Centrale Commissie Dierproeven

Form
Project proposal

- This form should be used to write the project proposal for animal procedures.
- The appendix 'description animal procedures' is an appendix to this form. For each type of animal procedure, a separate appendix 'description animal procedures' should be enclosed.
- For more information on the project proposal, see our website (www.centralecommissiedierproeven.nl).
- Or contact us by phone (0900-2800028).

1 General information

1.1	Provide the approval number of the 'Netherlands Food and Consumer Product Safety Authority'.	50100
1.2	Provide the name of the licenced establishment.	The Netherlands Organisation for Applied Scientific Research (TNO)
1.3	Provide the title of the project.	Investigating potential cardiovascular safety issues of drugs and other substances

2 Categories

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

- ☐ Basic research
- x Translational or applied research
- ☐ Regulatory use or routine production
- ☐ Research into environmental protection in the interest of human or
- ☐ Research aimed at preserving the species subjected to procedures
- ☐ Higher education or training
- ☐ Forensic enquiries
- ☐ Maintenance of colonies of genetically altered animals not used in other animal procedures

3 General description of the project

3.1 Background

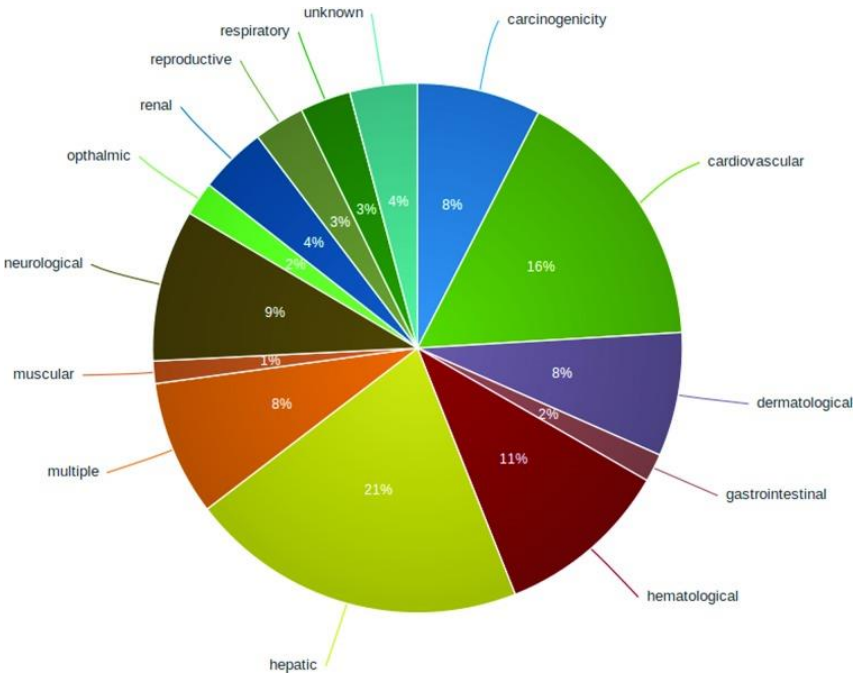
Describe the project (motivation, background and context) with respect to the categories selected in 2.

- For legally required animal procedures, indicate which statutory or regulatory requirements apply (with respect to the intended use and market authorisation).
- For routine production, describe what will be produced and for which uses.
- For higher education or training, explain why this project is part of the educational program and describe the learning targets.

Cardiovascular safety

Efficacy and safety are two decisive factors that affect the viability of new drugs. Over the decades, drug regulatory agencies, pharmaceutical companies and various clinical studies have reported the events of drug withdrawals due to severe adverse side-effects. In a recent publication (Siramshetty et al, Nucleic Acids Res 2016) a database was presented with an extensive list of withdrawn and discontinued drugs in the USA and Europe, including the reason for withdrawal, eq. the type of adverse effect. 16% of the withdrawn drugs were associated with cardiovascular safety issues.

Figure 1: Overview of safety/toxicity issues associated with drug withdrawal (see also database http://cheminfo.charite.de/withdrawn)



Also other substances (chemicals) could be associated with cardiovascular safety issues in humans, when (chronic) exposure of these substances to humans occur. As an example we mention the persistent perfluorinated hydrocarbons (PFOS and PFOA), which show a positive association in cross-sectional epidemiological studies with increased plasma cholesterol levels (a risk factor for cardiovascular disease; see also below).

These cardiovascular safety issues were not detected preclinically in the standard regulatory safety/toxicity studies. This is because standard regulatory safety/toxicity studies are primarily performed in young and healthy mice and rats. Since these animals do not have a predisposition for cardiovascular disorders, potential adverse (side) effects of new drugs and other substances (chemicals) on cardiovascular risk factors are not likely to be picked up in these healthy animals.

Cardiovascular disease and risk factors

Cardiovascular disease (CVD) is the leading cause of death worldwide. Its major underlying pathology is atherosclerosis, a complex, multi-factorial disease that is driven by dyslipidaemia and chronic inflammation. Obesity is strongly related to major cardiovascular risk factors such as raised blood pressure, glucose intolerance/insulin resistance, type 2 diabetes and dyslipidaemia. These metabolic abnormalities tend to cluster together and this combination is called Metabolic Syndrome. The liver plays a central role in the control of lipid metabolism and contributes to systemic inflammatory changes, insulin resistance and hyperlipidaemia determining progression of CVD. Recently, accumulating evidence suggests that obesity induced fattening of the liver (nonalcoholic fatty liver disease, NAFLD or

nonalcoholic steatohepatitis (NASH) in case the liver is also inflamed) may pose a cardiovascular risk above and beyond that is conferred by traditional cardiovascular risk factors. CVD is likely to continue to grow due to increased obesity rates and our aging society.

In the past 30 years, TNO has developed translational animal models that allow detailed analysis of different cardiovascular risk factors. In contrast to wild type mice, these animal models develop subclinical phenotypes of cardiovascular risk factors, in which (adverse) effects can be very well studied. In recent years, we have found that our translational animal models and technology are particularly suited to provide a meaningful contribution to research into the potential cardiovascular safety aspects of new drugs (which are already on the market or still in a preclinical or clinical study phase) and other substances with potential cardiovascular safety aspects.

Identifying the potential cardiovascular/metabolic safety issues allow our partners in the chemical & pharmaceutical industry to develop ways to prevent or mitigate these safety issues.

Through our portfolio, we support both the chemical & pharmaceutical industry as well as academia and medical centers. We strive to continuously incorporate new insights and technologies in our portfolio to offer the best possible preclinical research tools to perform cardiovascular safety studies in relevant translational animal models.

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?

Our aim is to provide a meaningful contribution to research into the potential cardiovascular/metabolic safety aspects of (new) drugs and other substances.

Based on preclinical, clinical or post-clinical adverse drug reactions or epidemiological studies (in case of (chronic) exposure of humans to chemicals) in association with cardiovascular safety, we (together with, or on behalf of our pharmaceutical or chemical industry partners) will investigate drugs with cardiovascular/metabolic safety aspects. The specific research question of a study will generally focus on the potential adverse effect of the drugs or other substances on one or more cardiovascular risk factors and/or the elucidation of the mechanism involved. We also further aid our pharmaceutical and chemical industry partners by screening other candidate drugs in a pre-clinical, clinical or post-clinical phase or other substances in order to select drugs or other substances that do not show these cardiovascular/metabolic safety aspects.

Approximately 70% of our studies will be applied research performed to investigate the adverse effects of drugs or substances on one or more cardiovascular risk factors. In addition, about 30% of our studies is translational research performed to investigate the underlying working mechanism of the adverse effects.

If for example in a study a new drug shows adverse effects on the cardiovascular risk factor hyperlipidaemia, the next study could be a further elucidation of the mechanisms involved. Or the next study could also be investigating potential adverse effects other drugs (in the same class) or investigating whether the drug also has adverse effects on atherosclerosis development.

This project has a high feasibility:
Within the strategy of TNO, Healthy Living is one of the five focus areas. Within the focus area of Healthy Living research is being done that varies from the development of healthy and safe food, children growing up healthily or working healthily to predictive health technologies. A substantial portion of the research is dedicated to education, prevention and treatment, either commissioned by the government or in collaboration with academia or industry. The research described in this project is embedded within the theme of predictive health technologies that aims to have a better understanding of health and diseases and better predict (adverse) effects of drugs and other substances.

Researchers within the group are already more than 20 years working in the field of cardiovascular and metabolic diseases (both efficacy studies, fundamental research as safety studies), and have an extensive track record of more than 150 peer-reviewed international publications. For the research described in this project, focusing on cardiovascular/metabolic safety aspects of drugs and other substances, we have performed numerous studies. For example, in a translational mouse model for hyperlipidemia and atherosclerosis, we have performed a number of studies in which the adverse effects of new drugs on lipid metabolism were investigated: HIV-inhibitors (against AIDS), JAK inhibitors (in development for rheumatoid arthritis), bexarotene (against metastatic differentiated thyroid carcinoma) and tyrosine kinase inhibitors (chronic myeloid leukemia), and torcetrapib (a CETP inhibitor with adverse cardiovascular side effects). We have also performed studies on the mechanism of action of the widely used and persistent perfluorinated hydrocarbons (PFOS and PFOA), which show a positive association in cross-sectional epidemiological studies with increased plasma cholesterol levels.

A large network has been built within both the academic world and the pharmaceutical industry, as well as biotech companies, academic medical centers, patient organizations and governments. Within this network we have conducted more than 150 cooperation projects over the past 15 years (both bilaterally and in larger consortia). Our previous achievements show that with the experiments described in this project we contribute to our main focus area Healthy Living.

3.3 Relevance

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

Over the decades, drug regulatory agencies, pharmaceutical companies and various clinical studies have reported the events of drug withdrawals due to severe adverse side-effects. 16% of the withdrawn drugs in the USA and Europe were associated with cardiovascular safety issues.

CVD represents a major economic burden on health care systems in terms of direct (eg, hospitalizations, rehabilitation services, physician visits, drugs) and indirect costs associated with mortality and morbidity (eg, losses of productivity due to premature mortality and short- or long-term disability). The effects of CVD are not limited to health, but can seep into social aspects of life as well (physical limitations, social limitations, decreased life expectancy).

Investigating potential cardiovascular safety issues of drugs (designed for treatment of other diseases) or other substances will be important for better predicting potential adverse cardiovascular effects which will allow our partners of the pharmaceutical and chemical industry to develop ways to prevent or mitigate these safety issues.

3.4 Research strategy

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

All studies that will be conducted in this project will be cardiovascular safety studies to evaluate the potential adverse effect of the new drugs or other substances on one or more cardiovascular risk factors and/or the elucidation of the mechanism involved.

Cardiovascular safety issues of drugs are mainly discovered in a clinical or post-clinical phase. Associations between cardiovascular adverse effects and other substances humans can be (chronically) exposed to are mainly discovered in epidemiological studies. Also if preclinical *in vitro, in vivo or in silico* (Target Safety Assessment) studies give potential cardiovascular safety issues, or if there are known properties of a (certain class of) compound(s) which could cause potential cardiovascular issues, this will be a reason to evaluate this further in relevant models. We (together with, or on behalf of our pharmaceutical or chemical industry partners) will investigate these drugs or other substances on their potential cardiovascular safety aspects and/or underlying mechanism(s). We will first perform a literature search what already is known about the specific drug/substance and what is known about the potential cardiovascular safety aspects of the drug. The partner will also be asked what is already known of the drug/substance. Then, based on this knowledge and the research question, we will decide and advise our partner whether the research question can be answered using in vitro (eg primary human cells), ex vivo (existing patient materials or materials available from previous animal studies) or in silico models (target safety assessment) or whether our translational in vivo models are necessary (or a combination of these models are necessary).

3 R developments in metabolic disorder research: possibilities and limitations
TNO has set up a research program to refine, reduce and replace animal testing. In this program, TNO collaborates with others to accelerate the process of developing alternatives. TNO is constantly looking for new insights and technologies that can reduce animal experiments. Our department has an extensive track record using primary human cells (a.o. primary human hepatocytes or HUVECs) for research questions in the field of cardiovascular and metabolic diseases. TNO also makes use of in silico models (target safety assessment) to predict potential safety liabilities. However, the development of cardiovascular disease and its underlying atherosclerosis is a complicated multifactorial process in which multiple organs interact and especially if the mechanism of potential adverse (side) effects of drugs on metabolic disorders is not clear, at present time it will not be possible to use only in vitro and in silico models to investigate these potential cardiovascular issues. Therefore, we also have a number of highly translational subclinical animal models in which we investigate potential cardiovascular issues of drugs and other substances.

For each individual in vivo study, the partner(s) will be advised on the optimal study design which includes aspects such as choosing the most suitable model (see also table 1), the experimental design, the route of administration, treatment frequency, power analysis, the concentration of choice, the primary and secondary read-out parameters, etc. If insufficient information is available, it will be decided to first perform a pilot experiment to obtain the desired information. For instance, a dose-finding pilot study could first be performed to find the dose in mice which correlates with a clinically relevant dose in humans (metabolic rate in mice is generally higher than in humans, so for equal plasma concentrations, mostly a higher dose is needed).

One of the most important selection points in our studies, is the choice of animal model to be used (see also table 1). This choice depends very much on which cardiovascular safety aspect(s) of the new drug is to be evaluated. Since each model has different cardiovascular risk factor characteristics, the combination of certain characteristics may fit better, dependent on the research question and any knowledge.

Study specific designs can vary and depend on the type of potential cardiovascular risk factor(s) of the new drug or substance are to be investigated and length of the study (for example, adverse effects on dyslipidaemia can be investigated fairly quickly (4 weeks, but atherosclerosis development in this mouse model takes much longer (16 weeks) to provide a window to investigate the potential adverse effects).

3.4.2 Provide a basic outline of the different components of the project and the type(s) of animal procedures that will be performed.

In order to induce the cardiovascular risk factors of interest a suitable mouse model from our portfolio is chosen (see table 1). The choice of the mouse model will depend on the research question. In general, the APOE*3Leiden(CETP) mouse is the preferred model as compared to the APOE -/- and LDLR-/- mouse, because, since in our view the APOE*3Leiden(CETP) mouse is the best translational model to evaluate potential cardiovascular issues. The APOE*3Leiden mouse contains the human APOE*3Leiden transgene which results a human-like (V)LDL clearance. The APOE*3Leiden.CETP mouse has the same characteristics as the APOE*3Leiden mouse in terms of (V)LDL metabolism, but also expresses human CETP, which results in a human-like HDL metabolism. So, if there are potential adverse effects on HDL metabolism (can be in addition to potential adverse effects on (V)LDL metabolism), the APOE*3Leiden.CETP mouse is the preferred model when compared to the APOE*3Leiden mouse. The APOE*3Leiden mouse would be preferred over the APOE*3Leiden.CETP mice if we want to exclude potential effects on CETP mediated HDL-metabolism or if the focus of the study is on adverse effects on chronic inflammation.

We would like to use the APOE- /- mouse or the LDLR -/- mouse model if, for example, we want an independent confirmation of data in the literature or if we want to exclude the involvement of ApoE or LDLR mediated (V)LDL clearance in potential cardiovascular issues.

The mice will be fed a western type diet to mimic the consumption in the Western World, thereby inducing the cardiovascular risk factors. The new drug or substance to be tested on its cardiovascular safety aspects will be administered via admix food, admix drinking water, gavages or injections or osmotic minipumps. During the study parameters will be measured:
Body weight and food intake will be monitored regularly during the study and blood samples will be taken regularly for measurement of lipids, inflammation markers etc. At the end of the study animals are euthanized and blood and tissues are collected for further analyses. More specific parameters can be measured during the study and are mentioned in the appendix. Diets and parameters to be measured are described in more detail in the appendix.

Table 1. Mouse models and cardiovascular risk factors to be studied

Mouse model	Cardiovascular risk factor	Notes
ApoE*3Leiden	hyperlipidemia	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 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.
	atherosclerosis	Only females are suitable to evaluate adverse effects on atherosclerosis development. Male mice do not/hardly develop atherosclerosis.
	chronic inflammation	Only females on a high cholesterol diet are suitable to evaluate adverse effects on chronic inflammation (plasma and in vessel wall).
APOE*3Leiden.CETP	hyperlipidemia, 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.
LDLR-/-	hyperlipidemia, atherosclerosis	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-/-	hyperlipidemia, atherosclerosis	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 as compared to all models above. This model is not to be used when ApoE could be involved in the adverse effect under investigation.

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.

Different cardiovascular risk factors can be induced in translational animal models: obesity, insulin resistance, Type 2 Diabetes, hyperlipidemia, atherosclerosis, NAFLD and NASH. Our department currently focuses on potential adverse effects on hyperlipidemia, inflammation and atherosclerosis, based on our expertise in this area and cardiovascular safety issues of drugs and other substances tested by us thus far. The coherence between the studies is that in all studies potential adverse effects on cardiovascular risk factors and/or underlying mechanism are being studied. Identifying the potential cardiovascular/metabolic safety issues allow our partners in the chemical & pharmaceutical industry to develop ways to prevent or mitigate these safety issues, in order to reduce CVD.

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	Cardiovascular safety study
2	
3	
4	
5	
6	
7	
8	
9	
10	
11	