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.	Prevention and treatment of metabolic disorders.

2 Categories

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

- X 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.

Excess intake of energy rich diets with high levels of sugars and saturated fat in combination with a sedentary lifestyle has resulted in a worldwide epidemic of obesity and associated metabolic disorders. Metabolism is the concerted interplay of several organs (gut, liver, adipose tissue, muscle, vascular system) that interact in an orchestrated way to provide energy from food and distribute it to the other

organs (e.g. heart, brain) or use it for storage purposes (typically in adipose tissue, liver, muscle). Importantly, each organ is responsible for a specific step in the metabolic chain and has a role in metabolic homeostasis (for instance to keep the concentration of glucose and lipids at constant levels). A disturbance in this chain of metabolic events results in metabolic disease indicators such as abdominal obesity (access storage of lipids), hyperlipidemia (high lipid levels), impaired glucose tolerance (uncontrolled glucose levels), hypertension (impaired vessel function) or insulin resistance (inadequate response of metabolic organs to insulin). These metabolic disorders often tend to cluster together and especially the combination, called Metabolic Syndrome, poses a major risk for further complications. While the precise definition of Metabolic Syndrome remains debated, it is unambiguously clear that combinations of metabolic disorders lead to an enhanced risk for the development cardiovascular disease and Type 2 Diabetes. These different metabolic disorders are undoubtedly related and interconnected (because metabolic dysfunction of one organ directly affects the function of the other organs in the metabolic chain) and are often sharing similar underlying mechanisms. Clustering of metabolic anomalies does not only lead to an enhanced risk for cardiovascular diseases and Type 2 Diabetes, but also other complications, like liver diseases as non-alcoholic steatohepatitis (NASH) and hepatic fibrosis, nephropathy (in kidney) and microvascular disease (in retina and brain) can occur. Since humans differ in the metabolic capacity of their organs (from person to person), the disease typically manifests in the 'weakest organ with lowest capacity' and most patients develop one or more of the different pathologies of the Metabolic Syndrome simultaneously. Due to this cohesion between the different metabolic disorders, their simultaneous occurrence and similar underlying mechanisms, the Metabolic Syndrome can be considered as one disease. Life-style changes are the preferred treatment, however the increasing incidence of these metabolic disorders and complications thereof, warrants further development of pharmacological approaches as well.

At our department, we work on the full spectrum of metabolic health, metabolic disease and complications thereof (Figure 1) and aim to attenuate their development (e.g. by lifestyle and nutritional or pharmacological interventions). In general, the overall theme of our research is to understand the transitions from metabolic healthy towards metabolic disorders and towards the development of complications, and to unravel the underlying mechanisms. All in order to investigate whether it is possible to prevent these transitions or intervene to reverse them. An essential step in developing novel therapies is to demonstrate efficacy on key risk factors and/or disease endpoints in a preclinical setting before authorities allow testing of a novel therapy in humans (e.g. a clinical trial). Although in vitro models and in silico models are most helpful in studying parts of the Metabolic Syndrome, the complex organ-organ interactions that are intrinsic to metabolism and metabolic disorders make it currently unavoidable to use animal models to study the Metabolic Syndrome and develop new therapies. In the past 30 years, TNO has developed a broad portfolio of in vivo models that allow detailed analysis of different aspects of metabolic disorders. For instance, an extensive historical database of preclinical studies allows an evidence-based selection of a diet with optimal composition in combination with an optimal mouse strain to address a specific question. Through our portfolio, we support both the nutrition & pharmaceutical industry as well as academia and medical centers in the development of new therapy options. We strive to continuously incorporate new insights and technologies in our portfolio to offer the best possible preclinical research tools.

Patients with Metabolic Syndrome may have different disease stages. Therefore, the research on metabolic disease development can be divided into two main (consecutive) stages (see Figure 1): **metabolic disorders** develop in first place (first stage) which give rise to **metabolic complications** that may emerge as a consequence (second stage). As a rule of thumb, metabolic disorder is thought to be (partially) reversible whereas second stage disease processes can involve tissue damage with a more irreversible phenotype.

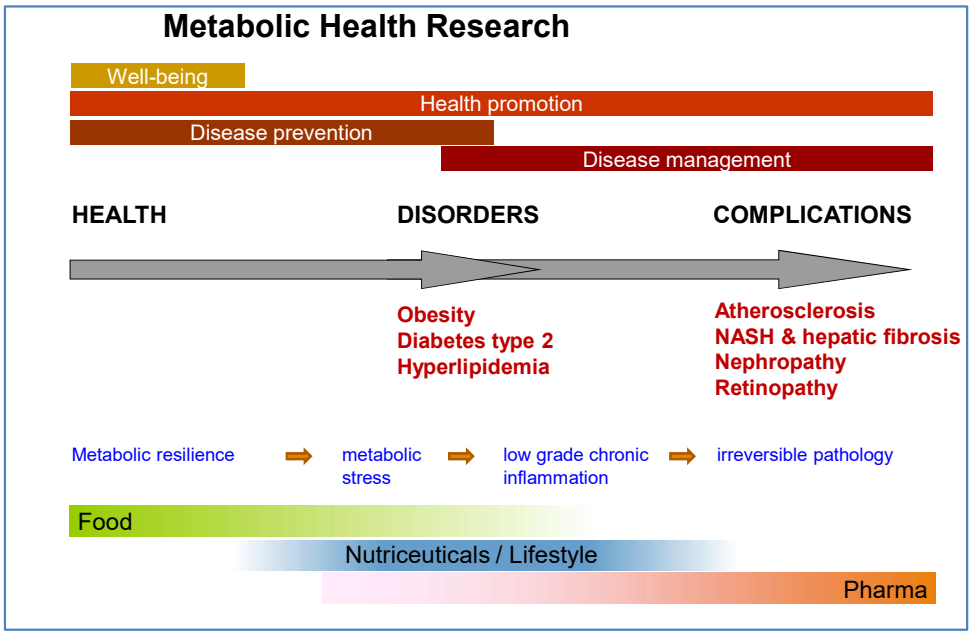


Figure 1. The research area of our department, Metabolic Health Research.

1. Metabolic disorders (first stage)
Nowadays, in our Western society food is in abundance and energy rich which contain high levels of sugars and saturated fat, and the same holds true for developing countries. For the first time in history, there are more subjects suffering from overweight and obesity than from hunger.
If a situation of nutritional abundance persists only shortly, the body can still cope with this metabolic stressor and we speak of metabolic resilience, which is still considered a healthy situation. The problems arise when the period of nutritional abundance is long-lasting or when there are too many metabolic stressors (e.g. from food, from drinks, at day and night etc). In this situation the body cannot cope with the metabolic stressor anymore, and metabolic stress occurs in the weakest organ (which varies from subject to subject) and metabolic disorders will develop. At our department we investigate the sequence of events during the development of metabolic disorders (for knowledge on improved diagnosis and monitoring) and try to attenuate pathological processes or reverse them via treatments that are based on this knowledge. Different metabolic disorders are studied, for instance: **obesity** (with fatty liver disease), **Type 2 Diabetes**, **hyperlipidemia** and/or combinations thereof (essentially as in patients with Metabolic Syndrome who develop these pathologies simultaneously).

2. Metabolic complications (second stage)
After a prolonged period of metabolic disorders, multiple complications can develop. For instance, when the hyperlipidemic time period becomes too long, **atherosclerosis** will develop. Likewise, chronic hyperglycemia and insulin resistance (Type 2 Diabetes) typically leads to complications like **nephropathy** or complications of the small blood vessels that supply the retina (microvascular disease) which manifests in patients as **retinopathy**. In the case of the liver, fatty liver disease can progress further towards non-alcoholic steatohepatitis (**NASH**), a condition characterized by both hepatic lipid accumulation and hepatic inflammation which leads to damage and (irreversible) liver fibrosis, because scar tissue will replace the normal liver cells.

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 ultimate aim is to maintain metabolic health and prevent the development of metabolic disorders and their complications. Our direct aim for the next 5 years is to gain better understanding of the

underlying mechanisms that drive metabolic disorders and to contribute to the prevention and/or treatment (via nutritional or pharmacological interventions) of metabolic disease development. To this end, novel therapeutic strategies will be tested in our translational models to contribute as much as possible to the prediction of the ultimate clinical effectiveness.

In general, the overall theme of our research is to understand the transitions from natural metabolic homeostasis towards metabolic disorders and towards the development of complications. In addition, to unravel the underlying mechanisms in order to investigate whether it is possible to prevent these transitions or intervene to reverse them.
Depending on the disease stage, the purpose of a study can be:

- To monitor metabolic disorder development and prevent, attenuate or reverse the disease development.
- To monitor the development of disease complications and try to prevent attenuate or reverse their development.

The specific research question of a study will depend on the type of metabolic disorder being studied and an example is shown in the appendix.
Approximately 70% of our studies will be applied research performed to investigate the efficacy of novel treatments against a metabolic disease and aim at investigating to which extent the treatment can prevent or improve the metabolic disease.
In addition, about 20% of our studies is translational research performed to investigate the underlying working mechanism of a novel candidate therapy or identify biomarkers that can be translated to clinic. For instance if a compound was shown to have beneficial effects on hyperlipidemia and could reduce plasma cholesterol and triglycerides, a next study could be performed to investigate the mechanism of this reduction.
Besides directly testing different prevention- or intervention-therapies, approximately 10% fundamental research studies are performed that aim to understand the underlying mechanisms of the disease. For understanding the disease development, time-course studies can be performed in which groups of animals are compared after different time periods on a metabolic stressor (for instance different periods of high fat diet feeding that lead to liver disease: steatosis, liver inflammation, NASH/fibrosis development). As mentioned before, we would also like to understand the underlying mechanisms that are involved in the transition from healthy homeostasis towards metabolic disorders and from metabolic disorders towards metabolic complications. For instance, why do some people with Diabetes develop certain complications and others do not (how do these group differ)? Animal studies that compare the animals that do develop complications after application of a certain metabolic stressor with animals that do not develop these complications (despite being exposed to the same stressor) can help to understand the underlying mechanisms that play a role, and unravel protective mechanisms. Also fundamental research studies can be performed to evaluate a novel therapeutic target. These studies are not directly aimed at treatment of disease but are first investigating the role of a specific pathway or protein within the disease development to see whether this could be a potential target for treatment.
For all of our studies, it is very important that we use translational models that reflect the disease situation in humans as much as possible. We are continuously working on improving our in vivo models. Therefore also fundamental studies can be performed aimed at developing a novel model or improving existing models. An improvement of the model could be via adaptations of the diets used to induce the disease or by genetic interventions, for instance via cross-breeding of different transgenic mice, resulting in a novel animal model with a desired phenotype. A cross-bred of ApoE*3Leiden with GK+/- mice (hyperglycemic and diabetic mice due to the diminished expression of glucokinase enzyme) can be expected. The latter cross-bred does not lead to discomfort. Similarly as in humans, that are often unaware of their metabolic disorders, until a very late stage. Other cross-breedings could be performed as well. Cross-breedings that will lead to discomfort will be requested in an amendment.

Although not every study in itself (especially the more fundamental research studies/studies designed for model development/improvement) is directly aimed at treatment of metabolic disease, all studies all together are aimed to prevent or to treat metabolic disorders and their complications.

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. For the research described in this project, focusing on metabolic disorders, TNO has an extensive track record, resulting in more than 150 peer-reviewed international publications. Researchers within the group are already more than 20 years working in the field of cardiovascular and metabolic diseases. As example, we mention several (mechanistical) studies with different statins, fibrates (PPAR-α agonists), niacin, CETP-inhibitors, PCSK9 antibodies, phytosterols, polyunsaturated fatty acids, GLP-1 inhibitors, exendin, glitazones (PPAR-γ agonists), bile acid sequestrants. Some of these studies have been used for FDA filing. A large network has been built within both the academic world and the pharmaceutical industry, nutritional companies, as well as biotech companies, academic medical centers, patient organizations and governments. Within this network we have conducted over the past 15 years more than 150 cooperation projects (both bilaterally and in larger consortia). Our previous achievements makes it very likely that with the experiments described in this project we will make large contributions to our main research questions.

3.3 Relevance

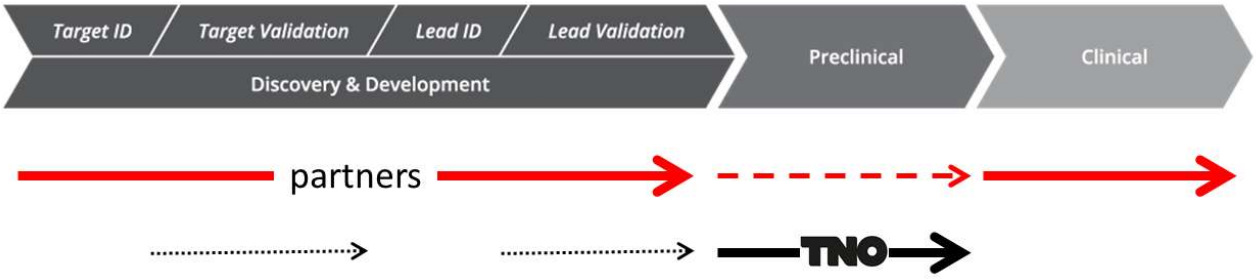
What is the scientific and/or social relevance of the objectives described above?

Presumably due to changes in lifestyle, the good food supply (no periods of scarcity) and the overall aging of the population, a strong increase in the incidence of obesity, leading to metabolic derangement has occurred during the last years. These metabolic abnormalities like abdominal obesity, hypertension, hyperlipidemia and impaired glucose tolerance of insulin resistance often tend to cluster together and this combination is called Metabolic Syndrome. The Metabolic Syndrome poses a major risk for further complications, like cardiovascular disease (atherosclerosis) and Type 2 Diabetes, but also complications, like liver disease (non-alcoholic steatohepatitis (NASH) and hepatic fibrosis), nephropathy, retinopathy and cognitive disorders can occur.
Worldwide obesity has more than doubled since 1980. The most recent numbers of the World Health Organization (WHO) are of 2014, in which more than 1.9 billion adults (18 years and older) were overweight. Of these over 600 million were obese. Forty-one million children under the age of 5 were overweight or obese in 2014. This has all led to a rapid increase in the number of people with associated metabolic diseases or complications. For instance, the number of people with Diabetes has risen from 108 million in 1980 to 422 million in 2014 and an estimated 17.5 million people died from cardiovascular diseases in 2012, representing 31% of all global deaths. (Source: WHO, updated June 2016). These numbers have major implications: An expert panel convened by the National Institutes of Health (NIH) stated that for the first time in history, the steadily improving worldwide life expectancy could level off or even decline as a result of increasing obesity. Also the economic costs are enormous: in the US the medical cost to treat obesity were estimated to be as high as $147 billion per year (Finkelstein et al., Annual medical spending attributable to obesity: Payer-and service-specific estimates. Health Aff (Millwood) 2009;28:w822–31).
To date, few people succeed in preventing the development of metabolic disorders and their complications by means of an improved lifestyle. Therefore, it is important to promote metabolic health and to develop new prevention and intervention strategies.
Due to the rapidly increasing numbers of people that develop a metabolic disorder, development of novel prevention- and intervention-strategies will have a large social and economic impact. The development of novel insights into the underlying mechanisms involved in metabolic disease development will also have scientific relevance and will contribute to the general knowledge on these metabolic disorders.

3.4 Research strategy

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

The development of new interventions such as nutritions or therapeutics follows a fixed pattern:



First of all, suitable targets will be identified on the basis of, for example, literature, human studies and / or in vitro testing. Subsequently, it will be evaluated whether modification of the target can indeed lead to a change of pathology (for example by in vitro testing, ex vivo tests with human tissue, in vivo testing in transgenic animals). Thereafter, leads will be developed that can affect the target (eg, nutritions, inhibitory antibodies, small molecules, RNA therapy) and those leads will be validated (which substance is most potent, what's the solubility or specificity?). The most promising candidates then enter the preclinical testing phase, which examines whether the candidates are good enough to be tested in humans (clinical phase). The ultimate goal is of course that candidates successfully complete the clinical phase and will be admitted as a new therapy.

TNO as Research and Technology Organization (RTO) supports for years both nutritional or pharmaceutical industry and academic partners in implementing and optimizing preclinical research in the field of cardiovascular and metabolic diseases. The majority of work under this project is in the preclinical phase, but work in the area of target validation (for example, show that a pathway is involved) or lead validation (eg comparing nutritions or different variants of a new compound in order to determine which variant will continue further development) also occurs.

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 better alternatives. TNO is constantly looking for new insights and technologies that can reduce animal experiments. For example, we evaluate drug efficacy with micro-dosing, where a very small dose of the drug is introduced into humans without any risk and measured with our advanced technology. Accelerator Mass Spectrometry (AMS) is used to establish whether the substance has reached the target and if it resides long enough to induce an effect. In addition, we currently focus on designing 'organ-on-a-chip' models. Based on human (stem) cells, we use these models to mimic organ functionality, with organs such as the liver, intestines and lungs. Therewith we want to increase the predictivity and translation of in vitro results to humans. These improved in vitro models provide increasingly better understanding of the underlying mechanisms of metabolic disorders, and reduce the need for animal testing. However, the development of metabolic disorders is a complicated multifactorial process in which multiple organs interact and at present it is not possible to obtain all the knowledge about these complex disorders using only in vitro models. Therefore, we also have a number of highly translational animal models in which we investigate these metabolic disorders.

The current project describes the research that we conduct using these animal models with the aim to 1) either attenuate the development of metabolic disorders or 2) to treat (established) metabolic disorders and their complications, via nutritional or pharmacological interventions. This approach (prevention and treatment) is in line with the present situation in primary care and in hospitals: doctors have to treat patients with different stages of metabolic disease with a combination of lifestyle, nutrition and pharmacotherapy.

The majority (>70%) of the studies that will be conducted in this project will be efficacy studies to evaluate nutritional interventions or new therapeutics. This will take place in conjunction with or on behalf of external partners. For each individual study, the partner(s) will be advised on the optimal study design. Each partner will always be asked what is already known of the compound to be tested, to

prevent needless animal use. Furthermore, also aspects such as choosing the most suitable model (usually depending on the type of metabolic disorder(s) being studied or based on the mechanism of action of the lead), the experimental design, the route of administration, treatment frequency, power analysis, the concentration of choice, the primary and secondary read-out parameters, etc. will be included. 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 optimal dose).

The remaining studies that will be carried out under this project application, will be aimed at improvement of the animal models or aim to obtain more knowledge about the models and/or the underlying mechanisms of the different metabolic diseases.Overall we use or develop translational animal models that reflect a certain metabolic disorder. Based on data generated in previous and ongoing studies, available data and information from nutritional or pharmaceutical partners, available literature and interactions with other scientists, we generate an idea on which disease processes are important and should be reflected in the translational models. We perform basic research studies to obtain more knowledge on the metabolic disorders, the disease development and the relevant processes and underlying mechanisms, but also on the models itself and the translational aspects. Ultimately, specific hypothesis regarding intervention in the disease development are evaluated in order to prevent or treat the metabolic disorders or to study the mode of action.

Study specific designs can vary and depend on the stage of the metabolic disease and the type of metabolic disease.

Expectation for the next 5 years:
All studies, animal models, interventions and measurements currently performed are described in this application. New developments in the research field and the focus of companies that develop new compounds or nutrients will strongly affect what kind of studies will be performed the coming years. At this moment, the expectation for the next 5 years is that a significant part of the studies will be NASH studies. Hyperlipidemia and atherosclerosis studies will also remain important. With the new cardiovascular therapies currently under development, it is possible to obtain a further reduction in hyperlipidemia via combination therapy. With this further reduction of hyperlipidemia regression of atherosclerotic plaques might be possible (in addition to further progression). A further shift to combination therapies and regression studies is therefore to be expected. In addition, it is expected that more attention will be paid to diabetic atherosclerosis. In the case of nephropathy, we expect less studies in the coming years: it seems to be difficult to obtain a translational model with similar pathology as observed in humans. For this reason the use of these models to study nephropathy is currently under discussion. Retinopathy is a relatively new field of research within our group, which is still in a somewhat exploratory phase. Depending on the results (development of translational model), we expect this to be a growth area. In addition, the cross-talk between organs (intestine, fat, muscle, brain and liver) and the research on biomarkers is a growing field of research, which we are expected to focus more on in the coming years.

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.

For fundamental research: a metabolic stressor will be applied in a mouse model to induce one or more of the following metabolic disorder(s): **obesity, Type 2 Diabetes, hyperlipidemia, atherosclerosis, NASH, nephropathy and retinopathy**.
For applied or translational research: again a metabolic stressor will be applied in a mouse model to induce one or more of these metabolic disorders, but a preventive or therapeutic intervention will be performed as well. Interventions will usually be nutritional/compound administration (via admix food, admix drinking water, gavages or injections or osmotic minipumps) but can also be other interventions (surgical removal of fat, exercise).
For either fundamental or applied/translational studies, 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 blood glucose, plasma insulin, lipids, inflammation markers etc. At the end of the study animals are sacrificed and blood and tissues are collected for further analyses. More specific

parameters can be measured during the study, but depend on the type of metabolic disorder studied and are mentioned in the appendix.
Metabolic stressors, interventions and parameters to be measured are described in more detail in the appendix.

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 stages of metabolic disorder(s) are being studied, as well as different metabolic disorders/complications: obesity, Type 2 Diabetes, hyperlipidemia, atherosclerosis, NASH, nephropathy and retinopathy. The coherence between the studies is that all studies are related to metabolic disease and contribute to our aim to develop therapeutic strategies to prevent metabolic disorders and their complications. The different metabolic disorders are not separate diseases but are related: the metabolic disorders coincide and the occurrence of one metabolic disorder enhances the risk for others. In addition, similar common pathways play a role in the different metabolic disorders.
One of the most important selection points in our studies, is the choice of animal model to be used. This choice depends very much on the primary and secondary research questions studied, ie. the nature of metabolic disorder endpoint studied and also the stage of the metabolic disorder or complications we would like to evaluate. Since each model has different phenotypic characteristics, the combination of certain characteristics may fit better, dependent on these primary and secondary research questions and the balance of the importance between those research questions. However, additional knowledge on for instance the working mechanism of the compound to be tested can affect the choice of animal model to be used as well. In table 1 the optional animal models are described and in the appendix the optional procedures will be outlined further for the different metabolic disorders and complications.

Table 1. Animal models per metabolic disorder.

Metabolic disorder	Model	Comments
Obesity & Diabetes type 2	C57BL6/j	When put on a high fat diet these mice become obese and insulin resistant.
	Ob/Ob	Leptin deficient mice, develop spontaneously obesity and type 2 diabetes after a short period. Fast and more severe model than diet induced C57BL6/j, but disadvantage is that etiology of type 2 diabetes is not translational to human situation.
	Db/Db	Leptin receptor deficient mice, develop spontaneously obesity and type 2 diabetes after a short period. Disadvantage is that etiology of type 2 diabetes is not translational to human situation. More severe model than ob/ob mice, after 3-4 months drop in insulin and transition to type 1 diabetes model. Only when used in long-lasting studies severe Diabetes with mild-moderate discomfort can occur.
	KKA^{y}	Develop type 2 diabetes of polygenic origin. When fed a high fat diet mice become obese and show additional hyperinsulinemia and hyperglycemia. Only when used in long-lasting studies severe Diabetes with mild-moderate discomfort can occur.
Hyperlipidemia & atherosclerosis	ApoE*3Leiden	Mice carrying a human APOE*3Leiden transgene that leads to a defective clearance of triglyceride-rich lipoproteins. While normal wild-type mice have a very rapid clearance of triglyceride rich lipoproteins, 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 therefore extremely suitable in combination / comparison studies. The animals also respond to lifestyle interventions, dietary supplements, anti-oxidants, omega-3 PUFAs, hormones and pre / probiotics. Males and females

		can be used for studies of lipids, only females are suitable for atherosclerosis research. Male mice do not/hardly develop atherosclerosis but do in turn develop insulin resistance and liver disease (NAFLD).
	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. Furthermore, this mouse has the same characteristics as the APOE*3Leiden mouse regarding its (V)LDL metabolism.
	ApoE*3Leiden.GK+/-	Cross-breeding of ApoE*3Leiden with GK+/- mouse (diabetic model due to the diminished expression of glucokinase enzyme). This particular model combines the dyslipidemic phenotype of ApoE*3Leiden mouse with diabetic phenotype of GK mouse and this model can therefore be used to study diabetic hyperlipidemia or diabetic atherosclerosis.
	LDLR-/- and LDLR-/-.Leiden	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). Both males and females can be used for lipids and atherosclerosis research. LDLR-/-.Leiden mice are an established substrain of LDLR-/- mice that are susceptible to become obese on energy dense diets and that activate proinflammatory and profibrotic pathways in response to diets with human-like composition of macronutrients.
	ApoE-/-	The mice lack apolipoprotein E. Both males and females can be used for lipid and atherosclerosis research. This is a more severe model than all the above models, with higher lipid levels and more atherosclerosis.
NASH	ApoE*3Leiden and ApoE*3Leiden.CETP	Mice that have a human-like lipoprotein metabolism (see also comments above) and when put on a high fat and high cholesterol diet these mice develop obesity, dyslipidemia, mild insulin resistance and several characteristics of NASH (steatosis, inflammation and hepatic fibrosis). The underlying mechanism of NASH induction probably involves the formation of hepatic cholesterol crystals, leading to hepatic inflammation and lipotoxicity. At this moment, we know that male mice develop NASH and fibrosis when put on the high fat and cholesterol diet. Male mice are more susceptible to become obese in response to high caloric diets and accumulate fat in the abdominal cavity (essentially as it is also the case in humans with metabolic disease). For ApoE*3Leiden.CETP mice we are currently evaluating whether the female mice also develop NASH and fibrosis (*in vivo* studies ongoing). Depending on the outcome, female mice might be used as well.
	LDLR-/-.Leiden	Ldlr deficient mice (see also comments above) that develop hyperlipidemia when treated with all sorts of high caloric diets. These mice develop pronounced obesity and insulin resistance and several characteristics of NASH (steatosis, inflammation and hepatic fibrosis). The underlying mechanism of NASH induction differs as compared to ApoE*3Leiden(.CETP) mice: in both models the increase in white adipose tissue is thought to be involved, but in the LDLR-/-.Leiden mice this mechanism plays a more prominent role. At this moment, we know that male mice develop NASH and fibrosis when put on the high caloric diets. Male mice are more susceptible to become obese in response to high caloric diets and accumulate fat in the abdominal cavity (essentially as it is also the case in humans with

		metabolic disease). For LDLR-/-.Leiden mice we are currently evaluating whether the female mice also develop NASH and fibrosis (*in vivo* studies ongoing). Depending on the outcome, female mice might be used as well.
Nephropathy	Ob/Ob	Obese and insulin resistant/diabetic mice (see also comments above) that can also be used for nephropathy studies.
	Db/Db	Obese and insulin resistant/diabetic mice (see also comments above) that can also be used for nephropathy studies. Only when used in long-lasting studies severe Diabetes with mild-moderate discomfort can occur.
	KKA^{y}	Obese and insulin resistant/diabetic mice (see also comments above) that can also be used for nephropathy studies. Only when used in long-lasting studies severe Diabetes with mild-moderate discomfort can occur.
Retinopathy	ApoE*3Leiden and ApoE*3Leiden.CETP	Mice that have a human-like lipoprotein metabolism (see also comments above) and when put on a high fat diet these mice also develop retinopathy.
	LDLR-/-.Leiden	Ldlr deficient mice (see also comments above) and when put on a high fat diet these mice also develop retinopathy.

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	Metabolic disorder(s) study
2	
3	
4	
5	
6	
7	
8	
9	
10	