Centrale Commissie Dierproeven

Appendix
Description animal procedures

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

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	List the serial number and type of animal procedure. *Use the serial numbers provided in Section 3.4.4 of the Project Proposal form.*	Serial number: 3.4.4.2 Type of animal procedure: Long-term metabolic health consequences of early life treatments

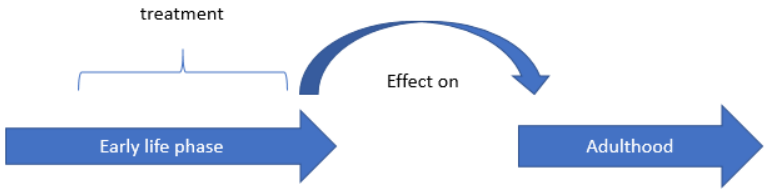
2 Description of animal procedures

A. Experimental approach and primary outcome parameters

Describe the general design of the animal procedures in relation to the primary outcome parameters. Justify the choice of these parameters.

The **general design** of studies that evaluate the long-term consequences of early life treatments for metabolic health in adulthood is shown in the figure below.

Typical design of appendix 2 study



During growth and development in early life mice are kept on a control diet with well-defined composition (e.g. a chow diet, or a low fat diet, AIN (American Institute of Nutrition) diet or any other diet). The control group receives just this diet, the treatment groups receive this diet plus early-life interventions. Early-life interventions are typically administered via the diet (e.g. infant nutrition; complex childhood foods; fibres) or orally (e.g. probiotics; milk oligosaccharides; milk fat glomerular membrane; antibiotics) and are often of interest for the growth and development of young children in terms of organ development, brain function and metabolic health. Depending on the research question, these interventions are administered to mice during a dedicated period within the early-life phase. The

interventions are thus, for example, administered between the time point of weaning (i.e. ~4 weeks after birth) and the time point at which most hormones and endocrine factors have stabilized in plasma (i.e. ~12 weeks after birth). The exact length of the intervention period depends on the research question and the nature of the intervention under investigation. Early life interventions can constitute macronutrients (e.g. casein protein or protein hydrolysates); dietary supplements (polyphenols, polyunsaturated fatty acids such as DHA); exercise (running wheel); prebiotics and fibres; probiotics; human milk oligosaccharides; short chain fatty acids; antibiotics or other pharmaceuticals with potential impact on metabolic health in later life) (e.g. Arnoldussen et al., J Biochem Nutr., 2016; Schoemaker et al., PLoS ONE 2017; Arnoldussen et al., Int J Obes., 2016). While some of the interventions are considered to have an persistent programming effect on DNA methylation and genes (e.g. polyunsaturated fatty acids like DHA) or on microflora (e.g. antibiotics), the mechanism of other interventions requires that they are continued until adulthood (e.g. exercise; prebiotics; short chain fatty acids; milk oligosaccharides) to reduce risk of metabolic diseases.
To induce metabolic overload and diseases of the gut-liver-brain axis and to assess the health effect of early life interventions for adulthood, mice will be treated with an energy-dense diet (e.g. high fat diet or high carbohydrate diet). The start of this metabolic challenge with an unhealthy diet depends on the exact research question and can either be already within early-life (i.e. mimicking children that are exposed to an unhealthy environment provided by their parents) or in early adulthood (e.g. from 12 weeks onwards; thus mimicking young adolescents/young adults with unhealthy lifestyle and food habits). The mice will be fed energy-dense diets until metabolic diseases along the gut-liver-brain axis have developed. With a 24% w/w high fat diet that is comparable to diets consumed in Finland or Greece, the development of metabolic diseases requires about 24-32 weeks. We typically use diets that have a macronutrient composition which is comparable to (unhealthy) diets of humans. As in humans, metabolic disease and cognitive impairment develop slowly on such diets (because they are not extreme). The **primary outcome parameters** (metabolic diseases) are non-alcoholic fatty liver disease (NAFLD) in liver, atherosclerosis in the vessels/heart, and cognitive dysfunction in brain. These diseases are interlinked and characterized by abnormal fat depositions and chronic inflammation within these organs. Typically, inflammation in adipose tissue and gut leakage are analyzed as secondary parameters because they constitutes potential drivers of pathogenesis.
The primary endpoints are chosen because they represent the most important causes of loss of quality of life (e.g. NAFLD and brain dysfunction) and the major cause of death (atherosclerosis in vessels of the heart and brain leads to myocardial infarction and stroke, respectively). A health effect on any of these primary outcome parameters is of high socio-economic importance. Furthermore, the identification of predictive biomarkers that circulate in blood and inform on the risk of future disease and development of a primary endpoint can be a research topic of some of our studies.

Choice of energy-dense diet and duration of treatment: The diets being used will depend on the exact research question(s) of the study: in general energy dense diets can be obtained with macronutrients from multiple sources (e.g. energy from milk fat is important for children in the Netherlands; energy from vegetable fat is of importance for children receiving infant formulations instead of breast milk; energy from carbohydrate-rich foods and drinks is relevant for children of low socio economic classes or in ethnic groups that consume a lot of sugar).
The duration of the exposure to the energy-dense diet depends on the type of diet being used, the sensitivity of a mouse strain towards this diet, and the desired stage of metabolic disease of interest (severity of the metabolic phenotype). For instance, in Ldlr-/-.Leiden mice, overweight develops after 6-10 weeks on a 24% w/w high fat diet (HFD), ectopic fat deposition in liver (steatosis) requires about 12-14 weeks of a HFD treatment, the development of fatty-inflamed liver (NASH) takes 24-30 weeks on a HFD, first structural brain changes are observed after about 12 weeks on HFD and it requires about 24-32 weeks to develop cognitive impairment on a HFD.
Importantly, the specific combination of animal model and dietary challenge (e.g. whether the calories are from carbohydrates or from fat) determines the speed and characteristics of the metabolic phenotype that will predominantly be induced as well as the biochemical pathways along the metabolic chain that will be challenged.

Typical study groups of Appendix 2 studies: A study can include the following groups:
1. Healthy reference diet group (baseline control): no metabolic overload. Mice are fed a low fat or low carbohydrate chow diet in early life phase and also in adulthood and serve as baseline control.
2. Negative control group: A metabolic disease phenotype induced by treatment with an energy-dense high caloric diet, and there is no intervention. The energy-dense diet is fed until adulthood.
Also an additional negative control group can be added that reflects the situation in early life at which the energy-dense diet treatment is started. Or an additional control group may be added prior to start of an intervention to investigate: 1) whether an intervention has a health effect relative to the situation at which it was started; or 2) whether an intervention can reverse a dysfunctional programming.
3. Positive control group or reference group: A metabolic disease phenotype along the gut-liver-brain axis is induced with energy-dense diets as in group 2, but mice receive also an early-life intervention with known beneficial (positive control) or well-established (reference control) health effect (e.g. a combination of the polyunsaturated fatty acids ARA/DHA as in Wielinga et al., Mol Food Res, 2012; Arnoldussen et al., J Nutr.Biochem. 2016). A positive control is, for instance, also necessary to validate newly identified biomarkers that may inform on a primary or secondary endpoint because the positive control should modulate this biomarker in the same way as it modulates the endpoint.
4. Treatment groups: A metabolic disease phenotype is induced with energy-dense diets as in group 3., but mice receive early-life treatments that have an unknown health effect (for instance, a new supplement that is tested for an infant nutrition formulation as reported Schoemaker et al., PloS ONE, 2017).

Extra groups may be added: for instance to obtain kinetical information if an additional research question is to obtain in-depth dynamical information about the treatments.
Furthermore, it can also be decided to first perform a pilot experiment if basic information is missing (for example, it may be necessary to first perform a dose-finding pilot study in order to establish an optimal dose of a treatment).

Besides directly testing the efficacy of prevention and intervention treatments (e.g. by lifestyle, nutrition, supplements, and pharmaceutical), also more basic research studies are performed aiming at understanding the sequence of events or underlying mechanisms in the early life period. For understanding the development of early-life programming events, time-course studies might be performed in which groups of animals are compared after increasing periods of time on a healthy (group 1.) versus an metabolic overload-inducing diet (group 2.). Such longitudinal studies enable us to unravel the biochemical mechanisms that precede a particular histological phenomenon of organ development or a particular dysfunction of an organ (for instance whether microbiota changes precede or follow the development of fatty liver disease in order to investigate whether changes in gut bacteria are cause or consequence).
Also animal studies can be performed that further improve the translational character of existing models, or compare the existing models to specific human childhood subtypes.

For all studies, it is very important that models are used that reflect the biochemical and metabolic-physiological processes of humans as much as possible. We are continuously aiming at validating our *in vivo* models by comparison with human studies and we are actively involved in human studies (ranging from biobanking initiatives of human tissue after obduction or after surgery to clinical metabolism studies with specific human subtypes (young vs old subjects) to bariatric surgery studies on weight loss to children cohort profiling by metabolomics). We use human tissues to validate mouse models and, for instance, to compare biomarker profiles in plasma (e.g. to assess whether the same biomarkers can be used in humans and mice). Typical comparative studies include: Liang et al. Establishment of a general NAFLD scoring system for rodent models and comparison to human liver pathology. PLoS ONE, 2014; van de Steeg et al., Combined analysis of pharmacokinetic and efficacy data of preclinical studies with statins markedly improves translation of drug efficacy to human trials. J Pharmacol Exp Ther. 2013; Liang et al., Metabolically induced liver inflammation leads to NASH and differs from LPS- or IL-1β-induced chronic inflammation. Lab Invest, 2014. An improvement of the animal model could be for instance a change in the composition of the energy-rich diet being used to induce the metabolic overload and mechanism-based evidence why a particular mouse strain or diet is translational to humans so that it can become broadly accepted in academia/industry.

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

General: Body weight and food intake will be monitored regularly and blood/plasma measurements will be performed to analyse circulating biomarkers of health and risk factors for metabolic dysfunction. At the end of each study, tissues will be collected for analysis of primary outcome parameters as explained above, viz. metabolic disease endpoints which can be assessed in liver (e.g.non-alcoholic fatty liver disease) or vasculature (e.g. atherosclerosis) or in brain (impaired function). Typically, histopathological analysis of other organs along the metabolic chain (e.g. gut and vasculature as secondary endpoints) can be performed because of their anatomic and physiologic connection to the primary endpoints. It is an intrinsic feature of metabolic disease that several organs become dysfunctional simultaneously, often requiring the analysis of a primary endpoint in context of secondary endpoints to understand the causes and mechanisms, and to develop effective interventions.

Detailed description of **animal procedures**:
For all studies applies that treatments (with foods, nutrients, dietary supplements and pharmaceutical small molecules) can have different administration routes:
- Oral administration via diet admix of via drinking water (most common form; expected to cover 80% of all early-life studies)
- Oral administration (p.o.) by gavage (15% of all studies)
- Intraperitoneal injection (i.p.) (5% of all studies)
- Subcutaneous injection (s.c.) (very unlikely)

During the study mice will be checked daily and body weight and food intake will be monitored regularly by weighing the mice individually and weighing food per cage.

During the study at several time-points blood samples will be taken to measure health markers in plasma. Blood samples can be taken:
- via tail vein after 4-5 hours fasting
- via tail vein after overnight fasting (in particular cases it may be necessary to collect blood after prolonged fasting, e.g. when ketone bodies or free fatty acids or other markers of fasting are measured or if blood samples that do not contain chylomicrons or any food derived components are needed).
- via tail vein non-fasted (in particular cases it may be necessary to collect non-fasted blood, e.g. after postprandial challenge tests after meal or to assess intestinal absorption of a food component, metabolite, gut integrity marker or nutrient).
Maximum volume / frequency to be used are according to what is considered good practice (Diehl *et al.*, 2001).

At the end of the experiment mice will be euthanized, and plasma and different tissues along the gut-liver-brain axis will be collected.

Depending on the research question, the following procedures and metabolism-tests can be performed to assess the long-term consequences for metabolic health and potential development of metabolic diseases:

Dietary and lifestyle interventions

Drinking water interventions
- Sugar-rich drinks are considered to induce obesity. Fructose in drinking water and other carbohydrate-containing liquids can stimulate VLDL production, and may result in steatosis of the liver as well as excess lipid accumulation in adipose tissue (obesity).
- Blood pressure is elevated in many obese children. This condition can be mimicked by administering a vasoconstrictor in drinking water. L-NAME and L-NNA (N (G) -nitro-L-arginine methyl ester) are safe NOS inhibitors that increases the blood pressure. In blood vessels endogenously formed NO relaxes the

smooth muscle cells which results in vasodilatation and in this way, it can reduce blood pressure. NO can be made from endogenous L-arginine and oxygen by means of various nitric oxide synthase (NOS) enzymes.

Alternate diet interventions
A generic loss of adipose tissue mass (unspecific and not limited to a particular depot) can be achieved with restricted or alternate diet feeding (switch from high fat diet to chow/low fat diet). This type of dietary challenge is a specific form of lifestyle intervention (which can be implemented at home and for children) to attenuate metabolic disease risk. This fat-loss treatment may be applicable in some of the studies, alone or in combination with other therapies (nutritional and pharmaceutical).

Exercise and training interventions
Generic weight loss may also be achieved with voluntary exercise (e.g. running wheels) as it is commonly advised in humans to strengthen muscles and burn excess energy/calories through intensified training. In some of our studies these generic lifestyle interventions can be employed and the calories burnt will be determined (e.g. via indirect calorimetry, analysis of the resultant of exercise in the form of muscle/grip strength).

Non-invasive readouts of metabolic health

During a study, metabolic health parameters are measured (in body fluids and excretions like urine or feces). Depending on the primary question related to the gut-liver-brain axis, a combination of readouts from the list below will be evaluated to answer the question most optimally. For each study, the overall number and combination of different procedures will be considered and the cumulative discomfort will not exceed above moderate.

Feces collection
For measurements of gut metabolites, to calculate an energy balance or perform a microbiota analysis.
- This involves collecting feces after several days from the cage bed (on group level) or after lifting mice (individual feces collection)
- This involves taking rectal swabs essentially as done in humans using small device (‘mini-wattenstaaf’).

Urine sampling (for measurements of metabolites reflecting healthy homeostasis and biomarker finding)
- Spontaneous excretion caused by lifting the mouse and collection of urine. If the mouse does not lose urine spontaneously, light bladder massage can be applied.
- Urine sampling overnight (for detailed measurement of excretion of proteins and metabolites). Mice will be housed individually in an excretion collection cage overnight (16 hours). Urine and feces can be sampled.

Non-invasive imaging measurements
- Body composition (fat, water and lean body mass) can be measured via placement of mice in Echo MRI (about 1-3 minutes). This does not require any anesthesia.
- Other imaging methods relevant for metabolic health assessment such as magnetic resonance imaging (MRI) and fluorescence imaging for instance could be used for more precise measurement of abdominal fat, ectopic fat or detection of early disease-related morphological organ changes, by imaging of reflected light or changes of electromagnetic fields under appropriate anesthesia.
- Functional fMRI analysis for non-invasive analysis of iron-ions using magnetic fields (Arnoldussen et al., J Nutr Biochem, 2016; Arnoldussen et al., Int J Obes., 2017), i.e. essentially the same magnetic scanners that is used for children and adults and applying the same working principle: analysis of the naturally circulating hemoglobin (via its iron) thereby detecting blood flow and allowing the analysis of cerebral flow, connectivity between brain areas non-invasively and using appropriate anesthesia.
- Indirect calorimetry using the TSE system. The mice are temporarily housed individually (max. one week) in sealed cages, which are comparable in terms of bedding and size with the normal housing cages. There is a constant air flow through the cage. The air goes into the cage and the air cage again is measured by VO_2 and VCO_2. From this the energy consumption and the oxidation of the substrate can be

measured as a measure of metabolic performance. In addition, the continuous activity is measured by infra-red light streams around the cage.
- Other innovative non-invasive imaging methods relevant for metabolic health assessment such as OCT, Raman and fluorescence imaging for instance could be used for more precise measurement of blood vessel function, detection of metabolic dysfunction-related morphological changes in the non-invasively accessible eye vasculature using retinal imaging (for example by imaging of reflected light by OCT or vascular leakage under appropriate anesthesia).

Blood pressure measurement
Carried out using non-invasive tail cuff measurement, in a restrainer without anesthesia.

Grip strength and inverted screen test (to an integral measure of overall metabolic health, and specifically muscle functioning)
Muscles with impaired metabolic supply of energy via gut and liver have reduced strength and may constitute a non-invasive secondary readout of health. Grip strength will be determined by placing mice with two or four limbs on a grid attached to a force gauge and steadily pulling the mice by their tails. Grip strength is defined as the maximum strength produced by the mouse before releasing the grid. On each occasion five trials will be performed for each mouse with a 1-minute resting period between the trials.
Inverted screen test will be performed by placing the mouse in the center of a wire mesh screen and then rotating the screen to an inverted position within 2 seconds. The time when the mouse lets go of the grid is noted or the mouse is removed when the predefined criterion time (60-120) seconds is reached. If a mouse lets go of the grid within 10 seconds, another trial will be performed with a maximum of three trials.

Dynamic tests of phenotypic metabolic flexibility

A) Challenge tests (frequently employed):

Challenge tests to assess whole-body phenotypic flexibility (a measure of metabolic resilience and plasticity)
- Administration of a metabolic-inflammatory stressor which is frequently a dietary challenge (i.a. a bolus of dietary fat, carbohydrate, dextran, cholesterol) to assess the metabolic and inflammatory response after set time points up to 7 days.
- Challenge tests to measure gut integrity and to detect early-life permeability (by administration of FITC-dextran (90% of cases), and ^{14}C-PEG200 or heat-inactivated bacteria (10% of cases for specific questions).
Blood samples can be taken before and after the challenge and may constitute one or more blood samples, depending on the required resolution of dynamics.

B) Metabolic flexibility *test (s*ometimes required):

Glucose tolerance or lipid tolerance test (a specific form of challenge test for assessment of energy handling and insulin sensitivity)
After 4-5 hours or overnight fasting a first blood sample is taken via tail vein (t=0 minutes). Thereafter, the glucose/lipid tolerance test will be started essentially as performed in humans. For this purpose, the mice receive a bolus of glucose/lipids via oral gavage or via ip injection, after which small blood samples will be taken within a period of maximally 6 hours according to a collection scheme (e.g. at 5, 15, 30, 45, 60, 120 and 240 minutes after the bolus) for the measurement of blood glucose and optional plasma insulin.

Insulin tolerance test (a specific form of challenge test for assessment of insulin clearance)
After 4-5 hours or overnight fasting a first blood sample is taken via tail vein (t=0 minutes). Thereafter, the insulin tolerance test will be started. For this purpose, the mice receive an insulin bolus (via ip injection), after which small blood samples will be taken after set time points (typically within 4 hours) for the measurement of plasma insulin (and optional blood/plasma glucose).

Hyperinsulinemic euglycemic clamp (measurement of insulin sensitivity of organs; non-recovery)

Mice are fasted overnight, and then anesthetized with appropriate anesthesia. During this clamp procedure insulin will be administered via a primed continuous intravenous infusion for 2 to 3 hours to attain steady-state circulating insulin levels. A variable glucose infusion will also be started and adjusted to maintain euglycemia, measured at 10-minutes intervals via tail bleeding. Both hepatic and peripheral insulin sensitivity as well as tissue-specific insulin sensitivity (in heart, fat, and muscle) can be determined (using a ^{3}H-glucose label to measure peripheral and hepatic insulin resistance, and in addition, ^{14}C-deoxyglucose label for assessment of tissue-specific insulin resistance). Blood samples will be taken during the procedure and at the end mice will be sacrificed via cervical dislocation and heart puncture will be performed and tissues will be collected.

Hyperglycemic clamp (measurement of insulin secretion; non-recovery)
Mice are fasted overnight, and then anesthetized with appropriate anesthesia. During this clamp procedure glucose will be administered via a primed continuous intravenous infusion for 2 to 3 hours to attain hyperglycemia. Blood samples will be taken at defined time points (e.g. 0, 2, 5, 10, 15, 40, 50 and 60 minutes) for measurement of plasma insulin and at the end mice will be sacrificed via cervical dislocation and heart puncture will be performed and tissues will be collected.

Molecular readouts of metabolic health
Deuterated water administration (D_2O) (allows to assess dynamics of a molecular conversion over the period in which mice receive deuterated water which behaves as regular water but has the advantage that it can be distinguished and thus analyzed with high-tech technologies).
To be able to trace newly formed proteins (lipids for instance) within a given period, D_2O can be given for a short period (number of days) or long (several weeks) period in our studies. Labeling can take place at various times of the study and depends on the specific question. D_2O will be built into all the newly synthesized proteins and in this way newly formed protein can be traced. On first day, the mice receive a single i.p. injection with body warm D_2O 100% / 0.9% NaCl to label the body water around 2-5% of the mouse. Then the D_2O body water levels will be maintained by adding D_2O in the drinking water (containing 4-8% D_2O) until sacrifice.

VLDL production (a measurement of liver health, i.e. specifically the ability to form VLDL particles which supply the other organs with lipids and nutrients; non-recovery)
Mice are fasted for 4 hours, and then anesthetized with appropriate injection anesthesia (i.p.). Under anesthesia Tran ^{35}S-label is injected intravenously in the tail for measurement of the apoB novo synthesis and 30 minutes thereafter the mice are injected with Triton WR 1339 (iv, tail) for complete blocking of VLDL clearance. At set time points (e.g. typically at 0, 15, 30, 60 and 90 minutes after Triton injection), a blood sample is taken via the tail for measurement of plasma triglycerides (VLDL production measurement). After 90 minutes, the mice are sacrificed via cervical dislocation and the remaining blood is collected via cardiac puncture for VLDL isolation and determination of the ApoB novo synthesis and lipid composition VLDL. Different tissues can be isolated as well.

VLDL clearance (a measurement of liver health, i.e. specifically of VLDL clearance, i.e. the ability of the liver to remove particles from the circulation for energy conversion; non-recovery)
For this purpose, mice are anesthetized intraperitoneally with appropriate injection anesthesia. Mice will receive VLDL-like particles labeled with ^{3}H-triolein and ^{14}C-cholesteryl oleate via an injection into the tail. At set time points (e.g. typically after 2, 5, 10 and 15 minutes), a blood sample is taken via the tail. The mice are sacrificed by cervical dislocation and blood is collected at the end of the experiment by means of a heart puncture and different tissues are isolated as well. ^{3}H and ^{14}C activity is measured in tissues and plasma samples.

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

All studies will require quantitative (and not qualitative) data as readouts. To estimate the required number of animals for an experiment, we use the effect size on basis of published data in the literature

or from our own historical study database which is based on several decennia of experience with similar types of experiments, or from pilot studies). We estimate the sample size needed to achieve the desired power (usually between 0.8-0.9), considering whether the endpoint is normally distributed or not, and using appropriate statistical tests (e.g. a t-test with a $p<0.05$ for comparison of two normally distributed groups).
As an indication: n=15-20 mice/group has been used for early life studies in the past. This number is required because physiological set points are not defined in early life resulting in a high variability because the rate of metabolic processes that determine the 'normal values' of plasma parameters will be defined in the early life (which is the subject of this project). Furthermore, and intrinsic to early-life studies, it is often not possible to identify low-responders to diet or biological outliers with the usual criteria (because lipids or glucose or other biochemical biomarkers as well as hormones are not adjusted yet). For instance, if a treatment starts before seventh week after birth, no pre-selection can be made. As animals develop further, plasma levels stabilize more and more, hence allowing identification of unusual subjects in early-life studies with focus on the later stages of development.

Another means to minimise the number of animals is to combine studies to share control groups or reference groups. Where possible we will combine experiments and study groups, so that for instance different treatment groups can share the same control groups.

B. The animals

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

Mice from the LDLR-/-.Leiden, ApoE*3Leiden or ApoE*3Leiden.CETP mice (on C57BL/6J background) of the breeding stocks at our facility will be used, preferably littermates. Early life studies start with animals that have an age of about 4-6 weeks (first weeks after weaning).
The final choice of animal model to be used for a study depends on the primary research question and the group of human subjects at risk that should be mimicked in first place. We anticipate to use two mouse strains predominantly because we already established the (non-invasive) technology platforms and basic experimental conditions for Ldlr-/-.Leiden mice (Arnoldussen et al., Int J Obes.,2017 and unpublished data from an ongoing EFRO consortium) and ApoE*3Leiden mice (Arnoldussen et al., J Nutr.Biochem. 2016) in previous years. As reported in these studies, these strains of mice are predisposed to develop features of metabolic dysfunction in humans (including cognitive dysfunction). LDLr-/-.Leiden mice lack a receptor required for lipid clearance as subjects with humans with familial dyslipidemia and ApoE*Leiden mice express a dysfunctional human APOE3 protein that prevents clearance of lipids representing another group of humans with predisposition to disease. Thus, through these genetic modulations the mice mimic specific and relevant human phenotypes each having an increased risk of metabolic dysfunction and disease. Since there is only little known about early-life programming in general, there is no specific scientific preference for either of these models.
Gender of the mice to be used: Male mice are typically used for diet-induced obesity studies because they are generally more sensitive to develop obesity on high caloric diets, and a recent study indeed showed that i) the gene expression response of male and female mice is very different (with only 10% of genes overlapping between the sexes) and ii) exclusively male pups respond to Western-style diet with increased weight gain (Mischke et al., Maternal Western-style high fat diet induces sex-specific physiological and molecular changes in two-week-old mouse offspring, PLoS One. 2013). Within an EU-project, we are currently performing experiments together with infant nutrition companies and several academic groups to establish the optimal experimental conditions to study obesity-associated cognitive dysfunction. In this program, we compare for the first time female and male LDLR-/-.Leiden mice and perform cognition and brain function experiments in the context of pronounced early life obesity. First results show that male mice develop brain dysfunction in response to high caloric diets whereas females do not. This may be related to the observed modest weight gain in females which became much less obese than male littermates. Altogether, these findings indicate that only males are suitable for use of gut-liver-brain studies as described herein. The female mice that become available after breeding are typically used for atherosclerosis studies because they develop high cholesterol levels upon Western type diet feeding.

Strain of mice to be used: We expect that 60% of the experiments will be conducted in male Ldlr-/-.Leiden mice, and 35% will be performed in male ApoE*3Leiden mice for which several brain function assays were established in context of modest overweight. (As mentioned above, female mice of both strains are typically used for atherosclerosis experiments.) Male ApoE*3Leiden.CETP mice will be used instead of the ApoE*3Leiden if HDL metabolism via the CETP enzyme is an integral part of the research question on early metabolic programming.
Incidentally, (5% of cases), if very basic epigenetic or metabolism questions are investigated (e.g. effect of specific food or nutrient on DNA methylation of specific genes), wildtype mice (from a commercial provider) with C57BL/6J background can be used on basis of a recent study of us specifically focussing on gene imprinting effects (e.g. Zwamborn et al., Nature Scientific Reports, 2017).

The required number of mice will be estimated to be 1.600 for a period of 5 years. This is based on an expected number of early-life studies of approx. 4 per year (based on historical data over the last years). The average study requires on average 80 mice because a study typically consist of 4 groups of 15-20 mice each. (n=1600 mice:= 4 studies x n=80 animals x 5 years).

C. Re-use

Will the animals be re-used?

X No, continue with question D.

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

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

X No

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

D. Replacement, reduction, refinement

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

Replacement: We continuously strive to replace animal studies with validated alternative methods, preferably using human tissue or human cell lines. We have replaced specific type of animal studies (namely certain dose optimization studies) by studies in humans using an novel analytical Accelerated Mass Spectrometry (AMS) approach that allows us to optimize drug dosing using very low quantities of radioactive label (comparable to the radioactive exposure during an intercontinental flight). In principle this AMS technology can also employed in children for dose-optimization (since drugs are typically developed for adults and doses for children rely on estimations rather than hard data). Thus, dose-optimization studies can be conducted in humans including children. However, AMS technology does not allow to test drug efficacy (because very low drug doses that do not have a physiological effect are used) and hence allow only determination of pharmacokinetical and pharmacodynamical data (e.g. clearance profiles, half-life in plasma etc).
Furthermore, we use in vitro systems and human biopsy assays if possible (typically if a very specific process has already been identified and has to be studied in more detail and when the research concerns 1 isolated organ. (Validated models for studying organ-organ interactions under physiologically meaningful conditions are not yet available.) TNO has recently launched a program Organ-on-a-Chip (see also Organ-on-a-Chip whitepaper: http://publications.tno.nl/publication/34622299/rkPoAJ/grootaers-2016-three.pdf) that aims at mimicking some organ functions and eventually combining them via a fluidic system and we are involved as partner in international consortia. The available tools do currently not mimic the organ-organ interactions and the elementary features of metabolic disease (e.g. ectopic fat accumulation, vascular dysfunction) and are still in their infancy.
Possible studies using human tissues comprise the identification of key hallmarks of disease and the identification of molecular pathways that characterize a particular patient subtype. Knowledge on these subtypes allows us to select and establish model conditions that mimic human subtypes. We have access to human biopsy material (e.g. liver, adipose tissue, muscle tissue but also human plasma and microbiota) because of the human studies we are involved in and the extensive collaborations with

hospitals and doctors. However, fresh human tissue is very scarce and often not fresh enough to quantify sensitive molecules with rapid turn-over or short half-life such as mRNAs, cytokines and hormones, bioactive lipids (see also Vital tissue whitepaper: http://mailing.tno.nl/ct/m8/k1/lG9BLvQKYu1MheK9DpQtFAFy3yeEoutjgfEr6Gj5ucJVVT1LUJPXZ6kSA7n8Q0sK4vv7T0WuHMUTxdHEBnJhNg/ZD8yND3CLIt58HW).
One of the main difficulties with human material is that it is obtained from unhealthy subjects that often are treated with multiple drugs. Healthy reference tissue is hardly available (e.g. from car accidents) but intrinsic to this, the tissue is not rapidly collected and frozen and often also disintegrated as a consequence of the mechanical impact of accidents. The tissue of subjects with disease pose another challenge to research because the biological pathways and processes are reflecting the multi-drug therapies (e.g. a lot of the tissue comes from cancer patients which receive multiple drugs at high doses) and/or the anesthesia which overshadow all physiological processes.
When we have discovered ways to replace animal testing, we use them ourselves and encourage others to apply these alternative tests. Non-testing techniques, such as computer modelling, are continually being developed and improved but also they rely on information of human tissue with its limitations as described above. We are currently launching an initiative together with academic partners and bariatric surgery groups to fully profile subjects including the tissues that can be collected during bariatric surgery. Although it is merely reflecting a specific group of subjects (namely very obese people), it is a first approach to generate a comprehensive data set of tissue, plasma, microbiota, and even brain function measurements and this data may be used to develop and train computer models.
Altogether, we believe that we contribute to the establishment of in vitro and in silico models that allow to replace testing and we are actively complying with the 3Rs (replacement, reduction and refinement) principles by keeping the animal numbers to a minimum.
The animal studies proposed herein are currently unavoidable to study complex early life organ-organ interactions that are intrinsic to all metabolic processes and metabolic pathologies such as cardiovascular disease, hepatic steatosis and cognitive decline. Early life growth and development processes are very complicated, multifactorial process in which multiple organs act in concert (gut, liver, adipose tissue, muscle, vascular system) to provide energy from food and distribute it thereby ensuring healthy growth and development. Hence, given the complexity of the energy (glucose and lipid) metabolism and metabolic health in general, the prediction of effects of treatments or interventions rely on an entire physiological organism.
Reduction: TNO aims to reduce the number of animals and has developed statistical protocols to optimize group sizes. Furthermore, we regularly review our testing models and methods to implement integrated testing strategies. This helps us to determine whether animal testing is needed or whether the same information can be obtained in other ways. Data simulations are performed to determine an optimal study design that will provide the most valuable information with the smallest number of animals in each experiment resulting in a minimum number of animals required. Experiments will be performed in a logical order and, so that decisions for the next steps are taken on basis of previous results. When possible different study groups from different studies will be combined, so that they can share the same control groups which results in a reduction of the number of animals required for control groups.
Refinement: We continuously aim to develop, validate and adapt (preferentially non-invasive) test methods in such a way that the test animals are exposed to as little discomfort and stress as possible (for instance, the use of echoMRI as a non-invasive method to measure body composition within one animal at multiple time-point). All animal handlings will be carried out by authorized and qualified persons. Where possible, all animals will be group housed and environmental enrichment will be provided as it is set forward in the respective regulations on animal housing.

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

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

Repetition and duplication

E. Repetition

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

Not applicable (alleen voor wetgevingsgereguleerd onderzoek).

Accommodation and care

F. Accommodation and care

Is the housing and care of the animals used in experimental procedures not in accordance with Annex III of the Directive 2010/63/EU?

X No

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

G. Location where the animals procedures are performed

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

X No > Continue with question H.

☐ Yes > Describe this establishment.

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

Classification of discomfort/humane endpoints

H. Pain and pain relief

Will the animals experience pain during or after the procedures?

☐ No > Continue with question I.

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

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

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

For procedures that require non-moving animals (e.g. fMRI, retinal imaging) appropriate anesthesia / analgesia will be used and some procedures (e.g. VLDL production) will also be terminal. In consultation with the attending veterinarian, surgery protocols, including appropriate anaesthesia and analgesia is determined and frequently reviewed and updated towards best practices.

I. Other aspects compromising the welfare of the animals

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

The treatment with energy-dense diets that reflect the composition of human diets (e.g. high caloric high fat or high carbohydrate diet) is typically not associated with marked discomfort; on the contrary, mice prefer these feeds above regular chow. The development of overweight/obesity or early symptoms of metabolic disease in early life like dyslipidemia, fatty liver, early vascular changes or cognitive impairment is also not associated with marked discomfort, and animals typically behave normal, look normal, move normal and eat and drink normally.

The assessment of early-life treatments on the development of metabolic disorders later in life like pronounced obesity, vascular disease and non-alcoholic fatty liver disease (NAFLD/NASH) or cognitive impairment do not lead to discomfort. Similarly as in humans, these metabolic-inflammatory disorders remain often unnoticed because of the overcapacity of most metabolic organs, and most subjects are often unaware of any metabolic disturbances until a very late stage (e.g. cancer or cardiovascular event, both of which is not reached in the experiments described herein). In all of our studies we will thus not reach any severe form of cancer or diabetes which can be accompanied by discomfort to the mice.
With respect to discomfort caused by interventions: most of the treatments are food or nutrition related interventions with potency to be applied in children (in infant nutrition) and we do not expect that these cause discomfort. It is possible that novel compounds (e.g. novel antibiotic or probiotica) or micronutrients may have an adverse effect on the early life growth and development endpoints analysed which is why these studies are conducted (i.e. to test whether a product can be applied to humans in a clinical trial or a study in infants/children).

Explain why these effects may emerge.

Although most novel compounds and food constituents to be tested are expected to have a beneficial effect, we cannot exclude the possibility that the combination of the intervention with the diet and the specific subtype of human mimicked by our models can lead to unexpected adverse effects. The likelihood (based on historical data over the last 5 years) is low and <0.1%.

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

Upon start of treatment/intervention mice will be closely monitored so that early signs of discomfort or potential adverse effects are noticed rapidly. The situation will then be discussed with the animal welfare officer or veterinarian and if possible adequate measures will be taken to relief animal discomfort or animals will be taken out of experiment and sacrificed. For instance, it may happen that an animal spontaneously develops a carcinoma or an organ abnormality ('water-nier') requiring a humane endpoint.

J. Humane endpoints

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

☐ No > Continue with question K.

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

In general the induction of the metabolic dysfunctions(s) in early life studies does not lead to animal discomfort, and cases of animal discomfort are rare as described above. Mice will be monitored daily and in case of discomfort, cages will be labeled and the affected mice will be more closely monitored to observe whether the health status is improving or deteriorating. Deterioration of the health status with unexpected weight loss, severe wounds and/or signs of general sickness and/or discomfort (eating habbits) will lead to the decision that a mouse will be euthanized. This decision will be taken together with an animal welfare officer or a veterinarian.

Indicate the likely incidence.

For early life studies: <2%.

K. Classification of severity of procedures

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

In general, the cumulative discomfort for early life studies will be mild to moderate and will not go beyond moderate. The different procedures that can be used, will all be within good practice and are assigned to the following categories:

Animal procedure:	**Level of discomfort:**
Diet interventions	mild
Drinking water interventions	mild
D_2O drinking water	mild
Voluntary exercise (running-wheel)	geen
Grip strength and inverted screen test	mild
4 or 5 hours fasting	mild
Overnight fasting	moderate

Multiple blood sampling via tail vein within good practice	mild - moderate*
Single or multiple gavage or injections (iv, ip, im, sc)	Mild - moderate**
Urine collection	mild - moderate***
Feces collection or fecal swabs	mild
Indirect calorimetry (TSE systems)	moderate
EchoMRI	mild
Non-invasive imaging (fMRI, retinal imaging)	mild – moderate****
Challenge tests	mild - moderate****
Glucose tolerance test	moderate
Insulin tolerance test	moderate
Blood pressure measurements	mild - moderate****
Hyperinsulinemic euglycemic clamps	non-recovery
Hyperglycemic clamp	non-recovery
VLDL production test	non-recovery
VLDL clearance test	non-recovery
Euthanasia	mild
*mild if sampling is bi-weekly or monthly and in total less than 6 blood collections.	
**mild if no more than 2 times per week and no longer than a total of 8 weeks.	
*** mild if spot urine collection and moderate if individual housing (16 h) is necessary to collect urine.	
**** mild if only once in a study	

End of experiment

L. Method of killing

Will the animals be killed during or after the procedures?

☐ No

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

The biochemical processes of early life metabolic programming manifest within tissues and cannot be analyzed only via plasma or observational and non-invasive measurements. To be able to investigate the mechanisms of early life programming and the molecules involved therein, tissues are collected and analysed (e.g. with various methods ranging from histology to gene expression analyses to DNA methylation analyses). The identification of biomarkers that could be used in the future as a marker of proper metabolic programming is subject of this research.

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

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

X Yes