

MODELS FOR CARDIOVASCULAR AND METABOLIC DISEASES



TNO innovation
for life

Metabolic health is the ability to adapt and to successfully cope with metabolic overload which is a consequence of modern lifestyle and food consumption.

Continuous metabolic overload leads to dyslipidemia, insulin resistance and metabolic stress within multiple organs and is the driving force of many disorders and pathologies including cardiovascular disease and type 2 diabetes, often with macro- and microvascular complications.

TNO's **in-house translational** models are superior to state-of-the art models allowing better prediction of effects in humans. As a default, we use the human scoring system to analyze pathologic endpoints like atherosclerosis or non-alcoholic fatty liver disease (NAFLD) and non-alcoholic steatohepatitis (NASH). Translational models are available for:

- › Risk factors of cardiometabolic disease including hyperlipidemia and chronic inflammation
- › (diabetic) Atherosclerosis
- › Metabolic syndrome
- › Insulin resistance and type 2 Diabetes
- › NAFLD/NASH
- › Diabetic nephropathy and chronic renal failure
- › Energy homeostasis, obesity and adipose tissue inflammation

Proprietary animal strains together with extensive knowledge of nutrition and dietary inducers of disease allow us to

establish model conditions that are relevant for humans and superior to standard models. Making use of sophisticated technologies (e.g. lipid and energy metabolism and metabolic cages, all kind of omics, advanced (immune-)histology, non-invasive monitoring of adiposity, hyperinsulinemic euglycemic clamp analyses and validation in human tissue biopsies), these models are valuable for pharmaceutical and nutrition companies to evaluate compound efficacy and safety and to understand mechanism of action of small molecules, biologicals and complex food mixtures.

Our track record consists of >200 studies for companies and >250 papers in international literature on disease etiology and mechanisms, compound efficacy and safety (new concepts and mechanism of action, combination therapies, drug testing, biologicals, miRNA etc), nutritional interventions and nutraceuticals.

TNO'S UNIQUE MOUSE MODELS:**APOE*3LEIDEN MOUSE**

APOE*3Leiden (E3L) transgenic mice were generated by the introduction of the human *apoε*3*-Leiden and *apoc1* genes. The primary effect of the dominant E*3-Leiden mutation is an impaired clearance of triglyceride-rich lipoproteins (chylomicron- and VLDL-remnants) caused by reduced affinity for the LDLR, whereas overexpression of APOC1 inhibits lipolysis. While normal wild-type mice have a very rapid clearance of apoB-containing lipoproteins, E3L mice show an impaired clearance and are thereby mimicking the slow clearance in humans. As a consequence, E3L mice exhibit a human-like lipoprotein profile comparable to that of patients with familial dysbetaalipoproteinemia (most of the circulating cholesterol is confined to (V)LDL particles), and develop atherosclerosis upon feeding with saturated fat and cholesterol. However, E3L mice (like wild-type mice) do not possess a Cholesteryl Ester Transfer Protein (*cetp*) gene, an essential component of human lipoprotein metabolism, and therefore these mice do not respond to HDL-modulating interventions.

APOE*3LEIDEN.CETP MOUSE

APOE*3Leiden E3L.CETP transgenic mice were generated by cross-breeding the E3L mice with CETP transgenic mice, which express the human *cetp* gene under control of its natural flanking regions. CETP transfers cholesteryl ester from HDL to the apoB-containing lipoproteins in exchange for triglycerides, resulting in a more human-like lipoprotein metabolism. As compared to the E3L mice, E3L.CETP mice are more prone to develop hyperlipidemia and atherosclerosis upon feeding a western type diet containing cholesterol, and very suited for (nutritional) interventions under human-like diet conditions.

Treatment with a high fat diet together with fructose in the drinking water, mimicking the unhealthy diet in the western world, induces characteristics of the metabolic syndrome with increased body weight and insulin resistance and diabetic dyslipidemia.

INFLAMMATION MODELS (HUMAN CRP TRANSGENIC MOUSE; NFkB TRANSGENIC MOUSE)

Elevated plasma CRP levels are highly predictive for the future development of diabetes and cardiovascular events. Human CRP transgenic mice carry the complete human CRP gene including its regulatory elements. Consequently, the CRP expression in these mice is liver-specific and comparable to humans. The model allows assessment of liver inflammation and testing of the anti-inflammatory properties of nutrients and drugs. CRP expression can be induced with diets (metabolic overload of liver) or by specific cytokines as desired (variable level of systemic inflammation or an acute inflammation). Mice develop diet-inducible NAFLD/NASH, kidney fibrosis and osteoarthritis.

TNO'S IN-HOUSE STRAIN OF LDL-RECEPTOR KNOCK-OUT MOUSE (LDLR-LEIDEN-/-), A MODEL FOR DIABETIC COMPLICATIONS.

The LDLR-Leiden-/- mouse is prone to develop adipose inflammation and diabetic complications when exposed to specific energy dense diets. Similar to humans, animals develop complications of type 2 diabetes in liver (NASH, including fibrosis), in kidney (microalbuminuria) and in the aorta (atherosclerosis) even in the absence of cholesterol-feeding. The model is sensitive to anti-diabetic drugs (TZDs) as well as anti-inflammatory interventions (e.g. anti-caspase-1; miRNAs) and specific

proteins and bioactive fatty acids. Mice develop the endpoints rapidly and sequentially allowing interventions at specific stages of the disease.

APPLICATIONS:**DRUG REGISTRATION**

Studies with the E3L(.CETP) mice have been used in dossiers for drug registration to FDA and safety dossiers to EPA.

CV-SAFETY

Due to its sensitivity for modulation of plasma lipids, parameters of the metabolic syndrome, inflammation and atherosclerosis adverse effects with respect to CV safety can be easily detected, e.g. the CETP-inhibitor torcetrapib, HIV-protease and reverse transcriptase inhibitors, drugs against Gaucher's Disease, 11 β -HSD1-inhibitor, tyrosine kinase-inhibitors (e.g. JAK-inhibitors), plant sterol derivative have been investigated.

With respect to environmental safety and related CV safety issues a series of studies towards the mechanism of action of perfluorosurfactants on lipid and lipoprotein metabolism were conducted.

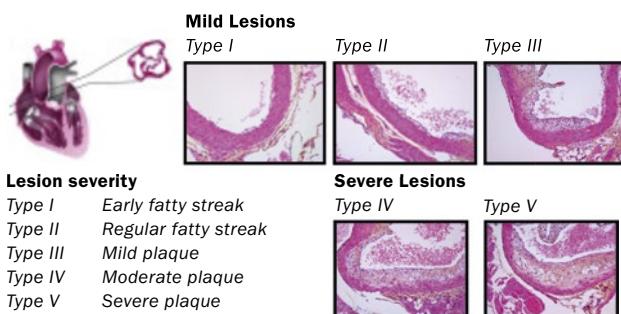
BIOLOGICALS

Studies have been performed with mAbs and vaccines against a.o. PCSK9, CETP, ox-LDL, IL-6, and with rec.HDL, GLP-1 and PYY analogues and an EPO-receptor agonist. Anti-PCSK9 antibodies demonstrated a significant additional effect on top of a statin with respect to lipid-lowering, similarly as in man, and atherosclerosis.

NON-LIPID MODULATION AND COMBINATION STUDIES

The E3L(.CETP) mice models are also very suitable to investigate the effect of (non-)lipid modulators, either alone or in combination with a statin on inflammation and atherosclerosis, e.g. studies have been performed with flavonoids and other anti-inflammatory compounds, anti-hypertensives as Ca-antagonists, and angiotensine II receptor and renin blockers, niacin, PCSK9 inhibition, CETP-inhibitors torcetrapib and anacetrapib etc. (list of publications upon request).

New drugs nowadays need to show additional effect on top of a statin. Since the E3L(.CETP) mice respond to statins as humans do, the model is very suited for combination studies.



Atherosclerosis development in APOE*3-Leiden.hu CETP transgenic mice.
Classification of lesion phenotype according to AHA

HYPERLIPIDEMIA, MIXED DYSLIPIDEMIA AND ATHEROSCLEROSIS

Atherosclerosis is a multifactorial, multistep disease with numerous etiologies which have to act in concert to initiate and promote the atherosclerotic process. In addition to traditional risk factors such as dyslipidemia and hypertension, inflammation is now accepted as a major driving force of atherosclerotic lesion development. TNO offers a wide range of models to study the various risk factors and the relative contribution of a risk factor can be optimized according to the type of intervention. The unique E3L(CETP) mice are established and well-recognized models for hyperlipidemia and the development of atherosclerosis as well as mixed dyslipidemia, a condition comparable with diabetic dyslipidemia. These models show the following favorable characteristics:

- (i) Responsiveness to all hypolipidemic drugs currently used in the clinic, such as statins, fibrates, ezetimibe and niacin, at similar dosages and in a similar way to humans;
- (ii) Suitable for testing the effect of combination therapy;
- (iii) The ability to titrate cholesterol and triglycerides to any desired level;
- (iv) Atherosclerosis studies can be performed in a progression (prevention) or a regression (therapeutic) design.

Moreover, the E3L.CETP mouse is very well suited to testing the effect of drugs that modulate HDL levels. The mice demonstrate reduced apoB-containing lipoproteins and increased HDL levels upon treatment with the registered drugs atorvastatin, fenofibrate and niacin and with CETP-inhibitors.

This model is also a predictive animal model: drugs and nutritional factors that failed in clinical studies, such as the GPR109a agonist MK-0354, the CETP-inhibitor torcetrapib, an experimental RCT-inducer, a plant sterol derivative and policosanol, also failed in this mouse model (publication list upon request).

METABOLIC SYNDROME

The metabolic syndrome comprises several metabolic abnormalities, including abdominal obesity, hypertension, hypertriglyceridemia, low levels of HDL cholesterol and impaired glucose tolerance or insulin resistance. These metabolic disorders tend to cluster

together and the combination poses a major risk for cardiovascular disease and type 2 diabetes.

In the E3L.CETP mouse on a high fat diet with fructose in drinking water, six important characteristics of the metabolic syndrome are combined in one animal model, i.e. increased body weight and insulin resistance (increased plasma glucose and insulin levels) and at the same time adverse changes in plasma lipids as observed in diabetic dyslipidemia, with increased triglycerides and apoB-containing lipoproteins and decreased HDL. The model also develops NAFLD and has been validated by intervention with the anti-diabetic and lipid-lowering drugs.

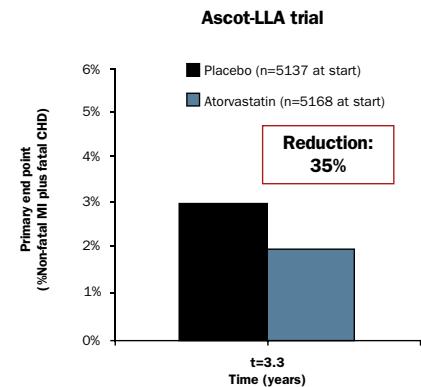
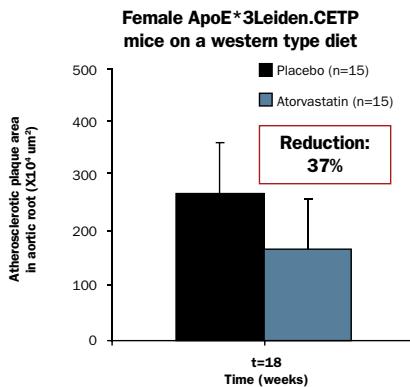
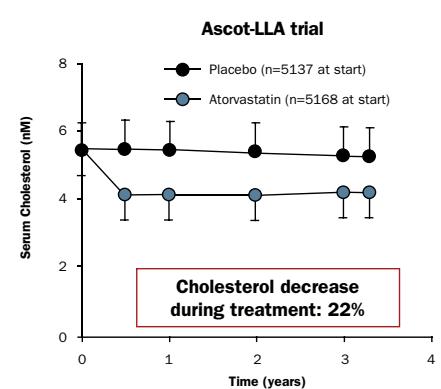
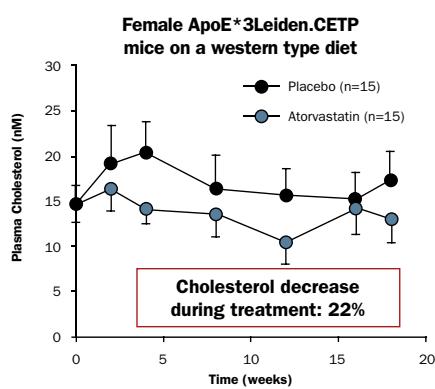
TYPE 2 DIABETES

Type 2 diabetes mellitus (T2DM) is a growing health problem worldwide. While peripheral insulin resistance is common during obesity and aging, progression to T2DM is partly due to insulin secretory dysfunction, leading to an inability to compensate for the insulin resistance. Besides conventional *in vivo* models for diabetes such as ob/ob mice, db/db mice and KKAY mice, TNO has developed specific mouse strains with diet-induced obesity, insulin resistance and diabetic dyslipidemia or a hyperlipidemic mouse

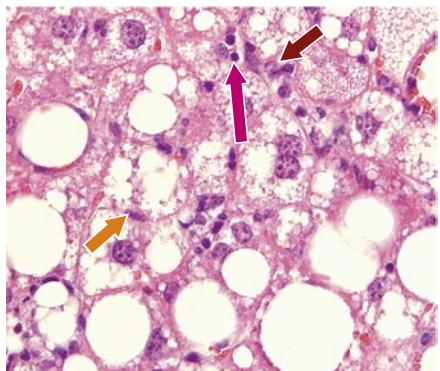
strain with diet-related obesity, T2DM and diabetic complications (see overview of TNO's unique mouse models above). Using specific diets we are able to evoke metabolic stress in specific organs. Knowledge of the sequence of pathologic events over time allows us to start intervention at a desired stage of the disease process.

NAFLD/NASH

NAFLD is becoming the most common chronic liver disease worldwide and can lead to NASH which is characterized by an inflamed fatty liver. NASH can become life-threatening when progressing towards liver fibrosis and cirrhosis. Human CRP transgenic mice and NFkB-luciferase.APOE*3-Leiden double transgenic mice allow to study lipotoxicity and liver inflammation, and to evaluate the effect of therapeutic interventions. By dietary intervention E3L(CETP) and LDLR-Leiden-/- mice develop all characteristics of human-like NASH as confirmed by human liver biopsies, although via different mechanisms. The lipid and cholesterol driven NASH observed in the E3L(CETP) mice is less severe than the high fat diet induced NASH observed in LDLR-Leiden-/- mice, in which fibrosis develops. In both animal models the development of NAFLD and the transition



Progression of atherosclerosis in APOE*3-Leiden.hu CETP transgenic mice treated with atorvastatin as compared to clinical outcome in patients treated with atorvastatin



— Kupffer cells
— Polymorphic nuclear cells/granulocytes
— Monocytes

Histopathology of NASH in mouse livers. As in human NASH, mixed inflammatory cell infiltration can be found.

of benign steatosis into NASH/fibrosis can be blocked with pharmaceuticals as well as with specific dietary supplements.

DIABETIC NEPHROPATHY AND CHRONIC RENAL FAILURE

Diabetic nephropathy, the most common cause of chronic kidney failure and end-stage kidney disease, develops in both type 1 and type 2 diabetics. Poorly controlled blood glucose levels but also high blood pressure and increased cholesterol levels increase the risk for the development and enhance the progression of disease.

TNO offers models which develop characteristics of diabetic nephropathy spontaneously (*db/db*) or upon dietary treatment (*KKA* or *LDLR-Leiden^{-/-}*) and chronic renal failure after partial nephrectomy. Disease status is measured functionally (Urinary albumin:creatinine ratio and glomerular filtration rate) and histologically.

ENERGY HOMEOSTASIS, OBESITY AND ADIPOSE TISSUE INFLAMMATION

White adipose tissue (WAT) stores energy for the body. Excessive availability of energy, e.g. due to modern lifestyle and food, expands WAT, leading to obesity. Continuous metabolic overload of WAT, may lead to adipose tissue inflammation. TNO offers models in which dietary induced adipose tissue inflammation develops. The health status of fat depots can be assessed and translated to human adipose tissue.

Histological analysis enable us to discriminate between safe fat storage and metabolic overload of adipose tissue including adipose inflammation.

These models are sensitive to stimulation of energy metabolism ('fat burners'; stimulation of brown fat), and involvement of the gut microbiota has been shown using probiotics and protein extracts. Tools are available to study the central regulation systems of satiety and to study energy expenditure in the brain.

ACTIVELY SEEKING CO-DEVELOPMENT PARTNERS

As an applied research institute, TNO participates in many public-private partnerships to accelerate the translation of new findings to applications (e.g. 290 European framework consortia as per November 2012). Within TNO there is a broad experience with translational mouse models and development technologies, efficiently applying internal and external capabilities, mostly in cardiovascular and metabolic diseases.

TNO's scientists have a wide network of external academic and industrial collaborators, thus ensuring that a solution to a specific biomedical problem can be identified, including innovation-driven pharmaceutical, nutraceutical, analytical and diagnostic companies, biobanks, clinicians, academic scientists and patient organizations. In addition to scientific excellence, TNO scientists possess distinguished operational skills and are equipped to deliver top-level scientific evidence in a professional and efficient manner.

TNO is actively seeking partners for co-development and collaboration in:

- › models for multi-factorial age-related end-stage cardiovascular and metabolic diseases on a background of its translational E3L mouse models (e.g. a vulnerable plaque model);
- › establishing combined disease models in E3L(.CETP) mice as translational animal models for the development of multiple chronic diseases in the ageing population (e.g. CVD-rheumatoid arthritis/osteoarthritis, CVD-chronic renal failure);
- › target discovery and functional characterization of newly discovered human target genes (e.g. from GWAS);
- › generation of humanized mouse models containing human receptor genes targeted by human-specific biologicals that do not interact with their mouse equivalents;
- › biomarker validation.



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TNO HEALTHY LIVING

TNO initiates technological and societal innovation for healthy living and a dynamic society.

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