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 the Guidelines to the project licence application form for animal procedures on our website (www.centralecommissiedierproeven.nl).
- Or contact us by phone (0800-789 0789).

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.

- ☒ Basic research
- ☒ Translational or applied research
- ☐ Regulatory use or routine production
- ☐ Research into environmental protection in the interest of human or animal
- ☐ 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 2.1.

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 (see also review paper of Grundy SM, Clin Cornerstone. 2005;7(2-3):36-45, PMID: 16473259). 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 of cardiovascular disease (CVD), Type 2 Diabetes (T2DM) and neurodegenerative diseases (NDD). 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 this is associated with poor long-term patient adherence and compliance, and 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). The overarching theme of our research is to understand and unravel the mechanisms of the transitions from a healthy state to metabolic disorders and ultimately to complications, 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 necessary to use animal models to study the Metabolic Syndrome and develop new therapies. Since our previous project proposal (AVD5010020172064), we have not modified our study design or general strategy, while with respect to reduction of discomfort our refinement several steps have been taken, especially for our nephropathy studies using KKA^{y} mice (a.o. reduction of handling and measurements, improved housing on half-heated cages, improved handling using tubes, urine collection using labsand and improved skin and wound care), see also Appendix G, refinement. In comparison to 5 years ago, our research on organ-organ interaction has intensified and we obtained more knowledge on the underlying mechanisms of organs interacting with each other during metabolic overload. For example in one study the effects of an intervention on both gut permeability and NASH were tested, in another study the effects on both adipose tissue and liver/NASH were evaluated whereas in another example intervention on both NASH and atherosclerosis was explored. Related to this, we also observed an increased interest from nutrition/pharma companies to evaluate the effect of an intervention on multiple organs instead of focusing on one organ/disease. In addition, we deepened our research on the effects of metabolic overload in our models on microvascular disease in brain (brain structure, function) and neuroinflammation, while we have not worked on retinopathy. In the past 30-40 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. During the last 5 years, we tested new interventions in our more established (atherosclerosis) models (Stokman et al., Liver Int. 2020, PMID: 32841505; Pouwer et al., J Lipid Res. 2020, PMID: 31843957; Keulen et al., PloS One. 2019, PMID: 31461490; Schuster et al., Sci

Rep. 2019, PMID: 31366894; Pouwer et al., Front Cardiovasc Med. 2018, PMID: 29946549). We are continuously working on improving our in vivo models and in the last 5 years we tested the translatability of our NASH models and compared them head-to-head to large cohorts of patients (e.g. Martinez-Arranz et al., Hepatology, 2022, PMID: 35220605; Morrison et al., Hepatol Communic, 2018, PMID: 30556039; van den Hoek et al., Cells, 2021, PMID: 32883049) and we used different high fat diets to emphasize certain disease characteristics (as described in van den Hoek et al., Cells, 2021, PMID: 32883049). In addition, we developed a nephropathy model with moderate to advanced renal damage (posters ASN meeting 2018 & 2022, manuscript in preparation). 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.

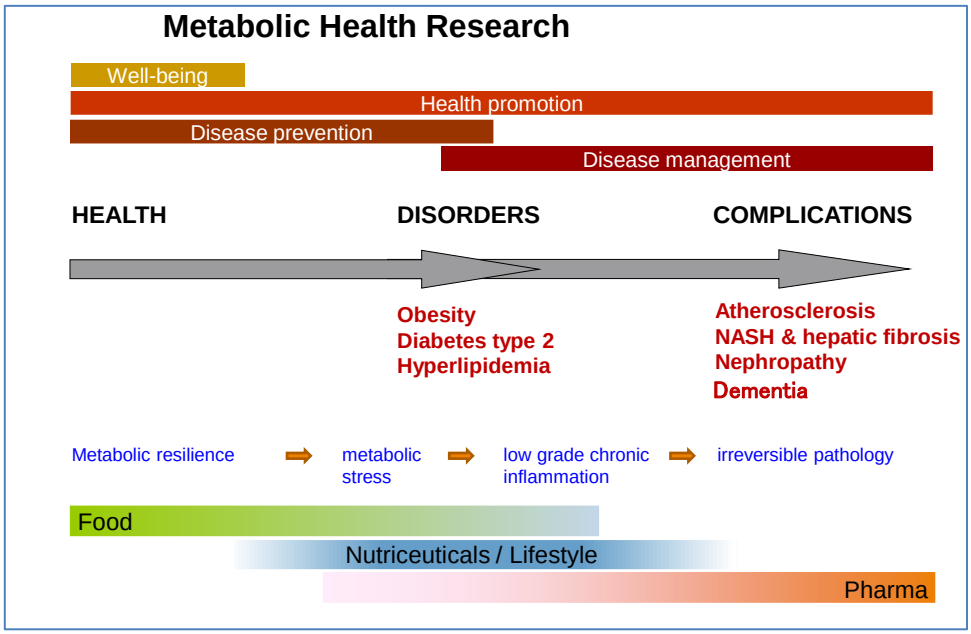


Figure 1. The research area of our department, Metabolic Health Research.

1. Metabolic disorders (first stage)

Nowadays, in our Western society food is in abundance and energy rich, containing 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 lead to complications like **nephropathy** or complications of the small blood vessels that supply the retina (microvascular disease)or the brain (small vessel disease) which manifests in patients as **retinopathy** or **dementia,** respectively. 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 replaces the normal liver cells. If this condition remains untreated, liver cells may switch on cell division and growth programs resulting in **hepatocellular carcinoma (HCC)**.

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 necessary to use animal models to study the Metabolic Syndrome and develop new therapies. 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 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. TNO has an active involvement in programs to replace animal testing: our department is involved in TPI (in Dutch: Transitie Proefdiervrije Innovatie) and is working on several projects, such as AdtoME and Mechpath (both with Stichting Proefdiervrij) to define whether, based on the disease mechanisms, in vitro models are representative for patients and whether we can develop a drug development approach that establishes the efficacy of new drug candidates, without the use of experimental animals. 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. In our translational models we do not only mimic (histo)pathological characteristics, but have similar underlying disease pathways as well. For instance, in our ApoE*3Leiden and ApoE*3Leiden.CETP mice the ApoE*3Leiden construct leads to a defective clearance of VLDL/LDL particles and are thereby mimicking the slow clearance of humans, while wild-type mice have a fast clearance. For our Ldlr-/-.Leiden NASH mouse model we demonstrated using transcriptomics, preoteomics and metabolomics level that underlying disease pathways are similar as for NASH patients. Our KKA^{y} nephropathy model not only has a similar kidney pathology as observed in patients, but has a similar process regarding organ function as well with first glomerular hyperfiltration followed by hypofiltration in a later phase.

3.2 Purpose

3.2.1 Describe the project's immediate and ultimate goals. Describe to which extent achieving the project's immediate goal will contribute to achieving the ultimate goal.

- If applicable, describe all subobjectives

Our ultimate aim is to help patients suffering from metabolic disease and understand how to maintain metabolic health and prevent the development of metabolic disorders and their complications. Our direct

aim for the next 5 years is to refine our 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.

The overarching theme of our research is to understand and unravel the mechanisms of the transitions from a healthy state to metabolic disorders and ultimately to complications, 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 will 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. A novel development we observed within the last 5 years with this type of research was that while previously the efficacy was typically evaluated by measuring plasma lipids or end-point disease stages via histology, we now also performed studies in which the efficacy was tested on transcriptome signature level (different fibrosis gene signatures were evaluated) and for future studies we might expect efficacy testing on biomarker level.
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. An example of this research in the last 5 years is a study investigating the mode of action, in which a compound was first shown to have beneficial effects on hyperlipidemia by reducing plasma cholesterol and triglycerides, followed by a next study to investigate the mechanism of this reduction (a decrease in VLDL-production or an increased postprandial TG-clearance? Or was lipase activity affected or fecal excretion?). Another example of the last 5 years is a currently ongoing mechanistic study with a compound currently tested in clinical trials for its lipid lowering effects when given on top of a statin. In our mouse study, we can also test the effect of monotherapy of this compound (independent of statin; for patients it was not an option to withdraw the statin treatment) and we added a matched low cholesterol control group (with lower dietary cholesterol to obtain similar plasma cholesterol levels) to evaluate whether this compound also has beneficial effects on atherosclerosis independent of its plasma cholesterol lowering effects. Similar mechanistic studies can be expected for the next 5 years.
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 groups 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 identify protective mechanisms. Fundamental research studies can also be performed to evaluate a novel therapeutic target/target validation. These studies are not directly aimed at treatment of disease but are 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. 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 (similar to the high fat diet vs. fast food diet evaluation we did during the last 5 years and described in van den Hoek, Cells, 2021, PMID 32883049) or by genetic interventions, for instance via cross-breeding of different transgenic mice, resulting in a novel animal model with a desired phenotype. For instance during the last 5 years a cross-breed of ApoE*3Leiden with GK+/- mice (hyperglycemic and diabetic mice due to the diminished expression of glucokinase enzyme) was made (and described in Pouwer et al., J Diabetes Res, 2019, PMID: 30949516). For the next 5 years, we might expect the use of this or another cross-breeding. More likely, instead of cross-breeding, we could use injections of adeno associated viruses (AAV) or siRNA to selectively target a gene of interest in order to emphasize a certain metabolic phenotype. For the next 5 years, we expect we might like to use this latter technology to emphasize the systemic inflammation in our ApoE*3Leiden(.CETP) mouse model in order to be able to discriminate between cholesterol lowering and anti-inflammatory effects of compounds with a beneficial effect on atherosclerosis. In addition, it might be expected that we will use FRG® KO mice (=a triple knockout model), as a translational mouse model in which the liver has been repopulated for 70-90% with human primary hepatocytes, thereby mimicking the human liver. This is an interesting translational model and we might like to use this model in the next 5 years as well as a NASH model.

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

3.2.2 Provide a justification for the project's feasibility.

The type of work we describe in this proposal has a high feasibility and is of great interest for stakeholders for which TNO delivers translational models and studies:
Within the strategy of TNO, Healthy Living is one of the 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 biomedical and digital health 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 200 peer-reviewed international publications. Researchers within the group are already more than 25 years working in the field of cardiovascular and metabolic diseases. As example, we mention several (mechanistic) 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 (e.g. TNO studies with rosuvastatin, plant stanols and evinacumab) and many of the studies have contributed to the development of pharmaceutical or nutritional strategies that reduced morbidity and mortality of patients. 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 more than 180 cooperation projects (both bilaterally and in larger consortia) over the past decades. Animal experiments are performed by a skilled team of technicians that are very experienced with the handlings described in this application. Our previous achievements make it very likely that with the experiments described in this project we can address the important research questions in the field of cardiometabolic diseases that will be relevant in the future to improve patient's health and attenuate disease.

3.2.3 Are, for conducting this project, other laws and regulations applicable that may affect the welfare of the animals and/or the feasibility of the project?

☒ No

☐ Yes > Describe which laws and regulations apply en describe the effects on the welfare of the animals and the feasibility of the project.

3.3 Relevance

3.3.1 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 or 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. The Metabolic Syndrome and its complications are debilitating and associated with impaired quality of life and an increased mortality.
Worldwide obesity has nearly tripled since 1975. The most recent numbers of the World Health Organization (WHO) in 2022 are of 2016, in which more than 1.9 billion adults (18 years and older) were overweight. Of these over 650 million were obese. Over 340 million children and adolescents aged 5-19 were overweight or obese in 2016. 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 17.9 million people died from cardiovascular diseases in 2019, representing 32% of all global deaths. (Source: WHO, updated June 2021). 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. The economic costs are enormous: in the US the medical costs 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, PMID: 19635784).
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 socio-economic impact. 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.

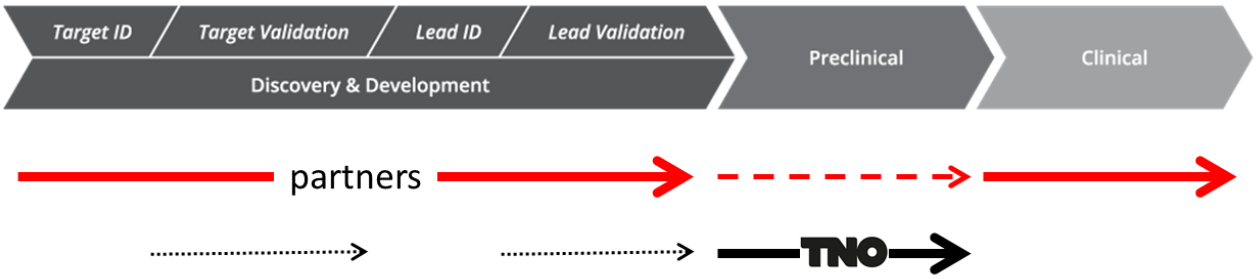
3.3.2 Who are the project's stakeholders? Describe their specific interests.

Through our portfolio, we support both the nutrition & pharmaceutical industry as well as academia and medical centers in the development of new therapy options. Scientists working in this research area may benefit from the information that will be made publicly available via peer-reviewed publications. TNO scientists are stakeholders and benefit from the scientific information and published articles. In the end, patients suffering from any of the cardio- and metabolic disorders mentioned in this proposal will benefit when successful therapies will become available for patients. While TNO is an independent non-profit organization, for individual studies TNO may have a commercial interest/profit as well. In addition, the experimental animals are a stakeholders. They will experience suffering and it is not in their interest to be used in these studies.

3.4 Strategy

3.4.1 Provide an overview of the overall design of the project (strategy). If applicable, describe the different phases in the project, the coherence, the milestones, selection points and decision criteria.

In general:
The development of new interventions such as nutraceuticals 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 (go/no-go decision), leads will be developed that can affect the target (eg, nutritions, inhibitory antibodies, small molecules, RNA therapy) and if this is indeed the case (go/no-go decision after proof of concept study) those leads will be validated (efficacy study will then be performed to evaluate which substance is most potent (go/no-go decision if there is no efficacy), what's the solubility or specificity?). If there is proven efficacy (go/no-go decision), mode of action can be evaluated in mechanistic study. 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.

Majority of our work in preclinical phase

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.

Aim/topic of our studies

The current project describes the research that we conduct using these animal models with the overall 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.

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 neurodegenerative disorders. The coherence between the studies is that all studies are part of metabolic disease and its complications 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 and shared mechanisms (e.g. oxidative stress, mitochondrial dysfunction, chronic inflammation) play a role in the different metabolic disorders.

Type of studies

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 dosing 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. **Approach**
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.

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 (also based on our experience of last 5 years) is that a significant part of the studies will be NASH studies, but 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. We already noticed this during the last 5 years that we have more studies for example with a novel atherosclerosis drug on top of a statin. We expect that this trend of combination therapy will continue in the next 5 years and might become more important in the NASH field as well. For atherosclerosis combination studies, the further reduction of hyperlipidemia makes regression of atherosclerotic plaques possible as well (in addition to further progression). A further shift to combination therapies and regression studies is therefore to be expected. In the case of nephropathy, we expect the contribution to the total number of studies will still be less in the coming years. During the last 5 years we have developed a translational animal model with moderate to advanced renal damage and now aim to further dissect the underlying pathways involved in disease onset and progression. A combination with cardiovascular damage (cardiorenal damage) is foreseen and will be further explored within the next years. Since, we have not been working on retinopathy during the last 5 years, we do not foresee retinopathy studies the next 5 years as well, while brain metabolism and neuroinflammation are a relative new field of research and will be a new growth area, still in a somewhat exploratory phase. In addition, the cross-talk between organs (intestine, fat, muscle, brain and liver), studies evaluating two end-points (e.g. NASH and atherosclerosis), and the research on biomarkers were foreseen to be a growing field of research during the last 5 years and we expect this to be continued/growing the coming 5 years as well.

Study specific designs can vary and depend on the stage of the metabolic disease and the type of metabolic disease.
The basic outline 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 neurodegenerative disorders**.

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, delivery of RNAi via AAV vectors or siRNA).
An example of a typical study design is provided in the appendix.

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 also described in more detail in the appendix.

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. When a sponsor is involved, the choice of animals will be discussed with the sponsor and we will specifically ask the sponsor about their primary and secondary research questions, aimed stage of metabolic disorder/complication, any knowledge on toxicity of compounds, prior experience with mouse studies. As an example, we know the bioavailability of compounds can be different in obese animals/animals with compromised livers or influenced via type of diet. For NASH studies we will specifically ask for prior knowledge such as whether the compounds have been tested in obese animals on a HFD as well. 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 target our working mechanism of the compound to be tested can affect the choice of animal model to be used as well. We will not perform all studies, for instance if a certain target is not available, we will not perform the study or recommend a different animal model. 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. Please note that not all these animal models are used simultaneously, but depending on the specific research question an appropriate choice will be made from this list of models.

Table 1. Animal models per metabolic disorder.

Metabolic disorder	Model	Comments
Obesity & Diabetes type 2	C57BL/6J	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 C57BL/6J, 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.
	MS-NASH mouse	Develops obesity, metabolic syndrome all in presumed presence of an intact leptin pathway. We would like to evaluate the MS-NASH model in comparison with KKA^{y} mice to see if this mouse model perhaps has comparable kidney damage (on both functional and pathological level), but without some of the discomfort issues observed in KKA^{y} mice in long-lasting studies.
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.
	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. Mice develop atherosclerosis in context of insulin resistance and obesity.
	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).
	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) recapitulating disease pathways and processes in humans as well as the metabolome of NASH patients (Morrison et al. Frontiers Physiol, 2018, PMID: 29527177 and Hepatol Communic 2018, PMID 30556039; Martinez-Arranz Hepatology 2022, PMID 35220605). 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 and gut permeability and dysbiosis are also involved (Gart et al. Frontiers Nutr. 2022, PMID: 35782914; Gart et al., Biomedicines 2021, PMID: 34944770; Gart et al., Int J Mol Sci 2019, PMID: 31491949). Both male and female mice develop NASH and fibrosis when put on the high caloric diets. However, males are to be preferred since the male mice are more susceptible to become obese and insulin resistant 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). Furthermore, only male mice develop obesity induced neuroinflammation (Jacobs et al., Nutrients 2019, PMID: 31405127).
	FRG® KO mice	A triple knock-out mouse model (fumarylacetoacetate hydrolase (Fah), recombination-activating gene-2 (Rag-2) and the common gamma chain of the interleukin receptor (Il2rg) have been disrupted/inactivated) in which the liver has been repopulated for 70-90% with human primary hepatocytes, thereby mimicking the human liver.
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.
	MS-NASH mouse	Develops obesity, metabolic syndrome all in presumed presence of an intact leptin pathway. We would like to evaluate the MS-NASH model in comparison with KKA^{y} mice to see if this mouse model perhaps has comparable kidney damage (on both functional and pathological level), but without some of the discomfort issues observed in KKA^{y} mice in long-lasting studies.
Neurodegenerative disease/ neuroinflammation	LDLR-/-.Leiden	This strain of mice develops neuroinflammation as a consequence of the aging process (already on standard chow diet) and when put on a high caloric diet these mice show disturbed cerebral blood flow and elevated blood pressure (Arnoldussen et al., Int J Obes. 2022, PMID: 34716425 and Tengeler & Gart et al, FASEB J 2020, PMID: 32472598). Also, the autonomous nervous system and peripheral nervous system is impacted in these mice (Mohanta et al. Nature 2022, PMID: 35477759).

3.4.2 Provide a justification for the strategy described above.

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. TNO has set up a research program to refine, reduce and replace animal testing.

In CRO projects such as those carried out by TNO, it is difficult to predict in advance what pharmaceutical targets will be tested. Efficacy studies are often part of the preclinical phase of the sponsor and are independent studies for each sponsor (no connection to studies of other sponsors). In general, first a proof-of-concept study will be performed, followed by dose-finding studies, efficacy studies, followed by mode-of-action studies to provide more insight into the underlying mechanisms. TNO can be requested to perform all these studies sequentially for the sponsor or one specific part (for instance efficacy study will be performed at TNO, but dose finding studies have been performed elsewhere).
For model optimalization, studies also follow a fixed pattern: first proof-of-concept study, followed by optimalization studies and validation studies.

In general we always use mouse models in which the animals develop a metabolic disease, intervention is applied and finally the effect of this intervention is determined. Because we use already known models and interventions, the cumulative inconvenience for each experiment can be estimated well in advance and will never be more than moderate. All genetic modified models described in this application do not have intrinsic discomfort.

In the strategy we will strive to first obtain as much insight as possible with regard to target engagement, for example, by means of in vitro/ex vivo techniques. Only when in vivo models are unavoidable, animal studies will be started.

The strategy described above allows us to answer key questions such as:
- Does the intervention work on prevention of the studied metabolic disease
- What are the mechanisms
- Are there any side effects

3.4.3 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	