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Titel der Masterarbeit

Regulation of the LDL-R gene family expression in the cells of the human coronary artery vascular wall

Verfasserin

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Abstract

Cardiovascular disease (CVD) is rated as a leading cause of death, particularly in industrialised countries. Atherosclerosis is a common cause of CVD. Therefore, several mechanisms leading to atherosclerosis development are covered in this thesis. The aim of the thesis project was to investigate the regulation of the low-density lipoprotein receptor (LDL-R) gene family expression in human coronary artery endothelial cells (HCAEC) and human coronary artery smooth muscle cells (HCASMC). The study focused on the LDL-R, VLDL-R, LRP1, and LRP2 and included the influence of several factors which have been suggested to play a role in atherosclerosis: modified lipoproteins (in this study carbamylated LDL), estrogen, and alterations of vascular shear stress.

Carbamylated LDL is considered as a risk factor since patients with chronic kidney disease are prone to develop CVD ("uremia-induced atherosclerosis"). Low shear stress is believed to act atherogenic because of its numerous unfavourable effects on endothelial function. Actually, atherosclerotic lesions occur preferentially at regions with low vascular shear stress. In contrast, estrogen is suggested to be responsible for the lower incidence of atherosclerosis in premenopausal women. Thus, atheroprotective properties are attributed to the steroid hormone.

Human coronary artery endothelial cells were cultivated under static and dynamic (high and low shear stress) conditions, human coronary artery smooth muscle cells were cultivated under static conditions. Then, the cells were treated with native and carbamylated lipoproteins and 17β-estradiol in different combinations. The receptor gene expression levels in these cells were analysed by real-time quantitative PCR. Furthermore, protein detection by Western-blot, lipoprotein modification analysis by relative electrophoretic mobility determination and cytotoxicity assays should complete the studies.

In summary, significant alterations in gene expression of both cell types could be determined. These findings provide further evidence for a crucial role of carbamylated LDL, estrogen, and shear stress in disease development.

Zusammenfassung

Kardiovaskuläre Erkrankungen zählen zu den führenden Todesursachen in den Industriestaaten. Sie werden häufig durch die multifaktorielle Erkrankung Atherosklerose verursacht, weswegen in dieser Arbeit einige Faktoren untersucht werden sollen, die zur Entwicklung dieser Krankheit beitragen. Das Ziel dieses Projekts ist die Untersuchung der Expression der LDL-Rezeptor-Genfamilie und ihrer Regulation in Endothelzellen und glatten Muskelzellen der menschlichen Koronararterien. Die Studie konzentrierte sich dabei auf den LDL-R, VLDL-R, LRP1 und LRP2 und behandelte den Einfluss von mehreren Faktoren, die für die Entstehung von Atherosklerose von Bedeutung sein sollen: modifizierte Lipoproteine (in dieser Studie carbamyliertes LDL), Östrogen und Veränderungen der auf die Gefäßwand wirkenden Scherkräfte („shear stress“).

Carbamyliertes LDL gilt als Risikofaktor, da Patienten mit chronischen Nierenerkrankungen eine kardiovaskuläre Erkrankung entwickeln („Urämie-induzierte Atherosklerose“). Verminderter „shear stress“ wird aufgrund seiner zahlreichen negativen Auswirkungen auf die Endothelfunktion ebenfalls als atherogen eingestuft. Tatsächlich treten Läsionen bevorzugt in Gefäßbereichen auf, in denen geringere Scherkräfte wirken. Östrogen werden zahlreiche atheroprotektive Funktionen zugeschrieben. Das Steroidhormon wird daher für die geringere Inzidenz von Atherosklerose in prämenopausalen Frauen verantwortlich gemacht.

Endothelzellen wurden unter statischen und dynamischen Bedingungen kultiviert, glatte Muskelzellen unter statischen Bedingungen. Anschließend wurden beide Zelltypen mit verschiedenen Kombinationen von nativen und carbamylierten Lipoproteinen und 17β-Estradiol behandelt. Die Genexpression der Rezeptoren wurde mittels quantitativer RT-PCR ermittelt. Ein Proteinnachweis mittels Western-blot sowie eine Lipoprotein-Modifikationanalyse mittels Elektrophorese („relative mobility“) und Zytotoxizitätstests sollten die Untersuchung ergänzen.

Zusammengefasst konnten signifikante Änderungen der Genexpression festgestellt werden. Diese Erkenntnisse sind als weiterer Hinweis auf eine wichtige Rolle der untersuchten Faktoren in der Krankheitsentstehung zu bewerten.

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1. Introduction

1.1 Atherosclerosis

Atherosclerosis has become a major health problem, especially in industrialised countries. This inflammatory disease is characterised by the progressive accumulation of lipids and the development of atheromatous plaques in the vessel wall. Resulting in cardiovascular disease like myocardial infarction and stroke, it is rated as a leading cause of death. This chapter will refer to disease development, mechanisms, and risk factors.

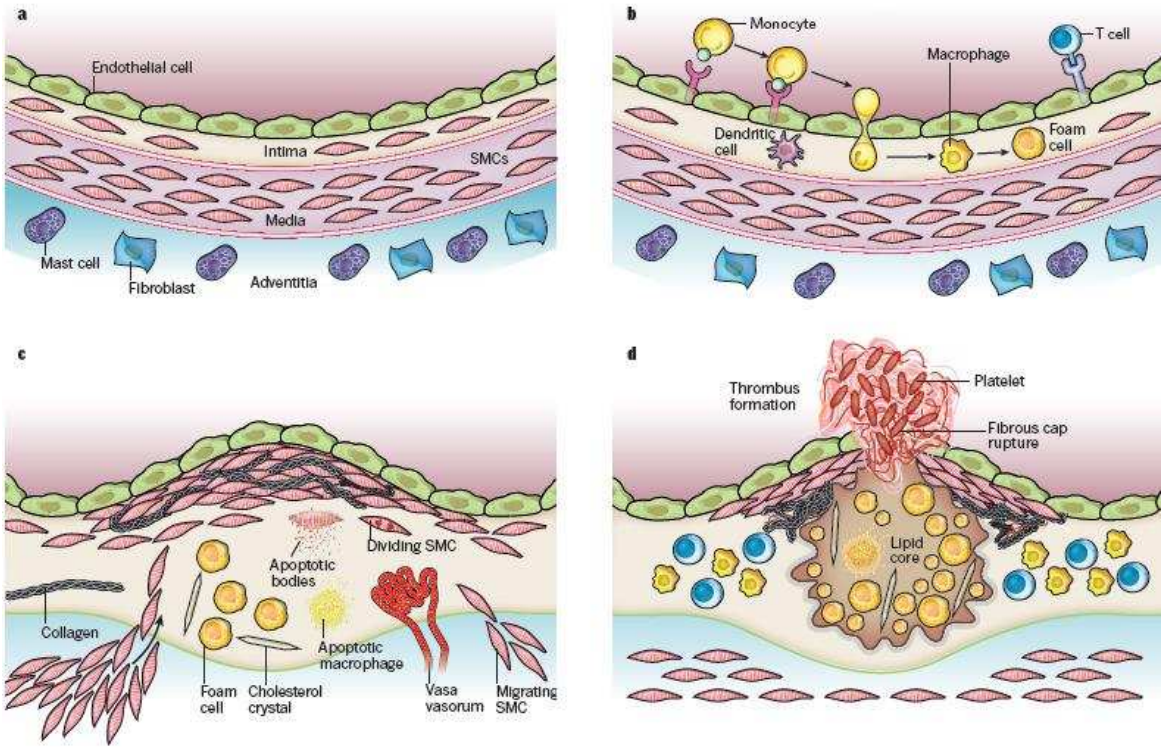

Fig. 1: Formation of atherosclerotic lesions, took from [1].

a) the normal artery wall

b) adhesion and entry of monocytes, foam cell formation by lipid uptake

c) migration and proliferation of smooth muscle cells, formation of a fibrous cap

d) plaque rupture and thrombus formation

1.1.1 The healthy artery wall and endothelial function

The normal arterial wall is built up of three layers (see fig. 1a). A monolayer of endothelial cells (EC) lines the tunica intima that contains resident smooth muscle cells (SMC). The middle layer, termed tunica media, comprises smooth

muscle cells in an extracellular matrix (ECM). Fibroblasts, mast cells, microvessels, and nerve endings are located in the outermost layer (adventitia) [1]. In the healthy artery, endothelial cells perform atheroprotective functions mediated by endothelial nitric oxide synthase (eNOS). They resist adhesion and aggregation of inflammatory cells and platelets, control the vascular tone and promote fibrinolysis [2]. More precisely, nitric oxide (NO) induces vasodilation, inhibits platelet activation, and blocks the expression of adhesion molecules (vascular cell adhesion molecule VCAM-1, intracellular adhesion molecule ICAM-1) as well as inflammatory molecules regulated by nuclear factor kappa B (NF-κB). However, constant exposure to risk factors (hypertension, hyperlipidemia, obesity, smoking, insulin resistance, inflammation) results in eNOS decrease, a feature of endothelial dysfunction [3].

1.1.2 The onset of atherosclerosis

The initial steps of the disease are characterised by the capture of monocytes on the EC surfaces and the entry of lipoprotein particles in the artery wall (see fig. 1b). The expression of adhesion molecules is provoked by irritating stimuli like dyslipoproteinemia, hypertension, and pro-inflammatory mediators. Simultaneous changes in ECM composition and endothelial permeability advance entry and retention of LDL particles in the artery wall [4]. LDL is supposed to show an increased affinity for ECM components, especially chondroitin sulphate proteoglycans, leading to interaction, retention and aggregation [5]. The resulting complexes of lipoproteins and proteoglycans are prone to modifications (aggregation, oxidation, enzymatic cleavage) and therefore considered as pro-atherogenic [3].

Monocytes are attracted by the chemokine MCP-1 (macrophage chemoattractant protein 1) [6]. After binding to the endothelial surface and migration into the tunica intima, they differentiate to macrophages by colony stimulating factor (CSF). Subsequently, they start to engulf LDL particles and become “foam cells” (intracellular lipid droplets formed by cholesterol esters cause a foamy appearance) [1]. The uptake of modified lipoprotein particles is mediated by scavenger receptors [7] and also by LRP1 [8]. Foam cells release growth factors, cytokines, matrix-metalloproteases, reactive oxygen species (ROS) and tissue factors (TF)

maintaining the inflammatory response. Moreover, these factors induce vascular remodelling and elevate the susceptibility to thrombus formation [3].

1.1.3 Disease progression and thrombosis

Lesion progression implies the recruitment of SMC from tunica media to the tunica intima (see fig. 1c), where SMC proliferation is induced by mediators such as platelet-derived growth factor (PDGF). Furthermore, the SMC form ECM molecules (interstitial collagen and elastin). As a consequence, the plaque is covered by a fibrous cap [1]. Dying foam cells located under this cap release lipids that accumulate extracellularly and contribute to necrotic core formation [9]. When influenced by atherogenic stimuli, SMC undergo a transformation from the non-proliferative, contractile phenotype into actively proliferating cells leading to vascular remodelling and intimal thickening [3].

Plaques cause medical problems by developing blood-flow limiting stenoses leading to tissue ischemia. In addition, they can interrupt the blood flow by eliciting thrombi. A thrombus arises when the fibrous cap ruptures (see fig. 1d) leading to an exposition of the core's pro-coagulant substances to coagulation proteins in the blood [10].

1.2 Lipoproteins

Lipoproteins are of particular importance for atherosclerosis since a strong association between CVD and plasma lipid levels has been observed [11].

1.2.1Structure and classification of lipoproteins

Lipoproteins facilitate the transport of hydrophobic lipids in the blood. As illustrated in fig. 2 and fig. 3, these spherical molecule complexes consist of a hydrophilic shell (phospholipids, cholesterol, apolipoproteins) and a hydrophobic core (triglycerides, cholesterol esters). They differ in density, size, place of assembly, lipid composition, and apolipoprotein classes. Usually, they are classified by their density (see fig. 4). Each lipoprotein class has its specific apolipoproteins, which serve as structural elements and stabilize lipid emulsions. Furthermore, they mediate synthesis and degradation of lipoproteins by enzyme

activation and serve as ligands for lipoprotein receptors in membranes of target cells [12, 13].

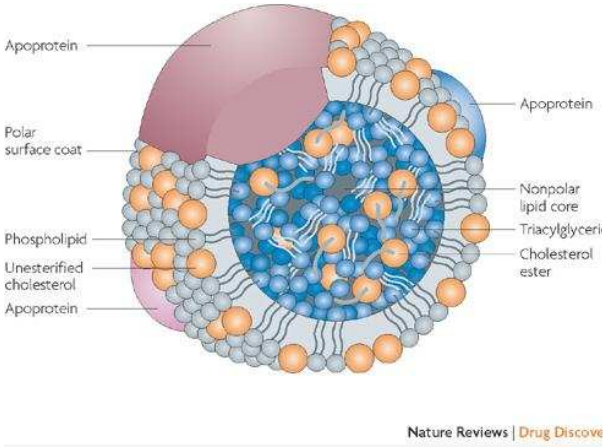

Fig. 2: Structure of a lipoprotein [14].

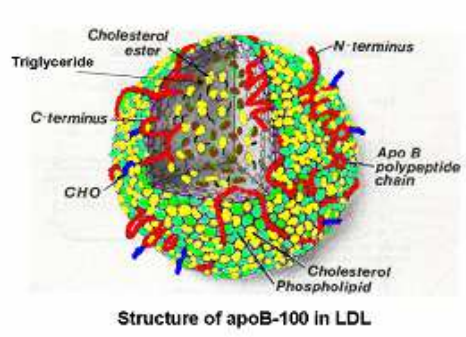

Fig.3: LDL and its apoprotein B100 [15].

Classes*	Density range (g/ml)	Particle size‡ (diameter, nm)	Flotation rate (Sf)	Apolipoproteins
CM	<0.95	75-1200 (0)	> 400	B-48, A-I, A-II, C†, E
VLDL	0.95-1.006	30-80 (preβ)	20-400	B-100, C†, E
IDL	1.006-1.019	25-35 (β)	12-20	B-100, C†, E
LDL	1.019-1.063	18-25 (β)	0-12	B-100
HDL	1.063-1.210	5-12 (α)		A-I, A-II, C†, E

*CM, chylomicrons; VLDL, very low density lipoproteins; IDL, intermediate density lipoproteins; HDL, high density lipoproteins.
C† There are three apoC subclasses: apoC-I, apoC-II, apoC-III.
‡ Electrophoretic mobilities in agrose are indicated in parentheses.
Source: Data from Zubay et al (1995), Schultz and Liebman (1997).

Fig. 4: Overview of lipoproteins and their characteristics [16].

1.2.2 The lipoprotein metabolism (illustrated in fig. 5)

Ingested lipids are transported by chylomicrons from the intestine into the periphery. Then, lipoproteinlipase (LPL) is activated by apolipoprotein (Apo) CII leading to a release of free fatty acids, which are absorbed by body cells. The cholesterol-rich chylomicron-remnants are taken up by the liver by binding to ApoE receptors. The liver exports triglycerides and cholesterol in very-low density lipoprotein (VLDL) particles, which deliver fatty acids to the periphery also using the mechanism of LPL activation. Approximately 50% of the VLDL-remnants (also called intermediate density lipoprotein (IDL)) are converted to low-density lipoprotein (LDL) by hepatic lipase. LDL is responsible for choles-

terol transport to peripheral tissues, where it regulates the de-novo cholesterol synthesis. LDL uptake by receptor-mediated endocytosis is shown in figure 6 and figure 7. It requires the binding of ApoB-100 to the LDL receptor. The receptor-LDL-complex is internalised in a vesicle that fuses with a lysosome. The LDL is hydrolysed whereby free cholesterol enters the cytoplasm and the LDL receptor goes back to the membrane. The resulting free cholesterol acts as regulator for cellular cholesterol homeostasis. The particular mechanisms are described in figure 6. High-density lipoprotein (HDL) is responsible for reverse cholesterol transport. ApoAI on HDL activates lecithin-cholesterol-acyltransferase (LCAT), which esterifies cholesterol. Thus, excess cholesterol is eliminated from the blood and transported to the liver. In addition, HDL exerts several other beneficial functions (see fig. 8) [12, 13].

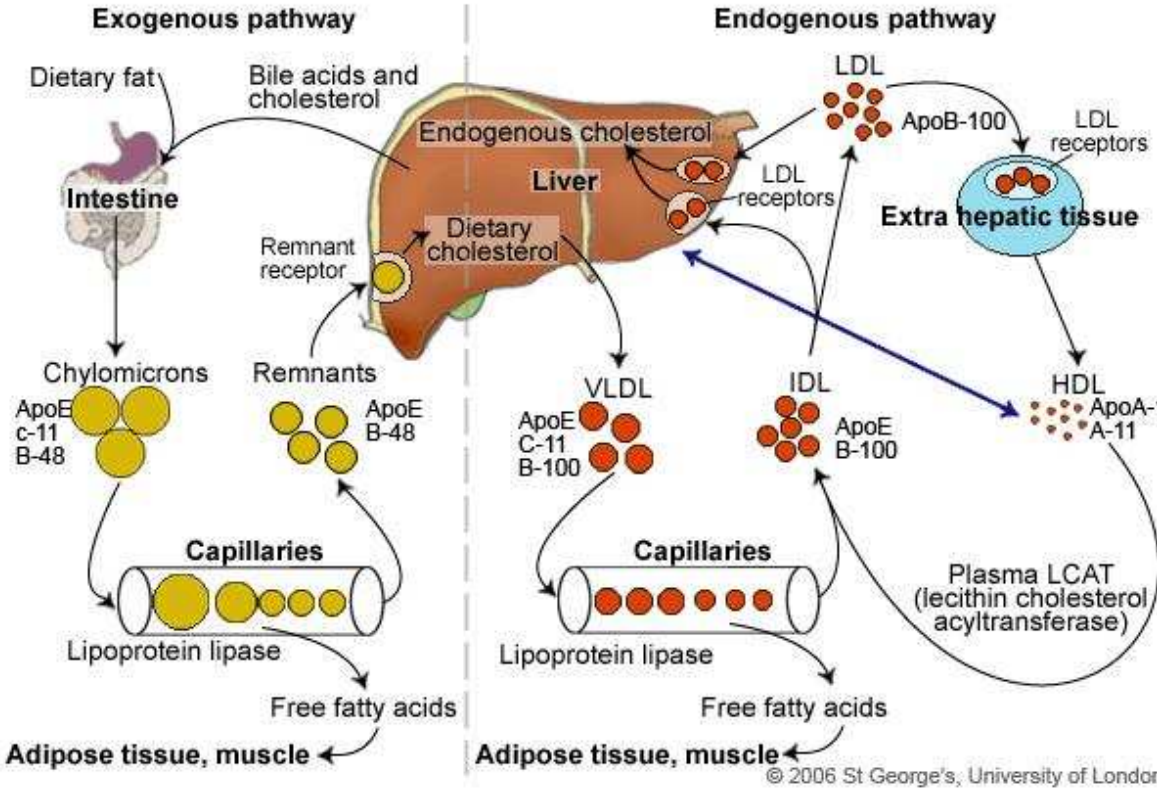

Fig. 5: Lipoprotein metabolism, took from [17].

Fatty acids are transported from the intestine to the blood via chylomicrons. These particles supply peripheral tissues with fatty acids. Chylomicron remnants are absorbed by the liver. The liver releases VLDL particles for delivering fatty acids to adipose tissue and muscles. Their remnants are partly converted to LDL, which is internalized by the LDL receptors of the liver or extrahepatic tissues. HDL mediates the transport of cholesterol back to the liver.

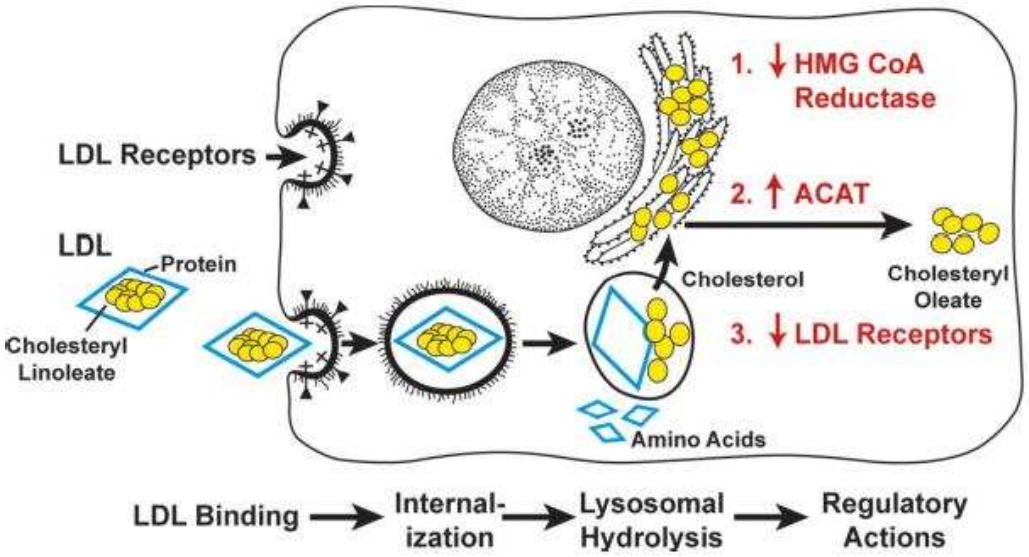

Fig. 6: Regulatory actions of dietary cholesterol [18].

The LDL receptor binds LDL via apoB100. This complex is internalized ("clathrin-coated pits") in a vesicle and hydrolysed in a lysosome. The released cholesterol executes regulatory functions: to maintain cellular cholesterol homeostasis, it accelerates cholesterol esterification by activating ACAT (Acyl-CoA-Cholesterin-Acyltransferase) and blocks HMG CoA reductase (3-hydroxyl-3-methyl-glutaryl-CoA reductase), the enzyme responsible for endogenous cholesterol synthesis. In addition, it reduces the number of LDL receptors to decrease further cholesterol uptake. The sterol regulatory element binding protein (SREBP) transcription factors have a crucial role in these regulatory actions.

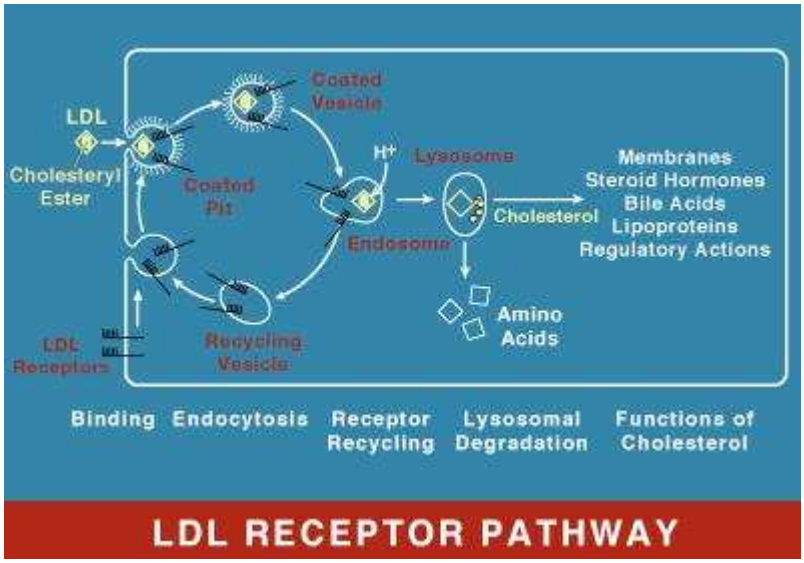

Fig. 7: The cycle of the LDL receptor [19].

After cholesterol release, the receptor is recycled and goes back to the membrane.

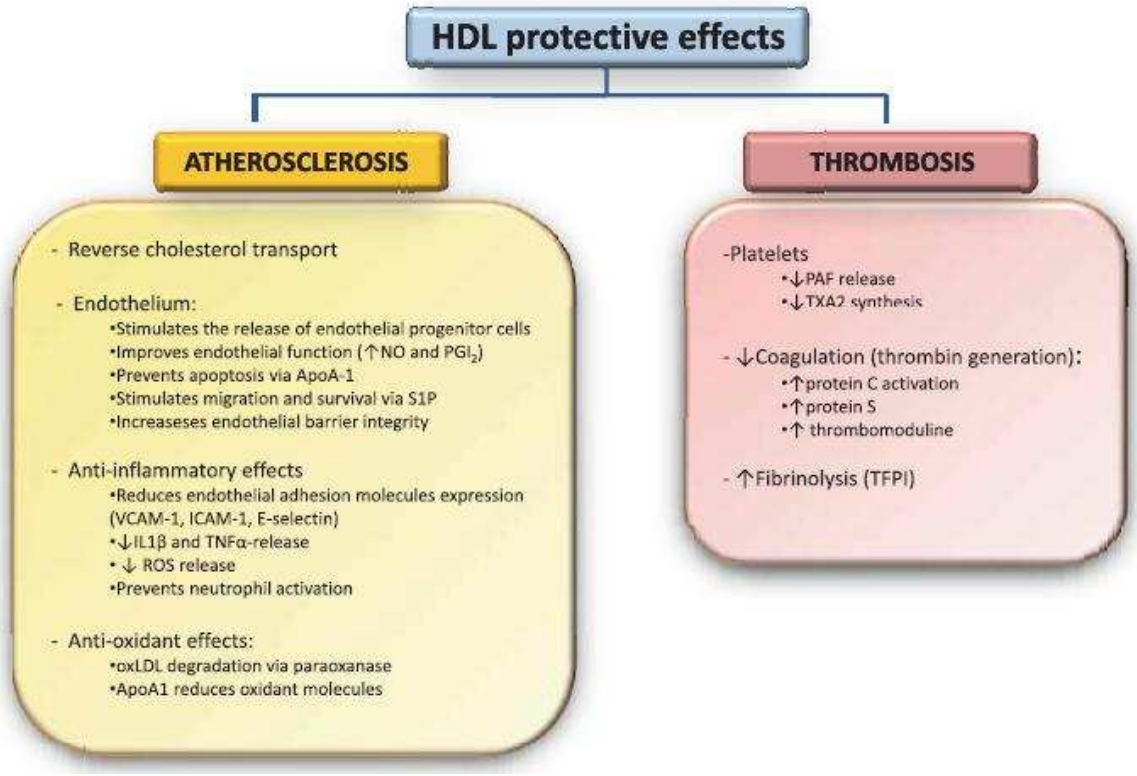

Fig. 8: Protective effects of HDL concerning atherosclerosis and thrombosis [3].

1.3 The LDL receptor gene family

The members of the LDL receptor gene family are transmembrane receptors that share structural and functional properties. They are expressed on the cell surface and interact with several ligands via specific extracellular binding sites. After receptor internalization by coated pits, these ligands are transported to lysosomes and degraded. Fig. 9 visualizes the structure of the receptor family: they consist of ligand binding repeats, epidermal growth factor (EGF) precursor repeats, YWTD repeats (“β-propeller”), a single transmembrane domain, and a short cytoplasmic tail containing internalization signals [20, 21].

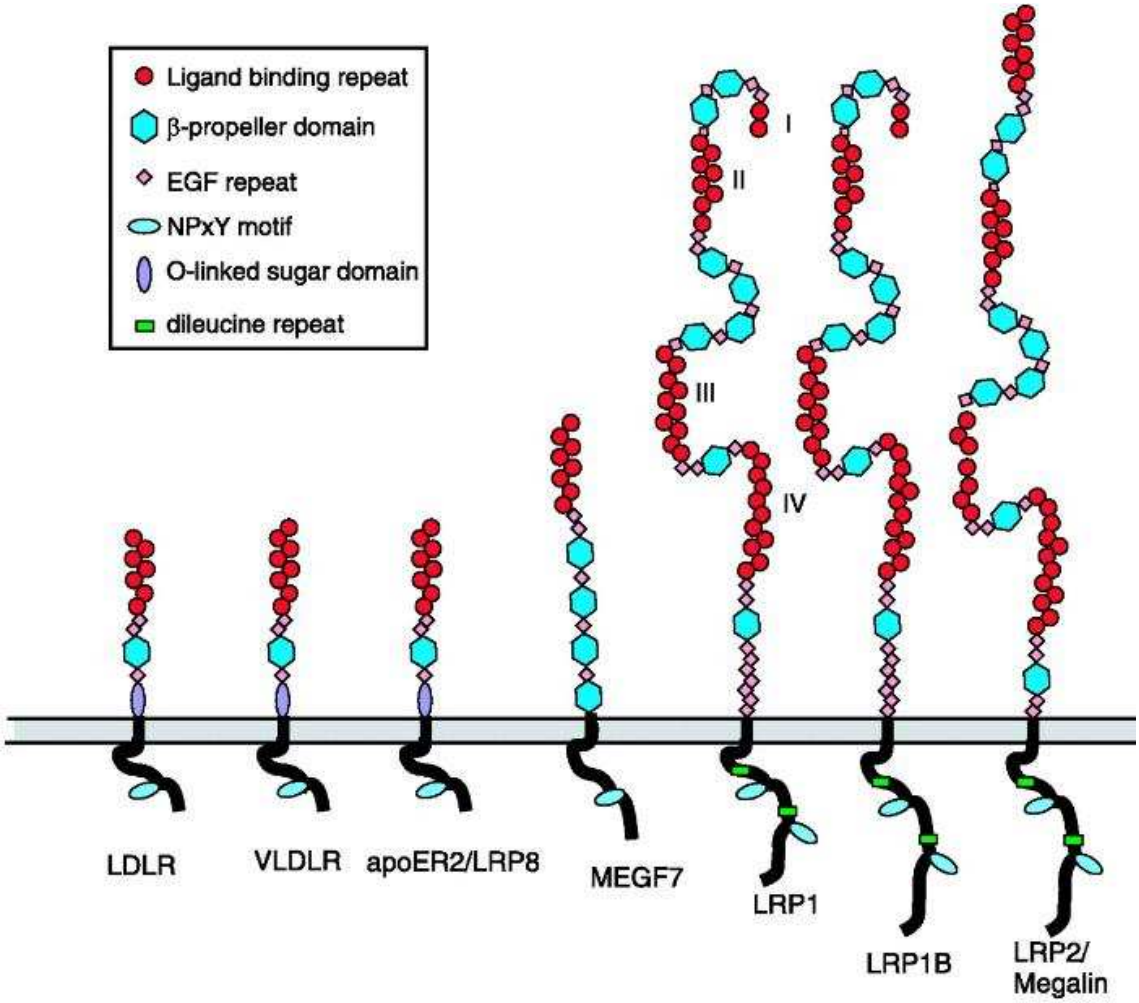

Fig. 9: The structure of the LDL receptor gene family [22]

1.3.1 The low-density lipoprotein receptor (LDL-R)

The LDL receptor was discovered in the 1970s by Brown and Goldstein, who did research on familial hypercholesteremia (FH) and the regulation of the lipoprotein metabolism in fibroblasts [23-29]. This integral membrane glycoprotein is made up of 839 amino acids and binds lipoproteins containing apoB-100 and apoE. Additionally, it contains an O-linked sugar domain, which is not shared by all family members. Its main responsibility is to mediate lipoprotein uptake of the cells (see fig. 6 and fig. 7). LDL receptor defects lead to disturbances of the lipoprotein metabolism. For example, patients with FH suffer from high cholesterol levels due to a lack of functional receptors [18, 30]. Therefore, it is self-evident that a sufficient lipoprotein clearance by the LDL receptor is crucial for mainte-

nance of the health of the arterial wall. The influence of various factors on the LDL receptor's expression level has to be investigated for that reason.

1.3.2 The very-low-density lipoprotein receptor (VLDL-R)

The VLDL receptor was discovered in the group of T. Yamamoto in 1992 [31]. This receptor consists of 846 amino acids and differs from the LDL receptor just by possessing one additional ligand-binding domain. It was found to be highly expressed in the heart, skeletal muscle, and adipose tissue but not in the liver. Therefore, it was suggested to supply peripheral fatty acid active tissues with triglyceride-rich lipoproteins. The VLDL receptor was reported to bind VLDL, IDL, and β-VLDL, but not LDL [32, 33].

VLDLR expression was detected in endothelial cells and smooth muscle cells of healthy arteries and veins as well as in plaques and macrophage-derived foam cells [34]. Furthermore, the expression of VLDLR (and scavenger receptor) mRNA was suggested to be strongly induced in atherosclerotic lesions. As well as LRP1, the VLDLR was supposed to play a role in formation of macrophage- and smooth muscle cell-derived foam cells [35]. In particular, it was reported to contribute to foam cell formation by β-VLDL uptake leading to an accumulation of cholesterol esters in the respective cells [36]. In contrast to the LDLR, the VLDLR is not downregulated by sterols. Beyond its role in lipoprotein metabolism and atherosclerosis, the VLDLR has been proposed to function in cardiac fatty acid metabolism, neuronal migration, insulin resistance, and obesity [37].

1.3.3 LDL receptor-related protein 1 (LRP1)

LRP1 has a relative molecule mass of 600kDa and has been detected in liver, lung, brain, hepatocytes, fibroblasts, neurons, macrophages, and smooth muscle cells [38]. It recognizes at least 40 different ligands. Several ApoE-containing lipoproteins (chylomicrons, VLDL-remnants, β-VLDL) are among them. Furthermore, it was reported to bind cytoplasmic adaptor proteins and to interact with other transmembrane receptors suggesting a role in signalling [22].

In addition, its deletion was shown to be lethal in mice. This finding indicates an important role in development [39]. Beyond its presumptive function as remnant receptor, LRP1 performs other atherosclerosis-relevant tasks. It regulates vascular smooth muscle cell growth and migration by controlling PDGF signalling. Thus, LRP1 contributes significantly to the protection of the vasculature [40-43]. In macrophages, LRP1 was reported to mediate the uptake of chylomicron remnants [44]. Furthermore, the higher intracellular lipid deposition in vascular smooth muscle cells (VSMC) from atherosclerotic plaques was attributed to increased LRP1 expression levels. Thus, LRP1-mediated LDL uptake was considered to contribute to lipid accumulation in the arterial wall [45]. Notably, LRP1 expression itself was reported to be elevated by exposure to high LDL concentrations and accumulation of cholesterol esters in SMC [46].

1.3.4 LDL receptor-related protein 2 (LRP2)

LRP2 (also termed megalin or gp330) is a large (600kDa) endocytic receptor expressed in epithelial cells, especially on their apical surface. Amongst others, it has been detected in kidney, lung and gallbladder. In the kidney, it is believed to execute a relevant function: facilitating the recapture of filtered molecules in the proximal tubules. Beyond internalization, LRP2 has been suggested to act in signal transduction pathways and to play a role in the central nervous system as well as in neurodegenerative diseases. ApoB and apoE also belong to the numerous known ligands of LRP2. Furthermore, it has been reported to internalize apoAI and apoAII. Thus, a contribution to the regulation of HDL metabolism is conceivable [47, 48]. In a complex with its partner cubilin, megalin was shown to internalize LDL [49] and HDL [50]. All these findings indicate a role in lipoprotein metabolism.

1.4 Carbamylation of LDL

Besides common risk factors, chronic kidney disease (CKD) has been recognised as severe preconditioning background for the development of atherosclerosis and, as a consequence, cardiovascular disease. Patients with end-stage renal disease frequently die from cardiovascular complications. Carbamylated LDL has been suggested to link CKD to uremia-induced atherosclerosis. Furthermore, modified lipoproteins are counted among the uremia-related risk factors like hyperhomocysteinemia, increased oxidative stress, endothelial injury and dysfunction, and chronic microinflammation [51-58].

In addition to carbamylated LDL (cLDL), the following chemical modifications of LDL have been reported: oxidized LDL (oxLDL) [59], acetylated LDL, ethylated LDL, methylated LDL [60], and glycated LDL [61]. They are commoly accepted to cause endothelial cell injury, thereby favouring atherosclerotic processes [62]. Moreover, they elevate the expression of adhesion molecules for monocyte binding [63] and increase macrophage scavenger receptor expression [64]. As a result, the proliferation of VSMC is stimulated, which is also mediated by oxLDL directly [65]. Recently, they were shown to lower the activity and expression of lysosomal acid lipase in EC and SMC [66].

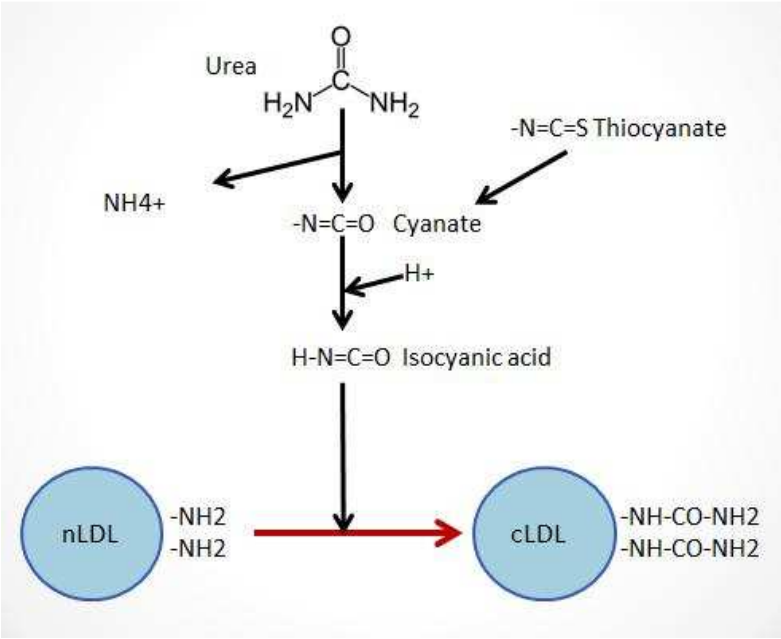

Fig. 10: Reaction scheme of LDL carbamylation, modified from [58].

Fig. 10 shows the formation of cLDL. Urea spontaneously dissociates to ammonia and cyanate. Isocyanic acid reacts irreversibly with the N-terminal amino groups of LDL forming epsilon-amino-carbamyl-lysines. These modifications can accumulate during a lipoprotein's lifespan [67]. This nonenzymatic reaction is proportional to the urea concentration in the blood plasma. Thus, uremic patients show a significantly elevated level of cLDL. Cyanate is also obtained by myeloperoxidase-catalyzed oxidation of thiocyanate, whose levels are elevated in smokers [68].

The clearance of mildly carbamylated LDL is decreased because of reduced LDL receptor binding. However, there is no binding to scavenger receptors yet. Hence, it remains longer in the bloodstream. With increasing carbamylation, the affinity to scavenger receptors rises and binding to the LDL receptor is reduced to a minimum [69-71]. In vitro studies revealed that carbamylated LDL causes injury and dysfunction of EC [72]. Furthermore, it is believed to induce endothelial cell death by mediation of cell proliferation via the mitogen-activated protein kinase (MAPK) pathway [73]. Recent studies suggest an involvement of endonuclease G in cLDL-induced cell death [74]. cLDL exposure to EC in vitro was also shown to induce ICAM-1 and VCAM-1 expression leading to monocyte adhesion [75]. In addition, cLDL affects smooth muscle cells by inducing monocyte adhesion (ICAM-1, VCAM-1 expression) and mediating proliferation [76, 77].

Increasing carbamylation lowers the affinity to the LDL-R, but increases binding to scavenger receptors [78]. These scavenger receptors are supposed to act pro-atherogenic. They bind several modified and native lipoproteins and partially promote inflammation, cell adhesion, oxidative stress and foam cell formation. cLDL is mainly scavenged by lectin-like oxidized LDL receptor 1 (LOX-1) and partially by CD36, SREC-1, and SR-A1. It was postulated to cause in vitro upregulation of LOX-1. However, the translocation of cLDL into the vascular wall is not mediated by LOX-1. Nevertheless, the LOX-1 upregulation was thought to contribute importantly to the cytotoxicity of cLDL to EC [79-83]. Scavenger receptor classes and their structures are illustrated in figure 11.

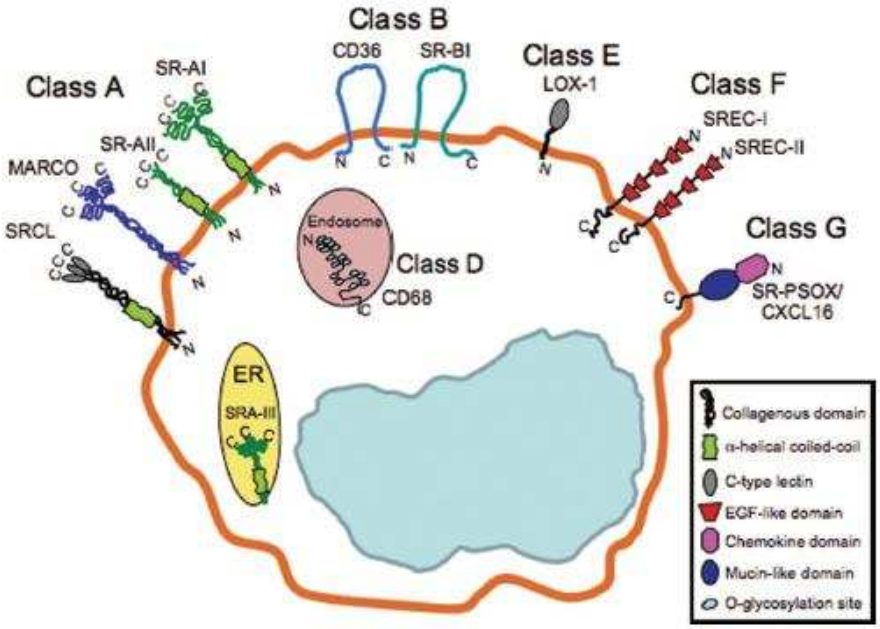

Fig. 11: Structures and classes of scavenger receptors [82].

1.5 The impact of estrogen

It is commonly accepted that premenopausal women have a lower risk for developing cardiovascular disease than men of the same age group. This sex-related difference is mainly attributed to estrogen [84-86]. 17β-estradiol (E_2, structure see figure 12) has been reported to fulfil several protective functions (see figure 13).

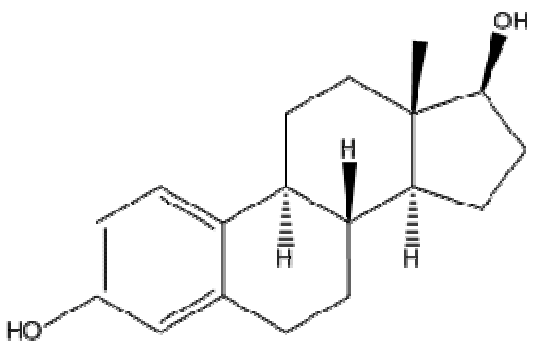

Fig. 12: The structure of the steroid hormone 17β-estradiol (E_2) [87].

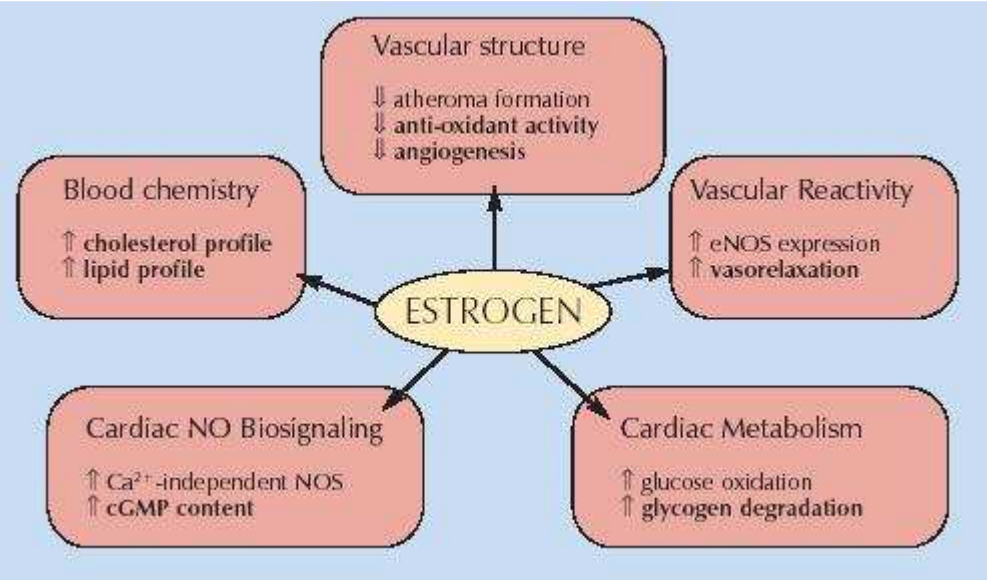

Fig. 13: Protective functions mediated by estrogen [88].

Vascular reactivity is influenced directly by targeting endothelial cells. Furthermore, estrogen modulates vascular tone by affecting the extracellular matrix and vascular smooth muscle cells. It impacts serum lipid concentrations, fibrinolysis, and coagulation. In addition, it causes vasodilation by increasing the release of vasodilatory substances (NO, prostacyclin) and decreasing vasoconstrictor levels (endothelin, angiotensin II) [89-91]. Moreover, it performs anti-oxidative actions [92]. A role in remodelling of the actin cytoskeleton and endothelial cell migration has also been revealed [93]. Recently, an estrogen-mediated reduction of cholesterol ester accumulation in human monocyte-derived macrophages has been demonstrated [94]. Sex differences in vascular contraction have been suggested to be directly related to the effects of estrogen on vasculature [95]. However, the application of hormone replacement therapy remains controversial as there are non-beneficial effects observed as well [96].

The effects of estrogen are regulated by genomic and non-genomic pathways (see fig.14). The genomic effects require transcriptional processes, whereas the non-genomic are so-called “rapid responses”. Three receptors are known: two ligand-activated transcription factors ERα and ERβ (estrogen receptor α and β) that belong to the nuclear hormone receptor superfamily and a G-protein coupled estrogen receptor (GPER or GPR30) [97]. ERα and ERβ were reported to act in EC and SMC [90], GPER was detected in the vasculature as well. GPER

is known to bind estrogen with high affinity and has been proposed to mediate some of the non-genomic effects [98]. It was recently described to relax coronary artery smooth muscle cells after activation by estrogen. Thus, it mediates estrogen signalling in coronary arteries leading to relaxation independent from endothelium and NO [99]. Simultaneously, another group studied the effects of estrogen on SMC and found that contraction was mediated by mineralocorticoid receptor, ERα and GPER [100]. A crucial role in regulating the atheroprotective action of estrogen in LDLR-deficient mice was assigned to the endothelial ERα by Billon-Galés et. al. [101]. However, Villablanca and co-workers assessed ERα as not essential for estrogen-mediated atheroprotection [102].

The stimulatory action of estrogen on LDL-R expression was first described in 1980 [103]. In the meantime, this effect has been investigated more closely. It has been suggested that a specific interaction between ERα and transcription factor Sp1 (specifity protein 1, transcription factor bound to the LDL-R promoter) leads to transcriptional activation of LDL-R by estrogen. Furthermore, an interaction of SREBP-1 with the ERα-Sp1 complex could be responsible for a transactivation of LDL-R [104]. Interestingly, the basal transcription of the LDL-R requires protein kinase C (PKC) activity, whereas the estrogen-stimulated way was found to depend on tyrosine kinase (TK) activity [105].

VLDL-R mRNA expression in the rabbit heart was reported to be strongly increased by estrogen [106]. Furthermore, LRP1 levels in the mouse brain were elevated by estradiol replacement [107] indicating that other LDL-R gene family members are influenced by estrogen as well.

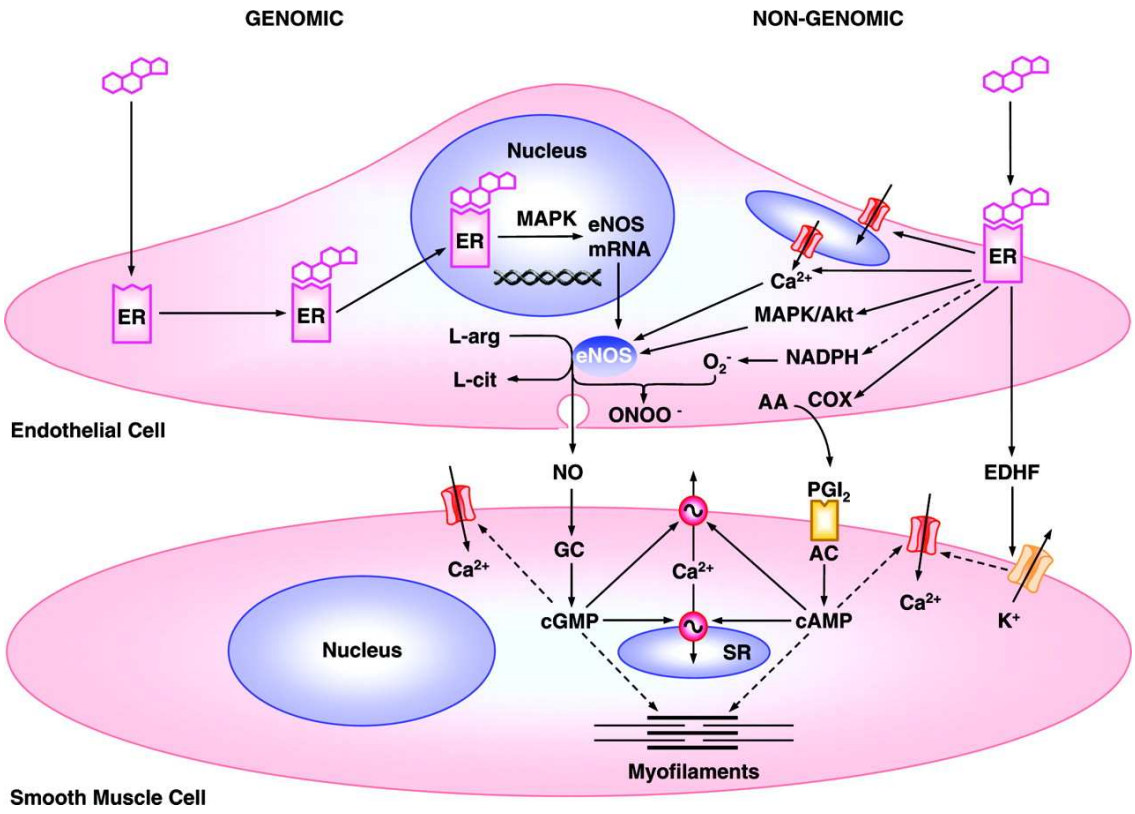

Fig. 14: Model of genomic and non-genomic estrogen actions on EC and SMC [89]. Interrupted arrows mean inhibition.

Genomic: estrogen is bound to endothelial cytosolic/nuclear ER leading to MAPK activation followed by an increase of gene transcription and elevated eNOS production. Non-genomic: estrogen binds to endothelial surface membrane ER resulting in raised Ca^{2+} release from the endoplasmic reticulum and stimulation of the MAPK/Akt pathway leading to eNOS activation.

NO diffuses into VSMC, where it increases cGMP (cyclic guanosine monophosphate). This leads to VSMC relaxation due to decreased intracellular Ca^{2+} concentration. Estrogen also increases endothelial prostacyclin (PGI_2) release leading to cAMP (cyclic adenosine monophosphate) elevation in VSMC and thus relaxation. Further consequences of estrogen action are inhibition of nicotinamide adenine dinucleotide phosphate (NADPH) production (resulting in blockage of NO inactivation and peroxynitrite formation) and endothelium-derived hyperpolarization factor (EDHF)-mediated VSMC relaxation due to hyperpolarization (K^+ channels).

1.6 Shear stress and endothelial function

The predominant development of atherosclerotic lesions in regions with altered blood flow has already been observed in 1969 [108]. Today, it is well-known that lesions preferentially occur at bifurcations, bends, and branches of the arterial tree. This is based on the exposure of these sites to lower or disturbed blood flow resulting in low or oscillatory shear stress on the vessel wall. In contrast, regions subjected to high shear stress resulting from unidirectional flow are protected from atherosclerotic lesion development and inflammation [109]. Shear stress is defined as *"the force per unit area created when a tangential force of blood flow acts on the endothelium"* [110]. It can be expressed as

$$\tau = 4\mu Q/\pi r^3$$

μ… viscosity

Q… flow rate

r… vessel radius

in units of dynes/cm² [111]. The mechanical forces acting on the endothelium are converted by mechanotransduction to intracellular biochemical signals (see figure 15). These signals subsequently influence gene expression. Cells are currently estimated to sense shear stress via ion channels, calveolae, G-protein coupled receptors, tyrosin-kinase receptors, cell-adhesion molecules, glycocalyx, and primary cilia [112].

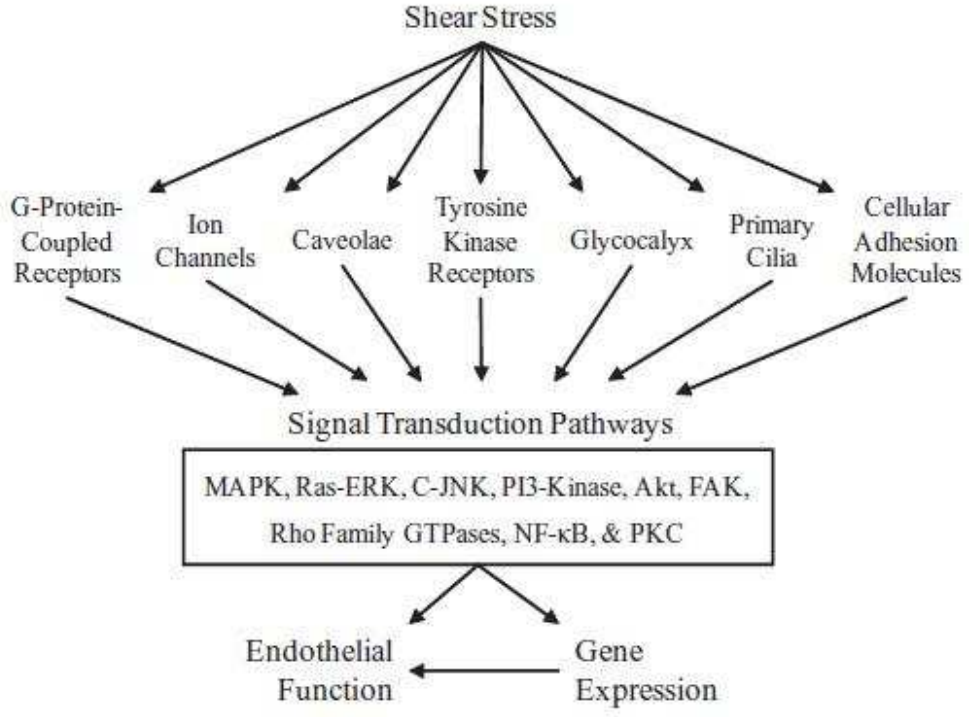

Fig. 15: Ways of shear stress mechanotransduction and involved signalling pathways [112].

Besides alterations in gene expression, the exposition of endothelial cells to shear stress causes changes in cell morphology. The cells align in flow direction (see fig. 16). This alignment has recently been suggested to be promoted by c-Jun N-terminal kinase 2 (JNK2) [113].

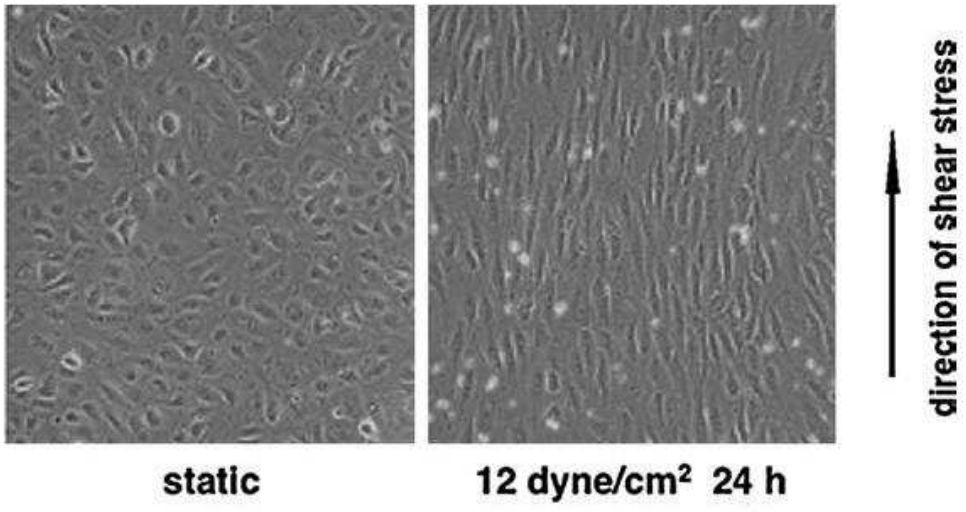

Fig. 16: Endothelial cells change morphology when subjected to shear stress [110].

Laminar shear stress is considered to influence gene expression in an atheroprotective way (see fig. 17). As summarized by Shi Pan [110], it inhibits tumor necrosis factor alpha (TNFα) -mediated inflammatory events and stimulates eNOS-mediated NO production and prostacyclin (PGI_2) secretion. Elevated NO levels result, among others, in decreased monocyte attraction/adhesion and suppression of VSMC proliferation. Furthermore, laminar shear stress activates antioxidative enzymes. Their action is of great relevance since ROS augment monocyte adhesion, SMC proliferation, and lipid oxidation [114]. In contrast, low shear stress supports monocyte binding and transmigration. In addition, it stimulates the production of ROS and SMC growth factors (PDGF, endothelin (ET-1)) [110]. The opposite actions of high and low shear stress are also illustrated in figures 18 and 19.

Shear stress was reported to elevate the expression of the LDL-R leading to an increase of LDL clearance [115]. In particular, the integrin-mediated activation of SREBP-1 by shear stress was suggested to cause LDL-R upregulation [116]. To date, nothing is known about a possible influence on other LDL-R gene family members. Coculture-experiments indicate a mutual affection of smooth muscle cells and shear stress-exposed endothelial cells [117]. As a rather exact reconstruction of the natural conditions, this finding is of great importance.

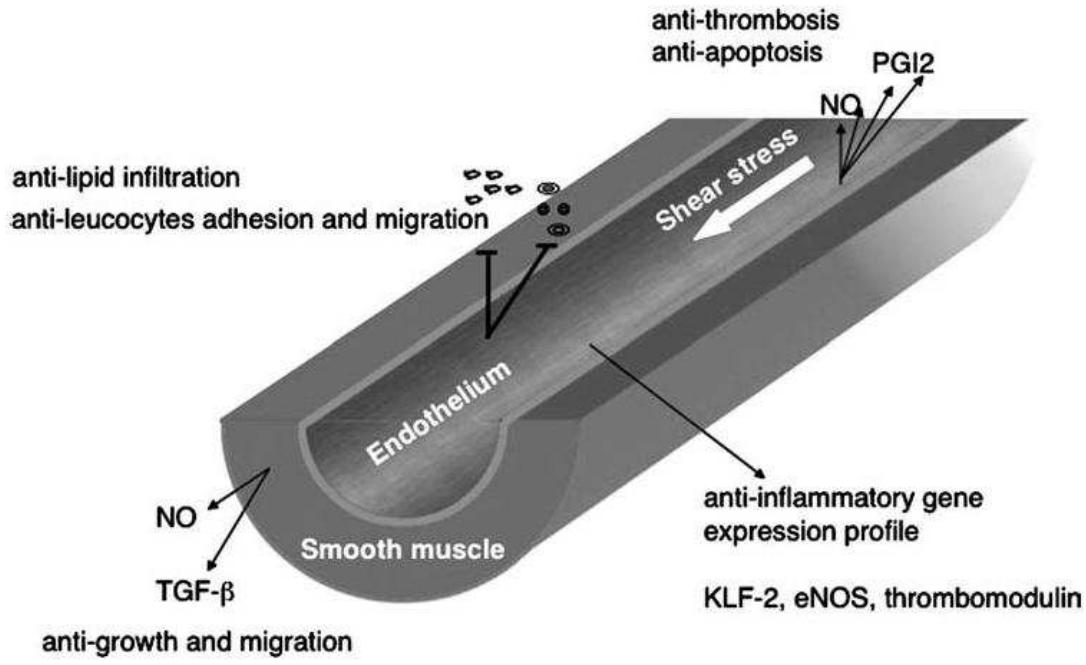

Fig. 17: The atheroprotective actions of high laminar shear stress [110].

Table I. Summary of effects of different flow patterns and associated shear stresses on EC and vascular biology.

Type of flow: Level of shear stress:	Laminar High	Disturbed Low
Vasoactive response	Vasodilation	Vasoconstriction
EC turnover	Lower	Higher
Macromolecular permeability	Lower	Higher
LDL uptake	Lower	Higher
DNA synthesis	Lower	Higher
Phenotype	Elongated, aligned	Polygonal
Expression of adhesion molecule, inflammatory, and chemokine genes	Lower	Higher
Expression of antioxidant genes	Higher	Lower
WBC adhesion and platelet aggregation	Inhibited	Promoted
Oxidative stress	Reduced	Sustained
Endothelialization	Promoted	Reduced

EC: endothelial cell; LDL: low-density lipoprotein; WBC: white blood cell.

Fig. 18: This table (took from [118]) summarizes the effects of shear stress on the vasculature.

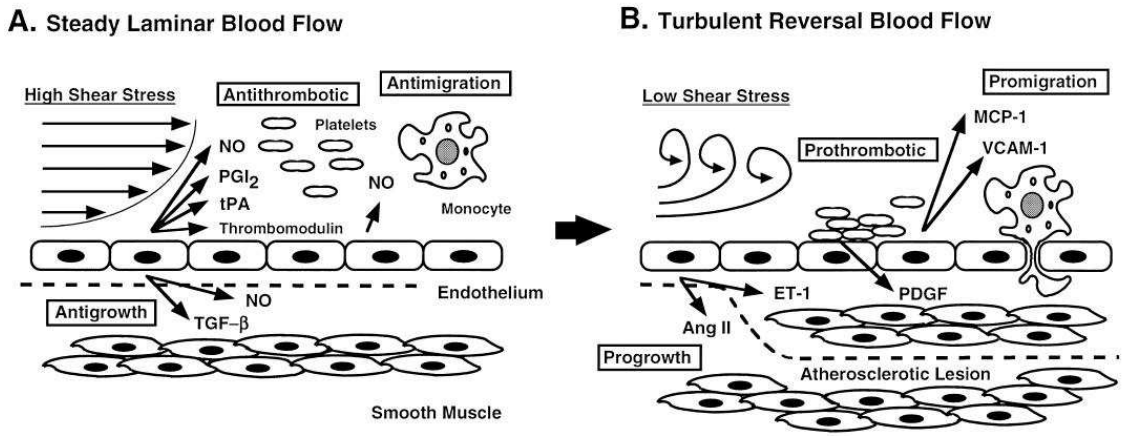

Fig. 19: Comparison of the consequences of high and low shear stress for the endothelium and smooth muscle cells [119]. A) High shear stress causes endothelial cells to release factors that counteract leukocyte migration, coagulation and VSMC proliferation. B) In contrast, low shear stress leads to a release of factors that favour the exact opposite. (tPA… tissue plasminogen activator, TGF-β… transforming growth factor beta, Ang II… angiotensin II)

Following up on research findings gained in previous diploma theses [122, 133], this thesis aims to investigate processes emerging by the influence of estrogen on the LDL-R gene family in HCAEC under high and low shear stress as well as static culture conditions and in HCASMC under static conditions. An essential part of the work was performed in the laboratory of Dr. Gürkan Sengölge (Medical University of Vienna, Department of Internal Medicine III).

2. Methods

2.1 Preparation and modification of lipoproteins

2.1.1 Preparation of lipoproteins (according to Goldstein et. al. [120])

Lipoproteins were separated and isolated by sequential density gradient ultra-centrifugation with potassium bromide. Therefore, human plasma was mixed with a calculated amount of KBr and put in a quick seal tube. The tube was heat-sealed and centrifuged over night at 14℃ as described in [120]. The amount of KBr was calculated using this formula:

$$m(KBr) = \frac{V(plasma) \times (D2 - D1)}{1 - (0{,}312 \times D2)}$$

m(KBr)…. mass of KBr in g
V(plasma)… volume of plasma in mL
D2… designated density
D1… current density
density of HDL: 1.063 – 1.210 g/mL
density of LDL: 1.019 – 1.063 g/mL
density of VLDL: 1.006 – 1.019 g/mL

At first, VLDL had to be removed. The EDTA-plasma (density of 1.006) was therefore adjusted to a density of 1.019 using a calculated amount of solid KBr. If necessary, fulfilling of the quick seal tube (37mL, Beckman) was done with KBr/EDTA buffer (density of 1,019). The tube was heat-sealed and centrifuged at 50K for 20h using a 50.2 Ti rotor from Beckman. Figure 20 visualizes the fractions formed during centrifugation.

After aspirating the VLDL fraction collected on the top of the tube, the bottom fraction was adjusted to a density of 1.063 and subsequently centrifuged at 60K at 14℃ for 24h using an 80 Ti rotor from Beckman.

The top fraction containing LDL was aspirated using a syringe and immediately desalted (PD-10 desalting column, GE healthcare). The column had previously been equilibrated with LDL buffer (150mM NaCl, 0,24mM EDTA, pH 7.4). At last, HDL was separated at a density of 1.210 for 36h at 55K and 14℃ and desalted in the same way.

After this, the concentrations of LDL and HDL were determined spectro-photometrically using the Modified Lowry Protein Assay Kit (Thermo Scientific, Pierce Protein Research Products) according to manufacturer's instructions. Standard solutions with defined concentrations of BSA (0, 10, 20, 40 and 80 mg) were used to generate a calibration line.

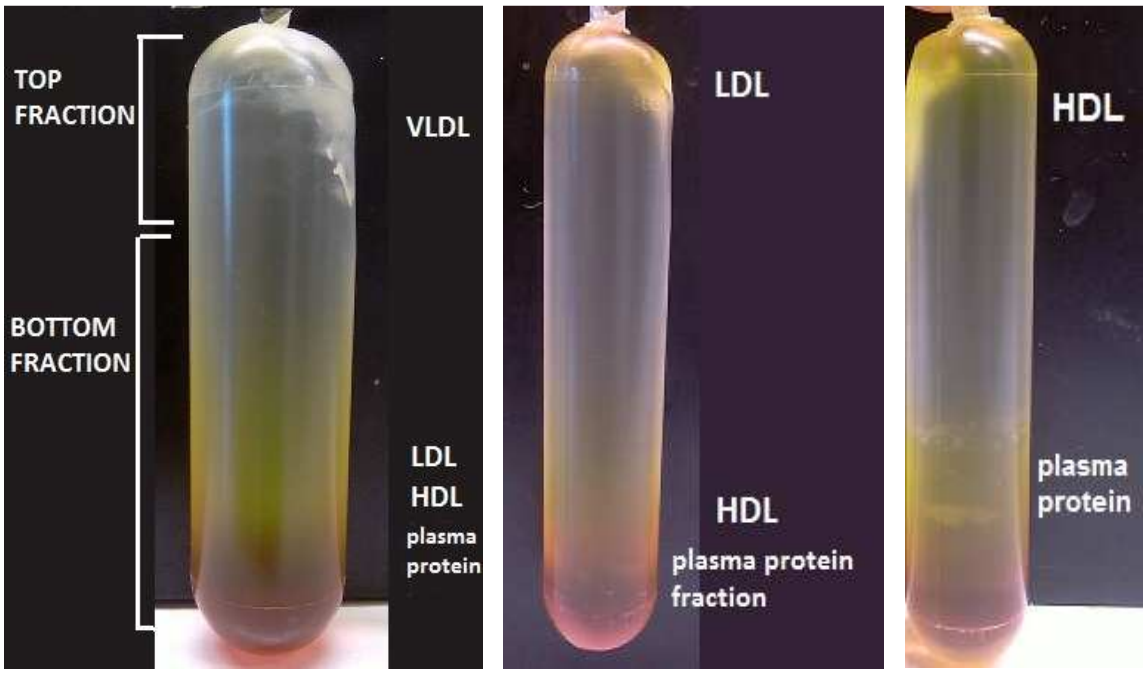

Fig. 20: Tubes with separated lipoprotein fractions after centrifugation.

2.1.2 Detection of modification

Carbamylated LDL was obtained from Prof. Bernhard Gmeiner (Institute of Medical Chemistry, Vienna). LDL was carbamylated by using potassium cyanate (KOCN).

The chemical modification should be visualized by a relative electrophoretic mobility assay. For this purpose, lipoprotein samples were diluted in 50mM Tris-HCl (pH 7.4), mixed with sample buffer and loaded onto a polyacrylamide gel. Subsequently, the gel was run at 150-180V for 1-2h (depending on experiment). Electrophoresis was performed using 4% native gels (composition of gel and materials see below) and SDS-containing gels (6% or gradient 4.5-18%). The composition of the SDS-containing gels and buffers is described in chapter "2.4.2 Western-blotting". Additionally, the lipoproteins were analysed on a 1.5% agarose gel.

Sample buffer (native)	Running buffer (native) pH 8.4
2.5 mL 125mM Tris/HCl pH 8.4	3.0g Tris
2mL Glycerine	14.4g Glycine
1mg Bromophenol blue	fill up to 1L with ddH_2O
ddH_2O added to 10Ml	

Reagent	stacking gel 4%	separation gel 4%
ddH_2O [mL]	1.55	3.10
0,5M Tris pH 6.8 [mL]	0.625	-
1,5M Tris pH 8.8 [mL]	-	1.25
PAA (30%) [mL] (29.2% Acrylamide, 0.8% Methylenbisacrylamide)	0.325	0.650
APS (10%) [µL]	12.5	25
TEMED [µL]	5	5

The gel was dyed in Coomassie Brilliant Blue (10% Acetic acid, 25% Isopropanol, 0.862g Coomassie Blue R250, and 1950 mL ddH_2O) for 1h on a shaker. Subsequently, it was destained in Destain Solution (30% Methanol, 10% Acetic

acid). For preservation, the gel was put on filter paper and dried at 70℃ for 2h in a vacuum gel dryer.

2.2 Cell culture techniques

2.2.1 Materials, media and standard operations

	Name	**manufacturer**
cell lines		
	Human coronary artery endothelial cells (HCAEC)	PromoCell
	Human coronary artery smooth muscle cells (HCASMC)	PromoCell
media		
	Endothelial Cell Growth Medium MV	PromoCell
	Smooth Muscle Cell Growth Medium 2	PromoCell
	Endothelial Medium without L-Glutamine (“serum-free medium” , SFM)	PAA
buffers		
	Trypsin-EDTA 1x	PAA
	Dulbecco’s Phosphate-Buffered Saline 1x (“PBS”), Gibco	Invitrogen
materials		
	Sterile cell culture flasks with filtered caps (25cm²)	BD Falcon
	Sterile cell culture flasks with filtered caps (75cm²)	Nunc
	Cell scraper	BD Falcon
	Centrifuge and test tubes 15mL, sterile	BD Falcon
	Disposable plastic pipettes (1/5/25mL)	BD Falcon
	FiberCell Endothelial Cartridges (PS+, C2025)	FiberCell Systems
	Microprocessor-controlled pump	FiberCell Systems

HCAEC and HCASMC were stored in liquid nitrogen. After rapid thawing in a water bath (37℃), they were washed in PBS and centrifuged carefully at 800rpm for 4min without brake (Hettich Rotana RTC). The cells were re-suspended in pre-warmed (37℃) growth medium (Endothelial Cell Growth Medium MV for HCAEC and Smooth Muscle Cell Growth Medium 2 for HCASMC) and transferred into a cell culture flask followed by incubation at 37℃ with 5%CO_2 and 95% relative humidity. Cell population was routinely controlled under the microscope.

For cell splitting, the medium was aspirated using a Pasteur pipette. The cells were subsequently washed with PBS followed by treatment with Trypsin-EDTA (up to 1min in incubator). When all cells were detached (control under microscope), the cells were suspended in fresh growth medium and distributed to several culture flasks.

2.2.2 Experiments under static flow conditions

These experiments were performed with both HCAEC and HCASMC. The experiments under static conditions were carried out in cell culture flasks. Cells were grown in growth medium until confluent, then they received serum-free medium. 24 hours later, this medium was replaced by serum-free medium with experiment-specific additions (50nM E_2, 25µg/mL nLDL or cLDL, 20µg/mL HDL) followed by incubation for 18 hours.

Then, the cells were harvested. Therefore, the medium was discarded and the cells were washed with PBS followed by scratching with a cell scraper. The obtained suspension of cells in PBS was collected in a 15mL tube and kept on ice till centrifugation at 1200 rpm for 5min, 4℃ (Hettich centrifuge, 46 RSC). The supernatant was discarded, except the last 0,5mL, in which the cell pellet was re-suspended and transferred into a 1,5mL Eppendorf tube. After centrifugation at 13000rpm for 2min (Eppendorf 5415D), the whole supernatant was aspirated and the pellet was frozen at -80℃.

2.2.3 Cartridges: cells under shear stress

Shear stress experiments were only performed with HCAEC. In a living organism, endothelial cells are always exposed to hemodynamic shear stress. Therefore, growing endothelial cells under static culture conditions is not a satisfying model for studying the gene expression behaviour of these cells. FiberCell cartridges allow more authentic culture conditions.

A bundle of hollow fibers (small, cylindrical filters with an internal diameter of 700µm) is surrounded by an extracapillary space. However, the inside of the fibers and the extracapillary space can be filled separately. The inside of the fibers is filled by liquid entering through the end ports (A and D), the extracapillary space by the side ports (B and C, see figure 22). The exchange between these two spaces is limited by the filter's pore size of 0.1µm. After seeding endothelial cells onto the inside of the fiber wall (see figure 21), medium is pumped through the fibers at a controllable flow rate to generate shear stress. One endothelial cartridge contains 20 fibers, which result in a total lumen surface area of 70cm². The FiberCell cartridges would also allow a co-culture (e.g. with smooth muscle cells) by growing the other cells in the extracapillary space [121].

First, the cartridge had to be activated. Therefore, the capillary space was flushed with 70% ethanol by using syringes which were attached to the ports. After that, the cartridge was rinsed with sterile water. Then, the inner membrane surface was coated with fibronectin (1 hour at 37°C). After coating, the protein solution was rinsed out with medium. Preculture was done overnight.

Freshly harvested cells were brought into the cartridge and incubated without flow. Every 30 minutes, the cartridge was rotated about 90° for 2h. After cell attachment and perfusion, the cartridges were connected to a pump. The cells were now exposed to shear stress for 10 days (low shear stress: 2.5 dynes/cm², high shear stress: 25 dynes/cm²). From day 9 to 10, the experiment-specific additions (50nM estrogen, 25µg/mL nLDL or cLDL) were added. Cells were harvested by flushing the cartridge with Trypsin-EDTA. The whole procedure of working with the cartridge is also described in [122].

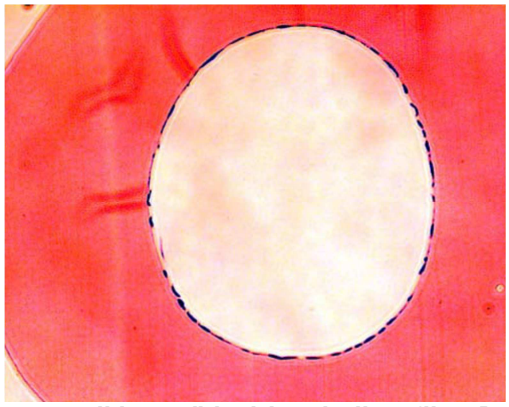

Fig. 21: Cell layer (blue) in a hollow fiber [121].

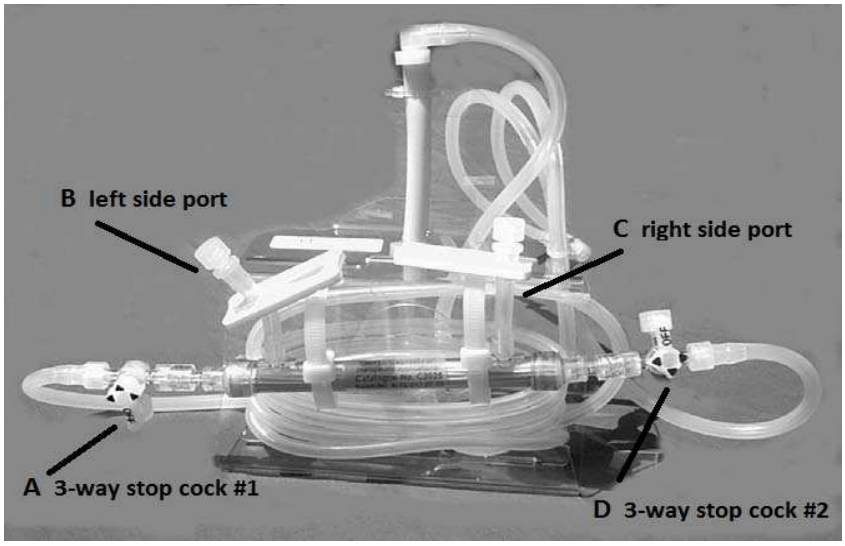

Fig. 22: FiberCell Endothelial Cartridge [121].

2.3 Gene expression analysis by RT-PCR

2.3.1 Theoretical background

The polymerase chain reaction was developed in the 1980ies in the group of Kary B. Mullis [123, 124]. This method facilitates rapid amplification of a desired DNA fragment and allows qualitative analysis of the DNA template (whether it contains a certain sequence). A conventional PCR master mix is at least composed of

- a DNA template
- two oligonucleotides, called “primers”, who flank the DNA sequence to be amplified
- a heat-stable polymerase
- reaction buffer containing appropriate concentrations of Mg^+ ions and the four nucleotide triphosphates [125].

The reaction itself is controlled by subjecting the reaction mix to a defined temperature/time programme (“temperature cycling”). At first, high temperature is applied to separate the DNA strands. However, primer annealing and elongation of the new strands occur at lower temperatures (see thermal profile).

A major disadvantage of the classic PCR is the necessity of a post-PCR analysis (e.g. by electrophoresis). That is why Higuchi et. al. [126] intended to solve this problem by showing that the increase of the PCR product can be monitored during the amplification process by adding an intercalating dye. The today commonly used real-time PCR is based on this observation. However, adding intercalating dyes is just one method to monitor product increase in real-time. In our experiment, Taqman probes were used. The foundation for this method was laid by Holland et. al. in 1991, when they postulated that the 5’ → 3’ exonuclease activity of Thermus aquaticus DNA polymerase could be used to detect specific PCR products [127]. In 1995, this finding was picked up by Livak et. al. who concentrated on the design of specific oligonucleotide probes for detection of PCR products [128]. They placed a reporter dye at the 5’ end of a specific oligonucleotide probe and a quencher dye at 3’. When the fluorescent reporter dye at 5’ gets excited by irradiation, the fluorescent emission will be quenched if the 3’ quencher dye is close enough (fluorescence resonance energy transfer). However, when the probe is cleaved by the Taq polymerase’s nucleolytic activity during strand extension, the dyes move away from each other and there is no quenching. Thus, fluorescence increases and can be measured (see also scheme in figure 23 and figure 24).

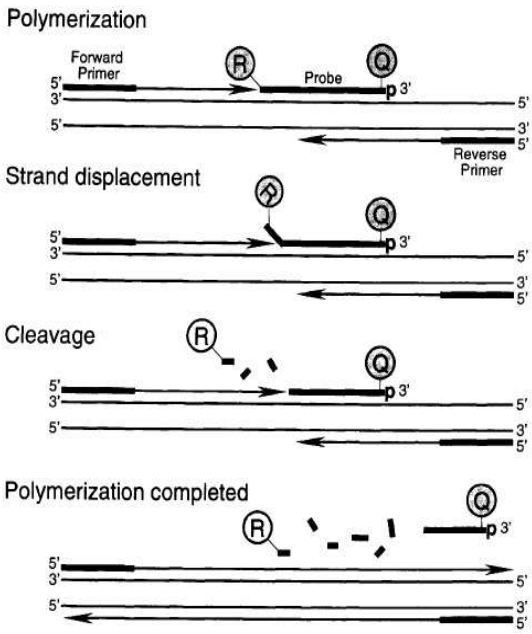

Fig. 23: The "5' nuclease assay" from Livak in 1995 [128].

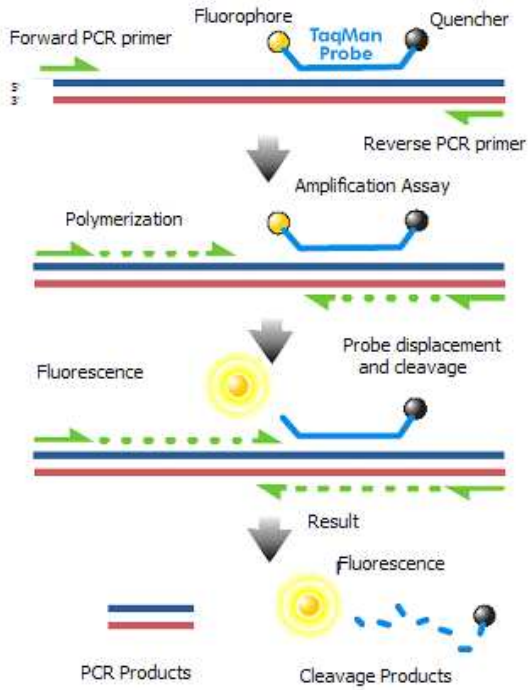

Fig. 24: The principle of the "modern Taqman" [129].

After completion of the PCR process, the CT (cycle threshold) values are displayed. "Cycle threshold" means the number of the cycles required to reach a certain threshold (see figure 25). How many cycles a sample needs depends on the initial amount of DNA. Thus, CT values can be compared for quantification. However, it has to be considered that the DNA concentration in the particular samples is usually not equal. To make them comparable, one can normalize to an internal reference gene (in our case the housekeeping gene β-actin). Hence, the gene expression ratio of our gene of interest is determined relative to the reference gene. Unlike the reference gene whose expression should stay constant, the gene of interest is expected to be possibly affected by the conditions studied [125]. The evaluation method of choice for such an experiment is the $2^{-\Delta\Delta CT}$ method. Its derivation was comprehensibly described by Livak and Schmittgen in 2001 [130].

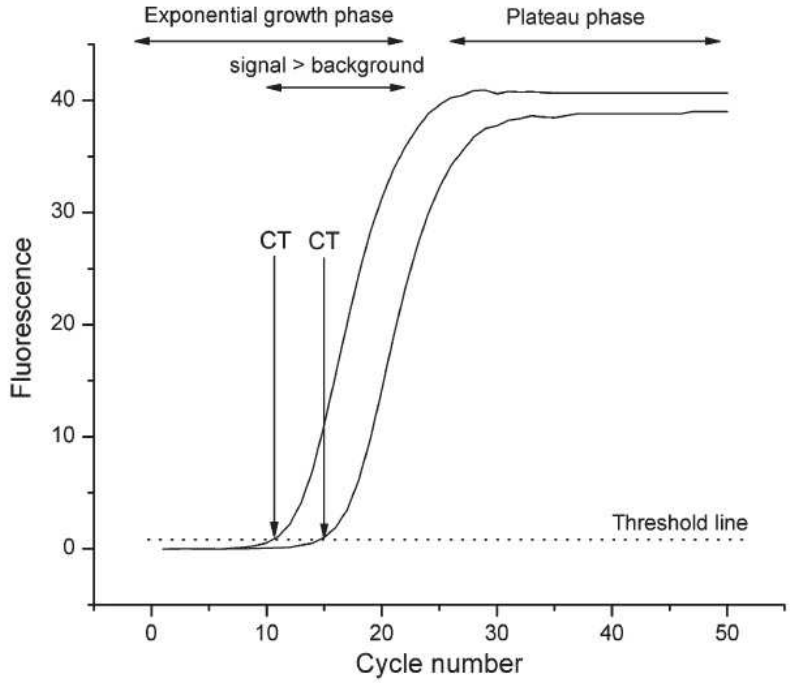

Fig. 25: PCR response curve, took from [125].

2.3.2 Reverse transcription

Since the expression of a gene has to be studied, not gDNA but cDNA (synthesized from RNA) is required for PCR. For this procedure, the FastLane Cell cDNA Kit (Qiagen) was utilized. The cell pellets were thawed and mixed with 1mL of Buffer FCP. After incubation at room temperature for 5-10 min, the "FastLane lysates" were ready to use for reverse transcription.

According to the protocol, the genomic DNA elimination reaction was set up as follows:

gDNA Wipeout Buffer 7x	4µL
FastLane lysate	20µL
RNAse-free water	4µL
Total volume	28µL

This mixture had to be incubated at 42℃ for 5 min (Eppendorf Thermomixer) and subsequently stored on ice.

The mixture for the reverse transcription reaction was composed as follows:

Quantiscript RT Buffer 5x	8µL
RT Primer Mix	2µL
FastLane lysate (Template RNA)	28µL
Quantiscript Reverse Transcriptase	2µL
Total volume	40µL

Incubation was done at 42℃ for 30min followed by heating at 95℃ for 3 min (enzyme inactivation).

2.3.3 PCR procedure

name of material	**manufacturer**
TaqMan Fast Universal PCR Master Mix (2X), No AmpErase UNG	Applied Biosystems
TaqMan Pre-Developed Assay Reagents Human ACTB 20x (probe dye: FAM-MGB)	Applied Biosystems
TaqMan Gene Expression Products “Assays-on-Demand” (probe dye: FAM) Hs01045922_m1 (human VLDL-R 20x) Hs01092523_m1 (human LDL-R 20x) Hs01059338_g1 (human LRP 20x) Hs00189742_m1 (human LRP2 20x)	Applied Biosystems
7500 Fast Real Time PCR System (Thermocycler)	Applied Biosystems
7500 Fast System SDS Software	Applied Biosystems
MicroAmp Fast Optical 96-well plates 0,1mL	Applied Biosystems

PCR mix composition:

cDNA	1 µL
Nuclease-free water	3,5 µL
Primer 20x (ACTB or receptor)	0,5 µL
TaqMan Master Mix 2x	5µL
Total volume	10µL

Negative controls were always included. Nuclease-free water was used instead of cDNA.

Thermal profile:

Step	Temperature [℃]	Duration [sec]	Repetition
Initial denaturation	95	20	1
Denaturation	95	3	40
Annealing/Elongation	60	30	40

2.3.4 Statistical analysis

PCR data were analysed using the $2^{-\Delta\Delta CT}$ method (derivation see [130]). For statistical analysis, at least 5 individual results were required. The end-results are expressed as the averages of these individual results +/- SEM (standard error of the mean). A two-tailed Student T-test was utilized to determine the statistical significance of the data. A p-value < 0.05 was considered as significant, p < 0.01 as highly significant.

2.4 Protein expression analysis

2.4.1 Protein extraction and quantification

The cell pellets (obtained from three 75cm² flasks) were mixed with 100µL of buffer B (200mM Tris maleate pH 6.5, 2mM $CaCl_2$, 1.4% Triton X-100). Before addition, the buffer had been completed with “complete EDTA-free” Protein Inhibitor Cocktail Tablets (Roche, 1 tablet per 50mL buffer solution). After keeping the mixture on ice for 10 min, it was centrifuged at 90000 rpm (Beckman Ultra-

centrifuge, TLA 100 rotor) and 4℃ for 40min. The supernatants were taken off and the pellets were discarded.

The Quick Start Bradford 1x Dye Reagent (BIORAD) was used for protein quantification. 1mL reagent mixed with 5µL of BSA (2mg/mL, BIORAD) served as standard. 1µL of sample protein solutions was added to 1mL reagent for protein analysis. The OD was determined with the spectrophotometer at 595nm. The following formula was utilized for calculation of protein concentration:

$$\frac{[\mu g]standard}{OD\ standard} \times \frac{OD\ sample}{[\mu L]sample} = sample\ conc.\ [\mu g\ per\ \mu L]$$

2.4.2 Western blotting

Western blotting is a method for protein detection first described by the group of George R. Stark and co-workers in Stanford in 1979 [131]. The principle has remained the same: proteins are separated by SDS-PAGE and subsequently transferred to a membrane, where they are detected using specific antibodies.

Name of required material:	**Source:**
Mini Trans-Blot Electrophoretic Transfer Cell	BIO-RAD
Mini-PROTEAN 3 electrophoresis chamber	BIO-RAD
PowerPac power supply	BIO-RAD
Filter papers	Whatman
Nitrocellulose membrane Hybond C-Extra	Amersham
Precision Plus Protein Unstained Standard	BIO-RAD
Anti- LDL receptor (from mouse) diluted 1:500	General Hospital
Anti-LRP2 (polyclonal, from mouse) diluted 1:1000	abcam
Anti-LDL receptor (from rabbit) diluted 1:500	self-made
Anti-rabbit HRP-conjugated, diluted 1:50000	Sigma
Anti-mouse HRP-conjugated, diluted 1:20000	Jackson
Super Signal West Pico Chemiluminescent Substrate	Thermo Scientific
CL-XPosure Films 5x7 inches	Thermo Scientific

Sample Buffer (2x)	Sample Buffer reducing (2x)	Running Buffer (1x)
31.2% Glycerine	31.2% Glycerine	25mM Tris-HCl
6% SDS	6% SDS	192mM Glycine
20mM Tris-HCl pH7.5	20mM Tris-HCl pH7.5	1% SDS
Bromophenol-blue	25mM Dithiothreitol (DTT)	
ddH_2O to 20mL	Bromophenol-blue	
	ddH_2O to 20mL	

1x Transfer buffer	Ponceau S solution	10x TBS
25mM Tris-HCl	0.2% Ponceau S	1.37 M NaCl
192mM Glycine	3% Trichloracetic acid	0.027 M KCl
		0.25 M Tris-HCl pH 7.4

Blocking solution	TBS-T
5% non-fat dry milk	1x TBS with
in 1x TBS-T	0.1% Tween 20

SDS-PAA gel reagents:	stacking gel 4%	separation gel 6%	separation gel 10%
ddH_2O [mL]	1.525	2.625	2.025
0,5M Tris pH 6.8 [mL]	0.625	-	-
1,5M Tris pH 8.8 [mL]	-	1.25	1.25
PAA (30%) [mL] (29.2% Acrylamide, 0.8% Methylenbisacrylamide)	0.325	1	1.65
SDS (10%) [µL]	25	50	50
APS (10%) [µL]	12.5	25	25
TEMED [µL]	5	5	5

An appropriate amount of protein from each cell extract was mixed 1:1 with loading buffer (in case of reduced samples: denaturation for 3min at 95℃) and loaded on the 1mm polyacrylamide gel (composition see table). The gel was run

at 150V for approximately one hour (until the blue dye reached the bottom). After that, a “sandwich” out of sponges, filter papers, membrane and gel was assembled. Blotting to the nitrocellulose membrane was carried out for 1h at 100V and 130mA. The blotting unit was kept in an ice box.

To check transfer efficiency, the membrane was shortly stained in Ponceau S solution and destained in ddH_2O. Subsequently, it was blocked in TBS-T/milk solution for 1h on a shaker. Then, the primary antibody was diluted appropriately in blocking solution and put on the membrane over night at 4°C with gentle agitation. After that, the membrane was washed three times in TBS-T for 10min followed by incubation with the secondary antibody (1-2h at room temperature). Finally, the membrane was washed again (3x10min) in TBS-T and treated with the detection reagents (Super Signal West Pico Chemiluminescent Substrate, Thermo Scientific).

The chemiluminescence reaction is illustrated in figure 26. HRP catalyses the reaction of Luminol to its light-emitting oxidized form. The two components of the detection kit were mixed 1:1 and put on the membrane for 2-3min. Then, the membrane was transferred into a film cassette together with a CL-Xposure film (Thermo Scientific). Exposure was done for at least 30min followed by development.

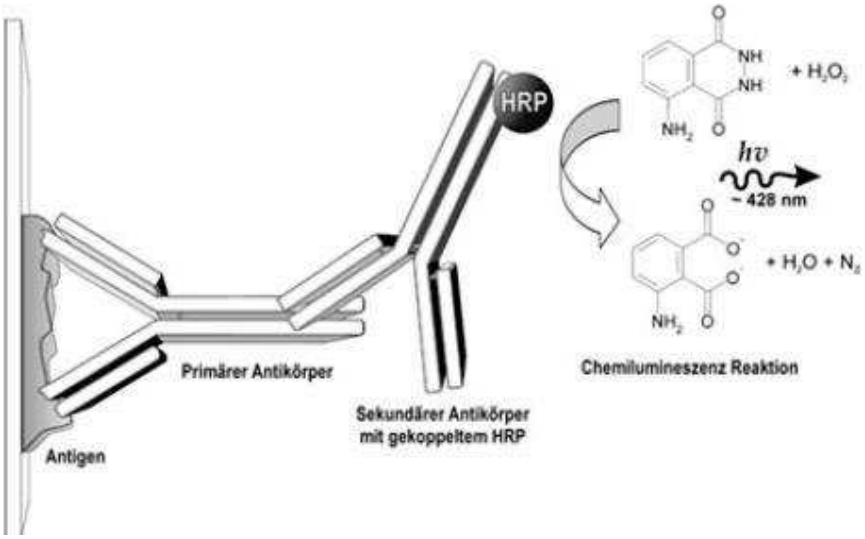

Fig. 26: The principle of the chemiluminescence reaction. Luminol oxidation is catalysed by HRP leading to light emission [132].

2.5 Cytotoxicity and apoptose assay

To determine whether our applied concentrations of lipoproteins and estrogen exert toxic effects on the cells, a cytotoxicity assay was performed. Therefore, HCAEC were cultured in 96-well plates and incubated with lipoproteins and estrogen in different combinations (25µg/mL nLDL or cLDL, 20µg/mL HDL, 50nM E_2) in serum-free medium. Furthermore, growth medium and serum-free medium without additions were used as controls. The tests were carried out as quadruplicates (n=4). Cytotoxicity was determined by measurement of spontaneous lactat dehydrogenase (LDH) in medium and total LDH release after cell lysis. Calculated was the percentage of spontaneous LDH release in medium in comparison to total LDH release in lysed cells. LDH release was detected using the CytoTox 96 Non-Radioactive Cytotoxicity Assay Kit (Promega) according to the manufacturer's recommendations. Absorbance was measured at the Victor³V microplate reader (Perkin-Elmer).

The apoptose assay required the same experimental setup as the cytotoxicity assay (HCAEC cultured in a 96-well plate, estrogen and lipoproteins as additions). Then, the plate was utilized to perform the Apo-ONE Homogenous Caspase -3/7 Assay (Promega) according to the manufacturer's instructions. This assay facilitates the detection of apoptosis by measurement of caspase activity. Fluorescence is caused by enzymatic cleavage of the substrate and is therefore proportional to caspase activity. Fluorescence emission was measured at the Victor³V microplate reader (Perkin-Elmer).

3. Results

3.1 Analysis of lipoprotein modification

The lipoproteins used for cell culture experiments were analysed on polyacrylamide gels to visualize their difference/modification. Figure 27 shows a native PAA gel with native and carbamylated LDL samples. A difference in electrophoretic mobility is recognizable.

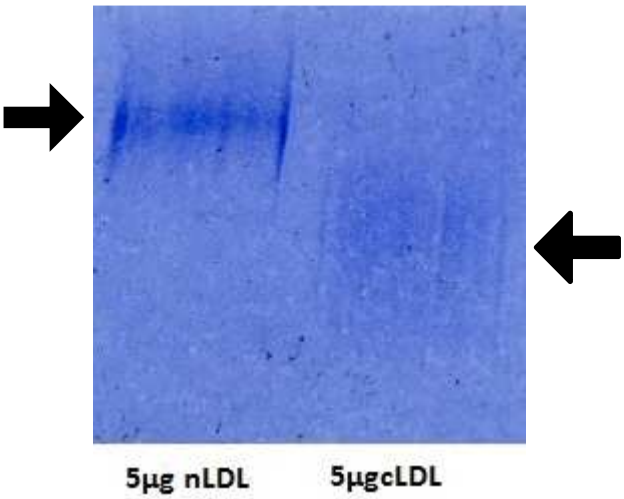

Fig. 27: Native LDL and carbamylated LDL run for 2h at 150V on a 4% native PAA gel.

In addition to electrophoretic mobility, lipoproteins should be separated according to their size. Therefore, further studies were performed on SDS-PAA gels (non-reducing and reducing conditions). Figure 28 visualizes various lipoproteins run on a gradient gel and compares non-reduced versus reduced condition.

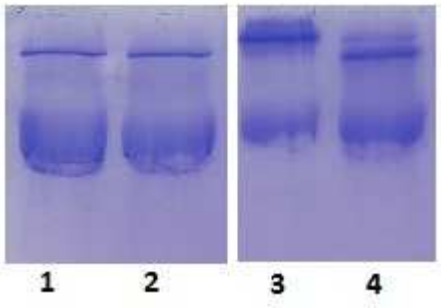

Fig. 28: Native and modified LDLs on a gradient PAA gel (4.5-18%), run for 2h at 180V.

1… 15µg nLDL 3… 15µg nLDL (reduced)

2… 15µg cLDL 4… 15µg cLDL (reduced)

In figure 29, the same samples were analysed on a 6% SDS-PAA gel. As already observable in figure 28, the individual modifications lead to a distinct behaviour. Interestingly, the reduction of the lipoproteins results in stronger formation of multiple bands.

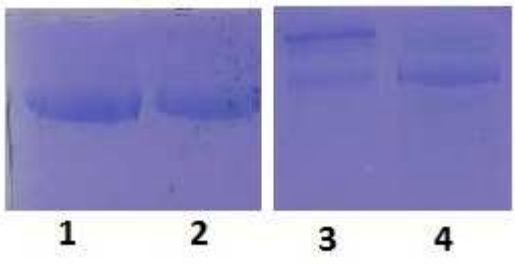

Fig. 29: Native and modified LDLs on a 6% PAA gel, run for ~1h at 180V.

1… 10µg nLDL 3… 10µg nLDL (reduced)

2… 10µg cLDL 4… 10µg cLDL (reduced)

Finally, figure 30 visualizes the clear difference in REM of native and carbamylated LDL on an agarose gel. Furthermore, the increasing degree of modification by treatment with higher concentrated KOCN is evident.

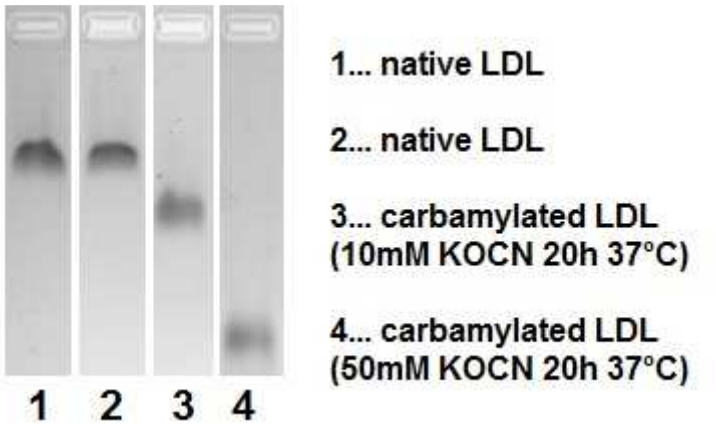

Fig. 30: Native and carbamylated LDL run on a 1.5% agarose gel. The LDL treated with a higher concentration of KOCN showed an increased change in relative electrophoretic mobility.

3.2 Regulation of the LDL-R gene expression

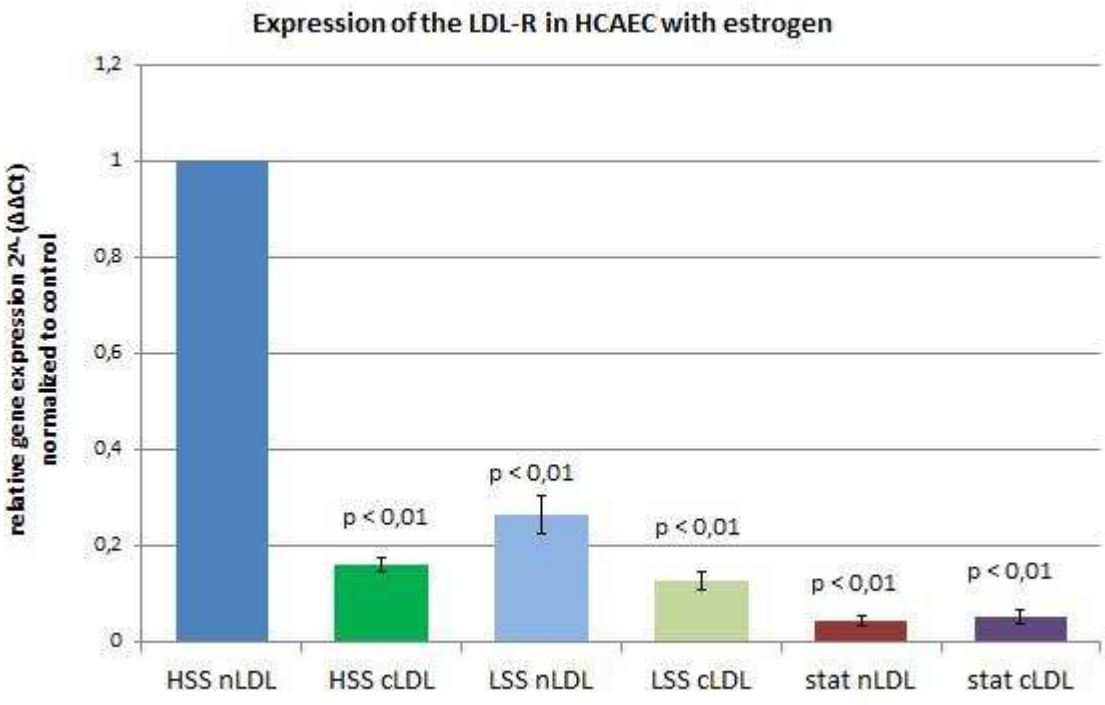

Fig. 31: LDL-R expression in EC with estrogen. All values are expressed as average +/- SEM and are relative to the condition HSS nLDL, which was set to 1. The p-values are calculated between HSS nLDL and the respective condition.
Abbreviations: HSS… high shear stress, LSS… low shear stress, stat… static culture

Figure 31 illustrates LDL-R gene expression in HCAEC under estrogen influence. Shear stress alterations and LDL carbamylation affected LDL-R expression profoundly. To determine the effect of estrogen, these results were compared with gene expression findings from studies without estrogen (see fig. 31). The results for HSS nLDL/cLDL and LSS nLDL/cLDL were generated in a former study [133].

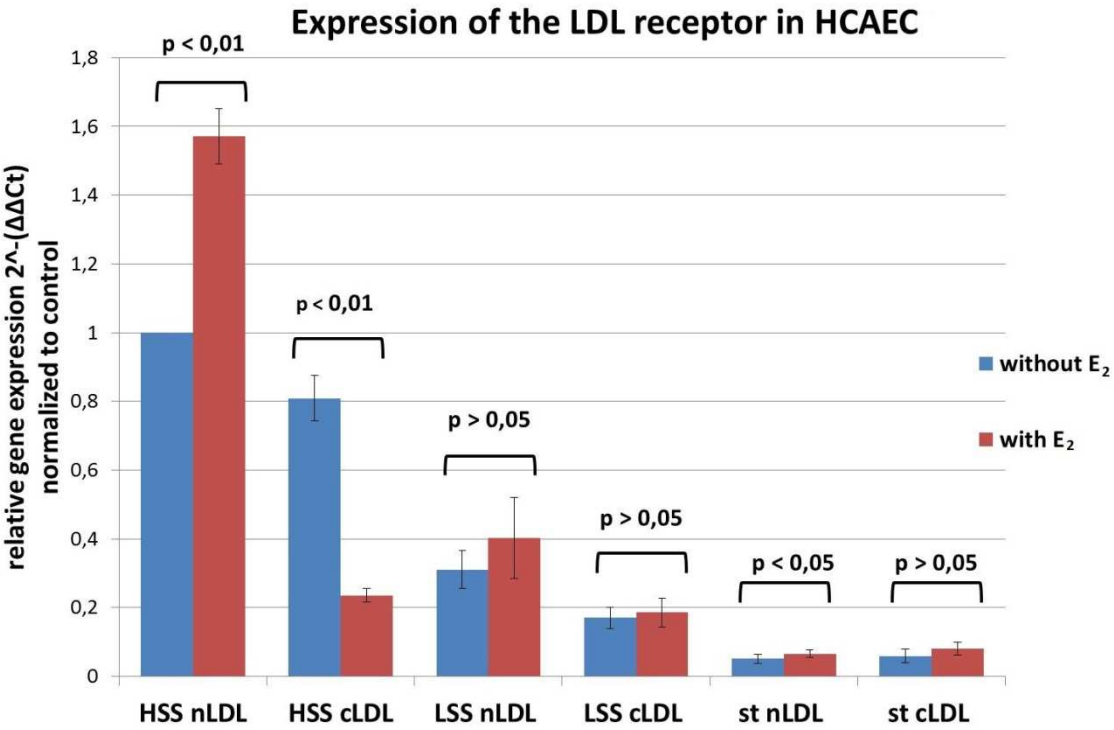

Fig. 32: All studies on LDL-R gene expression in endothelial cells without or with estrogen. A part of the test results without estrogen (HSS nLDL/cLDL, LSS nLDL/cLDL) were generated in former studies [133]. All values are expressed relative to HSS nLDL without E_2, which was set to 1. The p-values were calculated between the conditions without and with estrogen and should indicate the significance of estrogen influence.

Abbreviations: HSS… high shear stress, LSS… low shear stress, st… static culture conditions, nLDL… native LDL, cLDL… carbamylated LDL, E… 17β-estradiol (E_2)

Figure 32 provides an overview of all studied parameters. Strikingly, a tremendous difference between HSS, LSS and static culture conditions was observed. Certainly, the static culture is far away from the physiological conditions of coronary artery endothelial cells. However, the deterioration from HSS to LSS con-

stitutes an interesting finding. Carbamylation decreased LDL-R expression. Estrogen tended to have a positive effect, except for the condition HSS with cLDL.

Figure 33 illustrates LDL-R expression in HCASMC. cLDL lead to a significant reduction. Furthermore, estrogen decreased receptor expression in absence of lipoproteins and in presence of nLDL. However, it increased LDL-R expression in presence of cLDL.

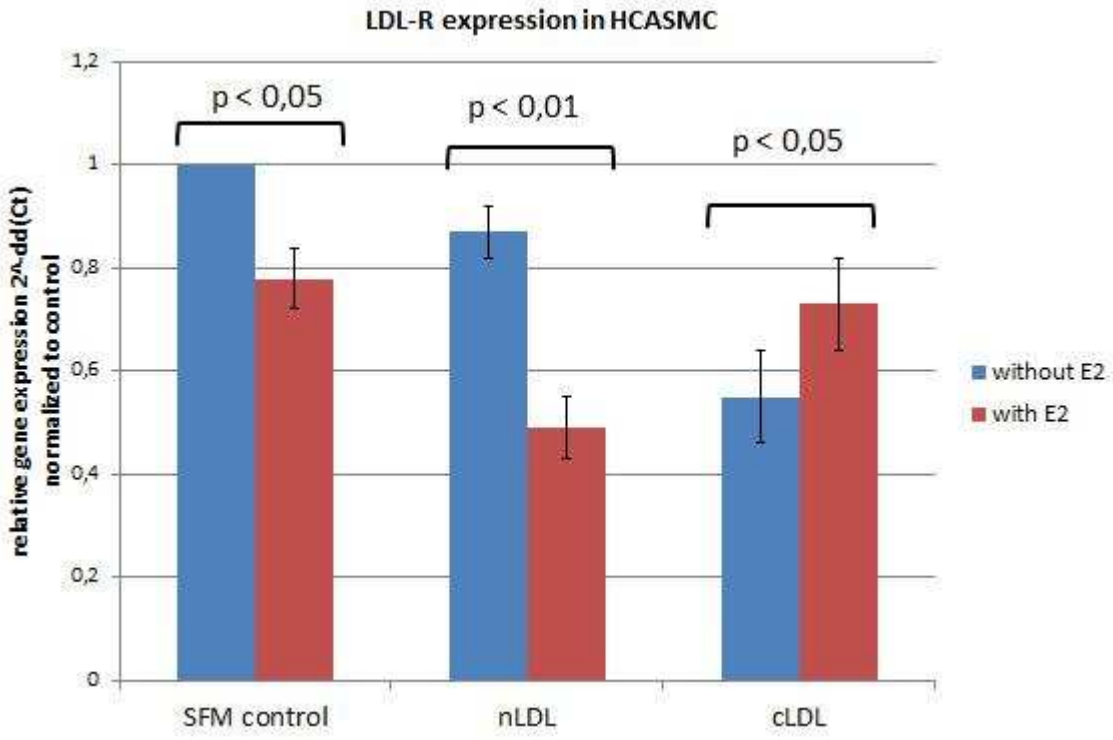

Fig. 33: Studies of LDL-R gene expression in coronary artery smooth muscle cells. All values are depicted relative to the control (SFM… serum-free medium), which was set to 1. The p-values express the significance of estrogen action.

3.3 Regulation of the VLDL-R gene expression

Figure 34 illustrates a strong reduction of VLDL-R expression by low shear stress in EC. In contrast, LDL carbamylation had no effect. The impact of estrogen is demonstrated in figures 35a and 35b. Estrogen treatment elevated VLDL-R gene expression under static conditions independently from lipoprotein type (see figure 35b). In contrast, estrogen seems to exert a negative effect on VLDL-R expression under shear stress conditions (see figure 35a). However, more data for shear stress without estrogen conditions are required for a statistical confirmation of these results.

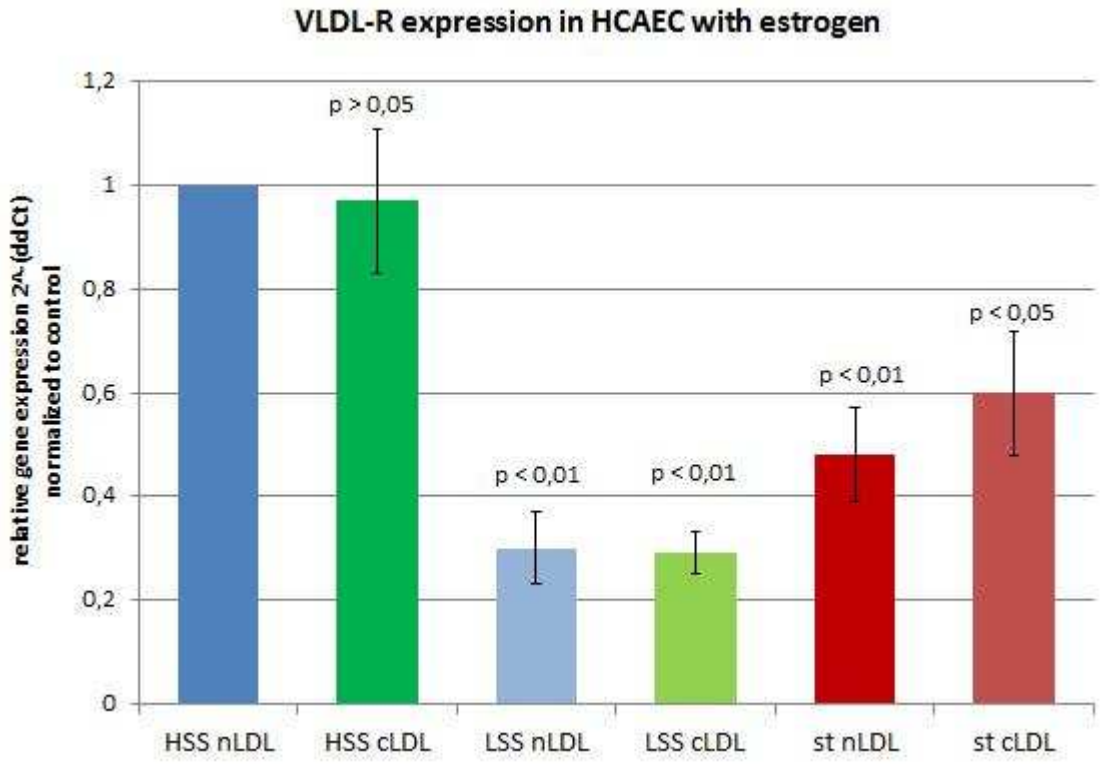

Fig. 34: Gene expression of the VLDL-R in endothelial cells under different culture conditions (all with E_2). All results are expressed relative to HSS nLDL, which was set to 1. The p-values indicate the significance of the comparison between the reference (HSS nLDL) and the particular condition.

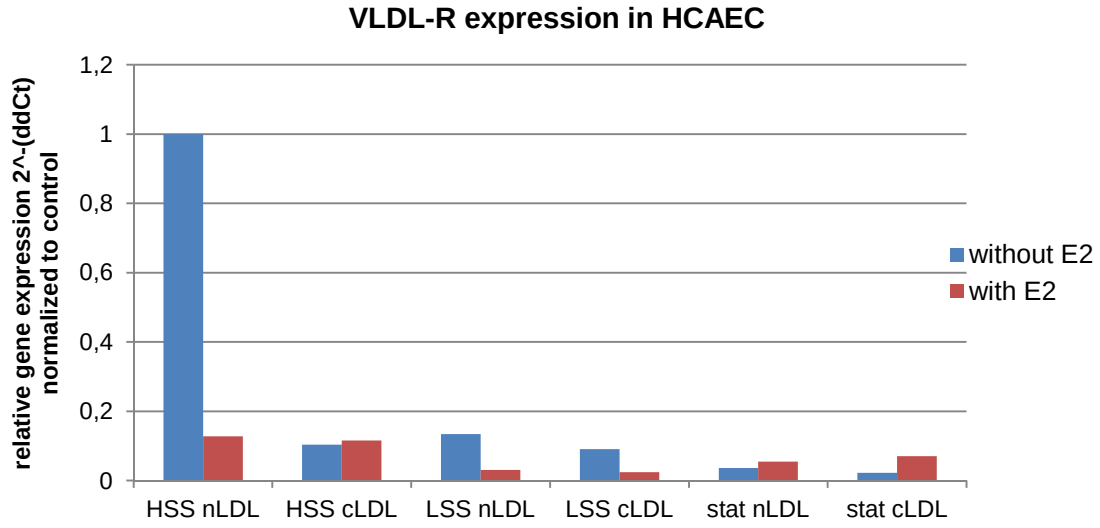

Fig. 35a: Summary of all studies performed on the VLDL-R gene expression in HCAEC. All results are expressed relative to HSS nLDL without estrogen. A part of the results without estrogen (HSS nLDL/cLDL, LSS nLDL/cLDL) were generated in former studies [133]. Only one data set was available for these conditions. Therefore, a solid statistical analysis could not be performed. The other results were generated in this study, at least five data sets were available. The mean values were compared to the single results.

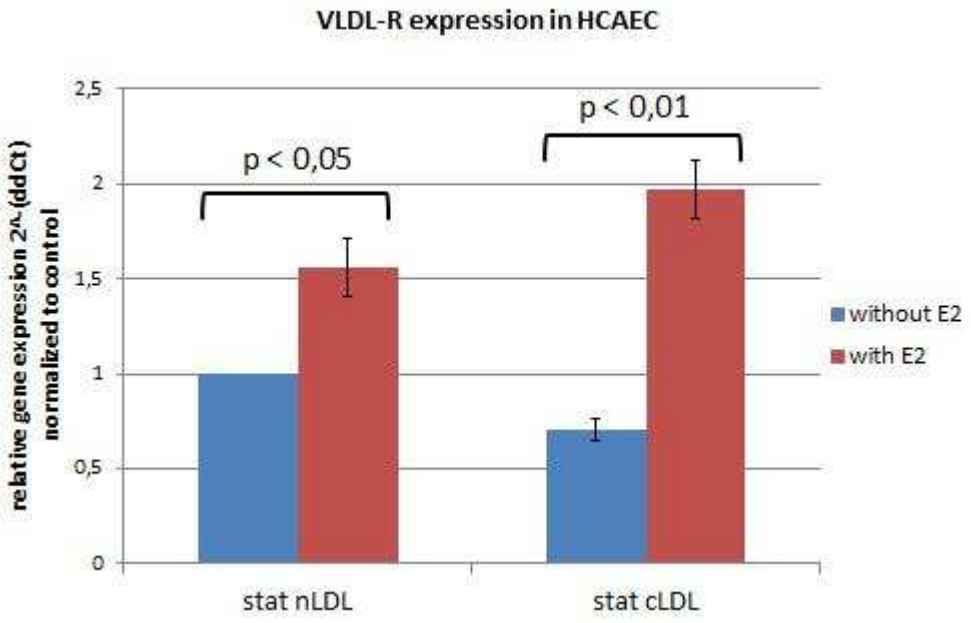

Fig. 35b: The influence of estrogen and carbamylation on VLDL-R expression in EC under static culture conditions. The condition stat nLDL was used as reference, p-values refer to the influence of estrogen.

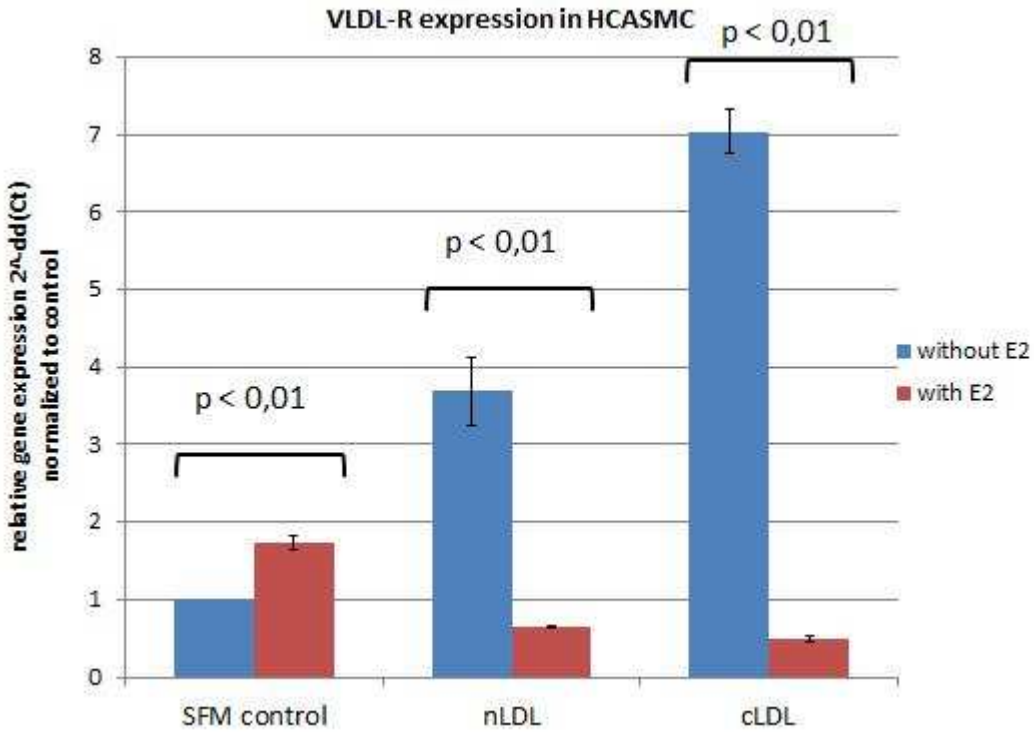

Fig. 36: VLDL-R gene expression in SMC. All results are expressed relative to the serum-free medium control, which was set to 1. The p-values were calculated between a condition without estrogen and the respective condition with estrogen.

In smooth muscle cells (see figure 36), VLDL-R expression was raised by lipoproteins. Especially carbamylated LDL lead to a massive increase. Estrogen caused an elevation in absence of lipoproteins. However, when combined with lipoproteins, the expression was diminished compared to the control.

3.4 Regulation of the LRP1 gene expression

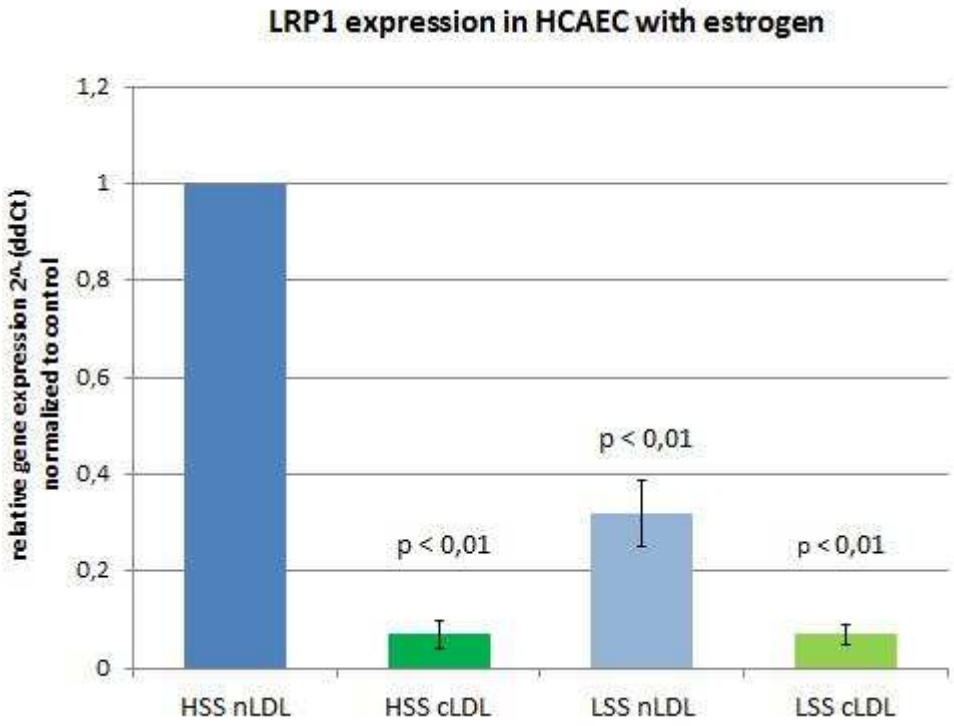

Fig. 37: The influence of carbamylation and shear stress on LRP1 expression in EC with estrogen. The condition HSS nLDL was set to 1. p-values express the significance of change in reference to HSS nLDL.

Carbamylated LDL nearly abolished LRP1 expression in EC subjected to shear stress (see figure 37). A decline of shear stress caused a reduction of gene expression as well. Under static conditions, estrogen elevated LRP1 expression in presence of carbamylated LDL (see figure 38b). In contrast, there was no significant difference when combined with native LDL. Figure 38a provides an overview over all studies of LRP1 expression in HCAEC. Estrogen may influence LRP1 expression in different ways depending on culture conditions.

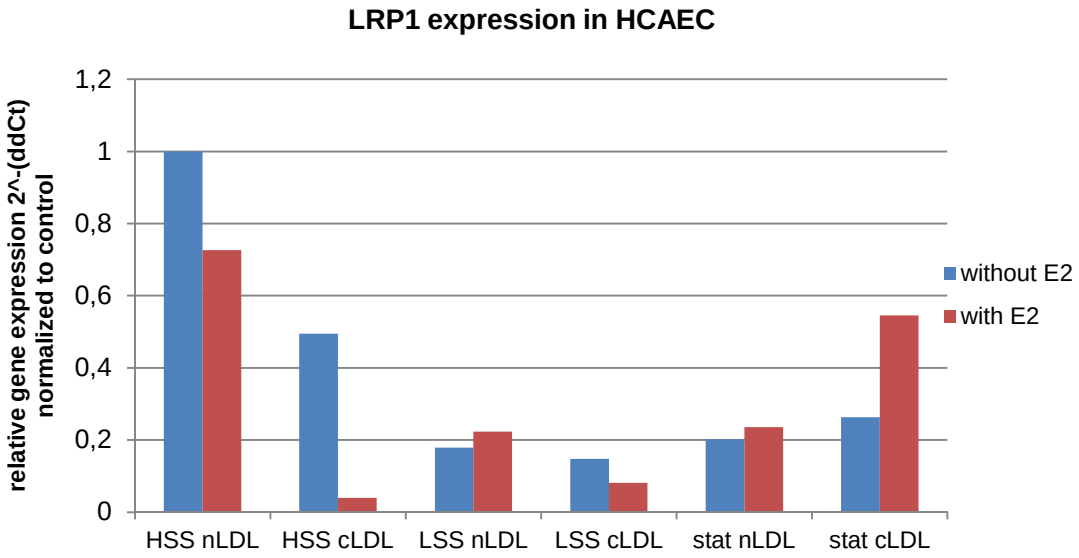

Fig. 38a: Summary of all studies performed on LRP1 gene expression in HCAEC. All results are expressed relative to HSS nLDL without estrogen. A part of the results without estrogen (HSS nLDL/cLDL, LSS nLDL/cLDL) were generated in former studies [133]. Only one data set was available for these conditions. Therefore, a solid statistical analysis could not be performed. The other results were generated in this study, at least five data sets were available. The mean values were compared to the single results.

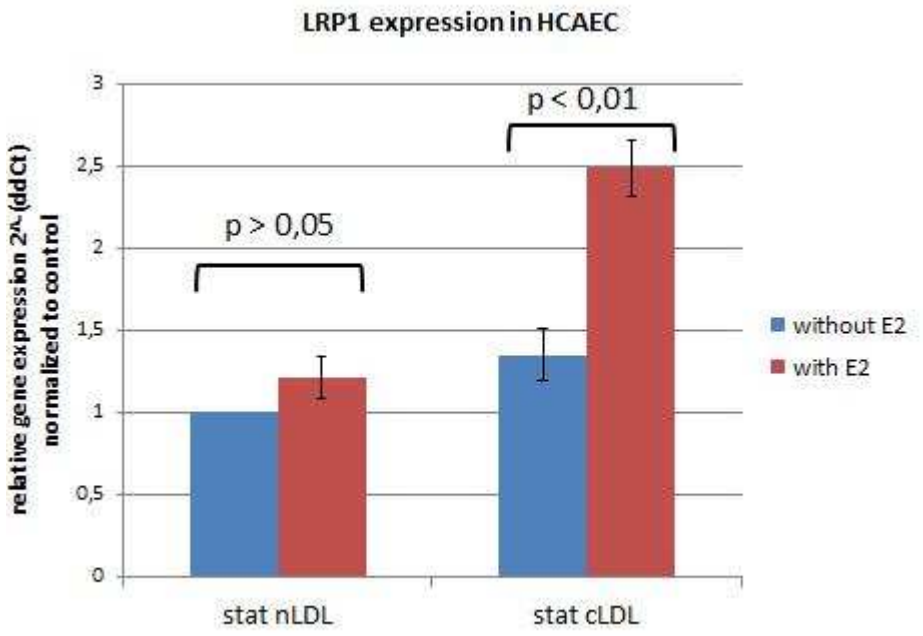

Fig. 38b: The influence of estrogen and carbamylation on LRP1 expression in EC under static culture conditions. P-values indicate the significance of estrogen's influence, stat nLDL serves as reference.

Figure 39 illustrates LRP1 expression levels in SMC. Native LDL decreased LRP1 expression. Furthermore, the combination of estrogen and lipoproteins (nLDL as well as cLDL) caused a strong reduction.

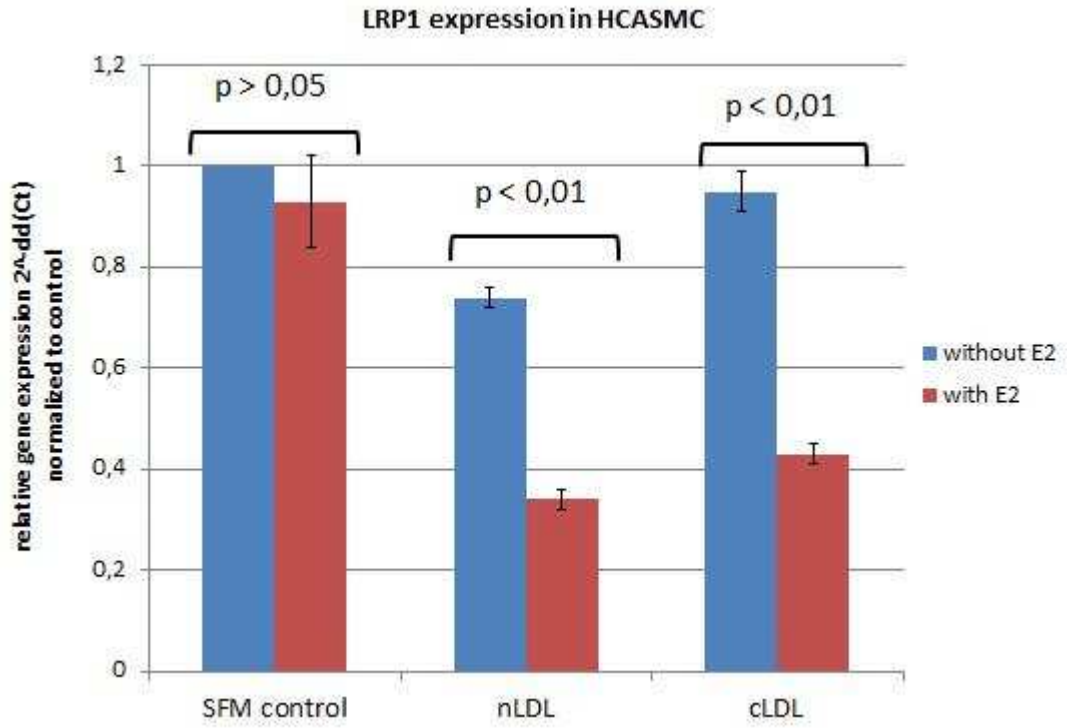

Fig. 39: LRP1 expression in SMC. The serum-free medium control serves as reference for all values. The significance of estrogen's effect is indicated by the p-value.

3.5 LRP2 expression

LRP2 gene expression was also investigated by RT-qPCR. It was neither detected in endothelial cells nor in smooth muscle cells (data not shown).

3.6 Protein detection

HCAEC lysates from static culture experiments were analysed by Western blotting to confirm the gene expression results on protein level. Consistent with the gene expression results, LRP2 was not detected in endothelial cells by Western-blot (data not shown). The LDL-R gene expression under static culture conditions was found to be highly reduced compared to shear stress experiments. Thus, the LDL-R was not detectable because of insufficient protein amounts (data not shown).

3.7 Cytotoxicity and apoptose assay

The cytotoxicity assay was performed to assure that the experimental additions do not act cytotoxic. It revealed that none of the studied additions executes a particular cytotoxic effect on HCAEC in the applied concentration. As illustrated in figure 40, the effects of the additions range in the same order of magnitude as the growth medium and the serum-free medium do.

Moreover, the additions were investigated with regard to a possible apoptose-causing potential. The results of the apopotose assay are shown in figure 41. None of the studied conditions leads to a noticeable increase in caspase activity.

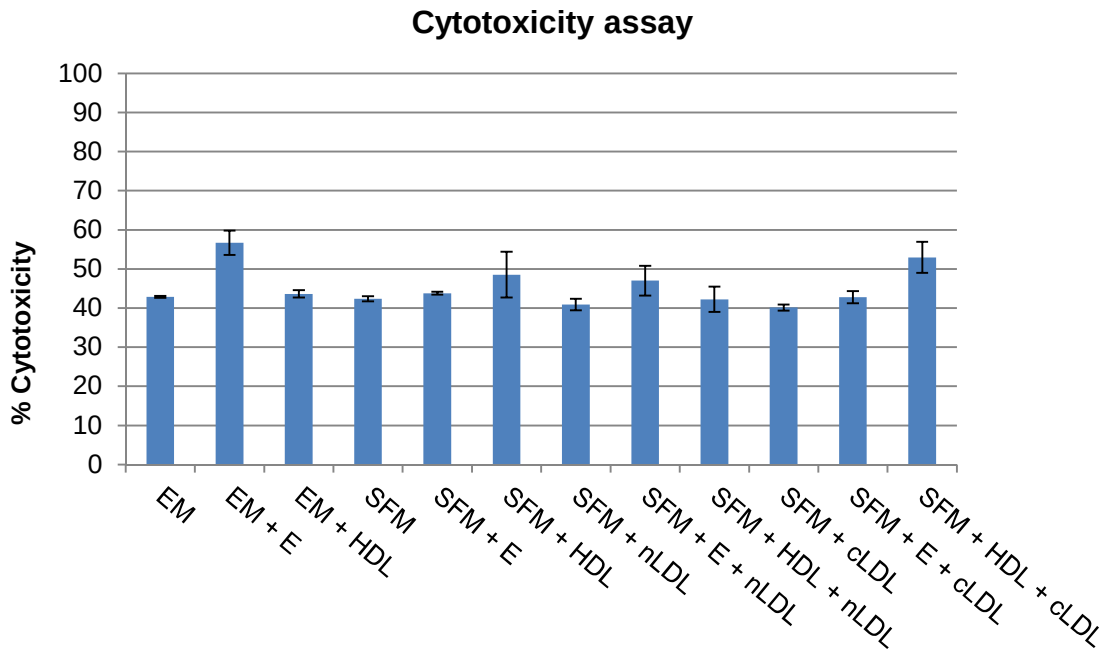

Fig. 40: Cytotoxicity assay. The results are expressed as averages +/- SEM.

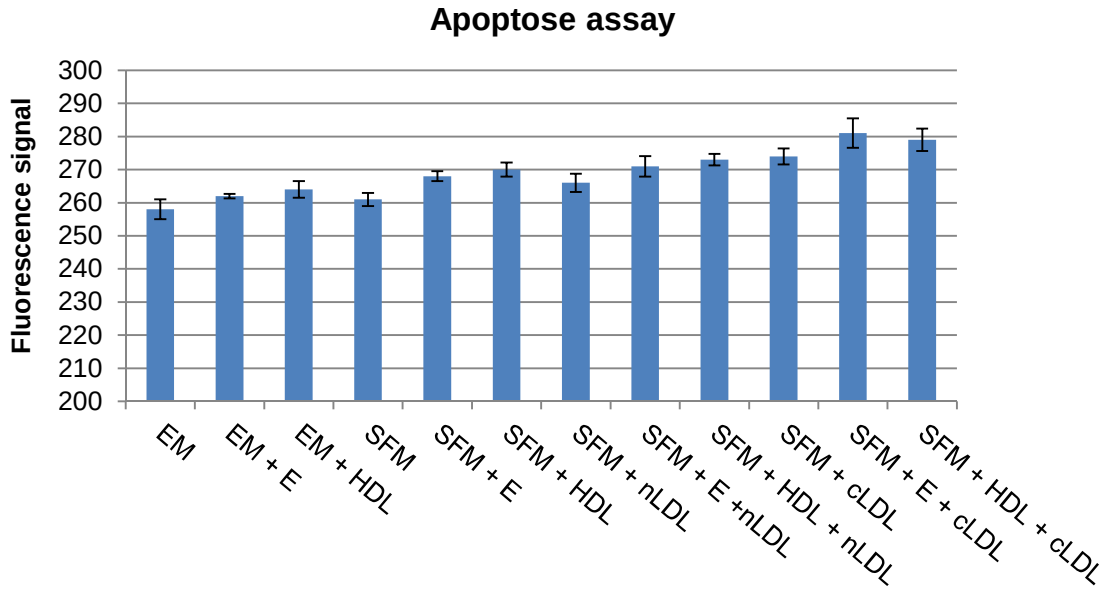

Fig. 41: Apoptose assay. The results are expressed as averages +/- SEM.

4. Discussion

The aim of the project was to discover how modified lipoproteins, estrogen and altered shear stress influence the gene expression of certain receptors in the cells of the coronary artery wall. Therefore, HCAEC were cultivated under static and dynamic (high/low shear stress) conditions and HCASMC were grown in static culture. In these experiments, native and carbamylated lipoproteins were used. Their modification was successfully visualized by electrophoresis. Cytotoxicity and apoptose assays showed that the experimental additions (17-β estradiol, native LDL, carbamylated LDL, HDL) did not function particularly toxic on endothelial cells in comparison to addition-free cell culture media.

cDNA was obtained from HCAEC (shear stress and static conditions) and HCASMC (static conditions) and used for expression analyses by real-time quantitative PCR. Gene expression levels of the LDL-R, VLDL-R, LRP1 and LRP2 (which are members of the LDL-R gene family) were investigated.

The LDL-R occurred in both cell types and was affected by all studied parameters. First, its expression in endothelial cells in static culture was dramatically reduced compared to more physiological conditions (shear stress). Thus, static culture is very far from the physiological situation of EC and therefore limited in informative value. Nevertheless, the LDL-R was downregulated with decreasing shear stress level (see fig.31 and fig. 32). This finding is consistent with previous knowledge [115, 116], as shear stress has been reported to cause an SREBP-1-mediated increase in LDL-R expression. However, these investigators applied shear stress for up to 24 or 48 hours. Our study investigates the effect of chronic shear stress (10 days). Furthermore, the results of our study also strengthen the hypothesis that high shear stress acts atheroprotective (increased LDL clearance) and low shear stress favours atherogenesis (decreased LDL clearance).

Our findings suggest that carbamylated LDL decreases LDL-R expression. Therefore, the carbamylation of LDL should be considered as atherogen since it reduces lipoprotein clearance. In contrast, estrogen affected LDL-R expression

positively. In combination with HSS and native LDL, the upregulation by estrogen was highly significant (see figure 32). This finding is consistent with literature, as an estrogen-mediated increase of LDL-R expression has already been reported in the rat liver and HepG2 cells [103, 104]. However, estrogen reduced LDL-R expression dramatically when combined with cLDL. This finding indicates that chronic kidney disease or smoking could have a particularly negative effect, more on women than men.

In HCASMC, LDL-R expression was decreased by cLDL in absence of estrogen. Moreover, estrogen reduced LDL-R expression in combination with serum-free medium and nLDL, but elevated expression when combined with cLDL (see fig. 33). Lipid uptake in SMC is an essential step in disease progression. Therefore, the observed reduction of LDL-R expression by estrogen has to be considered as protective. However, cLDL antagonises this effect. Our suggestion of estrogen's protective effect concerning lipid uptake in SMC is strengthened by Corcoran et. al. [94], who reported that estrogen reduced cholesteryl ester accumulation in human monocyte-derived macrophages.

The VLDL-R expression in estrogen-treated HCAEC was strongly affected by shear stress (see figure 34). However, LDL carbamylation had no effect. This is not astonishing as LDL is not recognized by the VLDL-R [32, 33]. The obvious effect of shear stress alteration on VLDL-R expression could possibly be mediated by the same mechanism as in the LDL-R. A regulation by SREBP-1 would be imaginable, as the VLDL-R gene contains two SRE-1-like sequences [33]. Estrogen had previously been reported to increase VLDL-R mRNA in rabbit heart [106]. Consistent with this finding, it elevated VLDL-R levels in EC in our experiment (static culture, see figure 35b). However, our studies provide a clue that estrogen could also exert an opposite influence on VLDL-R expression under shear stress conditions. As shown in figure 35a, estrogen radically diminished VLDL-R expression when subjected to high shear stress and treated with native LDL. In consideration of the recent suggestion that the VLDL-R promotes leukocyte transmigration and inflammation [134], estrogen's action is protective. However, a repetition of the shear stress experiment without estrogen is indispensable for statistics.

VLDL-R gene expression in HCASMC was affected by both lipoproteins and estrogen (see figure 36). Estrogen alone caused an increase, whereas the combination with lipoproteins led to a decrease of receptor expression. This observation can be explained as a protection mechanism against excess lipoprotein uptake. Furthermore, Lipoproteins alone strongly raised VLDL-R expression. This effect was stronger in cLDL than in nLDL. The VLDL-R was suggested to play a role in SMC-derived foam cell formation by contributing to lipoprotein accumulation [35, 36]. An estrogen-mediated decrease of VLDL-R expression can therefore be valuated as protective. However, the upregulation by nLDL and cLDL is not comprehensible as the VLDL-R is not regulated by LDL. Anyway, this effect could promote disease progression and is stronger induced by cLDL than by nLDL.

LRP1 expression in HCAEC is markedly reduced by carbamylation and low shear stress (see figure 37 and figure 38a). Since LRP1 fulfils protective tasks in the vasculature [40-43], LSS and cLDL act adversely. Under HSS conditions, we observed that LRP1 expression was slightly reduced by estrogen when combined with nLDL and strongly reduced in combination with cLDL. Again, we provide evidence for a particular negative effect of cLDL on women. Admittedly, more data are required to confirm these findings statistically. In contrast, estrogen increased LRP1 expression significantly when combined with cLDL in static culture (see figure 38b). A stimulatory effect of estradiol on LRP1 in the mouse brain has been reported in 2007 by Cheng et. al. [107].

LRP1 is supposed to contribute to lipid accumulation in the artery wall by mediating LDL uptake in smooth muscle cells [45]. In our experiment, estrogen reduced LRP1 expression in HCASMC significantly when combined with lipoproteins (see figure 39). In consideration of LRP1's role in SMC-derived foam cell formation, estrogen executes a protective function.

In contrast, LRP2 gene expression was neither found in endothelial cells, nor in smooth muscle cells. Furthermore, LRP2 was not detected on protein level. As literature provides no evidence of LRP2 expression in HCAEC and HCASMC, these results suggest an absence of LRP2 expression in these cells.

In conclusion, the results of our experiments provide meaningful clues concerning the role of the studied parameters in atherosclerosis development. Our data support the hypotheses of the atheroprotective action mediated by high shear stress and the unfavourable effect of low shear stress. In addition, we provide evidence for the harmful effect of carbamylated LDL by revealing its influence on the regulation of the LDL-R gene family expression. Furthermore, we point out several mechanisms of estrogen's protective action on endothelial cells and smooth muscle cells.

To obtain a sufficient amount of data for performing meaningful statistical analyses, some shear stress experiments without estrogen from a former study [133] will have to be repeated. Additionally, it remains open if all gene expression results can be confirmed on protein level. The static cell culture experiment was repeated to obtain more material for finishing LDL-R detection. Furthermore, it is intended to perform Western-blot experiments for detection of VLDL-R and LRP1 protein expression in HCAEC. The protein analyses are only performed with material from static experiments because the yield of cell material in shear stress experiments is extremely low. However, these procedures will be the subject of prospective theses.

The experimental design already aims to simulate the physiological conditions. However, it could be improved by establishing a co-culture (EC in hollow fibers and SMC in extracapillary space). Furthermore, modified lipoproteins isolated from CKD patients could be used for further experiments. These optimizations could improve the validity of the experimental data.

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Abbreviations

ACAT	acyl-CoA-cholesterol-acyltransferase
ACTB	beta-actin
AngII	angiotensin 2
apo	apolipoprotein
BSA	bovine serum albumin
cDNA	complementary DNA
CKD	chronic kidney disease
cLDL	carbamylated LDL
CM	chylomicrons
CSF	colony stimulating factor
CVD	cardiovascular disease
ddH2O	double-distilled / deionized water
E2	17β-estradiol (estrogen)
EC	endothelial cells
ECM	extracellular matrix
EDHF	endothelium-derived hyperpolarizing factor
EGF	epidermal growth factor
eNOS	endothelial nitric oxide synthase
ER	estrogen receptor
ESRD	end-stage renal disease
ET-1	endothelin
FH	familial hypercholesterolemia
gDNA	genomic DNA
gLDL	glycated LDL
GPER	G-protein coupled estrogen receptor
HCAEC	human coronary artery endothelial cells
HCASMC	human coronary artery smooth muscle cells
HDL	high density lipoprotein
HMG CoA reductase	3-hydroxy-3-methyl-glutaryl-CoA reductase
HRP	horseradish peroxidase
HSS	high shear stress
ICAM-1	intercellular adhesion molecule 1

IDL	intermediate density lipoprotein
JNK2	c-Jun N-terminal kinase 2
LCAT	lecithin-cholesterol-acyltransferase
LDL	low densitiy lipoprotein
LDL-R	low densitiy lipoprotein receptor
LOX-1	lectin-like oxidized LDL receptor-1
LPL	lipoprotein lipase
LRP1	low densitiy lipoprotein receptor-related protein 1
LRP2	low densitiy lipoprotein receptor-related protein 2
LSS	low shear stress
MAPK	mitogen-activated protein kinase
MCP-1	macrophage chemoattractant protein 1
mLDL	methylated LDL
NF-κB	nuclear factor kappa B
nLDL	native LDL
NO	nitric oxide
oxLDL	oxidized LDL
PAA	polyacrylamide
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PDGF	platelet-derived growth factor
PGI2	prostacyclin
PKC	protein kinase C
ROS	reactive oxygen species
RT-PCR	reverse transcriptase / real-time polymerase chain reaction
SDS	sodium dodecyl sulfate
SEM	standard error of the mean
SFM	serum-free medium
SMC	smooth muscle cells
Sp1	specifity protein 1 (transcription factor)
SR	scavenger receptor
SRE	sterol regulatory element
SREBP	sterol regulatory element binding protein
st	static

TF	tissue factor
TGF-β	transforming growth factor beta
TK	tyrosine kinase
TNFα	tumor necrosis factor alpha
tPA	tissue plasminogen activator
VCAM-1	vascular cell adhesion molecule-1
VLDL	very low density lipoprotein
VLDL-R	very low density lipoprotein receptor
VSMC	vascular smooth muscle cells

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