The interplay between cholesterol and inflammation in the evolution of atherosclerosis

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Chapter 1

General Introduction

1.1 Introduction

Despite significant advances in treatment and in understanding of its biology, coronary atherosclerosis remains the leading cause of morbidity and mortality of men and women in industrialized societies. Hypercholesterolemia, particularly of low-density lipoprotein (LDL) cholesterol and very low-density lipoprotein (VLDL) cholesterol, is a well-established risk factor of atherosclerosis and its pathologic complications, myocardial infarction and stroke. For the past 20 years, treatment of atherosclerosis was mainly directed at normalizing plasma cholesterol levels and the statin class of cholesterol-lowering drugs has been the mainstay for the treatment of hypercholesterolemia ([1], and references therein). However, despite effectively lowering cholesterol levels and reducing cardiovascular causes of death, two thirds of the statin-treated patients still experience adverse coronary events.[2] Consequently, in atherosclerosis research there is increasing attention to risk factors other than hypercholesterolemia. Also, atherosclerosis is now recognized as a multifactorial, multistep disease with numerous etiologies which have to act in concert to initiate and promote the atherosclerotic process.[3]

In addition to “classical” risk factors discovered by classical epidemiology, including hypercholesterolemia, hypertriglyceridemia, low high-density lipoprotein (HDL), hypertension, insulin resistance and type-2 diabetes, inflammation has been accepted as a major driving force in atherosclerotic lesion development.[4] There is increasing experimental evidence that (chronic) inflammation plays a causal role in atherosclerosis. Many studies have shown that raised levels of circulating inflammatory markers, and in particular C-reactive protein (CRP), are prospectively associated with increased cardiovascular disease risk.[5,6] As in many human inflammatory diseases, the inciting stimulus in each case remains uncertain. Also, the intricate interaction between cholesterol and inflammation in the pathogenesis of atherosclerosis is not exactly understood.

The general theme of this thesis is to further delineate the role of inflammation in the atherosclerotic process and to elucidate the interplay between cholesterol and inflammation in atherosclerotic lesion formation and progression. In this general introduction, first some background information is provided regarding the relevance of cholesterol and inflammation in atherogenesis. Next, some potential anti-atherosclerotic targets are described that have been studied in this thesis with respect to their effect on plasma cholesterol levels, systemic inflammation, and/or the local (vascular) inflammatory state. Subsequently, mouse models for inflammatory processes and atherosclerosis used in our studies are introduced. Finally, the scope, aims and outline of the thesis are given.

1.2 Role of cholesterol and lipoproteins in atherogenesis

Cholesterol and triglycerides are important lipids in human physiology and required for many physiological processes. For example, cholesterol plays an important role in the regulation of the fluidity and barrier function of cell membranes and is a precursor of bile acids and steroid hormones. The most important physiological role of triglycerides is to transport and store energy. Triglycerides can either be stored in adipose tissue (storage) or can be used to generate ATP by tissues that rely on lipids as energy source (e.g. cardiac and skeletal muscle). The uptake and transport of the lipids cholesterol and triglycerides is coupled as discussed briefly in the following.

characteristic	chylomicron	very-low-density lipoproteins	intermediate-density lipoproteins	low-density lipoproteins	high-density lipoproteins
abbreviation		VLDL	IDL	LDL	HDL
density (g/ml)	<0.95	0.95 - 1.006	1.006 - 1.019	1.019 - 1.063	1.063 - 1.210
diameter (nm)	75-1200	30-80	25-35	18-25	5-12
composistion (% dry wt)					
protein	2	8	19	22	47
triglycerides	86	55	23	6	4
cholesterol	5	19	38	50	19
phospholipid	7	18	20	22	30
apolipoproteins	A1, A2				A1, A2
	B-48	B-100	B-100	B-100	
	C1, C2, C3	C1, C2, C3	C1, C2, C3		C1, C2, C3
	E	E	E		E

Table 1. Physical properties and composition of human plasma lipoproteins

Most of the endogenous pool of cholesterol/cholesterol esters and triglycerides is taken up in the intestine. Since cholesterol and triglycerides are hydrophobic molecules they have to be transported in the form of soluble lipoproteins which can differ with respect to density, size, apolipoprotein composition and electrical charge (Table 1): chylomicrons, very low density lipoproteins (VLDL), low density lipoproteins (LDL), intermediate density lipoproteins (IDL), and high density lipoproteins (HDL).[7,8]

In the intestine, dietary cholesterol and triglycerides are packed into chylomicrons which enter the circulation via the lymph. Chylomicrons are the main transporters of dietary lipids towards the liver, while VLDL, IDL, LDL and HDL function to transport endogenous lipids throughout the body.[9] The liver plays an important role in the homeostasis of plasma lipoproteins and is the major assembling site of VLDL. Based on lipoprotein composition, lipoproteins can be divided into an apoB-containing lipoprotein group (VLDL, IDL, and

LDL) and a non-apoB-containing group (HDL). In particular, the apoB-containing lipoproteins are important for the delivery of cholesterol from the liver to peripheral tissues, whereas HDL appears to mediate the reverse process of movement of cholesterol from tissues back to the liver.[10]

Epidemiological studies have established that elevated levels of apoB-containing lipoproteins in humans are associated with an increase in the incidence of cardiovascular disease.[11, 12] Enzymatic modifications, enhanced retention and accumulation of these lipoproteins in the arterial wall are generally considered to be the first steps in the development of atherosclerosis. The end-product of enzymatic modification of LDL, oxLDL, acts as a potent pro-inflammatory chemoattractant for macrophages and T lymphocytes in the vasculature. Furthermore, oxLDL is cytotoxic for endothelial cells and stimulates the release of soluble inflammatory molecules, thus creating an inflamed environment in the human vasculature.[13]

1.3 Role of inflammation in atherogenesis

It is now well-accepted that atherosclerosis is not only a lipid disorder but also an inflammatory disease. Inflammatory processes have been shown to play important roles at all stages of the disease process. In fact, as depicted in Figure 1, inflammatory processes play a role in all stages of atherogenesis, from early endothelial activation to progression and, eventually, plaque rupture.[4,14] In early atherogenesis for example, adhesion molecules are upregulated on endothelial cells in response to inflammatory cytokines. Circulating monocytes can then attach to the activated endothelium cell layer and can migrate into the vessel wall.

Within the vessel wall, transformation of monocytes to macrophages takes place, followed by engulfment of oxidized LDL, resulting in foam cells. The lipid-laden foam cells further promote inflammation and atherosclerotic plaque progression via secretion of inflammatory mediators that attract additional leukocytes including T-cells. The newly attracted and immigrated T-cells secrete cytokines which subsequently further amplify the inflammatory response and promote the migration and proliferation of intimal smooth muscle cells. This self-perpetuating cycle further amplifies inflammation thereby accelerating the atherogenic process.[16] Later in the atherogenic process, inflammation-induced proteases can weaken the protective fibrous cap of the atheroma, ultimately leading to plaque rupture, thrombosis and the occurrence of acute coronary syndromes such as unstable angina pectoris and myocardial infarction (MI).

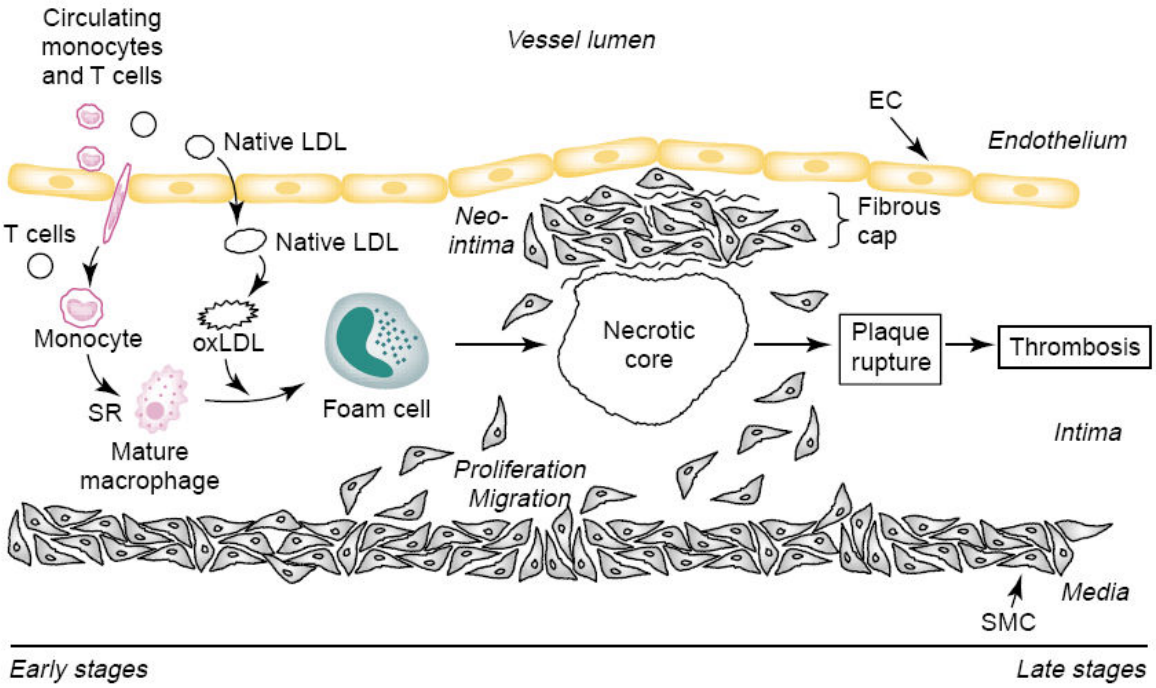

Figure 1. Schematic overview of inflammatory processes in various stages of atherosclerosis. [15]

In the past several years it has become clear that the inflammatory component of atherogenesis is not restricted to local inflammation in the vascular wall but that inflammation originating from other organs such as the liver and the adipose tissue can contribute significantly to progression of atherosclerotic disease.[17] This is also underlined by data describing increased incidence of cardiovascular disease in patients suffering from chronic inflammatory diseases such as systemic lupus erythematosus[18] and rheumatoid arthritis.[19,20]

1.4 Selected targets for treatment of atherosclerosis

1.4.1 HMG-CoA-reductase

Most circulating cholesterol is synthesized in the body, primarily from acetyl-CoA through the 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase pathway. Statins form a class of hypolipidemic drugs used to lower cholesterol levels in humans with or at risk of cardiovascular disease.[21] They lower cholesterol by inhibiting the enzyme HMG-CoA reductase, which catalyzes -what is thought to be- the rate-limiting step in cholesterol biosynthesis, the formation of mevalonate.[22,23] Reduction of mevalonate synthesis leads to reduction in the regulatory sterol pool, which in turn causes up-regulation of HMG-CoA reductase[24] and other enzymes of the cholesterol biosynthesis pathway[25, 26], and, most

importantly, the LDL receptor.[27,28] Although not the original target, the induction of the LDL receptor expression is crucial for the effectiveness of statins in lowering cholesterol.

In the past several years, very powerful new statins have been developed, such as rosuvastatin and atorvastatin. These statins have a higher affinity for HMG-CoA reductase and – because of different chemical properties – a different bioavailability than the first generation statins. Molecular differences among the statins also give rise to some important differences in their properties, including their anti-atherogenic and anti-inflammatory actions (among the so-called pleiotropic effects).

1.4.2 Nuclear hormone receptors

The nuclear hormone receptor superfamily of ligand-activated transcription factors regulates gene expression in such diverse processes as metabolism, development, and reproduction. The family has 48 members in humans, and includes, for example, retinoid, steroid, and thyroid hormone receptors.

The structure of nuclear hormone receptors (see Figure 2) consists of a variable NH2 terminal region and a highly conserved DNA-binding domain (DBD).[29,30] The DBD region contains a short motif responsible for DNA binding-specificity and is involved in dimerization of nuclear receptors. A linker region is situated between the DBD and the ligand binding domain (LBD). This region functions as a flexible hinge and contains the nuclear localization signal. The LBD is involved in the binding of ligand. This domain also contains a ligand-regulated transcriptional activation function-2 (AF-2) necessary for binding transcriptional co-activators which interact, among others, with the general transcriptional activation machinery. Most nuclear hormone receptors contain in the N-terminal variable domain a transcriptional activation function-1 (AF-1) that mediates ligand-independent transcriptional activation.

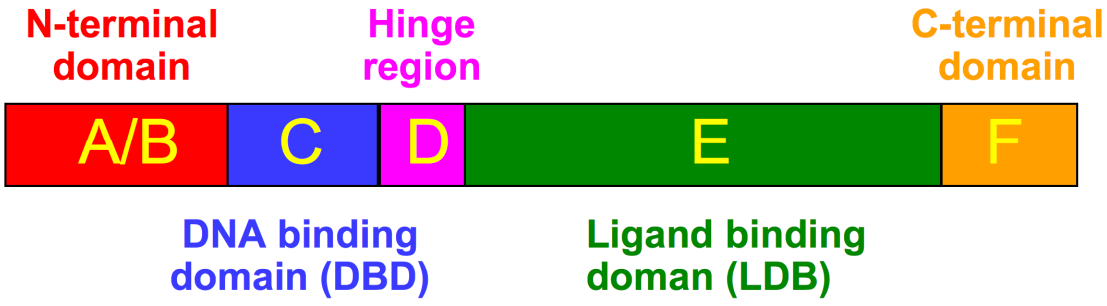

Figure 2: Schematic 1D amino acid sequence of a nuclear receptor.

The subfamilies known as peroxisome proliferator-activated receptor (PPAR) and liver-X-receptor (LXR) have emerged as dominant regulators of processes that influence cardiovascular risk, namely various aspects of lipid and glucose metabolism, insulin sensitivity, as well as inflammation.[31-36]

Notably, PPAR and LXR not only show these effects at the systemic level but also regulate lipid homeostasis and inflammation in macrophages, endothelial cells, and smooth muscle cells within the vessel wall. Agonists that specifically activate these receptors may therefore retard the development of atherosclerosis at several levels.[37]

Peroxisome Proliferator-Activated Receptor (PPAR)

PPARs are transcriptional factors belonging to the ligand-activated nuclear receptor superfamily.[38] There are three distinct PPAR subtypes, PPARα, PPARβ/δ, and PPARγ. Although there is overlap in natural ligands (fatty acids, eicosanoids) that are capable of activating the three PPARs, each receptor subtype has a tissue-specific expression pattern and exhibits both overlapping and distinct biological activities.[1,39]

In rodents and humans, PPARα is expressed in tissues involved in fatty acid oxidation, including liver, kidney, heart, skeletal muscle and brown fat[40] and in a range of vascular cells such as endothelial cells[41], vascular smooth muscle cells (VSMCs)[42] and monocytes/macrophages.[43]

When activated by its ligand, PPARα positively regulates the transcription of target genes through binding to specific gene promoter response elements (Figure 3). This mode of action is particularly important for the regulation of genes that control lipid and lipoprotein metabolism.[44] Nowadays, synthetic PPARα activators such as fibrates are clinically used to lower plasma lipid levels and treat hypertriglyceridemia and mixed dyslipidemia.[45] There is a large body of clinical evidence that fibrates lower plasma triglycerides and LDL cholesterol, and increase HDL cholesterol by up-regulating the hepatic gene expression and synthesis of ApoA1, the major apolipoprotein of HDL.[45] Independent of this mechanism, activation of PPARα can also act as a negative regulator of pro-inflammatory genes via antagonizing the activity of inflammatory transcription factors or co-activators (Figure 3).[46]

Liver X Receptor (LXR)

The liver X receptors are members of the nuclear receptor family of proteins that are critical for the control of lipid homeostasis in virtually all vertebrates.[47,48] Two LXR isoforms

have been described so far, LXRα and LXRβ. LXRβ has a ubiquitous tissue distribution, whereas LXRα predominates in liver, adipose tissue, and intestinal tissue, as well as macrophages. Both LXRα and LXRβ are activated by physiological concentrations of sterol metabolites such as 22(R)-hydroxycholesterol, 24(S)-hydroxycholesterol, 27-hydroxycholesterol, and 24(S), 25-epoxycholesterol.[49-51] Both isoforms appear to respond to the same natural and synthetic ligands. When activated by its ligand, LXR heterodimerize with RXR and positively regulates the transcription of target genes through binding to specific gene promoter response elements.

LXR activation has several reported beneficial effects on the expression of genes that are involved in cholesterol efflux in macrophages[52], hepatic bile acid synthesis[48], intestinal cholesterol absorption[53], inflammation[54], and glucose tolerance[55]. These features make LXRs attractive targets for the development of drugs for treatment of cardiovascular, metabolic and/or inflammatory diseases.

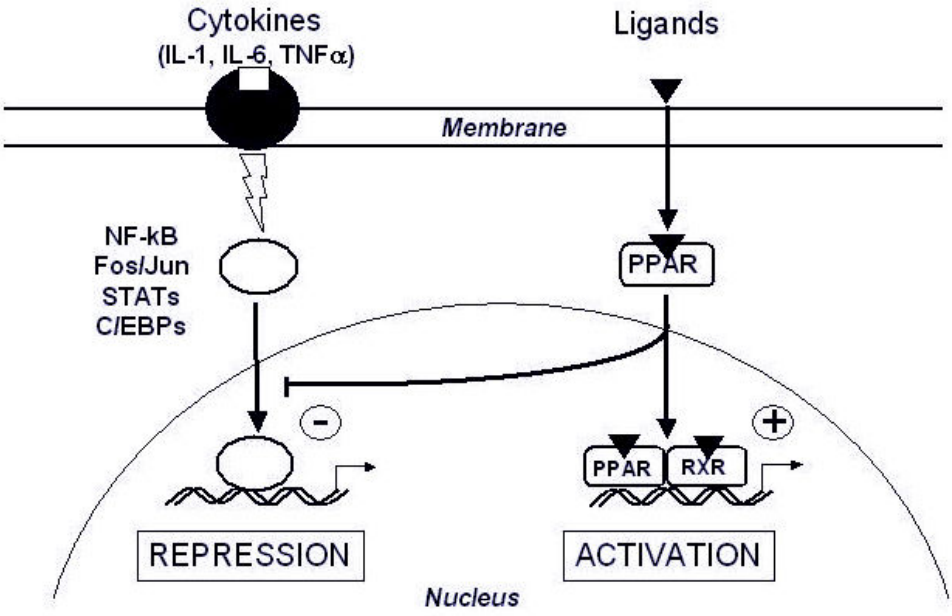

Figure 3: **Mechanisms of transcriptional regulation by PPARs.** Following activation, PPARs heterodimerize with retinoic X receptor (RXR) and bind to response elements in the promoter region of target genes, thereby activating their transcription. PPARs also repress gene transcription in a DNA-binding independent fashion by interfering directly with transcription factors or co-activators.

1.4.3 Macrophage Migration Inhibitory Factor (MIF)

MIF was discovered over 40 years ago and constitutes one of the first cytokines described in literature. In these early studies, MIF was found to inhibit the random migration of peritoneal macrophages which gave rise to its name.[56] In 1993, the protein was rediscovered as a pituitary-derived factor with hormone-like properties and a mediator of

endotoxic shock.[57] Today, MIF is known as a pleiotropic macrophage and T-cell cytokine[58] and endocrine factor[59] that is involved in the regulation of a great number of inflammatory processes[57, 60] and inflammatory diseases.[61] Recent studies have demonstrated that MIF expression is up-regulated in atherosclerotic lesions in humans[62] and animals.[63] In the vessel wall, increased MIF expression stimulates cells to produce pro-inflammatory cytokines such as TNFα and IL-1β.[58, 60] Increased TNFα and IL-1β levels in turn stimulate vascular cells to produce substantial amounts of MIF which is a good example for a self-perpetuating cycle in the vasculature that accelerates the atherogenic process.[16] Interference in this cycle by inhibition or blockage of MIF activity could retard the progression of atherosclerotic disease, but also other diseases in which this inflammatory cycle is of relevance (e.g. insulin resistance).

1.5 Mouse models

Detailed analyses of processes that contribute to human disease development are often hampered by the complex interactions and variation in environmental and genetic factors in humans. Therefore, mice with homogenous genetic background (inbred strains) that are housed in a standardized way (comparable environmental factors) have been proven to be a useful tool. Since regular wild-type mice such as C57BL/6 mice differ from humans with respect to lipid metabolism and inflammation (i.e. the two major risk factors of cardiovascular disease), transgenic mice have been developed that allowed us to better study the disease process and the risk factors. In the following, three models will be discussed in more detail: 1) Human CRP transgenic mice as a model to study the risk factor inflammation; 2) ApoE*3Leiden transgenic mice and 3) Ldlr-/- mice to study atherogenesis (lipid metabolism and inflammation).

1.5.1 Human C - reactive protein transgenic mice

Several clinical studies have shown that C-reactive protein (CRP), a liver-derived inflammation marker, is the strongest independent predictor of future cardiovascular events (summarized by Libby et al.[4] and Ridker et al.[64]). In response to infection or inflammation, CRP is highly induced in man but not in wild-type mice, where its serum levels never raise above 2-3mg/l.[65] To overcome this difference in response, transgenic mice have been generated that carry the full human CRP gene. This so called “human-CRP transgenic (huCRPtg)” mouse carries a 31-kb human DNA fragment containing the human *CRP* gene including all known *cis*-acting regulatory elements including the entire

human CRP promoter. Unlike its wild-type mouse counterpart, human CRP behaves as a major acute phase reactant when introduced into the mouse genome.[66] The expression of huCRP is highly inducible and tissue specific. The generation of this mouse model enabled us to monitor inflammatory responses in mice.

*1.5.2 ApoE*3Leiden (E3L) transgenic mice*

The APOE*3Leiden mutation is a rare, dominant negative mutation in the human *APOE3* gene. It is characterized by a tandem duplication of codons 120-126 and associated with familial dysbetalipoproteinemia in humans. ApoE*3Leiden transgenic (E3L) mice have been generated by introducing a human *APOE*3Leiden* gene construct into C57Bl/6 mice. Besides the *APOE*3Leiden* gene, this construct consists of the *APOC1* gene and a promoter element that regulates the expression of *APOE* and *APOC1* genes ([67,68], and references therein). Although E3L mice still express endogenous ApoE protein, the clearance of ApoE-containing lipoproteins is impaired, albeit less dramatically than in ApoE-/- mice. The introduction of the *APOC1* gene may further increase plasma lipid levels by diminished VLDL uptake through the LDL receptor and LRP.

E3L mice show significant elevations of plasma cholesterol and triglycerides on a regular chow diet and are, in contrast to wild-type mice, highly responsive to fat-, sugar-, and cholesterol-containing diets, resulting in strongly elevated plasma cholesterol and triglyceride levels, with a prominent increase in VLDL- and LDL-sized lipoprotein fractions.[67] Plasma lipid levels can easily be adjusted to a desired concentration by titrating the amount of cholesterol and sugar in the diet. As compared to ApoE-/- and Ldlr-/- mice, E3L mice represent a moderate mouse model for hyperlipidemia (cholesterol levels on chow are about 2 mM and do not exceed 25 mM on a high-cholesterol diet). In addition, the plasma cholesterol and triglyceride levels respond strongly to changes in hepatic VLDL production. Therefore, drugs and diets influencing the chylomicron and VLDL production show parallel effects on plasma cholesterol and triglyceride levels. In this respect, E3L mice are more sensitive than ApoE-/- and Ldlr-/- mice and respond to hypolipidemic compounds with cholesterol-lowering.

E3L mice develop atherosclerotic lesions with all the characteristics of human vascular pathology, varying from fatty streak to mild, moderate and severe plaques. Atherosclerosis development starts at the aortic root and progresses along the entire arterial tree in a time-dependent fashion.[69]

1.5.3 LDL receptor-deficient (Ldlr-/-) mice

In humans, mutations in the gene for the LDL-receptor (LDLr) cause familial hypercholesterolemia. Mice lacking the gene for LDLr display a modestly elevated plasma cholesterol level when maintained on a regular chow diet (about 5 mmol/l versus 2 mmol/l in wild-type animals), and they develop atherosclerosis only slowly. On HC diet feeding, Ldlr-/- mice show strongly elevated plasma cholesterol (>25mmol/l) and rapid development of atherosclerosis.[70] The plasma lipoprotein profile of Ldlr-/- mice resembles that of humans, with the cholesterol being confined mainly to the LDL fraction.

The morphology of the lesions in Ldlr-/- mice is comparable to that in ApoE-/- and ApoE*3Leiden mice, with the plaques developing in a time-dependent manner, starting from the proximal aorta. Quantification of atherosclerosis in Ldlr-/- mice shows mild to moderate atherosclerotic lesions in the aortic root and coronary arteries of mice fed a mild atherogenic diet lacking excessive amounts of cholesterol and cholate.[71]

Most interestingly, and in contrast with the ApoE*3Leiden transgenic mouse model, the Ldlr-/- mouse model does also develop diet-induced obesity, hypertriglyceridemia and insulin resistance, in parallel with the development of atherosclerotic lesion [72].

1.6 Scope, aims and outline of this thesis.

Atherosclerosis is a multifactorial disease of the large arteries and the leading cause of morbidity and mortality in industrialized countries.[73] There is ample evidence that hypercholesterolemia (i.e. elevated plasma levels of VLDL and LDL) is a major causative factor in atherogenesis.[74,75] It is equally clear that atherogenesis has an inflammatory component which is thought to drive the progression of the disease.[76,77] However, while the lipid component in atherosclerosis development is relatively well-understood, the origin and exact contribution of the inflammatory component remains largely unknown.

The aim of this thesis is to further define and delineate the contribution of the inflammatory component to the atherosclerotic process and to elucidate the link between cholesterol and inflammation in atherosclerotic lesion formation and progression. Since inflammatory markers associated with elevated cardiovascular risk are increased by cholesterol feeding in animal models, particular emphasis is devoted to cholesterol as an inflammation-evoking factor.

In **Chapter 2** we elucidated the link between dietary cholesterol intake and the onset of inflammation in the context of early atherosclerotic lesion formation using ApoE*3Leiden (E3L) mice. Since cholesterol feeding is associated with elevations in liver-derived

inflammation markers such as CRP in humans, we studied the effect of increasing dietary cholesterol concentrations on liver gene expression (genome-wide transcriptome) and the liver lipid profile (lipidome). Functional systems biology was applied to unravel key inflammatory pathways activated by cholesterol and to identify the molecules that link cholesterol metabolism to inflammation.

To further delineate the relative contribution of lipids versus inflammation to atherogenesis, different approaches have been applied in the following chapters.

First, in **Chapter 3** we investigated compounds with well-established lipid-modulating (anti-atherogenic) effects on their putative anti-inflammatory activity, *viz.* a statin, a PPARα agonist and an LXR-agonist. To characterize their lipid-independent anti-inflammatory capacity, we have evaluated these compounds in a mouse model for inflammation, huCRP transgenic mice**.**

Since additional anti-inflammatory effects were observed for all drug classes, one compound per class was further analyzed in the context of atherosclerosis. Dietary experimental conditions were established in the E3L model that enabled us to delineate the contribution of the lipid component and the inflammatory component to the overall atherosclerotic process. In **Chapter 4** we evaluated the pleiotropic anti-inflammatory effects of fenofibrate, a PPARα activator. The additional contribution of the inflammatory component to the atherosclerotic process could be deduced by comparing of the anti-atherogenic activity of fenofibrate with a dietary cholesterol-matched control group. In **Chapter 5** we analyzed the pleiotropic effects of atorvastatin, a HMG-CoA reductase inhibitor, in the context of lesion progression (i.e. the treatment of already established lesions). In **Chapter 6** we studied the anti-inflammatory effects of T0901317, an LXR-agonist in longitudinal studies of lesion progression and regression. Since T0901317 had no plasma cholesterol-lowering effect in E3L mice, this study in particular provided insight into the importance of (cellular) inflammation in atherogenesis.

In **Chapter 7** we used a different approach (genetic deletion of the pro-inflammatory factor Macrophage Migration Inhibitory Factor (MIF)), to study the role of inflammation on atherogenesis. The Ldlr-/- model not only enabled us to find further evidence for a role of inflammation alone in atherogenesis, it also allows to extent our findings to another disease area and combine atherosclerosis and insulin resistance. The relevance of MIF in atherosclerotic disease in the human situation was confirmed in **Chapter 8**, in which the involvement of MIF in the occurrence of an atherosclerosis-related disease, abdominal

aortic aneurysm, was evaluated. The general conclusions from the studies performed in this thesis and future perspectives are described in **Chapter 9**.

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