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Suvi Heinonen

Modeling Cardiovascular Complications of Diabetes Mellitus

Development of a New Mouse Model and Evaluation of a Gene Therapy Approach

Publications of the University of Eastern Finland
Dissertations in Health Sciences

SUVI HEINONEN

Modeling cardiovascular complications of diabetes mellitus

Development of a new mouse model and evaluation of a gene therapy approach

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A.I. Virtanen Institute for Molecular Sciences
Faculty of Health Sciences

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Author's address: Department of Biotechnology and Molecular Medicine
A.I. Virtanen Institute for Molecular Sciences
Faculty of Health Sciences
University of Eastern Finland
KUOPIO
FINLAND
E-mail: suvi.heinonen@uef.fi

Supervisors: Professor Seppo Ylä-Herttuala, M.D., Ph.D.
Department of Biotechnology and Molecular Medicine
A.I. Virtanen Institute for Molecular Sciences
Faculty of Health Sciences
University of Eastern Finland
KUOPIO
FINLAND

Docent Ivana Kholová, M.D., Ph.D.
Pathology, Laboratory Centre
Tampere University Hospital
TAMPERE
FINLAND

Reviewers: Docent Maria Gomez, Ph.D.
Department of Clinical Sciences
Clinical Research Center
Lund University
MALMÖ
SWEDEN

Docent Risto Kerkelä, M.D., Ph.D.
Department of Pharmacology and Toxicology
Institute of Biomedicine
University of Oulu
OULU
FINLAND

Opponent: Professor Markku Savolainen, M.D., Ph.D.
Department of Internal Medicine
Institute of Clinical Medicine
University of Oulu
OULU
FINLAND

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ABSTRACT

Cardiovascular complications are the major cause of morbidity and mortality in diabetic patients. Accelerated atherogenesis and related vascular diseases increase the need for revascularization procedures and for the development of improved therapeutical approaches. Proangiogenic gene therapy represents a promising treatment option for myocardial and limb ischemia. However, enhanced neovascularization can also contribute to certain pathogenic processes, such as atherosclerotic lesion progression and the development of diabetic retinopathy. Although clinical trials of therapeutic angiogenesis have shown a favourable safety profile, concerns have been raised based on animal studies. The aim of this thesis was to assess the safety and effects of vascular endothelial growth factor (VEGF) gene therapy with respect to atherosclerosis, and to develop better experimental models for use in preclinical research by establishing and characterizing a new mouse model replicating type 2 diabetes and its associated vascular complications.

In contrast to previous mouse studies, no evidence of increased atherosclerosis was found following systemic adenoviral gene transfers of different VEGFs in hypercholesterolemic $LDLR^{-/-}ApoB^{100/100}$ mice. In order to establish a mouse model simulating diabetic vascular complications, $LDLR^{-/-}ApoB^{100/100}$ mice were cross-bred with mice in which type 2 diabetes develops as a consequence to overexpression of insulin-like growth factor-II (IGF-II) in pancreatic beta cells. The resulting IGF-II/$LDLR^{-/-}ApoB^{100/100}$ model demonstrated typical type 2 diabetic metabolic characteristics as well as a worsening of the atherosclerotic lesion phenotype. Both the $LDLR^{-/-}ApoB^{100/100}$ and diabetic IGFII/$LDLR^{-/-}ApoB^{100/100}$ mice displayed extensive coronary artery disease leading to left ventricular dysfunction with structural and functional evidence suggestive of chronic myocardial hibernation. IGFII/$LDLR^{-/-}ApoB^{100/100}$ mice also exhibited altered retinal morphology and photoreceptor atrophy, but there were no microvascular changes typical of diabetic retinopathy.

In conclusion, adenovirus-mediated gene transfers of VEGFs had no proatherogenic effects in hypercholesterolemic mice. This supports the clinical trial data, and indicates that the testing and development of VEGF gene therapy for cardiovascular diseases can proceed without unnecessary concerns about accelerated atherogenesis. Furthermore, a promising new model of macrovascular complications in type 2 diabetes was developed and characterized, and is now available for experimental and translational research.

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Medical Subject Headings: Gene Therapy; Disease Models, Animal; Mice; Atherosclerosis; Vascular Endothelial Growth Factors/therapeutic use; Diabetes Mellitus, Type 2/complications; Safety; Gene Transfer Techniques; Adenoviridae; Hypercholesterolemia; Coronary Artery Disease; Ventricular Dysfunction, Left

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TIIVISTELMÄ

Tyypin 2 diabetes on kasvava kansanterveysongelma, johon liittyy merkittävä sydän- ja verisuonisairauksien riski. Kaikilla sydämen tai alaraajojen valtimoiden pallolaajennusta tai ohitusleikkausta tarvitsevilla potilailla perinteisillä menetelmillä ei saada riittävää hoitovastetta, tai menetelmiä ei voida soveltaa vaikean taudinkuvan vuoksi. Verisuonten uudismuodostuksen aikaansaaminen kasvutekijägeeninsiirron avulla (terapeuttinen angiogeneesi) on osoittautunut lupaavaksi uudeksi hoitomuodoksi sydänlihaksen ja alaraajojen verenkierron parantamisessa. Kliinisissä kokeissa terapeuttinen angiogeneesi on osoittautunut turvalliseksi, mutta muutamista eläinkokeista on saatu viitteitä valtimonkovettumataudin lisääntymisestä kasvutekijöiden vaikutuksesta. Tämän tutkimuksen tarkoituksena oli arvioida verisuonen endoteelikasvutekijöiden (VEGF) vaikutusta valtimonkovettumatautiin, sekä tuottaa prekliiniseen tutkimukseen parempia tautimalleja kehittämällä ja karakterisoimalla tyypin 2 diabetesta ja siihen liittyviä kardiovaskulaarisia sairauksia ilmentävä hiirimalli.

Aiemmista hiirillä tehdyistä tutkimuksista poiketen adenovirusvälitteisten VEGF-A, -B, -C tai -D geeninsiirtojen ei havaittu lisäävän valtimonkovettumatautia hyperkolesterolemisessa $LDLR^{-/-}ApoB^{100/100}$ hiirimallissa. Mallintaaksemme diabeettisia kardiovaskulaarikomplikaatioita, $LDLR^{-/-}ApoB^{100/100}$ hiiret risteytettiin hiirikantaan, jossa tyypin 2 diabetes aiheutuu insuliininkaltaisen kasvutekijä-II:n (IGFII) yli-ilmentämisestä haiman beetasoluissa. IGF-II/$LDLR^{-/-}ApoB^{100/100}$ hiirissä tyypin 2 diabetes aiheutti ateroskleroottisten leesioiden pahenemisen. Sekä $LDLR^{-/-}ApoB^{100/100}$ että IGF-II/$LDLR^{-/-}ApoB^{100/100}$ hiirille kehittyi lisäksi vakava sepelvaltimotauti, joka aiheutti sydämen vasemman kammion toimintahäiriön. IGF-II/$LDLR^{-/-}ApoB^{100/100}$ hiirillä esiintyi muutoksia myös silmän verkkokalvon rakenteessa ja fotoreseptorien atrofiaa, mutta diabeettiseen retinopatiaan viittaavia verisuonimuutoksia ei havaittu.

Yhteenvetona voidaan todeta, että VEGF:n tilapäinen yli-ilmentäminen ei lisännyt valtimonkovettumatautia hyperkolesterolemisissa $LDLR^{-/-}ApoB^{100/100}$ hiirissä. Tämä vahvistaa kliinisissä kokeissa saatuja tuloksia ja viittaa siihen, että VEGF-geeniterapiasovellusten tutkimus ja kehitys sydän- ja verisuonitauteihin voi edetä ilman merkittävää huolta valtimonkovettumataudin kiihtymisestä. Tutkimuksessa kehitetyssä uudessa hiirimallissa yhdistyvät aikuistyypin diabetekselle tyypilliset metaboliset häiriöt sekä valtimonkovettumataudista aiheutuvat sydän- ja verisuonisairaudet, ja mallia voidaan soveltaa sairauksien syntymekanismien tutkimiseen ja uusien hoitomuotojen kehittämiseen.

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Yleinen suomalainen asiasanasto: geeniterapia; eläinkokeet; hiiret; sydän- ja verisuonitaudit; ateroskleroosi; diabetes - - komplikaatiot; kasvutekijät; verisuonet

"If you can dream it, you can do it.
Always remember that this whole thing was started with a dream and a mouse."

- Walt Disney

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List of the original publications

This dissertation is based on the following original publications:

I Leppänen P, **Koota S**, Kholová I, Koponen J, Fieber C, Eriksson U, Alitalo K, Ylä-Herttuala S. Gene transfers of vascular endothelial growth factor-A, vascular endothelial growth factor-B, vascular endothelial growth factor-C, and vascular endothelial growth factor-D have no effects on atherosclerosis in hypercholesterolemic low-density lipoprotein-receptor/apolipoprotein B48-deficient mice. *Circulation 112:1347-1352 2005.*

II **Heinonen SE**, Leppänen P, Kholová I, Lumivuori H, Häkkinen SK, Bosch F, Laakso M, Ylä-Herttuala S. Increased atherosclerotic lesion calcification in a novel mouse model combining insulin resistance, hyperglycemia, and hypercholesterolemia. *Circulation Research 101:1058-1067, 2007.*

III **Heinonen SE**, Merentie M, Hedman M, Mäkinen PI, Loponen E, Kholová I, Bosch F, Laakso M, Ylä-Herttuala S. Left ventricular dysfunction with reduced functional cardiac reserve in diabetic and non-diabetic LDL-receptor deficient apolipoprotein B100-only mice. *Cardiovasular Diabetology 10:59, 2011.*

IV *Kinnunen K, ***Heinonen SE**, Kalesnykas G, Laidinen S, Uusitalo-Järvinen H, Uusitalo H, Ylä-Herttuala S. Ocular phenotype of type 2 diabetic $LDLR^{-/-}ApoB^{100/100}$ mice reveals photoreceptor atrophy and altered morphology of the retina. *Manuscript, 2011.*

* Authors with equal contribution

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Unpublished results are also presented.

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Abbreviations

AAV	Adeno-associated virus
AD	Atherogenic diet
Ad libitum	Free feeding with unlimited access to food and water
AGE	Advanced glycosylation end products
ALP	Alkaline phosphatase
AMDCC	Animal Models of Diabetic Complications Consortium
ANOVA	Analysis of variance
Apo	Apolipoprotein
Apobec-1	Apolipoprotein B mRNA editing enzyme, catalytic polypeptide 1
AR	Aldose reductase
α-SMA	Smooth muscle cell α-actin
AT-II	Angiotensin II
A^{y}/a	Agouti
β	Beta
Bax	Bcl-2-associated X protein
BMP-2	Bone morphogenetic protein 2
BP	Blood pressure
CAD	Coronary artery disease
CCA	Cholesterol/cholic acid containing diet
CD	Cholesterol diet
cDNA	Complementary DNA
CETP	Cholesterol ester transfer protein
DCM	Diabetic cardiomyopathy
DNA	Deoxyribonucleic acid
CD31	Cluster of differentiation 31, platelet endothelial cell adhesion molecule
CD36	Cluster of differentiation 36, a class B scavenger receptor
CMV	Cytomegalovirus
CPS	Contrast pulse sequence
CRP	C-reactive protein
CVD	Cardiovascular diseases
db	Diabetic
DD	Diabetogenic diet
DM	Diabetes Mellitus
ELISA	Enzyme-linked immunoassay
En face	Atherosclerotic lesion area analysis from longitudinally opened aortas
eNOS	Endothelial nitric oxide synthase
EPC	Endothelial progenitor cell
ER	Endoplasmic reticulum
ET-1	Endothelin-1
Ex vivo	Outside the living organism
FFA	Free fatty acids
GCL	Ganglion cell layer
GP	Glycoprotein
GPx1	Glutathione peroxidase-1
GTG	Gold thioglucose
GTT	Glucose tolerance test
HCD	High-cholesterol diet
HDL	High-density lipoprotein
HFD	High-fat diet
HFHC	High-fat/high-cholesterol diet
HLA	Human leukocyte antigen
HSP	Heat shock protein
HuB	Human apoB
IDL	Intermediate density lipoprotein
IFG	Impaired fasting glucose
IGF-II	Insulin-like growth factor II
IGT	Impaired glucose tolerance
ICAM	Intercellular adhesion molecule
IL-6	Interleukin 6
INL	Inner nuclear layer
Ins	Insulin (gene)

In vitro	In an artificial environment outside the living organism	PFA	Paraformaldehyde
		pfu	Plaque forming unit
In vivo	Within a living organism	PI-3K	Phosphatidylinositol-3 kinase
i.p.	Intraperitoneal	PKC	Protein kinase C
IPL	Inner plexiform layer	RAGE	Receptor for advanced glycosylation products
IR	Insulin resistance		
IRS	Insulin receptor substrate	rh	Recombinant human
IS	Inner segment	RNA	Ribonucleic acid
ITT	Insulin tolerance test	ROS	Reactive oxygen species
i.v.	intravenous	RT-PCR	Reverse transcription PCR
LacZ	Beta-galactosidase	SAA	Serum amyloid A
LDL	Low-density lipoprotein	SAP	Serum amyloid P
LDLR	Low-density lipoprotein receptor	s.c.	subcutaneous
L-PGDS	Lipocalin-type prostaglandin D_2 synthase	SD	Standard deviation
		sdLDL	Small dense low-density lipoprotein
LPL	Lipoprotein lipase		
LV	Left ventricle	SEM	Standard error of the mean
MAPK	Mitogen-activated protein kinase	SMC	Smooth muscle cell
MCP-1	Monocyte chemotactic protein-1	sRAGE	Soluble RAGE
mMQ	Mouse macrophage	STZ	streptozotosin
NF-κB	Nuclear factor kappa B (nuclear factor kappa-light-chain-enhancer of activated B cells)	SUMMIT	Surrogate markers for Micro- and Macro-vascular hard endpoints for Innovative diabetes Tools
NI	Neointima	T1D	Type 1 diabetes
NO	Nitric oxide	T2D	Type 2 diabetes
NOD	Non-obese diabetic	TC	Total cholesterol
ob	Obese	TF	Tissue factor
ONL	Outer nuclear layer	TG	Triglyceride
OPL	Outer plexiform layer	TNFα	Tumor necrosis factor alpha
OPN	Osteopontin	VLDL	Very low-density lipoprotein
OS	Outer segment	VEGF	Vascular endothelial growth factor
PAI-1	Plasminogen activator inhibitor-1		
		VCAM	Vascular cell adhesion molecule
PBS	Phosphate buffered saline	w/	With
PCNA	Proliferating cell nuclear antigen	WD	Western diet
PCR	Polymerase chain reaction	WHO	World Health Organization
PD	Paigen diet	w/o	Without

1 Introduction

The prevalence of diabetes is increasing and especially type 2 diabetes is starting to reach epidemic proportions in the Western world. Cardiovascular diseases (CVD) are the major cause of morbidity and mortality in diabetes, and patients suffer from both micro- and macrovascular complications. The high prevalence of concomitant cardiovascular risk factors, the predisposition to atherogenic lipid abnormalities, and other diabetes-related biochemical and metabolic perturbations, lead to accelerated atherosclerosis and arterial dysfunction. Since there are no curative treatments for either diabetes or atherosclerosis, therapeutical options are somewhat limited to symptom alleviation. In addition to pharmacological therapies, revascularization procedures are performed to improve blood flow in the heart and lower limbs. However, due to the often diffuse vascular disease and complex clinical picture of diabetic patients, the response to treatment is often not optimal or the conventional surgical procedures cannot be performed. As a new treatment modality, gene therapy applications show promise in the treatment of CVD. In clinical trials, therapeutical angiogenesis has proven to be safe and feasible. However, increased neovascularization can contribute also to pathogenic processes, such as atherosclerotic lesion growth and the development of diabetic retinopathy, and concerns about the safety of pro-angiogenic therapies have been raised based on results from some animal studies.

Animal models are essential tools in the preclinical research of complex metabolic disorders. In recent years, various mouse models combining disorders of lipid and glucose metabolism have been generated and studied, and they have provided new and valuable information. However, one major limitation of most mouse models is the fact that they do not experience diabetic cardiovascular complications without a concomitant increase in plasma lipid levels, which does not replicate the human situation. Therefore, new models are urgently needed for studying the pathophysiology of both micro- and macrovascular complications in diabetes, since this could help in the development of new therapeutic approaches and also improving the efficacy of the current procedures.

This thesis studied the safety and pharmacodynamics of a cardiovascular gene therapy approach in relation to atherosclerosis. Furthermore, in an attempt to create improved research tools better mimicking diabetic cardiovascular complications, a new mouse model was developed and characterized.

2 Review of the literature

2.1 DIABETES AND CARDIOVASCULAR DISEASES

2.1.1 Diabetes mellitus

Diabetes mellitus is a group of disorders characterized by abnormally high blood glucose levels resulting either from insulin deficiency or from resistance to the action of insulin, or from a combination of both. The two main forms are type 1 diabetes (T1D), where the insulin-producing pancreatic beta (β) cells have been destroyed, and type 2 diabetes (T2D), which is a metabolic disorder with a more complex etiology. Additional less prevalent subclasses include gestational diabetes, genetic forms and secondary conditions resulting from other pancreatic diseases or medications. The number of diabetic patients worldwide is currently over 220 million and the number is continuously increasing (WHO, 2011). The increase in prevalence has been most dramatic in T2D, which accounts for over 90 % of all diabetes cases (Zimmet, Alberti & Shaw 2001). The acceleration of the diabetes epidemic is naturally also accompanied by an increase in diabetic complications, which are the major causes of heart disease, blindness, renal failure and lower limb amputation.

The diagnosis of diabetes is based on the criteria recommended by the World Health Organization (Table 1) (WHO, 2005). Diagnostic cut-off points for diabetes are fasting plasma glucose ≥7.0 mmol/l or 2-hour plasma glucose concentration ≥11.1 mmol/l in an oral glucose tolerance test. However, glucose levels also below the diabetic threshold correlate with the probability of developing T2D and the risk of CVD (Unwin *et al.* 2002). Thus the terms impaired glucose tolerance (IGT) (WHO, 1980) and impaired fasting glucose (IFG) (WHO 1999) have been introduced to describe the status between normoglycemia and diabetes. This state is also called pre-diabetes, since it precedes full-blown disease in T2D patients. However, many individuals with pre-diabetic values do not progress to diabetes, and therefore the term "intermediate hyperglycemia" is recommended to describe IFG and IGT (WHO, 2005).

Table 1. Diagnostic criteria for diabetes and intermediate hyperglycemia (IFG and IGT) (WHO 2005). Criteria differ depending on the sample collection site: plasma values are about 11 % higher than in whole blood, and in non-fasting conditions venous whole blood gives higher results than capillary samples. Plasma glucose (in bold) is used as the standard parameter.

	Fasting glucose (mmol/l)				**2-hour post-glucose load (mmol/l)**		
	Plasma (venous)	**Whole blood** Venous	Capillary		**Plasma** (venous)	**Whole blood** Venous	Capillary
Normal	**<6.1**	<5.6	<5.6		**<7.8**	<6.7	<7.8
IFG	**6.1-6.9**	5.6-6.0	5.6-6.0	**and**	**<7.8**	<6.7	<7.8
IGT	**<7.0**	<6.1	<6.1	**and**	**7.8-11.0**	6.7-9.9	7.8-11.0
Diabetes	**≥7.0**	≥6.1	≥6.1	**or**	**≥11.1**	≥10.0	≥11.1

IFG, impaired fasting glucose; IGT, impaired glucose tolerance.

2.1.1.1 Type 1 diabetes

T1D results from the dysfunction and destruction of pancreatic β-cells which causes an absolute insulin deficiency and complete dependence on exogenous insulin to regulate blood glucose levels. T1D is commonly known as juvenile-onset diabetes, as it generally occurs before the age of 30, peaking around the time of puberty. The worldwide incidence of childhood-onset T1D varies significantly with the highest rates (>40/100 000) being reported from Finland (The DIAMOND Project Group 2006).

The cause of T1D remains unclear. Substantial evidence indicates that both genetic and environmental etiological factors are involved (Borchers, Uibo & Gershwin 2010). Numerous genetic loci have been associated with the risk for T1D and the major causal candidates have been located in the human leukocyte antigen (HLA) region (Pociot *et al.* 2010). HLA molecules are essential in the function of the immune system and account for antigen presentation to T-lymphocytes. Accordingly, in most cases of T1D, the β-cell destruction is autoimmune-mediated and autoantibodies exist for months or years before any clinical manifestation (Atkinson, Maclaren 1994). The pancreatic function deteriorates gradually - it has been estimated that at the time of diagnosis about 80-90 % of the β-cells have been destroyed, reaching total extinction in the following 1-2 years (Foulis *et al.* 1986). It has also been postulated that exposure to certain environmental factors during pregnancy, neonatally or in early childhood could trigger the disease onset. These include *e.g.* certain viral infections, exposure to cow's milk antigens and climatic factors (Borchers, Uibo & Gershwin 2010). In particular enterovirus infection has been shown to have a strong association with T1D (Yeung, Rawlinson & Craig 2011). However, the distinct causal roles of environmental triggers remain unclear. For example, early exposures to particular viral infections could promote the development of autoimmunity, and subsequent infections in later life would then accelerate the process. Furthermore, genetically susceptible individuals might have an altered immune system causing generally augmented reactivity to otherwise harmless antigens (Hirschhorn 2003).

Insulin deficiency has various effects on metabolism (Figure 1). Normally, insulin regulates blood glucose levels by stimulating glucose uptake in peripheral tissues (mainly skeletal muscle), increasing the storage of glucose as glycogen and by inhibiting hepatic glucose production (Bennett 2000, Aronoff *et al.* 2004). In the absence of insulin, the tissue uptake of glucose is impaired and blood glucose levels become elevated. As the glucose concentration exceeds the renal reabsorption capacity, dehydration develops via glucosuria and osmotic diuresis and these cause the first typical symptoms of diabetes: polyuria and thirst (Guyton, Hall 2006).

Insulin also promotes fat storage, inhibits lipid mobilization and regulates triglyceride (TG) utilization (Bennett 2000). In uncontrolled diabetes, the accelerated hydrolysis of stored TGs releases large quantities of free fatty acids (FFA) into the circulation. FFAs are then used in the hepatic synthesis of very low-density lipoproteins (VLDL) or ketone bodies. A prolonged excess of ketone bodies leads to ketoacidosis, which, if not corrected with exogenous insulin, can induce unconsciousness, coma or even death (Guyton, Hall 2006).

As an anabolic hormone, insulin also facilitates the transport of amino acids into tissues, decreases proteolysis and increases protein synthesis in liver and skeletal muscle (Bennett 2000). Thus when insulin is not available, protein catabolism is switched on and this leads to elevated amino acid concentrations in blood. Most of the excess amino acids are used as

substrates in hepatic gluconeogenesis, thus worsening hyperglycemia, or are excreted in urine, inducing weight loss (Guyton, Hall 2006).

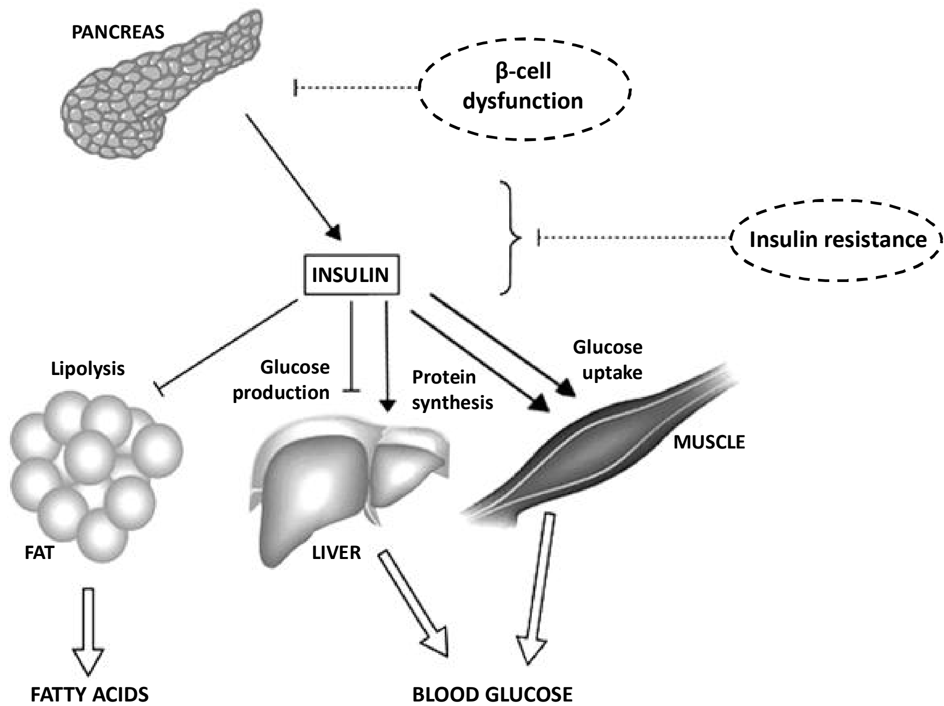

Figure 1. Metabolic actions of insulin. In normal conditions, blood glucose levels are controlled by insulin-stimulated glucose uptake and the suppression of hepatic glucose production, and lipolysis in fat tissue is inhibited. If insulin secretion is diminished (β-cell dysfunction) or the actions of insulin blunted (insulin resistance), hyperglycemia and increased levels of circulating fatty acids ensue. Modified from Stumvoll, Goldstein & van Haeften (2005).

2.1.1.2 Type 2 diabetes

T2D is a heterogeneous disorder characterized by peripheral insulin resistance (IR) and inadequate insulin secretion, both of which are usually present at the time of clinical manifestation. T2D accounts for by far the most diabetes cases and its global prevalence is continuously increasing. Moreover, although T2D, also known as the adult-onset diabetes, has long been regarded as a disease of the middle-aged and elderly, the numbers of younger, even pediatric patients, are also steadily growing (Alberti *et al.* 2004).

The increase in T2D seen during the last few decades clearly highlights the significant contribution of environmental, or lifestyle, factors – major determinants include obesity (especially central adiposity), diet, and physical inactivity, although also the intrauterine environment *(e.g.* low birth weight or mother’s diabetes) can influence the risk of future T2D (Bennett 2000). Thus, it seems that the pathogenesis of T2D is multifactorial – genetic factors determine the individual susceptibility but environmental factors dictate whether this predisposition will manifest as diabetes. The genetic risk profile consists of variations in

many genes, which on their own are not causative, but each add slightly to the overall genetic susceptibility (Das, Elbein 2006).

2.1.1.2.1 Insulin resistance

IR represents the hallmark of T2D. It is described as defective glucose uptake in skeletal muscle, liver and adipose tissue in response to a normal insulin concentration. The main mechanisms of IR involve alterations in insulin receptor expression and affinity, as well as insulin signaling defects (Schinner *et al.* 2005, Pessin, Saltiel 2000). Insulin signaling is transduced via two major pathways: metabolic and hemodynamic effects are mediated by phosphatidylinositol-3 kinase (PI-3K) and the Ras-mitogen-activated protein kinase (MAPK) pathway is mainly involved in gene expression regulation, cell growth and differentiation (Avruch 1998). In IR, only the PI-3K dependent signaling is impaired; other pathways are not affected (Cusi *et al.* 2000). These signaling defects can evolve as a consequence of mutations in the insulin signaling molecules, although the extent of their contribution is not certain (Schinner *et al.* 2005). Moreover, and since most insulin resistant individuals are obese, dysfunctions in adipose tissue may play a central role in the deterioration of insulin signaling through two distinct mechanisms (Saltiel, Kahn 2001). First, the release of excessive amounts of FFAs from adipose tissue leads to the accumulation of triglycerides and fatty acid-derived metabolites in skeletal muscle and liver cells, which suppresses insulin-signaling proteins. Secondly, in addition to being an energy storage site, adipose tissue also acts as an endocrine organ by secreting adipokines. The expression of the so-called insulin sensitizing adipokines, such as adiponectin, is decreased in obesity, while there is an increase in the expression of factors blunting the insulin signaling cascade, *e.g.* tumour necrosis factor α (TNFα).

Although many molecular events of IR have been identified, the exact course of events is still elusive. It is known that insulin sensitivity and insulin secretion are tightly linked and once one of them changes, the other process adapts (Kahn 2003). However, the sequence and pathophysiological mechanisms involving IR and hyperinsulinemia are not entirely clear. It is generally thought that hyperinsulinemia occurs as a compensatory reaction in order to maintain normoglycemia when peripheral insulin sensitivity is reduced. Nonetheless, also an alternative theory of hyperinsulinemia being the primary incident has been proposed. This is mainly based on insulin's ability to desensitize its target cells to its own actions (Shanik *et al.* 2008). Regardless of the precise pathogenesis, once IR has developed, even the hypersecretion of insulin is not sufficient to sustain normal glucose regulation and eventually mild postprandial hyperglycemia appears, resulting in IGT. As long as the β-cells are able to maintain a satisfactory insulin secretion, diabetes does not develop. However, after a period of hyperinsulinemia, insulin secretion diminishes in some individuals, leading to overt hyperglycemia and progression to clinical T2D.

2.1.1.2.2 Impaired insulin secretion

The mechanisms behind insulin deficiency in T2D include both an inefficient secretion of insulin and reduced β-cell mass (Weir, Bonner-Weir 2000). Impaired insulin secretion can result from β-cell exhaustion, which develops following hyperinsulinemia and prolonged glucose exposure. In fact, this is observed already in IGT and early T2D as a defective first phase insulin response to a rapid glucose load, *e.g.* in oral or intravenous glucose challenge,

and as a result, postprandial hyperglycemia ensues, since hepatic glucose production is not suppressed (Caumo, Luzi 2004). Chronic hyperglycemia further deteriorates β-cell function through glucotoxicity, which is postulated to be mediated by the production of reactive oxygen species (ROS), the activation of endoplasmic reticulum (ER) stress and increase in intracellular calcium (Chang-Chen, Mullur & Bernal-Mizrachi 2008). In the presence of hyperglycemia, also elevated levels of FFAs deteriorate β-cell function and survival (glucolipotoxicity). The overall effect of these processes impairs insulin secretion, reduces insulin gene expression and eventually results in the apoptosis of β-cells. The reduction of β-cell mass is a continuous process beginning already before overt diabetes. In fact, apoptosis-mediated reductions of 41 % and 63 % in β-cell volumes have been reported in individuals with IFG and T2D, respectively (Butler *et al.* 2003).

2.1.2 Cardiovascular complications in diabetes

After the onset of diabetes, most patients develop cardiovascular complications. Microvascular dysfunction can lead to significant morbidity and premature mortality, and macrovascular events are the leading cause of death in diabetic patients (UK Prospective Diabetes Study Group 1998).

2.1.2.1 Macrovascular disease in diabetes

Diabetes magnifies the risk of macrovascular diseases independently of conventional risk factors. Compared to non-diabetic individuals, the risks of developing coronary and peripheral artery disease are more than doubled in diabetes (Abbott, Brand & Kannel 1990, The Emerging Risk Factors Collaboration 2010). In addition, the risk of cerebrovascular events, such as stroke, is increased about 2-fold in the whole patient population and more than 10-fold in diabetic patients under the age of 55 (The Emerging Risk Factors Collaboration 2010, You *et al.* 1997). In addition to increasing the risk of developing macrovascular complications, diabetic patients also experience adverse outcomes with high rates of recurrence and mortality following myocardial infarction and stroke, as well as amputation of lower limbs (Beckman, Creager & Libby 2002).

2.1.2.1.1 Atherosclerosis

The principal pathophysiological process underlying macrovascular diseases is atherosclerosis. Atherosclerosis is a progressive disease of the large and medium-sized arteries with the accumulation of lipids and fibrous material in the arterial wall (Figure 2) (Ross 1999, Lusis 2000, Libby 2002). The classical risk factors include elevated level of low-density lipoprotein (LDL), low high-density lipoprotein (HDL) concentration, smoking, hypertension and diabetes (Lusis 2000).

Arteries consist of three layers: intima, media and adventitia. Intima is the innermost layer consisting of the endothelium lining the luminal surface and some smooth muscle cells (SMC) (Stary *et al.* 1992). Media is an elastic muscular layer formed mainly by SMCs. Adventitia is the outer layer of connective tissue, which stabilizes the artery and contains the innervation and blood supply for the vessel wall. Intact endothelium is crucial for the normal functioning of the artery and it plays important role in the regulation of blood clotting, inflammation and vascular tone. Atherosclerosis affects primarily the sites of

vasculature with turbulent blood flow and low shear stress, such as branches, bifurcations and curvatures (Gimbrone 1999). Altered blood flow increases endothelial permeability and induces the expression of adhesion molecules, thus rendering the site susceptible for the atherosclerotic process (Ross 1999).

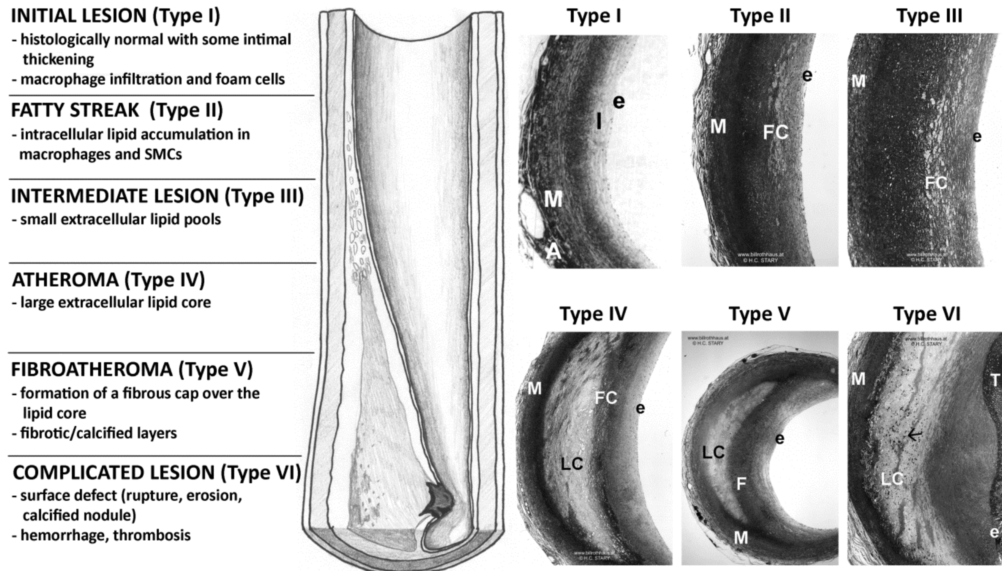

Figure 2. Schematic picture of different lesion types in the development of atherosclerosis and representative histological images from human coronary arteries. E, endothelium; F, fibrous cap; FC, foam cells; I, intima, LC, lipid core; M, media; SMC, smooth muscle cell; T, thrombus. Adapted from Stary *et al.* (1992, 1995), Pepine (1998) and Atherosclerosis Image Library/Stary (available for academic use at http://www.atherosclerosis-image-library.at).

The formation of an atherosclerotic lesion begins when LDL diffuses into the arterial wall from the bloodstream and accumulates in the subendothelial space. Trapped LDL undergoes oxidative modifications and stimulates the endothelial cells to produce proinflammatory molecules, which increase the influx of leukocytes and monocytes into the artery wall. Monocytes differentiate into macrophages that take up oxidized LDL, leading to the formation of foam cells. These early atherosclerotic lesions are called fatty streaks. If the inflammatory response caused by oxidized LDL does not cease, the atherosclerotic process continues with the migration and proliferation of medial SMCs within the inflammed intima. SMCs secrete extracellular matrix proteins, take up modified LDL and form a fibrous cap structure. The inflammatory environment also induces the death of lipid-loaded macrophages and SMCs, which form a necrotic core typical for more advanced lesions. Lesions can be further complicated by calcification and neovascularization. Although the progressive narrowing of the arterial lumen by the growing lesion can cause ischemic symptoms, acute and clinically more serious cardiovascular events, like myocardial infarction and stroke, usually result from plaque rupture and the following thrombosis. The atherosclerotic plaque can rupture if the fibrous cap breaks as a result of erosion caused by chronic inflammation. This most often happens in the shoulder regions of the plaque, which are the sites of macrophage influx, accumulation and apoptosis. When the thrombogenic lipid core is exposed and comes in contact with the coagulation factors of blood, thrombosis

occurs and can occlude the artery, blocking blood flow. (Lusis 2000, Libby 2002, Glass, Witztum 2001).

Endothelial dysfunction is considered to be the basis for the development of angiopathy in both the micro- and macrovasculature (Calles-Escandon, Cipolla 2001). It can be defined as disturbed adhesive properties, increased permeability and an imbalance between the production of contracting and relaxing substances by the endothelium. Under normal conditions, nitric oxide (NO) produced by the endothelial cells plays a pivotal role in mediating vasodilation and other antiatherogenic effects, such as the inhibition of platelet activation, inflammation, and SMC migration and proliferation. In the dysfunctional endothelium, impaired production of NO, as well as increased NO inactivation by ROS, cause decreased bioavailability of NO and the development of a proatherogenic state in the vessel wall. Although common etiological factors, such as elevated cholesterol levels (Drexler *et al.* 1991), smoking (Heitzer *et al.* 1996) and hypertension (Panza *et al.* 1990), contribute to the development of endothelial dysfunction and subsequent atherogenesis, there are also pathogenic mechanisms specific to diabetes (Figure 3) (Sitia *et al.* 2010).

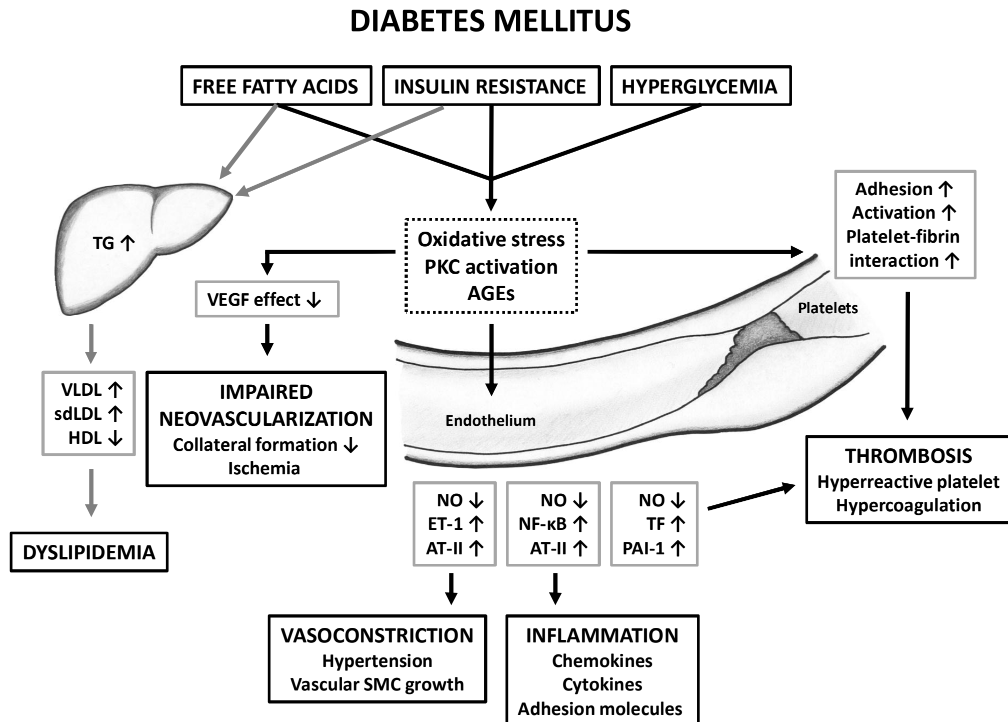

Figure 3. Diabetic metabolic abnormalities promote atherogenesis by inducing oxidative stress, activating signaling cascades (*e.g.* PKC) and inducing the formation of advanced glycation end products (AGEs), which lead to the dysfunction of the endothelium, platelets and vascular endothelial growth factors (VEGF). Together with diabetic dyslipidemia, these changes accelerate atherogenesis and create a pro-thrombotic state, exposing the individual to the risk of serious cardiovascular events. AT-II, angiotensin II; ET-1, endothelin-1; HDL, high-density lipoprotein; NF-κB, nuclear factor kappa B; NO, nitric oxide; PAI-1, plasminogen activator inhibitor-1; PKC, protein kinase C; sdLDL, small dense low-density lipoprotein; TF, tissue factor; TG, triglycerides; VLDL, very low-density lipoprotein. The conceptual idea for the figure was obtained from Creager *et al.* (2003).

The main factor underlying the increased risk of CVD in diabetes is accelerated atherosclerosis. In diabetes, atherosclerosis also develops earlier and is usually more severe and diffuse than in non-diabetic patients (Goraya *et al.* 2002). Although the precise mechanisms by which diabetes leads to more aggressive atherosclerosis are still undefined, several factors contributing to the pathogenesis of diabetic macrovasculopathy have been clarified.

2.1.2.1.1.1 Atherogenic effects of hyperglycemia

The correlation between blood glucose levels and the risk of cardiovascular events has been clearly demonstrated in epidemiological studies (Coutinho *et al.* 1999). Although the effect of intensive glycemic control on the prevention of macrovascular disease is less profound than on the reduction of microvascular complications (Ray *et al.* 2009), it is evident that hyperglycemia has many detrimental effects on the macrovasculature and it promotes atherogenesis. While most cell types are able to reduce glucose uptake when exposed to a high concentration of this carbohydrate, some other cells (such as endothelial cells, glomerular mesangial cells, neurons and Schwann cells in peripheral nerves) are not able to effectively regulate the intracellular glucose concentration, making them vulnerable to hyperglycemia-induced damage (Brownlee 2005).

Based on the current knowledge, the central mechanism mediating the harmful effects of a high glucose concentration on cells is thought to be increased oxidative stress and subsequent mitochondrial production of ROS (Brownlee 2005) (Figure 3). This decreases the metabolism of glucose through glycolysis, and the flux through the alternative polyol and hexosamine pathways is increased. The accumulation of glycolytic intermediates activates the protein kinase C (PKC) pathway, and this induces the formation of advanced glycation end products (AGE). The overall effects of these mechanisms are increased oxidative stress, apoptosis and vascular permeability. Pro-inflammatory gene expression is stimulated through the activation of transcription factors such as nuclear factor kappa B (NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells), leading to an increased production of adhesion molecules, leukocyte-attracting chemokines and cytokines activating inflammatory cells in the vascular wall. A pro-thrombotic state is generated by the increased production of lesion-based coagulants, such as tissue factor, and the inhibitors of fibrinolysis, such as plasminogen activator inhibitor-1. Vascular remodeling and vasomotor tone are enhanced through reduced NO and an increased activity and production of vasoconstrictors, *e.g.* endothelin-1. (Creager *et al.* 2003, Brownlee 2005, D'Souza *et al.* 2009). Although greatly induced by hyperglycemia, these central pathogenetic mechanims are not specific only to hyperglycemia but are promoted also by other metabolic abnormalities present in diabetes.

The glycosylation of proteins and lipids in the arterial wall is a normal and continuous physiological process, which is directly dependent on the blood glucose concentration. AGEs are irreversible compounds formed when reactive glucose intermediates non-enzymatically react with nucleic acids, transcription factors and intracellular or extracellular proteins (Brownlee 2001). Glycation alters the structure of the molecules and thus disturbs their function and receptor recognition properties. For example, the glycation of apolipoprotein B in the LDL particle reduces the clearance of LDL (Bucala *et al.* 1995), and glycated phospholipids render the LDL particle more susceptible to oxidative modifications (Bucala *et al.* 1993), thus making the LDL more atherogenic. Through the activation of their

receptor (RAGE), AGEs also initiate signaling cascades which potentiate the effects of hyperglycemia. (D'Souza *et al.* 2009, Brownlee 2001, Aronson, Rayfield 2002).

The consequences of hyperglycemia are seen also in the plaque structure, where medial SMCs play an important role. As the plaque develops, SMCs migrate from media to intima and strengthen the plaque by forming a fibrous cap and synthetizing collagen. Vascular SMCs from diabetic patients exhibit increased proliferation, adhesion and migration (Faries *et al.* 2001), which might provoke accelerated lesion development. However, it has been observed that ruptured plaques in general and also the advanced lesions of diabetic patients contain fewer SMCs than the stable plaques or lesions of non-diabetic patients (Fukumoto *et al.* 1998, Libby 2001). One possible reason for this is SMC apoptosis induced by oxidized LDL (Taguchi, Oinuma & Yamada 2000), a process which is enhanced by the hyperglycemic oxidant milieu. Additionally, hyperglycemia-induced endothelial cytokine and proteinase production have been reported to promote plaque destabilization through decreasing the production and increasing the breakdown of collagen (Death *et al.* 2003). Despite the possible stabilizing effect of SMCs, they are also believed to be essential in the development of intimal hyperplasia and the subsequent restenosis, as well as in the negative remodeling of arteries, both of which have been observed to be increased in diabetic patients (Kornowski *et al.* 1997, Gilbert, Raboud & Zinman 2004) and to worsen the outcome of revascularization procedures. Moreover, unlike in vulnerable atherosclerotic lesions, in intact diabetic human arteries, downregulated apoptosis and an excessive accumulation of extracellular matrix have been noted (Chung *et al.* 2007), emphasizing the complexity and versatility of the effects created by diabetes in different types and different phases of the vascular injury.

In addition to worsening the endothelial dysfunction and accelerating atherogenic processes, hyperglycemia predisposes the individual to thrombosis. This is mediated through the effects on the vascular wall and the plasma components of coagulation, but also through a direct influence on platelet function. Platelets of diabetic patients have been observed to represent a hyperreactive phenotype with increased adhesion, activation and aggregation. Hyperglycemia induces these changes by various mechanims including AGE formation and PKC activation, although also IR and dyslipidemia contribute to platelet hyperreactivity. (Ferreiro, Gomez-Hospital & Angiolillo 2010).

2.1.2.1.1.2 Insulin resistance in atherogenesis

IR is an independent risk factor for CVD (Bonora *et al.* 2002). IR causes proatherogenic effects via multiple mechanisms (Figure 3). Firstly, it promotes endothelial dysfunction and a prothrombotic state. Normally, insulin stimulates NO production in endothelial cells by activating NO synthase via the PI-3K pathway. In IR this pathway is impaired, and therefore the production of NO is diminished (Kim *et al.* 2006) and its beneficial effects are lost. In addition to the direct effects of IR on the endothelium, IR in adipose tissue is associated with elevated circulating levels of plasminogen activator inhibitor-1 (PAI-1), which contributes to the impaired fibrinolysis (Trost, Pratley & Sobel 2006). Secondly, IR leads to an excessive release of FFAs from adipose tissue, which evokes pathogenic gene expression through PKC activation and increased oxidative stress (Inoguchi *et al.* 2000). In parallel, the IR-induced excess of FFAs is essential also in the development of dyslipidemia, which further promotes the development of a proatherogenic lipid profile; this will be discussed in more detail below. Thirdly, at the cellular level, IR in macrophages promotes the uptake of atherogenic

lipoproteins and foam cell formation, as well as increases macrophage apoptosis and impairs their ability to phagocytose cellular waste in the atherosclerotic lesions (Liang *et al.* 2007), thus increasing the size of the necrotic core and making the plaques more prone to rupture.

2.1.2.1.1.3 Diabetic dyslipidemia

Both T1D and T2D are associated with lipid abnormalities that promote atherogenesis, although they are much more prevalent in T2D. While some differences exist, a common pattern of dyslipidemia is descriptive for both types of diabetes: elevated TGs, low total HDL and small, dense LDL (sdLDL) particles (Figure 3).

In T2D, IR is the main pathogenetic mechanism underlying the lipid abnormalities and its actions are mediated through effects on both adipose and hepatic tissues. An increased hepatic influx of FFAs enhances the production of VLDL, which when IR is present, cannot be suppressed by insulin. VLDL is normally catabolized by lipoprotein lipase (LPL), which hydrolyzes TG-rich particles. However, when VLDL levels are high, LPL becomes saturated, leading to reduced VLDL catabolism and a longer plasma half-life of the VLDL particles. As a consequence, there is accelerated lipid exchange between the TG-rich VLDL particles and lower density lipoproteins by cholesterol ester transfer protein (CETP). This results in highly atherogenic cholesterol-enriched VLDL remnants, and TG-rich LDL and HDL particles. These LDL and HDL particles are susceptible to lipolysis by hepatic lipase, with the end result being the formation of small, dense HDL and LDL. The HDL of this form is cleared more rapidly and is less able to perform reverse cholesterol transport, which is the main mechanism for removal of cholesterol from extrahepatic tissues to the liver for excretion. In turn, the sdLDL particles demonstrate increased atherogenicity – they enter the arterial wall more easily than larger LDL, their plasma half life is prolonged due to decreased receptor-mediated uptake and they are more susceptible to oxidative modifications. On the whole, the TG concentration is elevated, the levels and cardioprotective characteristics of HDL are decreased, and there is a shift towards an increased number of more atherogenic LDL particles and VLDL remnants. (Betteridge 2000).

In T1D, the lipid abnormalities are clearly related to the level of glycemic control. In untreated T1D, or diabetic ketoacidosis, the elevation in FFAs increases the VLDL concentration. Simultaneously, the catabolism of VLDL particles is diminished, because LPL activity is decreased due to the lack of insulin stimulation and consequently, the levels of LDL and HDL are also decreased. If diabetes is treated but glycemic control is poor, elevated TG levels may persist and in addition, since VLDL catabolism is less decreased due to the partly preserved LPL activity, also elevated LDL levels may occur. However, these abnormalities can be abolished by insulin treatment and in good glycemic control, the situation is normalized: TG and LDL levels are normal or even reduced, and the HDL concentration is normal, or sometimes elevated. (Verges 2009).

The atherogenic effects of diabetic dyslipidemia are diverse. In epidemiological studies, hypertriglyceridemia is found to be a strong and independent risk factor for CVD (Cullen 2000), although the association between sdLDL and CVD is also clear (Carmena, Duriez & Fruchart 2004, Rizzo, Berneis 2007). Both TG-rich lipoproteins and sdLDL particles have been shown to be atherogenic *in vitro*. Therefore, it is somewhat unclear if the increased risk observed in hypertriglyceridemia is caused by direct atherogenic effects of the cholesterol-

enriched VLDL particles, or if it reflects the overall effects of hypertriglyceridemia on the lipid metabolism, including decreased HDL levels and the formation of sdLDL (Cullen 2000).

2.1.2.1.2 Angiogenic paradox in diabetes

In conjunction with the excessive atherosclerosis, additional factors exist that increase the CVD burden in diabetic patients. An abnormal regulation of neovascularization plays an essential role in the vascular complications of diabetes (Simons 2005).

Enhanced angiogenesis, *i.e.* capillary vessel growth in response to hypoxia, can potentially promote the destabilization of atherosclerotic plaques (Khurana *et al.* 2005) and is the main mechanism underlying the development of diabetic retinopathy (Nguyen, Wong 2009). On the other hand, insufficient angiogenesis hinders wound healing and thus contributes to the emergence of diabetic ulcers (Brem, Tomic-Canic 2007). With respect to macrovascular diseases, the main abnormality worsening the clinical picture is defective arteriogenesis (Figure 3). Arteriogenesis is defined as an increase in the diameter of pre-existing arterioles and arteries, and is crucial in the development of collateral vessels in response to arterial occlusion both in the myocardium and lower extremities. A reduced number of collateral vessels has been reported in diabetic patients (Abaci *et al.* 1999, Waltenberger 2001) and there is experimental data showing that their number is also reduced in ischemic hind limbs of diabetic mice (Yan *et al.* 2009a). The defect seems to be more severe in T2D than in T1D (Yan *et al.* 2009a), probably reflecting the additional deteriorating effects of dyslipidemia and other metabolic disturbances which are more prevalent in T2D. Impaired collateral formation leads to worse outcome and poorer recovery after myocardial and peripheral ischemia, accounting to some extent for the increased cardiac mortality and the need for amputation.

The underlying mechanisms of unbalanced neovascularization in diabetes have not been fully elucidated. There are findings indicating defects in signaling of the most potent promoter of vascular growth, vascular endothelial growth factor (VEGF). VEGF-A is essential for embryonic vasculogenesis and in adults it is needed for the maintenance of vascular homeostasis as well as tissue regeneration after injury and ischemia (Ylä-Herttuala *et al.* 2007). It has been postulated that low levels of VEGF-A are crucial for its vasculoprotective effects, such as the maintenance of vascular integrity, NO production and the suppression of SMC proliferation, whereas angiogenic effects, either physiologic or pathologic, occur only with markedly higher concentrations of this factor. In diabetic CAD patients, increased myocardial VEGF expression, but a reduced expression and decreased activation of VEGF receptors have been observed as compared to the situation in non-diabetic subjects (Sasso *et al.* 2005). Glycosylation-mediated inactivation of signal transducers has been suggested as a possible mechanism for the impaired VEGF effects in endothelial cells (Luo, Soesanto & McClain 2008), and an elevated baseline activity of signaling effectors and subsequent resistance to further VEGF stimulation have been observed in monocytes (Tchaikovski *et al.* 2009). In addition to these disturbances with VEGF effects, diabetes is associated with a reduced number and impaired function of endothelial progenitor cells (EPC), which have been proposed to contribute to neovascularization in ischemic tissues (Tepper *et al.* 2002, Loomans *et al.* 2004). However, the net effect of this finding on CVD remains obscure, since in some animal studies, the EPCs have also been associated with the progression of atherosclerosis (Khurana *et al.* 2005, Aicher, Zeiher & Dimmeler 2005).

2.1.2.1.3 Metabolic abnormalities in the diabetic heart

Diabetes induces metabolic changes in the heart that contribute to the increased susceptibility to functional defects and to the poor recovery from ischemic events. The heart is capable of using a variety of substrates to fulfill its continuous need for energy and the metabolic preference varies depending on substrate availability and physiological conditions (e.g. workload, oxygen availability). Under normal conditions, 60-70 % of energy comes from FFAs with the rest being derived mainly from carbohydrates, whose influx into cardiomyocytes is mediated by insulin-stimulated glucose transporters. In diabetes, a switch from glucose further to FFAs occurs, so that up to 90-100 % of energy is being derived from FFAs (Lopaschuk 2002). This might partially be due to blunted insulin action affecting glucose transport as well as the regulation of fatty acid oxidation, although it has been noted that despite a profound peripheral IR, the myocardium of diabetic patients maintains relatively intact responsiveness to insulin (Utriainen *et al.* 1998). Since fatty acids are less efficient as an energy source requiring more oxygen than glucose to produce the equal amount of energy, this switch decreases cardiac efficiency. While in a stressed state, the heart normally switches to glucose usage, in diabetes it is no longer able to modulate its substrate selection, rendering it extremely sensitive to changes in oxygen availability. As the blood flow in ischemic conditions is reduced, this impairs the transport of substrates, and the energy is provided mainly by anaerobic glycolysis. If and when reperfusion occurs, the recovery will be the better the more energy can be produced by glucose oxidation (Lopaschuk 2002). Therefore, the diabetic heart is less able to resist ischemic conditions and the recovery after an ischemic event is impaired.

2.1.2.1.4 Diabetic cardiomyopathy

Although accelerated atherosclerosis plays a major role in the increased cardiovascular mortality in diabetes, there is epidemiological data indicating that diabetes increases the risk of cardiac dysfunction and heart failure also independently of the coexistence of coronary artery disease (CAD), hypertension, or other macrovascular risk factors (Fang, Prins & Marwick 2004). The term diabetic cardiomyopathy (DCM) has been introduced to describe this distinct clinical entity characterized by the functional (primarily diastolic dysfunction) and structural (hypertrophy) changes in the left ventricle, leading to heart failure.

Although the pathophysiological mechanisms leading to DCM are still elusive, several associated factors have been proposed. Hyperglycemia has been shown to strongly relate to the incidence of DCM (The Diabetes Control and Complications Trial Research Group 1993). It is considered to be the underlying factor triggering other contributing changes such as myocardial metabolic disturbances, microangiopathy and myocardial fibrosis. In fact, it is the microvascular changes that can cause reduced myocardial perfusion and subsequent ischemia, and therefore the categorization of DCM into micro- or macrovascular complications is unclear.

2.1.2.2 Microvascular disease in diabetes

The microvasculature consists of arterioles, capillaries and venules. Their function is to regulate tissue perfusion, provide exchange surface between plasma and tissues and to regulate blood pressure (Ko, Cao & Liu 2010). Hyperglycemia is the single most important

factor behind microvascular diseases and there is clear evidence about the benefit of good glycemic control in the prevention and delay of microvascular complications in both T1D and T2D (UK Prospective Diabetes Study Group 1998, The Diabetes Control and Complications Trial Research Group 1993).

The microvascular disease associated with diabetes particularly damages those organs whose blood supply depends heavily on the microvasculature: retina, kidneys and peripheral nerves. Despite the differences in target organs, the main pathophysiological characteristics in the development of microvascular complications are similar. As is the case in the macrovasculature, hyperglycemia disturbs the regulation of vascular tone and increases permeability also in the microvessels. After prolonged exposure to elevated levels of glucose, the overproduction of extracellular matrix and the deposition of extravasated plasma proteins start to occlude capillaries, and there is death of microvascular cells. In the retina, this leads to the loss of neuronal cells and pericytes, edema, ischemia and hypoxia-induced neovascularization, manifest as diabetic retinopathy. In the kidneys, podocyte loss, increase in mesangial matrix, glomerulosclerosis and glomerular hyperfiltration lead via microalbuminuria to proteinuria, manifest as diabetic nephropathy. In the peripheral nerves, a dysfunction of the microvasculature supplying the nerves causes neuronal ischemia and multifocal axonal degeneration, underlining the defects characterizing diabetic neuropathy. (Brownlee 2001, Orasanu, Plutzky 2009).

Microvascular dysfunction also affects the heart and skeletal muscle, which both contain an extensive microvascular network. Capillary rarefaction, *i.e.* the reduction in the number of perfused capillaries, has been observed in skeletal muscle samples of subjects with T2D (Levy *et al.* 2008). In addition to lowering the threshold for ischemia, impaired microvascular perfusion might further reduce glucose uptake in the muscle and thus worsen IR. Myocardial microvascular abnormalities have been associated with the development of diabetic cardiomyopathy and might also underlie the impairment in coronary flow reserve, which is characteristic to diabetic patients even in the absence of detectable coronary stenosis (Fang, Prins & Marwick 2004, Levy *et al.* 2008). This intersection of micro- and macrovascular pathologies is a concrete example highlighting the complexity of diabetes and emphasizes the nature of (especially type 2) diabetes as a vascular disease.

2.2 MOUSE MODELS OF DIABETES AND CARDIOVASCULAR DISEASE

Whereas cell culture and other *in vitro* studies provide information on specific matters relating to a certain disease, many etiological processes and therapeutical responses need to be studied in a living organism, *in vivo*, where relevant physiological conditions and interactions between different cell type and tissues exist. The animal model of a disease can be homologous, *i.e.* having the same cause, symptoms and treatment options as humans with the same disease. Predictive models, on the other hand, display the symptoms of a human disease without a known cause and can be used for etiological studies and the screening of candidate disease genes. However, most models are isomorphic, since the etiology of experimentally induced disease often differs from that of spontaneously occurring disease in man. Nevertheless, isomorphic models present symptomatic resemblance to the human disease, share the same treatment options and are thus useful in experimental and translational research.

In an attempt to simulate the pathophysiological events in humans, some modification of the animal model is often required due to physiological and metabolic differences. Thus, genetic manipulation, dietary and/or chemical induction are typically used to improve the applicability of laboratory animals as disease models. Mice are the most widely used experimental animals in biomedical research for various reasons: their genome has been sequenced (Mouse Genome Sequencing Consortium 2002), available and workable, and inbred strains are genetically uniform, which reduces variance in the study populations. Mice are cost-effective, easy to maintain, and suitable by their lifespan for research purposes. Their general biology is closely comparable to that of humans, although some relevant differences do exist that should be taken into consideration when using mice in diabetic cardiovascular research (Table 2). However, the wide availability of techniques to modify the mouse genome has provided an abundance of transgenic and knock-out models for different research applications.

Table 2. Major pathophysiological differences between human and mouse relevant in studying cardiovascular diseases. Modified from Goldberg & Dansky (2006) and Getz (2007).

Characteristic	**Human**	**Mouse**
Life span (years)	≈ 80	≈ 2-3
Heart rate (beats/minute)	60-80	> 300
Normal diet	High fat, high cholesterol	Low fat, low cholesterol
Plasma cholesterol (mmol/l)	≈ 5.0	≈ 2.0
Major lipoprotein	LDL	HDL
Cholesterol synthesis (mg/day/kg)	10	160
Hepatic LDL clearance (ml/day/kg)	12	500
ApoB subtypes in liver	B100	B48 and B100
Apo(a) expression	Yes	No
CETP	Yes	No
HDL in disease	Reduced in T2D	Not reduced in obesity/IR
Acute phase protein	CRP	SAA, SAP
Age of disease manifest		
Type 1 diabetes	Early adult	Young adult
Type 2 diabetes	Middle aged to older	Young adult
Cardiovascular diseases	Middle aged to older	Young adult
Aortic sinus atherosclerosis	No	Common
Coronary artery atherosclerosis	Common	Rare
Hypertension	Common	No

Apo, apolipoprotein; CETP, cholesterol ester transfer protein; CRP, C-reactive protein; HDL, high-density lipoprotein; LDL, low-density lipoprotein; SAA, serum amyloid A; SAP, serum amyloid P.

In recent years, considerable input has been put into developing animal models for diabetic complications. In addition to vigorous research, joint undertakings such as the "Animal Models of Diabetic Complications Consortium" (AMDCC) by the National Institute of Health in the United States, and the consortium of "Surrogate markers for Micro- and Macro-vascular hard endpoints for Innovative diabetes Tools" (SUMMIT) by Innovative Medicines Initiative in the European Union address this matter. Because of the variety of models used in the literature and the major impact of the model selection on study results, knowledge about the basic aspects of different models is crucial for choosing the appropriate model for each study set up and for the interpretation of the results.

2.2.1 Diabetic atherosclerosis models

Mice are naturally very resistant to atherosclerosis due to species-specific characteristics of lipoprotein metabolism. In humans, the most abundant apolipoprotein (apo) B subtype is

apoB100, which is produced only in the liver and is the major protein component in VLDL, LDL and intermediate-density lipoprotein (IDL) particles. The minor subtype is apoB48, which is synthetized in the intestine and functions as a part of chylomicrons transporting ingested lipids into the liver. However, some rodents, like rats and mice, are able to produce apoB48 also in the liver (Greeve *et al.* 1993). Therefore the majority (~70 %) of all apoB in mice is apoB48, in contrast to apoB100 in humans. In addition, in mice, the clearance of LDL is much faster than in humans (Dietschy, Turley 2002). Mice also lack CETP activity, which in humans transfers cholesterol esters from HDL to atherogenic LDL and VLDL particles, and effectively lowers HDL levels (Hogarth, Roy & Ebert 2003). Consequently, in mice ~90 % of cholesterol is carried in HDL and only a fraction in proatherogenic LDL and VLDL (Figure 4). Nevertheless, once mice have plasma cholesterol levels of over ~5.0 mmol/l and the cholesterol is primarily in non-HDL particles, they will eventually develop atherosclerosis. However, genetic modification is needed to achieve this status.

The most common hyperlipidemic mouse models include the apolipoprotein E knock-out mouse ($ApoE^{-/-}$) and the mouse deficient in LDL receptor ($LDLR^{-/-}$). $ApoE^{-/-}$ mouse was one of the first models developed to study atherosclerosis (Plump *et al.* 1992, Piedrahita *et al.* 1992). The depletion of apoE results in the accumulation of cholesterol-rich remnant particles, causing a ~4-fold increase in total cholesterol levels and the formation of atherosclerotic lesions throughout the aortic tree. However, when compared to humans, $ApoE^{-/-}$ mice have a very different remnant-like lipoprotein profile (Figure 4) and lack all the functions of apoE on cholesterol homeostasis, cellular cholesterol efflux and inflammatory responses (Curtiss, Boisvert 2000). The $LDLR^{-/-}$ mice were developed quite concurrently with $ApoE^{-/-}$ mice (Ishibashi *et al.* 1993). Although in humans LDLR deficiency leads to a syndrome called familial hypercholesterolemia with the onset of profound atherosclerosis already in childhood (Goldstein, Brown 1989), the phenotype of the $LDLR^{-/-}$ mouse is notably milder, since mice are able to uptake lipoproteins also via the LDLR related protein receptor. Thus, the resulting twofold increase in total cholesterol is not sufficient to induce lesion development in the $LDLR^{-/-}$ mice without a high-fat diet. Due to these shortcomings, modifications have been made into these models. The synthesis of apoB48, the predominant apoB form in mice, has been inhibited in both $ApoE^{-/-}$ and $LDLR^{-/-}$ backgrounds either by targeted mutations (Véniant *et al.* 1998, Véniant *et al.* 2000) or by preventing the enzymatic editing of apoB mRNA (Powell-Braxton *et al.* 1998, Osuga *et al.* 1997). If all these models are considered, then it is the $LDLR^{-/-}$ mouse synthetizing only apoB100 ($LDLR^{-/-}ApoB^{100/100}$ and $LDLR^{-/-}Apobec\text{-}1^{-/-}$ mice) that best represents the human-like lipoprotein profile (Figure 4).

The development of murine models describing diabetic macrovascular diseases has been challenging and at present there is no perfect model of the human disease. However, numerous models have been generated that each possess specific attributes making them useful for studying both the mechanistic and therapeutic aspects of the vascular complications of diabetes. In view of the expanding diabetes epidemic worldwide, any new information from animal models is positive, even if the model does not perfectly represent the human condition; however, translation into human conditions should be done cautiously.

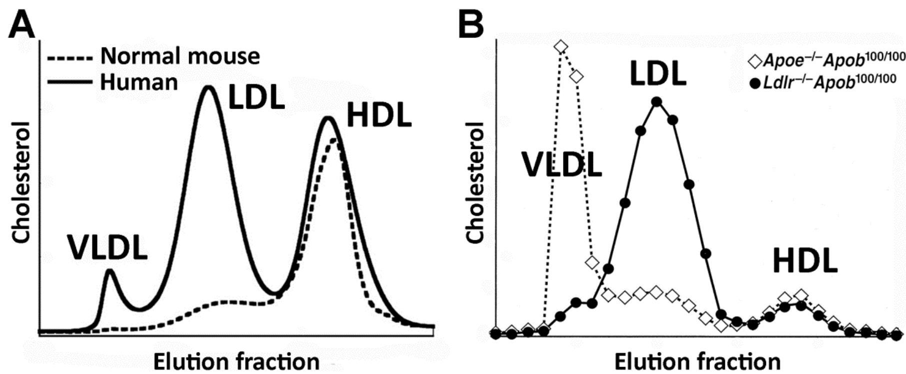

Figure 4. Distribution of cholesterol within the size-fractionated plasma lipoproteins in A) humans and a normal mouse, and B) in genetically modified hyperlipidemic mouse models with either ApoE$^{-/-}$ or LDLR$^{-/-}$ background. Adapted from Véniant, Withycombe & Young (2001) and Steenbergen *et al.* (2010).

2.2.1.1 Atherosclerosis models with chemically induced diabetes

Some chemical agents are able to induce a diabetic state in experimental animals. Alloxan and streptozotocin (STZ) are toxic glucose analogues, which accumulate in pancreatic β-cells. Their cytotoxicity is mediated via the production of ROS (alloxan) or alkylating properties (STZ), which cause β-cell death leading to insulinopenic diabetes with hyperglycemia. Because of the better stability, specificity and dose-response reproducibility, STZ is used more commonly (Lenzen 2008). However, STZ does not fully replicate T1D since it usually does not induce ketosis and treated mice do not require insulin therapy. Some of the atherosclerosis studies using chemically induced diabetes are summarized in Table 3.

Kunjathoor *et al.* (1996) were the first to study the effects of STZ-induced diabetes on atherosclerotic lesion development in mice. They found that STZ alone did not induce lesion formation, but when combined with an atherogenic diet, hyperglycemia accelerated lesion initiation in Balb/c but not in C57Bl/6 mice. However, in these strains, the observed lesions were limited only to the early stages of fatty streaks. Therefore, to generate a more human-like atherosclerosis, mice with modified lipid metabolism have mostly been used in subsequent experiments.

Park *et al.* (1998) reported the use of STZ in atherosclerotic ApoE$^{-/-}$ mice. Hyperglycemic ApoE mice showed accelerated lesion development and at the level of aortic sinus an over 5-fold increase in the lesion area. STZ treatment also caused a significant elevation in total plasma cholesterol level, which most probably had some atherogenic influence. However, accelerated lesion development could be fully suppressed by soluble RAGE in a lipid-independent manner. This was interpreted to highlight the atherogenic impact of hyperglycemia, although subsequently soluble RAGE has been established also to have general anti-inflammatory properties beyond its effects on hyperglycemia (Yan *et al.* 2009b). Since the study of Park and colleagues, ApoE$^{-/-}$ mice with STZ have been widely used in the studies of diabetic atheroclerosis and the model has been well characterized (reviewed in Wu & Huan 2007). The focus of these studies has been on exploring the effects of various anti-hypertensive, hypolipidemic and anti-diabetic agents on diabetic atherosclerosis and elucidating the mechanisms of accelerated atherosclerosis. The role of oxidative stress has been investigated in STZ-injected ApoE$^{-/-}$ mice lacking the antioxidant enzyme glutathione

peroxidase-1 ($ApoE^{-/-}GPx1^{-/-}$ mice) (Lewis *et al.* 2007). The lack of GPx1 further increased lesion development in the diabetic context, emphasizing the importace of antioxidant defense in diabetes-associated atherosclerosis. However, one major drawback of STZ diabetes is the abovementioned secondary hyperlipidemia - in most studies concomitant elevations in lipids in diabetic versus non-diabetic $ApoE^{-/-}$ mice have arisen and these changes are exacerbated if high-fat diets are used. Only in two studies, both with normal diets, did STZ diabetes not significantly increase cholesterol or TG levels, but nevertheless increased not only lesion development as such (Lewis *et al.* 2007, Tse *et al.* 1999) , but also the amount of calcified lesions (Tse *et al.* 1999).

When STZ was used in $LDLR^{-/-}$ mice for the first time, no diabetes-induced changes in plasma lipids or atherosclerosis were noted (Reaven *et al.* 1997). This was most probably due to the extremely long period of atherogenic diet (6 months), during which the lesion development perhaps plateaued in the presence of exceptionally high cholesterol levels and the possible effects of hyperglycemia were masked. Subsequently, by using a more short-term (6 weeks) dietary induction of hyperlipidemia, it was found that STZ does accelerate lesion initiation in $LDLR^{-/-}$ mice, although this was accompanied again by elevations in the levels of cholesterol and TG (Keren *et al.* 2000, Vikramadithyan *et al.* 2005). This has also been the case in $LDLR^{-/-}Apobec\text{-}1^{-/-}$ mice (Hammad *et al.* 2003). Since conclusions from studies with such lipid changes are suspect, other strategies have been pursued in attempts to avoid this problem. Goldberg *et al.* (2004) modified the $LDLR^{-/-}$ mice to present a more human-like lipoprotein profile by crossing them with apolipoprotein AI deficient mice. When the mice were fed a low-fat, high-cholesterol diet, STZ diabetes did not result in lipid changes, but neither did it affect lesion development.

To unravel potential mechanisms explaining the difficulties in reproducing diabetes-accelerated atherosclerosis in mice, the role of aldose reductase (AR) has been investigated in STZ-induced hyperglycemia. AR mediates the generation of toxic products from glucose and is normally expressed at much lower levels in mice than humans. Therefore the lack of AR was proposed to be one possible factor hindering the manifestation of the vasculotoxic effects of hyperglycemia. Vikramadithyan *et al.* (2005) used both $LDLR^{-/-}$ and $LDLR^{+/-}$ mice which were crossed with mice expressing human AR. They found that only in the presence of hyperglycemia, did the expression of hAR increase atherosclerosis and this occurred in both backgrounds. Recently, a similar finding was made also in $ApoE^{-/-}$ background (Vedantham *et al.* 2011). Despite these consistent results, the role of AR is not totally straightforward - conflicting results pointing to a protective role for AR in diabetes-associated atherosclerosis were reported by Srivastava *et al.* (2009), who found that AR deficiency reduced lesion formation in STZ diabetic $ApoE^{-/-}$ mice.

In addition to the traditional atherosclerosis models, the effects of STZ diabetes have been studied also in other settings. Kako *et al.* (1999) used mice over-expressing human apoB (HuB). When fed a Western diet, diabetic HuB mice had less lesions despite increased lipid levels. However, in a later study, this finding was not reproduced, as STZ caused no changes in lipids or atherosclerosis in HuB mice (Kako *et al.* 2002). The authors then sought to modify the model towards a more human-like lipid profile and generated HuB mice with a heterozygous deletion of LPL (Kako *et al.* 2002). In this model, both increased plasma lipids and greater lesion amount were observed after STZ. Surprisingly, the induction of CETP expression in these diabetic $HuB/LPL^{+/-}$ mice reduced both hyperlipidemia and atherosclerosis. It was speculated that although CETP expression had caused an expected

reduction in HDL levels, the HDL level was still sufficiently high to provide antiatherogenic protection.

Table 3. Atherosclerotic mouse models with chemically induced diabetes. The conceptual idea for the table was obtained from Goldberg & Dansky (2006) and Wu & Huan (2007).

Mouse model	Inducer	DM	Hyper-lipidemia	Athero-sclerosis	Comments	Reference
Diabetes does not affect atherosclerosis						
C57Bl/6J	STZ + AD (12-20 wks)	1	TC↑ (7.5 mM) TG↔ (0.2 mM)	↔	Cholate	Kunjathoor, 1996
LDLR$^{-/-}$	STZ + AD (6 mos)	1	TC↔ (25.1 mM) TG↔ (4.6 mM)	↔		Reaven, 1997
HuB	STZ + WD (18wks)	1	TC↑ (14.0 mM) TG↔ (2.5 mM)	↓		Kako, 1999
HuB	STZ + WD (20 wks)	1	TC↔ (9.7 mM) TG↔ (2.0 mM)	↔		Kako, 2002
HuB/LPL$^{+/-}$CETP	STZ + WD (20 wks)	1	TC↔ (8.8 mM) TG↔ (3.1 mM)	↔		Kako, 2002
LDLR$^{-/-}$ApoAI$^{-/-}$	STZ + CD (11 wks)	1	TC↔ (10.4 mM) TG↔ (0.9mM)	↔		Goldberg, 2004
LDLR$^{+/-}$	STZ + HFHC (1 mo)	1	TC↑ (21.4 mM) TG↔ (1.3 mM)	↔	Cholic acid	Berti, 2005
LDLR$^{+/-}$CETP	STZ + HFHC (1 mo)	1	TC↑ (23.4 mM TG↔ (1.3 mM)	↔	Cholic acid	Berti, 2005
ApoE$^{-/-}$	Gold thioglucose	2	TC↑ (19.8 mM) TG↑ (1.1 mM)	↓ x 2.5	Leptin ↑	Lyngdorf, 2003
Diabetes affects atherosclerosis and alters plasma lipid levels						
Balb/c	STZ + AD (12-20 wks)	1	TC↑ (9.8 mM) TG↑ (0.6 mM)	↑ x 17	Cholate	Kunjathoor, 1996
ApoE$^{-/-}$	STZ	1	TC↑ (35.3 mM) TG↔ (1.7 mM)	↑ x 5	sRAGE → lesions ↓	Park, 1998
LDLR$^{-/-}$	STZ + WD (6 wks)	1	TC↑ (44.3 mMl) TG↑ (6.3 mM)	↑ x 6.5		Keren, 2000
HuB/LPL$^{+/-}$	STZ + WD (20 wks)	1	TC↑ (13.8 mM) TG↑ (5.5 mM)	↑ x 14		Kako, 2002
LDLR$^{-/-}$Apobec-1$^{-/-}$	STZ	1	TC↑ (9.9 mM) TG↔ (1.6 mM)	↑ x 1.5		Hammad, 2003
LDLR$^{-/-}$	STZ + HCD (12 wks)	1	TC↑ (91.6 mM) TG↔ (1.3 mM)	↑ x 3		Vikramadithyan, 2005
LDLR$^{-/-}$hAR	STZ + HCD (12 wks)	1	TC↑ (95.2 mM) TG↔ (1.0 mM)	↑ x 4	vs. LDLR$^{-/-}$ + STZ: lipids ↔, lesions ↑ x 2	Vikramadithyan, 2005
Diabetes affects atherosclerosis without changes in plasma lipids						
ApoE$^{-/-}$	STZ	1	TC↔ (9.1 mM) TG↔ (1.3 mM)	↑ x 2	Calcified lesions	Tse, 1999
ApoE$^{-/-}$GPx1$^{-/-}$	STZ	1	TC↔ (13.8 mM) TG↔ (2.1 mM)	↑ x 4		Lewis, 2007
LDLR$^{+/-}$	STZ + CCA (12 wks)	1	TC↔ (14.1 mM) TG↔ (0.6 mM)	↑	Cholic acid	Vikramadithyan, 2005
LDLR$^{+/-}$hAR	STZ + CCA (12 wks)	1	TC↔ (13.6 mM) TG↔ (0.6 mM)	↑ x 2	Cholic acid; vs. LDLR$^{+/-}$ + STZ: lipids ↔, lesions ↑ x 2	Vikramadithyan, 2005
ApoE$^{-/-}$hAR	STZ	1	TC↔ (31.1 mM) TG↔ (3.1 mM)	↑ x 2		Vedantham, 2011

AD, atherogenic diet (12.5-40 % energy from fat, 0.075-1.5 % cholesterol, possibly with 0.5 % sodium cholate); CCA, cholesterol/cholic acid–containing diet (1.25 % cholesterol and 0.5 % sodium cholate); CD, cholesterol diet (normal chow with 0.5 % cholesterol); DM, diabetes mellitus; HCD, high-cholesterol diet (20 % energy from fat, 0.15-1.25 % cholesterol); HFHC, high-fat/high-cholesterol diet (20 % fat, 1.25 % cholesterol, and 0.5 % cholic acid); sRAGE, soluble receptor for AGEs; STZ, streptozotocin; TC, total cholesterol; TG, triglycerides; WD, Western diet (Harlan Teklad TD96125 or TD88137). For the other abbreviations, see text.

Although STZ has been widely and quite reproducibly used in studies of diabetic atherosclerosis, there are some drawbacks to its use. Firstly, STZ induces insulinopenic diabetes, which in humans is not usually found in conjunction with severe hypercholesterolemia. Secondly, STZ can produce non-specific toxic effects, especially if a single high-dose injection is used, making it difficult to interpret if changes are caused by hyperglycemia or by the toxin itself (Imaeda *et al.* 2002, Koulmanda *et al.* 2003, Palm *et al.* 2004, Inada *et al.* 2008). Thirdly, as noted previously, STZ diabetes evokes major changes in plasma lipoproteins and leads to more extensive hyperlipidemia in most mouse strains, making it difficult to examine the direct effects of hyperglycemia. The mechanism causing increased lipid levels seems to be reduced catabolism of remnant lipoproteins – STZ diabetes causes a defect in liver proteoglycans, which normally account for the trapping and rapid clearance of apoB48 containing lipoproteins from the plasma (Ebara *et al.* 2000, Goldberg *et al.* 2008). Here also diet selection plays a major role. A good example is the study of Vikramadithyan *et al.* (2005), where STZ diabetes increased both lipid levels and atherosclerosis when LDLR$^{-/-}$ mice were fed a high-fat diet. However, when lesion development in heterozygous LDLR$^{+/-}$ mice with milder hyperlipidemia was induced with a cholesterol/cholic acid-containing diet, hyperglycemia was able to accelerate atherosclerosis in the absence of lipid changes. Furthermore, a cholic acid-containing high-fat diet elevated cholesterol levels but nevertheless did not increase atherosclerosis in STZ-treated LDLR$^{+/-}$ mice with or without CETP expression (Berti *et al.* 2005). In addition to these general interpretation difficulties caused by different diets, cholate-containing diets are especially problematic, since in addition to inducing hypercholesterolemia by promoting cholesterol absorption and suppressing the conversion of cholesterol to bile acids, cholic acid has pleiotrophic and even toxic effects *e.g.* triggering inflammation and interfering with liver function and these may modify atherogenesis in ways not relevant to human disease. Therefore its use in atherogenic diets is no longer recommended (Getz, Reardon 2006).

Since STZ is not optimal in the study of diabetic atherosclerosis, other strategies using a chemical induction of T2D features have been implemented. One such approach is the induction of central hyperphagia by a single i.p. injection of gold thioglucose (GTG), which destroys the hypothalamic satiety center. As a result, mice develop significant obesity and subsequently T2D-like perturbations in glucose metabolism (Karasawa, Takaishi & Kumagae 2011). Lyngdorf *et al.* (2003) employed this strategy in ApoE$^{-/-}$ mice. Surprisingly, despite the diabetic phenotype and slightly increased plasma cholesterol and TG levels, GTG-treated ApoE$^{-/-}$ mice displayed significantly less atherosclerosis than control ApoE$^{-/-}$ mice.

As a summary, chemical induction of diabetes has mostly relied on the use of STZ. It is a well-established method for inducing T1D-like hyperglycemia and *e.g.* useful for investigating the roles of certain genes (knock-out strategies) or studying the specific effects of pharmacological agents. However, relevant control groups should always be included so that the contribution of hyperglycemia versus lipids as well as the possible non-specific effects of STZ to the end result can be controlled.

2.2.1.2 Atherosclerosis models with diet-induced diabetes

One very common way of developing mouse models with type 2 diabetic atherosclerosis is by diet induction. Models generated by this approach are summarized in Table 4. With a

Table 4. Atherosclerotic mouse models with diet-induced diabetes. The concept for the table was obtained and adapted from Goldberg & Dansky (2006) and Wu & Huan (2007).

Mouse model	Inducer	DM	Hyper-lipidemia	Athero-sclerosis	Comments	Reference
Diabetes does not affect atherosclerosis						
C57Bl/6	DD (6-14 wks)	2	TC↑ (4.8 mM) TG↓ (0.2 mM)	Fatty streaks	Insulin ↑, IR	Schreyer, 1998
NOD	PD (18 wks)	1	TC↑ (8.8 mM) TG↑ (1.3 mM)	None	Cholate; hyperglycemia	Keren, 2001
NOD	WD (18 wks)	1	TC↑ (6.8 mM) TG↑ (2.8 mM)	None	Hyperglycemia	Keren, 2001
ApoE$^{-/-}$	DD (16 wks)	2	TC↔ (10.2 mM) TG↔ (0.6 mM)	↔	No effect on glucose metabolism	Schreyer, 2003
LDLR$^{-/-}$	HFD (20 wks)	2	TC↑ (18.2 mM) TG↓ (2.8 mM)	↔	Glucose ↑, insulin ↑, IR	Wu, 2006
LDLR$^{-/-}$hAR	HFD (20 wks)	2	Similar as above	↔	Similar as above	Wu, 2006
Diabetes affects atherosclerosis and alters plasma lipid levels						
LDLR$^{-/-}$	WD (17 wks)	2	TC↑ (27.0 mM) TG↑ (4.3 mM)	↑	Glucose ↑, insulin ↑, vs. HFD w/o cholesterol + similar dysglycemia: lesions ↑	Towler, 1998
LDLR$^{-/-}$	WD (4.5 mos)	2	TC↑ (25.0 mM) TG↑ (2.6 mM)	↑	Insulin ↑, IR; vs. fructose diet w/ equal cholesterol + euglycemia: lesions ↔	Merat, 1999
LDLR$^{-/-}$	DD (16 wks)	2	TC↑ (17.9 mM) TG↑ (5.0 mM)	↑	Glucose ↑, insulin ↑, IR	Schreyer, 2003
ApoE$^{-/-}$	WD (5 wks)	2	TC↑ (33.8 mM) TG↑ (1.1 mM)	NI ↑	Glucose ↑, insulin ↑	Phillips, 2003
ApoE*Leiden	HCD (28 wks)	2	TC↑ (20 mM) TG↑ (1.7 mM)	↑	IR	Zadelaar, 2006
HuB	HFD (12 mos)	2	TC↑ (3.5 mM) TG↑ (1.0 mM)	Fatty streaks	Glucose ↑, insulin ↑, IR	Bartels, 2009
Diabetes affects atherosclerosis without changes in plasma lipids						
ApoE$^{-/-}$	HFD (17 wks)	2	TC↔ (15.6 mM) TG↔ (1.1 mM)	↑	Glucose ↑, IR vs. mice w/ equal lipids	King, 2010
Other potential models						
L-PGDS$^{-/-}$	HFD (20 wks)	2	-	↑ x 5	Nephropathy	Ragolia, 2005

DD, diabetogenic diet (Bioserve F1850: 35.5 % energy from fat); DM, diabetes mellitus; HCD, high-cholesterol diet (20 % energy from fat, 0.15-1.25 % cholesterol); HFD, high-fat diet (20-60 % energy from fat, usually no cholesterol); NI, neointima; PD, Paigen diet (1.25 % cholesterol, 0.5 % sodium cholate); TC, total cholesterol; TG, triglycerides; WD, Western diet (Harlan Teklad TD96125 or TD88137). For the other abbreviations, see text.

dietary induction of diabetes, results depend greatly on the diet composition: some diets induce obesity and IR, whereas others cause mostly hyperlipidemia without major alterations in glucose metabolism. For example, the high-fat Western diet elevates plasma cholesterol more than high-fat diets rich in carbohydrates, and this *per se* can affect lesion development (Temel, Rudel 2007). There is also extensive variation in the effects between different mouse strains and genetic models. C57BL/6 mice are the most atherosclerosis sensitive strain, but seem to be quite resistant to diabetes-accelerated atherosclerosis (Schreyer, Wilson & LeBoeuf 1998). The so-called diabetogenic diet induces obesity, mild diabetic phenotype, profound hyperlipidemia and increased atherosclerosis in LDLR$^{+/-}$ mice,

whereas $ApoE^{-/-}$ mice are resistant to changes other than obesity (Schreyer *et al.* 2002, Schreyer *et al.* 2003). In addition, when fed a Western diet, the effect on glucose metabolism is more marked in $LDLR^{-/-}$ mice (Towler *et al.* 1998, Merat *et al.* 1999, Karagiannides *et al.* 2008) than in $ApoE^{-/-}$ mice, which only develop profound hyperlipidemia but are mainly protected against the diabetic effects (Karagiannides *et al.* 2008, Phillips *et al.* 2003, Su *et al.* 2006). Overall, the diabetic features induced by diet are so subtle that it is likely that the increases noted in atherosclerosis are a consequence of the hyperlipidemia rather than to alterations in glucose metabolism. Consistently, in the study of Wu *et al.* (2006) the expression of hAR that aggravated the effects of STZ-induced hyperglycemia (Vikramadithyan *et al.* 2005) had no effect in a milder diabetic phenotype induced by diet. In the same study, diet-induced diabetic features did not affect lesions in $LDLR^{-/-}$ mice when compared to euglycemic $LDLR^{-/-}$ mice with equal lipid levels, supporting the superior effect of hyperlipidemia. Only when $ApoE^{-/-}$ mice were fed an extremely high-fat diet without cholesterol, glucose intolerance and increased lesion formation without significant elevation in total cholesterol occurred (King *et al.* 2010).

Aside from inducing diabetes in hyperlipidemic models, Ragolia *et al.* (2005) investigated prostaglandin D_2 synthase (L-PGDS) knock-out mice. These mice became insulin resistant on diabetogenic diet and developed more atherosclerosis compared to both C57Bl/6 controls and insulin sensitive $L\text{-}PGDS^{-/-}$ mice. However, the impact of IR remains elusive, since plasma lipid values were not reported. In non-obese diabetic (NOD) mice, a spontaneous model of T1D, high-fat diets with and without cholate have been used in an attempt to induce atherosclerosis (Keren *et al.* 2001). Although modest increases in lipid levels were attained in hyperglycemic versus normoglycemic animals, no lesion formation was observed. This reflects the genetically predetermined resistance to atherosclerosis in some strains and underlines the importance of background strain selection and genetic modification if atherosclerosis is to be studied in mice.

In conclusion, inducing diabetic traits by diet is a common method, although it usually results in a rather mild diabetic phenotype, which in most cases does not appear to affect atherosclerosis without concomitantly raised plasma lipid levels. In addition, notable variance in diet compositions and durations complicate direct comparisons of studies.

2.2.1.3 Genetic models of diabetic atherosclerosis

Attempts to model diabetic macrovascular disease by modifying genes involved in carbohydrate and/or lipid metabolism have produced numerous genetically modified mouse models (summarized in Table 5). In order to avoid the disadvantages of STZ, Renard *et al.* (2004) studied the effects of T1D on atherosclerosis in $LDLR^{-/-}$ mice expressing a viral glycoprotein (GP) under the insulin promoter ($LDLR^{-/-}$GP mouse). In these mice, T1D can be induced by lymphocytic choriomeningitis virus infection, which causes an autoimmune-mediated destruction of β-cells. When fed a cholesterol-free diet, $LDLR^{-/-}$GP mice did not develop diabetes-induced lipid abnormalities but nonetheless showed an increased lesion area. However, on cholesterol-rich diets diabetes aggravated hyperlipidemia and led to increased lesion amount. This suggested that in a setting of moderate hyperlipidemia (produced by the $LDLR^{-/-}$ background) hyperglycemia was likely to stimulate lesion initiation, whereas it is mainly the secondary lipid abnormalities that contribute to the progression of lesions. This was subsequently confirmed by Johansson *et al.* (2008), who observed that the vulnerable plaque phenotype found in $LDLR^{-/-}$GP mice was abolished

with a reduction of secondary hypertriglyceridemia. A model of spontaneous T1D, the Akita mouse ($Ins2^{Akita}$) (Yoshioka *et al.* 1997, Wang *et al.* 1999), has also been utilized in atherosclerosis studies. In these mice, apoptosis of β-cells is caused by ER stress following the improper folding of proinsulin (Yoshinaga *et al.* 2005). Recently, the Akita mice have been crossed with both $LDLR^{-/-}$ (Zhou *et al.* 2011) and $ApoE^{-/-}$ mice (Jun, Ma & Segar 2011). In both backgrounds, diabetes increased atherosclerosis with accompanying elevations in lipid levels, probably by altering the hepatic expression of genes regulating lipid homeostasis. Although not optimal, these genetic models provide better and more relevant tools for studying T1D-related macrovascular complications than the use of STZ.

Although hyperglycemia as such is a strong risk factor for diabetic vascular complications, additional factors contribute especially to the development of diabetic macrovascular diseases. Thus, it would be essential to achieve models representing the overall risk profile combining diabetic metabolic changes and atherosclerosis. To date, this has mostly been pursued by cross-breeding leptin-deficient (ob/ob) or leptin receptor–deficient (db/db) mice with atherosclerotic $ApoE^{-/-}$ (Wu *et al.* 2005, Gruen *et al.* 2006, Wendt *et al.* 2006) and $LDLR^{-/-}$ mice (Gruen *et al.* 2006, Wendt *et al.* 2006, Hasty *et al.* 2001). Recently the ob/ob mouse was cross-bred also with $ApoE^{-/-}ApoB^{100/100}$ and $LDLR^{-/-}ApoB^{100/100}$ mice (Lloyd *et al.* 2008), resulting in promising models of the metabolic syndrome. However, also in these models, the major shortcoming is that diabetes does not appear to affect atherosclerosis without large concomitant changes in plasma lipid levels (Goldberg, Dansky 2006, Kennedy *et al.* 2010). In fact, when compared to hyperlipidemic controls with similar cholesterol leves, leptin deficiency can actually protect from atherosclerosis in both $LDLR^{-/-}$ (Taleb *et al.* 2007) and $ApoE^{-/-}$ backgrounds (Chiba *et al.* 2008).

The effects of obesity-induced IR on atherosclerosis have been studied also in other models of obesity. When $LDLR^{-/-}$ mice were crossed with yellow agouti (A^{y}/a) mice, a spontaneous model of adult-onset obesity (Coenen, Hasty 2007), no increase in lesion formation was seen despite significant obesity, IR, and elevated cholesterol and TG levels compared to the respective $LDLR^{-/-}$ controls. A similar study was also carried out in $ApoE^{-/-}$ mice (Gao *et al.* 2007). Unexpectedly, the lack of apoE totally prevented the development of obesity and glucose intolerance, implicating the involvement of apoE in the pathogenesis of obesity and related disorders.

Other approaches include crossing the $ApoE^{-/-}$ mice with type 2 diabetic MKR-mice overexpressing a dominant negative insulin-like growth factor-I receptor in skeletal muscle (Kawashima *et al.* 2009). In this model, apoE deficiency surprisingly abrogated diabetic traits but nevertheless caused no reduction in atherosclerosis. This was likely due to the elevated cholesterol levels present in $ApoE^{-/-}$MKR mice compared to $ApoE^{-/-}$ mice, once again emphasizing the dominance of hypercholesterolemia in mouse atherogenesis. However, there are also genetic models without diabetes-induced lipid elevations. The role of IR in atherogenesis has been studied in $ApoE^{-/-}$ mice with a partial (Gonzalez-Navarro *et al.* 2008, Clough *et al.* 2005) or total deficiency of insulin receptor substrate 2 (IRS2) (Baumgartl *et al.* 2006, Gonzalez-Navarro *et al.* 2007). These mice do not exhibit a diabetic phenotype on normal diet but systemic IR is provoked by high-fat diet, accompanied with increased aortic lesion development when compared to $ApoE^{-/-}$ mice with similar plasma lipid levels. Since the plasma lipid levels of these diabetic mice resemble those of the respective hyperlipidemic background, it is possible to study and differentiate mechanisms behind the observed proatherogenic effects.

In short, genetic modification is the most potent way of achieving mouse models mimicking the overall metabolic disorders typically found in an average cardiovascular patient.

Table 5. Atherosclerotic mouse models with genetically induced diabetes. The conceptual idea for the table was obtained from Goldberg & Dansky (2006) and Wu & Huan (2007).

Mouse model	Diet	DM	Hyper-lipidemia	Athero-sclerosis	Comments	Reference
Diabetes does not increase atherosclerosis						
$LDLR^{-/-}A^{y}/a$	WD (12 wks)	2	TC↑ (38.9 mM) TG↑ (9.2 mM)	↔	Insulin ↑	Coenen, 2007
$LDLR^{-/-}$ob/ob		2	TC↔ (50.0 mM) TG↑ (15.0 mM)	↓	Vs. $LDLR^{-/-}$ mice w/ equal TC (HCD)	Taleb, 2007
$ApoE^{-/-}$ob/ob	AD (16 wks)	2	TC↔ (67.5 mM) TG↑ (1.0 mM)	↓ 50 %	± cholate	Chiba, 2008
$ApoE^{-/-}IRS2^{+/-}$		2	TC↔ (6.5 mM) TG↔ (1.5 mM)	↔		Gonzáles-Navarro, 2008
$ApoE^{-/-}$MKR	HCD (16 wks)	2	TC↑ (95 mM) TG↑ (2500 nM)	↔	IR ↓ vs. MKR	Kawashima, 2009
Diabetes affects atherosclerosis and alters plasma lipid levels						
$LDLR^{-/-}$ob/ob		2	TC↑ (44.5 mM) TG↑ (11.5 mM)	↑		Hasty, 2001
$LDLR^{-/-}$GP	AD (12 wks)	1	TC↑ (34.9 mM) TG↑ (23.8mM)	↑ x 2	Lesion hemorrhages ↑	Renard, 2004
$LDLR^{-/-}$GP	HFD (16 wks prior to DM)	1	TC↔ (15 mM) TG↑ (3.5 mM)	↔	Intraplaque hemorrhage ↑, plaque disruption ↑	Johansson, 2008
$LDLR^{-/-}$ob/ob		2	TC↑ (17.2 mM) TG↑ (3.4 mM)	↑ x 4		Gruen, 2006
$LDLR^{-/-}ApoB^{100/100}$ob/ob		2	TC↑ (44.8 mM) TG↑ (7.4 mM)	↑	Vs. C57Bl/6: Insulin ↑, BP ↑	Lloyd, 2008
$ApoE^{-/-}$db/db		2	TC↑ (15.1 mM) TG↑ (2.6 mM)	↑ x 3		Wu, 2005
$ApoE^{-/-}$db/db	WD (10 wks)	2	TC↑ (37.9 mM) TG↑ (10.1 mM)	↑		Wu, 2005
$ApoE^{-/-}$db/db		2	TC↑ (24.8 mM) TG↔ (0.2 mM)	↑ x 3		Wendt, 2006
$ApoE^{-/-}$ob/ob		2	TC↑ (17.7 mM) TG↔ (1.5 mM)	↑ x 3		Gruen, 2006
$ApoE^{-/-}ApoB^{100/100}$ob/ob		2	TC↑ (31.2 mM) TG↑ (3.8 mM)	↑	Vs. C57Bl/6: glucose ↑, insulin ↑, IR, BP ↑	Lloyd, 2008
$LDLR^{-/-}Ins2^{Akita}$	HCD (16 wks)	1	TC↑ (32.5 mM) TG↑ (5.3 mM)	↑ x 2	Phenotype stronger in males	Zhou, 2011
$ApoE^{-/-}Ins2^{Akita}$		1	TC↑ (22.5 mM) TG↑ (0.9 mM)	↑ x 3		Jun, 2011
Diabetes affects atherosclerosis without changes in plasma lipids						
$LDLR^{-/-}$GP		1	TC↔ (8.8 mM) TG↔ (2.1 mM)	↑ x 3		Renard, 2004
$ApoE^{-/-}IRS2^{-/-}$	WD (9-12 wks)	2	TC↔ (64.9 mM) TG↔ (0.8 mM)	↑		Baumgartl, 2006
$ApoE^{-/-}IRS2^{-/-}$	AD (4 wks)	2	TC↔ (15.6 mM)	↑		Gonzáles-Navarro, 2007

AD, atherogenic diet (10.8-40 % energy from fat, 0.075-1.5 % cholesterol, possibly with 0.5 % sodium cholate); BP, blood pressure; DM, diabetes mellitus; HCD, high-cholesterol diet (20 % energy from fat, 0.15-1.25 % cholesterol); HFD, high-fat diet (20-60 % energy from fat, usually no cholesterol); TC, total cholesterol; TG, triglycerides; WD, Western diet (Harlan Teklad TD96125 or TD88137). For the other abbreviations, see text.

2.2.2 Mouse models of other macrovascular complications in diabetes

In addition to general atherosclerosis, macrovascular complications in models of diabetic atherosclerosis have been studied to a lesser extent. CAD and/or myocardial infarction have been examined in hypercholesterolemic models (Calara *et al.* 2001, Nakashima *et al.* 1994, Chase *et al.* 2007, Coleman *et al.* 2006, Dworschak *et al.* 2005, Kuhlencordt *et al.* 2001, Caligiuri *et al.* 1999, Li *et al.* 2001, Saraste *et al.* 2008), but data from the diabetic conditions is lacking.

There is also little information available on peripheral arterial disease. Recently, therapeutical angiogenesis was studied in $ApoE^{-/-}$ mice with STZ-induced hyperglycemia (Balestrieri *et al.* 2010). It was found that hyperglycemia impaired the capillarization and perfusion of the ischemic hindlimbs. However, the impact of increased lipid levels in diabetic versus non-diabetic $ApoE^{-/-}$ mice must be taken into consideration before one can draw conclusions about the worsened ischemic response. In fact, when arteriogenesis in an ischemic hindlimb was compared in mouse models of T1D (STZ-induction and the NOD mice), IR (ob/ob) and hypercholesterolemia (APOE*Leiden mouse), hypercholesterolemia reduced collateral arterial growth significantly more than hyperglycemia or IR alone (van Weel *et al.* 2006), which is uniformly in line with the abovementioned observations from general atherosclerosis.

Because of the natural resistance of mice to atherosclerosis, diabetic mouse models provide a convenient tool for studying diabetic cardiomyopathy. The models of T1D present varying phenotypes with left ventricular diastolic dysfunction being the dominant feature in the Akita mice (Basu *et al.* 2009), whereas also systolic dysfunction is represented in OVE26 (Xie *et al.* 2011), NOD (Pacher *et al.* 2002) and STZ treated mice (reviewed in (Poornima, Parikh & Shannon 2006, Hsueh *et al.* 2007). Based on the cardiac phenotypes of these models, it seems that hyperglycemia and the absence of insulin are sufficient to cause functional defects but the structural changes of DCM might not be duplicated. In contrast to humans, where the clinical picture of DCM is similar in T1D and T2D, the cardiac phenotypes of T2D mouse models differs from T1D models in that systolic function is usually preserved. The most common finding in ob/ob and db/db mice is cardiac hypertrophy, with contractile disturbances and increased chamber stiffness seen in some cases (Poornima, Parikh & Shannon 2006).

2.2.3 Mouse models of diabetic microvascular complications

The studies and model development of diabetic retinopathy in mice have thus far limited to a few strains. Many models, such as STZ diabetic C57Bl/6 mice (Feit-Leichman *et al.* 2005), the Akita mice (Barber *et al.* 2005, Gastinger *et al.* 2008) and db/db mice (Cheung *et al.* 2005) develop vascular and neural changes typical of the early stages of diabetic retinopathy over a period of 6-9 months of diabetes. Despite the fact that the db/db mice with a $ApoE^{-/-}$ background display a more pronounced retinal phenotype (Barile *et al.* 2005), profound retinopathy with capillary and neuronal loss, capillary obliteration, retinal edema and preretinal neovascularization are absent in most models. Recently, Akita mice were crossed with Kimba mice, which transiently overexpress human VEGF in their photoreceptors (Rakoczy *et al.* 2010). In this "Akimba" mouse, the combination of hyperglycemia and short-term elevation in VEGF expression results in a more severe retinal pathology with also retinal neovascularization and persistent retinal edema. Although etiologically not identical

to the human disease, at present the Akimba mice are the only model displaying the more advanced stages of diabetic retinopathy.

The validation criteria for an ideal murine models of diabetic nephropathy suggested by the AMDCC are as follows: 1) over 50 % decline in glomerular filtration rate, 2) over 10-fold increase in albuminuria compared to controls, 3) mesangial matrix expansion, 4) arterial hyalinosis, 5) glomerular basement membrane thickening by >50 %, and 6) tubulointerstitial fibrosis (Brosius *et al.* 2009). Despite the fact that no model currently fulfills all of these criteria, many mouse models demonstrate a varying degree of characteristics resembling human diabetic nephropathy (Breyer *et al.* 2005). Although extensively used as a T1D model, STZ induced hyperglycemia induces only modest renal changes: mild increase in albuminuria and serum creatinine, as well as faint histopathological lesions (Tesch, Allen 2007). A stronger phenotype is seen in STZ diabetic ApoE$^{-/-}$ mice displaying albuminuria and glomerular and tubulointerstitial injury (Lassila *et al.* 2004), suggesting a contributory role for hyperlipidemia in the progression of diabetic renal disease. This approach has been widely used in different settings, *e.g.* with different genetic modifications such as the ApoE$^{-/-}$GPx1$^{-/-}$ mice, which showed further accelerated renal pathology (Chew *et al.* 2010). In type 2 diabetic nephropathy studies, the rapid onset of renal injury has made the db/db mouse a common model of choice (Tesch, Lim 2011). The nephropathic phenotype of db/db mice can be accelerated by removing one kidney or by genetic modification, *e.g.* deleting endothelial nitric oxide synthase (Zhao *et al.* 2006).

In order to properly mimic human diabetic neuropathy, experimental models should demonstrate the following key features: 1) sensory loss (analyzed by thermal sensitivity assessment), 2) electrophysically measured nerve impairment (nerve conduction measurement), and 3) nerve fiber loss (nerve fiber density) (Sullivan *et al.* 2007). Despite profound and persistent hyperglycemia, STZ-induced diabetes does not produce neuropathic disorders. In the Akita mouse, the phenotype is also mild. In fact, it has been noted that the genetic background is an important factor determining the susceptibility to diabetic nephropathy with the common C57Bl/6 strain being relatively resistant. Consistently, the most robust model of diabetic neuropathy is the db/db mouse (with its typical C57BKLS background) exhibiting an impaired response in all areas. (Sullivan *et al.* 2007).

2.3 ADVANCED THERAPIES FOR DIABETIC CARDIOVASCULAR COMPLICATIONS

Since the causes underlying the increased risk for CVD in diabetes are multifactorial, therapeutic interventions addressing each risk factor are needed. The benefits of nutritional and lifestyle guidance cannot be understated as significant non-pharmacological treatments in the primary prevention of CVD endpoints, and have been emphasized also in practice guidelines. Weight control by reducing the proportion of fat to ~30 % of daily energy, the preference of unsaturated fatty acids, and adequate fiber intake form the basis for nutritional management. Together with physical activity and the cessation of smoking, these changes profoundly reduce the risk of CVD in diabetic patients (Rydén *et al.* 2007, Buse *et al.* 2007).

The pharmacological management of CVD in diabetes consists of controlling blood glucose, lipid alterations, hypertension and achieving the prevention of thrombosis. The choice of glucose-lowering therapy is tailored individually. In T2D, oral antidiabetics are the

primary strategy before resorting to insulin replacement therapy. These drugs can be used for stimulating insulin secretion (sulphonylureas, glinides and gliptins), increasing insulin sensitivity in peripheral tissues (glitazones), reducing hepatic gluconeogenesis (biguanides), and slowing the digestion of carbohydrates (alpha-glucosidase inhibitors). The latest entrants are injectable incretin mimetics that increase insulin release and, unlike most other anti-diabetic agents, induce moderate weight loss by delaying gastric emptying and reducing food intake. For the treatment of dyslipidemia, lowering of LDL cholesterol levels with statins is the main intervention in both the primary and secondary prevention of CVD endpoints. The blood pressure recommendation in diabetes is <130/80 mmHg and usually this can only be achieved by a combination of several anti-hypertensive drugs. Inhibitors of the renin-angiotensin system (*i.e.* angiotensin-converting enzyme inhibitors and angiotensin receptor blockers) should always be included in the treatment, since they have been shown to be most effective in reducing CVD events and the progression of renal changes in diabetic patients. Despite the current uncertainty of the effect of the antithrombotic agent aspirin (acetylsalicylic acid) on the prevention of CVD events in diabetes, all current guidelines recommend the regular use of low dose aspirin (~100 mg/day). (Rydén *et al.* 2007, Buse *et al.* 2007, Pignone *et al.* 2010).

Alongside traditional pharmacology, the increasing knowledge of the molecular background and mechanisms of diseases gives rise to new opportunities for more targeted medical treatments. New therapeutic approaches are emerging in parallel to conventional small-molecule pharmaceuticals - since the year 2000 about one quarter of all genuinely new drugs approved have been biopharmaceuticals (Walsh 2010) and undoubtedly their share of products in the pipeline of pharmaceutical companies is even much higher. Biopharmaceuticals are medicinal products whose active substance is made by or derived from a living organism and they include vaccines, blood products, allergens, recombinant therapeutic proteins and the so-called advanced therapies: gene therapy, tissue engineered products and somatic cell therapy.

2.3.1 Gene therapy

Gene therapy is defined as a medicinal product whose prophylactic, diagnostic or therapeutic effects directly relate to the transferred genetic material itself or to the expression product of this genetic material (Directive 2001/83/EC part IV, revised Annex I). In practice, gene transfer is performed into somatic cells either removed from the body (*ex vivo* approach) or directly into cells in their normal location (*in vivo* approach) with an expression system containing the genetic material and a vector of viral or non-viral origin. When aiming at a therapeutic effect, the transgene can function by regulating, repairing, replacing, adding or deleting a genetic sequence in the transduced cell. For example, the transduced cell can be induced to produce the desired therapeutical protein or, on the contrary, the expression of a harmful or faulty protein can be prevented. The ideal gene transfer vector would exhibit low immunogenicity, have an acceptable safety profile, a high transduction efficacy and display expression of the transgene for a defined time period (Gupta, Tongers & Losordo 2009). Although non-viral vectors, such as plasmid DNA, possess a rather optimal safety profile, they also have very low efficacy. Therefore viral vectors with a naturally high gene transfer efficiency are preferred and have so far been used in ~70 % of gene therapy clinical trials (Journal of Gene Medicine, http://www.wiley.com/legacy/wileychi/ genmed/clinical/) (Young *et al.* 2006). Adenovirus

with a highly efficient transduction of both proliferating and non-proliferating cells and a transient expression of the therapeutic gene, has been the most used viral vector. Retroviruses (mostly originating from murine leukemia virus) transduce only proliferating cells and integrate into the host genome, inducing a permanent expression of the transgene but also causing the risk of activating unwanted genes, *e.g.* oncogenes, through insertional mutagenesis. Therefore, for therapeutic applications requiring long-term gene expression, vectors with a more favourable safety profile such as adeno-associated virus (AAV) and lentivirus are being developed. Overall, different viruses vary greatly in their characteristics, *e.g.* tropism, immunogenicity, transgene capacity and integration, and the duration of transgene expression. Thus the optimal vector needs to be chosen based on the given application.

Compared to traditional pharmacology, the major advantages of gene therapy are 1) the possibility of targeting the treatment only to selected tissues or cell types thus reducing systemic toxicity, and 2) the prospect of achieving a long treatment effect with a single administration. Applications of gene therapy have in the last 20 years broadened from monogenic diseases (*e.g.* severe immunodeficiencies) to the treatment of multifactorial acquired diseases. Since the first gene transfer made to a human being took place in 1989 (Rosenberg *et al.* 1990), over 1700 clinical trials of gene therapy have been conducted or are underway (http://www.wiley.com/legacy/wileychi/genmed/clinical/). A substantial proportion (65 % of all trials) has been cancer related, with CVD, monogenic disorders and infectious diseases each accounting for about 8 % of trials. However, so far no nucleic acid-based drug has been approved for clinical use in either Europe or the United States. Only in China are two products on the market for treating head and neck squamous cell carcinoma: Gendicine™ is based on an adenoviral transfer of the tumor suppressor gene p53, and Oncorine™ is an oncolytic adenovirus promoting the lysis of malignant cells. Two products that have been closest to marketing authorization approval in the Western world are the Dutch Glybera® for LPL deficiency and Anglo-Finnish Cerepro® for the treatment of malignant glioma. Glybera® is based on AAV carrying a variant of the human LPL gene. Cerepro® therapy contains adenoviral gene transfer of thymidine kinase, which when combined with ganciclovir medication induces the death of dividing tumor cells. However, only Cerepro® is currently in clinical use, since it has received a named patient permission in Finland and France.

2.3.1.1 Cardiovascular gene therapy

Many components of CVD are successfully managed with traditional pharmacotherapy, whereas in the treatment of fatal complications, these are usually not sufficient and revascularization procedures are needed. However, many patients are not candidates for conventional therapeutic modalities *e.g.* because of a diffuse disease or comorbidities, and a considerable proportion of those treated experience incomplete revascularization. Thus several gene therapy approaches have been designed with the most explored being the induction of therapeutic angiogenesis, the prevention of restenosis after angioplasty and in vein grafts, as well as the treatment of heart failure (Rissanen, Ylä-Herttuala 2007).

Therapeutic angiogenesis, *i.e.* the induction of therapeutic vascular growth, represents the most extensively studied approach in cardiovascular gene therapy and constitutes the majority of clinical trials performed so far. The aim is to increase perfusion in ischemic tissue by promoting the formation of supplemental collaterals for bypassing an occluded artery. In

preclinical studies performed in animal models of myocardial or hindlimb ischemia, several factors have been shown to induce angiogenesis (Rissanen, Ylä-Herttuala 2007). VEGF-A has demonstrated the highest angiogenic potency in experimental studies and therefore has been the main focus also in clinical trials ever since the first human patient with critical limb ischemia was treated in 1996 (Isner *et al.* 1996). Phase I studies have shown promising results in treating both peripheral and CAD (Ylä-Herttuala *et al.* 2007). The later stages of trials with controls and randomization have so far produced disappointing results in terms of efficacy, indicating the need for further optimization, although the experiences from approximately 2500 patients of placebo-controlled studies have proven angiogenic gene therapy to be safe and feasible (Zachary, Morgan 2011). Nevertheless, concerns about the role of VEGF-A in atherogenesis have been raised since there is much evidence for an association between neovascularization and the severity of atherosclerotic disease (Moreno *et al.* 2006). Studies in experimental animals have produced confusing and even conflicting data (Zachary, Morgan 2011), and thus the issue remains contentious and of high importance.

The non-angiogenic gene therapy of CVD includes the prevention of post-angioplasty restenosis and stenosis of the vein grafts used in by-pass surgery. Drug-eluting stents are currently the treatment of choice to alleviate balloon-angioplasty restenosis, but gene therapy approaches could enhance their benefits by introducing factors improving re-endothelialization and endothelial function. Vein grafts, on the other hand, offer a feasible option for *ex vivo* gene transfers, and pre-clinical studies introducing vasculoprotective factors inhibiting thrombosis, vasoconstriction, oxidative stress, inflammation and intimal thickening have been conducted (Rissanen, Ylä-Herttuala 2007). In addition to the vasculature, gene therapy has been targeted to cardiomyocytes to improve cardiac function and prevent heart failure. The enhancement of calcium metabolism to improve contractility, and targeting the myocardial β-adrenergic system to modulate electrical activity and prevent arrhythmias, have been the most extensively studied approaches (Rissanen, Ylä-Herttuala 2007, Njeim, Hajjar 2010). The concept of a biological pacemaker has also gathered interest (Cho, Marban 2010).

2.3.1.1.1 Angiogenic gene therapy in diabetes

Despite promising preclinical studies, angiogenic gene therapy has so far shown limited efficacy in clinical trials. One explanation might be the differences between human patient populations and the animals used in preclinical research. Since virtually all patients needing revascularization procedures are suffering from a variety of antiangiogenic problems, such as endothelial dysfunction, hypercholesterolemia and diabetes, it is not surprising that the response to treatment is not the same as in healthy, young animals that are usually used in the preclinical testing. This brings about awareness of both the need for modification of the therapeutic strategy (*e.g.* improving the effect of angiogenic agent by combined amelioration of the endothelial dysfunction) and the importance of animal model selection. (Boodhwani, Sellke 2009).

In addition to creating a challenging environment for successful angiogenic interventions, the fact that both excessive and insufficient angiogenesis contribute to different diabetic vascular complications, calls for further considerations in relation to the safety of pro- and anti-angiogenic gene therapy. In theory, systemic effects could worsen some other diabetic vascular complication outside the target tissue. One example can be seen in the use of systemically administered anti-VEGF antibodies and VEGF receptor inhibitors in cancer

treatment, where adverse cardiovascular effects such as hypertension and thromboembolic events are commonly encountered (Force, Kerkelä 2008). However, the protocols of pro-angiogenic gene therapy used in clinical trials typically include rather localized dosage with either intramuscular or intra-arterial administration near to the target site. Anti-angiogenic therapy for diabetic retinopathy can also be performed with local intravitreal injections and thus contain very little risk for systemic effects. (Duh, Aiello 1999). Nevertheless, the role of angiogenesis in the development of vulnerable atherosclerotic plaques remains a subject of debate and the controversial results emerging from animal studies warrant further investigations (Khurana *et al.* 2005).

3 *Aims of the study*

The aim of this thesis was to evaluate the impact and safety of VEGF gene therapy in relation to atherosclerosis and to develop a new mouse model for studying the cardiovascular complications related to type 2 diabetes.

The specific aims were as follows:

I To investigate adenoviral VEGF gene therapy with regard to its effect on atherosclerotic lesion development in a mouse model of hypercholesterolemia.

II To establish and characterize a new mouse model for type 2 diabetes and cardiovascular diseases.

III To study the status of coronary artery disease and cardiac function in mice manifesting atherosclerosis and type 2 diabetes.

IV To characterize the ocular phenotype of the established mouse model of type 2 diabetes and atherosclerosis.

4 Materials and methods

4.1 ANIMALS

Hypercholesterolemic LDLR$^{-/-}$ApoB$^{100/100}$ mice (Véniant *et al.* 1998) (studies I-IV) in a mixed genetic background (~75 % C57BL/6J and ~25 % 129/SvJae) were originally obtained from the Jackson Laboratory (stock no 003000; Bar Harbor, Maine, USA). The model was modified to manifest atherosclerosis with the characteristics of T2D by crossbreeding them for ten generations with C57BL6/SJL mice overexpressing insulin-like growth factor II (IGF-II) in pancreatic β-cells (Devedjian *et al.* 2000) (study II). The resulting IGF-II/LDLR$^{-/-}$ApoB$^{100/100}$ mice were then characterized in studies II-IV and IGF-II negative LDLR$^{-/-}$ApoB$^{100/100}$ littermates (II-IV), IGF-II mice (II) and C57Bl/6J mice (III) served as controls. The used mouse strains and diets are outlined in Table 6.

Table 6. Characteristics of the study groups.

Study	Mouse strains	Diet groups	Age of mice
I	LDLR$^{-/-}$ApoB$^{100/100}$	Western diet (3 months)	5 and 8 months
II	LDLR$^{-/-}$ApoB$^{100/100}$ IGF-II/LDLR$^{-/-}$ApoB$^{100/100}$ IGF-II	Chow diet Western diet (3 months)	6 months 15 months
III	LDLR$^{-/-}$ApoB$^{100/100}$ IGF-II/LDLR$^{-/-}$ApoB$^{100/100}$ C57Bl/6J	Western diet (3 months)	18 months
IV	LDLR$^{-/-}$ApoB$^{100/100}$ IGF-II/LDLR$^{-/-}$ApoB$^{100/100}$ C57Bl/6J	Chow diet Western diet (3 months)	3 months 15 months

Mice were fed *ad libitum* a normal chow diet (R36, Lactamin, Stockholm, Sweden) either throughout the study (II, IV) or were put on a high-fat Western diet (TD 88137, Harlan Teklad: 42 % of calories from fat and 0.15 % from cholesterol, no sodium cholate) for three (I-IV) or 5.5 months (I) before analyses Both female and male mice were used in all studies. The following anesthetics were used: a combination of fentanyl-fluanisone (3.15 and 10 mg/kg; Hypnorm®, Janssen) and midazolam (5 mg/kg; Dormicum®, Roche) s.c. (study I and for GTT in studies II-IV), or xylazine (10mg/kg; Rompun®, Bayer) and ketamine (80mg/kg; Ketalar®, Pfizer) s.c. (II-IV). In echocardiography and ultrasonography (III) inhalation anesthesia with isoflurane was used (induction: 4.5 % isoflurane, 450 ml air, maintenance: 2.0 % isoflurane, 200 ml air, Baxter International Inc., Deerfield, IL, USA). Mice were housed in groups and maintained in a temperature- and humidity-controlled environment with a 12-hour light/dark cycle at the National Laboratory Animal Centre in Kuopio. Euthanasia was performed with carbon dioxide. Experiments were approved by the Experimental Animal Committee of the University of Kuopio and the National Experimental Animal Board.

4.2 GENE TRANSFERS

First generation replication-deficient adenoviruses were used as gene transfer vectors. Systemic gene transfers via the tail vein were performed in anesthetized animals with adenoviruses encoding human VEGF-A_{165}, VEGF-B_{167}, VEGF-C or VEGF-$D^{\Delta N \Delta C}$ under CMV promoter (I). A viral dose of $1x10^9$ plaque forming units (pfu) in a total injection volume of 200 µl was used. Saline, recombinant human VEGF-A protein (rhVEGF-A, 2 µg/kg) and adenovirus encoding β-galactosidase (LacZ) under CMV promoter were used as controls. The functionality of the adenoviruses had been tested previously (Bhardwaj *et al.* 2003, Rissanen *et al.* 2003). Mice were consuming a Western diet before the gene transfer for either 6 or 16 weeks, and were sacrificed 6 weeks after the gene transfer.

4.3 METABOLIC ANALYSES AND CLINICAL CHEMISTRY

The analyses of glucose metabolism included blood glucose measurement in fed and overnight fasted animals (II-IV), intraperitoneal glucose tolerance test (GTT) (II-IV) and insulin tolerance tests (ITT) (II). GTT was performed in mice fasted overnight (15 h). Anesthetized animals received an intraperitoneal glucose injection (1.5 g/kg) and blood glucose was measured from the tail vein immediately before the injection and at time-points 30, 60, 90 and 120 min. For insulin tolerance tests (ITT) mice were fasted for 5 hours, anesthetized, injected i.p. with a solution of insulin (0.5 IU/kg; Ultratard®, Novo Nordisk A/S) and blood glucose was measured at baseline and 5, 15, 30, 45 and 60 min post-injection. Blood glucose determination was performed with Ascensia Elite XL glucometer (Bayer). Plasma insulin levels were measured using a kit based on enzyme-linked immunoassay (ELISA) (Linco Research Inc., Missouri, USA) (II).

The assessment of plasma lipids was done from samples after overnight fasting. Triglycerides were determined enzymatically using GPO-PAP (Ecoline® S+, Diasys, Diagnostic Systems Gmbh, Holzheim, Germany) (I-IV). The quantification of total cholesterol was performed with CHOD-PAP kit (Ecoline® 25, Merck KgaA, Darmstadt, Germany) (I-IV). Free fatty acids were measured by the acyl-CoA synthase and acyl-CoA oxidase method (Wako Chemicals GmbH, Neuss, Germany) (II). Plasma lipoprotein profiles were investigated by fractionating fasting plasma samples (200 µl) with fast-performance liquid chromatography (Yan *et al.* 2007).

The measurements of plasma cytokine concentrations were done with ELISA assays: hVEGF-A and -D (Quantikine®, R&D Systems) (I), hVEGF-C (Zymed) (I) and osteoprotegerin (Quantikine, R&D Systems) (II). A sandwich ELISA was developed for VEGF-B (I).

4.4 HISTOLOGICAL METHODS

For the histological analyses, mice were perfused via the left ventricle with phosphate-buffered saline (PBS) and 10 % formalin (I) or 4 % paraformaldehyde (PFA, pH 7.4) (II-IV). Tissue specimens were collected, post-fixed for 2-4 hours and processed for histology. Five micrometer paraffin sections were used for histological analyses performed with general

(hematoxylin-eosin) or special stainings (Masson's Trichrome, modified Movat's pentachrome (Movat 1955), Alizarin Red S, Biotinylated or fluorescence-conjugated Griffonia (Bandeiraea) Simplifonica Lectin I).

Immunohistochemical stainings were performed using the avidin-biotin-horseradish peroxidase system (Vector Elite, Vector Laboratories) with 3-3'-diaminobenzidine (Zymed) or alkaline phosphatase (Vector Laboratories) with fuchsin (Dako) as the chromogen. When using mouse primary antibodies, the Dako ARK™ (Animal Research Kit, peroxidase, Dako) was applied to minimize the reactivity of the secondary anti-mouse antibody with endogenous immunoglobulin possibly present in the sample. The antibodies used in the different studies are presented in Table 7.

Table 7. Primary antibodies used in immunohistochemical stainings.

Antibody	Specificity	Species	Dilution	Distributor	Study
mMQ	Macrophages	Rabbit anti-mouse pAb	1:5000-1:6500	Accurate Chemical & Scientific Corp.	I-II
CD-31	Endothelium	Rat anti-mouse mAb	1:20-1:50	BD Biosciences Pharmingen	I, II, IV
α-SMA	Smooth muscle α–actin	Mouse mAb	1:200	Sigma	I-II
PCNA	Proliferating cells	Mouse mAb	1:500	NeoMarkers, Lab Vision Ltd	II
Caspase-3	Apoptotic cells	Rabbit anti-human pAb	1:250	Promega Corp.	II, IV
OPN	Osteopontin	Goat anti-mouse pAb	1:500	R&D Systems	II
RAGE	Receptor for advanced glycation end products	Goat anti-mouse pAb	1:50	R&D Systems	II, IV
VEGF-A	Vascular endothelial growth factor A	Rabbit anti-mouse pAb	1:500	Serotec	II
eNOS	Endothelial nitric oxide synthase	Rabbit and human pAb	1:150	BD Biosciences Pharmingen	II
Nf-κB	Nuclear factor kappa B p65 subunit	Rabbit anti-human mAb	1:500	Nordic Biosite	II
ICAM-1	Intercellular adhesion molecule-1	Goat anti-mouse pAb	1:500	R&D Systems	II
VCAM-1	Vascular adhesion molecule-1	Rat anti-mouse mAb	1:100	Chemicon® International	II
IL-6	Interleukin 6	Rat anti-mouse mAb	1:300	Lifespan Biosciences	IV
Insulin	Insulin, cytoplasm of pancreatic β–cells	Guinea pig anti-porcine pAb	1:100	DakoCytomation Inc.	II
Glucagon	Glugacon, cytoplasm of pancreatic α–cells	Rabbit anti-human pAb	1:50	DakoCytomation Inc.	II
NG2	Retinal pericytes	Rabbit anti-rat pAb	1:200	Chemicon	IV
Calbindin	Amacrine cells in retina	Rabbit anti-rat pAb	1:1000	SWANT	IV
Calretinin	Amacrine cells in retina	Rabbit anti-human pAb	1:1000	SWANT	IV
HSP-25	Heat shock protein (25 kDa)	Rabbit anti-mouse pAb	1:500	Stressgen Bioreagents	IV
HSP-60	Heat shock protein (60 kDa)	Goat anti-human pAb	1:500	Stressgen Bioreagents	IV
HSP-70	Heat shock protein (70 kDa)	Rabbit anti-human pAb	1:200	Stressgen Bioreagents	IV
Rhodopsin	Photoreceptors (rod cells)	Rabbit anti-human pAb	1:500	Chemicon	IV

For ultrastructural analysis (III), the tissue samples were fixed overnight in 2.5 % glutaraldehyde, treated with 1 % osmium tetroxide, dehydrated and embedded in LX-112

resin and polymerized. 60-70 nm sections were contrasted with uranyl acetate and lead citrate and examined by transmission electron microscopy (JEM 1200EX, JEOL Ltd. Japan).

4.5 EVALUATION OF ATHEROSCLEROSIS

In the *en face* analysis of atherosclerotic lesions, the aortas from heart to the iliac bifurcation were collected after PBS and PFA perfusion and the adventitia was removed under a dissection microscope (I-II). The aortas were opened longitudinally, pinned onto a black wax dissection pan, imaged and the lesion area was quantified with an image analyzer (MCID/M4, Imaging Research) (Leppänen *et al.* 1998).

The evaluation of lesion composition and the degree of arterial stenosis were performed from serial paraffin cross-sections. Aortic lesions were studied in the level of sinuses to assure identical anatomical locus for comparisons. Cross-sectional lesion area was calculated as the percentage from the total aortic area limited by the tunica media (I-III). Plaque calcification and cholesterol clefts were measured as a percentage from the respective plaque area (II, III). In the assessment of coronary artery atherosclerosis, the arteries were followed and lesion areas quantified in serial sections from the origin of the arteries towards the apex of the heart (III). Analyses were performed in a blinded manner using AnalySIS software (Soft Imaging System GmbH).

4.6 MEASUREMENT OF CARDIAC FUNCTION

Cardiac function (III) was assessed by performing transthoracic echocardiography with a high-resolution imaging system for small animals (Vevo 770, VisualSonics Inc., Toronto, ON, Canada) equipped with a high-frequency ultrasound probe (RMV-707B) (Huusko *et al.* 2010). The dimensions and wall thicknesses of the left ventricle (LV) in diastole and systole were measured from parasternal short-axis M-mode images. Other parameters (ejection fraction, fractional shortening, LV volume and LV mass) were calculated by the Vevo770 software. Ejection fraction was calculated with the Teicholz formula (Huusko *et al.* 2010). The averaged values from 3 cardiac cycles were reported.

In the examination of myocardial viability, dobutamine stress echocardiography was performed to evaluate cardiac functional reserve (III). The β-adrenoceptor agonist, dobutamine, was injected intraperitoneally (1 μg/g) and echocardiography was performed at baseline and after injection. Regional LV wall motion was evaluated before and after dobutamine stress from electrocardiogram kilohertz-based visualization cine loops. A 12-segment model on two levels of short axis views (mid-ventricle and apical) comprising of 6 wall segments (anteroseptal, anterior, anterolateral, inferolateral, inferior, and inferoseptal) was used. Wall motion was scored 1=normal, 2=hypokinetic, 3=akinetic and 4=dyskinetic, and a score index was calculated as the sum of scores divided by the total number of segments. In addition to the regional evaluation of the myocardium in dobutamine stress echocardiography, LV wall motion was also assessed in all animals from the long-axis cine view.

Myocardial perfusion (III) was measured with Cadence™ contrast pulse sequence (CPS) ultrasound by Acuson Sequoia C256 using 15L8 probe (Siemens Medical Solutions,

Mountain View, CA, USA) and a second generation microbubble contrast agent (SonoVue, Bracco Diagnostics Inc., Princeton, NJ, USA) administered as a 50 µl bolus via the tail vein (Huusko *et al.* 2010). The quantification of CPS signal intensities was performed using Datapro software (v2.13, Noesis SA, Courtaboeuf, France).

4.7 GENE EXPRESSION ANALYSES

Gene expression levels were examined in aortas (II) (Table 8). Tissue samples were cut from both lesions and intact regions. Total RNA was extracted and reverse transcribed into cDNA. The quantitative measurements of gene expression with real-time quantitative RT-PCR were done using Assays-on-demand gene expression products (Applied Biosystems) with the ABI PRISM 7700 Sequence Detection System (Applied Biosystems). The expression levels were normalized to 18S ribosomal RNA (Applied Biosystems). All experiments were done in duplicate.

Table 8. Aortic gene expression analyses.

<table>
<tr><th>Gene</th><th>Taqman® gene expression assay</th><th>Biological function</th><th>Analyzed tissues</th></tr>
<tr><td>OPN (Osteopontin)</td><td>Mm00436767_m1</td><td rowspan="3">Regulation of vascular calcification</td><td rowspan="7">Intact vascular wall and atherosclerotic lesion</td></tr>
<tr><td>ALP (Alkaline phosphatase)</td><td>Mm00475831_m1</td></tr>
<tr><td>BMP-2 (Bone morphogenetic protein 2)</td><td>Mm01340178_m1</td></tr>
<tr><td>Bax (Bcl-2–associated X protein)</td><td>Mm00432050_m1</td><td>Apoptosis</td></tr>
<tr><td>IL-6 (Interleukin 6)</td><td>Mm00446190_m1</td><td rowspan="2">Inflammation</td></tr>
<tr><td>MCP-1 (Monocyte chemotactic protein 1)</td><td>Mm00441242_m1</td></tr>
<tr><td>CD36 (A class B scavenger receptor)</td><td>Mm00432403_m1</td><td>Uptake of oxidized LDL</td></tr>
</table>

4.8 ASSESSMENT OF OCULAR PHENOTYPE

In addition to the histological analyses of the ocular structures, retinal vasculature was studied in flat mounts with confocal microscopy (IV). Enucleated eyes were fixed in 4 % PFA for 10 minutes, after which the retinas were dissected to flat mounts, further fixed for 1 hour and stained with fluorescence-conjugated lectin. Whole mount retinas were imaged using a confocal microscope (Olympus IX70 inverted microscope). The *in vivo* evaluation of the eyes (IV) included examination with a slit lamp, ophthalmoscopy and fundus photography performed using a fundus camera (Nikon D70s, Nikon Corp., Tokyo, Japan) after dilating pupils with tropicamide (5 mg/ml) and phenylephrine hydrochloride (100 mg/ml).

4.9 STATISTICAL ANALYSES

In choosing the tests to evaluate statistical significance, analyses of distributions were performed. Most data passed the Kolmogorov-Smirnov test for normality. However, the power of the test to detect a Gaussian distribution is weak in a sample size less than a few dozen. Since most biological data is generally close to the Gaussian distribution, and because nonparametric tests lack power in small samples and can give misleading results from Gaussion populations, the data scatter was visually checked to approximately follow the Gaussian distribution, and parametric tests were chosen. Two-tailed Student's t-test for independent samples was used for comparing two groups, and paired t-test was applied to comparing values before and after dobutamine challenge. For more groups, the one-way analysis of variance (ANOVA) or ANOVA for repeated measures (for GTT and ITT) were used with Bonferroni's correction for multiple comparisons. The degree of association was measured with two-tailed Pearson's correlation coefficient (r) test. P values <0.05 were considered significant. Numerical values for each measurement are shown as a mean ± standard deviation (SD) or mean ± standard error of the mean (SEM) as stated. Statistical analyses were performed using SPSS (SPSS 7.5, SPSS Inc.) (I) or GraphPad Prism 4.0 (GraphPad Software, USA) (II-IV).

5 Results

5.1 EFFECTS OF VEGF GENE THERAPY ON ATHEROSCLEROSIS (I)

In the first study, the safety and effects of systemic adenoviral gene transfers with human VEGF-A, -B, -C and -D on atherosclerosis were examined in hypercholesterolemic LDLR$^{-/-}$ApoB$^{100/100}$ mice. LacZ was used as a control gene. In addition, a systemic administration of rhVEGF-A protein was included.

The plasma levels of gene expression products were detectable with ELISA until 4 (VEGF-B) to 6 weeks (VEGF-A, -C, and -D) after gene transfers, whereas the concentration of rhVEGF-A was below the detection limits already 15 minutes after administration. As the liver is the principal tissue of transduction and expression of systemically transferred genes (Smith *et al.* 1993), increased hepatic neovascularization was seen after VEGF-A and VEGF-D gene transfers. However, no changes were observed in the plasma levels of triglycerides or total cholesterol. Human VEGF-A was found present also in aortic tissue 5 days after gene transfer, but no effects on lesion development, composition or neovascularization in the adventitia were seen at the study end point 6 weeks after the gene transfer. Hence, it can be concluded that although some animal studies have pointed to a pro-atherogenic role for VEGF-A, a transient expression of VEGFs did not affect atherosclerosis in the LDLR$^{-/-}$ApoB$^{100/100}$ mouse model.

5.2 MOUSE MODEL OF DIABETIC VASCULAR COMPLICATIONS (II-IV AND UNPUBLISHED RESULTS)

5.2.1 Diabetic characteristics

To evaluate if the developed IGF-II/LDLR$^{-/-}$ApoB$^{100/100}$ model represented diabetic characteristics, metabolic and morphological analyses were performed. The basic metabolic parameters of the old animals used in the majority of studies (II-IV) are summarized in Table 9. Overall, IGF-II/LDLR$^{-/-}$ApoB$^{100/100}$ mice presented elevated fasting plasma glucose and insulin levels, whereas lipid values were not different from the hypercholesterolemic LDLR$^{-/-}$ApoB$^{100/100}$ mice. In addition, the distribution of lipids in the different lipoprotein fractions was similar in LDLR$^{-/-}$ApoB$^{100/100}$ and IGF-II/LDLR$^{-/-}$ApoB$^{100/100}$ mice (unpublished results).

Table 9. Metabolic parameters from old (age 15-18 months) mice after 3 months of Western diet.

Analyzed parameters	C57Bl/6J	LDLR$^{-/-}$ApoB$^{100/100}$	IGF-II/ LDLR$^{-/-}$ApoB$^{100/100}$
Weight (g)			
Females	34.35 ± 6.55	31.01 ± 6.25	33.58 ± 4.52
Males	44.77 ± 5.27	36.98 ± 6.35[a]	39.81 ± 4.35
Glucose (mmol/l)	3.52 ± 0.75	5.06 ± 1.86	7.94 ± 3.18[b]
Triglycerides (mmol/l)	0.99 ± 0.24	1.50 ± 0.74[a]	1.38 ± 0.53
Cholesterol (mmol/l)	4.02 ± 1.36	24.19 ± 8.37[c]	25.51 ± 6.37[c]

Glucose and lipid values are plasma concentrations after overnight fasting. Values denote mean ± SD. [a]$P<0.05$ vs. C57Bl/6J. [b]$P<0.001$ vs. C57Bl/6J and LDLR$^{-/-}$ApoB$^{100/100}$. [c]$P<0.001$ vs. C57Bl/6J.

Compared to the C57Bl/6J and LDLR$^{-/-}$ApoB$^{100/100}$ mice, IGF-II/LDLR$^{-/-}$ApoB$^{100/100}$ mice were markedly glucose-intolerant (II-III): intraperitoneal glucose injection did not induce a response in insulin secretion and glucose removal from blood was impaired (5A). In addition, significant insulin insensitivity in response to an intraperitoneal administration of insulin was seen in IGF-II/LDLR$^{-/-}$ApoB$^{100/100}$ mice (Figure 5B). These changes were present independently of age and diet.

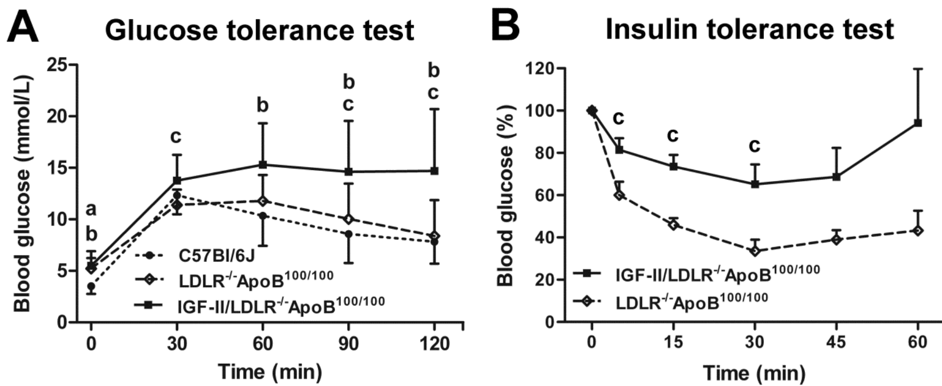

Figure 5. A, Glucose tolerance test in 18-month-old mice after 3 months of Western diet. B, Insulin tolerance test in 6-month-old mice on normal diet. Values denote means ± SD. [a] $P<0.05$ in C57Bl/6J vs. LDLR$^{-/-}$ApoB$^{100/100}$, [b] $P<0.05$ in C57Bl/6J vs. IGF-II/LDLR$^{-/-}$ApoB$^{100/100}$, [c] $P<0.05$ in LDLR$^{-/-}$ApoB$^{100/100}$ vs. IGF-II/LDLR$^{-/-}$ApoB$^{100/100}$.

In pancreas, immunohistochemical stainings with glucagon and insulin antibodies revealed an abnormal islet architecture in the IGF-II/LDLR$^{-/-}$ApoB$^{100/100}$ mice: the islets were irregular in shape and the distribution of α- and β-cells was disturbed (Figure 6) (unpublished results). Thus, the morphological changes seen in the original IGF-II mouse (Devedjian *et al.* 2000) had persisted. No such changes were observed in the LDLR$^{-/-}$ApoB$^{100/100}$ controls.

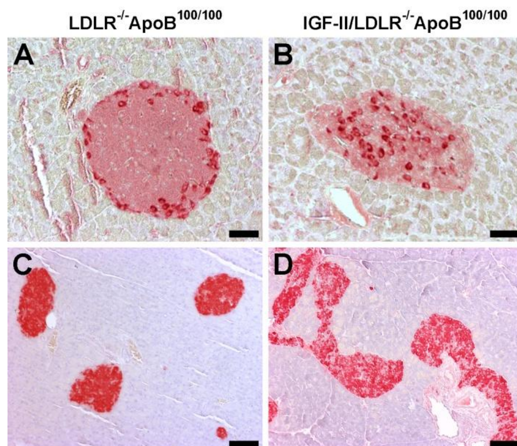

Figure 6. Immunohistochemical stainings of pancreatic sections in LDLR$^{-/-}$ApoB$^{100/100}$ (left panel) and IGF-II/LDLR$^{-/-}$ApoB$^{100/100}$ mice (right panel). In glucacon staining, a normal distribution of α-cells in the periphery of Langerhans islets was seen in LDLR$^{-/-}$ApoB$^{100/100}$ mice (A), whereas in IGF-II/ LDLR$^{-/-}$ApoB$^{100/100}$ mice they are randomly distributed (B). Compared to uniform islet morphology in LDLR$^{-/-}$ApoB$^{100/100}$ mice (C), irregularities in the shape and size of islets were visible in the insulin-stained sections of IGF-II/LDLR$^{-/-}$ApoB$^{100/100}$ mice (D). Original magnifications x200 (A, B) and x100 (C, D). Scale bars 50 μm (A, B), 100 μm (C, D).

5.2.2 Macrovascular disease

To study if T2D would affect the macrovasculature, atherosclerotic lesions in young (6 months) and old (15 and 18 months) mice on normal and Western diet were evaluated and quantified *en face* in longitudinally opened aortas (II) and in serial cross-sections from aortic sinus level (II-III). Combined results from studies II-III are presented in Figure 7. There were no differences in overall lesion areas between IGF-II/LDLR$^{-/-}$ApoB$^{100/100}$ and LDLR$^{-/-}$ApoB$^{100/100}$ mice: both models exhibited lesions covering of ~15 % of the total aortic area and cholesterol elevation as induced by Western diet had no significant effect. On the other hand, cross-sectional lesion areas were more responsive to aging and diet: in 6-month-old mice, the Western diet increased the lesion area over 3-fold, and in old animals, the increase was 1.5-fold. Despite the similar atheroma burden, plaques in diabetic IGF-II/LDLR$^{-/-}$ApoB$^{100/100}$ mice were found to be significantly more calcified in comparison to LDLR$^{-/-}$ApoB$^{100/100}$ mice (Figure 7B, E). In both diabetic and non-diabetic LDLR$^{-/-}$ApoB$^{100/100}$ mice, the extensive lesion formation at the aortic sinus was associated with a compensatory enlargement of the vessel area, resulting in the preservation of the lumen area.

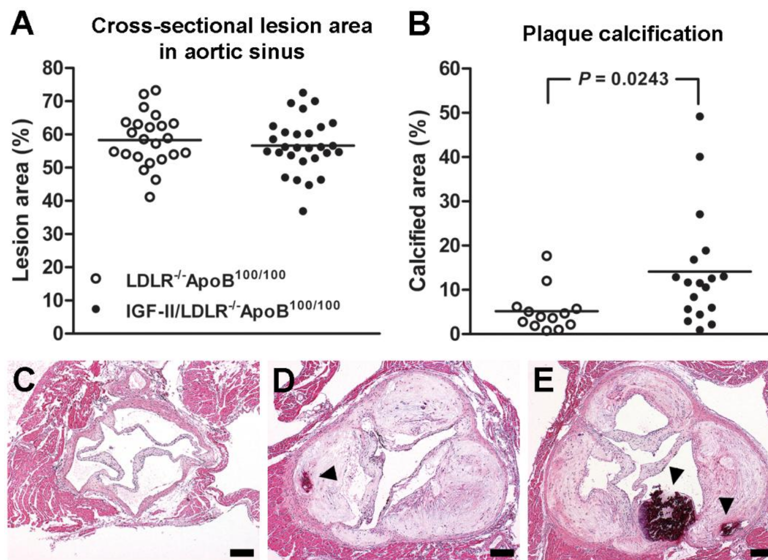

Figure 7. Quantification of atherosclerotic lesion areas (A) and plaque calcification (B) in old LDLR$^{-/-}$ApoB$^{100/100}$ and IGF-II/LDLR$^{-/-}$ApoB$^{100/100}$ mice after Western diet. Representative aortic cross-sections from C57Bl/6J (C), LDLR$^{-/-}$ApoB$^{100/100}$ (D) and IGF-II/LDLR$^{-/-}$ApoB$^{100/100}$ mice (E). Calcification is indicated with arrowheads. Original magnifications x40, scale bars 200 μm.

When lesions were studied in order to discover possible mechanisms behind the increased calcification, no quantifiable differences could be detected between the IGF-II/LDLR$^{-/-}$ApoB$^{100/100}$ and LDLR$^{-/-}$ApoB$^{100/100}$ mice in immunohistochemical analyses (II). Therefore gene expression studies were performed with quantitative real-time RT-PCR from lesions and intact aorta (II). In lesions, the gene expression levels were comparatively similar in both groups. Interestingly, in healthy aortas, the basal expression levels of genes related to calcification (OPN, ALP-2, BMP-2), inflammation (MCP-1) and oxidized LDL uptake (CD36) were higher in IGF-II/LDLR$^{-/-}$ApoB$^{100/100}$ mice, indicative of a more inflammatory vascular profile typical of diabetes.

In the evaluation of coronary atherosclerosis, severely stenotic coronary arteries were seen in both LDLR$^{-/-}$ApoB$^{100/100}$ and IGF-II/LDLR$^{-/-}$ApoB$^{100/100}$ mice (Figure 8) (III). Stenosis was most prominent in the left coronary artery in which the lumen area was significantly narrowed in all LDLR$^{-/-}$ApoB$^{100/100}$ and IGF-II/LDLR$^{-/-}$ApoB$^{100/100}$ mice (average luminal stenosis of ~80 %) despite outward remodeling. The right coronary and septal arteries were stenosed in fewer animals, although also there the narrowing was as severe as in the left coronary when it was present. Despite severe atherosclerosis in the proximal coronary arteries, no lesions were detected in the distal parts. In C57Bl/6J mice, no lesion formation was observed in any part of the coronary tree.

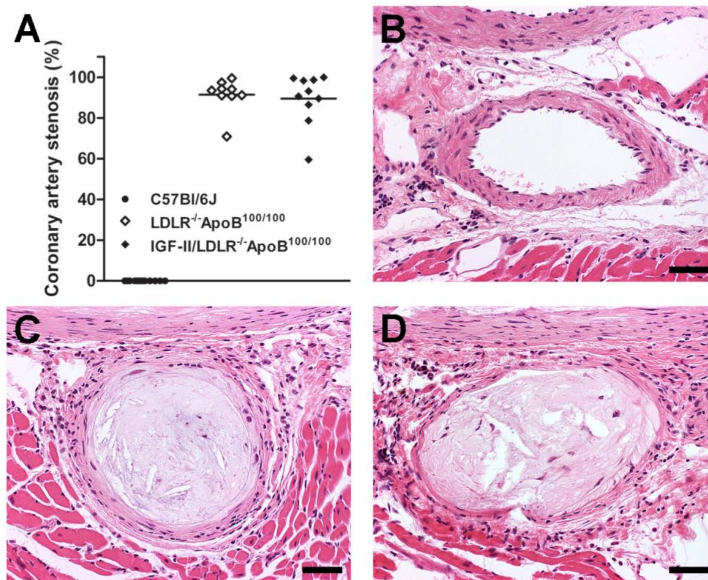

Figure 8. Assessment of coronary artery atherosclerosis. A) Stenoses of coronary arteries with atherosclerotic lesions in old C57Bl/6J, LDLR$^{-/-}$ApoB$^{100/100}$ and IGF-II/LDLR$^{-/-}$ApoB$^{100/100}$ mice after Western diet. Representative cross-sections from left coronary arteries in C57Bl/6J (B), LDLR$^{-/-}$ApoB$^{100/100}$ (C) and IGF-II/LDLR$^{-/-}$ApoB$^{100/100}$ mice (D). Original magnifications x200, scale bars 50 µm.

In order to determine whether T2D would affect cardiac performance, the function of the LV in old animals was examined (Figure 9) (III). Compared to C57Bl/6J mice, which represented a normal global heart function assessed by echocardiography, severe contractile dysfunction was evident in both LDLR$^{-/-}$ApoB$^{100/100}$ and IGF-II/LDLR$^{-/-}$ApoB$^{100/100}$ mice (LV ejection fraction 73.0 ± 9.5 % vs. 44.9 ± 14.7 % and 48.2 ± 11.0 %, respectively). In addition, these groups presented LV dilatation with an increased size and decreased thickness of the LV walls. The visual evaluation of 2-dimensional cine loops revealed also impaired LV wall motion and even akinesia in the anteroapical areas. Animals with the most severe dysfunction exhibited high aortic cross-sectional lesion areas (> 70 %), calcified plaques (4 - 12 % of the plaque area), severe coronary artery stenoses (> 93 % occlusion in left coronary artery) and LV ejection fraction of 22 - 47 %. β-adrenergic stimulation with dobutamine induced chronotropic and inotropic responses in all strains, but in relation to baseline values, the response was diminished in both LDLR$^{-/-}$ApoB$^{100/100}$ and

IGF-II/LDLR$^{-/-}$ApoB$^{100/100}$ mice. However, despite the poor regional wall motion at baseline especially in the LDLR$^{-/-}$ApoB$^{100/100}$ mice, all of the animals showed improvements in wall motion in response to the β-adrenergic stimulation, evidence of a viable myocardium.

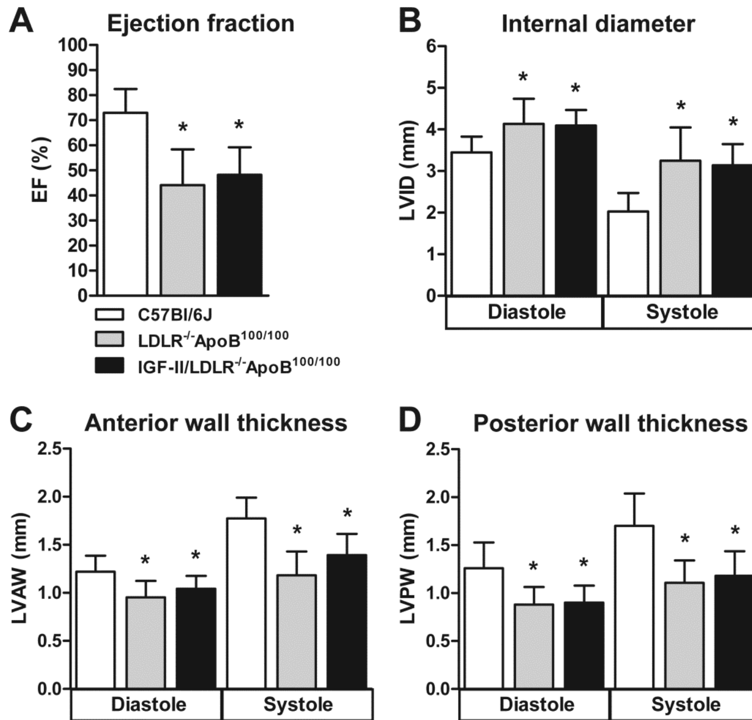

Figure 9. Left ventricular parameters assessed by echocardiography in old mice after Western diet are indicative of dysfunction with reduced pumping efficiency and dilatation. A) Reduced LV ejection fraction in LDLR$^{-/-}$ApoB$^{100/100}$ and IGF-II/LDLR$^{-/-}$ApoB$^{100/100}$ mice depicting a decreased amount of blood pumped out of the LV in systole. B) Increased internal LV diameter in LDLR$^{-/-}$ApoB$^{100/100}$ and IGF-II/LDLR$^{-/-}$ApoB$^{100/100}$ mice in both diastole and systole. Thinning of the anterior (C) and posterior (D) LV walls in LDLR$^{-/-}$ApoB$^{100/100}$ and IGF-II/LDLR$^{-/-}$ApoB$^{100/100}$ mice. Values denote mean ± SD. * $P<0.05$ vs. C57Bl/6J. AW, anterior wall; EF, ejection fraction; ID, internal diameter; LV, left ventricle; PW, posterior wall.

LV anterior wall perfusion was measured with contrast-enhanced ultrasound and a trend towards reduced perfusion was seen in LDLR$^{-/-}$ApoB$^{100/100}$ and IGF-II/LDLR$^{-/-}$ApoB$^{100/100}$ mice. In the histological investigation of the myocardium, no scarring indicative of myocardial infarction was detected. Nevertheless, electron microscopy revealed an ischemic yet viable myocardium and changes reflecting myocardial hibernation in LDLR$^{-/-}$ApoB$^{100/100}$ and IGF-II/LDLR$^{-/-}$ApoB$^{100/100}$ mice.

5.2.3 Ocular phenotype

As retinopathy is the most common microvascular complication of diabetes, the ocular phenotype of the IGF-II/LDLR$^{-/-}$ApoB$^{100/100}$ mice was investigated (IV). No signs of neovascularization or microvascular damage were observed in fundus photographs, retinal whole mounts or histological analysis. However, the retinal morphology of

IGF-II/LDLR$^{-/-}$ApoB$^{100/100}$ mice showed abundant alterations (Figure 9) not present in C57Bl/6J or LDLR$^{-/-}$ApoB$^{100/100}$ mice. In addition, atrophy of photoreceptors was observed in IGF-II/LDLR$^{-/-}$ApoB$^{100/100}$ mice, which was supported by an increased expression of caspase-3, and reduced positivity in rhodopsin staining. These findings were not dependent on aging or diet, but were present both in young (3 months) and old (15 months) animals fed either a normal or Western diet.

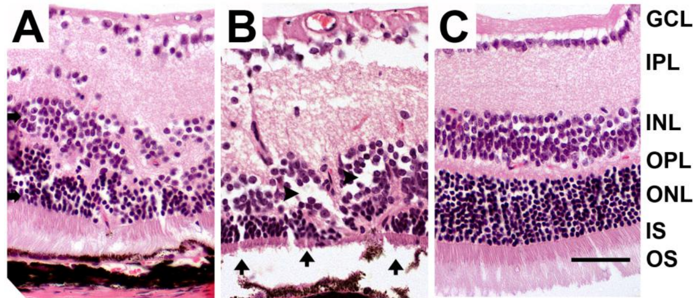

Figure 10. Hematoxylin-eosin stained sections showing retinal changes found in IGF-II/LDLR$^{-/-}$ApoB$^{100/100}$ mice. A) Lack of organized structure and displaced cells in the retina of IGF-II/LDLR$^{-/-}$ApoB$^{100/100}$ mice fed with normal diet. B) Photoreceptor atrophy and thinning of the outer nuclear layer (arrows) with large acellular areas (arrowheads) in outer nuclear layer. C) LDLR$^{-/-}$ApoB$^{100/100}$ mice demonstrated normal retinal morphology. Original magnification x400, scale bar 25 µm. GCL, ganglion cell layer; IPL, inner plexiform layer; INL, inner nuclear layer; OPL, outer plexiform layer; ONL, outer nuclear layer; IS, inner segment; OS, outer segment.

6 Discussion

6.1 VEGF GENE THERAPY AND ATHEROSCLEROSIS

As one of the cytokines shown to enhance angiogenesis, VEGF-A is considered as a potent therapeutic agent to stimulate blood vessel formation in myocardial and limb ischemia (Zachary, Morgan 2011). However, neovascularization is also associated with the growth and progression of atherosclerotic lesions (Moreno *et al.* 2006). Although to date, placebo-controlled clinical trials have not shown any indication that VEGF-A would promote or accelerate atherosclerosis and its clinical outcomes (Hedman *et al.* 2009, Muona *et al.* 2011), concerns about the safety of VEGF-A gene therapy and the role of VEGF-A in atherogenesis have been raised based on some animal studies. Celletti *et al.* (2001a, 2001b) reported increased atherosclerosis in $ApoE^{-/-}ApoB^{100/100}$ mice and cholesterol-fed rabbits after single intraperitoneal and intramuscular injections of low-dose (2 μg/kg) rhVEGF-A protein. Furthermore, a reduction in the lesion amount has been achieved in $ApoE^{-/-}$ mice by using endothelial cell -specific angiogenesis inhibitors (Moulton *et al.* 1999). Since clinical studies with products based on VEGF-A and other angiogenic factors for the treatment of tissue ischemia are currently ongoing, these observations from animal studies have raised concerns about the possibility of atherosclerosis progression. We addressed this question by studying the effects of systemic administration of human VEGF-A protein and adenoviral gene transfers with human VEGF-A, -B, -C and -D on atherosclerosis in hyperlipidemic $LDLR^{-/-}ApoB^{100/100}$ mice.

In the present study, the transient expression of VEGFs did not increase aortic atherosclerosis in $LDLR^{-/-}ApoB^{100/100}$ mice despite the fact that human VEGF-A was detected as being present in aortas with over 7-fold concentration compared to the endogenous mouse VEGF-A. The plasma levels of expressed gene products peaked around day 4 and diminished during the next two weeks, which is a typical expression pattern for adenovirus (Hiltunen *et al.* 2000). On the other hand, when rhVEGF-A protein was administered i.v. in the same amount as i.p. in the study of Celletti *et al.* (2001b), it was undetectable from plasma already after 15 minutes. This is in line with human pharmacokinetic studies showing a rapid clearance from the circulation within hours from intravenous administration. Indeed, in the "VEGF in Ischemia for Vascular Angiogenesis" clinical trial, an i.v. dosing of rhVEGF-A (12 μg/kg) was sufficient to saturate high-affinity receptors for 7 hours and elicit acute effects, such as transient hypotension caused by the activation of endothelial NO synthase and subsequent NO release (Eppler *et al.* 2002). However, this duration of saturation is probably not enough to stimulate any longer-term effects, like angiogenesis, and thus the biological efficacy of a single low-dose bolus of VEGF-A protein used in previous animal studies remains controversial. In fact, it has been noted that a duration of >2 weeks of high VEGF-A expression is needed to induce durable functional vessels in mice with reversible VEGF-A induction (Dor *et al.* 2002).

Although plaque neovascularization is a prominent feature of human atherosclerosis, its significance in mice is less certain. Since blood nutrients are capable of diffusing a distance of ~100 μm (Krogh 1919), only relatively large plaques exceed this limit in mice. Therefore it is logical that the incidence of intimal vessels in mice is actually quite low - *e.g.* only 15 of

114 advanced lesions in ApoE$^{-/-}$ mice were reported to contain intimal neovascularization (Moulton *et al.* 1999). A role for adventitia as a source of plaque microvessels has also been postulated (Moreno *et al.* 2006) and the inhibition of adventitial angiogenesis has been shown to reduce lesion area in mice (Drinane *et al.* 2009, Moulton *et al.* 2003) and pigs (Gössl *et al.* 2009). On the other hand, an antibody-blockade of the VEGF receptor 2, which mediates the angiogenic effects of VEGF-A, did not affect atherosclerosis in ApoE$^{-/-}$ mice (Luttun *et al.* 2002), suggesting that the overall role of angiogenesis is not superior in the complex process of atherogenesis. Moreover, these studies test the effects of angiogenesis inhibition, not the hypothesis that the administration of proangiogenic factors would enhance either adventitial neovascularization or atherosclerosis. In the present study, no changes were observed in the adventitial angiogenesis of intact or plaque-related aortic areas after VEGF-A gene transfers. These facts raise the question if the effects of VEGF-A seen on atherosclerosis in some studies are being mediated through angiogenesis or by some other mechanisms. The conflicting results obtained from different mouse models also imply that some model-dependent factors, such as differences in lipoprotein metabolism, may be underlying these discrepancies. Further studies comparing different models might help to elucidate this matter.

Even though in general the extrapolation from animals to humans is not straightforward, animal model selection can affect the results greatly and is of major importance in the preclinical research of potential human therapies. To date, the greatest problem in clinical trials of therapeutic angiogenesis is the lack of efficacy - promising results achieved in preclinical studies have not been translated into human trials (Zachary, Morgan 2011). A number of factors can contribute to this discrepancy. Firstly, in animal experiments, the study population is very homogenous with identical genetic background, living conditions and disease phenotype. In clinical trials, on the other hand, the patient population is very heterogenous with different clinical pictures and comorbidities, and responses to treatment vary. Additionally to high individual variation in revascularization potential, concomitant medications can contribute to the effectiveness of angiogenic therapy. For example, non-steroidal anti-inflammatory drugs possess some inhibitory effect on angiogenesis (Tarnawski, Jones 2003), and statins have been suggested to promote dose-dependently either pro- or antiangiogenic effects (Weis *et al.* 2002, Urbich *et al.* 2002, Boodhwani *et al.* 2006). Secondly, animal models might lack fidelity to the human clinical phenotype. It has been suggested that molecular, cellular and microvascular environments contribute to the efficacy of angiogenic gene therapy products. For example, NO has a crucial role in the angiogenic process and partly mediates the effects of VEGF-A. Decreased NO availability, a typical feature of endothelial dysfunction caused by various cardiovascular risk factors, can impair both endogenous and exogenous angiogenic responses and might thus attenuate also the effects of angiogenic therapy (Boodhwani, Sellke 2009). It is obvious that studies performed in healthy animals do not capture this situation. Since aging is also usually associated with more advanced disease and poorer recovery both in humans and animals (Faber *et al.* 2011), preclinical studies using young animals might provide an overoptimistic picture of the therapeutic efficacy. At present, the clinical trials have mostly been performed in no-option patients with end-stage diseases, in which measurable improvements might in general be hard to accomplish (Gupta, Tongers & Losordo 2009). Nevertheless, even if at some point clinical trials could enroll patients at an earlier disease stage, young and healthy animals are unlikely going to be relevant models for even their condition. Thirdly, in addition to species-, age- and heath-related issues, the differences can also be a consequence

of vector biology, leading to false conclusions about the efficacy of the therapeutic agent itself. For example, the transfection efficacy of adenovirus is in general markedly less in humans than in mice (Ylä-Herttuala, Alitalo 2003), and even in animals, it declines with aging (Communal *et al.* 2003).

6.2 MOUSE MODEL OF DIABETIC CARDIOVASCULAR COMPLICATIONS

T2D is globally approaching epidemic proportions and concurrently increases the number of CVD patients with a complex clinical picture. Better animal models are urgently needed for the study of disease mechanisms, development of new therapeutic approaches and for the improvement of the current treatments. The present study aimed to develop a new mouse model mimicking the cardiovascular complications encountered in T2D and to evaluate its applicability by performing a thorough phenotypic characterization.

Cross-breeding of the IGF-II transgenic mice with hyperlipidemic $LDLR^{-/-}ApoB^{100/100}$ mice resulted in a model exhibiting hyperglycemia, IR and after high-fat diet feeding also hyperinsulinemia. As an advantage, compared to most other models attempting to combine diabetes and hyperlipidemia, there were no diabetes-induced changes in the plasma lipid levels or lipoprotein profiles. Therefore the IGF-II/$LDLR^{-/-}ApoB^{100/100}$ mice offer the possibility to study the cardiovascular effects of diabetes in parallel to non-diabetic controls with identical lipid levels. There are few models which permit this comparison. The role of STZ-induced hyperglycemia can be assessed in $ApoE^{-/-}$ mice with a defective antioxidant defense (Lewis *et al.* 2007), and in mice overexpressing human AR in $LDLR^{+/-}$ or $ApoE^{-/-}$ backgrounds (Vikramadithyan *et al.* 2005, Vedantham *et al.* 2011). With diet-induced obesity and diabetes, only one study in $ApoE^{-/-}$ mice has reported increased lesion formation without concurrent changes in plasma lipids (King *et al.* 2010). From genetic models, the effects of IR without any hyperglycemia can be studied in $ApoE^{-/-}$ mice with IRS2 deficiency (Clough *et al.* 2005, Baumgartl *et al.* 2006). Although the problem of increased lipid levels is avoided in these models, a general downside is that models based on the $ApoE^{-/-}$ background are very different from humans in relation to lipoprotein metabolism and thus are not the optimal model for atherosclerosis studies.

T2D did not increase either the total aortic lesion area or the cross-sectional lesion thickness at the aortic sinus level in $LDLR^{-/-}ApoB^{100/100}$ mice. This is likely attributable to the high cholesterol level of the $LDLR^{-/-}ApoB^{100/100}$ background, and its widely recognized impact on mouse atherogenesis. However, in aged diabetic IGF-II/$LDLR^{-/-}ApoB^{100/100}$ mice, the lesion morphology was altered with an increased prevalence of calcified lesions, indicative of accelerated lesion progression. Atherosclerotic lesion calcification is a common phenomenon in advanced atherosclerotic lesions in humans (Stary *et al.* 1995) and is more prevalent in diabetic patients (Mielke, Shields & Broemeling 2001), probably reflecting the generally greater atheroma burden. It occurs also in hypercholesterolemic mouse models (Roselaar, Kakkanathu & Daugherty 1996), correlating with the severity of hyperlipidemia (Roselaar, Kakkanathu & Daugherty 1996, Qiao *et al.* 1994). In general, atherosclerotic calcification arises from dead and dying vascular SMCs with a contribution of following osteogenic and chondrogenic processes (Shao, Cai & Towler 2006). The procalcific process is initiated by stimuli related to inflammation and oxidative stress (Demer, Tintut 2008), which are typically pronounced in diabetes. However, data from mouse models of diabetic

atherosclerosis is limited - an up-regulation of osteogenic genes has been found in aortas of $LDLR^{+/-}$ mice with a diet-induced diabetes (Towler *et al.* 1998), and $ApoE^{-/-}$ mice with STZ-induced hyperglycemia display increased lesion calcification (Tse *et al.* 1999). In our study, the aortic vascular wall of IGF-II/$LDLR^{-/-}ApoB^{100/100}$ mice demonstrated a more inflammatory profile and an increased expression of calcification-related genes as compared to non-diabetic $LDLR^{-/-}ApoB^{100/100}$ mice. This finding is in line with studies reporting an increased expression of the BMP-mediated signaling system in different diabetic mouse model (Nett *et al.* 2006, Boström *et al.* 2011, Zervou *et al.* 2010). In addition, the fact that calcification was most profound in old animals is concordant with clinical studies, where especially age and the duration of diabetes have remained as independent risk factors for coronary artery calcification in patients with type 2 diabetes after multivariate analysis (Wagenknecht *et al.* 2001, Godsland *et al.* 2006). In contrast to humans, where plaque neovascularization is closely related to accelerated plaque progression in diabetic patients (Purushothaman *et al.* 2011), no differences were observed here in angiogenesis. This might be a consequence of the fact that lesion size *per se* was not increased, and that generally the significance of plaque angiogenesis in mice is not necessarily similar to humans.

Instead of an actual numeral lack of models with diabetic atherosclerosis, there is a lack of well characterized models. Although it is well-known that T2D significantly increases the risk of coronary events in humans, the main emphasis in mice has been on the assessment of aortic atherosclerosis and, somewhat surprisingly, data about the coronary artery status is missing and there is very little knowledge about their cardiac phenotype. In fact, the cardiac function has thus far been examined only in $LDLR^{-/-}$ob/ob mice, where increased ventricular stiffness was found (Verreth *et al.* 2004, Verreth *et al.* 2006, Van den Bergh *et al.* 2008, Van den Bergh *et al.* 2009). In the present study, CAD and cardiac function were characterized in diabetic and non-diabetic $LDLR^{-/-}ApoB^{100/100}$ mice. Irrespective of the more calcified aortic lesions, diabetes did not lead to worsened CAD: the proximal coronary arteries were found to be significantly stenosed in both models, despite an extensive positive remodeling of the vessels. Consequently, although aortic calcification could increase cardiac work by reducing aortic compliance, also the cardiac outcome was similarly impaired in both models and although regional akinesia was seen, no clear signs of myocardial infarction were detected. These results are in line with previous studies from different atherosclerosis models. Proximal lesions without signs of an infracted myocardium have been found in $LDLR^{-/-}ApoB^{100/100}$ mice also previously (Saraste *et al.* 2008) and in some studies using $ApoE^{-/-}$ mice (Coleman *et al.* 2006, Dworschak *et al.* 2005, Kuhlencordt *et al.* 2001), although in $ApoE^{-/-}$ mice a complete spectrum ranging from totally clean coronary arteries (Coleman *et al.* 2006) to coronary artery occlusion with myocardial infarction has also been reported (Nakashima *et al.* 1994, Chase *et al.* 2007). However, there is no previous data in the context of diabetes. Therefore a thorough characterization of the vascular and cardiac phenotype of a mouse model combining common metabolic disorders, CAD and chronic myocardial hypoperfusion in the present study is of value to future experimental research.

In an attempt to study the possible manifestation of diabetic microvascular complications, the ocular phenotype of the IGF-II/$LDLR^{-/-}ApoB^{100/100}$ mice was characterized. No vascular alterations indicative of diabetic retinopathy were observed in the IGF-II/$LDLR^{-/-}ApoB^{100/100}$ mice. In fact, features of advanced stages of diabetic retinopathy, such as retinal neovascularization, are generally missing in all diabetic mouse models. Only the recently developed Akimba mouse has demonstrated the characteristics of proliferative diabetic retinopathy, although even in that model the neovascular changes do not arise from

hyperglycemia, but from a transgenic up-regulation of VEGF in the photoreceptors (Rakoczy *et al.* 2010). Instead of diabetic retinopathy, the photoreceptor atrophy found in IGF-II/LDLR$^{-/-}$ApoB$^{100/100}$ mice points to some form of retinal degeneration. Dysfunction and a loss of photoreceptors are the major underlying causes of blindness in industrialized countries, and typically manifest in age-related macular degeneration and in complex hereditary disorders, such as retinitis pigmentosa (Wright *et al.* 2010). It has been observed both in humans and mice that photoreceptor degeneration offers protection from the reactive retinal neovascularization typically encountered in diabetic retinopathy (Arden 2001, Lahdenranta *et al.* 2001). Hence it is possible that the absence of diabetic vascular changes is a consequence of photoreceptor atrophy also in IGF-II/LDLR$^{-/-}$ApoB$^{100/100}$ mice. Nevertheless, the cause of the degenerative changes is unclear. Further studies assessing the ocular ultrastructure and retinal function with electroretinography could provide more information about the observed changes.

6.3 LIMITATIONS OF MOUSE MODELS IN ATHEROSCLEROSIS RESEARCH

Despite the facts that there are many similarities in the atherosclerotic process between genetically engineered mouse models and humans, and that mice are very common model animals for studying the cellular and molecular mechanisms of atherosclerosis, limitations do exist. Differences in vessel architecture impact also on lesion structures: human lesions are often more fibrous due to the intimal SMCs and connective tissue, whereas in mice, the intima is very thin, consisting mainly of only endothelium. Furthermore, early stage lesions, such as fatty streaks and intermediate lesions without a necrotic core, do not have clinical consequences in humans and can actually regress to normal instead of progressing into more mature plaques (Stary 2000). These foam cell lesions have even been suggested to be called as "non-atherosclerotic intimal lesions" (Virmani *et al.* 2000). Yet in many studies using mice it is the early lesions that prevail. Since the main goal of experimental studies is to investigate clinically significant atherosclerosis, the term atherosclerosis is used somewhat vaguely when it comes to mouse studies and should be used only when some hallmarks of mature or progressive lesions are present (Getz 2000). Due to the short duration of most mouse studies, it might also be that there is simply a general failure to observe clinically significant lesions. Therefore the characterization of older animals is of value and was performed in the present study. In fact, it was found that the effects of diabetes on atherosclerosis did not manifest themselves until later *i.e.* in older animals with a more advanced disease state.

Having said that the goal is to model clinically significant atherosclerosis, it is also somewhat uncertain if human-like plaque rupture and superimposed thrombosis take place in mice at all (Schwartz *et al.* 2007). Ruptured plaques have been reported in the brachiocephalic artery of fat-fed ApoE$^{-/-}$ mice (Jackson *et al.* 2007), but they do not fulfil all the criteria of the human situation. Although this is a shortcoming, it does not necessarily devalue the usefulness of the mouse as a model animal, since it is possible that the process in mice is simply markedly different in terms of histopathology or physiology (Jackson 2007). In fact, major variation appears in coagulation and thrombosis in different mouse strains (with C57Bl/6, the most widely used background strain in atherosclerosis studies, being rather resistant to thrombosis) (Peters *et al.* 2002, Hoover-Plow *et al.* 2006) as well as in

comparison to humans (Magallon *et al.* 2011). In addition, diabetic db/db and ob/ob mice actually demonstrate reduced blood coagulation and aggregation compared to their respective genetic controls, and thus do not reproduce the hypercoagulable state seen in human patients with T2D (Henry *et al.* 2008). The fact that mice are seemingly protected against the clinical consequences of thrombosis, such as ischemic heart disease, might also be attributable to the different patterns of lesion distribution also observed in the present study - *e.g.* in their coronary arteries, mice exhibit mainly only retrovalvular lesions and the sites usually diseased in humans (the first segment and branches) are free of atherosclerosis (Hu *et al.* 2005). However, strong data supporting a generally similar pathophysiology of atherosclerosis comes from the genetic analyses, where a number of corresponding quantitative trait loci have been discovered in both mice and men (Wang *et al.* 2005).

In general, the usage and selection of an animal model is always more or less of a compromise. Although non-human primates would be excellent model animals in CVD research, they are hardly the ideal choice for many reasons related to handling, expences and ethical concerns. Pigs share great anatomical, histological and mechanistical similarities with human atherosclerosis and strains with different stages of metabolic disorders are available, although overt diabetes has to be induced chemically (Bellinger, Merricks & Nichols 2006). Rabbits differ more from humans in relation to lipid metabolism and atherosclerotic lesions, although a spontaneously hyperlipidemic strain and some transgenic rabbits are available. However, also in rabbits, only chemically induced diabetes can be studied. (Singh *et al.* 2009). The rat has the advantage of a sequenced genome (Rat Genome Sequencing Project Consortium 2004), and the number of genetically modified strains is likely to increase. However, they offer little benefit over the mouse, especially since surgical and imaging techniques for small animals are constantly improving.

6.4 FUTURE DIRECTIONS IN MOUSE MODEL DEVELOPMENT

There is no perfect model available for diabetic cardiovascular complications, but many options for modeling different aspects of these complications. One can even speculate whether developing such a model is possible. However, it is possible to improve the present mouse models of atherosclerosis by further genetic optimization of lipid metabolism. It would be desirable to achieve conditions, where the use of very high-fat diets could be avoided and advanced atherosclerotic lesions would be obtained with physiological and clinically relevant plasma lipid levels. Although the LDLR$^{-/-}$ApoB$^{100/100}$ mice represent the most human-like lipoprotein profile so far, the fact that mice in general do not express CETP significantly contributes to their high HDL levels and subsequent resistance to atherosclerosis. Therefore a transgenic induction of CETP expression could be used for lowering their naturally high HDL levels. Since the plasma total cholesterol concentration of LDLR$^{-/-}$ApoB$^{100/100}$ mice is ~8 mmol/l and advanced atherosclerotic lesions are formed also on a normal diet, the expression of CETP could be used to fine-tune their lipid profile and to achieve a very satisfactory mouse model of human atheroslcerosis.

The situation with diabetes-induction and with modeling the subsequent increase in atherosclerosis is more challenging. Ultimately, if the less frequent monogenic forms are omitted, T2D and related metabolic disorders are complex and multifactorial, and thus their simulation in animal models is anything but simple. The possible mechanistical interspecies

differences underlying the lack of accelerated atherogenesis typically seen in diabetic human patients complicate the task further. The development of new animal models is slow, unpredictable and requires considerable resources. Thus a profound characterization establishing the relevance of the models for the human pathophysiology is a necessity. Therefore, as animal models represent an indispensable tool for a wide area of preclinical research, their development should also be supported by minimizing the publication bias in favor of experiments or models showing only positive results, so that all information would be available and lessons learned in these studies would not need to be duplicated.

7 Conclusions and future perspectives

The following conclusions can be drawn based on this thesis.

I Adenoviral gene transfers of human VEGF-A, -B, -C or -D do not increase atherosclerosis in hypercholesterolemic LDLR$^{-/-}$ApoB$^{100/100}$ mice. This supports the results from previous clinical trials indicating the safety of VEGF gene therapy.

II IGF-II/LDLR$^{-/-}$ApoB$^{100/100}$ mouse model represents T2D with insulin resistance, hyperglycemia and mild hyperinsulinemia, and more advanced aortic atherosclerosis with increased lesion calcification. This is one of the few models where the effects of diabetes can be studied uncomplicated by concomitant lipid changes.

III Hypercholesterolemic LDLR$^{-/-}$ApoB$^{100/100}$ mice with or without diabetes develop significant CAD and severe left ventricular dysfunction with regional akinesia but without infarction, and possess an ischemic but viable myocardium. Although cardiac function is not worsened by diabetes, the combination of common metabolic disorders, CAD and chronic myocardial hypoperfusion will be useful for future studies.

IV The ocular phenotype of IGF-II/LDLR$^{-/-}$ApoB$^{100/100}$ mice reveals alterations in retinal morphology with photoreceptor atrophy. No vascular alterations indicative of diabetic retinopathy were observed. Hence the model might be useful for studying changes related to early or pre-diabetes, and disorders involving retinal degeneration.

The results obtained in this thesis raise the question about the etiology of VEGF-induced atherogenesis observed in some studies using different mouse models. Further studies investigating the underlying mechanisms and possible model-dependent factors would elucidate this clinically highly relevant issue. Since diabetic patients experience poor recovery from myocardial ischemia and suffer from more severe peripheral arterial disease, it would be important to evaluate if the IGF-II/LDLR$^{-/-}$ApoB$^{100/100}$ mouse reproduces these features and the impairments in angiogenic potential. The developed model provides a valuable tool for preclinical research and the development of new therapeutic approaches for T2D related cardiovascular complications.

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Suvi Heinonen

Modeling Cardiovascular Complications of Diabetes Mellitus

Development of a New Mouse Model and Evaluation of a Gene Therapy Approach

Animal models are essential tools in the preclinical research of complex metabolic disorders. In this thesis, a new mouse model representing type 2 diabetes and associated vascular complications was developed and characterized. Furthermore, insight into the safety of cardiovascular gene therapy is given by in vivo evaluation of proangiogenic gene transfers in relation to atherosclerosis.

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