	Inventaris Wob-verzoek W16-01								
		wordt verstrekt				**weigeringsgronden**			
nr.	**document**	**reeds openbaar**	**niet**	**geheel**	**deels**	**10.1.c**	**10.2.e**	**10.2.g**	**11.1**
	NTS2015240								
1	Aanvraagformulier				x		x	x	
2	Niet-technische samenvatting	x							
3	Projectvoorstel				x		x	x	
4	Bijlage beschrijving dierproeven 1			x					
5	Bijlage beschrijving dierproeven 2				x			x	
6	Bijlage beschrijving dierproeven 3				x			x	
7	Bijlage beschrijving dierproeven 4			x					
8	DEC-advies				x		x	x	
9	Factuurinformatie				x		x	x	
10	Ontvangstbevestiging				x		x	x	
11	Telefoonnotitie				x		x	x	x
12	Advies CCD		x						x
13	Beschikking en vergunning				x		x	x	

15 SEP. 2015

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.zbo-ccd.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*	10300
☐ Nee > *U kunt geen aanvraag doen*	

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

Naam instelling of organisatie	Stichting Katholieke Universiteit Nijmegen
Naam van de portefeuillehouder of diens gemachtigde	[redacted]
KvK-nummer	4 1 0 5 5 6 2 9

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

Straat en huisnummer	Geert Grooteplein 10
Postbus	9101
Postcode en plaats	6500HB Nijmegen
IBAN	NL90ABNA0231209983
Tenaamstelling van het rekeningnummer	UMC St Radboud

1.4 Vul de gegevens in van de verantwoordelijke onderzoeker.

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

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

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

1.7 Is er voor deze projectaanvraag een gemachtigde?

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

☐ Nee

2 Over uw aanvraag

2.1 Wat voor aanvraag doet u?

☒ Nieuwe aanvraag > Ga verder met vraag 3

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

Vul uw vergunde projectnummer in en ga verder met vraag 2.2

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

Vul uw vergunde projectnummer in en ga verder met vraag 2.3

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

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

☐ Nee > Ga verder met vraag 3

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

☐ Nee > Ga verder met vraag 3

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

3 Over uw project

3.1 Wat is de geplande start- en einddatum van het project?	Startdatum: 10-10-2015
	Einddatum: 10-10-2020
3.2 Wat is de titel van het project?	The FGF23-klotho-vitamin D axis as a new instrumental target to combat the cardiovasc
3.3 Wat is de titel van de niet-technische samenvatting?	De FGF23-klotho-vitamine D as als een nieuwe instrumentale doelstelling om het cardiov
3.4 Wat is de naam van de Dierexperimentencommissie (DEC) aan wie de instellingsvergunninghouder doorgaans haar projecten ter toetsing voorlegt?	Naam DEC: RU DEC
	Postadres: Postbus 9101, 6500 HB Nijmegen [redacted]
	E-mailadres: [redacted]

4 Betaalgegevens

4.1 Om welk type aanvraag gaat het?

☑ Nieuwe aanvraag Projectvergunning € 741,00 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

☒ DEC-advies, factuurinformatie

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 1.6). 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	[redacted]
Plaats	Nijmegen
Datum	10-09-2015
Handtekening	[redacted]

Form
Project proposal

- This form should be used to write the project propsal of 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.zbo-ccd.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'.	10300
1.2	Provide the name of the licenced establishment.	Stichting Katholieke Universiteit Nijmegen
1.3	Provide the title of the project.	The FGF23-klotho-vitamin D axis as a new instrumental target to combat the cardiovascular risk of chronic kidney disease

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 of routine production
[] Research into environmental protection in the interest of human or animal health or welfare dier
[] 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.

This project is part of [redacted] granted by [redacted]) CP 10.11).
Chronic kidney disease (CKD) has a high prevalence currently affecting millions of people, and is a major public health issue. Accumulating data suggest that in patients with CKD, in addition to well-known risk factors like oxidative stress, renin-angiotensin-aldosterone system (RAAS) activation and proteinuria, a disrupted FGF23-klotho-vitamin D axis contributes to progressive renal failure and a severely increased cardiovascular risk (1). CKD, with its consequent imbalance between renal function and dietary calcium and phosphate load, triggers a vicious circle of the FGF23-klotho-vitamin D axis (2). When phosphate is in excess, FGF23 is secreted from bone and acts on the kidney to promote phosphate excretion into urine and to suppress vitamin D synthesis, thereby inducing a negative phosphate balance. One critical feature of FGF23 is that it requires klotho, a single-pass transmembrane protein expressed in renal tubules, as an obligate co-receptor to bind and activate FGF receptors. Increased plasma FGF23 levels and loss of renal klotho are early hallmarks of CKD (3). While ensuring renal phosphate excretion despite renal function impairment, the rise in FGF23 contributes to vitamin D deficiency (4). The latter blunts renin suppression, which by increased angiotensin II further impairs renal klotho generation.
In addition, it has been demonstrated that klotho protects against oxidative stress induced damage and that klotho deficiency leads to increased oxidative stress. Renal klotho deficiency impairs FGF23 signaling in the kidney, thus contributing to a further increase in FGF23, and may, moreover, lead to a systemic klotho deficiency (4). Increased FGF23 levels and klotho deficiency adversely affect vascular function and structure by effects on vitamin D metabolism, direct vascular toxicity/calcification, or both (5-7). Vascular calcification in CKD patients is strongly linked to deregulated mineral metabolism, mainly reflected in increased serum phosphate levels and transient states of hypercalcemia. Recently, high serum magnesium (Mg^{2+}) was associated with a reduced risk for cardiovascular events (8). The beneficial effects of Mg^{2+} have not been examined in detail, but it has been suggested that these effects can be attributed to reduced vascular calcifications. Using magnesium as a novel treatment strategy, we aim to improve outcome in CKD patients suffering from vascular calcification. Therefore, by uncovering the molecular mechanism of Mg^{2+} and how it results in vascular calcification, we aim to increase awareness among clinicians to routinely measure magnesium in CKD, and if needed, give magnesium supplementation to CKD patients. For the experiments proposed here, the *in vitro* part has been performed and the data are very

promising. In short: high phosphate is the hallmark of CKD and to mimic the calcification in CKD patients, human smooth muscle cells were incubated and cultured in high phosphate levels (3mmol) in the absence or presence of magnesiumchloride (final concentration 2mmol). After 14 days, clear calcium phosphate deposits were observed under high phosphate conditions, that were completely absent in the cells co-cultured with high concentrations of Mg2+.
Targeting FGF23 and/or klotho may provide a novel treatment paradigm with the potential to improve outcome in CKD. Therefore, our overall hypothesis is that derangement of the FGF23-klotho-vitamin D axis, in cross talk with the renin- angiotensin-aldosterone-system, is involved in the cardiovascular complications of CKD from its earliest stages onwards.
FGF23 seems to function as a protective factor, as it triggers adaptive changes that maintain a normal body phosphate homeostasis. Thus, modulation of the FGF23-klotho-vitamin D axis could represent a promising therapeutic target that might improve the fatal prognosis of patients with CKD and cardiovascular diseases. Assuming that the study goals are met and it is demonstrated that elevated FGF23 levels and/or klotho deficiency are important in the treatment of CKD patients, they can become novel targets in the treatment of CKD patients.
References:
(1) Rika Jimbo, Tatsuo Shimosawa. Cardiovascular Risk Factors and Chronic Kidney Disease—FGF23: A Key Molecule in the Cardiovascular Disease. Int J Hypertens. 2014; 381082.
(2) John GB1, Cheng CY, Kuro-o. Role of Klotho in aging, phosphate metabolism, and CKD. Am J Kidney Dis. 2011 ;58(1):127-34.
(3) Sakan H, Nakatani K, Asai O, Imura A, Tanaka T, Yoshimoto S, Iwamoto N, et al.,. Reduced renal alpha-Klotho expression in CKD patients and its effect on renal phosphate handling and vitamin D metabolism. PloS one 9: e86301, 2014.
(4) Yoon HE, Ghee JY, Piao S, Song JH, Han DH, Kim S, Ohashi N, Kobori H, et al., Angiotensin II blockade upregulates the expression of Klotho, the anti-ageing gene, in an experimental model of chronic cyclosporine nephropathy. Nephrol Dial Transplant 2011; 26: 800-13.
(5) Wang TJ, Pencina MJ, Booth SL, Jacques PF, Ingelsson E, Lanier K, Benjamin EJ, et al., Vitamin D deficiency and risk of cardiovascular disease. Circulation 2008; 117: 503-11.
(6)Dobnig H, Pilz S, Scharnagl H, Renner W, Seelhorst U, Wellnitz B, Kinkeldei J, et al.,Independent association of low serum 25-hydroxyvitamin D and 1,25-dihydroxyvitamin D levels with all-cause and cardiovascular mortality. Arch Intern Med. 2008; 168: 1340-9.
(7) Gutierrez OM, Mannstadt M, Isakova T, Rauh-Hain JA, Tamez H, Shah A, et al.,. Fibroblast growth factor 23 and mortality among patients undergoing hemodialysis. N Engl J Med. 2008; 359: 584-92.
(8) Kanbay M, Yilmaz MI, Apetrii M, Saglam M, Yaman H, Unal HU, Gok M, et al., Relationship between serum magnesium levels and cardiovascular events in chronic kidney disease patients. Am J Nephrol. 2012;36:228-237.

3.2 Purpose

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

- If the project is focussed on one or more research objectives, which research questions should be addressed during this project?
- If the main objective is not a research objective, which specific need(s) does this project respond to?

The aim of this project is to gain more insight in the underlying mechanism involved in the development of CKD and its extra renal consequences in the vascular bed. Our research group has a great expertise in the field of renal disease and ion handling. In the past we have used CKD mouse

models (either genetically induced or via surgery) and performed dietary interventions. Therefore, we have already generated a strong foundation for future experiments.
In CKD, intrarenal derangements in the FGF23-klotho-vitamin D axis occur in a vicious circle with increased RAAS activity and pathways of oxidative stress. The extrarenal consequences of these derangements are involved in the vascular complications of CKD.
Therefore we hope to meet these sub-aims:
· To delineate the role of RAAS blockade on the FGF23-klotho-vitamin D axis.
· To delineate effect of oxidative stress modulation on the FGF23-klotho-vitamin D axis.
· To unravel the role of deregulation of the FGF23-klotho-vitamin D axis in the increased cardiovascular risk of progressive CKD.
· To unravel the underlying mechanisms involved in vascular calcification alleviation by magnesium.

3.3 Relevance

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

Scientific relevance
Low levels of klotho hormone and elevated levels of FGF23 hormone may serve as an early warning sign of the presence of kidney disease and its deadly cardiovascular complications. Abnormalities of klotho and FGF23 levels may be involved in the development of these severe complications. To be able to adequately react to these threats, by prevention or treatment, it is of the utmost importance that we will get new insights into the mechanisms leading to CKD and derangements in these substances. Initial data suggests a potential role of klotho that may be beneficial in the prevention of CKD. Together with FGF23, klotho enhances renal phosphate excretion in order to maintain serum phosphate levels within the normal range. In addition, vitamin D is also an important hormone, produced by the kidney, to regulate phosphate balance. Hyperphosphatemia and hypercalcemia induce vascular calcification and atherosclerosis of the main arteries, including the aorta, which in turn cause increased stiffness of the vessel wall. Combined results of clinical, *in vitro* and *in vivo* studies show the beneficial effects of Mg2+ on the pathogenesis of vascular calcification. It will be our hope that this and future research will ultimately lead to better ways to slow down the progression of CKD.
Medical & Societal relevance
Chronic kidney disease (CKD) is currently a major medical problem worldwide, of which the incidence is still increasing which is accompanied by a high degree of morbidity and mortality. In line with that, cardiovascular complications originated from CKD are greatly increasing. It would be worthwhile, therefore, to explore a strategy and to test potential therapeutic options to treat these disturbed electrolyte homeostasis originated by CKD. In addition, administration of magnesium to treat vascular calcification could strongly reduce treatment costs and improve patient health in a simple and non-invasive manner. Therapeutic possibilities to treat the progression of CKD, would be a huge step forward in order to inhibit the progression and the development of CKD. Finding alternative treatments that will relatively alleviate these burdens and provide a better living condition.

3.4 Research Strategy

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

We will use a variety of approaches:
In the first 3 subprojects, the impact of pharmacological interventions (RAAS-blockade, antioxidants) on the FGF23-klotho-vitamin D axis will be studied using various mouse models.
- Specific preservation of renal klotho in CKD: role of RAAS, genetically altered model
- Specific preservation of renal klotho in CKD: role of oxidative stress
Some genetically altered animal models in our research question (i.e, FGF23 KO mice) are not a CKD model and therefore they need an extra approach to induce CKD. Among the available experimental models for CKD, the 5/6 nephrectomy has been a mainstay for studies of kidney disease and is performed by unilateral nephrectomy and removing of the poles of the remaining kidney after one week from the first surgery [1,2]. 5/6 nephrectomy shows common features to CKD observed in humans [3]. It has been used to test new therapies [4,5] and has been proven to be clinically relevant [6]. A less invasive model like adenine treatment is also available which does not require surgery. The major disadvantage is that the adenine model produces a more severe form of bone disease than 5/6 nephrectomy does [7]. Here, we are looking at the effect of the FGF23, Klotho and vitamin D axis, which are bone and kidney originated genes/hormones. Therefore, the adenine diet is not useful for our study. In addition, we performed a pilot study ([redacted]), to check whether chronic injection of Doxorubicin can lead to CKD in C57bl/6 mice. We selected C57bl/6 mice since most of our KO mice are generated in this mice strain. We found that this model is not severely inducing CKD and this model is not optimal to study CKD. Therefore using a surgical approach like 5/6 nephrectomy will be used to induce sufficient amount of CKD in a genetically altered mouse model which are interesting to our research question.
- Specific preservation of renal klotho in CKD: role of RAAS, surgically induced model
- Specific preservation of renal klotho in CKD: role of oxidative stress, surgically induced model
In the last subproject, the impact of Mg2+ interventions on the CKD induced vascular calcification will be studied using various CKD mouse models:
- Genetically modified CKD mouse models (i.e, klotho knock-out mice) + high magnesium diet
References:
1. Kang DH, Nakagawa T, Feng L, Johnson RJ. Nitric oxide modulates vascular disease in the remnant kidney model. Am J Pathol. 2002;161:239–248.
2. Santos LS, Chin EW, Ioshii SO, Tambara Filho R. Surgical reduction of the renal mass in rats: morphologic and functional analysis on the remnant kidney. Acta Cir Bras. 2006;21:252–257.
3. Kren S, Hostetter TH. The course of the remnant kidney model in mice. Kidney Int. 1999;56:333–337.
4. Fujihara CK, Malheiros DM, Zatz R. Losartan-hydrochlorothiazide association promotes lasting blood pressure normalization and completely arrests long-term renal injury in the 5/6 ablation model. Am J Physiol Renal Physiol. 2007;292:F1810–1818.
5. Terzi F, Burtin M, Hekmati M, Jouanneau C, Beaufils H, Friedlander G. Sodium restriction decreases AP-1 activation after nephron reduction in the rat: role in the progression of renal lesions. Exp Nephrol.2000;8:104–114.
6. Waanders F, Vaidya VS, van Goor H, Leuvenink H, Damman K, Hamming I, Bonventre JV, Vogt L, Navis G. Effect of renin-angiotensin-aldosterone system inhibition, dietary sodium restriction, and/or diuretics on urinary kidney injury molecule 1 excretion in nondiabetic proteinuric kidney disease: a post hoc analysis of a randomized controlled trial. Am J Kidney Dis. 2009;53:16–25.
7. Ferrari, Guaraciaba O; Ferreira, Juliana C; Cavallari, Raquel T; Neves, Katia R; dos Reis, Luciene M et al. Mineral bone disorder in chronic kidney disease: head-to-head comparison of the 5/6 nephrectomy and adenine models. (2014)
BMC nephrology vol. 15 (1) p. 69

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.

To elucidate the mechanism of CKD development and the role of FGF23, klotho and vitamin D, we will use four different approaches.

RAAS blockade, role of FGF23, klotho and vitamin D in CKD

A diet/water intervention with RAAS blockade combined with a genetic mouse model of CKD.

The interplay between the molecular pathways involved in RAAS and the FGF23-klotho-vitamin D axis will be studied. To this end, FGF23 and 1a-hydroxylase knockout mice will undergo a diet/water intervention with RAAS- blockades. Using this approach we will be able to determine the importance of FGF23, klotho and vitamin D and its interaction with the RAAS system in CKD. In addition we will elucidate the underlying mechanism involved in the CKD progression by using special KO mice and the role of FGF23, klotho and vitamin D in this prospective.

Oxidative stress modulation, role of FGF23, klotho and vitamin D in CKD

A dietary intervention with antioxidant combined with a genetic mouse model of CKD.

The interplay between the molecular pathways involved in oxidative stress and the FGF23-klotho-vitamin D axis will be studied. To this end, FGF23 KO, klotho KO and 1a-hydroxylase KO mice or other strains will be given a diet with an antioxidant (i.e, vitamin E) incorporated. With this, we want to dissect the role of oxidative stress pathways and their importance in FGF23, klotho and vitamin D modulation in CKD. In addition, we will elucidate the underlying mechanism involved in CKD progression, and the role of FGF23, klotho and vitamin D in this prospect, by using special KO mice.

RAAS blockade, role of FGF23, klotho and vitamin D in CKD

A genetically altered mouse model with surgically induced CKD with a diet/water intervention with RAAS blockade.

The interplay between the molecular pathways involved in RAAS and the FGF23-klotho-vitamin D axis will be studied. To this end, WT and KO mice will undergo a 5/6x nephrectomy to mimic CKD and to study the effect of RAAS modulation in the FGF23-klotho-vitamin D axis. Using this approach we will be able to determine the importance of FGF23, klotho and vitamin D and its interaction with the RAAS system in CKD. The 5/6 nephrectomy is known as the golden standard model for CKD. Therefore, CKD progression and the role of FGF23, klotho and vitamin D will be studied in this prospect.

High Mg2+ diet to reduce vascular calcification

A dietary intervention with high magnesium combined with a genetic mouse model of CKD.

To check the effect on vascular calcification in the presence of high magnesium in CKD mouse models (i.e, klotho KO) and in control (WT) mice. Using this approach we will be able to understand the role of magnesium homeostasis in calcified vascular smooth muscle cells, in the situation where FGF23, vitamin D and/or klotho are altered. Specific attention will be awarded to calcium and the calcium sensing receptor, since it is shown that activation of calcium sensing receptor reduces vascular calcification.

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

Part 1) Using RAAS blockades/inhibitors, incorporated in the diet (either food or water), we expect to improve the CKD phenotype and normalize mineral (ion) levels in the serum of genetically modified CKD mice. An example of this approach is using an angiotensin II blocker in klotho or 1a-hydroxylase (mice with vitamin D deficiency) knock-out (KO) mice.

Part 2) Using antioxidants (e.g. vitamin E), incorporated in the diet (either food or water), we expect to normalize the mineral (ion) homeostasis and to improve the CKD phenotype in genetically modified CKD mice (e.g. Klotho or 1a-hydroxylase KO mice). We expect to see a reduction in oxidative

stress pathways and rather a normal level of FGF23, vitamin D or klotho in CKD mice. If no effects are observed on ion-levels, especially phosphate levels, or in the expression of ion-transporters in the kidney, this part of the study will be only limited to a maximum of 3 antioxidants.
Part 3) Genetically modified mice might not totally represent the CKD as is seen in humans. Therefore, we want to use the 5/6 nephrectomy, to induce CKD properly in the mice. By administering RAAS inhibitors, we expect to see a normalization of renin, angiotensin and aldosteron levels, and rather normal levels of FGF23, klotho and vitamin D. The 5/6 nephrectomy model shows a progressive CKD phenotype over time and is a golden standard to study CKD. However, if no effects are observed in part 1, on mineral levels (especially phosphate levels) or on the expression of ion-transporters in the kidney, this part (part 3) of the project will not be performed (go/no-go point).
Part 4) CKD mice develop hyperphosphatemia and hypercalcemia. Mg2+ is shown to be effective in the attenuation of vascular calcification. Using a dietary intervention (high Mg2+ diet) in genetically modified CKD mice (e.g. Klotho KO or 1a-hydroxylase KO), this attenuation will be assessed. Mg2+ treatment is expected to reduce vascular calcification, especially at the level of vascular smooth muscle cells, in the absence of klotho or vitamin D. Based on our in vitro data (explained further on), we are expecting to see a reduction in phosphate and Ca2+ levels, at least in the klotho KO mice. If this expected result does not appear, we will not use other KO strains for CKD and we will not continue (go/no-go).
It is of the utmost importance that we will get new insights into the underlying mechanisms leading to CKD. Therefore, the deregulation of the FGF23-klotho-vitamin D axis is being investigated by RAAS and oxidative stress modulation.
The extra renal consequences of CKD will be investigated by testing Mg2+ intervention. We will study its possible beneficial effect in the vascular bed due to a deregulated FGF23-klotho-vitamin D axis. We will check if this non-invasive and cost-effective treatment is suitable in the improvement of vascular calcification.

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	RAAS blockades, genetic model, and FGF23, Klotho and vitamin D in CKD
2	Oxidative stress and FGF23, Klotho and vitamin D in CKD
3	RAAS blockades, surgical model, and FGF23,Klotho and vitamin D in CKD
4	CKD induced calcification treatment with Mg2+

4

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.zbo-ccd.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'.	10300	
1.2	Provide the name of the licenced establishment.	Stichting Katholieke Universiteit Nijmegen	
1.3	List the different types of animal procedures. Use the serial numbers provided in Section 3.4.4 of the Project Proposal form.	Serial number 1	Type of animal procedure RAAS blockades, genetic model, and FGF23, Klotho and vitamin D in CKD

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.

The aim of this project is to characterize the role of RAAS on the FGF23-klotho-vitamin D axis. We will use adult genetically modified mice and a pharmacological approach. RAAS has a key role in the regulation of blood pressure, sodium and water balance, and cardiovascular and renal homeostasis. RAAS blockade using angiotensin-converting-enzyme (ACE) inhibitors or angiotensin-receptor blockers (ARB) is the cornerstone in the treatment of renal disease. Alternative RAAS-blockade strategies include renin inhibition and aldosterone blockade. The effect of different RAAS blockades in the FGF23-klotho-vitamin D axis is not known. The FGF23-klotho-vitamin D axis can be regulated differently using different targets for blockades like renin, angiotensin-converting-enzyme, angiotensin-receptor or aldosterone. It is therefore important to investigate the effect of different blockades in the FGF23-klotho-vitamin D axis. Here we will use a maximum of 4 different RAAS blockades, in which they are blocking one of the above-mentioned parts of RAAS.
The groups will consist of CKD mouse models of (e.g. klotho KO mice, 1a-hydroxylase KO mice, or other available mice strains in the coming 5 years) and will be given ACE inhibitors or ARB blockers, in either drinking water or incorporated with diet.
- CKD genetically modified mouse model + RAAS blockades incorporated in food/water
- CKD genetically modified mouse model + normal food/water
- Wildtype littermate mice + RAAS blockades in incorporated in food/water
- Wildtype littermate mice + normal food/water

Animals coming from the same family will be randomly distributed across the groups, we will then check the body weight because we want to be sure that we don’t have one group with all the smallest/biggest animals. For this reason based on their body weight we correct the distribution of the animals when it is necessary. We don't expect to find any big differences since they have similar age.
To quantify the improved phenotype of the CKD mice after using RAAS blockades, several outcome parameters will be investigated.
The primary outcome parameters are serum and urine urea, createnine, calcium and phosphate concentrations. In addition, we will check the levels of FGF23, klotho and vitamin D in serum, urine and the kidneys of the mice. RAAS blockades are the first-line therapy for renoprotection in patients with CKD, as recommended by current guidelines. However, despite the proven benefits of this treatment strategy, the interactions between the FGF23-klotho-vitamin D axis and RAAS blockades in CKD are yet to be investigated. To more thoroughly investigate the effect on kidney function we will also check Glomerular Filtration Rate (GFR).
We will also study more in depth the effects on several proteins involved in the intracellular pathways involved in FGF23, klotho and vitamin D handling.
We will also determine the expression of Ca2+ and phosphate transporters in the intestines and kidney, as well as other transporters involved in renal ion homeostasis. There is great expertise in our department regarding CKD and ion handling.

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

We will use a genetic mouse model of CKD (i.e, klotho KO, 1 alpha-hydroxylase KO and others) and we will use RAAS blockades (ARB or ACE inhibitor) for example Losartan, incorporated with diet or with drinking water.

To study the effects of RAAS on the FGF23-klotho-vitamin D axis in CKD, we will use RAAS blockades (ARB or ACE inhibitor).
Before and during the animal experiments, mice will be housed in metabolic cages regularly (maximum of 1 period of 48hrs at the start, middle and end of the experiment) in order to collect faeces and urine, and determine food and water intake. Additionally, blood will be collected at different time intervals (start, middle and end of experiment). This will allow us to follow serum phosphate concentrations over time to see how RAAS blockade is effecting to inhibit CKD.
At the end of the experiment, the mice will be sacrificed under anesthesia by cervical dislocation. At this point blood is collected by eye extraction or heart puncture. Organs will be collected and used for subsequent analyses.
From the collected serum and urine we can measure the creatinine levels and there is a formula to measure GFR with that this will give us an indication how well a kidney is working.
The duration that animals will be in the experiment is different depending on the strain of mice (3 to 8 weeks). For instance, klotho KO mice have short life span and therefore they will be shorter (3 to 4 weeks) in the experiment in comparison to the other strains of mice.

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

Subsequently in each individual experiment, independent statistics will be carried out by using power calculation as below, to reduce the amount of mice used to a minimum:

$$N = \frac{2(Z\alpha + Z\beta)^2 s^2}{d^2}$$

With a power of 80% and a significance of 5%, and specific variations per primary outcome parameter, the minimal amount of mice needed for a significant difference will result from this calculation.

B. The animals

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

Adult: max. 480
We will make use of adult mice (mus musculus). We will use KO mouse models of CKD, often of a C57bl/6 background as our previous data are generated using this strain, making our results easy to compare to previous results.
To study CKD, the mouse is a well-established model in this field of research and a lot of literature is available. Mice are a highly relevant model because it will support (or contradict) the currently widely adopted hypothesis that FGF23 is a novel target in chronic kidney disease. In addition, given the fact that our research questions are largely mechanistically and involves the study of renal tissue, the use of animals in this type of study is unavoidable. We will therefore make use of mice because 1) the mouse is well-established in this field of research and 2) the mouse offers superior genetic models to study the molecular genetic background of complex diseases. Furthermore, mice resemble the situation in humans with CKD much better than cultured cells. Therefore, *in vivo* studies in animals cannot be avoided. By choosing male mice, the potential distribution due to hormonal influences, is kept as low as possible. It is currently not known whether changing levels of sex hormones may exert an influence on the regulation of calcium / phosphorus balance.
The number of mice per experiment will be based on statistical analysis (power analysis), our experience with similar type of experiments and/or (un)published data as below:
Quantitative analysis: prior to performing an experiment we perform statistical analysis to ensure that we use the minimum number of mice per group that will be statistically sound and biologically relevant.
Qualitative analysis (most of our experiments): the number is based on literature and/or years of experience with similar types of experiments. Moreover, these types of experiments will be performed sequentially via which we ensure that we will use the minimum number of mice per group that will be necessary.
It is currently impossible to exactly mention the number of mice required for these experiments over the next 5 years or per experiment. We have calculated the total number of mice based on experience over the past 5 years with similar type of experiments.
To that aim 40 mice are required for a complete and thorough analysis: 10 (number of mice per group) * 2 (different condition: with normal food/drink or RAAS inhibitor (i,e., losartan) diet/drink) * 2 (KO and WT mice) . However, the (simultaneously) performance where two genes are involved, requires 80 mice and not less. Wild-type littermates of genetically modified mice are a better control group than normal bl/6 mice. Therefore it is not possible to reduce the amount of wild-type mice in the case that we study two different knock-out mice. Here we will use a maximum of 4 different RAAS blockades: 40 (mice need per experiment) * 4 (RAAS blockades) * 3 (different mouse models of CKD: klotho KO mice, 1a-hydroxylase KO mice, or other available mice strain suitable for our research question) so in total we will use a maximum of 480 mice.
Importantly, before we will start our experiments we will write an application to the IVD. In this application we will exactly describe (among others) which considerations, facts and results have led to the proposition of the experiments, which specific question(s) we are trying to answer with the proposed animal experiments and what the ultimate goal is for the proposed experiments. Moreover we will describe the experimental design, argue the number of animals in the experiments, describe (i.a.) human endpoints, alternatives, nature of discomfort and ethical considerations. Experiments will only start upon IVD approval.
We will decide which CKD models are the most suitable strains and which RAAS blockade is the most effective, based on the primary outcome parameters (altered serum and urine urea, creatinine, calcium and phosphate concentrations).

Species	Origin	Maximum number of animals	Life stage
Mus musculus	Own breeding & Commercial supplier	480	5-15 weeks old mice (adult male mice; unless indicated otherwise)

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'?

[] 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.

Refinement
During the experiment the mice will be housed together in groups with free access to food and water. However, short periods of solitary housing in metabolic cages are necessary, because of the necessity of the measurement of key parameters in the urine. When the mice are in metabolic cages they will be placed in the same room as the other mice, to reduce stress levels. Before starting any experiment, extensive literature search and possibly a pilot-study will ensure that no sudden severe unwanted side-effects arise during the experiment. To obtain the best results possible, and reduce the amount of discomfort, mice will only be handled by skilled researchers and (bio)technicians. In addition, prior to sacrifice, the animals will be anesthetized. Moreover, our department is experienced in research regarding ion transport. The genetically altered mice are well-established models of CKD.
Replacement
The use of animals is essential to understand the basis of human disease at the systems and whole organism level, and to provide a link between in vitro studies and clinical disease. For a multi-organ disease, like chronic kidney, there is no substitute for animal studies. It would be neither permissible nor ethical to carry out the necessary procedures in humans, and simulations cannot provide answers to the questions we seek to address. In addition, the simple conditions for ion transport *in vitro* are not able to reproduce the complex ionic (micro-) gradients encountered *in vivo*. Moreover, the complicated interplay of organs, in which regulatory mechanisms and hormonal regulation play a crucial role, cannot be mimicked in a lower animal species.

For the majority of the proposed studies, the mouse is the most appropriate animal model because: (1) it is a mammal; (2) physiology is more extensively characterized in mice than in other mammalian model species; (3) mice are amenable to transgenic manipulation; (4) a large number of relevant transgenic and knock out lines are already available.
Reduction
A power calculation will be performed to determine the minimal amount of mice required to achieve significant differences in the primary outcome. If necessary, because not all information is available, a pilot study will be performed to provide good data on variation and effect size of the proposed RAAS blockade intervention and the type of KO mice to use. If the pilot experiment generates unfavourable results, the actual experiment will not be performed (go/no-go). Based on provided calculations the minimum number of animals will be used for our experiments.
Alteration and blocking of RAAS from different sites might have a different effect on the FGF23-klotho-vitamin D axis. This, however, has not yet been tested and therefore in our research we would like to make use of RAAS blockades using angiotensin-converting-enzyme (ACE) inhibitors or angiotensin-receptor blockers (ARB), renin inhibition and aldosterone blockade. Therefore all 4 types of RAAS inhibitors will be tested in DAP 1 and if the effect is similar in our primary outcome data, then we will only continue with ARB inhibitors. In DAP 1, RAAS blockades will be tested independently but as mentioned before we cannot reduce the amount of WT mice, since different littermates can act differently. There is published data from my thesis (chapter 5) showing the huge different effect of C57BL/6 mice and littermate mice. Hence the separate littermates for every KO mice are always essential.

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

In general, the discomfort of the mice is likely to be moderate.
In some cases, the combination of different mild experiments will lead to a higher degree of discomfort, which may be considered as moderate in combination. Often we will use diet/water intervention, genetically altered CKD models, blood sampling, housing in a metabolic cage for minimum possible days. Animal handling will be performed by skilled researchers and/or (bio)technicians at the animal facility to reduce the adverse effects due to stress.
Moreover, animals will only be housed together, unless when the collection of urine and faeces is desired.
When the mice are in the metabolic cages, they will be housed in the same room as the other mice to reduce stress levels.
When experiments will be performed with unknown (side)effects, a pilot will be performed prior to the actual study.
In short: all possibilities will be used to reduce pain, fear or suffering.

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 of the Directive 2010/63/EU?

[] No
[X] Yes > If this may adversely affect animal welfare, describe how the animals will be housed and provide specific justifications for these choices.

Preferably mice are group housed but in some cases this will not be possible:
Mice will be individually placed in metabolic cages for a duration of 48 hours (24 hours adaptation, 24 hours collection) that is necessary for the collection of urine and faeces, and to determine food and water intake. To reduce the discomfort, the cages will be placed in the room together with the normal cages.
Mice will get a RAAS blockade and this is incorporated with their food or water. It is still be possible to group house the mice.

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, analgesiaor other pain relieving methods be used?

[X] No > Justify why pain relieving methods will not be used.

Blood collection can be painful, but because of the stress and risk of death by anesthesia we will not use this, as is custom for blood collection. Anesthesia might have a possible effect on the parameter we want to measure in the blood.

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

I. Other aspects compromising the welfare of the animals

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

The main adverse effects on welfare of the genetic modification is the development of CKD and involved side-effects hereof. Klotho KO and 1a-hydroxylase KO mice will experience moderate discomfort due to the development of CKD. Therefore, KO mice are not bred unless needed for an experiment (a breeding DEC is necessary, and already approved for our department). Klotho KO mice do not grow normal and the size is half of the size of normal mice and their average survival is approximately 16 weeks. These mice are an accelerated model of ageing. 1-alpha hydroxylase KO mice are fragile due to lack of vitamin D and the bones are soft, therefore the chance of fractured bones is higher than in normal mice.
There is no side effect after administration of RAAS blockade in water/food. However, on the basis of clinical signs (appearance: fur and behaviour assessed daily, weight: more than 15% decrease of the initial bodyweight during experiment) mice will be removed from the study and will be sacrificed by a biotechnician or the responsible investigator. By monitoring the mice daily, we will make sure to exclude those mice from the study in a timely manner.
In general, the procedures with expected adverse effects on welfare include genetically altered mice to develop CKD, blood sampling, solitary housing (metabolic cages), and adverse effects from the interventions.
There will not be any surgical approaches in this subproject.

Explain why these effects may emerge.

- Development of CKD due to genetically altered model
- Solitary housing in metabolic cage
- Handling
- Collection of blood

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

The genetically modified mice are actually CKD models, therefore the discomfort they undergo cannot be prevented. They require a DEC to breed due to the discomfort. Klotho KO mice develop a hunch back in early life and die early (life length approximately 16 weeks). To minimize the number of animals experiencing discomfort due to phenotype, KO mice will only be bred when needed for an experiment, and in the quantity needed for the experiment.
The RAAS blockades incorporated to diet/water will not cause any discomfort.
Under normal circumstances, mice will be housed together in groups. Discomfort is mainly related to metabolic cages, in which they are housed solitary. However, it is not possible to avoid the use of these cages, because of the necessity of the measurement of key parameters in the urine. When the mice are in metabolic cages they will be placed in the same room as the other mice, to reduce stress levels.
Mice will be monitored closely. Daily inspection will take place, by the animal care takers of the animal facility and inspection by the responsible researcher. This may include animal behaviour, mice fur, body weight, colour of urine and other behavioural changes as a result of the induced intervention. When unexpected changes are found, the mice will be removed from the experiment to prevent further discomfort.
To obtain the best results possible, and reduce the amount of discomfort, mice will only be handled by skilled researchers and (bio)technicians.
Prior to sacrifice, the animals will be anaesthetised.

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 an humane endpoint are:
1. The animal experiences more than little, additional, discomfort as a result of conditions not related to the experiment.
2. Mice will be excluded from the experiments and immediately euthanised under general anaesthesia in case of visible signs of complications or discomfort like loosing weight which is more than 15% of the initial weight during experimental period. When these complications occur or when an animal shows other signs of discomfort such as hunchback and bad fur or does not respond adequately to stimuli (very slow in movements), they will be sacrificed.
3. The animal experiences more discomfort than justified for the purpose of the experiment and weighed by the CCD, DEC or IvD.
4. (Reliable and applicable) results cannot be achieved because of conditions not related with the experiment.

5. Klotho KO and 1a-hydroxylase KO mice are less tolerable to any intervention than normal mice and therefore those mice will be closely monitored and in the case of observed complications, they will be excluded from study. Hunchback and bone fractures are both humane endpoints which can be observed with these mice.
6. The objective of the experiment has been reached.
The responsible researcher will contact the Animal Welfare Body (IvD) if criteria 1, 2, 3, 4, 5 and/or 6 are applicable then animal will be taken out of the experiment.
If criteria 1, 2, 3, 4, 5 and/or 6 are applicable and the responsible researcher is not available, an animal caretaker or biotechnician has the obligation, (preferably after internal communication) to take the animal out of the experiment and euthanise it under general anaesthesia.

Indicate the likely incidence.

The incidence of humane endpoints due to genetic modification is present since specific CKD models of KO mice is being used in this procedure lead to discomfort; this is approximately 3-5%, according to previous experiments using klotho KO mice performed in our group.
Mostly, in case of dietary intervention/water intervention (RAAS blockades incorporated in food/water), the chance of reaching a humane endpoint is almost zero.
Metabolic cages for 48h periodically have also almost zero human endpoint.
Therefore, as half of the animals will develop CKD due to their KO phenotype, the chance of reaching a humane endpoint over the whole experiment is 1.5-2.5%.

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).

Due to either induction of CKD or the cumulative effect of mild procedures, 100% of the mice will experience moderate discomfort.

End of experiment

L. Method of killing

Will the animals be killed during or after the procedures?

L. Method of killing

[] No > Continue with Section 3: 'Signatures'.

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

Mice are killed at the end of the experiment, to collect blood and organs.

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.zbo-ccd.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'.	10300	
1.2	Provide the name of the licenced establishment.	Stichting Katholieke Universiteit Nijmegen	
1.3	List the different types of animal procedures. Use the serial numbers provided in Section 3.4.4 of the Project Proposal form.	Serial number 2	Type of animal procedure Oxidative stress and FGF23, Klotho and vitamin D in CKD

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.

The aim of this project is to characterize the role of oxidative stress on the FGF23-klotho-vitamin D axis. We will use adult mice and pharmacological approach by using antioxidants to inhibit the oxidation (i.e vitamin E) for this purpose. These groups will consist of CKD mouse models (i.e. klotho KO mice, 1a-hydroxylase KO mice, or other available mice strains) and will be given antioxidants, in either drinking water or incorporated with diet. Hence, the setup of groups might look like this:
- Wild-type mice + antioxidants (i.e vitamin E) incorporated in food/water
- Wild-type mice + normal food/water
- Transgenic CKD mouse model + antioxidants (i.e vitamin E) incorporated in food/water
- Transgenic CKD mouse model + normal food/water

Animals coming from the same family will be randomly distributed across the groups, we will then check the body weight because we want to be sure that we don't have one group with all the smallest/biggest animals. For this reason based on their body weight we correct the distribution of the animals when it is necessary. We don't expect to find any big differences since they have similar age.
To quantify the improved phenotype of CKD mice model after using antioxidants, several outcome parameters will be investigated.
The primary outcome parameters are serum and urine urea, creatinine and phosphate concentrations. Since CKD alleviation and its effect on the FGF23-klotho-vitamin D axis is the main outcome of our experiments, we will use several other additional parameters to determine this. We therefore will check the levels of FGF23, klotho and vitamin D in serum, kidney and urine of mice. To more thoroughly investigate the effect on kidney function we will check Glomerular Filtration Rate (GFR).
We will also study more in depth the effects on several proteins involved in the intracellular pathways involved in FGF23, klotho and vitamin D handling.
We will also determine the expression of Ca2+ and phosphate transporters in the intestines and kidney, as well as other transporters involved in renal ion homeostasis. There is great expertise in our department regarding this part.

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

We will use a genetic mouse model of CKD (i.e, klotho KO, 1 a-hydroxylase KO and others) and we will use antioxidants (e.g. vitamin E).
To study the effects of oxidative stress modulation on the FGF23-klotho-vitamin D axis in CKD, we will make use of antioxidants.
Before and during the animal experiments, the mice will be housed in metabolic cages regularly (maximum of 1 period of 48hrs at start, middle and end of the experiment) in order to collect faeces and urine, and determine food and water intake. Additionally, blood samples will be collected at

different time intervals (start, middle and end). This will allow us to follow serum phosphate concentrations over time to see how the antioxidant is effecting to inhibit CKD.
At the end of the experiment, the mice will be sacrificed under anesthesia by cervical dislocation. At this point blood is collected by eye extraction or heart puncture. Organs will be collected and used for subsequent analyses.
The duration that animals will be in the experiment is different depending on the strain of mice (3 to 8 weeks). For instance klotho KO mice have short life span and therefore they will be shorter (3 to 4 weeks) in the experiment in comparison to the other strains of mice.

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

Subsequently in each individual experiment, independent statistic will be carried out by using power calculation as below, to reduce the amount of mice used to a minimum:

$$N = \frac{2(Za + Zß)2\ s2}{d2}$$

With a power of 80% and a significance of 5%, and specific variations per primary outcome parameter, the minimal amount of mice needed for a significant difference will result from this calculation.

B. The animals

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

Adult: max. 480
We will make use of adult mice (mus musculus). We will use KO mouse models of CKD, often of a C57bl/6 background as our previous data are generated using this strain, making our results easy to compare to previous results.
To study CKD, the mouse is a well-established model in this field of research and a lot of literature is available. Mice are a highly relevant model because it will support (or contradict) the currently widely adopted hypothesis that FGF23 is a novel target in chronic kidney disease. In addition, given the fact that our research questions are largely mechanistically and involves the study of renal tissue, the use of animals in this type of study is unavoidable. We will therefore make use of mice because 1) the mouse is well-established in this field of research and 2) the mouse offers superior genetic models to study the molecular genetic background of complex diseases. Furthermore, mice resemble the situation in humans with CKD much better than cultured cells. Therefore, *in vivo* studies in animals cannot be avoided.
By choosing male mice, the potential distribution due to hormonal influences, is kept as low as possible. It is currently not known whether changing levels of sex hormones may exert an influence on the regulation of calcium/phosphorus balance.
The number of mice per experiment will be based on statistical analysis (power analysis), our experience with similar type of experiments and/or (un)published data as below:

Quantitative analysis: prior to performing an experiment we perform statistical analysis to ensure that we use the minimum number of mice per group that will be statistically sound and biologically relevant.
Qualitative analysis (most of our experiments): the number is based on literature and/or years of experience with similar types of experiments. Moreover, these types of experiments will be performed sequentially via which we ensure that we will use the minimum number of mice per group that will be necessary.
It is currently impossible to exactly mention the number of mice required for these experiments over the next 5 years or per experiment. We have calculated the total number of mice based on experience over the past 5 years with similar types of experiments.
To that aim 40 mice are required for a complete and thorough analysis: 10 (number of mice per group) * 2 (different condition: with normal food/water or antioxidant (e.g. vitamin E) * 2 (KO and WT mice). However, the (simultaneously) performance where two genes are involved requires 80 mice and not less. Wild-type littermates of genetically modified mice are a better control group than normal bl/6 mice. Therefore it is not possible to reduce the amount of wild-type mice in the case that we study two different knock-out mice. Here we will use 2-4 different antioxidants, due to variation in the effects of antioxidants reported in the literature. Therefore: 40 (mice need per experiment) * 4 (antioxidants) * 3 (different mice model of CKD: klotho KO mice, 1a-hydroxylase KO mice, or other available mice strain suitable for our research question) so in total we will use a maximum of 480 mice.
Importantly, before we will start our experiments we will write an application to the IVD. In this application we will exactly describe (among others) which considerations, facts and results have led to the proposition of the experiments, which specific question(s) we are trying to answer with the proposed animal experiments and what the ultimate goal is for the proposed experiments. Moreover we will describe the experimental design, argue the number of animals in the experiments, describe (i.a.) human endpoints, alternatives, nature of discomfort and ethical considerations.
Experiments will only start upon IVD approval.

Species	Origin	Maximum number of animals	Life stage
Mus Musculus	Own breeding & commercial supplier	480	5-15 weeks old mice (adult male mice; unless indicated otherwise)

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'?

[] 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.

Refinement
During the experiment the mice will be housed together in groups with free access to food and water. However, short periods of solitary housing in metabolic cages are necessary, because of the necessity of the measurement of key parameters in the urine. When the mice are in metabolic cages they will be placed in the same room as the other mice, to reduce stress levels. Before starting any experiment, extensive literature search and possibly a pilot-study will ensure that no sudden severe unwanted side-effects arise during the experiment. To obtain the best results possible, and reduce the amount of discomfort, mice will only be handled by skilled researchers and (bio)technicians. In addition, prior to sacrifice, the animals will be anesthetized. Moreover, our department is experienced in research regarding ion transport. The genetically altered mice are well-established models of CKD.
Replacement
The use of animals is essential to understand the basis of human disease at the systems and whole organism level, and to provide a link between in vitro studies and clinical disease. For a multi-organ disease, like chronic kidney disease, there is no substitute for animal studies. It would be neither permissible nor ethical to carry out the necessary procedures in humans, and simulations cannot provide answers to the questions we seek to address. In addition, the simple conditions for ion transport *in vitro* are not able to reproduce the complex ionic (micro-) gradients encountered *in vivo*. Moreover, the complicated interplay of organs, in which regulatory mechanisms and hormonal regulation play a crucial role, can not be mimicked in a lower animal species.
For the majority of the proposed studies, the mouse is the most appropriate animal model because: (1) it is a mammal; (2) physiology is more extensively characterized in mice than in other mammalian model species; (3) mice are amenable to transgenic manipulation; (4) a large number of relevant transgenic and knock out lines are already available.
Reduction
A power calculation will be performed to determine the minimal amount of mice required to achieve significant differences in the primary outcome. If necessary, because not all information is available, a pilot study will be performed to provide good data on variation and effect size of the proposed antioxidant intervention. If the pilot experiment generates unfavorable results, the actual experiment will not be performed. Based on provided calculation the minimum number of animals will be used for our experiments.

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

In general, the discomfort of the mice is likely to be mild to moderate. In some cases, the combination of different procedures will lead to a higher degree of discomfort, which may be considered as moderate in combination. Often we will use diet/water intervention, genetically altered CKD models, blood sampling, housing in a metabolic cage for minimum possible days. Animal handling will be performed by skilled researchers and/or (bio)technicians at the animal facility to reduce the adverse effect.

Moreover, animals will only be housed together, unless when the collection of urine and faeces is desired. When the mice are in the metabolic cages, they will be housed in the same room as the other mice to reduce stress levels.
When experiments will be performed with unknown side-effects, a pilot will be performed prior to the actual study.

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 of the Directive 2010/63/EU?

[] No
[X] Yes > If this may adversely affect animal welfare, describe how the animals will be housed and provide specific justifications for these choices.

Preferably mice should be group housed but in some cases this will not be possible:
Mice will be individually placed in metabolic cages for a duration of 48 hours (24 hours adaptation, 24 hours collection) that is necessary for the collection of urine and faeces, and to determine food and water intake. To reduce the discomfort, the cages will be placed in the room together with the normal cages.
Mice will get an antioxidant and this is incorporated with their food or rarely water. It is still possible to group house the mice.

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.

G. Location where the animals procedures are performed

[] 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, analgesiaor other pain relieving methods be used?

[X] No > Justify why pain relieving methods will not be used.

[redacted]

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

I. Other aspects compromising the welfare of the animals

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

The main adverse effects on welfare of the genetic modification is the development of CKD and involved side-effects hereof. Klotho KO and 1a-hydroxylase KO mice will experience moderate discomfort due to the development of CKD. Klotho KO mice do not grow normal and the size is half of the size of normal mice and average age expectation is approximately 16 weeks. These mice are an accelerated model of ageing and most of the

ageing symptoms are present. 1a-hydroxylase KO mice are fragile due to lack of vitamin D and the bones are soft, therefore the chance of fractured bones is higher than normal mice.
There is no side effect after administration of antioxidants. However, on the basis of clinical signs (appearance: fur and behavior assessed daily, weight: more than 15% of the initial bodyweight during experiment) mice might be removed from the study and will be sacrificed by a biotechnician or the responsible investigator. By monitoring the mice daily, we will make sure to exclude those mice from the study in a timely manner.
In general, the procedures with expected adverse effects on welfare include genetically altered mice to develop CKD, blood sampling, solitary housing (metabolic cages), and adverse effects from the interventions.
There will not be any surgical approaches in this subproject.

Explain why these effects may emerge.

- Development of CKD due to genetically altered model
- Solitary housing in metabolic cage
- Handling
- Collection of blood

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

The genetically modified mice are actually CKD models, therefore the discomfort they undergo cannot be prevented. They require a DEC to breed due to discomfort. Klotho KO mice develop a hunch back in early life and die early (life length approximately 16 weeks). To minimize the number of animals experiencing discomfort due to phenotype, KO mice will only be bred when needed for an experiment, and in the quantity needed for the experiment.
The antioxidants incorporated in food/diet will not cause any discomfort.
Under normal circumstances, mice will be housed together in groups. Discomfort is mainly related to metabolic cages, in which they are housed in solitary. However, it is not possible to avoid the use of these cages, because of the necessity of the measurement of key parameters in the urine. When the mice are in metabolic cages they will be placed in the same room as the other mice, to reduce stress levels.
Mice will be monitored closely. Daily inspection by the animal care takers of the animal facility and inspection by the responsible researcher. This may include animal behavior, mice fur, body weight, colour of urine and other behavioral changes as a result of the induced intervention. When unexpected changes are found, the mice will be removed from the experiment to prevent further discomfort.
To obtain the best results possible, and reduce the amount of discomfort, mice will only be handled by skilled researchers and (bio)technicians.
Prior to sacrifice, the animals will be anesthetized.

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 an humane endpoint are:
1. The animal experiences more than little, additional, discomfort as a result of conditions not related to the experiment.
2. Mice will be excluded from the experiments and immediately euthanised under general anaesthesia in case of visible signs of complications or discomfort like loosing weight which is more than 15% of the initial weight during experimental period. When these complications occur or when an animal shows other signs of discomfort such as hunchback and bad fur or does not respond adequately to stimuli (very slow in movements), they will be sacrificed.
3. The animal experiences more discomfort than justified for the purpose of the experiment and weighed by the CCD, DEC or IvD.
4. (Reliable and applicable) results cannot be achieved because of conditions not related with the experiment.
5. Klotho KO and 1a-hydroxylase KO mice are less tolerable to any intervention than normal mice and therefore those mice will be closely monitored and in the case of observed complications, they will be excluded from study. Hunchback and bone fracture are both humane endpoints which can be observed with these mice.
6. The objective of the experiment has been reached.
The responsible researcher will contact the Animal Welfare Body (IvD) if criteria 1, 2, 3, 4, 5 and/or 6 are applicable and the animal will be taken out of the experiment.
If criteria 1, 2, 3, 4, 5 and/or 6 are applicable and the responsible researcher is not available, an animal caretaker or biotechnician has the obligation, (preferably after internal communication) to take the animal out of the experiment and euthanise it under general anaesthesia.

Indicate the likely incidence.

The incidence of humane endpoints due to genetic modification is present since specific CKD models of KO mice are being used in this procedure lead to discomfort; this is approximately 3-5%, according to previous experiments using klotho KO mice performed in our group.
The chance of reaching an humane endpoint due to the antioxidant incorporated in the diet is almost zero.
Metabolic cages for 48h periodically have also almost zero human endpoint.
Therefore, the chance of reaching a humane endpoint is 1.5-2.5%.

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).

Due to either induction of CKD or the cumulative effect of mild procedures, 100% of the mice will experience moderate discomfort.

End of experiment

L. Method of killing

Will the animals be killed during or after the procedures?

[] No > Continue with Section 3: 'Signatures'.
[X] Yes > Explain why it is necessary to kill the animals during or after the procedures.

Mice are killed at the end of the experiment, to collect blood and organs.

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

[] No > Descripe 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.zbo-ccd.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'.	10300	
1.2	Provide the name of the licenced establishment.	Stichting Katholieke Universiteit Nijmegen	
1.3	List the different types of animal procedures. Use the serial numbers provided in Section 3.4.4 of the Project Proposal form.	Serial number 3	Type of animal procedure RAAS blockades, surgical model, and FGF23,Klotho and vitamin D in CKD

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.

The overall aim of this project is to characterize the role of RAAS on the FGF23-klotho-vitamin D axis. Some genetically altered animal models in our research question (i.e, FGF23 KO mice) are not a CKD model and therefore they need an extra approach, like 5/6x nephrectomy surgical approach to complete as a valid model. To that aim, next to genetic alteration, a surgical approach is necessary to have an appropriate model to study CKD. We will use adult genetically modified mice with pharmacological intervention using RAAS blockade. Here, we will make use of the most effective RAAS blockades based on our primary outcome obtained in DAP1. RAAS blockade will be given in either drinking water or incorporated with diet. Hence, the setup of groups will look like this:

- Sham operated KO mice + RAAS blockades in incorporated in food/water
- Sham operated KO mice + normal food/water
- 5/6X nephrectomy KO mice + RAAS blockades in incorporated in food/water
- 5/6X nephrectomy KO mice + normal food/water

- Sham operated WT mice + RAAS blockades in incorporated in food/water
- Sham operated WT mice + normal food/water
- 5/6X nephrectomy WT mice + RAAS blockades in incorporated in food/water
- 5/6X nephrectomy WT mice + normal food/water

Animals coming from same family will be randomly distributed across the groups, we will then check the body weight because we want to be sure that we don't have one group with all the smallest/biggest animals. For this reason based on their body weight we correct the distribution of the animals when it is necessary. We don't expect to find any big differences since they have similar age.
To quantify the improved phenotype of the surgically induced CKD mice after using RAAS blockades, several outcome parameters will be investigated.
The primary outcome parameters are serum and urine urea, creatinine and phosphate concentrations. Also other proteins might have an effect on the FGF23-klotho-vitamin D axis. Therefore we will check klotho and vitamin D levels in serum, kidney and urine of mice. To more thoroughly investigate the effect on kidney function we will check Glomerular Filtration Rate (GFR).
We will study more in depth the effects on several proteins involved in the intracellular pathways involved in FGF23, klotho and vitamin D handling. In addition, we will determine the expression of Ca2+ and phosphate transporters in the intestines and kidney, as well as other transporters involved in renal ion homeostasis. There is great expertise in our department regarding this part.

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

We will use a surgical approach (5/6X nephrectomy) to induce CKD in mice and we will use RAAS blockades, incorporated with diet or with drinking water.
The 5/6X nephrectomy consists in a ligation of the lower branch of the left renal artery to produce about one third area with visible renal ischemia; the upper pole of the left kidney is removed by cautery and the right kidney is decapsulated and nephrectomized to induce a total 5/6X nephrectomy. It is a severe surgery that can lead to weight loss/lack of weight gain, anemia, increase in blood pressure and kidney damage of course. Some animals can die during the experiments. To avoid discomfort during surgery, animals will be pre- and post-operatively supplied with analgesics (temgesic, due to the mainly hepatic elimination, this medication does not accumulate when renal function is compromised such as an ischemia injury) and surgery will be performed under anesthesia, while the animal rests on a heating mattress to maintain its body temperature. To aid a faster recovery of the mice after surgery, the animals will be placed in a heated incubator for 1,5 h. After the surgery pain killers will be injected every 12 hours for 2 days to reduce post-operative pain.
To study the effects of RAAS on the FGF23-klotho-vitamin D axis in CKD, we will use the most effective RAAS blockades obtained from DAP1.
Before and during the animal experiments, mice will be housed in metabolic cages regularly (maximum of 1 period of 48hrs at start, middle and end) in order to collect faeces and urine, and determine food and water intake. Additionally, blood will be collected at different time intervals (start, middle and end). This will allow us to follow serum phosphate concentrations over time to see how RAAS blockade is effecting to inhibit CKD.
At the end of the experiment, the mice will be sacrificed under anesthesia by cervical dislocation. At this point blood is collected by eye extraction or heart puncture. Organs will be collected and used for subsequent analyses.
From the collected serum and urine we can measure the creatinine levels and there is a formula to measure GFR with that this will give us an indication how well a kidney is working.
The duration of animals will be in the experiment is 8 to 14 weeks in total; since after surgery we need some time that they develop CKD.

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

Subsequently in each individual experiment, independent statistic will be carried out by using power calculation as below, to reduce the amount of mice used to a minimum:

$$N = \frac{2(Z\alpha + Z\beta)^2 s^2}{d^2}$$

With a power of 80% and a significance of 5%, and specific variations per primary outcome parameter, the minimal amount of mice needed for a significant difference will result from this calculation.

B. The animals

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

Adult: max. 360
Species and origin: We will make use of adult mice (mus musculus). We will use WT and KO mice, often of a C57bl/6 background as our previous data are generated using this strain, making our results easy to compare to previous results. The mice are obtained from our own breeding or from a commercial licensed breeder.
Choice of mice: To study CKD, the mouse is a well-established model in this field of research and a lot of literature is available. Mice are a highly relevant model because it will support (or contradict) the currently widely adopted hypothesis that FGF23 is a novel target in chronic kidney disease. In addition, given the fact that our research questions are largely mechanistically and involves the study of renal tissue, the use of animals in this type of study is unavoidable. We will therefore make use of mice because 1) the mouse is well-established in this field of research and 2) the mouse offers superior genetic models to study the molecular genetic background of complex diseases. Furthermore, mice resemble the situation in humans with CKD much better than cultured cells. Therefore, *in vivo* studies in animals cannot be avoided.
Choice of gender: By choosing the male mice, the potential distribution due to hormonal influences, is kept as low as possible. It is currently not known whether changing levels of sex hormones may exert an influence on the regulation of calcium/phosphorus balance.
Justification: The number of mice per experiment will be based on statistical analysis (power analysis), our experience with similar type of experiments and/or (un)published data as below:
Quantitative analysis: prior to performing an experiment we perform statistical analysis to ensure that we use the minimum number of mice per group that will be statistically sound and biologically relevant.
Qualitative analysis (most of our experiments): the number is based on literature and/or years of experience with similar types of experiments. Moreover, these types of experiments will be performed sequentially via which we ensure that we will use the minimum number of mice per group that will be necessary.
It is currently impossible to exactly mention the number of mice required for these experiments over the next 5 years or per experiment. We have calculated the total number of mice based on experience over the past 5 years with similar type of experiments.
For RAAS blockade experiment 80 mice are required for a complete and thorough analysis: 10 (number of mice per group) * 2 (different food/water: with normal food/water or RAAS inhibitor in food/water) * 2 (with sham operated or 5/6X nephrectomy operated) * 2 (KO and WT mice). However, the (simultaneously) performance where two genes are involved requires 160 mice and not less. Wild-type littermates of genetically modified mice are a better control group than normal bl/6 mice. Therefore it is not possible to reduce the amount of wild-type mice in the case that we study two different knock-out mice.
Given that we will study approximately 2 different KO mice and maximally 2 different RAAS blockades which this will make it: 80 mice per study * 2 genotypes * 2 inhibitors = 320 mice. Due to 10-15% post-surgery mortality we would like to have 40 extra mice in total. Given those reasons: we will have in total 360 mice for this experiment.
Importantly, before we will start our experiments we will write an application to the IVD. In this application we will exactly describe (among others) which considerations, facts and results have led to the proposition of the experiments, which specific question(s) we are trying to answer with the proposed animal experiments and what the ultimate goal is for the proposed experiments. Moreover we will describe the experimental design, argue the number of animals in the experiments, describe (i.a.) human endpoints, alternatives, nature of discomfort and ethical considerations. Experiments will only start upon IVD approval.
First we will use FGF23 KO mice to perform the experiment. However 5 years is a long time and there will be certainly new interesting genes for us to have another KO mice models. Therefore we would like to keep the possibility to be able to use another newly generated mice model. This model will be important and interesting to our research questions and potentially will help us in understanding more about the FGF23-klotho-vitamin D axis

in the CKD in the coming 5 years. In addition, according to the data obtained from DAP (subproject 1), we will decide which RAAS blockades are the most effective, based on the primary outcome parameters (altered serum and urine urea, creatinine, calcium and phosphate concentrations).

Species	Origin	Maximum number of animals	Life stage
Mus Musculus	Own breeding & commercial supplier	360	5-15 weeks old mice (adult male mice; unless indicated otherwise)

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'?

[] 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.

Refinement
Some genetically altered animal models in our research question (i.e, FGF23 KO mice) are not a complete CKD model and therefore they need an extra approach to induce CKD. Among the available experimental models for CKD, the 5/6 nephrectomy has been a mainstay for studies of kidney disease and is performed by unilateral nephrectomy and removing the poles of the remaining kidney after one week from the first surgery [1,2]. 5/6 nephrectomy shows common features to CKD observed in humans [3]. It has been used to test new therapies [4,5] and has been proven to be clinically relevant [6]. A less invasive model like adenine treatment is also available which does not require surgery. The major disadvantage is that the adenine model produces a more severe form of bone disease than 5/6 nephrectomy does[7]. Here, we are looking at the effect of the FGF23-klotho-vitamin D axis, which are bone and kidney originated genes/hormones. Therefore, the adenine diet is not useful for our study. In addition, we performed a pilot study ([redacted]), to check whether chronic injection of Doxorubicin can lead to CKD in C57bl/6 mice. We selected C57bl/6

mice since most of our KO mice are generated in this mice strain. We found that this model is not severely inducing CKD and this model is not optimal to study CKD. Therefore using a surgical approach like 5/6 nephrectomy will be used to induce sufficient amount of CKD in a genetically altered mice model which are interesting to our research question.
During the experiment the mice will be housed together in groups with free access to food and water. However, short periods of solitary housing in metabolic cages are necessary, because of the necessity of the measurement of key parameters in the urine. When the mice are in metabolic cages they will be placed in the same room as the other mice, to reduce stress levels. Before starting any experiment, extensive literature search and possibly a pilot-study will ensure that no sudden severe unwanted side-effects arise during the experiment. To obtain the best results possible, and reduce the amount of discomfort, mice will only be handled by skilled researchers and (bio)technicians. In addition, prior to sacrifice, the animals will be anesthetized. Moreover, our department is experienced in research regarding ion transport. The surgically induced CKD is a well-established model of CKD.
Replacement
The use of animals is essential to understand the basis of human disease at the systems and whole organism level, and to provide a link between *in vitro* studies and clinical disease. For a multi-organ disease, like chronic kidney disease, there is no substitute for animal studies. It would be neither permissible nor ethical to carry out the necessary procedures in humans, and simulations cannot provide answers to the questions we seek to address. In addition, the simple conditions for ion transport *in vitro* are not able to reproduce the complex ionic (micro-) gradients encountered in vivo. Moreover, the complicated interplay of organs, in which regulatory mechanisms and hormonal regulation play a crucial role, cannot be mimicked in a lower animal species.
For the majority of the proposed studies, the mouse is the most appropriate animal model because:
- it is a mammal;
- physiology is more extensively characterized in mice than in other mammalian model species;
- mice are amenable to transgenic manipulation;
- a large number of relevant transgenic and knock out lines are already available.

Reduction
A power calculation will be performed to determine the minimal amount of mice required to achieve significant differences in the primary outcome. If necessary, because not all information is available, a pilot study will be performed with the 5/6X nephrectomy technique and the intervention with RAAS blockades. If the pilot experiment generates unfavourable results, the actual experiment will not be performed. Based on provided calculation the minimum number of animals will be used for our experiments.
1. Kang DH, Nakagawa T, Feng L, Johnson RJ. Nitric oxide modulates vascular disease in the remnant kidney model. Am J Pathol. 2002;161:239–248.
2. Santos LS, Chin EW, Ioshii SO, Tambara Filho R. Surgical reduction of the renal mass in rats: morphologic and functional analysis on the remnant kidney. Acta Cir Bras. 2006;21:252–257.
3. Kren S, Hostetter TH. The course of the remnant kidney model in mice. Kidney Int. 1999;56:333–337.
4. Fujihara CK, Malheiros DM, Zatz R. Losartan-hydrochlorothiazide association promotes lasting blood pressure normalization and completely arrests long-term renal injury in the 5/6 ablation model. Am J Physiol Renal Physiol. 2007;292:F1810–1818.
5. Terzi F, Burtin M, Hekmati M, Jouanneau C, Beaufils H, Friedlander G. Sodium restriction decreases AP-1 activation after nephron reduction in the rat: role in the progression of renal lesions. Exp Nephrol.2000;8:104–114.
6. Waanders F, Vaidya VS, van Goor H, Leuvenink H, Damman K, Hamming I, Bonventre JV, Vogt L, Navis G. Effect of renin-angiotensin-aldosterone system inhibition, dietary sodium restriction, and/or diuretics on urinary kidney injury molecule 1 excretion in nondiabetic proteinuric kidney disease: a post hoc analysis of a randomized controlled trial. Am J Kidney Dis. 2009;53:16–25.

7. Ferrari, Guaraciaba O; Ferreira, Juliana C; Cavallari, Raquel T; Neves, Katia R; dos Reis, Luciene M et al. Mineral bone disorder in chronic kidney disease: head-to-head comparison of the 5/6 nephrectomy and adenine models. (2014) BMC nephrology vol. 15 (1) p. 69

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

In general, [redacted]. 5/6X nephrectomy [redacted], but it is the known golden standard to induce CKD in mice. (in this DAP, the mice undergo to 5/6 nephrectomy have severe discomfort and the sham operated mice experience moderate discomfort).
Often we will use food/water intervention, surgically induced (5/6X nephrectomy) CKD models, blood sampling, housing in a metabolic cage for minimum possible days. Animal handling will be performed by skilled researchers and/or (bio)technicians at the animal facility to reduce the adverse effect.
Moreover, animals will be housed together, unless when the collection of urine and faeces is desired. When the mice are in the metabolic cages, they will be housed in the same room as the other mice to reduce stress levels.

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 of the Directive 2010/63/EU?

[] No

F. Accommodation and care

[X] Yes > If this may adversely affect animal welfare, describe how the animals will be housed and provide specific justifications for these choices.

Preferably mice should be group housed but in some cases this will not be possible:
Mice will be individually placed in metabolic cages for a duration of 48 hours (24 hours adaptation, 24 hours collection) that is necessary for the collection of urine and faeces, and to determine food and water intake. To reduce the discomfort, the cages will be placed in the room together with the normal cages.
Mice will get a RAAS blockade and this is incorporated in their food or water. It is still possible to group house the mice.

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, analgesiaor 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.

The analgesic temgesic (0,05 mg/kg) will be administrated 15 minutes before the surgery and further anesthetized to stage 1.5% to 3.0% (percentage of isoflurane) by the inhalation of isoflurane (21% O2; 68% N2) in an induction chamber. During surgery time, animals will be in a temperature control bed. After the surgery the temgesic will be injected every 12 hours for 2 days to reduce post-operative pain according to normal procedure.

I. Other aspects compromising the welfare of the animals

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

The adverse effects on welfare of the surgical approach mice model is the development of CKD and involved side-effects hereof.
The 5/6X nephrectomy consists of a ligation of the lower branch of the left renal artery to produce about one third area with visible renal ischemia; the upper pole of the left kidney is removed by cautery and the right kidney is decapsulated and nephrectomized to induce a total 5/6th nephrectomy. It is a severe surgery that can lead to weight loss/lack of weight gain, anemia, increase in blood pressure and kidney damage of course. Some animals can die during the experiments. To avoid discomfort during surgery, animals will be pre- and post-operatively supplied with analgesics (temgesic, due to the mainly hepatic elimination, this medication does not accumulate when renal function is compromised such as an ischemia injury) and surgery will be performed under anesthesia, while the animal rests on a heating mat to maintain its body temperature. To aid the mice recover after surgery, the animals will be placed for in a heated incubator for 1,5 h. After the surgery the pain killer will be injected every 12 hours for 2 days to reduce post-operative pain.
There is a 10-15% chance of post-surgical mortality.
There is no side effect after administration of RAAS blockades. However, on the basis of clinical signs (appearance: fur and behaviour assessed daily, weight: more than 15% of the initial bodyweight during experiment) mice might be removed from the study and will be sacrificed by a biotechnician or the responsible investigator. By monitoring the mice daily, we will make sure to exclude those mice from the study in a timely manner.
In general, the procedures with expected adverse effects on welfare include surgery to induce CKD, blood sampling, solitary housing (metabolic cages), and adverse effects from the surgery.
There is no phenotypical adverse effect of KO mice (i.e, FGF23) which will be used in this experiments.

Explain why these effects may emerge.

- Development of CKD due to surgical intervention (5/6X nephrectomy)
- Death due to surgery (10-15% chance)
- Solitary housing in metabolic cage
- Handling
- Collection of blood

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

The 5/6X nephrectomy results in severe discomfort. Mice will get pre- and post-surgical analgesics.
The RAAS blockades incorporated in food/water will not cause any discomfort.
Under normal circumstances, mice will be housed together in groups. Discomfort is mainly related to metabolic cages, in which they are housed solitary. However, it is not possible to avoid the use of these cages, because of the necessity of the measurement of key parameters in the urine.
When the mice are in metabolic cages they will be placed in the same room as the other mice, to reduce stress levels.
Mice will be monitored closely. Daily inspection by the animal care takers of the animal facility and inspection by the responsible researcher. This may include animal behavior, mice fur, body weight, colour of urine and other behavioral changes as a result of the induced intervention. When unexpected changes are found, the mice will be removed from the experiment to prevent further discomfort.
To obtain the best results possible, and reduce the amount of discomfort, mice will only be handled by skilled researchers and (bio)technicians.
Prior to sacrifice, the animals will be anesthetized.

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 an humane endpoint are:

1. The animal experiences more than little, additional, discomfort as a result of conditions not related to the experiment.

2. Mice will be excluded from the experiments and immediately euthanised under general anaesthesia in case of visible signs of complications or discomfort like loosing weight which is more than 15% of the initial weight during experimental period. When these complications occur or when an animal shows other signs of discomfort such as bad fur or does not respond adequately to stimuli (very slow in movements), they will be sacrificed.

3. The animal experiences more discomfort than justified for the purpose of the experiment and weighed by the CCD, DEC or IvD.

4. (Reliable and applicable) results cannot be achieved because of conditions not related with the experiment.

5. The objective of the experiment has been reached.

6. In 5/6x nephrectomy, by daily monitoring we will exclude and sacrifice mice which are slow in movements, feels in pain and in the case of observation blood in urine, in the case of observed fluid faeces.

The responsible researcher will contact the Animal Welfare Body (IvD) if criteria 1, 2, 3, 4, 5 and/or 6 are applicable and the animal will not be taken out of the experiment.

If criteria 1, 2, 3, 4, 5 and/or 6 are applicable and the responsible researcher is not available, an animal care taker or biotechnician has the obligation, (preferably after internal communication) to take the animal out of the experiment and euthanise it under general anaesthesia.

Indicate the likely incidence.

The 5/6x nephroctomy surgical approach, which is being used in this project proposal, might cause to an humane endpoint. The incidence is approximately 10-15%, according to previous experiments performed in [redacted].
The chance of reaching an humane endpoint due to the RAAS blockades is almost zero.
Metabolic cages for 48h periodically have also almost zero human endpoint.
The chance of reaching an humane endpoint due to the gene KO is almost zero (no adverse phenotype).
Therefore, the chance of reaching a humane endpoint is 5-7.5%.

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).

Due to the induction of CKD via a surgical procedure (5/6X nephrectomy), 50% of the mice will experience more discomfort. 5/6x nephrectomy has a 10-15% post-surgery mortality. So 5/6 nephrectomy will result in a severe discomfort.
Due to a sham-operation and the accumulation of procedures with a mild discomfort, 50% of the mice will experience moderate discomfort.

End of experiment

L. Method of killing

Will the animals be killed during or after the procedures?

[] No > Continue with Section 3: 'Signatures'.
[X] Yes > Explain why it is necessary to kill the animals during or after the procedures.

Mice are killed at the end of the experiment, to collect blood and organs.

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

[] No > Descripe 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.zbo-ccd.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'.	10300	
1.2	Provide the name of the licenced establishment.	Stichting Katholieke Universiteit Nijmegen	
1.3	List the different types of animal procedures. Use the serial numbers provided in Section 3.4.4 of the Project Proposal form.	Serial number 4	Type of animal procedure CKD induced calcification treatment with Mg2+

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.

Vascular calcification and atherosclerosis is mainly due to hyperphosphatemia and hypercalcemia. Magnesium as 'a natural calcium antagonist' is known to adjust the activity of calcium ions. In addition, vitamin D, fibroblast growth factor 23 (FGF-23), and klotho represent an endocrine axis involved in the regulation of calcium and phosphate metabolism. A rapidly increasing body of evidence supports involvement of the FGF23-klotho-vitamin D axis in vascular calcification. However, not so much information related to the effect of magnesium on FGF23-klotho-vitamin D axis levels in suppression or retardation of vascular calcification in CKD, is available. Therefore, here we propose to investigate the effect of magnesium on calcification in CKD mice models (e.g. klotho KO, 1a-hydroxylase KO).
To investigate this, mice will be under a dietary intervention with magnesium. This diet contains either a normal or high amount of magnesium. Groups are as below:
- Wild-type mice + high Mg2+ diet
- Wild-type mice + normal diet
- Transgenic CKD mouse model + high Mg2+ diet
- Transgenic CKD mouse model + normal diet

Animals coming from the same family will be randomly distributed across the groups, we will then check the body weight because we want to be sure that we don't have one group with all the smallest/biggest animals. For this reason based on their body weight we correct the distribution of the animals when it is necessary. We don't expect to find any big differences since they have similar age.
To quantify the improved phenotype of the CKD mice with high Mg2+ diet, several outcome parameters will be investigated.
The primary outcome parameters are serum and urine calcium, magnesium and phosphate concentrations. Serum magnesium values will be determined, as well as several markers for calcification. To determine the reduced calcification, calcium deposition, transdifferentiation of cells and apoptosis will be measured by applying quantification of calcium. Moreover, using metabolic cages differences in urinary excretion of magnesium, calcium and other ions (Na, K, …) can be determined.
One other important outcome is to check the reduction in calcification of vascular smooth muscle cells and reduction in CKD stage. We will also determine the expression of Ca2+ and phosphate transporters in the intestines and kidney. In addition, we will check the level of FGF23, klotho and vitamin D levels in serum, urine and the kidneys of the mice. We will also study more in depth the effects on several proteins involved in the intracellular pathways involved in FGF23, klotho and vitamin D handling. There is a great expertise in our department regarding CKD and ion handling.

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

We will use a genetic mouse model of CKD (i.e, klotho KO and possibly others) and we will use high Mg2+ diet to reduce the calcification.
Using dietary intervention, the effect of magnesium on vascular calcification in the WT and KO mice models of CKD (e.g. klotho KO) can be thoroughly assessed. Mice will get the magnesium in their diet. Blood samples will subsequently be collected at different time intervals (start, middle and end) to determine the concentration of the substance. Blood withdrawal will be performed by cheek puncture or via tail vein.
This will also allow us to follow serum Ca2+ concentrations over time to see how Mg2+ is effecting to inhibit CKD. It is necessary that animals are housed in a metabolic cage in several stages of the experiment: at the start, middle and end of the experiment, to collect faeces and urine samples, and thus determine the concentration of the substance and/or metabolites. Finally mice will be sacrificed so organs and blood can be harvested. The organs are used for immunostainings, immunoblot and RNA isolations.
The duration that animals will be in the experiment is different depending on the strain of mice (3 to 8 weeks). For instance, klotho KO mice have short life span and therefore they will be shorter (3 to 4 weeks) in the experiment in comparison to the other strains of mice.

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

Subsequently in each individual experiment, independent statistics will be carried out by using a power calculation as below, to reduce the amount of mice used to a minimum:

$$N = \frac{2(Za + Z\beta)2\ s2}{d2}$$

With a power of 80% and a significance of 5%, and specific variations per primary outcome parameter, the minimal amount of mice needed for a significant difference will result from this calculation.

B. The animals

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

Max. 150 adult mice.
We will make use of adult male mice (mus musculus). We will make use of mice which are genetically modified to develop CKD and vascular calcification (e.g. klotho KO). These mice are often of C57bl/6 background as our previous data are generated using this strain, making our results easy to compare to previous results. To study cardiovascular complications of CKD the mouse is a well-established model in this field of research and a lot of literature is available. In addition, given the fact that our research questions are largely mechanistically and involves the study of cardio/renal tissue, the use of animals in this type of study is unavoidable. We will therefore make use of mice because 1) the mouse is well-established in this field of research and 2) the mouse offers superior genetic models to study the molecular genetic background of complex diseases.

Furthermore, mice resemble the situation in humans with CKD much better than cultured cells. Therefore, *in vivo* studies in animals cannot be avoided.
By choosing to male mice, the potential distribution due to hormonal influences is kept as low as possible. It is currently not known whether changing levels of sex hormones may exert an influence on the regulation of calcium/phosphorus balance.
This year we only will use klotho KO mice, but we expect that this experiment will include approximately 2 to 3 different mouse strains as a CKD model. Given that approximately 40-50 mice per strain will be suitable therefore approximately 150 mice will suit this experimental design.
To reduce the amount of mice used to a minimum, we performed a very decent *in vitro* experiment as below: In short: high phosphate is the hallmark of CKD and to mimic the calcification in CKD patients, human smooth muscle cells were incubated and cultured in high phosphate levels (3mmol) in the absence or presence of magnesium chloride (final concentration 2mmol). After 14 days, clear calcium phosphate deposits were observed under high phosphate conditions, which were completely absent in the cells co-cultured with high concentrations of mg2+.

Species	Origin	Maximum number of animals	Life stage
Mus Musculus	Own breeding & Commercial supplier	150	5-15 weeks old mice (adult mice; unless indicated otherwise)

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'?

[] 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.

Refinement

During the experiment the mice will be housed together in groups with free access to food and water. However, short periods of solitary housing in metabolic cages are necessary, because of the necessity of the measurement of key parameters in the urine. When the mice are in metabolic cages they will be placed in the same room as the other mice, to reduce stress levels. Before starting any experiment, extensive literature search and possibly a pilot-study will ensure that no sudden severe unwanted side effects arise during the experiment. To obtain the best results possible, and reduce the amount of discomfort, mice will only be handled by skilled researchers and (bio)technicians. In addition, prior to sacrifice, the animals will be anesthetized. Moreover, our department is experienced in research regarding ion transport.
Replacement
For a multi-organ disease, like chronic kidney disease, there is no substitute for animal studies. It would be neither permissible nor ethical to carry out the necessary procedures in humans, and simulations cannot provide answers to the questions we seek to address. In addition, the simple conditions for ion transport in vitro are not able to reproduce the complex ionic (micro-) gradients encountered in vivo, therefore use of a mouse model is needed. Moreover, the complicated interplay of organs, in which regulatory mechanisms and hormonal regulation play a crucial role, cannot be mimicked in a lower animal species or cell models.
Reduction
Statistics will be used to determine the minimal amount of mice required to achieve significant differences in the primary outcome. When necessary, a pilot study will be performed to ensure that the results obtained from the study will be valuable. This will minimize the chances of having to re-perform a certain experiment. If the pilot experiment generates unfavourable results, the actual experiment will not be performed. Based on provided calculation the minimum number of animals will be used for our experiments. Our *in vitro* data will reduce the amount of mice: instead of using mice and giving them a high phosphate diet, we performed this part *in vitro* to find out whether magnesium has any effect in the vascular calcification and after this observation we can directly test magnesium in the mice, we believe this is a part of reduction that reduced the amount of mice.

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

In general, the discomfort of the mice is likely to be moderate.
In some cases, the combination of different mild experiments will lead to a higher degree of discomfort, which may be considered as moderate in combination. Often we will use diet/water intervention, genetically altered CKD models, blood sampling, housing in a metabolic cage for minimum possible days. Animal handling will be performed by skilled researchers and/or (bio)technicians at the animal facility to reduce the adverse effects due to stress.
Moreover, animals will only be housed together, unless when the collection of urine and faeces is desired.
When the mice are in the metabolic cages, they will be housed in the same room as the other mice to reduce stress levels.
When experiments will be performed with unknown (side)effects, a pilot will be performed prior to the actual study.

Repetition and Duplication

E. Repetition

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 of the Directive 2010/63/EU?

[] No
[X] Yes > If this may adversely affect animal welfare, describe how the animals will be housed and provide specific justifications for these choices.

Preferably mice should be group housed but in some cases this will not be possible:
Mice will be individually placed in metabolic cages for a duration of 48 hours (24 hours adaptation, 24 hours collection) that is necessary for the collection of urine and faeces, and to determine food and water intake. To reduce the discomfort, the cages will be placed in the room together with the normal cages.
Mice will get a dietary intervention with high Mg2+ and this is incorporated in their food. It is still possible to group house the mice.

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, analgesiaor other pain relieving methods be used?

[X] No > Justify why pain relieving methods will not be used.

Blood collection can be painful, but because of the stress and risk of death by anesthesia we will not use this, as is custom for blood collection. Anesthesia might have a possible effect on the parameter we want to measure in the blood.

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

I. Other aspects compromising the welfare of the animals

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

The main adverse effects on welfare of the genetic modification is the development of CKD and involved side effects hereof. Klotho KO and in the case of using 1a-hydroxylase KO mice, both will experience moderate discomfort due to the development of CKD. Therefore, KO mice are not bred unless needed for an experiment (a breeding DEC is necessary, and already approved for our department). Klotho KO mice do not grow normal and the size is half of the size of normal mice and their average survival is approximately 16 weeks. These mice are an accelerated model of ageing. 1a-hydroxylase KO mice are fragile due to lack of vitamin D and the bones are soft, therefore the chance of fractured bones is higher than in normal mice.
A magnesium-rich diet has been done before in our department and the amount and percentage is already optimized and no adverse effects are being reported.
On the basis of clinical signs (appearance: fur and behavior assessed daily, weight: more than 15% of the initial bodyweight during experiment) mice might be removed from the study and will be sacrificed by a biotechnician or the responsible investigator. By monitoring the mice daily, we will make sure to exclude those mice from the study in a timely manner.

In general, the procedures with expected adverse effects on welfare include genetically altered mice to develop CKD, blood sampling, solitary housing (metabolic cages), and adverse effects from the interventions.
There will not be any surgical approaches in this subproject.

Explain why these effects may emerge.

- Development of CKD due to genetically altered model
- Solitary housing in metabolic cage
- Handling
- Collection of blood

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

The genetically modified mice are actually CKD models, therefore the discomfort they undergo cannot be prevented. They require a DEC to breed due to discomfort. Klotho KO mice develop a hunch back in early life and die early (life length approximately 16 weeks). To minimize the number of animals experiencing discomfort due to phenotype, KO mice will only be bred when needed for an experiment, and in the quantity needed for the experiment.
The Mg2+ incorporated to diet will not cause any discomfort.
Under normal circumstances, mice will be housed together in groups. Discomfort is mainly related to metabolic cages, in which they are housed in solitary. However, it is not possible to avoid the use of these cages, because of the necessity of the measurement of key parameters in the urine. When the mice are in metabolic cages they will be placed in the same room as the other mice, to reduce stress levels.
Mice will be monitored closely. Daily inspection by the animal care takers of the animal facility and inspection by the responsible researcher. This may include animal behavior, mice fur, body weight, colour of urine and other behavioral changes as a result of the induced intervention. When unexpected changes are found, the mice will be removed from the experiment to prevent further discomfort.
To obtain the best results possible, and reduce the amount of discomfort, mice will only be handled by skilled researchers and (bio)technicians.
Prior to sacrifice, the animals will be anesthetized.

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 an humane endpoint are:

1. The animal experiences more than little, additional, discomfort as a result of conditions not related to the experiment.

2. Mice will be excluded from the experiments and immediately euthanised under general anaesthesia in case of
visible signs of complications or discomfort like loosing weight which is more than 15% of the initial weight during experimental period. When these complications occur or when an animal shows other signs of discomfort such as hunchback and bad fur or does not respond adequately to stimuli (very slow in movements), they will be sacrificed.
3. The animal experiences more discomfort than justified for the purpose of the experiment and weighed by the CCD, DEC or IvD.

4. (Reliable and applicable) results cannot be achieved because of conditions not related with the experiment.

5. Klotho KO are less tolerable to any intervention than normal mice and therefore those mice will be closely monitored and in the case of observed complications, they will be excluded from study.
Eearly hunch back phenotype is an humane end points which can be observed with these mice.

6. The objective of the experiment has been reached.

The responsible researcher will contact the Animal Welfare Body (IvD) if criteria 1, 2, 3, 4, 5 and/or 6 are applicable and the animal will be taken out of the experiment.

If criteria 1, 2, 3, 4, 5 and/or 6 are applicable and the responsible researcher is not available, an animal care taker or biotechnician has the obligation, (preferably after internal communication) to take the animal out of the experiment and euthanise it under general anaesthesia.

Indicate the likely incidence.

The incidence of humane endpoints due to genetic modification is present since specific CKD models of KO mice is being used in this procedure lead to discomfort; this is approximately 3-5%, according to previous experiments using klotho KO mice performed in our group.
The chance of reaching an humane endpoint due to the magnesium diet is almost zero.
Metabolic cages for 48h periodically have also almost zero human endpoint.
Therefore, the chance of reaching a humane endpoint is 1.5-2.5%.

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).

Due to either induction of CKD or the cumulative effect of mild procedures, 100% of the mice will experience moderate discomfort.

End of experiment

L. Method of killing

Will the animals be killed during or after the procedures?

[] No > Continue with Section 3: 'Signatures'.
[X] Yes > Explain why it is necessary to kill the animals during or after the procedures.

Mice are killed at the end of the experiment, to collect blood and organs.

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

[] No > Descripe 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 2015-0076
2. Titel van het project: The FGF23-klotho-vitamin D axis as a new instrumental target to combat the cardiovascular risk of chronic kidney disease.
3. Titel van de NTS: Het gebruik van de 'FGF23-klotho-vitamine D as' als middel om het cardiovasculaire risico van chronische nierziekte te bestrijden.
4. Type aanvraag:
   - ☐ nieuwe aanvraag projectvergunning
5. Contactgegevens DEC:
   - Naam DEC: RUDEC
   - Telefoonnummer contactpersoon: [redacted], bereikbaar op maandag, dinsdag, en donderdag van 9:00 tot 15:00 uur
   - Mailadres contactpersoon: [redacted]
6. Adviestraject:
   - ☐ ontvangen door DEC: 23-04-2015
   - ☐ aanvraag compleet
   - ☐ in vergadering besproken: 12-05-2015
   - ☐ anderszins behandeld
   - ☐ termijnonderbreking(en) van 18-05-2015 tot 28-05-2015 en van 24-06-2015 tot 10-08-2015
   - ☐ besluit van CCD tot verlenging van de totale adviestermijn met maximaal 15 werkdagen n.v.t.
   - ☐ aanpassing aanvraag: 28-05-2015 en 10-08-2015
   - ☐ advies aan CCD: 07-09-2015
7. Eventueel horen van aanvrager
   - Datum
   - Plaats
   - Aantal aanwezige DEC-leden
   - Aanwezige (namens) aanvrager
   - Strekking van de vraag / vragen
   - Strekking van het (de) antwoord(en)
   - Het horen van de aanvrager heeft geleid tot aanpassing van de aanvraag
8. Correspondentie met de aanvrager
   - Datum: 18-05-2015
   - Strekking van de vragen:
   - **Niet-technische samenvatting:**

     - 1.1 De titel is niet te begrijpen voor de doelgroep. De onderzoekers worden verzocht dit te herformuleren. Wat is een instrumentale doelstelling?

     - 3.1 De commissie mist een korte uitleg over FGF23 en klotho.

     - 3.5 De categorie matig tot ernstig bestaat niet meer. Zal 10% van de dieren ernstig ongerief ervaren?

- - 4.1 De onderzoekers stellen dat er grote overeenkomsten zijn tussen muis en mens. Kunnen zij duidelijker uitleggen wat ze daarmee bedoelen?
- **Project Proposal:**
  - 3.1 De FGF23-klotho-vitamine D as staat aan de basis van de projectaanvraag. Kunnen de onderzoekers beter uitleggen wat FGF 23 en klotho zijn, en waarom zij samen met vitamine D een as vormen? Voorts is niet duidelijk waarom klotho deficiëntie via verzwakking van FGF23 signaling in de nier leidt tot een toename in plaats van een afname van FGF23. De uitleg die in 3.2 bij de doelstelling wordt gegeven, zou beter toegevoegd kunnen worden aan 3.1, zodat de doelstelling logischer aansluit op de gegeven achtergrond.
  - 3.4.1 Dit is geen overall design c.q. stappenplan. Waarom willen de onderzoekers zowel genetische als chirurgische modellen voor CKD gebruiken? De rationale achter deze aanpak ontbreekt, terwijl het chirurgische model ernstig ongerief voor de dieren oplevert. De onderzoekers kunnen deze experimenten alleen doen indien zij dit afdoende onderbouwen. In het algemeen vertonen de onderdelen 3.4.1 en 3.4.2 een grote mate van overlap. De commissie geeft de aanvragers in overweging 3.4.1 vooral te gebruiken voor het schetsen van het overall design (zonder te specifiek te worden).
- **Description of Animal Procedures:**
  - DAP 1, onderdeel A eerste vraag: Waarom willen de onderzoekers het effect van meerdere remmers onderzoeken, terwijl zij allen zorgen voor blokkade van RAAS?
  - DAP 1, onderdeel A derde vraag: Op basis waarvan wordt het meest geschikte model gekozen uit de drie genetische modellen?
  - DAP 1, onderdeel B. De onderbouwing van het aantal dieren wordt gegeven bij onderdeel A derde vraag, terwijl zij bij onderdeel B gevraagd wordt. Het is niet duidelijk waarom deze experimenten gedurende 5 jaar elk jaar herhaald moeten worden.
  - DAP2, onderdeel A eerste vraag: Waarom is er onderzoek in het chirurgisch model nodig als dezelfde vraagstelling al in het genetische model wordt onderzocht?
  - DAP 2, onderdeel B: De onderbouwing van het aantal dieren wordt gegeven bij onderdeel A derde vraag, terwijl zij bij onderdeel B gevraagd wordt. De onderzoekers gebruiken dezelfde redenering als in dierproef 1, maar komen nu uit op 200 dieren in plaats van 400 dieren. Wederom is niet duidelijk waarom dit experiment gedurende 5 jaar elk jaar herhaald moet worden.
  - DAP2, onderdeel K. De commissie is van mening dat de muizen ernstig ongerief ervaren als gevolg van de 5/6 nefrectomie.
  - DAP 2, onderdeel J, laatste vraag: Op deze manier geformuleerd leidt de operatie altijd tot het humane eindpunt. De onderzoekers worden verzocht de eerste zin te herformuleren.
  - DAP 3, onderdeel A derde vraag: Op basis waarvan wordt het meest geschikte model gekozen uit de drie genetische modellen?
  - DAP 3, onderdeel B. De onderbouwing van het aantal dieren wordt gegeven bij onderdeel A derde vraag, terwijl zij bij onderdeel B gevraagd wordt. Voorts is het is niet duidelijk waarom deze experimenten gedurende 5 jaar elk jaar herhaald moeten worden.
  - DAP 4, onderdeel A tweede vraag: Hoe gaan de onderzoekers bloed afnemen bij de dieren? Deze informatie ontbreekt bij de beschrijving van de experimentele handelingen.
  - DAP4, onderdeel A derde vraag: Hier wordt uitgelegd waarom de onderzoekers het effect van Mg-suppletie willen onderzoeken. Deze informatie hoort thuis in onderdeel 3.1 van het onderzoeksvoorstel.
- Datum antwoord: 22-06-2015
- Strekking van de antwoorden:
- **Niet-technische samenvatting:**

- 1.1: Het gebruik van de 'FGF23-klotho-vitamine D as' als middel om het cardiovasculaire risico van chronische nierziekte te bestrijden.
- 3.1: FGF23 is een hormoon dat calcium en fosfaat reguleert, voornamelijk in de nieren en de bijschildklier. Klotho is essentieel voor de fosfaat balans in het lichaam en het reguleert de uitscheiding van fosfaat via FGF23-signalering.
- 3.5: Ernstig ongerief: 10% van de muizen.
- 4.1: Het is gebleken dat tijdens de evolutie, veel genen in de nieren, en dus ook eiwitten, hetzelfde zijn gebleven tussen muis en mens, zogenaamde conservering. Omdat de genexpressie en de daaropvolgende eiwitexpressie heel belangrijk zijn in onderzoek naar de nieren, kan de muis goed gebruikt worden als model voor de mens.

- **Project Proposal:**

- 3.1: When phosphate is in excess, FGF23 is secreted from bone and acts on the kidney to promote phosphate excretion into urine and suppress vitamin D synthesis, thereby inducing negative phosphate balance. One critical feature of FGF23 is that it requires Klotho, a singlepass transmembrane protein expressed in renal tubules, as an obligate coreceptor to bind and activate FGF receptors.
FGF23 seems to function as a protective factor, as it triggers adaptive changes that maintain a normal body phosphate homeostasis. Thus, modulation of the FGF23-klotho-vitamin D axis could represent a promising therapeutic target that might improve the fatal prognosis of patients with CKD and cardiovascular diseases. Assuming that the study goals are met and it is demonstrated that elevated FGF23 levels and/or klotho deficiency are important in the treatment of CKD patients, they can become novel targets in the treatment of CKD patients. Using magnesium as a novel treatment strategy, we aim to improve outcome in CKD patients suffering from vascular calcification. Therefore, by uncovering the molecular mechanism of Mg2+ and how it results in vascular calcification, we aim to increase awareness among clinicians to routinely measure magnesium in CKD, and if needed, give magnesium supplementation to CKD patients.
- 3.4.1: Some genetically altered animal models in our research question (i.e, FGF23 KO mice) are not a CKD models and therefore they need an extra approach like 5/6x nephrecotmy surgical approach to complete as a valid model. To that aim next to genetic alteration, a surgical approach is necessary to have an appropriate model to study CKD.

- **Description of Animal Procedures:**

- DAP 1, onderdeel A eerste vraag: The renin–angiotensin–aldosterone system (RAAS) has a key role in the regulation of blood pressure, sodium and water balance, and cardiovascular and renal homeostasis. RAAS blockade using angiotensin-converting-enzyme inhibitors or angiotensin-receptor blockers is the cornerstone of treatment of renal disease. Alternative RAAS-blockade strategies include renin inhibition and aldosterone blockade. The effect of different RAAS blockades in FGF23, Klotho and vitamin D axes is not known. FGF23, Klotho and vitamin D axes can regulate differently when different parts like rennin, angiotensin-converting-enzyme, angiotensinreceptor or aldosterone is blocked. It is therefore important to investigate the effect of different blockade in FGF23, Klotho and vitamin. D axes. A better knowledge of the pathophysiology of the RAAS is crucial to fully understand the mechanisms of action of RAAS blockers and to exploit their renoprotective effects.
- DAP 1, onderdeel A derde vraag: We will decide which KO of CKD models are the most suitable strains, based on the primary outcome parameters (altered serum and urine urea, createnine, calcium and phosphate concentrations) after RAAS blockade intervention. This will be tested via pilot study. *[this part shifted to DAP 1, onderdeel B as requested]*

- DAP 1, onderdeel B: The number of mice is based on a project duration of 5 years. Each individual study group will include on average 40 mice allowing for 2-4 parallel experiments where RAAS blockades will be used in CKD mice. Terminal experiments will be carried out at 2 or 3 time points. We will decide which KO of CKD models are the most suitable strains, based on the primary outcome parameters (altered serum and urine urea, createnine, calcium and phosphate concentrations), after RAAS blockade intervention. To investigate RAAS and the FGF23-klotho-vitamin D axis in genetic CKD models, we expect to use 40 mice per experiment (approximately 4 groups of 10 mice). This is from several strains of WT and knock-out CKD mouse models. To reduce the amount of mice that will be used in total, first a pilot experiment will be carried out and for that we need 40 extra mice. So in total 440 mice is needed. Given those reasons: The estimated total number of mice is therefore 40 (mice) * 2 (experiments) * 5 (year) = 400.
- DAP2, onderdeel A eerste vraag: [also answered above]Some genetically altered animal models in our research question (i.e, FGF23 KO mice) are not a CKD models and therefore they need an extra approach like 5/6x nephrecotmy surgical approach to complete as a valid model. To that aim next to genetic alteration, a surgical approach is necessary to have an appropriate model to study CKD.
- DAP 2, onderdeel B: The number of mice is based on project duration of 5 years. Each individual study group will include on average 80 mice allowing for 2-4 parallel experiments where RAAS blockades will be used in CKD mice. Terminal experiments will be carried out at 2 or 3 time points. To investigate RAAS and the FGF23-klotho-vitamin D axis in CKD models, we expect to use 40 mice per experiment (approximately 4 groups of 10 mice). Due to 10-15% post-surgery mortality we would like to have 40 extra mice in total. Given those reasons: The estimated total number of mice is therefore 40 (mice) * 2 (experiments) * 5 (year) = 400. So in total 440 mice is needed.

[redacted]

- DAP 3, onderdeel A derde vraag: *[this part shifted to DAP 3, onderdeel B as requested]*
- DAP 3, onderdeel B.: The number of mice is based on a project duration of 5 years. Each individual study group will include on average 40 mice allowing for 2-4 parallel experiments where antioxidant will be used in CKD mice. Terminal experiments will be carried out at 2 or 3 time points. We will decide which KO of CKD models are the most suitable strains, based on the primary outcome parameters (altered serum and urine urea, createnine, calcium and phosphate concentrations), after an antioxidant intervention. To investigate pathways of oxidative stress and the FGF23-klotho-vitamin D axis in genetic CKD models, we expect to use 40 mice per experiment (approximately 4 groups of 10 mice). This is from several strains of WT and KO CKD mouse models. To reduce the amount of mice that will be used in total, first a pilot experiment will be carried out (with different mice strains) and for that we need 40 extra mice. Given those reasons:The estimated total number of mice is therefore 40 (mice) * 2 (experiments) * 5 (year) =400. So in total 440 mice is needed.
- DAP 4, onderdeel A tweede vraag: Blood withdrawal will be performed by cheek puncture or via tail vein.
- DAP4, onderdeel A derde vraag: This part moved to onderdeel 3.1 now.

- De antwoorden hebben geleid tot aanpassing van de aanvraag.

- Datum: 24-06-2015
- Strekking van de vragen:

  De onderzoekers wordt aangeraden nogmaals kritisch te kijken naar de engelse formuleringen en grammaticale fouten te verbeteren.

  -NTS:

  -3.5. Uit dierproef 2 blijkt dat 50 % van 440 muizen ernstig ongerief zullen ondervinden. Dit is 15% van het totaal aantal (1470) muizen. De onderzoekers worden verzocht de percentages aan te passen.

  -Project proposal:

  -[redacted]

*Er worden meerdere RAAS blokkades gebruikt. Hoeveel RAAS blokkades willen de onderzoekers hanteren en waarop is de keuze voor dit aantal gebaseerd? Wanneer de onderzoekers meerdere blokkades tegelijk onderzoeken in één experiment, kunnen zij volstaan met minder controles. Kunnen de onderzoekers aangeven of zij dit zullen doen, of beredeneren waarom dit niet mogelijk is? Zijn in het laatste geval toch elke keer weer dezelfde controles noodzakelijk?
-DAP2, onderdeel A. Zullen deze experimenten alleen met de FGF23 KO (en WT als controle) worden uitgevoerd? Zo nee, kunnen de onderzoekers uitleggen waarom de andere KO-stammen nodig zijn?
-DAP3, onderdeel B. Om het aantal muizen tot een minimum te beperken, is een in vitro experiment uitgevoerd. Kunnen de onderzoekers toelichten om welk in vitro experiment het gaat en uitleggen hoe dit het aantal muizen tot een minimum beperkt?
DAP 2 en 3, onderdeel B. De aantallen dieren in de tekst en in de tabel komen niet overeen. De onderzoekers worden verzocht deze aantallen in overeenstemming met elkaar te brengen.

- Datum antwoord: 10-08-2015
- Strekking van de antwoorden:
- [redacted]

1. *Kang DH, Nakagawa T, Feng L, Johnson RJ. Nitric oxide modulates vascular disease in the remnant kidney model. Am J Pathol. 2002;161:239–248.*

2. *Santos LS, Chin EW, Ioshii SO, Tambara Filho R. Surgical reduction of the renal mass in rats: morphologic and functional analysis on the remnant kidney. Acta Cir Bras. 2006;21:252–257.*
3. *Kren S, Hostetter TH. The course of the remnant kidney model in mice. Kidney Int. 1999;56:333–337.*
4. *Fujihara CK, Malheiros DM, Zatz R. Losartan--hydrochlorothiazide association promotes lasting blood pressure normalization and completely arrests long--term renal injury in the 5/6 ablation model. Am J Physiol Renal Physiol. 2007;292:F1810–1818.*
5. *Terzi F, Burtin M, Hekmati M, Jouanneau C, Beaufils H, Friedlander G. Sodium restriction decreases AP--1 activation after nephron reduction in the rat: role in the progression of renal lesions. Exp Nephrol.2000;8:104–114.*
6. *Waanders F, Vaidya VS, van Goor H, Leuvenink H, Damman K, Hamming I, Bonventre JV, Vogt L, Navis G. Effect of renin--angiotensin--aldosterone system inhibition, dietary sodium restriction, and/or diuretics on urinary kidney injury molecule 1 excretion in nondiabetic proteinuric kidney disease: a post hoc analysis of a randomized controlled trial. Am J Kidney Dis. 2009;53:16–25.*
7. *Ferrari, Guaraciaba O; Ferreira, Juliana C; Cavallari, Raquel T; Neves, Katia R; dos Reis, Luciene M et al. Mineral bone disorder in chronic kidney disease: head--to--head comparison of the 5/6 nephrectomy and adenine models. (2014) BMC nephrology vol. 15 (1) p. 69*

- **Description Animal Procedures:**

  -DAP 1, onderdeel B: Adult: max. 480

  The number of mice per experiment will be based on statistical analysis (power analysis), our experience with similar type of experiments and/or (un)published data as below:

  <u>Quantitative analysis</u>: prior to performing an experiment we perform statistical analysis to ensure that we use the minimum number of mice per group that will be statistically sound and biologically relevant.

  <u>Qualitative analysis</u> (most of our experiments): the number is based on literature and/or years of experience with similar types of experiments. Moreover, these types of experiments will be performed sequentially via which we ensure that we will use the minimum number of mice per group that will be necessary.

  It is currently impossible to exactly mention the number of mice required for these experiments over the next 5 years or per experiment. We have calculated the total number of mice based on experience over the past 5 years with similar type of experiments.

  *To that aim 40 mice are required for a complete and thorough analysis: 10 (number of mice per group) * 2 (different condition: with normal food/water or RAAS inhibitor in food/water) * 2 (KO and WT mice). However, the (simultaneously) performance where two genes are involved requires 80 mice and not less. Wild--type littermates of genetically modified mice are a better control group than normal bl/6 mice. Therefore it is not possible to reduce the amount of wild--type mice in the case that we study two different knock--out mice. Here we will use a maximum of 4 different RAAS blockades [each blockade may have a different effect]: 40 (mice need per experiment) * 4 (RAAS blockades) * 3 (different mouse models of CKD: klotho KO mice, 1α--hydroxylase KO mice, or other*

available mice strain suitable for our research question) so in total we will use a maximum of 480 mice in DAP1.
Importantly, before we will start our experiments we will write an application to the IVD. In this application we will exactly describe (among others) which considerations, facts and results have led to the proposition of the experiments, which specific question(s) we are trying to answer with the proposed animal experiments and what the ultimate goal is for the proposed experiments. Moreover we will describe the experimental design, argue the number of animals in the experiments, describe (i.a.) human endpoints, alternatives, nature of discomfort and ethical considerations. Experiments will only start upon IVD approval.
-DAP 2, onderdeel B: Adult: max. 480
The number of mice per experiment will be based on statistical analysis (power analysis), our experience with similar type of experiments and/or (un)published data. It is currently impossible to exactly mention the number of mice required for these experiments over the next 5 years or per experiment. We have calculated the total number of mice based on experience over the past 5 years with similar type of experiments.
*To that aim 40 mice are required for a complete and thorough analysis: 10 (number of mice per group) * 2 (different condition: with normal food/water or antioxidant (i,e., vitamin E in food/water) * 2 (KO and WT mice). However, the (simultaneously) performance where two genes are involved requires 80 mice and not less. Wild--type littermates of genetically modified mice are a better control group than normal bl/6 mice. Therefore it is not possible to reduce the amount of wild--type mice in the case that we study two different knock--out mice. Here we will use 2--4 different antioxidants, due to variation in the effects of antioxidants reported in the literature. Therefore: 40 (mice need per experiment) * 4 (antioxidants) * 3 (different mice model of CKD: klotho KO mice, 1α--hydroxylase KO mice, or other available mice strain suitable for our research question) so in total we will use a maximum of 480 mice in DAP2.*
Importantly, before we will start our experiments we will write an application to the IVD. In this application we will exactly describe (among others) which considerations, facts and results have led to the proposition of the experiments, which specific question(s) we are trying to answer with the proposed animal experiments and what the ultimate goal is for the proposed experiments. Moreover we will describe the experimental design, argue the number of animals in the experiments, describe (i.a.) human endpoints, alternatives, nature of discomfort and ethical considerations. Experiments will only start upon IVD approval.
-DAP3, onderdeel B: Adult, max. 360.
The number of mice per experiment will be based on statistical analysis (power analysis), our experience with similar type of experiments and/or (un)published data. It is currently impossible to exactly mention the number of mice required for these experiments over the next 5 years or per experiment. We have calculated the total number of mice based on experience over the past 5 years with similar type of experiments.
*For the RAAS blockade experiments 80 mice are required for a complete and thorough analysis: 10 (number of mice per group) * 2 (different food/water: with*

*normal food/water or RAAS inhibitor in food/water) * 2 (with sham operated or 5/6X nephrectomy operated) * 2 (KO and WT mice). However, the (simultaneously) performance where two genes are involved requires 160 mice and not less. Wild--type littermates of genetically modified mice are a better control group than normal bl/6 mice. Therefore it is not possible to reduce the amount of wild--type mice in the case that we study two different knock--out mice.*
*Given that we will study approximately 2 different KO mice and maximally 2 different RAAS blockades which this will make it: 80 mice per study * 2 genotypes * 2 inhibitors = 320 mice. Due to 10--15% post--surgery mortality we would like to have 40 extra mice in total. Given those reasons: we will have in total* ***360*** *mice for this experiment.*
Importantly, before we will start our experiments we will write an application to the IVD. In this application we will exactly describe (among others) which considerations, facts and results have led to the proposition of the experiments, which specific question(s) we are trying to answer with the proposed animal experiments and what the ultimate goal is for the proposed experiments. Moreover we will describe the experimental design, argue the number of animals in the experiments, describe (i.a.) human endpoints, alternatives, nature of discomfort and ethical considerations. Experiments will only start upon IVD approval.
At this moment (this year) we are aiming to use klotho, 1α-hydroxylase and FGF23 KO mouse models. However, 5 years is a long time and there certainly will be new interesting genes for us to generate a new KO mouse model with that. Therefore we would like to keep the possibility to be able to use other newly generated mouse models. These models will be important and interesting to our research questions and potentially will help us in understanding more about the FGF23--klotho--vitamin D axis in CKD in the coming 5 years. This new KO mice must have a proofed/potential relations with FGF23, klotho and vitamin D,to be interesting to our research question.
Before starting the experiments, we will write an application to the IVD. In this application we will exactly describe (among others) which considerations, facts and results have led to the choice of mice model, proposition of the experiments, which specific question(s) we are trying to answer with the proposed animal experiments and what the ultimate goal is for the proposed experiments. Moreover we will describe the experimental design, argue the number of animals in the experiments, describe (i.a.) human endpoints, alternatives, nature of discomfort and ethical considerations. All the experiments will only start upon IVD approval.
Alteration and blocking of RAAS from different sites might have a different effect on the FGF23--klotho--vitamin D axis. This, however, has not yet been tested and therefore in our research we would like to make use of RAAS blockades using angiotensin--converting--enzyme (ACE) inhibitors or angiotensin--receptor blockers (ARB), renin inhibition and aldosterone blockade. Therefore all 4 types of RAAS inhibitors will be tested in DAP 1 and if the effect is similar in our primary outcome data then we will only continue with ARB inhibitors. In DAP 1, RAAS blockades will be tested independently but as mentioned before we cannot reduce the amount of WT mice, since different littermates can act differently. There is published data from my thesis [redacted] *showing the huge different effect of C57BL/6*

mice and littermate mice. Hence the separate littermates for every KO mice are always essential.
-DAP3, onderdeel A. *As mentioned above in DAP3, first we will use FGF23 KO mice to perform the experiment. However 5 years is a long time and there will be certainly new interesting genes for us to generate KO mice models. Therefore we would like to keep the possibility to use other newly generated mouse models. These models will be important and interesting to our research questions and potentially will help us in understanding more about the FGF23--klotho--vitamin D axis in CKD in the coming 5 years.*
-DAP3, onderdeel B. *An in vitro study has been performed and the data are very promising. In short: high phosphate is the hallmark of CKD and to mimic the calcification in CKD patients, human smooth muscle cells were incubated and cultured in high phosphate levels (3mmol) in the absence or presence of magnesium chloride (final concentration 2mmol). After 14 days, clear calcium phosphate deposits were observed under high phosphate conditions, which were completely absent in the cells co--cultured with high concentrations of mg2+.*
This in vitro study will reduce the total amount of mice needed in our project proposal. It is no longer necessary to investigate whether magnesium has any effect on vascular calcification, during a high phosphate diet. Instead, we can directly test magnesium as a possible intervention for CKD.
-DAP 2 en 3, onderdeel B. *The number of animals in the text and in the table are adjusted.*

- De antwoorden hebben geleid tot aanpassing van de aanvraag.

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

B. Beoordeling (adviesvraag en behandeling)

1. Het project is vergunningplichtig.
2. De aanvraag betreft een nieuwe aanvraag.
3. De DEC is competent om hierover te adviseren.

C. Beoordeling (inhoud):

1. Het project is:
   - ☐ uit wetenschappelijk oogpunt verantwoord

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

3. De DEC onderschrijft het belang van de doelstelling, namelijk ‘to gain more insight in the underlying mechanism involved in the development of CKD and its extra renal consequences in the vascular bed’. Dit onderzoeksdoel is onderverdeeld in vier daar logisch uit voortvloeiende subdoelen, namelijk ‘to delineate the role of RAAS blockade on the FGF23-klotho-vitamin D axis’,

'to delineate the effect of oxidative stress modulation on the FGF23-klotho-vitamin D axis', 'to unravel the role of deregulation of the FGF23-klotho-vitamin D axis in the increased cardiovascular risk of progressive CKD', en 'to unravel the underlying mechanisms involved in vascular calcification alleviation by magnesium'. Een verstoorde FGF23-klotho-vitamine D as is een risicofactor voor progressief nierfalen en brengt een sterk verhoogd risico op cardiovasculaire complicaties bij patiënten met chronische nierziekte (CKD) met zich mee. Bovendien hebben veranderingen in deze FGF23-klotho-vitamine D as effect op twee andere bekende risicofactoren, namelijk oxidatieve stress en activatie van het zogenaamde RAAS. Hoge Mg2+ concentraties in het serum lijken te beschermen tegen het verhoogde cardiovasculaire risico. De te behalen onderzoeksresultaten in muismodellen voor CKD zullen duidelijk maken wat de rol is van klotho, FGF23 en vitamine D in het verloop van CKD en in het ontstaan van vaatschade op andere plaatsen in het lichaam, en op welke manier Mg2+ vaatschade kan voorkomen. Voorts zal duidelijk worden of een farmacotherapie gestoeld op deze kennis het verloop van CKD en de daarbij optredende cardiovasculaire complicaties kan beïnvloeden bij muismodellen voor CKD. CKD kent een toenemende prevalentie in de bevolking. De DEC vindt het beschikbaar komen van nieuwe therapieën die het verloop van de ziekte en de ernst van de cardiovasculaire complicaties kunnen beïnvloeden van groot belang. Dit onderzoek kan daaraan bijdragen en vertegenwoordigt in de ogen van de DEC een substantieel belang.

4. De gekozen strategie en experimentele aanpak kunnen leiden tot het behalen van de doelstelling binnen het kader van het project. Deze groep heeft veel ervaring in dit onderzoeksveld en met de voorgestelde dierproeven. De gekozen aanpak leidt tot meer inzicht in de rol van klotho, FGF23 en vitamine D en de moleculaire mechanismen betrokken bij het verloop van CKD en het ontstaan van cardiovasculaire complicaties. Verder zal het effect van farmacotherapie gebaseerd op deze inzichten bij muizen onderzocht worden.
5. Er is geen sprake van bijzonderheden op het gebied van categorieën van dieren, omstandigheden of behandeling van de dieren.
6. Het ongerief als gevolg van de dierproeven is realistisch ingeschat en geclassificeerd. Het ongerief wordt hoofdzakelijk bepaald door het ontstaan van CKD en de gevolgen daarvan voor de dieren. De DEC schat het ongerief als gevolg van de benodigde bloedafnames en de toevoeging van bio-actieve stoffen aan het drinkwater of het voedsel in als licht. De herhaalde solitaire huisvesting gedurende 48 uur, het ontstaan van CKD in muizen met een genetische aanleg hiervoor, en de schijn-nefrectomie zullen naar verwachting matig ongerief veroorzaken. Het ongerief als gevolg van de 5/6 nefrectomie schat de commissie in als ernstig. Het cumulatief ongerief voor de muizen in de beschreven vergunningaanvraag is dus juist ingeschat als matig voor 88% van de dieren en ernstig voor 12% van de dieren.
7. Er zijn geen methoden die de voorgestelde dierproeven geheel of gedeeltelijk zouden kunnen **vervangen.** De doelstelling van het project kan niet gerealiseerd worden zonder proefdieren of door gebruik van minder complexe diersoorten. Een ziekte waarbij meerdere organen zijn betrokken kan niet goed bestudeerd worden zonder proefdiermodellen.
8. In het project wordt optimaal tegemoet gekomen aan de vereiste van de **vermindering** van dierproeven. Het maximale aantal te gebruiken dieren is realistisch ingeschat en proportioneel ten opzichte van de gekozen strategie en de looptijd. De DEC is het eens met het beschreven onderzoeksmodel en de onderbouwing van het aantal benodigde dieren. Door de stapsgewijze aanpak waarin de resultaten uit eerdere proeven worden gebruikt voor het design van vervolgexperimenten wordt onnodig gebruik van proefdieren voorkomen. Wanneer niet alle

informatie voor het berekenen van de benodigde groepsgrootte bekend is, zal eerst een pilot experiment gedaan worden. Indien hieruit ongunstige resultaten blijken, zal het experiment niet uitgevoerd worden. De DEC is van oordeel dat het project kan worden uitgevoerd met maximaal 1470 muizen.

9. Het project is in overeenstemming met de vereiste van de **verfijning** van dierproeven. De experimentele handelingen bij de dieren zullen worden uitgevoerd door hierin getrainde onderzoekers, waardoor de stress voor de dieren zoveel mogelijk wordt beperkt. De commissie heeft de aanvrager tot twee keer toe kritisch bevraagd op de validiteit en noodzaak van het gebruik van het 5/6 nefrectomie model voor CKD, omdat dit model ernstig ongerief veroorzaakt. De commissie is er van overtuigd dat dit het juiste model is voor de voorgestelde dierproeven, dat het model alleen ingezet wordt in die delen van het project waar genetische modellen voor CKD niet gebruikt kunnen worden en dat in die gevallen minder belastende modellen ook geen uitkomst bieden. Verdere verfijning is op dit punt dus niet mogelijk. Dagelijkse controles van de dieren zorgen ervoor dat bij onverwacht optredend ongerief tijdig kan worden ingegrepen. Waar nodig wordt een pilot experiment gedaan om onbekende ernstige bijwerkingen zoveel mogelijk te voorkomen in het geplande experiment. De DEC is ervan overtuigd dat de dierproeven zo humaan mogelijk worden uitgevoerd.
Er is geen sprake van belangwekkende milieueffecten.

10. De niet-technische samenvatting is een evenwichtige weergave van het project, zelfstandig leesbaar, beknopt en begrijpelijk geformuleerd.

D. Ethische afweging

Op basis van de onder C genoemde overwegingen komt de DEC tot de volgende ethische afweging.

Met dit onderzoek worden belangrijke wetenschappelijke inzichten verworven in de rol van klotho, FGF23 en vitamine D op het verloop van CKD en het ontstaan van cardiovasculaire complicaties bij muizen, en in de moleculaire mechanismen die hierbij betrokken zijn. Die inzichten zouden aanleiding kunnen vormen om nieuwe therapeutische strategieën te ontwikkelen voor mensen met CKD. Het belang van meer inzicht in de moleculaire mechanismen die betrokken zijn bij het ontstaan van CKD en het beschikbaar komen van nieuwe interventies acht de DEC substantieel, gezien de toenemende prevalentie van CKD en de daarmee gepaard gaande cardiovasculaire complicaties in de bevolking.

Tegenover dit substantiële belang staat het gegeven dat 88% van de dieren matig en 12% van de dieren ernstig ongerief zullen ondervinden als gevolg van de inductie van CKD in combinatie met de benodigde handelingen. De commissie is er van overtuigd dat bij de dierproeven adequaat invulling gegeven zal worden aan de vereisten op het gebied van de vervanging, vermindering en/of verfijning van dierproeven. Het gebruik van de dieren en het daarbij optredende ongerief is onvermijdelijk, wil men de doelstellingen kunnen realiseren.

De DEC is van oordeel dat het hier boven geschetste belang de onvermijdelijke nadelige gevolgen van dit onderzoek voor de dieren, in de vorm van angst, pijn of stress, rechtvaardigt. Aan de eis dat het belang van de experimenten op dient te wegen tegen het ongerief dat de dieren wordt berokkend, is voldaan.

E. Advies

1. Advies aan de CCD

- 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

2. Het uitgebrachte advies is gebaseerd op consensus.

9

Radboud universitair medisch centrum
Concernstaf, sectie Kwaliteit en Veiligheid

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KvK 41055629/4

Geert Grooteplein 10
Postbus 9101
6500 HB Nijmegen

Datum [redacted]
29 april 2015

Onderwerp
Factuurinformatie Projectaanvraag

Geachte CCD,

Hierbij sturen wij u de administratieve gegevens behorend bij de ingediende projectaanvraag. Wij verzoeken u de factuur te versturen naar [redacted] gemachtigde van de vergunninghouder. Hiervoor AUB het bij u bekend e-mailadres gebruiken [redacted]

Om verwerking door de financiële afdeling mogelijk te maken verzoeken wij u tevens ***op de factuur*** de volgende gegevens te vermelden:

Factuuradres:	Radboudumc 28 F&A crediteuren Postbus 9101 6500HB, Nijmegen
Kostenplaats en kostensoort:	040823-461220
CDL projectnummer:	2015-0076
Verantwoordelijk onderzoeker:	[redacted]

Bij voorbaat dank.

Met vriendelijke groeten

[redacted]

Centrale Commissie Dierproeven

> Retouradres Postbus 20401 2500 EK Den Haag

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Onze referentie
Aanvraagnummer
AVD103002015240

Bijlagen
2

Datum 15 september 2015
Betreft Ontvangstbevestiging Aanvraag projectvergunning Dierproeven

Geachte [redacted]
Wij hebben uw aanvraag voor een projectvergunning dierproeven ontvangen op 10 september 2015.
Het aanvraagnummer dat wij aan deze aanvraag hebben toegekend is AVD103002015240. 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).

Met vriendelijke groet,

Centrale Commissie Dierproeven

Deze brief is automatisch aangemaakt en daarom niet ondertekend.

Bijlagen:
- Gegevens aanvraagformulier
- Factuur

Gegevens aanvrager

Uw gegevens	
Deelnemersnummer NVWA:	10300
Naam instelling of organisatie:	Radboud Universiteit Nijmegen
Naam portefeuillehouder of diens gemachtigde:	[redacted]
KvK-nummer:	41055629
Straat en huisnummer:	Geert Grooteplein 10
Postbus:	3101
Postcode en plaats:	6500 HB NIJMEGEN
IBAN:	NL90ABNA0231209983
Tenaamstelling van het rekeningnummer:	UMC St. Radboud

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

Gegevens plaatsvervangende verantwoordelijke onderzoeker

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

Gegevens verantwoordelijke uitvoering proces

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

Gegevens gemachtigde

BSN:	[redacted]
Naam:	[redacted]
Postbus:	9101
Postcode en plaats:	6500 HB NIJMEGEN
Wilt u een nieuwe machtiging afgeven?	Nee
Wat mag de gemachtigde doen?	[ ] Een projectvergunning aanvragen [ ] Een wijziging op een verleende projectvergunning aanvragen [ ] Een melding doorgeven op een verleende projectvergunning [ ] Een bezwaarschrift indienen en daarover communiceren met de Centrale Commissie Dierproeven en alle andere handelingen verrichten die nodig zijn voor een goede afwikkeling van het bezwaarschrift [x] Alle bovenstaande opties

Over uw aanvraag

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

Over uw project

Geplande startdatum:	10 oktober 2015
Geplande einddatum:	10 oktober 2020
Titel project:	The FGF23-klotho-vitamin D axis as a new instrumantal target to combat the cardiovasc
Titel niet-technische samenvatting:	De FGF23-klotho-vitamine D as als een nieuwe instrumentale doelstelling om het cardiov
Naam DEC:	RU DEC
Postadres DEC:	Postbus 9101, 6500 HB Nijmegen [redacted]
E-mailadres DEC:	[redacted]

Betaalgegevens

De leges bedragen:	€ 741,-
De leges voldoet u:	na ontvangst van de factuur

Checklist bijlagen

Verplichte bijlagen:	[x] Projectvoorstel [x] Beschrijving Dierproeven [x] Niet-technische samenvatting
Overige bijlagen:	[x] Melding Machtiging [x] DEC-advies

Ondertekening

Naam:	[redacted]
Functie:	[redacted]
Plaats:	Nijmegen
Datum:	10 september 2015

Centrale Commissie Dierproeven

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Aanvraagnummer
AVD103002015240

Bijlagen
2

Datum 15 september 2015
Betreft Factuur aanvraag projectvergunning Dierproeven

Factuur
Factuurdatum: 15 september 2015
Vervaldatum: 15 oktober 2015
Factuurnummer: 15700240
Ordernummer: 2015-0076 / [redacted]

Omschrijving	**Bedrag**
Betaling leges projectvergunning dierproeven Betreft aanvraag AVD103002015240	€ 741,00

Wij verzoeken u het totaalbedrag vóór de gestelde vervaldatum over te maken op rekening NL28RBOS 056.99.96.066 onder vermelding van het factuurnummer en aanvraagnummer, ten name van Centrale Commissie Dierproeven, Postbus 20401, 2500 EK te 's Gravenhage.

Centrale Commissie Dierproeven i.o.

Centrale Commissie Dierproeven

Postbus 20401
2500 EK Den Haag
www.zbo-ccd.nl

T 0900 28 000 28 (10 ct /min)

info@zbo-ccd.nl

Contactpersoon

memo

Telefoonnotitie [redacted]
Betreft: AVD103002015240
Datum: 02-10-2015

Datum

Bijlagen

De aanvrager neemt in het onderzoek alleen manlijke dieren mee.

Het Secretariaat is het met de aanvrager eens dat [redacted]
Daar staat tegenover dat [redacted], het Secretariaat een dilemma ziet [redacted] Hierover is met de onderzoeker telefonisch contact geweest.

Deze heeft verklaard dat [redacted]

Centrale Commissie Dierproeven

> Retouradres Postbus 20401 2500 EK Den Haag

Radboud Universiteit Nijmegen
[redacted]
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Onze referentie
Aanvraagnummer
AVD103002015240

Uw referentie

Bijlagen
1

16 OKT. 2015

Betreft Beslissing Aanvraag projectvergunning dierproeven

Geachte heer/mevrouw,

Op 10 september 2015 hebben wij uw aanvraag voor een projectvergunning dierproeven ontvangen. Het gaat om uw project "The FGF23-klotho-vitamin D axis as a new instrumental target" met aanvraagnummer AVD103002015240. Wij hebben uw aanvraag beoordeeld.

Beslissing

Wij keuren uw aanvraag goed op grond van artikel 10a van de Wet op de Dierproeven (hierna: de wet). Hierbij gelden de voorwaarden zoals genoemd in de vergunning. U kunt met uw project "The FGF23-klotho-vitamin D axis as a new instrumental target " starten. De vergunning wordt afgegeven van 10 oktober 2015 tot en met 9 oktober 2020. Deze termijn is anders dan in uw aanvraag, omdat De periode tot en met 09 oktober 2020 de maximaal vergunbare periode van 5 jaar is. Er is niet aangevraagd om nieuwe genetisch gemodificeerde dieren te mogen fokken, creëren of houden. Er wordt vanuit gegaan dat dit geen onderdeel vormt van de projectaanvraag. Overige wettelijke bepalingen blijven van kracht.
De CCD wil u graag attenderen op het gegeven dat er in uw projectaanvraag verhoudingsgewijs weinig in combinatie met in vitro studies of andere proefdierbesparende technieken wordt gewerkt, als deze studie wordt afgezet tegen andere projecten die de CCD onder ogen krijgt. De CCD wil graag benadrukken dat er een groot maatschappelijk belang wordt gediend als er blijvend wordt gewerkt aan van vernieuwende onderzoeksmethodes die een vermindering van ongerief en aantal proefdieren kunnen realiseren.

Beoordeling achteraf

Na afloop van het project zal er een beoordeling plaatsvinden, zoals bedoeld in artikel 10a1, lid 1d en lid 3, in de wet. Meer informatie over de eisen bij een beoordeling achteraf vindt u in de bijlage.

Procedure

Bij uw aanvraag heeft u een advies van de Dierexperimentencommissie RU DEC gevoegd. Dit advies is opgesteld op 7 september 2015. 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. Wij nemen dit advies van de commissie over, inclusief de daaraan ten grondslag liggende motivering.
Dit 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).

Met vriendelijke groet,

Centrale Commissie Dierproeven
namens deze:

[redacted]

ir. G. de Reuter
Algemeen Secretaris

Bijlagen:
- Vergunning

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

Dit besluit is genomen met inachtneming van het Besluit mandaat, volmacht en machtiging van de Centrale Commissie Dierproeven CCD 2014 zoals de Centrale Commissie Dierproeven heeft vastgesteld op 19 december 2014, ref 2014-04 en is gepubliceerd in de Staatscourant van 2 januari 2015, Nr. 163

Projectvergunning

gelet op artikel 10a van de Wet op de dierproeven

Verleent de Centrale Commissie Dierproeven aan
Naam: Radboud Universiteit Nijmegen
Adres: Postbus 3101
Postcode en plaats: 6500 HB NIJMEGEN
Deelnemersnummer: 10300

deze projectvergunning voor het tijdvak 10 oktober 2015 tot en met 9 oktober 2020, voor het project "The FGF23-klotho-vitamin D axis as a new instrumental target" met aanvraagnummer AVD103002015240, volgens advies van Dierexperimentencommissie RU DEC. De functie van de verantwoordelijk onderzoeker is Hoogleraar. Voor de uitvoering van het project is Instantie voor dierenwelzijn verantwoordelijk.
De aanvraag omvat de volgende bescheiden:
1 een aanvraagformulier projectvergunning dierproeven, ontvangen op 10 september 2015
2 de bij het aanvraagformulier behorende bijlagen:
a Projectvoorstel, zoals ontvangen per digitale indiening op 10 september 2015;
b Niet-technische Samenvatting van het project, zoals ontvangen per digitale indiening op 10 september 2015;
c Advies van dierexperimentencommissie d.d. 7 september 2015, ontvangen op 10 september 2015

Dierproeven

Naam dierproef	Diersoort	Aantal dieren	Ernst	Opmerkingen
1: Type of animal procedure RAAS blockades, genetic model, and FGF23, Klotho and vitamin D in CKD	Muizen (*Mus musculus*) ook genetisch gemodificeerde muizen. Alleen manlijke dieren.	480	Matig	Zie opmerkingen
2:Type of animal procedure Oxidative stress and FGF23, Klotho and vitamin D in CKD	Muizen (*Mus musculus*) ook genetisch gemodificeerde muizen. Alleen manlijke dieren.	480	Matig	Zie opmerkingen
Type of animal procedure RAAS blockades, surgical model, and FGF23,Klotho and vitamin D in CKD	Muizen (*Mus musculus*) ook genetisch gemodificeerde muizen. Alleen manlijke dieren.	360	Ernstig	Zie opmerkingen
4: Type of animal procedure CKD induced calcification treatment with Mg2+	Muizen (*Mus musculus*) ook genetisch gemodificeerde muizen. Alleen manlijke dieren.	150	Matig	Zie opmerkingen

Opmerkingen

Alleen manlijke dieren worden gebruikt omdat er sexeverschillen in resultaten verwacht worden. Er is niet aangevraagd om nieuwe genetisch gemodificeerde dieren te mogen fokken, creëren of houden. Er wordt vanuit gegaan dat dit geen onderdeel vormt van de projectaanvraag. Overige wettelijke bepalingen blijven van kracht.

De CCD wil u graag attenderen op het gegeven dat er in uw projectaanvraag verhoudingsgewijs weinig in combinatie met in vitro studies of andere proefdierbesparende technieken wordt gewerkt, als deze studie wordt afgezet tegen andere projecten die de CCD onder ogen krijgt. De CCD wil graag benadrukken dat er een groot maatschappelijk belang wordt gediend als er blijvend wordt gewerkt aan van vernieuwende onderzoeksmethodes die een vermindering van ongerief en aantal proefdieren kunnen realiseren.

Voorwaarden
Op grond van artikel 10a1 lid 2 Wod zijn aan een projectvergunning voorwaarden te stellen
De vergunning wordt verleend onder de voorwaarde dat eventuele go/no go momenten worden afgestemd met de IvD. In artikel 10, lid 1a 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 overleg met de IvD te melden bij de CCD. De CCD kan in een dergelijke situatie aan de vergunning nieuwe voorwaarden verbinden en gestelde voorwaarden wijzigen of intrekken.

Voorschriften

in verband met ernstig ongerief is een beoordeling achteraf vereist.

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.

Beoordeling achteraf
Volgens artikel 10a1, lid 1d en lid 3 van de wet worden projecten waarbij niet-menselijke primaten worden gebruikt, projecten die als ernstig ingedeelde dierproeven omvatten of een dierproef die leidt tot ernstige mate van pijn, lijden, angst of blijvende schade die waarschijnlijk langdurig zal zijn en niet kan worden verzacht, achteraf beoordeeld worden. In dit project worden dierproeven toegepast waarbij die vallen in de categorie ernstig volgens artikel 10b van de wet en wordt daarom voorzien van beoordeling achteraf.
Deze beoordeling zal uiterlijk januari 2021 plaatsvinden. Er zal dan beoordeeld worden of de doelstellingen van het project werden bereikt. Daarnaast wordt bekeken of de schade die de dieren hebben ondervonden, het aantal en soorten proefdieren en de ernst van lijden van de proevendieren conform de vergunning waren.