Centrale Commissie Dierproeven

Appendix
Description animal procedures

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

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General information

1.1	Provide the approval number of the 'Netherlands Food and Consumer Product Safety Authority'.	50100
1.2	Provide the name of the licenced establishment.	The Netherlands Organisation for Applied Scientific Research (TNO)
1.3	List the serial number and type of animal procedure. *Use the serial numbers provided in Section 3.4.4 of the Project Proposal form.*	Serial number: 3.4.4.1 Type of animal procedure: Investigating potential cardiovascular safety issues of drugs and other substances

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.

Different mouse models and conditions can be used. The general design of cardiovascular safety studies is treatment of (transgenic) mice with a western type diet (a cholesterol and fat rich diet) to mimic the consumption in the Western World, thereby inducing the cardiovascular risk factors and giving the necessary window to examine potential adverse effects on the risk factors. Fructose in drinking water could also be added which will result in a more atherogenic lipoprotein profile. The detailed study design being used depends on which cardiovascular safety issues are being investigated: hyperlipidemia, chronic inflammation, atherosclerosis.

The study duration and subsequently the length of the study ranges from approx. 8-24 weeks and will depend on the diet being used, the sensitivity of a mouse strain towards this diet, induction time of cardiovascular risk factor and the new drug under investigation (as an example: female ApoE*3Leiden.CETP mice fed cholesterol containing western type diet develop hyperlipidemia very quickly (3-4 weeks), but it takes a longer time to develop atherosclerosis (16-20 weeks).

Importantly, the specific combination of animal model + diet (e.g. the percentage of cholesterol) determines which cardiovascular safety issues can be investigated (see also table 1 in the general part of the project proposal).

A study can include the following groups:
1. control group (mice are fed a western type diet, no intervention).
2. reference control group (mice are fed a western type diet; intervention is with a drug or substance known to have adverse cardiovascular effects (reference control).
3. Treatment group (mice are fed a western type diet; and new drugs or substances with potential cardiovascular safety issues are applied)

After a run-in period on the western type diet, the mice are matched into groups based on body weight, age plasma lipids or inflammation markers (which parameters to be used is based on research question). Hereafter treatment with the drugs or substances with potential cardiovascular safety issues starts. The study varies in duration based on which cardiovascular safety issues are under investigation

Additional groups can be added as well: additional groups for PK/PD analysis could be added if an additional research question is also to obtain more PK/PD information on the administered compounds. Furthermore, it can also be decided to first perform a pilot experiment if crucial information is lacking (for example, first a dose-finding pilot study could be performed to find the optimal dose in mice which correlate to clinical dose in humans (= clinically relevant plasma levels of compound)).

Besides directly testing the potential adverse effects, also more basic research studies are performed aiming at understanding the underlying mechanisms of the potential adverse effect. For all studies, it is very important that we use translational models that reflect the cardiovascular risk factors in humans as much as possible. These studies provide necessary information for the development of drugs that do not have CVD (cardiovascular disease) side effects

The primary outcome parameters will depend on the specific research question:
- For investigating potential cardiovascular adverse effects on **hyperlipidemia,** the primary outcome parameters will be plasma lipids.
- For investigating potential cardiovascular adverse effects on **chronic inflammation,** the primary outcome parameters will be plasma inflammation markers and/or inflammation markers in the vessel wall).
- For investigating potential cardiovascular adverse effects on **atherosclerosis**, the primary outcome parameters will be histological analysis of plaque size and severity in aortic root area or aorta.

In general, body weight and food intake will be monitored and during the study blood/plasma measurements will be performed to analyse plasma parameters. At the end of each study, tissues will be collected for analysis of atherosclerosis.

Maximum volume and frequency injections/oral gavages/blood samples to be taken are according to what is considered good practice (Diehl et al., J Appl Toxicol. 2001 Jan-Feb; 21(1):15-23).

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

As mentioned above, the precise study design depends on which cardiovascular safety issues are to be studied.

During every study body weight and food intake are monitored regularly, different blood samples will be taken during the study and at the end mice will be euthanized and tissues will be collected. Depending on the research questions, extra procedures for measurement of additional parameters could be added to the study (see below for details).

There are different possibilities for administration routes of new drugs of substances:

- Oral administration via diet admix of via drinking water
- Oral administration (p.o.) by gavage
- Intraperitoneal injection (i.p.)
- Subcutaneous injection (s.c.)
- Intravenous injection (i.v.)
- Intramuscular injection (i.m.)
- Via osmotic mini-pump (s.c.): Using appropriate anesthesia and analgesia, osmotic minipumps suitable for mice will be placed subcutaneously. During an experiment, it might be necessary to replace the minipump.

During the study at several time-points blood samples will be taken to for plasma measurements. Blood samples can be taken:
- via tail vein after 4-5 hours fasting
- via tail vein after overnight fasting (in particular cases it may be necessary to collect blood after prolonged fasting, e.g. when ketone bodies or free fatty acids or other markers of fasting are measured or if we need blood samples that do not contain chylomicrons or any food derived markers).
- via tail vein non-fasted (in particular cases it may be necessary to collect non-fasted blood, e.g. after postprandial challenge tests after meal).
The frequency and volume of blood taken will be within good practice limits as described in Diehl et al. (2001)

At the end of the experiment mice will be euthanized, and plasma and different tissues will be collected.

During the study the following procedures could be added:

Deuterated water administration (D_2O)
To be able to trace newly formed proteins (lipids for instance) within a given period, D_2O can be given for a short period (number of days) or long (several weeks) period in our studies. Labeling can take place at various times of the study and depends on the specific question. D_2O will be built into all the newly synthesized proteins and in this way newly formed protein can be traced. On the first day, the mice receive a single i.p. injection with body warm D_2O 100% / 0.9% NaCl to label the body water around 2-5% of the mouse. Then the D_2O body water levels will be maintained by adding D_2O in the drinking water (containing 4-8% D_2O) until the end of the study.

Feces collection (for measurements of fatty acids, sterols, for example, to be able to evaluate the effect on lipid excretion)
- By collecting feces after several days from the cage bed (on group level) or after lifting mice (individual feces collection)
- By taking rectal swabs

VLDL production (measurement of VLDL production; non-recovery)
Mice are fasted for 4 hours, and then anesthetized with appropriate injection anesthesia. Under anesthesia Tran 35S-label is injected intravenously in the tail vein for measurement of the apoB novo synthesis and 30 minutes thereafter the mice are injected with Triton WR 1339 (iv, tail) for complete blocking of VLDL clearance. 0, 15, 30, 60 and 90 minutes after Triton injection, a blood sample (40 µl) is taken via the tail for measurement of plasma triglycerides (VLDL production measurement). After 90 minutes, the mice are euthanized via cervical dislocation and the remaining blood is collected via cardiac puncture for VLDL isolation and determination of the ApoB novo synthesis and lipid composition VLDL. Different tissues can be isolated as well.

VLDL clearance (measurement of VLDL clearance; non-recovery)
For this purpose, mice will be fasted for 4 hours after which mice will receive VLDL-like particles labeled with 3 H-triolein and 14C-cholesteryl oleate via an injection into the tail vein. After 2, 5, 10 and 15 minutes, a blood sample is taken via the tail (40 µl per time point). The mice are euthanized by cervical

dislocation and blood is collected at the end of the experiment by means of a heart puncture and different tissues are isolated as well. 3H and 14C activity is measured in tissues and plasma samples.

Challenge measurement (to measure potential adverse effects of new drugs or other substances after application of acute metabolic stressor)
- Administration of dietary challenge (bolus of dietary fat, lipids, bile acids, fatty acids, cholesterol) to assess the metabolic response after set time points.
- Challenges with heparin administration (iv or ip injection) for determination of lipoprotein lipase (LPL) activity.
Blood samples can be taken before, during and after the challenge (one or more blood samples, depending on the specific research question).

Blood pressure measurement (to measure potential adverse effects of new drugs or other substances on blood pressure).

In principle all procedures, except VLDL clearance and production (both non recovery procedures), could be performed in the same mouse. The study will be designed in a way that the mice will be given time between these procedures (good practice).

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

To estimate the number of animals to be used in an experiment, we use the adverse effect size (if known, e.g., from data in the literature, clinical studies and post clinical observations, from our own historical data or years of experience with similar type of experiments or if unknown from pilot studies) to estimate the sample size needed to achieve a certain power (usually between 0.8-0.9) with appropriate statistical tests like the t-test with a $p<0.05$.
As an indication: usually n=8 mice will be used to investigate the adverse effects on plasma lipids and plasma inflammation markers, and n=15 mice per group for to investigate the adverse effects on atherosclerosis and/or inflammation in the vessel wall.

In addition, there could be low-responders / outliers. For ApoE*3Leiden and ApoE*3Leiden.CETP mice it is well known that a certain percentage of the mice does not respond to a cholesterol containing diet with respect to the development of hyperlipidemia. For lipid and atherosclerosis studies, these mice will be excluded in the beginning of the study to reduce the variation and thus the number of animals required per group. If possible different study groups will be combined, so that different treatment groups can share the same control groups.

B. The animals

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

Mice, ApoE*3Leiden, ApoE*3Leiden.CETP mice from our own breeding facility, 8-22 weeks old will be used. In addition, ApoE-/-, LDLR-/-, 8-22 weeks old can be used.

*ApoE*3Leiden*: Mice carrying a human APOE*3Leiden transgene that leads to a defective clearance of Triglyceride and cholesterol-rich lipoproteins (VLDL and LDL). While normal wild-type mice have a very rapid clearance of VLDL and LDL, ApoE*3Leiden (E3L) mice have an impaired clearance and are thereby mimicking the slow clearance observed in humans. APOE*3-Leiden transgenic mice are highly responsive to fat, sugar and cholesterol feeding with respect to the effects on plasma cholesterol and triglyceride levels. APOE*3Leiden animals have proven to be responsive to the most of the drugs that are also used in the clinic, and also very suitable for cardiovascular safety studies. Males and females can be

used to evaluate adverse lipid effects, however they respond very different on dietary cholesterol induced hyperlipidemia. Therefore there is a preference per study to use female or male mice only.
Only females are suitable to evaluate adverse effects on atherosclerosis development. Male mice do not/hardly develop atherosclerosis.

*APOE*3Leiden.CETP*: In contrast to humans, wild type mice express no CETP (which transfers cholesterol from HDL to (V)LDL). The double transgenic ApoE3*Leiden.CETP mouse brings CETP to expression and therefore this model is translational to the human situation regarding HDL metabolism, so suitable to test potential adverse effects on HDL-cholesterol. Furthermore, this mouse has the same characteristics as the APOE*3Leiden mouse regarding its (V)LDL metabolism.

The two mice models above are, in our view the most translational mouse models to investigate potential cardiovascular safety issues of compounds, however, based on the research question and/or what is known from literature, the commercially available LDLR-/- and ApoE-/- mice could also be used to investigate potential cardiovascular safety issues.

LDLR-/-: both males and females can be used to evaluate adverse effects on lipids and atherosclerosis. The mice lack a specific receptor (Ldlr) and reflect a particular group of patients that have the same genetic impairment (patients with defective or absent Ldlr). This model is not to be used when LDLR could be involved in (side) adverse effect of new drug.

ApoE-/-mice: both males and females can be used to evaluate adverse effects on lipid and atherosclerosis. This is a model with higher lipid levels and more atherosclerosis than the models mentioned above. This model is not to be used when ApoE could be involved in the adverse effect under investigation.

Please note that all mouse models show a subclinical phenotype and do not lead to discomfort. Similarly as in humans, that are often unaware of these disturbances until a very late stage. Also the adverse effects are aimed and expected to show a subclinical phenotype without any discomfort.

The required number of mice for cardiovascular/metabolic safety studies will be estimated to be 1500 for a period of 5 years. This is based on an expected number of cardiovascular safety studies of approx. 5 per year The average study will consist of 3-6 groups of 8-15 mice each, so on average 50 mice per study. This leads to a required total number of 1250 mice (5 studies x 50 animals / study x 5 years). Expected low/non-responders are 250 mice, so the required number of mice will be estimated to be 1500 mice

C. Re-use

Will the animals be re-used?

X No, continue with question D.

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

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

X No

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

D. Replacement, reduction, refinement

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

Replacement: We continuously seek to replace animal studies with other methods, preferably using human tissue or cells. We work with human cells and in silico models, where possible, since that helps us to make better predictions about potential cardiovascular issues. When we have discovered ways to

replace animal testing, we use them ourselves and encourage others to apply these alternative tests. Non-testing techniques, such as computer modelling (target safety assessment), are continually being developed and improved.
Before we consider to perform a novel cardiovascular safety study, we will first analyse appropriate cell lines, existing patient materials or materials available from previous animal studies, which contributes to a reduction of animal numbers. The use of human material and in vitro cultures allows this project to comply with the 3Rs (replacement, reduction and refinement) by keeping the animal numbers to a minimum.
However, animal studies are currently unavoidable to study the complex organ-organ interactions that are intrinsic to all CVD. The development of CVD is a too complicated multifactorial process in which multiple organs and cells interact. Hence, given the complexity of the lipid metabolism, the potential cardiovascular safety issues of drugs can be examined only in intact animal models with intact vascular system and there are currently no established alternative methods to investigate these processes.

Reduction: TNO aims to reduce the number of animals involved in testing. We regularly review our testing methods and implement integrated testing strategies. This helps us to determine whether animal testing is needed or whether the same information can be obtained in other ways. Power analyses are made for each experiment. Whenever possible, we will perform pilot studies with the minimum number of animals possible. Experiments will be done sequentially, where on basis of the results, decisions will be taken for the next steps. If possible different study groups from different studies will be combined, so that they can share the same control groups.
Refinement: We continuously aim to develop, validate and adapt (preferentially non-invasive) test methods in such a way that the test animals are exposed to as little discomfort and stress as possible. All animal handlings will be carried out by authorized and qualified persons. Where possible, all animals will be group housed and environmental enrichment will be provided.

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

Appropriate possibilities to reduce pain, fear or suffering will be used. All work will be carried out by a small number of highly trained bio-technicians. Injection fluids will be brought to room or body temperature before injection. In consultation with the attending veterinarian, for surgery appropriate peri-operative care, including appropriate anaesthesia and analgesia, is determined and frequently reviewed and updated towards best practices. The welfare of the animals will be observed daily by different people. If there are clear signs of unexpected severe discomfort, the animals will be euthanized. There are no negative environmental effects; all mice will be housed under strict D1 conditions.

Repetition and duplication

E. Repetition

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

N.a.

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?

X No

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

G. Location where the animals procedures are performed

Will the animal procedures be carried out in an establishment that is not licenced by the NVWA?

X No > Continue with question H.

☐ Yes > Describe this establishment.

Provide justifications for the choice of this establishment. Explain how adequate housing, care and treatment of the animals will be ensured.

Classification of discomfort/humane endpoints

H. Pain and pain relief

Will the animals experience pain during or after the procedures?

☐ No > Continue with question I.

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

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

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

For more invasive procedures (placing of osmotic pumps), appropriate anesthesia / analgesia will be used or the procedure will be terminal. In consultation with the attending veterinarian, surgery protocols, including appropriate anaesthesia and analgesia is determined and frequently reviewed and updated towards best practices.

I. Other aspects compromising the welfare of the animals

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

The induction of the cardiovascular risk factors: hyperlipidemia, chronic inflammation and atherosclerosis do not lead to discomfort. Similarly as in humans, they are often unaware of these disturbances until a very late stage. Based on historical data, we expect the risk of clinical symptoms developing in the animals to be <0.1%

With respect to discomfort caused by drugs and other substances: Although the drugs/substances to be tested are expected to have adverse effects on cardiovascular safety, we aim in our studies that the adverse effects will not cause any discomfort to these animal models. We will perform studies predominantly in earlier phases of disorders and work with efficacy/clinically relevant doses of the drugs or other substances (far below acute toxic levels)

The drugs and chemicals have already been tested in standard regulatory safety/tox preclinical studies and often also clinical safety studies.

Although we do not expect discomfort, we cannot exclude this. Mice will be monitored daily and in case of discomfort, cages will be labelled and the affected mice will be closely monitored to observe whether the health status is improving or deteriorating. Deterioration of the health status with unexpected weight loss, severe wounds and/or signs of general sickness and/or discomfort, will lead to the decision that mice will be euthanized.

Explain why these effects may emerge.

We cannot exclude the possibility that the combination of the compounds in our models leads to unexpected adverse effects. The likelihood (based on historical data of last 5 years) is <0.1%.

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

Upon start of treatment mice will be closely monitored and upon signs of animal discomfort, the situation will be discussed with the animal welfare officer or veterinarian and if possible proper measurements will be taken to relief the animal discomfort or animals will be taken out of experiment and euthanized.

J. Humane endpoints

May circumstances arise during the animal procedures which would require the implementation of humane endpoints to prevent further distress?

x No > Continue with question K.

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

Indicate the likely incidence.

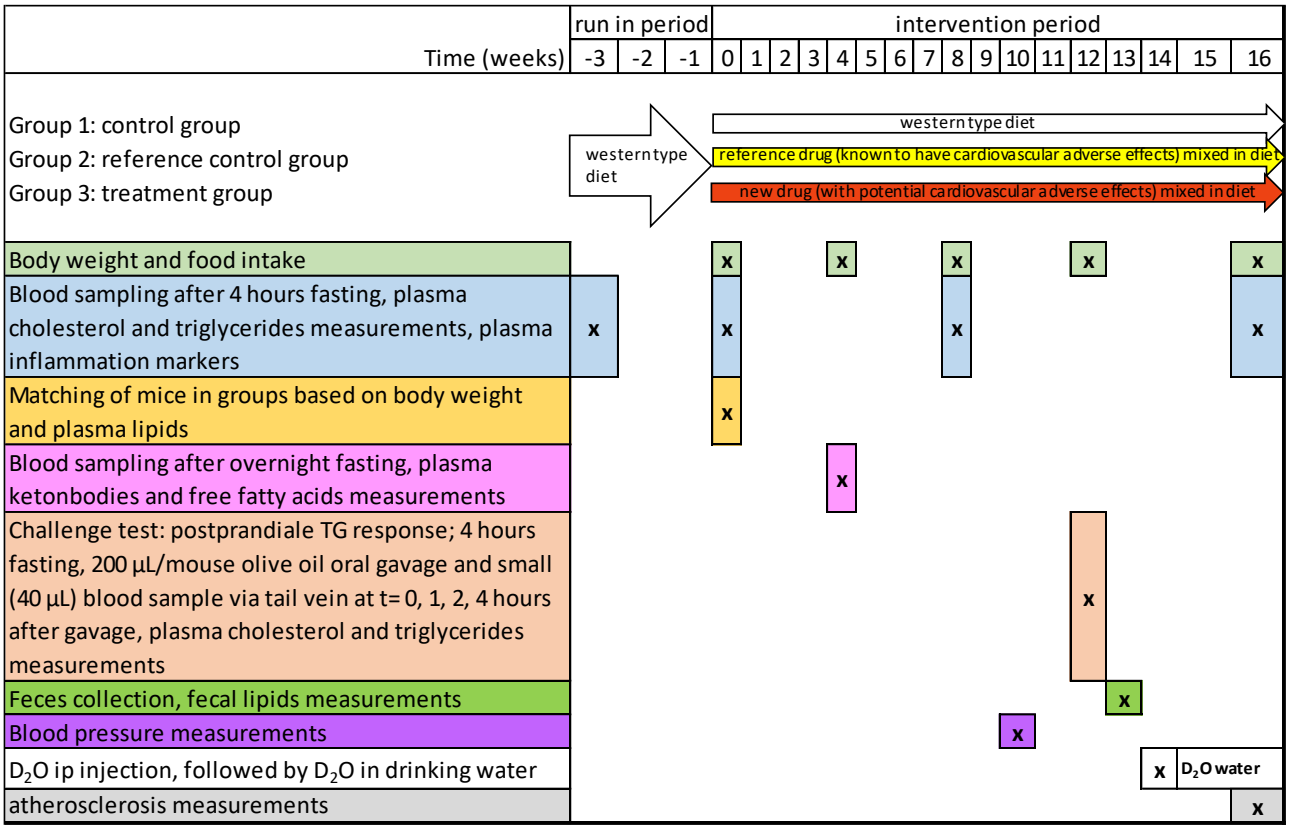
K. Classification of severity of procedures

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

In general, the cumulative discomfort for cardiovascular safety studies will be mild or moderate and will not extend beyond moderate. We expect that 95% of the mice will reach a mild cumulative discomfort and 5% of the mice will reach a moderate cumulative discomfort. The different procedures that can be used, will all be within good practice (Diehl et al., J Appl Toxicol. 2001 Jan-Feb; 21(1):15-23) and are assigned to the following categories:

Animal procedure:	**Level of discomfort:**
Diet interventions	mild
Drinking water interventions	mild
D_2O drinking water	mild
Blood sampling, non-fasted or after 4 or 5 hours fasting period or after overnight fasting period	mild
Multiple blood sampling via tail vein within good practice	mild
Single or multiple gavage or injections (iv, ip, im, sc)	mild, incidentally extending to moderate
Feces collection or fecal swabs	mild
VLDL production test	non-recovery
VLDL clearance test	non-recovery
Challenge tests	mild or moderate
Surgery: s.c. osmotic minipumps	moderate
Euthanasia	mild

Depending on the research question, different procedures will be performed in a study. In principle all procedures, except VLDL clearance and production (both non recovery procedures), could be performed in the same mouse. The study will be designed in a way that the mice will be given time to recover between these procedures. Example of an experimental set up:



End of experiment

L. Method of killing

Will the animals be killed during or after the procedures?

☐ No

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

Cardiovascular disease manifests within tissues and cannot be analyzed only via plasma. Therefore, the tissues are needed for measurements: heart, aortic root or aorta for atherosclerosis analysis

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

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

X Yes