysis is needed in secondary organs, we will already have them and the need to include more animals may not be necessary. Lastly, based on power calculations and experience with these types of procedures, we have determined the optimal amount of animals to include in these studies so that a significant difference between experimental conditions can be found (if one exists) and no repetition likely will be needed. We have also contemplated whether another experimental design can be used in order to minimize the requested numbers or the overall distress to the animals. However, as this is a characterization of the novel genetic *Slc44a2* mouse line from a haemostasis perspective, it is not

possible if we do not collect blood and organs related to haemostasis.

Refinement:
Several practices are put in place in order to minimize any pain or discomfort for the animals during the proposed studies. Anaesthesia will be administered prior to the collection of materials, such that no pain will be felt during these procedures and for mice receiving bone marrow transplantation, anaesthesia will be administered prior to the irradiation of recipient mice . These studies will also be conducted by trained researchers with experience performing these specific techniques. We will closely monitor the reactions of the animals at all times and if we observe any discomfort associated with humane endpoints such as changes in behaviour (posture, grooming, activity) or pathophysical reactions (rapid breath, unusual heart rate, weight loss) we will end the experiment immediately. In addition, as the irradiation may weaken the animals, we will provide special housing conditions including longer nipples on the water bottles, which will contain antibiotics, as well as soft food to ease accessibility for eating and drinking.

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

The measures taken to minimize animal suffering, pain and fear in regards to the proposed studies include pre-treatment of the animals with the appropriate anaesthesia before irradiation or the collecting any blood or tissues to be used in the characterization of these knockout animals. There are no adverse effects on the environment resulting from these studies.

## 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.

Not applicable.

## 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

X 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?

X 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.

x Yes > Will anaesthesia, analgesia or other pain relieving methods be used?

☐ No > Justify why pain relieving methods will not be used.

x Yes > Indicate what relieving methods will be used and specify what measures will be taken to ensure that optimal procedures are used.

All experimental animals will be anaesthetised prior to the collection of blood or tissues which will subsequently be used in the characterization of the global *Slc44a2* knockout mice. In regards to the characterization of animals after bone marrow transplantation, animals will be anaesthetised prior to the irradiation procedure and the collection of blood or tissues which will subsequently be used for characterization of the mice. These procedures are deemed optimal based on experience with such techniques within the department.

**I. Other aspects compromising the welfare of the animals**

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

A possible adverse effect that may result from this procedure is the sensation of pain and fear if the administration of the anaesthesia is somehow faulty before the start of the procedure. However, such an event would be very difficult to mistake, as symptoms of an effective administration are readily visible. Since these procedures will be performed by skilled researchers, with years of experience with mouse models, we do not expect that such a mistake would go unobserved and all mice will be determined to be thoroughly sedated before the onset of any technique. Adverse effects that may arise due to the bone marrow transplant procedure are either graft versus host disease or alternatively, host versus graft disease in which T-cells become reactive and attack the tissues of the host or graft, respectively. In addition, the effects of irradiation may also result in anaemia, immunosuppression, diarrhea and damage to the mucosal membranes.

Explain why these effects may emerge.

Such an effect may occur if the compounds that make up the anaesthetic regimen were improperly administered. In regards to adverse effects associated with bone marrow transplantation, this occurs when the MHC molecules are different between the host and donor mice.

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

In order to prevent incomplete anaesthetization of mice before the start of procedure, we will have experienced handlers present who can identify when the mouse is safely ready for blood collection. In order to minimize the chance of rejection of transplantation, we are using litter mates both as donor and recipient in mice that are backcrossed to C57BL/6 background for at least 6 generations. We expect the genetic variability to be very limited between such animals which will reduce the chance of mismatched MHC expression on the tissues. Animals will also be placed in special housing conditions (see section D, refinement) 3 weeks post irradiation in preparation for the adverse effects associated with the procedure.

**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.

X Yes > Describe the criteria that will be used to identify the humane endpoints.

Criteria for humane endpoints during this part of the study include rapid breathing during the laparotomy of the mouse which may indicate an abnormal response to the anaesthesia. If this occurs, we will not proceed with puncture of the vena cava and euthanize the mouse immediately as opposed to after blood collection. Criteria for humane endpoints for bone marrow transplantation include behavioural changes in posture, grooming or activity levels in addition to pathophysical reactions such as rapid breathing, weight loss greater than 15% or HBV activation following radiation.

Indicate the likely incidence.

We estimate a very low incidence (<5%) for general characterization and approximately 10% for bone marrow transplantations.

**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 procedures for the general characterization of the knockout animals fall under the category of "non-recovery" as the animals will be euthanized after the procedure, which is performed entirely under general anaesthesia. With respect to bone marrow transplantation, donor animals fall under the category of "non-recovery." Recipient animals may experience mild (90%) to moderate (10%) discomfort. Therefore, the mice will be given the appropriate anaesthesia and analgesia to minimize this as much as possible.

## End of experiment

**L. Method of killing**

Will the animals be killed during or after the procedures?

☐ No

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

Animals are killed and used amongst others for full pathological analysis

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

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

x 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. | [redacted] |
| 1.3 | List the serial number and type of animal procedure.<br><br>*Use the serial numbers provided in Section 3.4.4 of the Project Proposal form.* | Serial number: 3.4.4.2<br>Type of animal procedure: Effect of SLC44A2 antibodies on haemostasis |

## 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.

Blood and tissue samples from several organs central to the process of haemostasis will be collected from animals injected with SLC44A2 targeting antibodies, originally isolated from human donors. This may include, but is not limited, to material such as blood, liver, lungs and venous tissue. Subsequently, these samples can be used to determine the effect that SLC44A2 antibodies have on haemostasis. Additionally, these studies will help to determine the appropriate amount of antibodies needed to study their contributions to venous thrombosis, as described in appendices 3.4.4.3-4. Using such procedures we will be able to measure differences in the capacity of the blood to coagulate and related cells to produce coagulation factors, in addition to measuring changes in the immunological profile. In this way we can begin to answer how SLC44A2 targeting antibodies affect both normal and pathological haemostasis.

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

Mice will receive an infusion of varying concentrations of anti-SLC44A2 antibodies purified from human serum, in volumes set forth by the directive Diehl et al 2001, to determine a sufficient amount which allows for the presence of antibodies 72 hours post administration, without causing an acute response in the mouse. In order to minimize unwanted side effects, we start with the lowest concentration first and then raise it incrementally until the antibodies can be detected back after 72 hours. We will also compare the efficacy of using two time points versus one, namely at either 72 hours or both 72 and 24 hours before collection for tissue analysis. The goal of this study is twofold: 1) to determine the effect of anti-SLC44A2 antibodies on haemostasis as well as 2) determine the amounts of antibody necessary to

have adequate coverage present at the time of thrombosis induction (appendices 3.4.4.3-4). We base these time points and the initial amounts that will be injected on established studies of a similar nature used to study the role of anti-phospholipid antibodies in venous thrombosis (Pierangeli et al. Thromb. Haemost. 1995).

Following 72 hours of the initial administration, the mice will receive general anaesthesia and the abdomen will be opened and blood will be collected directly from the vena cava into a syringe containing sodium citrate so as to preserve the sample. Blood must be collected directly from the vein in order to avoid contamination with factors that activate coagulation (i.e. tissue factor) as it will be used for testing exactly that at a later time. The amount of blood retrieved will be the maximal amount possible, therefore following collection, the diaphragm of the mouse will be punctured, euthanizing the mouse in a humane manner. After this point, several tissues will be collected and snap frozen. These can then be used to determine if mice exhibit any differences in haemostatic activity both at the mRNA and protein level.

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

Group size: To determine the number of animals necessary per group we based our POWER calculation on previous work carried out to characterize mice treated with autoantibodies in the context of venous thrombosis. To this end, we use the recorded values for tissue factor (TF) activity in which mice treated with a control IgG antibody had a mean value of TF activity of 149.0 pM/mg/ml and mice treated with an autoantibody a mean value of 402.5 pM/mg/ml with a SD of 165.2. Using POWER calculation (http://biomath.info/power/ttest.htm) with an alpha of 0.05 and a power 0.80 we determined the appropriate group size to be 8.

Sex consideration: As it is unknown whether sex will influence the effect of the *SLC44A2* targeting antibodies and their role in haemostasis, we will include both for this initial characterization study. If no significant differences are presented between genders then we will continue only with females for the remainder of the project involving antibodies as there is generally less biological variation such as size/weight between age matched mice. Our rationale for starting with both sexes is due to the well described findings that hormones can play a role in haemostasis (i.e. contraception & thrombotic events), but because no differences in gender were described in the initial GWAS study linking *SLC44A2* to venous thrombosis, we do not expect sex to play a role. However, until we can rule this out we find it prudent to include both groups. In addition, as several studies report sex differences in biological response to murine models of VT (Alvarado et al Thromb Res 2011; Vanlangenvelde et al Comp Med 2005), we cannot justifiably group males and females together, as such bi-distributional readouts may obscure true differences which may be due to the antibodies alone.

**B. The animals**

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

The animals used in these studies are *Mus musculus,* from a C57BL/6J background, containing either a deleted region (exon 3-10) of the *Slc44a2* gene on both alleles (KO), one allele (HET) or both wild type alleles (WT). They originate from the lab of [redacted] at the University of Michigan but have been breed at the LUMC for many generations. To control for genetic variation as much as possible, we will make use of matched litter mates, using both WT, HET and KO siblings, generated from parents who are heterozygous for the KO allele. The mice will be included between ages of 8-14 weeks, which is the same age that will be used in the experimental disease models described in appendices 3.4.4.3-4. We estimate that the characterization of these knockout animals from a haemostatic perspective will involve approximately 64-384 animals, based on the calculation below:

Antibody titration:
1 genotypes (WT~~, HET, KO~~) x 8 (group size) x 1 sex (female) x 2 injection timepoints (72, 72 & 24hr) x 4 concentrations = 64

If we can find a working dosage, then we will continue with multiple genotypes for characterization:
3 genotypes (WT, HET, KO) x 8 (group size) x 2 sex (male,female) x 2 injection timepoints (72, 72 & 24hr) x 4 concentrations = 384

**C. Re-use**

Will the animals be re-used?

X 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'?

X 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:
Due to the complex nature of haemostasis, which involves several different biological systems including the circulatory, immune and digestive system, as well as the physical interaction between flowing blood and the vessel wall, it cannot be accurately replicated *in vitro*. In addition, it is still unclear which cell type can be targeted by these SLC44A2 antibodies. Thus an *in vivo* system, which includes interactions between all such factors, is essential in studying this process. However, once we have conducted the studies contained in this proposal, it is likely that we will be able to pinpoint the cellular players and signalling pathways that are central to this process, specifically in relation to SLC44A2 antibodies, and we can then follow up using *in vitro* models with human tissues to confirm and therefore strengthen our conclusions.

Reduction:
In order to reduce the number of animals necessary, we have taken several steps and carefully considered all options. First, we are making use of a pilot study to determine the proper dosing of antibodies before going further in using mice with variable genotypes. Second, we will also save most organs and materials from the mice and not only the tissues that are classically involved in haemostasis. In this way, if the data points to a novel finding and further analysis is needed in secondary organs, we will already have them and the need to include more animals may not be necessary. Lastly, based on power calculations, we have determined the optimal amount of animals to include in these studies so that a significant difference between experimental conditions can be found (if one exists) and no repetition likely will be needed. We have also contemplated whether another experimental design can be used in order to minimize the requested numbers or the overall distress to the animals. However, the models available to study the effect of antibodies in haemostasis and thrombosis are few, and thus we have chosen the most established system in order to cover the aspects that may be involved in these processes.

Refinement:
Several practices are put in place in order to minimize any pain or discomfort for the animals during the proposed studies. To start, we will begin by testing the lower concentrations of antibodies first in order to minimize the possible discomfort. We will also follow the guidelines in regards to injections set forth by Diehl et al 2001. Anaesthesia will also be administered prior to the final collection of tissues, such so that no pain will be felt during these procedures. These studies will also be conducted by trained researchers with experience performing these specific techniques. As there may be some adverse reactions to these treatments, we will closely monitor the response of the animals at several time points and if we observe any discomfort associated with humane endpoints such as changes in behaviour (posture, grooming, activity) or pathophysical reactions (rapid breath, unusual heart rate, weight loss) we will end the experiment immediately.

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

The measures taken to minimize animal suffering, pain and fear in regards to the proposed studies include pre-treatment of the animals with the appropriate anaesthesia before collecting any blood or tissues to be used in the characterization of these animals. There are no adverse effects on the

environment resulting from these studies.

## 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.

Not applicable.

## 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

X 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?

X 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.

x Yes > Will anaesthesia, analgesia or other pain relieving methods be used?

☐ No > Justify why pain relieving methods will not be used.

x Yes > Indicate what relieving methods will be used and specify what measures will be taken to ensure that optimal procedures are used.

All experimental animals will be anaesthetised prior to the collection of blood or tissues which will subsequently be used for characterization of the mice. The procedures are deemed optimal based on several publications in which a similar approach was used in addition to in house experience with these techniques .

**I. Other aspects compromising the welfare of the animals**

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

There is a chance that the animals may respond negatively to the infusion of SLC44A2 targeting antibodies by having an acute immune response, for example, the activation of neutrophils which may cause vascular damage.

Explain why these effects may emerge.

These effects may emerge if SLC44A2 targeting antibodies have a strong pathological influence on the general haemostasis of mice (unknown).

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

We will start by using the lowest doses of antibody until a sufficient amount in the blood is reached and maintained for 72 hours. We will observe the animals every 2 hours over the first 12 hours and then every 12 hours until 72 hours post injection in order to detect any acute response to the antibodies that results in a distressful effect to the mice (humane endpoints), at which point, we will end the experiments immediately, as this is not the goal of this study. In an effort to avoid any unwanted discomfort for the animals, we will be using titers that have been established in literature and have not been reported to cause severe distress to mice.

**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.

X Yes > Describe the criteria that will be used to identify the humane endpoints.

Criteria for humane endpoints during this part of the study include behavioural changes in posture, grooming or activity levels in addition to pathophysical reactions such as rapid breathing or weight loss greater than 15% as determined by weighing the mice before the start of the experiment and following up at 24 and 48 hours post injection.

Indicate the likely incidence.

We estimate a very low incidence (<5%).

**K. Classification of severity of procedures**

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

Depending on the response of the animals to SLC44A2 antibodies, levels of discomfort may vary, however we estimate the severity to range from mild (90%) to moderate (10%). Because we cannot say for sure, we will monitor the animals closely every 2 hours over the first 12 hours and then every 12 hours until 72 hours post injection to ensure the minimal amount of distress as determined by the humane endpoints described above.

## End of experiment

**L. Method of killing**

Will the animals be killed during or after the procedures?

☐ No

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

Animals are killed and used amongst others for full pathological analysis

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

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

x 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. | [redacted] |

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 | Type of animal procedure |
|---|---|
| 3.4.4.3 | Venous thrombosis through partial ligation of the caval vein |

## 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.

Mice lacking SLC44A2 expression, either whole body, in certain cellular compartments (through bone marrow transplantation), cell type specific (endothelial cells or neutrophils) or in mice that have been transfused with SLC44A2 targeting antibodies, will undergo a partial ligation procedure in order to mimic the process of venous thrombosis (VT) *in vivo* and to dissect out the cell types and signalling pathways which link SLC44A2 to disease pathogenesis. Blood and tissue samples from several organs central to the process of VT will be collected at different time points in order to record changes during key stages of disease progression. This may include, but is not limited to, material such as blood, liver, lungs, thrombus and venous tissue. Subsequently, these samples can be used to determine the contributions of SLC44A2 to VT. Using such procedures we will be able to measure differences in the capacity of the blood to coagulate and related cells to produce coagulation factors, changes in the immunological profile as well as characterize changes in thrombus formation/clearance and fibrin deposition.

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

Following anaesthetization of the mice and the administration of analgesia, a midline laparotomy will be made and the small bowel exteriorized and placed to the left of the animal. The inferior vena cava (IVC or caval vein) is then exposed by careful blunt dissection while sterile saline is applied at regular intervals to the exteriorized bowel to prevent its desiccation. Non-reactive prolene suture is looped around the IVC immediately caudal to the origin of the renal veins, with a space holder included on the outside of the vessel. Next the ligature is closed and the space holder is removed to avoid complete vessel occlusion.

Side branches will not be ligated or manipulated and IVC blood flow will be reduced immediately after the restriction. Lastly, the median laparotomy is sutured and the mice will be placed on a warming mat until anaesthesia wears off. Mice will additionally receive peri-operative analgesia until collection at at the 12, 24, 36 and 48hr designated time points . For the collection of blood and tissues, mice will receive a lethal dose of anaesthesia and subsequently several tissues will be collected, including the vena cava with the newly formed thrombus, and snap frozen. These can then be used to detect differences in expression levels of both mRNA and protein, as well as histological analysis of thrombus formation and fibrin deposition. The mice receiving anti-SLC44A2 antibodies will undergo all procedures described above in addition to receiving an infusion of antibodies prior to the surgery (appendix 3.4.4.2).
For the studies involving bone marrow transplantation, donor mice will be euthanized using standard techniques and bone marrow cells isolated and prepared for injection into the recipient mice. Bone marrow transplant recipients will begin receiving acidified, antibiotic water one week prior to the irradiation procedure. At least six hours before receiving the transplantation, they will be sub lethally irradiated at a dose suitable for their background (C57BL/6 mice). Following radiation, mice will receive a tail vein injection of the donor mouse bone marrow cells and subsequently housed for 21 days until the repopulation of hematopoietic cells is complete. At this point, the mice will then be ready to include in the ligation model described above.

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

Group size: The calculation of the experimental group size is based on the parameter 'thrombosis formation' (and not mRNA analysis) as this is the primary outcome and the parameter with the largest standard deviation. Previously, at 24 hours post thrombosis induction, we observed effects on thrombus size and weight ranging from 75% decrease up to 200% increase in size/weight as compared to control reference groups. Using these observations we can estimate that the control group has an average thrombi weight of 1g and a second group representing the knockout mice would have less thrombi, around .25g and a third group may have increased thrombi, up to double the weight of group 1, being 2g. As the readout will be comparing three groups, we will use a one way ANOVA test which will control for the type I error. Using the G*Power statistical software (http://www.gpower.hhu.de/) program to perform a POWER calculation with an alpha of 0.05 and a power of 0.80, we find the effect size f to be 0.55143 which would require a sample size of 36, signifying 12 animals per group. Because we want to rule out endothelial injury as a trigger for venous thrombosis, mice with bleedings or any injury of the IVC during surgery need to be excluded from further analysis. We therefore include an extra of 2 animals per group to compensate for animals displaying bleedings or any injury during the IVC during surgery. These 2 extra animals will only be included when necessary. For now we will use a maximum of 14 animals per experimental group.

Sex consideration: Unless we determine a clear link between the *Slc44a2* gene and sex in the initial characterization described in appendices 3.4.4.1-2, we will only use females for the following procedures. We opt for female over male since there is less biological variability in measures such as size and weight. Female mice can also be housed together even though they are not littermates (males cannot) which also helps to minimize any differences that may arise due to housing conditions. In addition, as several studies report sex differences in biological response to murine models of VT (Alvarado et al Thromb Res 2011; Vanlangenvelde et al Comp Med 2005), we cannot justifiably group males and females together, as such bi-distributional readouts may obscure true differences which may be due to the gene or antibodies alone.

**B. The animals**

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

The initial studies will include *Mus musculus* from a C57BL/6J background, containing either a deleted region (exon 3-10) of the *Slc44a2* gene on both alleles (KO), one allele (HET) or both wild type alleles (WT), globally in all tissues of the animal. They originate from the lab of [redacted] at the University of Michigan but have been breed at the LUMC for many generations. In addition, the final studies will employ mice lacking the *Slc44a2* gene only in specific cell types. They will be generated by cross breeding *Slc44a2* floxed mice, containing loxP sites on either side of exons 3-10, with mice expressing Cre-recombinase under the control of either the *Tie-2* or *LysM* promoter, resulting in mice lacking SLC44A2 in endothelial cells or neutrophils, respectively. To control for genetic variation as much as

possible, we will make use of matched litter mate siblings, generated from parents who are heterozygous for the KO allele. The mice will be included between ages of 8-14 weeks. We estimate that the maximal number of animals needed to complete all aspects of the studies included in this proposal regarding IVC ligation to be approximately 1008. If the response of mice in appendices 3.4.4.1-2 is determined to be dependent on sex (unlikely), then we will also include 1008 males.

Training and set up of procedure:
10 WT animals

IVC ligation-induced thrombosis with global knockout animals:
3 genotypes (WT, HET, KO) x 14 group size x 1 sex (female) x 4 timepoints (12, 24, 36, 48hr) = 168 animals

IVC ligation-induced thrombosis with anti-SLC44A2 antibodies:
3 genotypes (WT, HET, KO) x 14 group size x 1 sex (female) x 4 timepoints (12, 24, 36, 48hr) = 168 animals

IVC ligation-induced thrombosis with cell specific knockout animals:
2 genotypes (WT or KO) x 2 cell type (endothelial or neutrophil) x 14 group size x 1 sex (female) x 4 timepoints (12, 24, 36, 48hr) = 224 animals

IVC ligation-induced thrombosis with bone marrow transplantation :
4 systems (WT, WT hemo/KO stromal, KO hemo/WT stromal, KO) x 2 (donor, recipient) x 14 group size x 1 sex (female) x 4 timepoints (12, 24, 36, 48hr) = 448 animals

In the case that we receive a request from reviewers to repeat the IVC ligation procedure with a minor addition such as a therapeutic compound, neutralizing antibody or additional stimuli etc., we estimate that we would need approximately 168 additional animals, based on the calculations above. These animals will be used only if specifically requested by an editor/reviewer in order to increase the chances of a successful publication which would ensure the dissemination of these data within the scientific community.

**C. Re-use**

Will the animals be re-used?

X 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'?

X 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: Due to the complex nature of VT, which involves several different biological systems including the circulatory, immune and digestive system, as well as the physical interaction between flowing blood and the vessel wall, it cannot be accurately replicated *in vitro*. Thus an *in vivo* system, which includes interactions between all such factors shortly after the induction of injury and thrombi formation, is essential in studying this process. In addition, the ability to isolate the actual thrombi for analysis will provide critical clues as to the mechanism driving pathogenesis. However, once we have conducted the studies contained in this proposal, it is likely that we will be able to pinpoint the cellular players and signalling pathways that are central to this process, specifically in relation to SLC44A2, and we can then follow up using *in vitro* models with human tissues to confirm and therefore strengthen our conclusions.

Reduction:
In order to reduce the number of animals necessary, we have taken several steps and carefully considered all options. First, we are making use of littermates so as to reduce the chance of genetic variability. Second, we will save most organs and materials from the mice and not only the tissues that are classically involved in haemostasis. In this way, if the data points to a novel finding and further analysis is needed in secondary organs, we will already have them and the need to include more animals may not be necessary. Lastly, based on power calculations and experience with these types of procedures, we have determined the optimal amount of animals to include in these studies so that a significant difference between experimental conditions can be found (if one exists) and no repetition will likely be needed. Additionally, we plan to only use females so as to reduce the number of mice included. We have also contemplated as to whether another experimental design can be used in order to minimize the requested numbers or the overall distress to the animals. However, the model proposed in this study is one of the best available in the field to study venous thrombosis and when combined with the secondary model of spontaneous induced thrombosis (appendix 3.4.4.4), we believe it will cover all aspects that may be key in VT pathogenesis.

Refinement:
Several practices are put in place in order to minimize any pain or discomfort for the animals during the proposed studies. Anaesthesia will be administered prior to the surgery as well as before the final collection of tissues, so that no pain will be felt during these procedures and for mice receiving bone marrow transplantation, anaesthesia will be administered prior to the irradiation of recipient mice. In addition, to minimize any post-operative discomfort, analgesia will also be given peri-operatively and up until the time of collection. These studies will also be conducted by trained researchers with experience performing these specific techniques. As there may be complications related to the surgery, we will closely monitor the response of the animals at several time points and if we observe any discomfort associated with humane endpoints such as changes in behaviour (posture, grooming, activity) or pathophysical reactions (rapid breath, unusual heart rate, weight loss) we will end the experiment immediately. In addition, as the irradiation may weaken the animals, we will provide special housing conditions including longer nipples on the water bottles, which will contain antibiotics, as well as soft food to ease accessibility for eating and drinking.

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

The measures taken to minimize animal suffering, pain and fear in regards to the proposed studies include pre-treatment of the animals with the appropriate anaesthesia before surgery or collection of tissues in addition to peri-operative treatment with analgesia. There are no adverse effects on the environment resulting from these studies.

## 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.

Not applicable.

## 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

X 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?

X 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.

x Yes > Will anaesthesia, analgesia or other pain relieving methods be used?

☐ No > Justify why pain relieving methods will not be used.

x Yes > Indicate what relieving methods will be used and specify what measures will be taken to ensure that optimal procedures are used.

All experimental animals will be anaesthetised prior to surgery, irradiation or the collection of blood or tissues which will subsequently be used for characterization of the mice. In addition, mice will receive analgesia peri-operatively up until the time of sacrifice. The procedures are deemed optimal based on in house experience with these techniques.

**I. Other aspects compromising the welfare of the animals**

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

Possible adverse effects that may occur during these procedures include bleeding during the IVC surgery or infections after the surgery. Adverse effects that may arise due to the bone marrow transplant procedure are either graft versus host disease or alternatively, host versus graft disease in which T-cells become reactive and attack the tissues of the host or graft, respectively. In addition, the effects of irradiation may also result in anaemia, immunosuppression, diarrhea and damage to the mucosal membranes.

Explain why these effects may emerge.

These effects may emerge if there is an accidental puncture of vasculature tissue or the surgical environment is not completely aseptic. In regards to adverse effects associated with bone marrow transplantation, this occurs when the MHC molecules are different between the host and donor mice.

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

In order to minimize surgical errors during, we have included a number of animals in order to refine our techniques before the final studies begin. To minimize the chance of infection, we plan to follow established protocols to ensure the surgery is aseptic as possible. In order to minimize the chance of rejection of transplantation, we are using litter mates both as donor and recipient in mice that are backcrossed to C57BL/6 background several generations. We expect the genetic variability to be very limited between such animals which will reduce the chance of mismatched MHC expression on the tissues. Animals will also be placed in special housing conditions (see section D, refinement) 3 weeks post irradiation in preparation for the adverse effects associated with the procedure.

**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.

X Yes > Describe the criteria that will be used to identify the humane endpoints.

Criteria for humane endpoints during this part of the study include behavioural changes in posture, grooming or activity levels in addition to pathophysical reactions such as rapid breathing, weight loss greater than 15% or HBV activation following radiation.

Indicate the likely incidence.

We estimate the incidence to be approximately 15% for IVC ligation alone and approximately 10% for bone marrow transplantations.

**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 levels of discomfort are moderate due to the inclusion of a surgery which involves suturing the abdomen of the mice. In addition, the possibility for a thrombosis formation may also cause discomfort, especially if there is a rapid onset, and is considered to cause moderate discomfort. With respect to bone marrow transplantation, donor animals fall under the category of "non-recovery." Recipient animals will undergo surgery and will therefore experience moderate discomfort. The mice will be given the appropriate anaesthesia and analgesia to minimize this as much as possible.

## End of experiment

**L. Method of killing**

Will the animals be killed during or after the procedures?

☐ No

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

Animals are killed and used amongst others for full pathological analysis

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

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

x 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. | [redacted] |
| 1.3 | List the serial number and type of animal procedure.<br><br>*Use the serial numbers provided in Section 3.4.4 of the Project Proposal form.* | Serial number: 3.4.4.4<br>Type of animal procedure: Spontaneous venous thrombosis through siRNA |

## 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.

Mice lacking SLC44A2 expression, either whole body, in certain cellular compartments (through bone marrow transplantation) or cell type specific (endothelial cells or neutrophils), in addition to mice that have been transfused with SLC44A2 targeting antibodies, will receive an injection of siRNA targeting pro-coagulation proteins (antithrombin and protein C), which will induce formation of spontaneous venous thrombosis (VT). This will help to further determine the role of SLC44A2 in the pathogenesis of VT and overcome the limitations of the partial ligation model (appendix 3.4.4.3), namely the retrograde formation of thrombi and overestimation of the involvement of the immune system, as it is a major surgery. Blood and tissue samples from several organs central to the process of VT will be collected at different time points in order to record changes during key stages of disease progression. This may include, but is not limited to, material such as blood, liver, lungs, thrombus and venous tissue. Subsequently, these samples can be used to determine the contributions of SLC44A2 to VT. Using such procedures we will be able to measure differences in the capacity of the blood to coagulate and related cells to produce coagulation factors, changes in the immunological profile as well as characterize changes in thrombus formation/clearance and fibrin deposition in the liver.

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

The mice will receive an injection of solution containing siRNA lipidoids (based on the volumes set forth by the directive Diehl et al 2001) into the tail vein. After 36 hours, the mice will begin to receive analgesia and will be monitored regularly for signs of discomfort up until the time of sacrifice. Collection of the mice will occur at 24, 48 and 72 hours post injection. These time points are chosen in order to

observe the stages of VT propagation such as onset/initiation and progression. At these stages, mice are anesthetized and a midline laparotomy will be made. For the collection of blood, it will be taken directly from the vena cava into a syringe containing sodium citrate so as to preserve the sample. Blood must be collected directly from the vein in order to avoid contamination with factors that activate coagulation (i.e. tissue factor) as it will be used for testing exactly that at a later time. The amount of blood retrieved will be the maximal amount possible, therefore following collection, the diaphragm of the mouse will be punctured, euthanizing the mouse in a humane manner. After this point, several tissues will be collected and snap frozen, including the head which will contain the newly formed thrombus. These can then be used to detect differences both at the mRNA and protein level in addition to histological analysis which can be used to characterize changes in the thrombi as well as fibrin deposition. For the studies that involve antibody treatment, the mice will undergo the relevant procedures (detailed in appendices 3.4.4.2 ~~or 3.4.4.5~~) prior to the injection of siRNA.
For the studies involving bone marrow transplantation, donor mice will be euthanized using standard techniques and bone marrow cells isolated and prepared for injection into the recipient mice. Bone marrow transplant recipients will begin receiving acidified, antibiotic water one week prior to the irradiation procedure. At least six hours before receiving the transplantation, they will be sub lethally irradiated at a dose suitable for their background (C57BL/6 mice). Following radiation, mice will receive a tail vein injection of the donor mouse bone marrow cells and subsequently housed for 21 days until the repopulation of hematopoietic cells is complete. At this point, the mice will then be ready to include in the spontaneous siRNA model described above.

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

Group size: For the calculation of experimental group size we will use the parameter 'development of clinical phenotype' (and not a parameters like liver fibrin deposition), as this is the typical hallmark of the spontaneous thrombosis model and almost always coincides with the presence of thrombi in the large veins of the heads. Previously, 48 hours after siRNA injection, we observed effects of onset and incidence of the clinical phenotype ranging from 1. nearly full absence of phenotype as compared to siRNA injected control animals) to 2. accelerated onset for nearly all animals of a given condition as compared to siRNA injected control animals (factor XII inhibition, presence of Factor V Leiden). Typical group size in these experiments was 10 animals per group. Using Fisher's exact based POWER calculations, statistically significant changes are detected when in one group 8 out of 10 animals follow the black and white response which is the display of visible symptoms of the spontaneous thrombotic phenotype, while in the reference group 8 out of 10 do not respond and remain fully normal (resulting in P=0.023 (2-tail)). http://www.langsrud.com/stat/fisher.htm. Subsequently, these numbers (n=10) also allow to detect statistically significant changes in liver fibrin deposition (using non-parametric Mann Witney tests) and allow to describe biologically relevant changes in thrombus incidence (Fisher's exact tests), severity, structure and cellular composition.

Sex consideration: Unless we determine a clear link between the *Slc44a2* gene and sex in the initial characterization described in appendices 3.4.4.1-2, we will only use females for the following procedures. We opt for female over male since there is less biological variability in measures such as size and weight. This is particularly relevant for this model as we will inject the same amount of siRNA per mice so they must be as closely weight matched as possible. Female mice can also be housed together even though they are not littermates (males cannot) which also helps to minimize any differences that may arise due to housing conditions. In addition, as several studies report sex differences in biological response to murine models of VT (Alvarado et al Thromb Res 2011; Vanlangenvelde et al Comp Med 2005), we cannot justifiably group males and females together, as such bi-distributional readouts may obscure true differences which may be due to the gene or antibodies alone.

**B. The animals**

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

The initial studies will include *Mus musculus* from a C57BL/6J background, containing either a deleted region (exon 3-10) of the *Slc44a2* gene on both alleles (KO), one allele (HET) or both wild type alleles (WT), globally in all tissues of the animal. They originate from the lab of [redacted] at the University of Michigan but have been breed at the LUMC for many generations. In addition, the final studies will employ mice lacking the *Slc44a2* gene only in specific cell types. They will be generated by cross breeding *Slc44a2* floxed mice, containing loxP sites on either side of exons 3-10, with mice expressing Cre-recombinase under the control of either the *Tie-2* or *LysM* promoter, resulting in mice lacking

SLC44A2 in endothelial cells or neutrophils, respectively. To control for genetic variation as much as possible, we will make use of matched litter mate siblings, generated from parents who are heterozygous for the KO allele. The mice will be included between ages of 8-14 weeks. We estimate that the maximal number of animals needed to complete all aspects of the studies included in this proposal regarding siRNA spontaneous induced thrombosis to be approximately 540. If the response of mice in appendices 3.4.4.1-2 is determined to be dependent on sex (unlikely), then we will also include 540 males.

Training and set up of procedure:
10 WT animals

siRNA spontaneous induction of thrombosis in global knockout animals:
3 genotypes (WT, HET, KO) x 10 group size x 1 sex (female) x 3 timepoints (24, 48, 72hr) = 90 animals

siRNA spontaneous induction of thrombosis with anti-SLC44A2 antibodies:
3 genotypes (WT, HET, KO) x 10 group size x 1 sex (female) x 3 timepoints (24, 48, 72hr) = 90 animals

siRNA spontaneous induction of thrombosis in cell specific knockout animals:
2 genotypes (WT or KO) x 2 cell type (endothelial or neutrophil) x 10 group size x 1 sex (female) x 3 timepoints (24, 48, 72hr) = 120 animals

Spontaneous model of thrombosis:
4 systems (WT, WT hemo/KO stromal, KO hemo/WT stromal, KO) x 2 (donor, recipient) x 10 group size x 1 sex (female) x 3 timepoints (24, 48, 72hr) = 240 animals

In the case that we receive a request from reviewers to repeat the siRNA spontaneous induction of thrombosis procedure with a minor addition such as a therapeutic compound, neutralizing antibody or additional stimuli etc., we estimate that we would need approximately 90 additional animals, based on the calculations above. These animals will be used only if specifically requested by an editor/reviewer in order to increase the chances of a successful publication which would ensure the dissemination of these data within the scientific community.

**C. Re-use**

Will the animals be re-used?

X 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'?

X 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: Due to the complex nature of VT, which involves several different biological systems including the circulatory, immune and digestive system, as well as the physical interaction between flowing blood and the vessel wall, it cannot be accurately replicated *in vitro*. Thus an *in vivo* system, which includes interactions between all such factors, especially with spontaneous formation of thrombi, is essential in studying this process. In addition, the ability to isolate the actual thrombi for analysis will provide critical clues as to the mechanism driving pathogenesis. However, once we have conducted the studies contained in this proposal, it is likely that we will be able to pinpoint the cellular players and signalling pathways that are central to this process, specifically in relation to SLC44A2, and we can then follow up using *in vitro* models with human tissues to confirm and therefore strengthen our conclusions.

Reduction:
In order to reduce the number of animals necessary, we have taken several steps and carefully

considered all options. First, we are making use of littermates so as to reduce the chance of genetic variability. Second, we will save most organs and materials from the mice and not only the tissues that are classically involved in haemostasis. In this way, if the data points to a novel finding and further analysis is needed in secondary organs, we will already have them and the need to include more animals may not be necessary. Lastly, based on power calculations and experience with these types of procedures, we have determined the optimal amount of animals to include in these studies so that a significant difference between experimental conditions can be found (if one exists) and no repetition will likely be needed. Additionally, we plan to only use females so as to reduce the number of mice included. We have also contemplated as to whether another experimental design can be used in order to minimize the requested numbers or the overall distress to the animals. However, we believe that the two models proposed in this study (also appendix 3.4.4.3), used in combination, are the best strategy to research venous thrombosis and cover all aspects that may be key in VT pathogenesis.

Refinement:
Several practices are put in place in order to minimize any pain or discomfort for the animals during the proposed studies. Anaesthesia will be administered prior to the final collection of tissues, so that no pain will be felt during these procedures and for mice receiving bone marrow transplantation, anaesthesia will be administered prior to the irradiation of recipient mice. We also follow the guidelines set forth in the directive Diehl et al 2001 when determining the appropriate injection volumes. In addition, to minimize any discomfort that may arise due to the induction of VT, analgesia will also be given 36 hours post-injection and up until the time of collection, we do not expect full thrombi formation to occur before 36 hours . These studies will also be conducted by trained researchers with experience performing these specific techniques. We also plan to closely monitor the response of the animals at several time points after the injection and if we observe any discomfort associated with humane endpoints such as changes in behaviour (posture, grooming, activity) or pathophysical reactions (rapid breath, unusual heart rate, weight loss) we will end the experiment immediately. In addition, as the irradiation may weaken the animals, we will provide special housing conditions including longer nipples on the water bottles, which will contain antibiotics, as well as soft food to ease accessibility for eating and drinking.

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

The measures taken to minimize animal suffering, pain and fear in regards to the proposed studies include follow up treatment with analgesia continuously until sacrifice in addition to pre-treatment of the animals with the appropriate anaesthesia before collection of tissues. There are no adverse effects on the environment resulting from these studies.

## 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.

Not applicable.

## 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

X 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?

X 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.

x Yes > Will anaesthesia, analgesia or other pain relieving methods be used?

☐ No > Justify why pain relieving methods will not be used.

x Yes > Indicate what relieving methods will be used and specify what measures will be taken to ensure that optimal procedures are used.

All experimental animals will be anaesthetised prior to irradiation and the collection of blood or tissues which will subsequently be used for characterization of the mice. Mice will also begin to receive analgesia 36 hours post-injection and continuously up until the point of sacrifice. These procedures are deemed optimal based on experience with such techniques within the department.

**I. Other aspects compromising the welfare of the animals**

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

As we do not fully know the extent to which SLC44A2 is contributing to VT, it is possible that it plays a role in anticoagulation, in which case, the onset of the thrombus formation in knockout mice may be accelerated. This would result in visible symptoms including exophthalmos, peri-ocular haemorrhages or edema in the mandibular region or possibly death prior to the planned time points. Adverse effects that may arise due to the bone marrow transplant procedure are either graft versus host disease or alternatively, host versus graft disease in which T-cells become reactive and attack the tissues of the host or graft, respectively. In addition, the effects of irradiation may also result in anaemia, immunosuppression, diarrhea and damage to the mucosal membranes.

Explain why these effects may emerge.

These effects emerge as anticoagulation proteins are lowered and may be accelerated in the case that SLC44A2 plays a role in VT. In regards to adverse effects associated with bone marrow transplantation, this occurs when the MHC molecules are different between the host and donor mice.

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

We are prepared for such an outcome and have planned to minimize the severity of such an event by administering analgesia after the injection of siRNAs in case of discomfort from bleeding, in addition to regular monitoring of the mice for any signs of severe discomfort associated with humane endpoints. In order to minimize the chance of rejection of transplantation, we are using litter mates both as donor and recipient in mice that are backcrossed to C57BL/6 background several generations. We expect the genetic variability to be very limited between such animals which will reduce the chance of mismatched MHC expression on the tissues. Animals will also be placed in special housing conditions (see section D, refinement) 3 weeks post irradiation in preparation for the adverse effects associated with the procedure.

**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.

X Yes > Describe the criteria that will be used to identify the humane endpoints.

Criteria for humane endpoints during this part of the study include behavioural changes in posture,

grooming or activity levels in addition to pathophysical reactions such as rapid breathing, weight loss greater than 15% compared to starting weight prior to injection, exophthalmos, peri-ocular haemorrhages or edema in the mandibular region indicating a possible veinous rupture in the head. Additionally for mice with bone marrow transplant, HBV activation following radiation.

Indicate the likely incidence.

The incidence can range anywhere from 20% to 80% depending on the importance of SLC44A2 in the propagation of thrombosis, which is currently unknown. In the case that SLC44A2 does not play a role in VT we expect to see approximately 80% incidence as this is generally what we expect to observe in the control group. Similarly, if it exacerbates the VT phenotype, we expect to observe similar occurrence (80%), only at an earlier time point which is why we include several observation points after injection. If the loss of SLC44A2 has a protective role then we expect to observe incidence of approximately 20%. At the onset of any sign of exophthalmos, peri-ocular haemorrhages or edema in the mandibular region, the animal will be euthanized immediately.

**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 siRNAs targeting anticoagulation proteins will result in the formation of large thrombi in large veins of the head of the mouse which may result in intra/periocular haemorrhages. Based on these known outcomes, the procedures are deemed to include moderate discomfort and will thus include the proper analgesia to prevent the discomfort as much as possible. With respect to bone marrow transplantation, donor animals fall under the category of "non-recovery." Recipient animals will undergo the siRNA model and will therefore experience moderate discomfort. The mice will be given the appropriate anaesthesia and analgesia to minimize this as much as possible.

## End of experiment

**L. Method of killing**

Will the animals be killed during or after the procedures?

☐ No

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

Animals are killed and used amongst others for full pathological analysis

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

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

x Yes

# DEC-advies

## A. Algemene gegevens over de procedure

1. Aanvraagnummer: AVD116002016607

2. Titel van het project: The Role of the Solute Carrier Transporter 44A2 (SLC44A2) Gene in the Pathophysiology of Venous Thromboembolism.

3. Titel van de NTS: De Rol van de Erfelijke Factor 'Solute Carrier Transporter 44A2' (SLC44A2) in de Ontwikkeling van de Ziekte Veneuze Trombose.

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: 21-02-2017
   - ✓ aanvraag compleet: 21-02-2017
   - ✓ in vergadering besproken: 02-03-2017 & 13-04-2017
   - ☐ anderszins behandeld
   - ✓ termijnonderbreking(en) van 09-03-2017 t/m 04-04-2017 en 18-04-2017 t/m 21-04-2017
   - ☐ besluit van CCD tot verlenging van de totale adviestermijn met maximaal 15 werkdagen
   - ✓ aanpassing aanvraag: 04-04-2017 en 21-04-2017
   - ✓ advies aan CCD: 25-04-2017

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: 09-03-2017
   - Strekking van de gestelde vragen:
     De DEC heeft bij de aanvrager aanvullende informatie ingewonnen met betrekking tot de gekozen strategie, de go/no-go momenten, het ongerief, de berekening van het aantal dieren, de keuze van het geslacht, de analgesie, de 3 V's en de humane eindpunten.
     Naar aanleiding van deze vragen is het projectvoorstel inclusief bijlages en de NTS door de aanvrager aangepast.
   - Datum: 13-04-2017
   - Strekking van de gestelde vragen:
     De DEC heeft vragen gesteld over de aantallen en het gebruik van persoonlijke namen.

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 hoofddoelstelling te behalen. Het is helder welke handelingen individuele dieren zullen ondergaan. Hierdoor is ook duidelijk welk ongerief individuele dieren zullen ondergaan. De aanvrager heeft zowel binnen de doelstellingen en bijlagen dierproeven als tussen de doelstellingen 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 is bepalen hoe SLC44A2 de interactie tussen bloed en de veneuze vaatwand moduleert en daarmee ook het ontstaan en/of de verspreiding van veneuze tromboze. Het uiteindelijke doel is het verkrijgen van nieuwe therapeutische inzichten voor de ontwikkeling van verbeterde VTE behandelingen. Het betreft hier een fundamenteel onderzoek. 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 meerdere vorderingen zijn gemaakt met de identificatie van genetische varianten die verband houden met VTE, verklaren ze in veel gevallen slechts een klein percentage van de VTE risico’s. Uit de aanvraag blijkt tevens dat de huidige therapeutische strategieën voor VTE gericht zijn op het antagoneren van coagulatiefactoren wat als bijwerking tot levensbedreigende bloedingen kan leiden. Daarom is er een dringende behoefte aan verbeterde en/of

nieuwe therapeutische strategieën voor patiënten met VTE. 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 analyseren van de functie van SLC44A2, inclusief de interacties met antilichamen, en het ontrafelen van de onderliggende mechanismes tussen SLC44A2 en veneuze trombo-embolie (VTE) zijn de proefdieren, de onderzoekers en de patiënt en diens naasten. 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 onderzoekers bevorderd worden: De onderzoekers zullen kennis verkrijgen. Ook zullen de carrièremogelijkheden van de onderzoekers verbeteren door publicaties. Waarden die voor patiënten bevorderd worden: De gezondheid van patiënten zal verbeterd worden. Hierdoor zal de kwaliteit van leven verbeterd worden van patiënten en hun naasten.

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. De onderzoeksgroep heeft veel expertise op het gebied van dierexperimenteel onderzoek en de beschreven diermodellen. In de afgelopen jaren zijn volgens vergelijkbare strategieën en aanpak belangrijke wetenschappelijke resultaten behaald.

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 de dieren als gevolg van de chirurgische ingreep en de vorming van trombose cumulatief maximaal matig ongerief zullen ervaren. De donormuizen vallen naar inziens van de DEC in de categorie non-recovery. 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 genetisch gemodificeerd zijn. De integriteit zal ook gedragsmatig worden aangetast. Gedurende het project worden de dieren namelijk beperkt in hun bewegingsvrijheid. Hierdoor zullen de dieren geen 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. 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. De onderzoeksgroep heeft jarenlange ervaring met dit soort experimenten. Bovendien beschikt de onderzoeksgroep over een team van biotechnici die de benodigde ervaring hebben met proefdieronderzoek. De stapsgewijze opbouw zorgt voor een minimaal gebruik van proefdieren. Zo wordt er eerst een pilotexperiment uitgevoerd om de juiste dosering van antilichamen te bepalen voordat wordt verder gegaan in muizen met verschillende genotypes. Door gebruik te maken van nestgenoten wordt de kans op genetische variatie verkleind. Tevens worden organen en weefsels bewaard zodat deze wanneer nodig gebruikt kunnen worden voor verdere analyse. 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 het gebruik van adequate anesthesie en analgesie waar nodig. Tevens zullen eerst de lage concentraties antilichaam getest worden om de kans op ongerief zo laag mogelijk te houden. Tot slot krijgen de bestraalde dieren antibiotica door het drinkwater in flessen met extra lange drinktuitjes en weekvoer op de bodem van de kooi. 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. De aanvrager zal in het project in eerste instantie gebruik maken van zowel mannelijke als vrouwelijke muizen. Indien uit de resultaten blijkt dat er geen significante verschillen zijn tussen de geslachten zullen alleen vrouwelijke muizen gebruikt worden voor de rest van het project. 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. Na het doden worden bloed- en weefselmonsters uit verschillende organen die centraal staan bij het proces van VT

op verschillende tijdstippen worden verzameld om veranderingen te kunnen registreren tijdens belangrijke fases van de ziekte. 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 het bepalen hoe SLC44A2 de interactie tussen bloed en de veneuze vaatwand moduleert en daarmee ook het ontstaan en/of de verspreiding van veneuze tromboze met als uiteindelijk doel het verkrijgen van nieuwe therapeutische inzichten voor de ontwikkeling van verbeterde VTE behandelingen, het ongerief dat de dieren wordt aangedaan?

2. Project gericht op het analyseren van de functie van SLC44A2, inclusief de interacties met antilichamen, en het ontrafelen van de onderliggende mechanismes tussen SLC44A2 en veneuze trombo-embolie (VTE).
   Waarden die voor proefdieren in het geding zijn: matig nadeel.
   Waarden die voor onderzoekers bevorderd worden: gering voordeel.
   Waarden die voor de doelgroep (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. VTE is de derde voornaamste oorzaak van sterfte als gevolg van hart- en vaatziekte wereldwijd en is verantwoordelijk voor ongeveer een half miljoen sterfgevallen in West-Europa elk jaar. Bovendien zijn de niet lethale gevolgen die in verband worden gebracht met VTE verantwoordelijk voor een duidelijke vermindering van de kwaliteit van leven van miljoenen mensen. Tevens hebben de huidige therapeutische strategieën voor VTE grote bloedingen als bijwerking. De DEC acht het daarom van essentieel belang dat er nieuwe behandelstrategieën ontwikkeld worden zonder deze negatieve bijwerkingen. 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 vergunningplichtig 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.

9.

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
AVD116002016607

**Bijlagen**
2

Datum 12 mei 2017
Betreft Ontvangstbevestiging aanvraag projectvergunning Dierproeven

Geachte mevrouw Leids Universitair Medisch Centrum,

Wij hebben uw aanvraag voor een projectvergunning dierproeven ontvangen op 11 mei 2017. Het gaat om uw project "The Role of the Solute Carrier Transporter 44A2 (SLC44A2) Gene in the Pathophysiology of Venous Thromboembolísm". Het aanvraagnummer dat wij aan deze aanvraag hebben toegekend is AVD116002016607. 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:**
12 mei 2017
**Aanvraagnummer:**
AVD116002016607

Met vriendelijke groet,

Centrale Commissie Dierproeven

Deze brief is automatisch aangemaakt en daarom niet ondertekend.

Bijlagen:
- Gegevens aanvraagformulier
- Factuur

**Datum:**
12 mei 2017
**Aanvraagnummer:**
AVD116002016607

## 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: | [redacted] |
| Functie: | Associate Professor |
| Afdeling: | [redacted] |
| Telefoonnummer: | [redacted] |
| E-mailadres: | [redacted] |

**Datum:**
12 mei 2017
**Aanvraagnummer:**
AVD116002016607

Gegevens plaatsvervangende verantwoordelijke onderzoeker

| | |
|---|---|
| Naam: | [redacted] |
| Functie: | Post Doctoral Researcher |
| Afdeling: | [redacted] |
| Telefoonnummer: | [redacted] |
| E-mailadres: | [redacted] |

**Over uw aanvraag**

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

**Over uw project**

| | |
|---|---|
| Geplande startdatum: | 1 juni 2017 |
| Geplande einddatum: | 1 juni 2022 |
| Titel project: | The Role of the Solute Carrier Transporter 44A2 (SLC44A2) Gene in the Pathophysiology of Venous Thromboembolísm |
| Titel niet-technische samenvatting: | De Rol van de Erfelijke Factor Solute Carrier Transporter 44A2' (SLC44A2) in de Ontwikkeling van de Ziekte Veneuze Trombose |
| Naam DEC: | DEC Leiden |
| Postadres DEC: | [redacted], [redacted] |
| E-mailadres DEC: | [redacted] |

**Betaalgegevens**

| | |
|---|---|
| De leges bedragen: | € 1.684,- |
| De leges voldoet u: | na ontvangst van de factuur |

**Checklist bijlagen**

| | |
|---|---|
| Verplichte bijlagen: | [x] Projectvoorstel<br>[x] Beschrijving Dierproeven<br>[x] Niet-technische samenvatting |
| Overige bijlagen: | [x] DEC-advies |

**Datum:**
12 mei 2017
**Aanvraagnummer:**
AVD116002016607

**Ondertekening**

| | |
|---|---|
| Naam: | [redacted] |
| Functie: | Gemandateerd vergunninghouder |
| Plaats: | Leiden |
| Datum: | 8 mei 2017 |

Centrale Commissie Dierproeven

> Retouradres Postbus 20401 2500 EK Den Haag

Academisch Ziekenhuis Leiden h.o.d.n. LUMC
Leids Universitair Medisch Centrum
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centralecommissiedierproeven.nl
0900 28 000 28 (10 ct/min)
info@zbo-ccd.nl

**Onze referentie**
Aanvraagnummer
AVD116002016607

**Bijlagen**
2

Datum 12 mei 2017
Betreft Factuur aanvraag projectvergunning Dierproeven

**Factuur**
Factuurdatum: 12 mei 2017
Vervaldatum: 11 juni 2017
Factuurnummer: 170607

| **Omschrijving** | **Bedrag** |
|---|---|
| Betaling leges projectvergunning dierproeven<br>Betreft aanvraag AVD116002016607 | € 1.684,00 |

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.

10.

[redacted]

**Van:** [redacted]
**Verzonden:** woensdag 31 mei 2017 12:07
**Aan:** [redacted]
**CC:** [redacted]
**Onderwerp:** AVD116002016607: Aanhouden beoordelen.

Geachte [redacted],

Op 11 mei 2017 hebben wij uw aanvraag voor een projectvergunning dierproeven ontvangen. Het gaat om uw project 'The Role of the Solute Carrier Transporter 44A2 (SLC44A2) Gene in the Pathophysiology of Venous Thromboembolísm' met aanvraagnummer AVD116002016607. Wij hebben nog een aantal vragen over uw aanvraag. In deze e-mail leest u wat wij nog nodig hebben en wanneer u een beslissing kunt verwachten.

-Uit uw aanvraag (bijlagen 3.4.4.1, 3.4.4.2 en 3.4.4.4) wordt niet duidelijk hoeveel procent van de dieren matig ongerief ondergaan. U wordt verzocht dit te specificeren. U wordt ook verzocht om aan te geven welke handelingen de donordieren zullen ondergaan (eerst doden en daarna het uitnemen van beenmerg?). Dit in verband met de controle op de inschatting van de ongeriefsclassificatie van deze dieren. In dat geval moet het ongerief namelijk als licht worden geclassificeerd.

-U geeft in uw aanvraag aan een deel van het onderzoek in 1 geslacht te willen uitvoeren. U geeft aan dat het te onderzoeken gen op zich niet onderhevig is aan geslachtsspecifieke verschillen, maar dat wel bekend is dat hormonen een rol spelen in haemostasis en dat er geslachtsspecifieke verschillen zijn beschreven voor muizenmodellen voor veneuze trombose. Dit zijn op zich valide redenen om het onderzoek met 1 geslacht te willen uitvoeren als het gebruik van beide geslachten zou leiden tot een significante toename van het benodigd aantal dieren. Om te zien of er inderdaad geslachtsspecifieke verschillen zijn, en deze verschillen te kunnen onderzoeken, zullen initiële proeven in bijlagen 3.4.4.1. en 3.4.4.2 met beide geslachten worden uitgevoerd. Indien blijkt dat die er niet zijn, kiest u er vervolgens voor het onderzoek met 1 geslacht voort te zetten. Dit is in tegenspraak met het beleid van de CCD dat beide geslachten gebruikt moeten worden indien er geen geslachtsspecifieke verschillen zijn. De door u gebruikte onderbouwing en gekozen strategie is niet geheel duidelijk en lijkt op punten tegenstrijdig. U wordt daarom verzocht uw strategie op dit punt beter toe te lichten. Mocht u uw strategie niet beter kunnen onderbouwen, kan de CCD een voorwaarde voor het evenredig gebruik van beide geslachten aan een eventuele vergunning verbinden.

-U wordt verzocht de NTS in overeenstemming te brengen met de aanvraag wat betreft het aantal dieren en de ongeriefsclassificatie. In de NTS gebruikt u de term 'onverwacht'. Wij willen u er op wijzen dat het gebruik van een dergelijke term tot vragen zou kunnen leiden bij het publiek over de noodzaak van het uitvoeren van het onderzoek. Wij adviseren u daarom die zin in de NTS aan te passen.

**Opsturen binnen veertien dagen**
De CCD zou uw aanvraag graag in de eerstvolgende vergadering willen bespreken. Wij zouden het daarom op prijs stellen als u de informatie uiterlijk vrijdagochtend 2 juni 2017 kunt aanleveren. Mocht u niet in de gelegenheid zijn zo snel te reageren, is dit geen probleem, u heeft veertien dagen de tijd om de ontbrekende informatie aan te leveren. U kunt dit aanleveren via NetFTP. Wij ontvangen ook graag de aangepaste formulieren.

**Wanneer een beslissing**
De behandeling van uw aanvraag wordt opgeschort tot het moment dat wij de aanvullende informatie hebben ontvangen. Als u goedkeuring krijgt op uw aanvraag, kunt u daarna beginnen met het project.

**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).

Met vriendelijke groet,

[redacted]

Namens

**Centrale Commissie Dierproeven www.centralecommissiedierproeven.nl**

.....

Postbus 20401 | 2500 EK | Den Haag
**T: 0900 2800028**
**E:** info@zbo-ccd.nl (Let op: nieuw e-mail adres)

11.

June 01, 2017

Dear CCD evaluators,

Thank you for your feedback on our project proposal AVD116002016607, entitled “The Role of the Solute Carrier Transporter 44A2 (SLC44A2) Gene in the Pathophysiology of Venous Thromboembolism.” Please find attached the revised documents with the requested changes, highlighted in red throughout the files, as well as a summary of the additions below.

Thank you again for your advice and we hope this adapted version is suitable for approval by the commission.

In response to :

*-Uit uw aanvraag (bijlagen 3.4.4.1, 3.4.4.2 en 3.4.4.4) wordt niet duidelijk hoeveel procent van de dieren matig ongerief ondergaan. U wordt verzocht dit te specificeren. U wordt ook verzocht om aan te geven welke handelingen de donordieren zullen ondergaan (eerst doden en daarna het uitnemen van beenmerg?). Dit in verband met de controle op de inschatting van de ongeriefsclassificatie van deze dieren. In dat geval moet het ongerief namelijk als licht worden geclassificeerd.*

In all appendices, we have further specified the percentage of animals that will fall under each type of pain classification and included it within section K. With regards to the animals acting as bone marrow donors, we have now included how they will be euthanized (carbon dioxide asphyxiation) and stated that this will be done before the collection of the appropriate tissues (femur, tibia and humerus bones). These changes can be found in sections A, H and K. We consider the severity classification for these donor animals to be “non-recovery” since they will be euthanized before the isolation of bone marrow and will never recover consciousness, which is in line with the definition of “non-recovery” included in Appendix VIII of Directive 2010/63/EU.

*-U geeft in uw aanvraag aan een deel van het onderzoek in 1 geslacht te willen uitvoeren. U geeft aan dat het te onderzoeken gen op zich niet onderhevig is aan geslachtsspecifieke verschillen, maar dat wel bekend is dat hormonen een rol spelen in haemostasis en dat er geslachtsspecifieke verschillen zijn beschreven voor muizenmodellen voor veneuze trombose. Dit zijn op zich valide redenen om het onderzoek met 1 geslacht te willen uitvoeren als het gebruik van beide geslachten zou leiden tot een significante toename van het benodigd aantal dieren. Om te zien of er inderdaad geslachtsspecifieke verschillen zijn, en deze verschillen te kunnen onderzoeken, zullen initiële proeven in bijlagen 3.4.4.1. en 3.4.4.2 met beide geslachten worden*

*uitgevoerd. Indien blijkt dat die er niet zijn, kiest u er vervolgens voor het onderzoek met 1 geslacht voort te zetten. Dit is in tegenspraak met het beleid van de CCD dat beide geslachten gebruikt moeten worden indien er geen geslachtsspecifieke verschillen zijn. De door u gebruikte onderbouwing en gekozen strategie is niet geheel duidelijk en lijkt op punten tegenstrijdig. U wordt daarom verzocht uw strategie op dit punt beter toe te lichten.*

*Mocht u uw strategie niet beter kunnen onderbouwen, kan de CCD een voorwaarde voor het evenredig gebruik van beide geslachten aan een eventuele vergunning verbinden.*

We agree that this point can be further clarified. The use of both sexes during the initial characterization (appendix 3.4.4.1 and 3.4.4.2) is to rule out any gender specific interactions of SLC44A2 in regards to normal hemostasis. As stated previously, we do not expect there to be a link between SLC44A2 and gender. However, even if there is no association between SLC44A2 and gender, this does not mean we can pool together male and female animals in the models of venous thrombosis since it has been well established, both in our hands as well as by various other researchers in the field, that there is a clear bi-distributional sex response to thrombosis in murine models. A few examples of readouts that are significantly different between males and females are thrombus size, cytokine expression and blood cell counts (Alvarado et al Thromb Res 2011; Vanlangenvelde et al Comp Med 2005). If we pool the sexes, then we may miss the effect of the gene itself due to the large variation between mixed animals, which would imply that we would need to further increase the number of mice to find significant differences. We believe this to be counterproductive to the aim of *Reduction* and therefore, if there is no interaction between SLC44A2 and gender in hemostasis, we propose to continue with only one sex so as to decrease the number of animals needed for experimentation while at the same time ensuring that we can draw solid conclusions from the animals that are included. Additional text on this point has been added to all appendices.

*-U wordt verzocht de NTS in overeenstemming te brengen met de aanvraag wat betreft het aantal dieren en de ongeriefsclassificatie. In de NTS gebruikt u de term 'onverwacht'. Wij willen u er op wijzen dat het gebruik van een dergelijke term tot vragen zou kunnen leiden bij het publiek over de noodzaak van het uitvoeren van het onderzoek. Wij adviseren u daarom die zin in de NTS aan te passen.*

Here we have revised section 3.5 to include both the description of animals receiving bone marrow transplantation as well as the percentage of animals in each category of severity classification. Additionally, we've adjusted the number of animals to be used in section 3.3 to more accurately reflect the range that is possible in this proposal. Lastly, the word 'onverwacht' has been removed from sections 3.2 and 4.3.

# 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. | [redacted] |
| 1.3 | Provide the title of the project. | The Role of the Solute Carrier Transporter 44A2 (SLC44A2) Gene in the Pathophysiology of Venous Thromboembolism |

## 2 Categories

2.1 Please tick each of the following boxes that applies to your project.

X 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.

Venous thromboembolism (VTE) is the third leading cause of cardiovascular mortality worldwide and is responsible for approximately half a million associated deaths in Western Europe each year (Cohen et al

*Thromb Haemost* 2007). In addition, adverse events linked to VTE, i.e. deep vein thrombosis (DVT) and pulmonary embolism (PE), are major contributing factors to the growing number of years lived with a disability and results in a marked reduction in the quality of life for millions of people every year. VTE is a complex thrombotic disorder influenced both by environmental aspects as well as in particular by genetic predisposition. As part of [redacted], within the Leiden University Medical Center (LUMC), our research mainly focuses on the underlying mechanisms associated with VTE and places a strong emphasis on the role of genetics in this process. This has led to seminal contributions in the VTE field including the discovery of the factor V Leiden mutation in the mid-nineties and subsequently several other genetic variations which can predetermine thrombotic risk. Although there have been several advances in the field with the identification of many genetic variants linked to VTE, they still only explain a minor percentage of VTE risk in many cases.

Recently, as part of the international INVENT consortium, we undertook a meta-analysis of genome-wide association studies (GWAS) to identify additional VTE susceptibility genes (Germain et al. 2015 Am. J. Human Genetics). Twelve GWAS totalling 7,507 VTE cases and 52,632 controls formed our discovery stage where 6,751,884 single nucleotide polymorphisms (SNPs) were tested for association with VTE. Nine loci reached the genome-wide significance level including 6 already known to associate with VTE (ABO, F2, F5, F11, FGG and PROCR) and 3 unsuspected genes. SNPs mapping to the latter genes were selected for replication in 3 independent case-control studies totalling 3,009 VTE patients and 2,586 controls. This strategy lead to the identification of *SLC44A2* as a new risk locus ($p$ value = $2.75 \times 10^{-15}$). The lead SNP at the *SLC44A2* SNP locus is the non-synonymous rs2288904 previously shown to associate with transfusion related acute lung injury (TRALI). Importantly, a second independent GWAS study found that a locus also located within the *SLC44A2* gene is a genetic risk factor for both stroke and cardiovascular disease, further substantiating a possible role for SLC44A2 in VTE pathogenesis (Hinds et al. 2016 Hum Mol Genet.) SLC44A2 has not been described to associate with known haemostatic markers, thus interestingly, SLC44A2 does not belong to conventional pathways for thrombosis, nor has it been associated with other cardiovascular diseases or related quantitative biomarkers.

The VTE lead SNP, *SLC44A2* rs2288904 (A or G), coincides with an amino acid substitution in the extracellular domain of the Solute Carrier 44 A2 protein (SLC44A2 R154 or Q154, respectively) and is known to trigger antibody formation in carriers of the minor A allele, both during pregnancy and upon exposure to the major G allele variant. Upon transfusion, these antibodies induce neutrophil activation, sequestration, and finally, endothelial barrier damage which can result in possibly fatal transfusion related acute lung injury (TRALI). SLC44A2 is expressed on neutrophils, which is the postulated target cell for the anti-SLC44A2 antibodies in TRALI, although SLC44A2 is also expressed on vascular endothelial cells. Our collaborators at Giessen University, Germany, demonstrated *in vivo* that the anti-SLC44A2 antibodies induce loss of endothelial barrier integrity. In this model, neutrophils aggravated the destructive effects of anti-SLC44A2. Furthermore, they demonstrated *in vitro* that SLC44A2 can act as a receptor for von Willebrand Factor (vWF), a factor expressed on the endothelium and a key molecule important for normal haemostasis. In addition, it has become evident that two isoforms of SLC44A2 exist (i.e P1 and P2) with endothelial cells and neutrophils showing variable expression (P1 and P2 vs P1 only) possibly affecting SLC44A2 functionality.

Initial work characterizing the SLC44A2 global knockout mouse from a haemostasis perspective was performed in the laboratory of our collaborators at the University of Michigan in Ann Arbor, Michigan, USA. Preliminary testing found increased transcript levels of tissue plasminogen activator (tPA) in the lung of SLC44A2 knock out mice indicating that these mice may have a better capacity for clearing blood clots. Furthermore, upon laser induced injury of the cremaster muscle, SLC44A2 knockouts exhibited a reduction in fibrin formation, again suggesting that the mice have a faster clearance of clotting factors. These data point to a possible role for SLC44A2 in the process of haemostasis, which has not been previously described, however, the mechanisms behind these observations remain unclear.

Also of particular interest is the recent identification of autoantibodies against SLC44A2 in patients with autoimmune hearing loss (Kommareddi et al. 2009 Laryngoscope). The binding of these antibodies to SLC44A2 expressing cells in the inner ear leads to a detrimental effect on hair cell survival and hearing, again demonstrating that anti-SLC44A2 antibodies can have a disruptive effect on normal cellular

function. Several autoantibodies against haemostatic proteins such as prothrombin and thrombomodulin, in addition to antibodies targeting phospholipids, have been identified and linked to VTE, showing that autoantibodies can also contribute to the pathophysiology of VTE. We believe it is possible, as an alternative hypothesis, that a similar mechanism may also be occurring in the context of SLC44A2 and preliminary work by collaborators has even identified potential SLC44A2 autoantibodies in patients with VTE.

Altogether SLC44A2 appears to be relevant in both TRALI and VTE, two diseases where endothelial cells, neutrophils and their interactions are considered key to vascular pathophysiology. Importantly, antibodies can also play a destructive role in both of these conditions. Based on this knowledge, the current project proposal aims to further analyse the function of SLC44A2, including the interactions with autoantibodies, and unravel the mechanism underlying the association between SLC44A2 and VTE. We will focus in particular on the role of SLC44A2 in endothelial cells, neutrophils as well as with autoantibodies. We expect that the proposed project will provide novel therapeutic insight for the development of improved VTE treatments. Since all the current therapeutic strategies for VTE are directed at targeting coagulation factors and consequently have major bleeding as an unwanted side-effect and SLC44A2 has not been linked to traditional haemostasis, further understanding into its function may lead to the discovery of a unique treatment strategy without the adverse side effects of the current therapeutics.

**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?

SLC44A2 has been identified as a new VTE-associated locus, one with a strongly significant association with the disease ($p=2.75x10^{-15}$). However, at present, it is not understood how SLC44A2 and the allelic variation at amino acid 154 play a role in VTE pathophysiology. To further elucidate how SLC44A2 contributes to VTE pathogenesis, we formulated the following research questions: 1. What is the role of SLC44A2 during haemostasis? 2. How do autoantibodies targeting SLC44A2 affect haemostasis? 3. How does SLC44A2 contribute to VTE pathophysiology? 4. Do SLC44A2 targeting antibodies exacerbate the effects of VTE? 5. Are the contributions of SLC44A2 to VTE cell specific? Finding the answer to these questions will help us achieve our main objective which is to determine how SLC44A2 modulates the interactions between the blood and venous vessel wall, and thereby the initiation and/or propagation of venous thrombotic disease.

The current proposal is the result of a unique collaboration between [redacted], LUMC, The Netherlands and [redacted], Giessen, Germany. Thus, expertise from both institutes will join at the pre-clinical and basic science level to clarify the function of SLC44A2 and the relevance of SLC44A2 and of SLC44A2 antibodies in VTE. Recently, mice with a conditional floxed *Slc44a2* allele (with loxP sites flanking *Slc44a2* exon 3-10) were generated by a research group at [redacted] at the University of Michigan in Ann Arbor, Michigan, USA. We have established a collaboration with this group, and in March 2016 we received embryos of these conditional *Slc44a2* mice that will be used for the present project. Cryorecovery of the embryos was performed in April 2016 and the first offspring carrying the conditional allele became available in Leiden as of May 2016. As all parties involved have extensive experience with the experimental disease models included in this proposal, the principles of replacement, reduction and refinement will be wholly implemented in an effort to prevent as many negative consequences for the involved animals as possible.

Upon completion of this project, we expect to have a coherent answer to all of the above mentioned research questions and thus a much better understanding of the role of SLC44A2 not only in normal haemostasis, but in the pathological condition of VTE as well. With this knowledge, we can then begin to translate our observations into those from a clinical perspective with an aim to design better therapeutics

with less detrimental side effects for patients and ultimately improve the quality of life for those living with the symptoms associated with VTE.

### 3.3 Relevance

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

SLC44A2 is a novel susceptibility gene for VTE, however, very little is known about the protein and thus, at this juncture, it does not belong to any conventional pathways for VTE or have a role in haemostasis pathways. Therefore, the proposed objectives create an opportunity to elucidate the function of SLC44A2 in the context of physiological and pathological haemostasis. In regards to the impact this may have on the field as a whole, these studies may discover previously unknown mechanisms involved in the pathogenesis of VTE as well as establish a unique biomarker for the disease. Moreover, these findings may further prove significant in the clinical setting as targeting SLC44A2 might provide a more effective treatment strategy in VTE, as it is not linked to traditional haemostasis and would reduce the chance of adverse side effects such as bleeding, which accompanies all of the current therapeutics available.

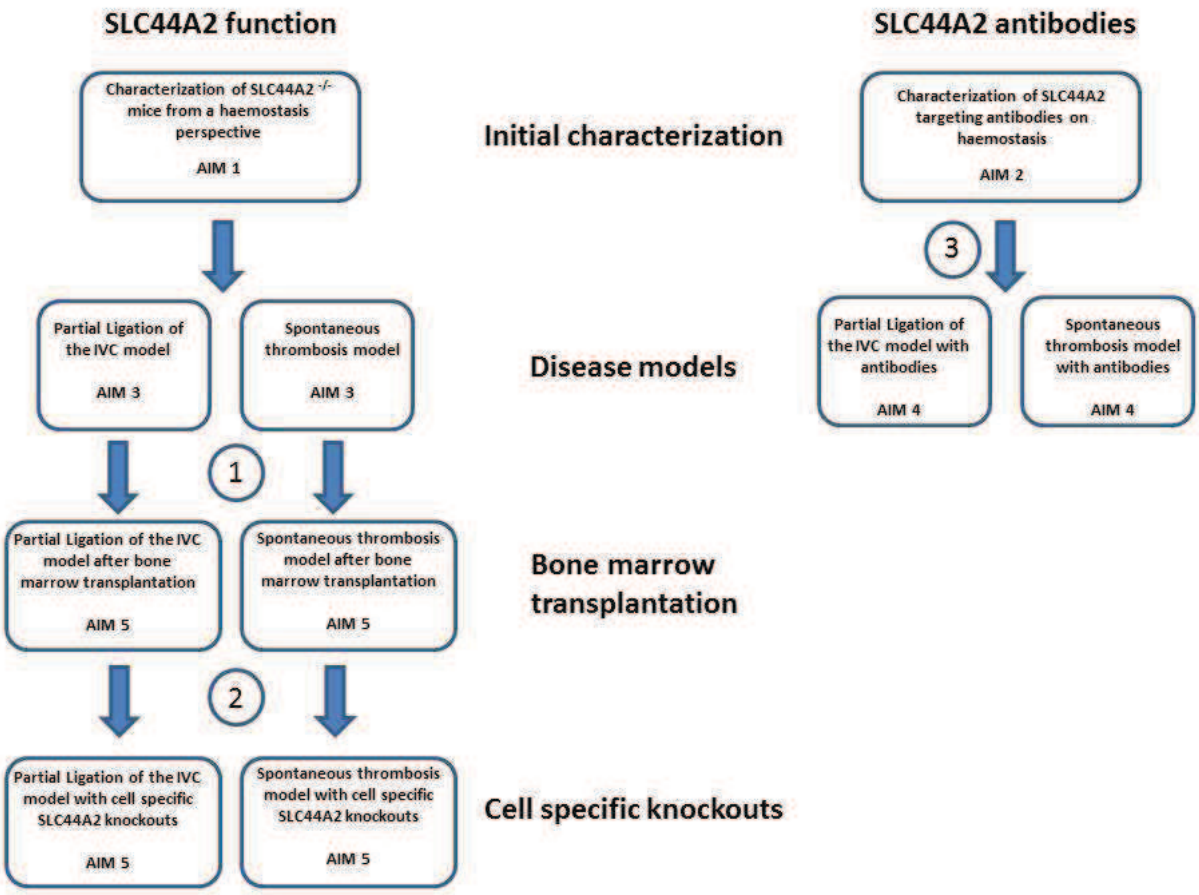
### 3.4 Research strategy

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

Haemostasis and subsequently VTE, are complex processes which involve various cell types and cell products from different biological systems including endothelial cells (circulatory), neutrophils (immune) and the production of coagulation factors by hepatocytes (digestive). There is also the added element of continuous blood flow, a physical factor that is difficult to replicate precisely *in vitro*, in addition to the possible changes in the immune profile. Therefore, it is only possible to truly define key interactions central to these processes using a model organism. In order to investigate the role of SLC44A2 in the pathogenesis of VTE we will make use of the recently generated *Slc44a2* exon 3-10 deficient knock out mice which allow for conditional deletions using the Cre-Lox recombination system. Our collaborator demonstrated that the *EIIa* Cre-mediated germ line inactivation of *Slc44a2* results in mice globally lacking *Slc44a2* (full knockouts) that appear viable and healthy with no spontaneous discomfort other than hearing loss upon a hearing challenge. This phenotype is only observed in the FVB genetic background and not the C57Black/6J background which we intend to use.

Using the mice in this manner we can then achieve the following aims:
1. Establish the role of SLC44A2 (if any) during traditional haemostasis
2. Determine if autoantibodies targeting SLC44A2 affect traditional haemostasis
3. Detect mechanisms which connect SLC44A2 to VTE pathophysiology
4. Test if SLC44A2 targeting antibodies exacerbate the effects of VTE
5. Dissect out the cell specific (i.e. endothelial cells or neutrophils) contributions of SLC44A2 to VTE

The following aims are summarized in the design overview below:




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.

In order to determine the importance of SLC44A2 function during normal haemostasis we will begin by characterizing the global *Slc44a2* knockout mice from a haemostasis perspective, focusing on the organs that are central to this process such as blood, liver and immune cells (AIM 1). It is at this point we will also determine whether or not it is necessary to include both male and female mice in the subsequent studies, as the response is well described to be bi-distributional and therefore the two genders cannot be combined. As obvious differences may not be detected in these mice under normal homeostatic conditions, regardless of the findings from this characterization, upon completion, we will move forward to the disease models (AIM 3). In this respect, we will employ two different models of venous thrombosis (VT), namely the partial ligation model and the spontaneous induced thrombosis model. With these two systems, we will cover all relevant aspects which are crucial to the induction of VT and overcome the limitations that each model presents such as retrograde thrombus formation in the ligation model and the lack of influence by neutrophils in the spontaneous model. The results from AIM 3 will then determine our first go/no go moment (arrow 1). If the disease models point to a clear effect of SLC44A2 in VT pathophysiology, we will then proceed to first part of AIM 5, which is the use of bone marrow transplantation in order to identify the cellular compartment expressing SLC44A2 that is contributing to the induction of VT, i.e. the stromal or hematopoietic compartment. The outcomes from these studies will then comprise our second go/no go moment (arrow 2). If the loss of SLC44A2 in either the stromal or hematopoietic compartment does not elicit differences in the propagation of VT, then we will conclude that ubiquitous expression of SLC44A2 is important in VT pathogenesis and no further study will be needed. However, if the findings point to a certain compartment as being the main contributor in the disease, then we will continue to pinpoint the specific cell type that is central to this process. To achieve this final objective of AIM 5, we will generate either endothelial cell (stromal) or neutrophil (hematopoietic) specific SLC44A2 knockout mice and then induce disease using the models described in

AIM 3.

In parallel to the above studies, we will also explore the contributions of SLC44A2 targeting antibodies in haemostasis and possibly VT. In order to do this, we must first find a working concentration of SLC44A2 antibody that can be tolerated by the mice without being cleared from the blood within a 72 hour time frame, specifically the duration of the disease model. In addition to titrating the amount of antibody that is suitable for later models, we will also determine the effect that these molecules have on haemostasis, These are the two main goals of AIM 2. The outcome of these studies will then form a go/no go moment for this portion of the project (arrow 3). If we can find an amount of SLC44A2 antibody that circulates freely within in the mouse without causing an acute reaction, we will proceed to use disease models in combination with autoantibodies (AIM4). However, if the mice have a severe response to the antibodies (which we do not expect) or if the antibodies do not remain in circulation long enough, then we will conclude this half of the project.

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.

Altogether the different components of this project culminate to form an explanation as to how SLC44A2 contributes to VTE. The initial study proposed in aim 1 will determine if SLC44A2 is involved in traditional haemostasis pathways through characterization of the *Slc44a2* global knockout mouse from a haemostasis perspective. We will evaluate whether the loss of SLC44A2 has any effect on the production of coagulation factors and subsequently blood clotting, which is the source of clinically manifested events associated with VTE. As the effects of the loss of SLC44A2 may not be evident under normal homeostatic conditions and only upon vascular injury, regardless of the outcome of this aim, we will proceed to the disease models described in aims 3-5 Additionally, in aim 2, we will also investigate the effect that SLC44A2 targeting antibodies have on haemostasis as these corresponding antibodies have already been implicated in vascular disease such as transfusion related acute lung injury (TRALI). Together these findings will form the first milestone of the project as such a detailed examination has yet to be described and is crucial before studying further aspects of SLC44A2 involvement in VTE. These results may also help us to hone our focus on certain mechanisms that may be amplified at later stages in the disease models included in aims 3-5. Moreover, the initial trials studying SLC44A2 targeting antibodies are of particular importance as these will establish the appropriate concentration of antibodies as well as administration schedule that will be used in aim 4. Upon completion of this initial characterization, we will move forward with aim 3 and determine the importance of SLC44A2 in the propagation of VTE using two independent disease models of thrombosis, namely the partial ligation and spontaneous model. It is vital to utilize both models of VTE as each has its disadvantages such as extensive surgical handling and retrograde thrombi formation in the ligation model and no role for neutrophils in the spontaneous model. Thus, using these systems in combination is essential and will ensure that we fully cover all aspects that are key to VTE pathophysiology. These data will then mark the second milestone of the project as it will become clear as to whether SLC44A2 is a central contributor in the induction of thrombosis. If mice lacking SLC44A2 are protected or exhibit a less severe thrombotic phenotype, we will conclude that this is directly linked to the loss of SLC44A2. However, if we do not observe such an effect, we will conclude that SLC44A2 does not play a role in VTE and will not proceed with experiments associated with aim 5. In addition, as detailed in aim 4, in order to distinguish the importance of SLC44A2 targeting antibodies in VTE pathogenesis we will include such antibodies, as defined by aim 2, in addition to the two thrombosis models and measure the additive effect that antibodies may have on vessel injury. The outcome from these studies will constitute another major milestone of the project as it would establish whether autoantibodies against SLC44A2 in particular can exacerbate the symptoms associated with VTE. This has yet to be described and may have strong implications for the clinical setting. Lastly, when the evidence from aim 3 supports further study of SLC44A2, we will continue to dissect out its importance by defining the cellular players that are contributing to disease induction, as described in aim 5. We will first utilize bone marrow isolated from WT and *Slc44a2* global knockout mice which is then transferred back to either WT or mice lacking *Slc44a* before the challenge of thrombosis. In this way we can begin to identify the cellular compartments (stromal or hematopoietic) that support the propagation of VTE as it relates to SLC44A2. Concurrently, we will also breed *Slc44a2* floxed mice with *Tie-2* Cre and *LysM* Cre in order to generate cell specific knockout mice, lacking SLC44A2 in either endothelial cells or neutrophils, respectively. Thrombosis will then be promoted in these mice and based on these results, we will be able to identify which of these cell types is driving the pathogenesis of VTE. This will be the final milestone of

the project as not only will we have determined the importance of SLC44A2 in VTE, but identified the principal cell types that are essential in SLC44A2 associated VTE pathogenesis.

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 | Haemostasis characterization of *Slc44a2* knockout mice |
| 2 | Effect of SLC44A2 targeting antibodies on haemostasis |
| 3 | Venous thrombosis through partial ligation of the caval vein |
| 4 | Spontaneous venous thrombosis through RNAi |
| 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. | [redacted] |

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 | Type of animal procedure |
|---|---|
| 3.4.4.1 | Haemostasis characterization of *Slc44a2* knockout mice |

## 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.

Blood and tissue samples from several organs central to the process of haemostasis will be collected from wild type and *Slc44a2* deficient animals, including 1) global knockout 2) cell specific knockout (endothelial or neutrophil) or 3) bone marrow transplanted animals which creates mice lacking SLC44A2 in either the stromal or hematopoietic compartment in addition to mice containing or lacking SLC44A2 in both compartments. This may include, but is not limited, to material such as blood, liver, lungs and venous tissue. Subsequently, these samples can be used to phenotype the *Slc44a2* knockout mouse (either global or cell/compartment specific) from a haemostasis perspective by measuring differences in the capacity of the blood to coagulate and related cells to produce coagulation factors, in addition to measuring changes in the immunological profile. In this way we can begin to answer how SLC44A2 affects both normal and pathological haemostasis. As it is difficult to isolate such material without causing injury to the animals, a total isolation of all tissues upon anesthetization, followed by euthanization, is the most humane procedure.

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

While under general anaesthesia, the abdomen of the mice will be opened and blood will be collected directly from the vena cava into a syringe containing sodium citrate so as to preserve the sample. Blood must be collected directly from the vein in order to avoid contamination with factors that activate coagulation (i.e. tissue factor) as it will be used for testing exactly that at a later time. The amount of blood retrieved will be the maximal amount possible, therefore following collection, the diaphragm of the

mouse will be punctured, euthanizing the mouse in a humane manner. At this point, several tissues will be collected and snap frozen. These can then be used to determine if *Slc44a2* mice exhibit any differences in haemostatic activity both at the mRNA and protein level.

For the mice undergoing bone marrow transplantation, donor mice will be euthanized using carbon dioxide asphyxiation and bone marrow cells from femur, tibias and humerus bones will be isolated and prepared for injection into the recipient mice. Bone marrow transplant recipients will begin receiving acidified, antibiotic water one week prior to the irradiation procedure. At least six hours before receiving the transplantation, they will be sub lethally irradiated at a dose suitable for their background (C57BL/6 mice). Following radiation, mice will receive a tail vein injection of the donor mouse bone marrow cells and subsequently housed for 21 days until the repopulation of hematopoietic cells is complete.

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

Group size: For the calculation of determining how many animals are necessary per group to establish any meaningful differences, we use the parameter of thrombin generation as it has the largest standard deviation as compared to other readouts of haemostasis, such as gene expression by qPCR. After reviewing the most recent literature, we have determined that a reasonable mean for group 1, the wild type (WT) group in a C57BL/6 background, for endogenous thrombin potential (ETP) is approximately 220 nM*min and for group 2, a knockout mouse that is reasonably healthy as compared to its WT littermates, a value of 290 nM*min. We estimate the heterozygous mice to fall somewhere in between and therefore we use the mean value of 255 nM*min and an average standard deviation of 45. Because the readout will be comparing three groups, we will use a one way ANOVA test which will control for the type I error. Using the G*Power statistical software (http://www.gpower.hhu.de/) program to perform a POWER calculation with an alpha of 0.05 and a power of 0.80, we find the effect size f to be 0.63505 which would require a sample size of 30, signifying 10 animals per group.

Sex consideration: As it is unknown whether sex will influence the effect of the *SLC44A2* gene and its role in haemostasis, we will include both for this initial characterization study. If no significant interactions between gender and SLC44A2 in aspects of hemostasis (thrombin generation, coagulation gene transcripts, etc.) are found, then we will continue only with females for the remainder of the project as there is generally less biological variation such as size/weight between age matched mice. Our rationale for starting with both sexes is due to the well described findings that hormones can play a role in haemostasis (i.e. contraception & thrombotic events), but because no differences in gender were described in the initial GWAS study linking *SLC44A2* to venous thrombosis, we do not expect sex to play a role in VT in the context of *SLC44A2* genotype status. However, until we can rule this out we find it prudent to include both groups. Furthermore, the reason we choose to continue with only one sex (female) and not pooled sexes is that it is well established (Alvarado et al Thromb Res 2011; Vanlangenvelde et al Comp Med 2005) that the biological response of males vs. females in murine models of VT is significantly different as demonstrated by several key factors including thrombus size, cytokine expression, blood cell counts, etc. Therefore, regardless of the characterization of SLC44A2 in hemostasis between the sexes, we cannot justifiably group males and females together in the subsequent VT models, as the bi-distributional readouts may obscure true differences which may be due to the gene alone.

**B. The animals**

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

The animals used in these studies are *Mus musculus,* from a C57BL/6J background, containing either a deleted region (exon 3-10) of the *Slc44a2* gene (KO) or a wild type (WT) allele. They originate from the lab of [redacted] at the University of Michigan but have been breed at the LUMC for many generations. In addition, final studies may also employ mice lacking the *Slc44a2* gene only in specific cell types. These will be generated in two distinct ways. First, we will use bone marrow transplants from WT or *Slc44a2* deficient mice to either WT or *Slc44a2* KO mice to create mice lacking SLC44A2 expression in either the stromal or hematopoietic compartments. Alternatively, we will also cross breed *Slc44a2* floxed mice, containing loxP sites on either side of exons 3-10, with mice expressing Cre-recombinase under the control of either the *Tie-2* or *LysM* promoter, resulting in mice lacking SLC44A2 in endothelial cells or neutrophils, respectively. To control for genetic variation as much as possible, we will make use of matched litter mates, using WT, HET and KO siblings, generated from parents who are heterozygous for the KO allele. The mice will be included between ages of 8-14 weeks, which is the same age that will be

used in the experimental disease models described in appendices 3.4.4.3-4. We estimate that the characterization of these knockout animals from a haemostatic perspective will involve approximately 180-290 animals, based on the calculations below:

General characterization of global knockout animals:
3 genotypes (KO, HET, WT) x 10 (group size) x 2 sex (males and females) = approximately 60 animals

General characterization of cell specific knockout animals:
3 genotypes (KO, HET, WT) x 10 (group size) x 1 sex (female) = approximately 30 animals
If sex differences are found in the general characterization of global knockouts then we will also use approximately 30 males.

Training and set up of bone marrow transplantation procedure:
5 WT and 5 KO animals

General characterization of bone marrow transplant animals:
4 systems (WT, WT hemo/KO stromal, KO hemo/WT stromal, KO) x 2 transfer (donor, recipient) x 10 (group size) x 1 sex (female) = approximately 80 animals
If sex differences are found in the general characterization of global knockouts then we will also use approximately 80 males.

**C. Re-use**

Will the animals be re-used?

X 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'?

X 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:
Due to the complex nature of haemostasis, which involves several different biological systems including the circulatory, immune and digestive system, in addition to the physical interaction between flowing blood and the vessel wall, it cannot be accurately replicated *in vitro*. Thus an *in vivo* system, which includes interactions between all such factors, is essential in studying this process. In addition, the ability to target deletion in cellular compartments through bone marrow transplantation and cell specific knockout animals and continue to study the interaction between the different biological systems is a very unique tool that cannot be readily reproduced. However, once we have conducted the studies contained in this proposal, it is likely that we will be able to pinpoint the cellular players and signalling pathways that are central to this process, specifically in relation to SLC44A2, and we can then follow up using *in vitro* models with human tissues to confirm and therefore strengthen our conclusions.

Reduction:
In order to reduce the number of animals necessary, we have taken several steps and carefully considered all options. First, we are making use of littermates so as to reduce the chance of genetic variability. Second, we will also save most organs and materials from the mice and not only the tissues that are classically involved in haemostasis. In this way, if the data points to a novel finding and further analysis is needed in secondary organs, we will already have them and the need to include more animals may not be necessary. Lastly, based on power calculations and experience with these types of procedures, we have determined the optimal amount of animals to include in these studies so that a

significant difference between experimental conditions can be found (if one exists) and no repetition likely will be needed. We have also contemplated whether another experimental design can be used in order to minimize the requested numbers or the overall distress to the animals. However, as this is a characterization of the novel genetic *Slc44a2* mouse line from a haemostasis perspective, it is not possible if we do not collect blood and organs related to haemostasis.

Refinement:
Several practices are put in place in order to minimize any pain or discomfort for the animals during the proposed studies. Anaesthesia will be administered prior to the collection of materials, such that no pain will be felt during these procedures and for mice receiving bone marrow transplantation, anaesthesia will be administered prior to the irradiation of recipient mice . These studies will also be conducted by trained researchers with experience performing these specific techniques. We will closely monitor the reactions of the animals at all times and if we observe any discomfort associated with humane endpoints such as changes in behaviour (posture, grooming, activity) or pathophysical reactions (rapid breath, unusual heart rate, weight loss) we will end the experiment immediately. In addition, as the irradiation may weaken the animals, we will provide special housing conditions including longer nipples on the water bottles, which will contain antibiotics, as well as soft food to ease accessibility for eating and drinking.

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

The measures taken to minimize animal suffering, pain and fear in regards to the proposed studies include pre-treatment of the animals with the appropriate anaesthesia before irradiation or the collecting any blood or tissues to be used in the characterization of these knockout animals. There are no adverse effects on the environment resulting from these studies.

## 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.

Not applicable.

## 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

X 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?

X 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.

x Yes > Will anaesthesia, analgesia or other pain relieving methods be used?

☐ No > Justify why pain relieving methods will not be used.

x Yes > Indicate what relieving methods will be used and specify what measures will be taken to ensure that optimal procedures are used.

All experimental animals will be anaesthetised prior to the collection of blood or tissues which will subsequently be used in the characterization of the global *Slc44a2* knockout mice. In regards to the characterization of animals after bone marrow transplantation, animals will be anaesthetised prior to the irradiation procedure and the collection of blood or tissues which will subsequently be used for characterization of the mice. Donor animals will be euthanized using carbon dioxide asphyxiation before the collection of bone marrow. These procedures are deemed optimal based on experience with such techniques within the department.

**I. Other aspects compromising the welfare of the animals**

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

A possible adverse effect that may result from this procedure is the sensation of pain and fear if the administration of the anaesthesia is somehow faulty before the start of the procedure. However, such an event would be very difficult to mistake, as symptoms of an effective administration are readily visible. Since these procedures will be performed by skilled researchers, with years of experience with mouse models, we do not expect that such a mistake would go unobserved and all mice will be determined to be thoroughly sedated before the onset of any technique. Adverse effects that may arise due to the bone marrow transplant procedure are either graft versus host disease or alternatively, host versus graft disease in which T-cells become reactive and attack the tissues of the host or graft, respectively. In addition, the effects of irradiation may also result in anaemia, immunosuppression, diarrhea and damage to the mucosal membranes.

Explain why these effects may emerge.

Such an effect may occur if the compounds that make up the anaesthetic regimen were improperly administered. In regards to adverse effects associated with bone marrow transplantation, this occurs when the MHC molecules are different between the host and donor mice.

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

In order to prevent incomplete anaesthetization of mice before the start of procedure, we will have experienced handlers present who can identify when the mouse is safely ready for blood collection. In order to minimize the chance of rejection of transplantation, we are using litter mates both as donor and recipient in mice that are backcrossed to C57BL/6 background for at least 6 generations. We expect the genetic variability to be very limited between such animals which will reduce the chance of mismatched MHC expression on the tissues. Animals will also be placed in special housing conditions (see section D, refinement) 3 weeks post irradiation in preparation for the adverse effects associated with the procedure.

**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.

X Yes > Describe the criteria that will be used to identify the humane endpoints.

Criteria for humane endpoints during this part of the study include rapid breathing during the laparotomy of the mouse which may indicate an abnormal response to the anaesthesia. If this occurs, we will not proceed with puncture of the vena cava and euthanize the mouse immediately as opposed to after blood collection. Criteria for humane endpoints for bone marrow transplantation include behavioural changes in posture, grooming or activity levels in addition to pathophysical reactions such as rapid breathing, weight loss greater than 15% or HBV activation following radiation.

Indicate the likely incidence.

We estimate a very low incidence (<5%) for general characterization and approximately 10% for bone marrow transplantations.

**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 procedures for the general characterization of the knockout animals fall under the category of "non-recovery" as 100% of the animals will be euthanized after the procedure, which is performed entirely under general anaesthesia. With respect to bone marrow transplantation, 100% of the donor animals fall under the category of "non-recovery" since they will be euthanized before the bone marrow is isolated. Recipient animals may experience mild (90%) to moderate (10%) discomfort. Therefore, the mice will be given the appropriate anaesthesia and analgesia to minimize this as much as possible.

## End of experiment

**L. Method of killing**

Will the animals be killed during or after the procedures?

☐ No

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

Animals are killed and used amongst others for full pathological analysis

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

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

x 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. | [redacted] |

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 | Type of animal procedure |
|---|---|
| 3.4.4.2 | Effect of SLC44A2 antibodies on haemostasis |

## 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.

Blood and tissue samples from several organs central to the process of haemostasis will be collected from animals injected with SLC44A2 targeting antibodies, originally isolated from human donors. This may include, but is not limited, to material such as blood, liver, lungs and venous tissue. Subsequently, these samples can be used to determine the effect that SLC44A2 antibodies have on haemostasis. Additionally, these studies will help to determine the appropriate amount of antibodies needed to study their contributions to venous thrombosis, as described in appendices 3.4.4.3-4. Using such procedures we will be able to measure differences in the capacity of the blood to coagulate and related cells to produce coagulation factors, in addition to measuring changes in the immunological profile. In this way we can begin to answer how SLC44A2 targeting antibodies affect both normal and pathological haemostasis.

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

Mice will receive an infusion of varying concentrations of anti-SLC44A2 antibodies purified from human serum, in volumes set forth by the directive Diehl et al 2001, to determine a sufficient amount which allows for the presence of antibodies 72 hours post administration, without causing an acute response in the mouse. In order to minimize unwanted side effects, we start with the lowest concentration first and then raise it incrementally until the antibodies can be detected back after 72 hours. We will also compare the efficacy of using two time points versus one, namely at either 72 hours or both 72 and 24 hours before collection for tissue analysis. The goal of this study is twofold: 1) to determine the effect of anti-SLC44A2 antibodies on haemostasis as well as 2) determine the amounts of antibody necessary to

have adequate coverage present at the time of thrombosis induction (appendices 3.4.4.3-4). We base these time points and the initial amounts that will be injected on established studies of a similar nature used to study the role of anti-phospholipid antibodies in venous thrombosis (Pierangeli et al. Thromb. Haemost. 1995).

Following 72 hours of the initial administration, the mice will receive general anaesthesia and the abdomen will be opened and blood will be collected directly from the vena cava into a syringe containing sodium citrate so as to preserve the sample. Blood must be collected directly from the vein in order to avoid contamination with factors that activate coagulation (i.e. tissue factor) as it will be used for testing exactly that at a later time. The amount of blood retrieved will be the maximal amount possible, therefore following collection, the diaphragm of the mouse will be punctured, euthanizing the mouse in a humane manner. After this point, several tissues will be collected and snap frozen. These can then be used to determine if mice exhibit any differences in haemostatic activity both at the mRNA and protein level.

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

Group size: To determine the number of animals necessary per group we based our POWER calculation on previous work carried out to characterize mice treated with autoantibodies in the context of venous thrombosis. To this end, we use the recorded values for tissue factor (TF) activity in which mice treated with a control IgG antibody had a mean value of TF activity of 149.0 pM/mg/ml and mice treated with an autoantibody a mean value of 402.5 pM/mg/ml with a SD of 165.2. Using POWER calculation (http://biomath.info/power/ttest.htm) with an alpha of 0.05 and a power 0.80 we determined the appropriate group size to be 8.

Sex consideration: As it is unknown whether sex will influence the effect of the *SLC44A2* targeting antibodies and their role in haemostasis, we will include both for this initial characterization study. If no significant interactions between gender and SLC44A2 in aspects of hemostasis (thrombin generation, coagulation gene transcripts, etc.) are found then we will continue only with females for the remainder of the project involving antibodies as there is generally less biological variation such as size/weight between age matched mice. Our rationale for starting with both sexes is due to the well described findings that hormones can play a role in haemostasis (i.e. contraception & thrombotic events), but because no differences in gender were described in the initial GWAS study linking *SLC44A2* to venous thrombosis, we do not expect sex to play a role in VT in the context of *SLC44A2* genotype status. However, until we can rule this out we find it prudent to include both groups. Furthermore, the reason we choose to continue with only one sex (female) and not pooled sexes is that it is well established (Alvarado et al Thromb Res 2011; Vanlangenvelde et al Comp Med 2005) that the biological response of males vs. females in murine models of VT is significantly different as demonstrated by several key factors including thrombus size, cytokine expression, blood cell counts, etc. Therefore, regardless of the characterization of SLC44A2 in hemostasis between the sexes, we cannot justifiably group males and females together in the subsequent VT models, as the bi-distributional readouts may obscure true differences which may be due to the antibodies alone.

**B. The animals**

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

The animals used in these studies are *Mus musculus,* from a C57BL/6J background, containing either a deleted region (exon 3-10) of the *Slc44a2* gene on both alleles (KO), one allele (HET) or both wild type alleles (WT). They originate from the lab of [redacted] at the University of Michigan but have been breed at the LUMC for many generations. To control for genetic variation as much as possible, we will make use of matched litter mates, using both WT, HET and KO siblings, generated from parents who are heterozygous for the KO allele. The mice will be included between ages of 8-14 weeks, which is the same age that will be used in the experimental disease models described in appendices 3.4.4.3-4. We estimate that the characterization of these knockout animals from a haemostatic perspective will involve approximately 64-384 animals, based on the calculation below:

Antibody titration:
1 genotype (WT) x 8 (group size) x 1 sex (female) x 2 injection timepoints (72, 72 & 24hr) x 4 concentrations = 64

If we can find a working dosage, then we will continue with multiple genotypes for characterization:

3 genotypes (WT, HET, KO) x 8 (group size) x 2 sex (male,female) x 2 injection timepoints (72, 72 & 24hr) x 4 concentrations = 384

**C. Re-use**

Will the animals be re-used?

X 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'?

X 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:
Due to the complex nature of haemostasis, which involves several different biological systems including the circulatory, immune and digestive system, as well as the physical interaction between flowing blood and the vessel wall, it cannot be accurately replicated *in vitro*. In addition, it is still unclear which cell type can be targeted by these SLC44A2 antibodies. Thus an *in vivo* system, which includes interactions between all such factors, is essential in studying this process. However, once we have conducted the studies contained in this proposal, it is likely that we will be able to pinpoint the cellular players and signalling pathways that are central to this process, specifically in relation to SLC44A2 antibodies, and we can then follow up using *in vitro* models with human tissues to confirm and therefore strengthen our conclusions.

Reduction:
In order to reduce the number of animals necessary, we have taken several steps and carefully considered all options. First, we are making use of a pilot study to determine the proper dosing of antibodies before going further in using mice with variable genotypes. Second, we will also save most organs and materials from the mice and not only the tissues that are classically involved in haemostasis. In this way, if the data points to a novel finding and further analysis is needed in secondary organs, we will already have them and the need to include more animals may not be necessary. Lastly, based on power calculations, we have determined the optimal amount of animals to include in these studies so that a significant difference between experimental conditions can be found (if one exists) and no repetition likely will be needed. We have also contemplated whether another experimental design can be used in order to minimize the requested numbers or the overall distress to the animals. However, the models available to study the effect of antibodies in haemostasis and thrombosis are few, and thus we have chosen the most established system in order to cover the aspects that may be involved in these processes.

Refinement:
Several practices are put in place in order to minimize any pain or discomfort for the animals during the proposed studies. To start, we will begin by testing the lower concentrations of antibodies first in order to minimize the possible discomfort. We will also follow the guidelines in regards to injections set forth by Diehl et al 2001. Anaesthesia will also be administered prior to the final collection of tissues, such so that no pain will be felt during these procedures. These studies will also be conducted by trained researchers with experience performing these specific techniques. As there may be some adverse reactions to these treatments, we will closely monitor the response of the animals at several time points and if we observe any discomfort associated with humane endpoints such as changes in behaviour (posture, grooming, activity) or pathophysical reactions (rapid breath, unusual heart rate, weight loss) we will end the experiment immediately.

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

on the environment.

The measures taken to minimize animal suffering, pain and fear in regards to the proposed studies include pre-treatment of the animals with the appropriate anaesthesia before collecting any blood or tissues to be used in the characterization of these animals. There are no adverse effects on the environment resulting from these studies.

## 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.

Not applicable.

## 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

X 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?

X 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.

x Yes > Will anaesthesia, analgesia or other pain relieving methods be used?

☐ No > Justify why pain relieving methods will not be used.

x Yes > Indicate what relieving methods will be used and specify what measures will be taken to ensure that optimal procedures are used.

All experimental animals will be anaesthetised prior to the collection of blood or tissues which will subsequently be used for characterization of the mice. The procedures are deemed optimal based on several publications in which a similar approach was used in addition to in house experience with these techniques .

**I. Other aspects compromising the welfare of the animals**

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

There is a chance that the animals may respond negatively to the infusion of SLC44A2 targeting antibodies by having an acute immune response, for example, the activation of neutrophils which may

cause vascular damage.

Explain why these effects may emerge.

These effects may emerge if SLC44A2 targeting antibodies have a strong pathological influence on the general haemostasis of mice (unknown).

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

We will start by using the lowest doses of antibody until a sufficient amount in the blood is reached and maintained for 72 hours. We will observe the animals every 2 hours over the first 12 hours and then every 12 hours until 72 hours post injection in order to detect any acute response to the antibodies that results in a distressful effect to the mice (humane endpoints), at which point, we will end the experiments immediately, as this is not the goal of this study. In an effort to avoid any unwanted discomfort for the animals, we will be using titers that have been established in literature and have not been reported to cause severe distress to mice.

**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.

X Yes > Describe the criteria that will be used to identify the humane endpoints.

Criteria for humane endpoints during this part of the study include behavioural changes in posture, grooming or activity levels in addition to pathophysical reactions such as rapid breathing or weight loss greater than 15% as determined by weighing the mice before the start of the experiment and following up at 24 and 48 hours post injection.

Indicate the likely incidence.

We estimate a very low incidence (<5%).

**K. Classification of severity of procedures**

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

Depending on the response of the animals to SLC44A2 antibodies, levels of discomfort may vary, however we estimate the severity to range from mild (90%) to moderate (10%). Because we cannot say for sure, we will monitor the animals closely every 2 hours over the first 12 hours and then every 12 hours until 72 hours post injection to ensure the minimal amount of distress as determined by the humane endpoints described above.

## End of experiment

**L. Method of killing**

Will the animals be killed during or after the procedures?

☐ No

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

Animals are killed and used amongst others for full pathological analysis

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

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

x 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. | [redacted] |

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 | Type of animal procedure |
|---|---|
| 3.4.4.3 | Venous thrombosis through partial ligation of the caval vein |

## 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.

Mice lacking SLC44A2 expression, either whole body, in certain cellular compartments (through bone marrow transplantation), cell type specific (endothelial cells or neutrophils) or in mice that have been transfused with SLC44A2 targeting antibodies, will undergo a partial ligation procedure in order to mimic the process of venous thrombosis (VT) *in vivo* and to dissect out the cell types and signalling pathways which link SLC44A2 to disease pathogenesis. Blood and tissue samples from several organs central to the process of VT will be collected at different time points in order to record changes during key stages of disease progression. This may include, but is not limited to, material such as blood, liver, lungs, thrombus and venous tissue. Subsequently, these samples can be used to determine the contributions of SLC44A2 to VT. Using such procedures we will be able to measure differences in the capacity of the blood to coagulate and related cells to produce coagulation factors, changes in the immunological profile as well as characterize changes in thrombus formation/clearance and fibrin deposition.

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

Following anaesthetization of the mice and the administration of analgesia, a midline laparotomy will be made and the small bowel exteriorized and placed to the left of the animal. The inferior vena cava (IVC or caval vein) is then exposed by careful blunt dissection while sterile saline is applied at regular intervals to the exteriorized bowel to prevent its desiccation. Non-reactive prolene suture is looped around the IVC immediately caudal to the origin of the renal veins, with a space holder included on the outside of the vessel. Next the ligature is closed and the space holder is removed to avoid complete vessel occlusion.

Side branches will not be ligated or manipulated and IVC blood flow will be reduced immediately after the restriction. Lastly, the median laparotomy is sutured and the mice will be placed on a warming mat until anaesthesia wears off. Mice will additionally receive peri-operative analgesia until collection at at the 12, 24, 36 and 48hr designated time points . For the collection of blood and tissues, mice will receive a lethal dose of anaesthesia and subsequently several tissues will be collected, including the vena cava with the newly formed thrombus, and snap frozen. These can then be used to detect differences in expression levels of both mRNA and protein, as well as histological analysis of thrombus formation and fibrin deposition. The mice receiving anti-SLC44A2 antibodies will undergo all procedures described above in addition to receiving an infusion of antibodies prior to the surgery (appendix 3.4.4.2).
For the studies involving bone marrow transplantation, donor mice will be euthanized using standard techniques and bone marrow cells isolated and prepared for injection into the recipient mice. Bone marrow transplant recipients will begin receiving acidified, antibiotic water one week prior to the irradiation procedure. At least six hours before receiving the transplantation, they will be sub lethally irradiated at a dose suitable for their background (C57BL/6 mice). Following radiation, mice will receive a tail vein injection of the donor mouse bone marrow cells and subsequently housed for 21 days until the repopulation of hematopoietic cells is complete. At this point, the mice will then be ready to include in the ligation model described above.

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

Group size: The calculation of the experimental group size is based on the parameter 'thrombosis formation' (and not mRNA analysis) as this is the primary outcome and the parameter with the largest standard deviation. Previously, at 24 hours post thrombosis induction, we observed effects on thrombus size and weight ranging from 75% decrease up to 200% increase in size/weight as compared to control reference groups. Using these observations we can estimate that the control group has an average thrombi weight of 1g and a second group representing the knockout mice would have less thrombi, around .25g and a third group may have increased thrombi, up to double the weight of group 1, being 2g. As the readout will be comparing three groups, we will use a one way ANOVA test which will control for the type I error. Using the G*Power statistical software (http://www.gpower.hhu.de/) program to perform a POWER calculation with an alpha of 0.05 and a power of 0.80, we find the effect size f to be 0.55143 which would require a sample size of 36, signifying 12 animals per group. Because we want to rule out endothelial injury as a trigger for venous thrombosis, mice with bleedings or any injury of the IVC during surgery need to be excluded from further analysis. We therefore include an extra of 2 animals per group to compensate for animals displaying bleedings or any injury during the IVC during surgery. These 2 extra animals will only be included when necessary. For now we will use a maximum of 14 animals per experimental group.

Sex consideration: Unless we determine a clear link between the *Slc44a2* gene and sex in the initial characterization described in appendices 3.4.4.1-2, we will only use females for the following procedures. We opt for female over male since there is less biological variability in measures such as size and weight. Female mice can also be housed together even though they are not littermates (males cannot) which also helps to minimize any differences that may arise due to housing conditions. Furthermore, the reason we choose to continue with only one sex (female) and not pooled sexes is that it is well established (Alvarado et al Thromb Res 2011; Vanlangenvelde et al Comp Med 2005) that the biological response of males vs. females in murine models of VT is significantly different as demonstrated by several key factors including thrombus size, cytokine expression, blood cell counts, etc. Therefore, regardless of the characterization of SLC44A2 in hemostasis between the sexes, we cannot justifiably group males and females together in the subsequent VT models, as the bi-distributional readouts may obscure true differences which may be due to the gene or antibodies alone.

**B. The animals**

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

The initial studies will include *Mus musculus* from a C57BL/6J background, containing either a deleted region (exon 3-10) of the *Slc44a2* gene on both alleles (KO), one allele (HET) or both wild type alleles (WT), globally in all tissues of the animal. They originate from the lab of [redacted] at the University of Michigan but have been breed at the LUMC for many generations. In addition, the final studies will employ mice lacking the *Slc44a2* gene only in specific cell types. They will be generated by cross

breeding *Slc44a2* floxed mice, containing loxP sites on either side of exons 3-10, with mice expressing Cre-recombinase under the control of either the *Tie-2* or *LysM* promoter, resulting in mice lacking SLC44A2 in endothelial cells or neutrophils, respectively. To control for genetic variation as much as possible, we will make use of matched litter mate siblings, generated from parents who are heterozygous for the KO allele. The mice will be included between ages of 8-14 weeks. We estimate that the maximal number of animals needed to complete all aspects of the studies included in this proposal regarding IVC ligation to be approximately 1008. If the response of mice in appendices 3.4.4.1-2 is determined to be dependent on sex (unlikely), then we will also include 1008 males.

Training and set up of procedure:
10 WT animals

IVC ligation-induced thrombosis with global knockout animals:
3 genotypes (WT, HET, KO) x 14 group size x 1 sex (female) x 4 timepoints (12, 24, 36, 48hr) = 168 animals

IVC ligation-induced thrombosis with anti-SLC44A2 antibodies:
3 genotypes (WT, HET, KO) x 14 group size x 1 sex (female) x 4 timepoints (12, 24, 36, 48hr) = 168 animals

IVC ligation-induced thrombosis with cell specific knockout animals:
2 genotypes (WT or KO) x 2 cell type (endothelial or neutrophil) x 14 group size x 1 sex (female) x 4 timepoints (12, 24, 36, 48hr) = 224 animals

IVC ligation-induced thrombosis with bone marrow transplantation :
4 systems (WT, WT hemo/KO stromal, KO hemo/WT stromal, KO) x 2 (donor, recipient) x 14 group size x 1 sex (female) x 4 timepoints (12, 24, 36, 48hr) = 448 animals

In the case that we receive a request from reviewers to repeat the IVC ligation procedure with a minor addition such as a therapeutic compound, neutralizing antibody or additional stimuli etc., we estimate that we would need approximately 168 additional animals, based on the calculations above. These animals will be used only if specifically requested by an editor/reviewer in order to increase the chances of a successful publication which would ensure the dissemination of these data within the scientific community.

**C. Re-use**

Will the animals be re-used?

X 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'?

X 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: Due to the complex nature of VT, which involves several different biological systems including the circulatory, immune and digestive system, as well as the physical interaction between flowing blood and the vessel wall, it cannot be accurately replicated *in vitro*. Thus an *in vivo* system, which includes interactions between all such factors shortly after the induction of injury and thrombi formation, is essential in studying this process. In addition, the ability to isolate the actual thrombi for analysis will provide critical clues as to the mechanism driving pathogenesis. However, once we have conducted the studies contained in this proposal, it is likely that we will be able to pinpoint the cellular players and signalling pathways that are central to this process, specifically in relation to SLC44A2, and

we can then follow up using *in vitro* models with human tissues to confirm and therefore strengthen our conclusions.

Reduction:
In order to reduce the number of animals necessary, we have taken several steps and carefully considered all options. First, we are making use of littermates so as to reduce the chance of genetic variability. Second, we will save most organs and materials from the mice and not only the tissues that are classically involved in haemostasis. In this way, if the data points to a novel finding and further analysis is needed in secondary organs, we will already have them and the need to include more animals may not be necessary. Lastly, based on power calculations and experience with these types of procedures, we have determined the optimal amount of animals to include in these studies so that a significant difference between experimental conditions can be found (if one exists) and no repetition will likely be needed. Additionally, we plan to only use females so as to reduce the number of mice included. We have also contemplated as to whether another experimental design can be used in order to minimize the requested numbers or the overall distress to the animals. However, the model proposed in this study is one of the best available in the field to study venous thrombosis and when combined with the secondary model of spontaneous induced thrombosis (appendix 3.4.4.4), we believe it will cover all aspects that may be key in VT pathogenesis.

Refinement:
Several practices are put in place in order to minimize any pain or discomfort for the animals during the proposed studies. Anaesthesia will be administered prior to the surgery as well as before the final collection of tissues, so that no pain will be felt during these procedures and for mice receiving bone marrow transplantation, anaesthesia will be administered prior to the irradiation of recipient mice. In addition, to minimize any post-operative discomfort, analgesia will also be given peri-operatively and up until the time of collection. These studies will also be conducted by trained researchers with experience performing these specific techniques. As there may be complications related to the surgery, we will closely monitor the response of the animals at several time points and if we observe any discomfort associated with humane endpoints such as changes in behaviour (posture, grooming, activity) or pathophysical reactions (rapid breath, unusual heart rate, weight loss) we will end the experiment immediately. In addition, as the irradiation may weaken the animals, we will provide special housing conditions including longer nipples on the water bottles, which will contain antibiotics, as well as soft food to ease accessibility for eating and drinking.

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

The measures taken to minimize animal suffering, pain and fear in regards to the proposed studies include pre-treatment of the animals with the appropriate anaesthesia before surgery or collection of tissues in addition to peri-operative treatment with analgesia. There are no adverse effects on the environment resulting from these studies.

## 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.

Not applicable.

## 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

X 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?

X 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.

x Yes > Will anaesthesia, analgesia or other pain relieving methods be used?

☐ No > Justify why pain relieving methods will not be used.

x Yes > Indicate what relieving methods will be used and specify what measures will be taken to ensure that optimal procedures are used.

All experimental animals will be anaesthetised prior to surgery, irradiation or the collection of blood or tissues which will subsequently be used for characterization of the mice. Donor animals will be euthanized using carbon dioxide asphyxiation before the collection of bone marrow. In addition, mice will receive analgesia peri-operatively up until the time of sacrifice. The procedures are deemed optimal based on in house experience with these techniques.

**I. Other aspects compromising the welfare of the animals**

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

Possible adverse effects that may occur during these procedures include bleeding during the IVC surgery or infections after the surgery. Adverse effects that may arise due to the bone marrow transplant procedure are either graft versus host disease or alternatively, host versus graft disease in which T-cells become reactive and attack the tissues of the host or graft, respectively. In addition, the effects of irradiation may also result in anaemia, immunosuppression, diarrhea and damage to the mucosal membranes.

Explain why these effects may emerge.

These effects may emerge if there is an accidental puncture of vasculature tissue or the surgical environment is not completely aseptic. In regards to adverse effects associated with bone marrow transplantation, this occurs when the MHC molecules are different between the host and donor mice.

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

In order to minimize surgical errors during, we have included a number of animals in order to refine our techniques before the final studies begin. To minimize the chance of infection, we plan to follow established protocols to ensure the surgery is aseptic as possible. In order to minimize the chance of rejection of transplantation, we are using litter mates both as donor and recipient in mice that are backcrossed to C57BL/6 background several generations. We expect the genetic variability to be very limited between such animals which will reduce the chance of mismatched MHC expression on the tissues. Animals will also be placed in special housing conditions (see section D, refinement) 3 weeks post irradiation in preparation for the adverse effects associated with the procedure.

**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.

X Yes > Describe the criteria that will be used to identify the humane endpoints.

Criteria for humane endpoints during this part of the study include behavioural changes in posture, grooming or activity levels in addition to pathophysical reactions such as rapid breathing, weight loss greater than 15% or HBV activation following radiation.

Indicate the likely incidence.

We estimate the incidence to be approximately 15% for IVC ligation alone and approximately 10% for bone marrow transplantations.

**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 levels of discomfort are moderate for all animals (100%) due to the inclusion of a surgery which involves suturing the abdomen of the mice. In addition, the possibility for a thrombosis formation may also cause discomfort, especially if there is a rapid onset, and is considered to cause moderate discomfort. With respect to bone marrow transplantation, 100% of donor animals fall under the category of "non-recovery" since they will be euthanized before the bone marrow is isolated. Recipient animals will undergo surgery and will therefore experience moderate discomfort (100%). The mice will be given the appropriate anaesthesia and analgesia to minimize this as much as possible.

## End of experiment

**L. Method of killing**

Will the animals be killed during or after the procedures?

☐ No

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

Animals are killed and used amongst others for full pathological analysis

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

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

x 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. | [redacted] |
| 1.3 | List the serial number and type of animal procedure.<br><br>*Use the serial numbers provided in Section 3.4.4 of the Project Proposal form.* | Serial number: 3.4.4.4<br>Type of animal procedure: Spontaneous venous thrombosis through siRNA |

## 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.

Mice lacking SLC44A2 expression, either whole body, in certain cellular compartments (through bone marrow transplantation) or cell type specific (endothelial cells or neutrophils), in addition to mice that have been transfused with SLC44A2 targeting antibodies, will receive an injection of siRNA targeting pro-coagulation proteins (antithrombin and protein C), which will induce formation of spontaneous venous thrombosis (VT). This will help to further determine the role of SLC44A2 in the pathogenesis of VT and overcome the limitations of the partial ligation model (appendix 3.4.4.3), namely the retrograde formation of thrombi and overestimation of the involvement of the immune system, as it is a major surgery. Blood and tissue samples from several organs central to the process of VT will be collected at different time points in order to record changes during key stages of disease progression. This may include, but is not limited to, material such as blood, liver, lungs, thrombus and venous tissue. Subsequently, these samples can be used to determine the contributions of SLC44A2 to VT. Using such procedures we will be able to measure differences in the capacity of the blood to coagulate and related cells to produce coagulation factors, changes in the immunological profile as well as characterize changes in thrombus formation/clearance and fibrin deposition in the liver.

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

The mice will receive an injection of solution containing siRNA lipidoids (based on the volumes set forth by the directive Diehl et al 2001) into the tail vein. After 36 hours, the mice will begin to receive analgesia and will be monitored regularly for signs of discomfort up until the time of sacrifice. Collection of the mice will occur at 24, 48 and 72 hours post injection. These time points are chosen in order to

observe the stages of VT propagation such as onset/initiation and progression. At these stages, mice are anesthetized and a midline laparotomy will be made. For the collection of blood, it will be taken directly from the vena cava into a syringe containing sodium citrate so as to preserve the sample. Blood must be collected directly from the vein in order to avoid contamination with factors that activate coagulation (i.e. tissue factor) as it will be used for testing exactly that at a later time. The amount of blood retrieved will be the maximal amount possible, therefore following collection, the diaphragm of the mouse will be punctured, euthanizing the mouse in a humane manner. After this point, several tissues will be collected and snap frozen, including the head which will contain the newly formed thrombus. These can then be used to detect differences both at the mRNA and protein level in addition to histological analysis which can be used to characterize changes in the thrombi as well as fibrin deposition. For the studies that involve antibody treatment, the mice will undergo the relevant procedures (detailed in appendices 3.4.4.2 ~~or 3.4.4.5~~) prior to the injection of siRNA.
For the studies involving bone marrow transplantation, donor mice will be euthanized using carbon dioxide asphyxiation and bone marrow cells from femur, tibias and humerus bones will be isolated and prepared for injection into the recipient mice. Bone marrow transplant recipients will begin receiving acidified, antibiotic water one week prior to the irradiation procedure. At least six hours before receiving the transplantation, they will be sub lethally irradiated at a dose suitable for their background (C57BL/6 mice). Following radiation, mice will receive a tail vein injection of the donor mouse bone marrow cells and subsequently housed for 21 days until the repopulation of hematopoietic cells is complete. At this point, the mice will then be ready to include in the spontaneous siRNA model described above.

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

Group size: For the calculation of experimental group size we will use the parameter 'development of clinical phenotype' (and not a parameters like liver fibrin deposition), as this is the typical hallmark of the spontaneous thrombosis model and almost always coincides with the presence of thrombi in the large veins of the heads. Previously, 48 hours after siRNA injection, we observed effects of onset and incidence of the clinical phenotype ranging from 1. nearly full absence of phenotype as compared to siRNA injected control animals) to 2. accelerated onset for nearly all animals of a given condition as compared to siRNA injected control animals (factor XII inhibition, presence of Factor V Leiden). Typical group size in these experiments was 10 animals per group. Using Fisher's exact based POWER calculations, statistically significant changes are detected when in one group 8 out of 10 animals follow the black and white response which is the display of visible symptoms of the spontaneous thrombotic phenotype, while in the reference group 8 out of 10 do not respond and remain fully normal (resulting in P=0.023 (2-tail)). http://www.langsrud.com/stat/fisher.htm. Subsequently, these numbers (n=10) also allow to detect statistically significant changes in liver fibrin deposition (using non-parametric Mann Witney tests) and allow to describe biologically relevant changes in thrombus incidence (Fisher's exact tests), severity, structure and cellular composition.

Sex consideration: Unless we determine a clear link between the *Slc44a2* gene and sex in the initial characterization described in appendices 3.4.4.1-2, we will only use females for the following procedures. We opt for female over male since there is less biological variability in measures such as size and weight. This is particularly relevant for this model as we will inject the same amount of siRNA per mice so they must be as closely weight matched as possible. Female mice can also be housed together even though they are not littermates (males cannot) which also helps to minimize any differences that may arise due to housing conditions. Furthermore, the reason we choose to continue with only one sex (female) and not pooled sexes is that it is well established (Alvarado et al Thromb Res 2011; Vanlangenvelde et al Comp Med 2005) that the biological response of males vs. females in murine models of VT is significantly different as demonstrated by several key factors including thrombus size, cytokine expression, blood cell counts, etc. Therefore, regardless of the characterization of SLC44A2 in hemostasis between the sexes, we cannot justifiably group males and females together in the subsequent VT models, as the bi-distributional readouts may obscure true differences which may be due to the gene alone.

**B. The animals**

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

The initial studies will include *Mus musculus* from a C57BL/6J background, containing either a deleted region (exon 3-10) of the *Slc44a2* gene on both alleles (KO), one allele (HET) or both wild type alleles (WT), globally in all tissues of the animal. They originate from the lab of [redacted] at the University of Michigan but have been breed at the LUMC for many generations. In addition, the final studies will

employ mice lacking the *Slc44a2* gene only in specific cell types. They will be generated by cross breeding *Slc44a2* floxed mice, containing loxP sites on either side of exons 3-10, with mice expressing Cre-recombinase under the control of either the *Tie-2* or *LysM* promoter, resulting in mice lacking SLC44A2 in endothelial cells or neutrophils, respectively. To control for genetic variation as much as possible, we will make use of matched litter mate siblings, generated from parents who are heterozygous for the KO allele. The mice will be included between ages of 8-14 weeks. We estimate that the maximal number of animals needed to complete all aspects of the studies included in this proposal regarding siRNA spontaneous induced thrombosis to be approximately 540. If the response of mice in appendices 3.4.4.1-2 is determined to be dependent on sex (unlikely), then we will also include 540 males.

Training and set up of procedure:
10 WT animals

siRNA spontaneous induction of thrombosis in global knockout animals:
3 genotypes (WT, HET, KO) x 10 group size x 1 sex (female) x 3 timepoints (24, 48, 72hr) = 90 animals

siRNA spontaneous induction of thrombosis with anti-SLC44A2 antibodies:
3 genotypes (WT, HET, KO) x 10 group size x 1 sex (female) x 3 timepoints (24, 48, 72hr) = 90 animals

siRNA spontaneous induction of thrombosis in cell specific knockout animals:
2 genotypes (WT or KO) x 2 cell type (endothelial or neutrophil) x 10 group size x 1 sex (female) x 3 timepoints (24, 48, 72hr) = 120 animals

siRNA spontaneous induction of thrombosis in bone marrow transplantation animals:
4 systems (WT, WT hemo/KO stromal, KO hemo/WT stromal, KO) x 2 (donor, recipient) x 10 group size x 1 sex (female) x 3 timepoints (24, 48, 72hr) = 240 animals

In the case that we receive a request from reviewers to repeat the siRNA spontaneous induction of thrombosis procedure with a minor addition such as a therapeutic compound, neutralizing antibody or additional stimuli etc., we estimate that we would need approximately 90 additional animals, based on the calculations above. These animals will be used only if specifically requested by an editor/reviewer in order to increase the chances of a successful publication which would ensure the dissemination of these data within the scientific community.

**C. Re-use**

Will the animals be re-used?

X 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'?

X 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: Due to the complex nature of VT, which involves several different biological systems including the circulatory, immune and digestive system, as well as the physical interaction between flowing blood and the vessel wall, it cannot be accurately replicated *in vitro*. Thus an *in vivo* system, which includes interactions between all such factors, especially with spontaneous formation of thrombi, is essential in studying this process. In addition, the ability to isolate the actual thrombi for analysis will provide critical clues as to the mechanism driving pathogenesis. However, once we have conducted the studies contained in this proposal, it is likely that we will be able to pinpoint the cellular players and signalling pathways that are central to this process, specifically in relation to SLC44A2, and we can then follow up using *in vitro* models with human tissues to confirm and therefore strengthen our conclusions.

Reduction:
In order to reduce the number of animals necessary, we have taken several steps and carefully considered all options. First, we are making use of littermates so as to reduce the chance of genetic variability. Second, we will save most organs and materials from the mice and not only the tissues that are classically involved in haemostasis. In this way, if the data points to a novel finding and further analysis is needed in secondary organs, we will already have them and the need to include more animals may not be necessary. Lastly, based on power calculations and experience with these types of procedures, we have determined the optimal amount of animals to include in these studies so that a significant difference between experimental conditions can be found (if one exists) and no repetition will likely be needed. Additionally, we plan to only use females so as to reduce the number of mice included. We have also contemplated as to whether another experimental design can be used in order to minimize the requested numbers or the overall distress to the animals. However, we believe that the two models proposed in this study (also appendix 3.4.4.3), used in combination, are the best strategy to research venous thrombosis and cover all aspects that may be key in VT pathogenesis.

Refinement:
Several practices are put in place in order to minimize any pain or discomfort for the animals during the proposed studies. Anaesthesia will be administered prior to the final collection of tissues, so that no pain will be felt during these procedures and for mice receiving bone marrow transplantation, anaesthesia will be administered prior to the irradiation of recipient mice. We also follow the guidelines set forth in the directive Diehl et al 2001 when determining the appropriate injection volumes. In addition, to minimize any discomfort that may arise due to the induction of VT, analgesia will also be given 36 hours post-injection and up until the time of collection, we do not expect full thrombi formation to occur before 36 hours . These studies will also be conducted by trained researchers with experience performing these specific techniques. We also plan to closely monitor the response of the animals at several time points after the injection and if we observe any discomfort associated with humane endpoints such as changes in behaviour (posture, grooming, activity) or pathophysical reactions (rapid breath, unusual heart rate, weight loss) we will end the experiment immediately. In addition, as the irradiation may weaken the animals, we will provide special housing conditions including longer nipples on the water bottles, which will contain antibiotics, as well as soft food to ease accessibility for eating and drinking.

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

The measures taken to minimize animal suffering, pain and fear in regards to the proposed studies include follow up treatment with analgesia continuously until sacrifice in addition to pre-treatment of the animals with the appropriate anaesthesia before collection of tissues. There are no adverse effects on the environment resulting from these studies.

## 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.

Not applicable.

## 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

X 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?

X 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.

x Yes > Will anaesthesia, analgesia or other pain relieving methods be used?

☐ No > Justify why pain relieving methods will not be used.

x Yes > Indicate what relieving methods will be used and specify what measures will be taken to ensure that optimal procedures are used.

All experimental animals will be anaesthetised prior to irradiation and the collection of blood or tissues which will subsequently be used for characterization of the mice. Donor animals will be euthanized using carbon dioxide asphyxiation before the collection of bone marrow. Mice will also begin to receive analgesia 36 hours post-injection and continuously up until the point of sacrifice. These procedures are deemed optimal based on experience with such techniques within the department.

**I. Other aspects compromising the welfare of the animals**

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

As we do not fully know the extent to which SLC44A2 is contributing to VT, it is possible that it plays a role in anticoagulation, in which case, the onset of the thrombus formation in knockout mice may be accelerated. This would result in visible symptoms including exophthalmos, peri-ocular haemorrhages or edema in the mandibular region or possibly death prior to the planned time points. Adverse effects that may arise due to the bone marrow transplant procedure are either graft versus host disease or alternatively, host versus graft disease in which T-cells become reactive and attack the tissues of the host or graft, respectively. In addition, the effects of irradiation may also result in anaemia, immunosuppression, diarrhea and damage to the mucosal membranes.

Explain why these effects may emerge.

These effects emerge as anticoagulation proteins are lowered and may be accelerated in the case that SLC44A2 plays a role in VT. In regards to adverse effects associated with bone marrow transplantation, this occurs when the MHC molecules are different between the host and donor mice.

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

We are prepared for such an outcome and have planned to minimize the severity of such an event by administering analgesia after the injection of siRNAs in case of discomfort from bleeding, in addition to regular monitoring of the mice for any signs of severe discomfort associated with humane endpoints. In order to minimize the chance of rejection of transplantation, we are using litter mates both as donor and recipient in mice that are backcrossed to C57BL/6 background several generations. We expect the genetic variability to be very limited between such animals which will reduce the chance of mismatched MHC expression on the tissues. Animals will also be placed in special housing conditions (see section D, refinement) 3 weeks post irradiation in preparation for the adverse effects associated with the procedure.

**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.

X Yes > Describe the criteria that will be used to identify the humane endpoints.

Criteria for humane endpoints during this part of the study include behavioural changes in posture, grooming or activity levels in addition to pathophysical reactions such as rapid breathing, weight loss greater than 15% compared to starting weight prior to injection, exophthalmos, peri-ocular haemorrhages or edema in the mandibular region indicating a possible veinous rupture in the head. Additionally for mice with bone marrow transplant, HBV activation following radiation.

Indicate the likely incidence.

The incidence can range anywhere from 20% to 80% depending on the importance of SLC44A2 in the propagation of thrombosis, which is currently unknown. In the case that SLC44A2 does not play a role in VT we expect to see approximately 80% incidence as this is generally what we expect to observe in the control group. Similarly, if it exacerbates the VT phenotype, we expect to observe similar occurrence (80%), only at an earlier time point which is why we include several observation points after injection. If the loss of SLC44A2 has a protective role then we expect to observe incidence of approximately 20%. At the onset of any sign of exophthalmos, peri-ocular haemorrhages or edema in the mandibular region, the animal will be euthanized immediately.

**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 siRNAs targeting anticoagulation proteins will result in the formation of large thrombi in large veins of the head of the mouse which may result in intra/periocular haemorrhages. Based on these known outcomes, the procedures are deemed to include moderate discomfort for 100% of the animals and will thus include the proper analgesia to prevent the discomfort as much as possible. With respect to bone marrow transplantation, 100% of the donor animals fall under the category of "non-recovery" since they will be euthanized before the bone marrow is isolated. Recipient animals will undergo the siRNA model and will therefore experience moderate discomfort (100%). The mice will be given the appropriate anaesthesia and analgesia to minimize this as much as possible.

## End of experiment

**L. Method of killing**

Will the animals be killed during or after the procedures?

☐ No

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

Animals are killed and used amongst others for full pathological analysis

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

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

x 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. | [redacted] |

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 | Type of animal procedure |
|---|---|
| 3.4.4.3 | Venous thrombosis through partial ligation of the caval vein |

## 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.

Mice lacking SLC44A2 expression, either whole body, in certain cellular compartments (through bone marrow transplantation), cell type specific (endothelial cells or neutrophils) or in mice that have been transfused with SLC44A2 targeting antibodies, will undergo a partial ligation procedure in order to mimic the process of venous thrombosis (VT) *in vivo* and to dissect out the cell types and signalling pathways which link SLC44A2 to disease pathogenesis. Blood and tissue samples from several organs central to the process of VT will be collected at different time points in order to record changes during key stages of disease progression. This may include, but is not limited to, material such as blood, liver, lungs, thrombus and venous tissue. Subsequently, these samples can be used to determine the contributions of SLC44A2 to VT. Using such procedures we will be able to measure differences in the capacity of the blood to coagulate and related cells to produce coagulation factors, changes in the immunological profile as well as characterize changes in thrombus formation/clearance and fibrin deposition.

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

Following anaesthetization of the mice and the administration of analgesia, a midline laparotomy will be made and the small bowel exteriorized and placed to the left of the animal. The inferior vena cava (IVC or caval vein) is then exposed by careful blunt dissection while sterile saline is applied at regular intervals to the exteriorized bowel to prevent its desiccation. Non-reactive prolene suture is looped around the IVC immediately caudal to the origin of the renal veins, with a space holder included on the outside of the vessel. Next the ligature is closed and the space holder is removed to avoid complete vessel occlusion.

Side branches will not be ligated or manipulated and IVC blood flow will be reduced immediately after the restriction. Lastly, the median laparotomy is sutured and the mice will be placed on a warming mat until anaesthesia wears off. Mice will additionally receive peri-operative analgesia until collection at at the 12, 24, 36 and 48hr designated time points . For the collection of blood and tissues, mice will receive a lethal dose of anaesthesia and subsequently several tissues will be collected, including the vena cava with the newly formed thrombus, and snap frozen. These can then be used to detect differences in expression levels of both mRNA and protein, as well as histological analysis of thrombus formation and fibrin deposition. The mice receiving anti-SLC44A2 antibodies will undergo all procedures described above in addition to receiving an infusion of antibodies prior to the surgery (appendix 3.4.4.2).
For the studies involving bone marrow transplantation, donor mice will be euthanized using standard techniques and bone marrow cells isolated and prepared for injection into the recipient mice. Bone marrow transplant recipients will begin receiving acidified, antibiotic water one week prior to the irradiation procedure. At least six hours before receiving the transplantation, they will be sub lethally irradiated at a dose suitable for their background (C57BL/6 mice). Following radiation, mice will receive a tail vein injection of the donor mouse bone marrow cells and subsequently housed for 21 days until the repopulation of hematopoietic cells is complete. At this point, the mice will then be ready to include in the ligation model described above.

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

Group size: The calculation of the experimental group size is based on the parameter 'thrombosis formation' (and not mRNA analysis) as this is the primary outcome and the parameter with the largest standard deviation. Previously, at 24 hours post thrombosis induction, we observed effects on thrombus size and weight ranging from 75% decrease up to 200% increase in size/weight as compared to control reference groups. Using these observations we can estimate that the control group has an average thrombi weight of 1g and a second group representing the knockout mice would have less thrombi, around .25g and a third group may have increased thrombi, up to double the weight of group 1, being 2g. As the readout will be comparing three groups, we will use a one way ANOVA test which will control for the type I error. Using the G*Power statistical software (http://www.gpower.hhu.de/) program to perform a POWER calculation with an alpha of 0.05 and a power of 0.80, we find the effect size f to be 0.55143 which would require a sample size of 36, signifying 12 animals per group. Because we want to rule out endothelial injury as a trigger for venous thrombosis, mice with bleedings or any injury of the IVC during surgery need to be excluded from further analysis. We therefore include an extra of 2 animals per group to compensate for animals displaying bleedings or any injury during the IVC during surgery. These 2 extra animals will only be included when necessary. For now we will use a maximum of 14 animals per experimental group.

Sex consideration: Unless we determine a clear link between the *Slc44a2* gene and sex in the initial characterization described in appendices 3.4.4.1-2, we will only use females for the following procedures. We opt for female over male since there is less biological variability in measures such as size and weight. Female mice can also be housed together even though they are not littermates (males cannot) which also helps to minimize any differences that may arise due to housing conditions. Furthermore, the reason we choose to continue with only one sex (female) and not pooled sexes is that it is well established (Alvarado et al Thromb Res 2011; Vanlangenvelde et al Comp Med 2005) that the biological response of males vs. females in murine models of VT is significantly different as demonstrated by several key factors including thrombus size, cytokine expression, blood cell counts, etc. Therefore, regardless of the characterization of SLC44A2 in hemostasis between the sexes, we cannot justifiably group males and females together in the subsequent VT models, as the bi-distributional readouts may obscure true differences which may be due to the gene or antibodies alone.

**B. The animals**

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

The initial studies will include *Mus musculus* from a C57BL/6J background, containing either a deleted region (exon 3-10) of the *Slc44a2* gene on both alleles (KO), one allele (HET) or both wild type alleles (WT), globally in all tissues of the animal. They originate from the lab of [redacted] at the University of Michigan but have been breed at the LUMC for many generations. In addition, the final studies will employ mice lacking the *Slc44a2* gene only in specific cell types. They will be generated by cross

breeding *Slc44a2* floxed mice, containing loxP sites on either side of exons 3-10, with mice expressing Cre-recombinase under the control of either the *Tie-2* or *LysM* promoter, resulting in mice lacking SLC44A2 in endothelial cells or neutrophils, respectively. To control for genetic variation as much as possible, we will make use of matched litter mate siblings, generated from parents who are heterozygous for the KO allele. The mice will be included between ages of 8-14 weeks. We estimate that the number of animals needed to complete all aspects of the studies included in this proposal regarding IVC ligation to be approximately 1008 (no sex response in appendices 3.4.4.1-2) to 2016 (yes sex response in appendices 3.4.4.1-2).

Training and set up of procedure:
10 WT animals

IVC ligation-induced thrombosis with global knockout animals:
3 genotypes (WT, HET, KO) x 14 group size x 1 sex (female) x 4 timepoints (12, 24, 36, 48hr) = 168 animals

IVC ligation-induced thrombosis with anti-SLC44A2 antibodies:
3 genotypes (WT, HET, KO) x 14 group size x 1 sex (female) x 4 timepoints (12, 24, 36, 48hr) = 168 animals

IVC ligation-induced thrombosis with cell specific knockout animals:
2 genotypes (WT or KO) x 2 cell type (endothelial or neutrophil) x 14 group size x 1 sex (female) x 4 timepoints (12, 24, 36, 48hr) = 224 animals

IVC ligation-induced thrombosis with bone marrow transplantation :
4 systems (WT, WT hemo/KO stromal, KO hemo/WT stromal, KO) x 2 (donor, recipient) x 14 group size x 1 sex (female) x 4 timepoints (12, 24, 36, 48hr) = 448 animals

In the case that we receive a request from reviewers to repeat the IVC ligation procedure with a minor addition such as a therapeutic compound, neutralizing antibody or additional stimuli etc., we estimate that we would need approximately 168 additional animals, based on the calculations above. These animals will be used only if specifically requested by an editor/reviewer in order to increase the chances of a successful publication which would ensure the dissemination of these data within the scientific community.

**C. Re-use**

Will the animals be re-used?

X 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'?

X 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: Due to the complex nature of VT, which involves several different biological systems including the circulatory, immune and digestive system, as well as the physical interaction between flowing blood and the vessel wall, it cannot be accurately replicated *in vitro*. Thus an *in vivo* system, which includes interactions between all such factors shortly after the induction of injury and thrombi formation, is essential in studying this process. In addition, the ability to isolate the actual thrombi for analysis will provide critical clues as to the mechanism driving pathogenesis. However, once we have conducted the studies contained in this proposal, it is likely that we will be able to pinpoint the cellular players and signalling pathways that are central to this process, specifically in relation to SLC44A2, and

we can then follow up using *in vitro* models with human tissues to confirm and therefore strengthen our conclusions.

Reduction:
In order to reduce the number of animals necessary, we have taken several steps and carefully considered all options. First, we are making use of littermates so as to reduce the chance of genetic variability. Second, we will save most organs and materials from the mice and not only the tissues that are classically involved in haemostasis. In this way, if the data points to a novel finding and further analysis is needed in secondary organs, we will already have them and the need to include more animals may not be necessary. Lastly, based on power calculations and experience with these types of procedures, we have determined the optimal amount of animals to include in these studies so that a significant difference between experimental conditions can be found (if one exists) and no repetition will likely be needed. Additionally, we plan to only use females so as to reduce the number of mice included. We have also contemplated as to whether another experimental design can be used in order to minimize the requested numbers or the overall distress to the animals. However, the model proposed in this study is one of the best available in the field to study venous thrombosis and when combined with the secondary model of spontaneous induced thrombosis (appendix 3.4.4.4), we believe it will cover all aspects that may be key in VT pathogenesis.

Refinement:
Several practices are put in place in order to minimize any pain or discomfort for the animals during the proposed studies. Anaesthesia will be administered prior to the surgery as well as before the final collection of tissues, so that no pain will be felt during these procedures and for mice receiving bone marrow transplantation, anaesthesia will be administered prior to the irradiation of recipient mice. In addition, to minimize any post-operative discomfort, analgesia will also be given peri-operatively and up until the time of collection. These studies will also be conducted by trained researchers with experience performing these specific techniques. As there may be complications related to the surgery, we will closely monitor the response of the animals at several time points and if we observe any discomfort associated with humane endpoints such as changes in behaviour (posture, grooming, activity) or pathophysical reactions (rapid breath, unusual heart rate, weight loss) we will end the experiment immediately. In addition, as the irradiation may weaken the animals, we will provide special housing conditions including longer nipples on the water bottles, which will contain antibiotics, as well as soft food to ease accessibility for eating and drinking.

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

The measures taken to minimize animal suffering, pain and fear in regards to the proposed studies include pre-treatment of the animals with the appropriate anaesthesia before surgery or collection of tissues in addition to peri-operative treatment with analgesia. There are no adverse effects on the environment resulting from these studies.

## 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.

Not applicable.

## 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

X 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?

X 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.

x Yes > Will anaesthesia, analgesia or other pain relieving methods be used?

☐ No > Justify why pain relieving methods will not be used.

x Yes > Indicate what relieving methods will be used and specify what measures will be taken to ensure that optimal procedures are used.

All experimental animals will be anaesthetised prior to surgery, irradiation or the collection of blood or tissues which will subsequently be used for characterization of the mice. Donor animals will be euthanized using carbon dioxide asphyxiation before the collection of bone marrow. In addition, mice will receive analgesia peri-operatively up until the time of sacrifice. The procedures are deemed optimal based on in house experience with these techniques.

**I. Other aspects compromising the welfare of the animals**

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

Possible adverse effects that may occur during these procedures include bleeding during the IVC surgery or infections after the surgery. Adverse effects that may arise due to the bone marrow transplant procedure are either graft versus host disease or alternatively, host versus graft disease in which T-cells become reactive and attack the tissues of the host or graft, respectively. In addition, the effects of irradiation may also result in anaemia, immunosuppression, diarrhea and damage to the mucosal membranes.

Explain why these effects may emerge.

These effects may emerge if there is an accidental puncture of vasculature tissue or the surgical environment is not completely aseptic. In regards to adverse effects associated with bone marrow transplantation, this occurs when the MHC molecules are different between the host and donor mice.

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

In order to minimize surgical errors during, we have included a number of animals in order to refine our techniques before the final studies begin. To minimize the chance of infection, we plan to follow established protocols to ensure the surgery is aseptic as possible. In order to minimize the chance of rejection of transplantation, we are using litter mates both as donor and recipient in mice that are backcrossed to C57BL/6 background several generations. We expect the genetic variability to be very limited between such animals which will reduce the chance of mismatched MHC expression on the tissues. Animals will also be placed in special housing conditions (see section D, refinement) 3 weeks post irradiation in preparation for the adverse effects associated with the procedure.

**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.

X Yes > Describe the criteria that will be used to identify the humane endpoints.

Criteria for humane endpoints during this part of the study include behavioural changes in posture, grooming or activity levels in addition to pathophysical reactions such as rapid breathing, weight loss greater than 15% or HBV activation following radiation.

Indicate the likely incidence.

We estimate the incidence to be approximately 15% for IVC ligation alone and approximately 10% for bone marrow transplantations.

**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 levels of discomfort are moderate for all animals (100%) due to the inclusion of a surgery which involves suturing the abdomen of the mice. In addition, the possibility for a thrombosis formation may also cause discomfort, especially if there is a rapid onset, and is considered to cause moderate discomfort. With respect to bone marrow transplantation, 100% of donor animals fall under the category of "non-recovery" since they will be euthanized before the bone marrow is isolated. Recipient animals will undergo surgery and will therefore experience moderate discomfort (100%). The mice will be given the appropriate anaesthesia and analgesia to minimize this as much as possible.

## End of experiment

**L. Method of killing**

Will the animals be killed during or after the procedures?

☐ No

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

Animals are killed and used amongst others for full pathological analysis

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

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

x 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. | [redacted] |
| 1.3 | List the serial number and type of animal procedure.<br><br>*Use the serial numbers provided in Section 3.4.4 of the Project Proposal form.* | Serial number: 3.4.4.4<br>Type of animal procedure: Spontaneous venous thrombosis through siRNA |

## 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.

Mice lacking SLC44A2 expression, either whole body, in certain cellular compartments (through bone marrow transplantation) or cell type specific (endothelial cells or neutrophils), in addition to mice that have been transfused with SLC44A2 targeting antibodies, will receive an injection of siRNA targeting pro-coagulation proteins (antithrombin and protein C), which will induce formation of spontaneous venous thrombosis (VT). This will help to further determine the role of SLC44A2 in the pathogenesis of VT and overcome the limitations of the partial ligation model (appendix 3.4.4.3), namely the retrograde formation of thrombi and overestimation of the involvement of the immune system, as it is a major surgery. Blood and tissue samples from several organs central to the process of VT will be collected at different time points in order to record changes during key stages of disease progression. This may include, but is not limited to, material such as blood, liver, lungs, thrombus and venous tissue. Subsequently, these samples can be used to determine the contributions of SLC44A2 to VT. Using such procedures we will be able to measure differences in the capacity of the blood to coagulate and related cells to produce coagulation factors, changes in the immunological profile as well as characterize changes in thrombus formation/clearance and fibrin deposition in the liver.

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

The mice will receive an injection of solution containing siRNA lipidoids (based on the volumes set forth by the directive Diehl et al 2001) into the tail vein. After 36 hours, the mice will begin to receive analgesia and will be monitored regularly for signs of discomfort up until the time of sacrifice. Collection of the mice will occur at 24, 48 and 72 hours post injection. These time points are chosen in order to

observe the stages of VT propagation such as onset/initiation and progression. At these stages, mice are anesthetized and a midline laparotomy will be made. For the collection of blood, it will be taken directly from the vena cava into a syringe containing sodium citrate so as to preserve the sample. Blood must be collected directly from the vein in order to avoid contamination with factors that activate coagulation (i.e. tissue factor) as it will be used for testing exactly that at a later time. The amount of blood retrieved will be the maximal amount possible, therefore following collection, the diaphragm of the mouse will be punctured, euthanizing the mouse in a humane manner. After this point, several tissues will be collected and snap frozen, including the head which will contain the newly formed thrombus. These can then be used to detect differences both at the mRNA and protein level in addition to histological analysis which can be used to characterize changes in the thrombi as well as fibrin deposition. For the studies that involve antibody treatment, the mice will undergo the relevant procedures (detailed in appendices 3.4.4.2) prior to the injection of siRNA.
For the studies involving bone marrow transplantation, donor mice will be euthanized using carbon dioxide asphyxiation and bone marrow cells from femur, tibias and humerus bones will be isolated and prepared for injection into the recipient mice. Bone marrow transplant recipients will begin receiving acidified, antibiotic water one week prior to the irradiation procedure. At least six hours before receiving the transplantation, they will be sub lethally irradiated at a dose suitable for their background (C57BL/6 mice). Following radiation, mice will receive a tail vein injection of the donor mouse bone marrow cells and subsequently housed for 21 days until the repopulation of hematopoietic cells is complete. At this point, the mice will then be ready to include in the spontaneous siRNA model described above.

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

Group size: For the calculation of experimental group size we will use the parameter 'development of clinical phenotype' (and not a parameters like liver fibrin deposition), as this is the typical hallmark of the spontaneous thrombosis model and almost always coincides with the presence of thrombi in the large veins of the heads. Previously, 48 hours after siRNA injection, we observed effects of onset and incidence of the clinical phenotype ranging from 1. nearly full absence of phenotype as compared to siRNA injected control animals) to 2. accelerated onset for nearly all animals of a given condition as compared to siRNA injected control animals (factor XII inhibition, presence of Factor V Leiden). Typical group size in these experiments was 10 animals per group. Using Fisher's exact based POWER calculations, statistically significant changes are detected when in one group 8 out of 10 animals follow the black and white response which is the display of visible symptoms of the spontaneous thrombotic phenotype, while in the reference group 8 out of 10 do not respond and remain fully normal (resulting in P=0.023 (2-tail)). http://www.langsrud.com/stat/fisher.htm. Subsequently, these numbers (n=10) also allow to detect statistically significant changes in liver fibrin deposition (using non-parametric Mann Witney tests) and allow to describe biologically relevant changes in thrombus incidence (Fisher's exact tests), severity, structure and cellular composition.

Sex consideration: Unless we determine a clear link between the *Slc44a2* gene and sex in the initial characterization described in appendices 3.4.4.1-2, we will only use females for the following procedures. We opt for female over male since there is less biological variability in measures such as size and weight. This is particularly relevant for this model as we will inject the same amount of siRNA per mice so they must be as closely weight matched as possible. Female mice can also be housed together even though they are not littermates (males cannot) which also helps to minimize any differences that may arise due to housing conditions. Furthermore, the reason we choose to continue with only one sex (female) and not pooled sexes is that it is well established (Alvarado et al Thromb Res 2011; Vanlangenvelde et al Comp Med 2005) that the biological response of males vs. females in murine models of VT is significantly different as demonstrated by several key factors including thrombus size, cytokine expression, blood cell counts, etc. Therefore, regardless of the characterization of SLC44A2 in hemostasis between the sexes, we cannot justifiably group males and females together in the subsequent VT models, as the bi-distributional readouts may obscure true differences which may be due to the gene alone.

**B. The animals**

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

The initial studies will include *Mus musculus* from a C57BL/6J background, containing either a deleted region (exon 3-10) of the *Slc44a2* gene on both alleles (KO), one allele (HET) or both wild type alleles (WT), globally in all tissues of the animal. They originate from the lab of [redacted] at the University of Michigan but have been breed at the LUMC for many generations. In addition, the final studies will

employ mice lacking the *Slc44a2* gene only in specific cell types. They will be generated by cross breeding *Slc44a2* floxed mice, containing loxP sites on either side of exons 3-10, with mice expressing Cre-recombinase under the control of either the *Tie-2* or *LysM* promoter, resulting in mice lacking SLC44A2 in endothelial cells or neutrophils, respectively. To control for genetic variation as much as possible, we will make use of matched litter mate siblings, generated from parents who are heterozygous for the KO allele. The mice will be included between ages of 8-14 weeks. We estimate that the number of animals needed to complete all aspects of the studies included in this proposal regarding siRNA spontaneous induced thrombosis to be approximately 540 (no sex response in appendices 3.4.4.1-2) to 1080 (yes sex response in appendices 3.4.4.1-2).

Training and set up of procedure:
10 WT animals

siRNA spontaneous induction of thrombosis in global knockout animals:
3 genotypes (WT, HET, KO) x 10 group size x 1 sex (female) x 3 timepoints (24, 48, 72hr) = 90 animals

siRNA spontaneous induction of thrombosis with anti-SLC44A2 antibodies:
3 genotypes (WT, HET, KO) x 10 group size x 1 sex (female) x 3 timepoints (24, 48, 72hr) = 90 animals

siRNA spontaneous induction of thrombosis in cell specific knockout animals:
2 genotypes (WT or KO) x 2 cell type (endothelial or neutrophil) x 10 group size x 1 sex (female) x 3 timepoints (24, 48, 72hr) = 120 animals

siRNA spontaneous induction of thrombosis in bone marrow transplantation animals:
4 systems (WT, WT hemo/KO stromal, KO hemo/WT stromal, KO) x 2 (donor, recipient) x 10 group size x 1 sex (female) x 3 timepoints (24, 48, 72hr) = 240 animals

In the case that we receive a request from reviewers to repeat the siRNA spontaneous induction of thrombosis procedure with a minor addition such as a therapeutic compound, neutralizing antibody or additional stimuli etc., we estimate that we would need approximately 90 additional animals, based on the calculations above. These animals will be used only if specifically requested by an editor/reviewer in order to increase the chances of a successful publication which would ensure the dissemination of these data within the scientific community.

**C. Re-use**

Will the animals be re-used?

X 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'?

X 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: Due to the complex nature of VT, which involves several different biological systems including the circulatory, immune and digestive system, as well as the physical interaction between flowing blood and the vessel wall, it cannot be accurately replicated *in vitro*. Thus an *in vivo* system, which includes interactions between all such factors, especially with spontaneous formation of thrombi, is essential in studying this process. In addition, the ability to isolate the actual thrombi for analysis will provide critical clues as to the mechanism driving pathogenesis. However, once we have conducted the studies contained in this proposal, it is likely that we will be able to pinpoint the cellular players and signalling pathways that are central to this process, specifically in relation to SLC44A2, and we can then follow up using *in vitro* models with human tissues to confirm and therefore strengthen our conclusions.

Reduction:
In order to reduce the number of animals necessary, we have taken several steps and carefully considered all options. First, we are making use of littermates so as to reduce the chance of genetic variability. Second, we will save most organs and materials from the mice and not only the tissues that are classically involved in haemostasis. In this way, if the data points to a novel finding and further analysis is needed in secondary organs, we will already have them and the need to include more animals may not be necessary. Lastly, based on power calculations and experience with these types of procedures, we have determined the optimal amount of animals to include in these studies so that a significant difference between experimental conditions can be found (if one exists) and no repetition will likely be needed. Additionally, we plan to only use females so as to reduce the number of mice included. We have also contemplated as to whether another experimental design can be used in order to minimize the requested numbers or the overall distress to the animals. However, we believe that the two models proposed in this study (also appendix 3.4.4.3), used in combination, are the best strategy to research venous thrombosis and cover all aspects that may be key in VT pathogenesis.

Refinement:
Several practices are put in place in order to minimize any pain or discomfort for the animals during the proposed studies. Anaesthesia will be administered prior to the final collection of tissues, so that no pain will be felt during these procedures and for mice receiving bone marrow transplantation, anaesthesia will be administered prior to the irradiation of recipient mice. We also follow the guidelines set forth in the directive Diehl et al 2001 when determining the appropriate injection volumes. In addition, to minimize any discomfort that may arise due to the induction of VT, analgesia will also be given 36 hours post-injection and up until the time of collection, we do not expect full thrombi formation to occur before 36 hours . These studies will also be conducted by trained researchers with experience performing these specific techniques. We also plan to closely monitor the response of the animals at several time points after the injection and if we observe any discomfort associated with humane endpoints such as changes in behaviour (posture, grooming, activity) or pathophysical reactions (rapid breath, unusual heart rate, weight loss) we will end the experiment immediately. In addition, as the irradiation may weaken the animals, we will provide special housing conditions including longer nipples on the water bottles, which will contain antibiotics, as well as soft food to ease accessibility for eating and drinking.

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

The measures taken to minimize animal suffering, pain and fear in regards to the proposed studies include follow up treatment with analgesia continuously until sacrifice in addition to pre-treatment of the animals with the appropriate anaesthesia before collection of tissues. There are no adverse effects on the environment resulting from these studies.

## 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.

Not applicable.

## 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

X 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?

X 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.

x Yes > Will anaesthesia, analgesia or other pain relieving methods be used?

☐ No > Justify why pain relieving methods will not be used.

x Yes > Indicate what relieving methods will be used and specify what measures will be taken to ensure that optimal procedures are used.

All experimental animals will be anaesthetised prior to irradiation and the collection of blood or tissues which will subsequently be used for characterization of the mice. Donor animals will be euthanized using carbon dioxide asphyxiation before the collection of bone marrow. Mice will also begin to receive analgesia 36 hours post-injection and continuously up until the point of sacrifice. These procedures are deemed optimal based on experience with such techniques within the department.

**I. Other aspects compromising the welfare of the animals**

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

As we do not fully know the extent to which SLC44A2 is contributing to VT, it is possible that it plays a role in anticoagulation, in which case, the onset of the thrombus formation in knockout mice may be accelerated. This would result in visible symptoms including exophthalmos, peri-ocular haemorrhages or edema in the mandibular region or possibly death prior to the planned time points. Adverse effects that may arise due to the bone marrow transplant procedure are either graft versus host disease or alternatively, host versus graft disease in which T-cells become reactive and attack the tissues of the host or graft, respectively. In addition, the effects of irradiation may also result in anaemia, immunosuppression, diarrhea and damage to the mucosal membranes.

Explain why these effects may emerge.

These effects emerge as anticoagulation proteins are lowered and may be accelerated in the case that SLC44A2 plays a role in VT. In regards to adverse effects associated with bone marrow transplantation, this occurs when the MHC molecules are different between the host and donor mice.

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

We are prepared for such an outcome and have planned to minimize the severity of such an event by administering analgesia after the injection of siRNAs in case of discomfort from bleeding, in addition to regular monitoring of the mice for any signs of severe discomfort associated with humane endpoints. In order to minimize the chance of rejection of transplantation, we are using litter mates both as donor and recipient in mice that are backcrossed to C57BL/6 background several generations. We expect the genetic variability to be very limited between such animals which will reduce the chance of mismatched MHC expression on the tissues. Animals will also be placed in special housing conditions (see section D, refinement) 3 weeks post irradiation in preparation for the adverse effects associated with the procedure.

**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.

X Yes > Describe the criteria that will be used to identify the humane endpoints.

Criteria for humane endpoints during this part of the study include behavioural changes in posture, grooming or activity levels in addition to pathophysical reactions such as rapid breathing, weight loss greater than 15% compared to starting weight prior to injection, exophthalmos, peri-ocular haemorrhages or edema in the mandibular region indicating a possible veinous rupture in the head. Additionally for mice with bone marrow transplant, HBV activation following radiation.

Indicate the likely incidence.

The incidence can range anywhere from 20% to 80% depending on the importance of SLC44A2 in the propagation of thrombosis, which is currently unknown. In the case that SLC44A2 does not play a role in VT we expect to see approximately 80% incidence as this is generally what we expect to observe in the control group. Similarly, if it exacerbates the VT phenotype, we expect to observe similar occurrence (80%), only at an earlier time point which is why we include several observation points after injection. If the loss of SLC44A2 has a protective role then we expect to observe incidence of approximately 20%. At the onset of any sign of exophthalmos, peri-ocular haemorrhages or edema in the mandibular region, the animal will be euthanized immediately.

**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 siRNAs targeting anticoagulation proteins will result in the formation of large thrombi in large veins of the head of the mouse which may result in intra/periocular haemorrhages. Based on these known outcomes, the procedures are deemed to include moderate discomfort for 100% of the animals and will thus include the proper analgesia to prevent the discomfort as much as possible. With respect to bone marrow transplantation, 100% of the donor animals fall under the category of "non-recovery" since they will be euthanized before the bone marrow is isolated. Recipient animals will undergo the siRNA model and will therefore experience moderate discomfort (100%). The mice will be given the appropriate anaesthesia and analgesia to minimize this as much as possible.

## End of experiment

**L. Method of killing**

Will the animals be killed during or after the procedures?

☐ No

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

Animals are killed and used amongst others for full pathological analysis

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

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

x Yes

19.

Centrale Commissie Dierproeven

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**Onze referentie**
Aanvraagnummer
AVD116002016607
**Bijlagen**
1

Datum 12 juni 2017
Betreft Beslissing aanvraag projectvergunning Dierproeven

Geachte [redacted]

Op 11 mei 2017 hebben wij uw aanvraag voor een projectvergunning dierproeven ontvangen. Het gaat om uw project "The Role of the Solute Carrier Transporter 44A2 (SLC44A2) Gene in the Pathophysiology of Venous Thromboembolism" met aanvraagnummer AVD116002016607. Wij hebben uw aanvraag beoordeeld.

Op 02 juni 2017 en 09 juni 2017 heeft u uw aanvraag aangevuld. Op 31 mei 2017 hebben wij u vragen gesteld over het ongerief dat de dieren ondergaan, uw onderbouwing voor het gebruik van 1 geslacht en de NTS. Op 08 juni 2017 hebben wij u een vraag gesteld over het aantal dieren. Wij kunnen ons vinden in uw toelichting.

**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.
Met het oog op artikel 10a, lid 1, zijn er algemene voorwaarden gesteld.

U kunt met uw project "The Role of the Solute Carrier Transporter 44A2 (SLC44A2) Gene in the Pathophysiology of Venous Thromboembolism" starten. De vergunning wordt afgegeven van 12 juni 2017 tot en met 1 juni 2022.

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 25 april 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:**
12 juni 2017
**Aanvraagnummer:**
AVD116002016607

Centrale Commissie Dierproeven
namens deze:

[redacted]

Algemeen Secretaris

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

Centrale Commissie Dierproeven

# Projectvergunning

**gelet op artikel 10a van de Wet op de Dierproeven**

Verleent de Centrale Commissie Dierproeven aan

| | |
|---|---|
| Naam: | Academisch Ziekenhuis Leiden |
| Adres: | Postbus 9600 |
| Postcode en plaats: | 2300 RC LEIDEN |
| Deelnemersnummer: | 11600 |

deze projectvergunning voor het tijdvak 12 juni 2017 tot en met 1 juni 2022, voor het project "The Role of the Solute Carrier Transporter 44A2 (SLC44A2) Gene in the Pathophysiology of Venous Thromboembolism" met aanvraagnummer AVD116002016607, volgens advies van Dierexperimentencommissie DEC Leiden.
De functie van de verantwoordelijk onderzoeker is Associate Professor.
De aanvraag omvat de volgende bescheiden:

1 een aanvraagformulier projectvergunning dierproeven, ontvangen op 11 mei 2017
2 de bij het aanvraagformulier behorende bijlagen:
a Projectvoorstel, zoals ontvangen per digitale indiening op 2 juni 2017;
b Niet-technische Samenvatting van het project, zoals ontvangen per digitale indiening op 2 juni 2017;
c Advies van dierexperimentencommissie d.d. 25 april 2017, ontvangen op 11 mei 2017.
d De aanvullingen op uw aanvraag, ontvangen op 02 juni 2017 en 09 juni 2017

**Aanvraagnummer:**
AVD116002016607

| Naam proef | Diersoort/ Stam | Aantal dieren | Ernst |
|---|---|---|---|
| **3.4.4.1. Haemostasis characterization of Slc44a2 knockout mice** | | | |
| | Muizen (Mus musculus) / | 290 | Beenmerg donoren:<br>100% Terminaal<br>Ontvanger dieren:<br>10% Matig<br>90% Licht<br>Karakterisatie knockout dieren:<br>100% Matig |
| **3.4.4.2. Effect of SLC44A2 antibodies on haemostasis** | | | |
| | Muizen (Mus musculus) / | 384 | 10% Matig<br>90% Licht |
| **3.4.4.3. Venous thrombosis through partial ligation of the caval vein** | | | |
| | Muizen (Mus musculus) / | 2016 | Beenmerg donoren:<br>100% Terminaal<br>Overige dieren:<br>100% Matig |
| **3.4.4.4. Spontaneous venous thrombosis through siRNA** | | | |
| | Muizen (Mus musculus) / | 1080 | Beenmerg donoren:<br>100% Terminaal<br>Overige dieren:<br>100% Matig% |

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

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

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:**
AVD116002016607

# 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.