universität wien

DIPLOMARBEIT

The role of lipoprotein subclasses HDL_2 and HDL_3 in their interaction with human platelets

angestrebter akademischer Grad

Magistra der Naturwissenschaften (Mag. rer.nat.)

Verfasserin	Sabine Ettinger
Matrikel-Nummer:	0305465
Studienrichtung	Ernährungswissenschaften (A474)
Betreuer:	Univ. Prof. Dr. Ibrahim Elmadfa

Wien, am 05.03.09

This diploma thesis was performed at:

University of Vienna

Department of nutritional sciences

Althanstraße 14

1090 Vienna, Austria

Head: Univ. Prof. Dr. Ibrahim Elmadfa

and

Medical University of Vienna

Institute of Physiology

Schwarzspanierstraße 17

1090 Vienna, Austria

Head of the laboratory: Univ. Prof. Dr. Ivo Volf

Acknowledgements

First I would like to thank Univ. Prof. Dr. Ivo Volf as my study supervisor for scientific advice, continuous support and for the possibility to work on such an interesting project. I am very grateful to Dr. Alice Assinger, who instructed me so well, for her big support and for her encouraging words during my work at the laboratory. In addition, thanks to all members of the institute. I also want to thank Univ. Prof. Dr. Ibrahim Elmadfa for his support.

I dedicate special thanks to my parents, Josefine and Rudolf, who supported my studies financially, and even more supported me personally throughout my life. Many thanks especially to my sister Monika!

1 Table of contents

2 List of figures

3 List of tables

4 Introduction and aim of the study

It is well known that high density lipoproteins (HDL) and low density lipoproteins (LDL) play a crucial role in regulation of platelet function and that circulating HDL cholesterol is reducing the risk of atherosclerosis. It is important to understand the molecular and cellular mechanisms that lead to the development of atherosclerosis for identifying strategies to limit disease progression before it leads to clinical consequences. HDL inclose several subclasses, mainly HDL_2 and $HDL_{3,}$ which diversify in size and composition and these subclasses exert different functions in the body. We aimed to evaluate differences in their effects on P-selectin expression in platelets and additionally we wanted to detect the influence of HDL on phosphorylated VASP (VASP-P). In summary, within this diploma thesis evaluations of the impact of native and hypochlorite modified HDL_2 and HDL_3 subclasses on degranulation and on the intracellular degree of VASP-P in human blood platelets were performed. Hypochlorite was used because of its proven in vivo relevance. It is formed in vivo by myeloperoxidase (MPO) from activated neutrophiles and monocytes. Moreover, MPO is present in human atherosclerotic lesions (Daugherty *et al.* 1994;Malle *et al.* 2006a;Marsche *et al.* 2008). Furthermore, the relevance of 2-Chlorohexadecanal (2-ClHDA) on platelet function was determined, because studies show its importance in mediating the inhibition of vasculoprotective nitric oxide synthase (NOS) in endothelial cells (Marsche *et al.* 2004). 2-ClHDA is produced in consequence of myocardial infarction (Thukkani *et al.* 2005) and findings also show that 2-ClHDA is able to manipulate neutrophil recruitment in vitro (Thukkani *et al.* 2002). Trials were carried out with FACS analysis to identify the differences between HDL_2 and HDL_3 in their interaction with human platelets.

5 Literature overview

5.1 Clinical picture of atherosclerosis

At present time, cardiovascular disease is the main cause for death and illness in developed countries. It will emerge as an outstanding health problem worldwide in future (Libby 2002). Various research studies tried to investigate the initiating factors that lead to atherosclerosis. Ross formulated the response to injury hypothesis, which first stated that endothelial denudation represents the first step of atherosclerosis. A more recent version says that endothelial dysfunction, and not denudation is the key event (Ross 1999). Other studies indicate that retention of lipoproteins within the vessel wall is initiating atherosclerosis, from which the response to retention hypothesis followed (Williams and Tabas 1995). In 1989, Steinberg et al. proposed the oxidative modification hypothesis**,** which was supported by many studies subsequently. This hypothesis points out modified lipoproteins in the vascular wall, built as a consequence of oxidative stress, as the crucial factor (Nakajima *et al.* 2006). These different hypotheses still evoke discussions, but one can definitely say that oxidative stress, lipoproteins and platelets play a pivotal role in the development of atherosclerosis. Atherosclerosis is a form of chronic inflammation resulting from interaction between modified lipoproteins, monocyte derived macrophages, T-cells, and the endothelium (Glass and Witztum 2001). It is not simply a disease, but rather a process contributing to the pathogenesis of various diseases, which in fact is a protective response to insults to the endothelium and smooth muscle cells of the arterial wall (Ross 1993).

5.1.1 Development

Epidemiologic studies identify genetic and environmental risk factors for the development of atherosclerosis. Elevated levels of serum cholesterol are proven to lead to this development in humans and experimental animals, even in the absence of other known risk factors (Glass and Witztum 2001). Oxidative stress and free radicals, caused by cigarette smoking, hypertension, diabetes mellitus, genetic alterations, elevated plasma homocysteine concentrations, and infectious microorganisms can cause endothelial dysfunction and hence are ranked among risk factors. Different phases in the chronic in-

flammatory process in the artery are noticeable (Ross 1999). At the beginning the atherosclerotic process is a protective response to an injury to the endothelium and smooth muscle cells of the arterial wall (Noll 1998). Under ordinary circumstances, the normal arterial endothelium is in contact with the flowing blood, but resists adhesion of leucocytes including blood monocytes. If endothelial cells are activated by inflammation, their expression of various leukocyte adhesion molecules is enhanced. The monocytes adhere to the activated endothelial layer and then diapedese between intact endothelial cells to reach the tunica intima and innermost layer of the arterial wall. Monocytes differentiate within the intima into tissue macrophages. Macrophages in the atheroma express scavenger receptors that bind oxidized lipoprotein particles (Libby 2002). Oxidized LDL lose their specificity for the apo-B receptor during their modification and thus are taken up by scavenger receptors that are found on macrophages. This process converts the macrophage into a foam cell, which is the hallmark of the arterial lesion. Growth factors, cytokines and other factors are expressed by foam cells. Cytokines stimulate endothelial cells to express adhesion molecules, which result in the adherence of more blood monocytes to the endothelial cell layer. The accumulation of foam cells and the proliferation of smooth muscle cells in response to growth factors released from macrophages lead to the formation of fatty streaks. This lesion is widely regarded as precursor of the mature atherosclerotic plaque (Barter *et al.* 2003a). This earliest type of lesion is pure inflammatory and is only made up of monocyte derived macrophages and T-lymphocytes (Ross 1999). Macrophages congregate in a central core in the typical atherosclerotic plaque. When the plaque ruptures, blood gets in contact with the potent procoagulant protein tissue factor, which is another macrophage product (Libby 2002).

5.1.2 Clinical consequences

Plaque rupture and thrombosis are considerable complications of advanced lesions that result in unstable coronary syndromes or myocardial infarctions (Falk *et al.* 1995). Generally large and medium sized elastic and muscular arteries are affected by lesions of atherosclerosis and these lesions can lead to ischemia of the heart, brain, or extremities, resulting in infarction. Myocardial infarction often occurs after erosion or uneven thinning and rupture of the fibrous cap, which is situated at lesion where macrophages enter,

accumulate, are activated, and where apoptosis may occur. Macrophage accumulation may be associated with increased plasma concentrations of both fibrinogen and C-reactive protein. Because of this, markers of inflammation may represent early signs of atherosclerosis. Degradation of the fibrous cap is the consequence of elaboration of metalloproteinases, such as collagenases, elastases, and stromelysins. Plaque instability and immune response are promoted by activated T cells possibly through stimulation of metalloproteinase production by macrophages in lesions. Further changes are the production of tissue-factor and other hemostatic factors (Ross 1999).

5.1.3 Atherosclerosis in context of HDL

There is increasing evidence that enhanced HDL levels diminish cardiovascular risk (Castelli 1996;Lamarche *et al.* 1997;Watts and Burnett 2004). HDL have protective effects against atherosclerosis and cardiovascular disease. These effects are mediated by enhanced reverse cholesterol transport and by direct antiatherosclerotic mechanisms. Epidemiological studies reveal that a 0.026 mmol/L increase in plasma HDL reduces the risk of coronary heart disease (CHD) by 2% in men and by 3% in women. These effects of HDL are independent of triglyceride and LDL concentrations. Another study shows that a 0.15 mmol/L increase in plasma HDL is associated with an 11% decrease in CHD. Significant data also demonstrate that HDL have intrinsic antiatherosclerotic properties, such as anti-inflammatory (Chrysohoou *et al.* 2007), antioxidant, antimitotic, anticoagulant, antiaggregatory, and profibrinolytic properties (Watts and Burnett 2004). In contrast, LDL are one of the most powerful and independent risk factors for CHD (Castelli 1996). According to results of several studies it is considered positive to have a high concentration of HDL in plasma together with a low level of LDL. In contrast, studies show that patients with almost total deficiencies of HDL are not more in danger of atherothrombotic cardiovascular disease than control subjects. An explanation could be that the relationship between the concentration of HDL and atherothrombotic cardiovascular disease might only be secondary to alterations in the metabolism of triglyceride rich lipoproteins or that these HDL deficiencies usually occur with a concomitant considerable reduction in LDL (Sastre *et al.* 2002).

5.2 Human blood platelets

Platelets play an important role in atherosclerosis because platelet adhesion and thrombosis are omnipresent in the initiation and generation of atherosclerotic lesions. They are important for vascular integrity in the absence of injury and protecting against spontaneous hemorrhage. Moreover, they can stick to dysfunctional endothelium and upon activation platelets release their granules, which contain cytokines and growth factors. Arachidonic acid liberated by activated platelets can be converted into prostaglandins such as thromboxane A_2, which is one of the most potent vasoconstricting and platelet aggregating substances known or into leukotrienes, which can amplify the inflammatory response. These factors and also thrombin may account for the migration and proliferation of smooth muscle cells and monocytes. Activated platelets can accumulate on the walls of arteries and recruit additional platelets into an expanding thrombus (Ross 1999).

5.2.1 Structure

Human platelets are the smallest cells in the blood and they are built by fragmentation of megakaryocytes. Platelets also enclose 2 unique organelles, the alpha- and dense granules and have a life span of 7 to 10 days. Only a few are consumed in the process of hemostasis, the others circulate until they are removed by phagocytic cells. The normal blood platelet count of a healthy human is 150 000 to 450 000 cells/mm^3. Platelets play a crucial role in thrombosis, hemorrhage, inflammation, tumor growth and defence, as well as antimicrobial activity (Chaer *et al.* 2006).

5.2.2 Signal transduction

The most important role of platelets is the process of primary hemostasis that is responsible for initiation and perpetuation of the hemostatic cascade. Primary hemostasis is characterized by vascular contraction, platelet adhesion and formation of a soft aggregate plug. It begins immediately after endothelial disruption. Secondary hemostasis is defined as the formation of fibrin and is responsible for stabilizing the soft clot and for maintaining vasoconstriction (Özüyaman 2003).

5.2.2.1 Platelet activation

Surface receptors regulate many functions and thereby control platelet activity. Various agonists and adhesive proteins have an impact on platelet receptors. In general, after stimulation of platelet receptors, different processes are activated (Freedman 2005). These processes are shape change, aggregation, secretory processes, and liberation of arachidonic acid. Arachidonic acid is rapidly converted into prostaglandins and lipoxygenase products (Willoughby *et al.* 2002). Adhesion occurs when circulating von Willebrand factor (vWf) attaches to the subendothelium. Glycoproteins (GPs) on the platelet surface adhere to the vWf. This results in an increase of intracellular calcium (Ca^{2+}) through tyrosine kinases and in an activation of the GP IIb/IIIa receptor (Özüyaman 2003). The interaction between platelet activating agonists, such as adenosine diphosphate (ADP), epinephrine, collagen, thrombin, serotonin, platelet activating factor, and their receptors evoke rapid mobilization of signalling molecules within the platelet. These signalling molecules are in particular Ca^{2+}, diacylglycerol, and inositol 1.4.5-trisphosphate, which are sufficient to activate and conclude shape change and aggregation responses (Willoughby *et al.* 2002).

5.2.2.1.1 Transmembrane domain receptors

Thrombin receptors

There are several protease activated receptors (PAR) like PAR-1 and PAR-4 on platelets. A clear mechanism of activation including a specific cleavage of the N-terminus acting as a ligand to the receptor is characteristic for the PAR class of receptors (Freedman 2005). The GPIb-IX complex is also ranked among this family and thrombin stimulation of human platelets results in its reduced surface expression (Kovacsovics and Hartwig 1996).

ADP receptors

ADP plays a crucial role in erythrocytes and endothelial cells. ADP is an important factor in hemostasis, thrombus formation, and vascular occlusion because it leads to platelet aggregation. ADP is secreted from the dense granules of stimulated platelets and is

able to amplify platelet aggregation. Furthermore, ADP evokes a rapid influx of external Ca^{2+} and mobilizes Ca^{2+} from intracellular stores. Attenuation of the inhibition of adenyl cyclase in platelets is also initiated by ADP. The transduction of ADP induced signals is possible through binding of ADP to purinergic receptors on the platelet surface. ADP receptors can be classified as P2X1, P2Y1, and P2Y12 (Freedman 2005).

5.2.2.1.2 Integrins

Glycoprotein IIb/IIIa

GP IIb/IIIa is an $\alpha_{IIb}\beta_3$ integrin and is only expressed on platelets (Freedman 2005). Every inactive platelet has approximately 80 000 copies of GP IIb/IIIa on its surface and on its α-granule membranes, which are dislocated to the surface after activation. Previous to activation, GP IIb/IIIa within the α-granule membranes is not on the surface (Özüyaman 2003). The GP IIb/IIIa is characterized by an inactive conformation and functions as a low-affinity adhesion receptor for fibrinogen in inactivated platelets. After stimulation intracellular bridges between platelets are formed by interactions between fibrinogen and GP IIb/IIIa (Freedman 2005). The affinity between this complex and fibrinogen and the number of receptors on the platelet surface are increased through thrombotic stimuli (Özüyaman 2003). GP IIb/IIIa mediates outside-in signalling and initiates intracellular signalling that further stabilizes the aggregate immediately after binding of fibrinogen. Some consequences of activation of the GP IIb/IIIa complex are Ca^{2+} mobilization, tyrosine phosphorylation, activation of phosphoinositide metabolism, and cytoskeletal reorganisation. These events change platelet aggregation from a reversible to an irreversible process. Platelet aggregation is completely inhibited by blockade of 80% of the surface GP IIb/IIIa receptors (Freedman 2005).

Glycoprotein Ia/IIa

This collagen receptor is an $\alpha_2\beta_1$ integrin. Damaged blood vessels expose the subendothelium to the bloodstream and thus platelets therein react with collagen. Platelets adhere to the damaged surface and form aggregates. Collagen has a strong ability to induce platelet adhesion and aggregation and there is a high content of collagen in the

subendothelium. A cascade of reactions is initiated after activation, which is important for activating the integrins and adhesive proteins. Integrin $\alpha_2\beta_1$ exists in three different conformations, such as a resting, non-active conformation (R0) having no affinity for soluble collagen and activated states R1 and R2, where R2 has higher affinity for soluble collagen than R1. ADP is secreted by other activated platelets and converts R0 to R1. Platelets release ADP due to low agonist concentrations. In platelets activated by a high agonist concentration also other systems are activated, such as protein kinase-C, phosphatidyl inositol 3-kinase, and protein tyrosine kinase. These signals result in the conversion of the R1 state activated integrin $\alpha_2\beta_1$ to the further activated form R2 (Jung and Moroi 2000).

5.2.2.2 Platelet secretion

Additional circulating platelets are activated by substances released from stimulated and aggregated platelets. Thereafter a thrombus is formed by fibrinogen bridging with the adherent platelets. The process of attracting additional platelets is called recruitment. Impairment of this process affects normal hemostasis, thrombosis and vascular remodelling. Synthesis of thromboxane (TXA_2) is stimulated by platelet activation and causes recruitment. Arachidonic acid is released from membrane phospholipids by phospholipase A_2 and C after platelet activation. Cyclooxygenase converts arachidonic acid to TXA_2 that binds to platelet thromboxane receptors and thereby further activates platelets what leads to activation of phospholipase C and liberation of intracellular Ca^{2+}. Both ADP and TXA_2 participate in a positive feedback loop that leads to platelet aggregation and recruitment. Platelets also release antithrombotic substances that may provide negative feedback for thrombus formation like nitric oxide (NO). Platelet function is inhibited by stimulating soluble guanylyl cyclase to produce cyclic guanosine monophosphate (cGMP), which results in the stimulation of cGMP dependent protein kinase (Freedman 2005).

5.2.2.3 Thrombus stability

The final phase of thrombus formation occurs after the receptors have been desensitized. This phase is essential for stabilising the platelet thrombus and for preventing premature disaggregation. For thrombus stability a sustained ligand binding to activated GP IIb/IIIa may be required. This binding is probably influenced by Ca^{2+}.The release of Ca^{2+} is induced by continuous stimulation of specific signalling pathways. PI3-kinase inhibition causes progressive reversal of aggregation after initial aggregation (Freedman 2005;Goto *et al.* 2006).

5.2.3 Endogenous influences on platelet function

5.2.3.1 Prostacyclin and VASP

Eicosanoids are generated from arachidonic acid by cyclooxygenase in endothelial cells and platelets. Endothelial cells include prostacyclin synthetase, which converts endoperoxides to prostacyclin (PGI_2). Another tissue hormone of the body is PGE_1, which also shows platelet stabilizing effects (Chang *et al.* 1990). A chemically synthesized form of PGE_1 was used within our project. PGI_2 stimulates adenylyl cyclase and is linked to a specific receptor on the surface of platelets. This leads to an increase of cAMP and results in Ca^{2+} reuptake by the dense tubular system (Best *et al.* 1977;Walter *et al.* 1993). Both PGE_1 and PGE_2 independently increase platelet cyclic AMP (McDonald and Stuart 1974). In consequence of this reuptake, intracellular Ca^{2+} content decreases and thus phosphorylation of vasodilator stimulated phosphoprotein (VASP) is increased and stabilized. VASP is a major substrate of protein kinase A and is a 46/50 kDa phosphoprotein, which is phoshorylated dependent on cGMP and cAMP dependent protein kinases. Three phosphorylation sites, Ser157, Ser239 and Thr278, have been identified. The phosphorylation of VASP (VASP-P) closely correlates with the inhibition of platelets, in particular with the inhibition of the fibrinogen bond to GP IIb/IIIa and thereby is used as an indicator for inhibition of platelets (Halbrugge *et al.* 1990;Horstrup *et al.* 1994). Due to these mechanisms PGI_2 inhibits platelet activation, platelet granule secretion, platelet aggregation, and the development of platelet procoagulant activities, in part through inhibition of release of P-selectin (Kroll and Schafer 1989;Willoughby *et al.* 2002).

5.2.3.2 Nitric oxide

NO is released by the intact endothelium and is mostly involved in the regulation of vascular tone and has antithrombotic properties. NO is able to decrease vascular smooth muscle tone and to inhibit platelet responses, like platelet adhesion, platelet granule secretion, and platelet aggregation (Freedman *et al.* 1997;Loscalzo 2001). It prevents thrombosis and inhibits the normal activation dependent increase in the expression of platelet surface GPs including P-selectin and the integrin GP IIb/IIIa complex. Furthermore, NO decreases the oxidation of arachidonate and inhibits the agonist dependent increase in platelet cytosolic free Ca^{2+} (Freedman 2005). NO activates soluble guanylyl cyclase, which results in an increase in cGMP production. Furthermore, this leads to phoshorylation of VASP through cGMP dependent proteinkinase (Eigenthaler *et al.* 1999;Lawrence and Pryzwansky 2001). Activation of cGMP dependent protein kinase also results in phosphorylation of Ca^{2+} transporters that decrease intracellular Ca^{2+} concentration. NO is produced during platelet aggregation and platelets themselves have constitutive and inducible NOS (cNOS and iNOS). Platelet derived NO pool also limits platelet recruitment to the growing platelet thrombus, possibly limiting the selfamplification of platelet thrombus formation in vivo (Freedman *et al.* 1997).

5.2.4 Interaction of platelets and lipoproteins

Platelets and lipoproteins are intimately connected with the pathogenesis of a wide variety of diseases, including atherosclerosis, thrombosis, and coronary heart disease. Results of studies suggest a direct relationship between plasma lipoproteins and the hemostatic function of platelets. Data also show that platelet hypersensitivity to stimulation is associated with increased cholesterol and that lipoproteins can augment the aggregation response of platelets. Thrombin-induced platelet aggregation is decreased by HDL and increased by LDL (Curtiss and Plow 1984). Platelets exposed to oxidized LDL show immediate platelet shape change and pseudopodia formation that increase adhesion of platelets to endothelial cells under flow conditions (Dardik *et al.* 2000). Collagen-stimulated platelets induce LDL modification, which is noticeable by increased conjugated dienes and lysophosphatidylcholine formation, enhanced electrophoretic mobility, Apo B-100 degradation, and monocyte LDL uptake. It is also shown a reduction of vi-

tamin E in LDL induced by activated platelets. There is evidence that platelets modify LDL via nicotinamide adenine dinucleotide phosphate (NADPH) oxidase mediated oxidative stress. This phenomenon could be regulated by arachidonic acid liberation. It is suggested that platelets play a role in favouring LDL accumulation within atherosclerotic plaque (Carnevale *et al.* 2007). Studies also report that very low density lipoproteins (VLDL) and remnant lipoproteins are able to activate platelets and the coagulation pathway and they can support the assembly of the prothrombinase complex. VLDL increase the expression and activity of the plasminogen activator inhibitor-1 gene and plasminogen activator inhibitor-1 antigen in platelets. This process is characterized by platelet aggregation and clot formation (Olufadi and Byrne 2006).

5.3 Human plasma lipoproteins

5.3.1 Plasma lipoprotein subclasses

Lipoproteins are divided into four major classes based on their chemical composition and their physical characteristics, like electrophoretic mobility, density, and diameter. One can distinguish between chylomicrons, VLDL, LDL, and HDL (McPherson *et al.* 2007). HDL particles are further subdivided into HDL_2 and HDL_3. Apart from the predominant HDL_2 and HDL_3 fractions, there also exists a subclass called HDL_1, a very minor fraction in humans, less dense than HDL_2 (Assmann and Schriewer 1980).

5.3.2 Classification of HDL

There exist various techniques to subfractionate HDL, including precipitation, electrophoresis, affinity chromatography, and ultracentrifugation. Within my diploma thesis ultracentrifugation was employed, which is the most often used method (McPherson *et al.* 2007). HDL can also be classified according to their apolipoprotein content. ApoA-I is quantitatively the most abundant protein in HDL, followed by apoA-II. HDL are classified in HDL with apoA-I and without apoA-II, HDL with apoA-I and with apoA-II, and HDL with apoA-II and without apoA-I. HDL populations can also be divided according to their electrophoretic mobility in agarose gels. These are classified

as α, pre-β and γ HDL. Separation for size by non denaturing gel electrophoresis in polyacrylamide gradient gels is possible. The populations obtained by this method are similar to those obtained by ultracentrifugation and are called (from smaller to greater size) HDL3c, HDL3b, HDL3a, HDL2a and HDL2b (Skinner 1994).

5.3.3 Metabolic pathway of HDL

The inside of HDL is made up of hydrophobic lipids such as cholesteryl esters (CEs) and triglycerides (TG), surrounded by an outer shell in which free cholesterol is interspersed with phospholipids, and the surface contains mostly apolipoproteins (Watts and Burnett 2004). The formation of HDL_2 from HDL_3 is continual and reversible. This is necessary for transport of cholesterol, of other lipids from extrahepatic cells and of catabolized triglyceride rich lipoproteins. Hepatic lipase converts HDL_2 to HDL_3 and is inhibited by estrogen and stimulated by androgens. Because of this, men have lower HDL_2 levels than women (Nestel 1987). The average HDL cholesterol plasma level is 55mg/dl for men and 65mg/dl for women (Kleuser 2007). ApoA-I and apoA-II are synthesized more in women than in men. HDL turnover is also influenced by nutrition. ApoA-I and HDL_2 are increased by carbohydrates, whilst synthesis is impeded by polyunsaturated fatty acids. Low fat and low cholesterol diets primarily result in lower HDL levels. Disorders such as alcoholic liver disease or Tangier disease can change HDL composition and force HDL removal (McPherson *et al.* 2007;Nestel 1987). The enzyme lecithin cholesterol acyltransferase (LCAT) accounts for conversion of HDL_3 in HDL_2. LCAT partially re-esterifies cholesterol. The enzyme cholesteryl ester transfer protein (CEPT) facilitates the transport of CEs, formed by the LCAT reaction, and TG between the lipoproteins. CEs can also be delivered to the liver for biliary secretion or reutilization during lipoprotein assembling. However, not only phospholipids, but also apoA-I is involved in the process of removing cellular cholesterol, where distinct regions within apoA-I play an important role (Oram and Yokoyama 1996).

5.3.4 Apolipoproteins in HDL

5.3.4.1 ApoA-I

68% of HDL protein is made up of apoA-I, which is synthesized in liver and intestine, is composed of 243 amino acids and its molecular weight is 28 kDa. Zones of the α-helical properties, called amphipathic helices, can interact with phospholipids because they are formed by one hydrophobic and one hydrophilic side. The hydrophobic side is in contact with the aliphatic chains of fatty acids. The hydrophilic part is in contact with the aqueous medium and interacting with the most polar region of phospholipids and with free cholesterol. Pre-pro-apoA-I, which is the precursor form, is characterized by high molecular weight. It is converted into pro-apoA-I after passing to cytoplasm. In the bloodstream and lymph, it is converted into the mature protein through the action of a plasmatic converter enzyme. The deficiency of apoA-I is associated with HDL deficiency. There exist various models of apoA-I structures in nascent discoidal HDL. The spheroidal particles of HDL contain 2 to 4 molecules of apoA-I. Their structure is presumably similar to that of discoidal HDL. ApoA-I plays a cardinal role in the first steps of the efflux of cholesterol from cells and it is the cofactor of the enzyme LCAT (Sastre *et al.* 2002).

5.3.4.2 ApoA-II

Plasma concentration of apoA-II in normolipaemic individuals is, on average, 33-35 mg/dl. ApoA-II constitutes approximately 20% of HDL protein and thus is the second most abundant HDL apolipoprotein. It is synthesized in the liver and the percentage of α-helix in apoA-II is 62% which has a closer affinity for lipid monolayers than that of apoA-I. This is the reason why apoA-I can be displaced from the HDL surface by an excess of apoA-II (Sastre *et al.* 2002). Results of studies show that apoA-II levels in plasma are mostly regulated by apoA-II production. In contrast, apoA-I levels are controlled by apoA-I catabolism (Ikewaki *et al.* 1995). Different findings demonstrate that apoA-II either activates or inhibits the action of hepatic lipase and CETP. Results also show that apoA-I lipid complexes can serve as substrates for LCAT and apoA-II might not (Forte *et al.* 1995). Overexpression studies of apoA-II have not, however, consis-

tently reported favourable effects on atherosclerosis in various experimental models (Watts and Burnett 2004).

5.3.4.3 Apo-E

The concentration of human apo-E in plasma is comparatively low with 3-6 mg/dl (Mahley *et al.* 1984), this is approximately 1-2% of total apolipoproteins and almost half can disappear during isolation of lipoproteins by ultracentrifugation. Most of the characteristics of apo-E are not related to a function in HDL. The existence of pre-β migrating HDL is interesting because it contains apo-E and seems to be able to induce the early steps of reverse transport of cholesterol from cell membranes to plasma. There exist apo-E polymorphisms, which could also influence reverse cholesterol transport. Furthermore, the presence of apo-E in HDL is related to the effectiveness of the transfer of HDL cholesterol esters to the SR-BI receptor (Arai *et al.* 1999). Studies reveal that apo-E shows a potent, but not unique effect in HDL, which acts inhibitory on platelets (Higashihara *et al.* 1991a). Apo-E acts as a receptor ligand and plays a role in immuno-modulation and nerve regeneration (Schiele *et al.* 2000). Furthermore, findings show that it markedly elevates platelet NO synthase activity and intraplatelet levels of cGMP. The inhibitory action of apo-E is noticeable limited by NO synthase inhibitors (Riddell *et al.* 1997).

5.3.5 Functions and cellular actions of HDL

5.3.5.1 Task of cholesterol transport

The most important function of HDL is reverse cholesterol transport, which represents a HDL mediated collection and transport of cholesterol from peripheral tissues to the liver for excretion, also called cholesterol efflux from peripheral tissues (Fielding and Fielding 1995;Sastre *et al.* 2002). Thus HDL have the ability to enhance the removal of excess cholesterol from peripheral tissues (Oram 2003). The fact that HDL represent an inverse risk factor for atherosclerosis (O'Connell and Genest, Jr. 2001;Okamura *et al.* 2006;Sastre *et al.* 2002) is probably related to the ability of HDL to induce cholesterol efflux from vascular cells.

5.3.5.2 Antioxidant properties

Another important function of HDL is the prevention of oxidative modification of LDL. This function is partially referred to enzymatic processes (Kontush *et al.* 2003;Kontush and Chapman 2006;Sastre *et al.* 2002). Morever**,** these antioxidant properties can be attributed to the apoA-I content of HDL because it contains methionine residues which are preferably oxidized (Garner *et al.* 1998). HDL can take up oxidized phospholipids and can then undergo oxidative modification and their protective functions can be manipulated. Results of studies report that HDL_2 and HDL_3 protect against oxidative modification of LDL. These modifications were detected via thiobarbituric acid reactive substances, lipid hydroperoxides and conjugated dienes (Huang *et al.* 1998;Singh *et al.* 1997). Findings show that HDL_2 protect LDL more effectively against oxidation by enzymic formation of OH^- than HDL_3 (Singh *et al.* 1997). HDL significantly inhibit the acceleration of oxidative modification of LDL by ascorbic acid (Sakuma *et al.* 2005). Moreover, HDL inhibit the transmigration of monocytes induced by oxidized LDL, the cytotoxicity induced by oxidized LDL and the oxidized LDL induced adhesion of monocytes to endothelial cells in vitro. ApoA-I also prevents the cytotoxicity induced by oxidized LDL. Exact mechanisms are currently unknown (Barter *et al.* 2003a;Kontush and Chapman 2006).

5.3.5.3 Influence on synthesis of nitric oxide

HDL also play a crucial role in the synthesis and bioavailability of NO. NO synthase catalyses the oxidation of L-arginine to L-citrulline and NO radicals. In specific situations it is possible that eNOS becomes uncoupled and reduces molecular oxygen and it does not transfer electrons to L-arginine. Thus O_2^- radicals are generated. This can lead to the generation of $ONOO^-$, which represents a potent oxidant that induces lipid and protein oxidation (Stocker and Keaney, Jr. 2005). Moreover, HDL containing apo-E elevate platelet NO synthase activity through interaction with platelet surface and therefore inhibit platelet aggregation (Riddell *et al.* 1997;Riddell and Owen 1999). HDL also elevate endothelial NO synthase expression. HDL enhance NO production via upregulating the expression of eNOS and stimulating eNOS via activation of kinase cascade (Mineo *et al.* 2006). Another study reveals that NO synthase activity is enhanced by

HDL and apoA-I via protein association and multisite phosphorylation (Drew *et al.* 2004).

5.3.5.4 Influence on liberation of mediators

HDL stimulate protein kinase-C, demonstrated by a transient increase in membrane associated kinase activity. It is proposed that kinase activation is dependent on binding of HDL_3 to the HDL receptor because tetranitromethane modified HDL_3, which do not bind to the receptor, is without effect (Mendez *et al.* 1991). It is also possible that HDL have an effect on the production of various mediators, which are inclosed in the development of atherosclerosis. One mediator is endothelin, a vasconstricting mitogenic peptide, thats secretion from endothelial cells is inhibited by HDL (Fan *et al.* 2000). Studies show that intracellular Ca^{2+} is increased by HDL in endothelial cells (Honda *et al.* 1999) and that the Ca^{2+} release from internal stores is maintained by the activation of phospholipase C (Mineo *et al.* 2006). Moreover, HDL are able to induce vasodilation in isolated aortae through an increase in Ca^{2+}(Nofer *et al.* 2004).

5.3.5.5 Antithrombotic and antiatherogenic effects

The antiatherogenic capacity of increased HDL cholesterol concentrations in plasma is demonstrated also in animal models. Many of the studies on HDL attempt to assign the antiatherogenic action of HDL to specific particle subpopulations (Sastre *et al.* 2002). The thrombogenic risk might be reduced by counteracting or interfering with thrombogenic and atherogenic LDL, or by any other interaction with platelets. Likewise it is assumed that apo-E and apo-E containing HDL might limit lipase mediated LDL retention in the subendothelial matrix (Saxena *et al.* 1993). Moreover, results of a study carried out with co-cultures of human aortic cells show that HDL and antioxidants can inhibit the transmigration of monocytes across an endothelial cell monolayer induced by oxidized LDL (Navab *et al.* 1991). Results also demonstrate that HDL and modified HDL partially regulate vascular tone (Martin-Nizard *et al.* 1995). ApoA and paraoxonase contents as well as the phospholipid composition define antiatherogenic effects of HDL. Antithrombotic properties are also defined by the abilities of HDL to extenuate expression of tissue factor and selectins. Purified HDL increase the inactiva-

tion of coagulation factor Va by activated protein C and protein S in vitro that results in decreased thrombin generation (Griffin *et al.* 1999). Furthermore, HDL enhance endothelial anticoagulant thrombomodulin that facilitates the generation of activated protein C. HDL directly and indirectly play a role in inhibiting platelet activation (Mineo *et al.* 2006). Moreover, results show protective effects of HDL, because HDL are able to restore endothelial function in hypercholesterolemic men (Spieker *et al.* 2002).

5.3.6 Established differences between HDL_2 and HDL_3

The relative proportion of HDL_3 to HDL_2 in plasma is known to be dependent on different factors, including sex, alcohol intake, exercise, smoking, obesity, and hypercholesterolemia (Krauss 1982).

5.3.6.1 Varieties in composition

HDL_2 are composed of 60% lipids and 40% protein, whereas HDL_3 are made up of 45% lipids and 55% protein. The ratio of phosphatidylcholin to sphingomyelin and the ratio of free to esterified cholesterol are higher in HDL_2 than in HDL_3. Approximately 90% of proteins in HDL consist of apoA-I and apoA-II. The composition of isolated HDL is dependent on the method of preparation (Assmann and Schriewer 1980).

5.3.6.2 Differences in metabolism

The metabolism of HDL_2 is faster than the catabolism of HDL_3. The conversion of HDL_2 to HDL_3 by means of hepatic lipase is inhibited by oestrogen and stimulated by androgens. Due to this, men have lower HDL_2 levels than women. ApoA-I and apoA-II are synthesized more in women than in men (Nestel 1987).

5.3.6.3 Discrepancies in race and sex

The Bogalusa heart study demonstrates a difference in levels of HDL_2 and HDL_3 in black and white adults. Whites show significantly higher levels of VLDL, LDL and

lower levels of HDL_2 and HDL_3 than blacks. White females have significantly higher levels of HDL_2 than white males. HDL_2 and HDL_3 are favorably affected by female sex and alcohol, whereas cigarette smoking shows unfavorable effects on VLDL and HDL_3. The race and sex specific differences are explained by the variation in their triglyceride metabolic capacity. It is shown that females have a greater activity of lipoprotein lipase than males and males have a greater activity of hepatic lipase than females. The study demonstrates that blacks compared with whites have significantly higher lipoprotein lipase and lower hepatic lipase activities (Srinivasan *et al.* 2001). Another study demonstrates that women have higher levels of HDL, HDL_2, HDL_3, and apoA-I and lower levels of ApoB than men (Gardner *et al.* 2000).

5.3.6.4 Differences in apolipoprotein content

Higashihara et al. and Desai et al. show that HDL_2 inhibit collagen- and thrombin-induced platelet aggregation more effectively than HDL_3. The authors suggest that most of the inhibiting effects are situated in the HDL_2 fraction due to their content of apo-E (Desai *et al.* 1989;Higashihara *et al.* 1991b). A study found that plasma levels of HDL containing both apoA-I and apoA-II (apoA-I/apoA-II) are 2.5 times higher than levels of apoA-I without apoA-II (apoA-I). The proportion of apoA-I associated with HDL_2 is greater than that of apoA-I/apoA-II. Furthermore, an increase in levels of HDL_2 causes an enhancement of the proportion of apoA-I in HDL_2. ApoA-I/apoA-II show a positive association with plasma LCAT activity within HDL_3, but not within HDL_2. Besides, apoA-I demonstrates an inverse association with plasma CETP mass, which was quantified using an immunoradiometric assay with polyclonal anti-CEPT antibody (Mowri *et al.* 1994).

5.3.7 HDL and various determinants

5.3.7.1 Impact of alcohol

A highly significant increase of lipids and apolipoproteins in HDL and its subfractions in alcohol consumers was measured. The increase in HDL_2 is more than twice of the increase in the HDL_3 subclass, which shows a higher HDL lipid load in alcohol con-

sumers (Schafer *et al.* 2007). Another study demonstrates an increase in total HDL cholesterol and apoA-I due to alcohol. This finding is referred to HDL_3 cholesterol, because the association seems to be linear. The study reveals that HDL_2 are increased just after high alcohol consumption levels and that they are diminished significantly among moderate drinking women (10–20 g/day) (Sillanaukee *et al.* 2000).

5.3.7.2 Effect of sports

Within a study on effects of sports changes in lipid profiles were measured and among other things blood samples were collected 24h post exercise and compared to samples collected pre exercise. It is shown a significant increase in 24h post exercise HDL at lactate threshold intensity, possibly due to increases in both HDL_2 and HDL_3 subfractions. Although 24h free cholesterol is increased significantly as well. The increase in HDL involves decreases in TG and VLDL at 24h post exercise. Hence the lactate threshold intensity might appear to be the threshold intensity of acute aerobic exercise necessary to promote a significant increase in HDL (Park and Ransone 2003). Studies reveal that exercise is associated with a significant increase in apoA-I and apoA-I/apoA-II and unchanged apoA-II. These findings support the assumption that exercise increases HDL_2 fraction (Kullmer and Kindermann 1985). Aerobic physical exercise is the exercise of choice to increase HDL (Watts and Burnett 2004).

5.3.7.3 Implications of selected diseases

5.3.7.3.1 Breast cancer

Michalaki et al. show that breast cancer patients have a higher risk for developing other chronic diseases including cardiovascular disease. In patients with breast cancer compared to the controls an increase in TG and a decrease in HDL, especially in the HDL_2 subfraction, is shown. Untreated breast cancer patients have unfavourable lipid profiles with high atherosclerosis indexes (Michalaki *et al.* 2005).

5.3.7.3.2 Non insulin dependent diabetics

There seems to exist a statistically significant decrease in HDL, HDL_2 and HDL_3 in comparison to non diabetic controls. A higher decrease of cholesterol is shown in HDL_2 than in HDL_3 subclass in the comparisons between non insulin dependent diabetes mellitus (NIDDM) with CAD and CAD subjects without NIDDM (Bakogianni *et al.* 2001).

5.3.7.3.3 Myocardial infarction

ApoA-I and apoA-II concentrations are both significantly lower in patients with myocardial infarction (MI). These differences remain even after adjustment for coronary risk factors and lipids. Apo-B and apo-E concentrations are not significantly related to MI after these adjustments. Both HDL_2 and HDL_3 cholesterol levels are significantly associated with MI. Especially apoA-I and apoA-II may give considerable information to determination of risk of MI (Buring *et al.* 1992).

5.3.7.4 Rising HDL by means of medication

5.3.7.4.1 Fibrates

TG are reduced by fibrates up to 50%, whereas HDL are enhanced by 5-25% due to the increase in the concentration of apoA-I and apoA-II. Fibrates activate peroxisome proliferator activated receptor alpha (PPARα). PPARα activators increase the synthesis of apoA-I, apoA-II, lipoprotein lipase, ATP binding cassette A1 (ABCA1) and scavenger receptor type B1 (SR-B1). The selective hepatic uptake of CEs is enhanced due to the increase in SR-B1. Increases in ABCA1 and SR-B1 both have the capacity to increase reverse cholesterol transport. Fibrates may thereby enhance the antiatherogenic function of HDL and they have a direct antiinflammatory effect by virtue of their ability to activate PPARα in the artery wall (Barter *et al.* 2003a).

5.3.7.4.2 Statins

The increase in the concentration of HDL by statins is generally in the range of 5-10%. This increase is achieved at relatively low doses of statins. In some cases the effect is

already maximal at the lowest recommended dose. Statins activate PPARα as do fibrates, but the mechanism of activation is different and the inhibition of the Rho family of small GTP proteins is involved. Rho activity is upregulating the activity of PPARα. There exists a synergistic activation of PPAR by simultaneous treatment with statins and fibrates. Statins also have the ability to inhibit CETP (Barter *et al.* 2003a).

5.3.7.4.3 Niacin

The synthesis of both apoA-I and apoA-II is stimulated by niacin. HDL are thus commonly increased by 30%. There exist unpleasant side effects, but when added at very low doses to statin therapy, niacin significantly increases the concentration of HDL, though the mechanism by which niacin increases the concentration of HDL is not known (Barter *et al.* 2003a).

5.3.7.4.4 Inhibitors of CETP

A deficiency of CETP is associated with increased HDL levels, decreased LDL levels and decreased apo-B levels. Small molecule inhibitors of CETP are able to provoke this profile in human subjects (Barter *et al.* 2003b).

5.3.8 Oxidative modification of HDL

Lipid and protein oxidation in the vascular wall is accepted to contribute to atherosclerosis. It is not known if oxidative stress in general or the oxidative modification of LDL in particular is jointly responsible for pathogenesis of atherosclerosis. The fact that LDL are linked to atherosclerosis is well established because reducing high cholesterol represents a very effective therapy (Stocker and Keaney, Jr. 2005). Sources for oxidative stress are hypercholesterolemia, diabetes, hypertension, cigarette smoking, aging, and nitrate tolerance. These risk factors increase the production of reactive oxygen species (ROS). ROS lead to altered vasomotion, matrix metalloproteinase activation, lipoprotein oxidation, apoptosis, vascular smooth muscle cell growth, and adhesion molecule expression via various enzyme systems, xanthine oxidase, mitochondrial sources, and NOS, respectively (Harrison *et al.* 2003). The oxidative modification hypothesis supposes that oxidation of proteins and lipids, mostly modification of LDL, lead to devel-

opment of atherosclerosis and that these changes can be prevented by antioxidants (Jessup *et al.* 2004). It is not known how and where LDL get oxidized and if results obtained by trials in vitro are also of importance in vivo (Chisolm and Steinberg 2000). Several studies with β-carotene and vitamin E were conducted, but no beneficial effects of these antioxidants on cardiovascular events are shown (Knekt *et al.* 2004;Steinberg and Witztum 2002;Vivekananthan *et al.* 2003).

5.3.8.1 The importance of myeloperoxidase derived hypochlorite

The MPO - H_2O_2 – Cl^- system forms hypochlorite (OCl^-) in activated neutrophils and monocytes in vivo and may react with lipids, antioxidants and proteins. OCl^- represents a most important proinflammatory chlorinating oxidant generated by MPO (Malle *et al.* 2006b;Marsche *et al.* 2008). The presence of enzymatically active MPO in human atherosclerotic lesions, the presence of hypochlorous acid (HOCl) modified lipoproteins and proteins in advanced human atherosclerotic lesions and inflammatory kidney tissues demonstrate the importance of MPO as a potent in vivo oxidant. MPO catalyzes oxidative reactions in the vascular wall and thereby may play a part in the development of atherosclerosis (Daugherty *et al.* 1994). Another finding, why MPO presumably is associated with atherosclerosis, is that atheroprotective activities of HDL are decreased and linked to lesions in HDL, which are modified by MPO and HOCl (Nicholls *et al.* 2005). Furthermore, MPO catalyzed oxidation products are highly enriched in circulating HDL from individuals with cardiovascular diseases where MPO concentrations are also increased and thus elevated MPO levels are linked to occurrence of CAD (Malle *et al.* 2006b;Zhang *et al.* 2001). Oxidation of HDL is shown to result in decreased cholesterol efflux in macrophages and findings referred to changes of the apolipoprotein domain (Bergt *et al.* 1999). Moreover, studies show that these changes result in modifications in the protein moiety apoA-I and in the conversion of HDL into a proatherogenic lipoprotein particle. Furthermore, also biophysical trials show that MPO binding is possible through this specific interactions with apoA-I. Specific targets for nitration and chlorination of specific tyrosine residues catalyzed by MPO are identified on apoA-I (Zheng *et al.* 2005). The ability of HDL to promote cellular cholesterol efflux via the ABCA1

transport system is detracted after the MPO catalyzed oxidation of apoA-I (Nicholls *et al.* 2005).

5.3.8.2 Relevance of 2-Chlorohexadecanal

2-ClHDA is derived from HOCl mediated oxidative cleavage of HDL associated plasmalogen. 2-ClHDA represents the main α-chloro fatty aldehyd build from cell associated plasmalogen (Thukkani *et al.* 2002). Plasmalogens are situated in the plasma membranes of mammalian cells and in lipoproteins and are thought to be antioxidants because the vinyl ether bond is susceptible to attack by oxidants. Findings demonstrate that the vinyl ether bonds of plasmalogens are highly amenable to reactive chlorinating species generated by the MPO hydrogen peroxide chloride system. Lysophosphatidylcholine and α-chlorinated fatty aldehyds are generated (Albert *et al.* 2001). Furthermore, results of studies suggest that 2-ClHDA is able to modulate eNOS expression and activity. A time dependent and concentration dependent decrease of NO production in human umbilical venous endothelial cells was measured (Marsche *et al.* 2004). Moreover, studies show that 2-ClHDA is produced as a result of a neutrophil dependent MPO based mechanism, which constitutes an answer to regional myocardial infarction and it might also contribute to cardiac dysfunction (Thukkani *et al.* 2005). Neutrophil recruitment is shown to be induced by physiologically relevant concentrations of 2-ClHDA in vitro. Because of this, the authors assume that α-chloro fatty aldehydes take part in neutrophil recruitment of neutrophil mediated inflammation in vivo (Thukkani *et al.* 2002).

6 Materials and laboratory equipment

6.1 Material

6.1.1 Chemicals

Sodium hypochlorite (Sigma-Aldrich)

Methionine (Sigma-Aldrich)

ADP (Sigma-Aldrich)

Collagen reagens Horm (Nycomed)

Thrombin (Sigma-Aldrich)

PGE_1 (Sigma-Aldrich)

Sepharose 4B (GE Healthcare)

Antibodies:

Polyclonal anti-mouse IgG antibody (cat. 349031, FITC conjugated, Becton Dickinson)

Monoclonal anti CD62 (cat. 348107, PE conjugated, Becton Dickinson)

Monoclonal anti-phospho-VASP (cat. 0153S0202, pSer 239, clone 22E11, nanoTools)

6.1.2 Buffers and Solutions

10% formaldehyde solution (Sigma Aldrich) in 0.9% sodium chloride solution

0.2% triton solution (Triton x-100, Sigma Aldrich) in PBS

Tyrode-HEPES buffer (with glucose and albumin)

140mM sodium chloride

3mM potassium chloride

1mM magnesium hydrogen carbonate

10mM HEPES

(5.5mM D-glucose)

(0.5% human serum albumin)

pH: 7.4

Borate buffer with EDTA

0.1M borate

100µM EDTA

pH: 7.2

Borate buffer with sodium chloride

0.1M borate

0.05M sodium chloride

pH: 7.2

Dulbecco´s phosphate buffered saline (PBS)

1.5mM potassium phosphate monobasic

2.7mM potassium chloride

137mM sodium chloride

8.3mM sodium phosphate dibasic (anhydrous)

pH: 6.8

Solutions for Lowry procedure

A1: 1g $CuSO_4*5H_2O$ in 100ml water distilled

A2: 2g K-Na-tartrat in 100ml water distilled

B: 50ml 2% (w/v) sodium carbonate + 1ml A

(A: 500µl A1 + 500µl A2)

C: Folin reagent + water distilled (1:1)

D: 1 N sodium hydroxide

6.2 Laboratory equipment

- Ultracentrifuge - Optima LXP Series with a 50.2 Ti rotor (Beckman Coulter)
- Centrifuge - Allegra X12R (Beckman Coulter)
- Analytic Flow Cytometer - FACSCalibur dual-laser (Argon-488nm and 635nm diode) (Becton Dickinson)
- Spectrophotometer - U-3010 (Hitachi)
- Microplate Reader - Anthos HT III (Anthos)
- Spectra Max Gemini XS (Molecular Devices)

7 Methods

7.1 Lipoprotein preparation

Blood of 24 healthy donors was centrifuged at 2000g for 20min. Blood plasma was separated as supernatant from the remaining blood and immediately frozen. Blood plasma was centrifuged in the presence of salt having a density suitable for separating the lipoproteins.

First VLDL were isolated. A density of 1.019g/ml was required, this equated to a 2.85% potassium bromide solution. The density of the blood plasma had to be adjusted, it was already one of a 1.1% potassium bromide solution, so the difference value amounted to 1.75%. Addition of potassium bromide: g (salt) = g (plasma) * 1.75 / 98.25

Each centrifuge tube was filled up with 15ml plasma and was overlaid by a potassium bromide solution of identical density (2.85%). The centrifuge tubes were centrifuged at 100 000g at 4 C° for 16 hours. Then VLDL could be removed as supernatant from the remaining plasma with a syringe. The centrifuge tubes were always kept on ice.

Then LDL were isolated. KBr was added to the remaining plasma until it reached a density of 1.063g/ml, this equated to a 8.6% potassium bromide solution. Addition of potassium bromide: g (salt) = g (plasma) * 5.75 / 94.25

Each centrifuge tube was filled up with 15ml plasma and was overlaid by a potassium bromide solution of identical density (8.6%). The centrifugation at this point took 20 hours at 100 000g and at 4C°. LDL could be separated as aforementioned for VLDL, and were filtered with Rotilabo syringe filter (0.45µm, PVDF, Roth).

Afterwards HDL_2 were isolated. KBr was added to the remaining plasma until a density of 1.125g/ml, which equated to a 16.1% potassium bromide solution. Addition of potassium bromide: g (salt) = g (plasma) * 7.5 / 92.5

Each centrifuge tube was filled up with 15ml plasma and was overlaid by a potassium bromide solution of identical density (16.1%). Accordingly the plasma was centrifuged for 36 hours at 100 000g and at 4C°. HDL_2 could be separated as aforementioned and were filtered with Rotilabo syringe filter (0.45µm, PVDF, Roth).

Thereafter HDL_3 were isolated. KBr was added to the remaining plasma until a density of 1.21g/ml, which equated to a 25.2% potassium bromide solution. Addition of potassium bromide: g (salt) = g (plasma) * 9.1 / 90.9

Each centrifuge tube was filled up with 15ml plasma and was overlaid by a potassium bromide solution of identical density (25.2%). The plasma was centrifuged for 40 hours at 100 000g and at 4C°. HDL_3 could be separated as aforementioned and had to be filtered with Rotilabo syringe filter (0.45µm, PVDF, Roth) as well. Lipoproteins were stored at 4C°.

Lipoprotein fractions used were HDL_2 and HDL_3.

7.2 Native HDL

Native HDL (nHDL) obtained by ultrazentrifugation contained potassium bromide, which was eliminated by rebuffering in Econo-Pac 10DG columns equilibrated with borate buffer containing 0.9% sodium chloride.

7.3 Modification of HDL

First nHDL were rebuffered in Econo-Pac 10DG columns equilibrated with borate buffer and EDTA to avoid oxidative modification of HDL by iron. Then lipoproteins were modified with hypochlorite. Different degrees of oxidatively modified HDL were obtained by using different molar excess of hypochlorite:

Maximal oxidation: hypochlorite was added until HDL were completely bleached. Hypochlorite was added in steps of 1µl and promptly mixed on vortex.

Medium oxidation: half of the volume of hypochlorite needed for maximal oxidation was added.

Minimum oxidation: fourth of the volume needed for maximal oxidation was added.

Reduction: maximally oxidized HDL were incubated with methionine [final concentration (f.c.): 12mM] for 10 minutes.

Afterwards each state of modification had to be rebuffered in Econo-Pac 10DG columns equilibrated with borate buffer containing 0.9% sodium chloride.

7.4 Protein determination - Lowry procedure

Protein content of lipoproteins was quantified according to Lowry. The Lowry method is sensitive to low concentrations of protein. Under alkaline conditions the divalent copper ion forms a complex with peptide bonds in which it is reduced to a monovalent ion. Monovalent copper ion and the radical groups of tyrosine, tryptophan, and cysteine react with Folin reagent to produce an unstable product that becomes reduced to molybdenum/tungsten blue.

Procedure for proteins in solution:

Different dilutions of proteins were prepared. 250µl of the protein containing solutions were added to 125µl of solution D. The test tubes were incubated for 30 minutes at room temperature. Then 1.25ml of solution B were added. After an incubation of 10 minutes 125µl of solution C were added. Each tube was mixed immediately and incubated for another 30 minutes. The absorbance of each sample was determined at 750nm. Data were analysed with a calibration curve constructed by known concentrations of bovine serum albumin.

7.5 Platelet isolation

Blood was obtained from healthy humans, either subjects from the centre of Physiology and Pathophysiology or platelet donors from the Vienna general hospital, who were free of medication for the last 2 weeks. Fresh human blood was drawn into acid citrate dextrose (3.8%). Platelet rich plasma (PRP) was obtained by centrifugation at 125g for 20min. The obtained PRP was gel filtrated. Gel filtration was performed on a Sepharose 4B column, which was equilibrated with HEPES-Tyrode buffer containing 0.5% human serum albumin. This was essential to get non activated platelets and to obtain platelet counts comparable to that observed in PRP. Gel-filtrated platelets (GFP) were eluted

with HEPES-Tyrode buffer. Subsequently platelets were kept at room temperature and used immediately for experiments.

7.6 FACS analysis

7.6.1 Quantification of vasodilator stimulated phosphoprotein

VASP-P was induced by PGE_1 and was used as indicator for inhibition of platelets (5.2.3.1).

300µl of GFP were incubated with HDL_2 and HDL_3 (7.2, 7.3) in Eppendorf tubes for 10 minutes at room temperature. Final concentrations of HDL added mounted up to 100µg/ml. Trials were also carried out with 2-ClHDA (f.c.: 2µM and 1µM), which was incubated with platelets in Eppendorf tubes for 10 minutes at room temperature.

All tests were performed in Cellstar TC-plates (96well, V-shape, Greiner Bio-One). Procedure is the same for HDL_2 and HDL_3 as well as for 2-ClHDA. PGE_1 (f.c.: 6.7nM, 16.7nM, 33.3nM) was added to 30µl of platelets and incubated for exact 2 minutes at room temperature. PGE_1 (f.c.: 33.3µM) was used as control for maximal possible VASP-P. Subsequently incubation with ADP (f.c.: 83.3µM), which was used as control because phosphorylation should be low after ADP activation in platelets, for a duration of 5 minutes started. Fixing of platelets was arranged at a final concentration of 1.6% formaldehyde solution for 15 minutes. Membranes of platelets were permeabilized by triton solution (f.c.: 0.02%). After centrifugation at 1700g for 10 minutes pellets were suspended in 70µl of PBS. There were two steps of labelling of platelets. First 5µl of pre-dilution of anti-phospho-VASP (Ser 239) (43µg/ml) were added to each sample and incubated for 45 minutes. Then there was another centrifugation for eliminating unbound antibody and subsequently platelets were re-suspended in 35µl of PBS. FITC labelled GAM antibody was added and incubated with platelets for 15 to 30 minutes in the dark. There were used 4µl of antibody solution per sample. Samples were transferred into FACS test tubes and diluted with 300µl of PBS. Finally measurement by flow cytometry was performed. Emitted light was collected in the fluorescence 1 (FL1) channel.

7.6.2 Quantification of P-selectin positive cells

P-selectin, stored in α-granules, is expressed on the surface of platelets after activation. Percentage of CD62 antibody positive platelets was determined.

First 300µl of GFP were incubated with HDL_2 and HDL_3 (see methods 6.2 native HDL and 6.3. modification of HDL) in Eppendorf tubes for 10 minutes at room temperature similar to quantification of VASP. Final concentrations were 100µg/ml for nHDL and 150µg/ml for hypochlorite modified HDL (hyp-OxHDL). Additionally two different trials with 2-ClHDA were carried out. For the first trial, 300µl of platelets were incubated with 2-ClHDA (f.c.: 0µM, 0.05µM, 0.1µM, 0.5µM, 1µM) in Eppendorf tubes for 10 minutes at room temperature. For the second one, 2-ClHDA (f.c.: 1.27nM, 7.6nM) was shortly incubated with $nHDL_2$ and $nHDL_3$ (f.c.: both 100µg/ml) in Eppendorf tubes and afterwards platelets were added and incubated for another 10 minutes at room temperature.

All tests were performed in Cellstar TC-plates (96well, V-shape, Greiner Bio-One). Procedure is equal for HDL_2 and HDL_3 as well as for 2-ClHDA. Subsequent incubation with different platelet agonists, such as ADP, collagen, or thrombin at different concentrations with 30µl of platelets started for 5 minutes at room temperature.

Different final concentrations:

ADP	1.7µM	4.2µM	8.3µM
Collagen	40µg/ml	50µg/ml	100µg/ml
Thrombin	6.7U/ml	16.7U/ml	33.3U/ml

Table 1: Final concentrations of platelet agonists

Fixing of platelets was arranged at a final concentration of 1.6% formaldehyde solution for 15 minutes. Then centrifugation at 1700g for 10 minutes was performed and pellets were suspended in 30µl of PBS. 2µl of CD62 antibody were added to each test and an incubation of one hour was performed. Samples were transferred into FACS test tubes and diluted with 300µl of PBS. The fluorescence was measured in FL2.

7.7 Determination of platelet aggregation using microplate reader

The principle of determination of platelet aggregation is based upon changes in light transmission. This light transmission increases with aggregation and was measured at a wavelength of 405nm. Light is sent through a microtiter well and the beam of light modified by platelet aggregation in the microplate well is measured by a detector.

Cellstar tissue culture plates (96well, flat bottom, Greiner Bio-One) were used. 2-ClHDA (f.c.: 1µM and 2µM) and ADP (f.c.: 50µM) as control were added to 50µl of GFP and aggregation was immediately measured at 37C°. There were forty cycles per twenty seconds (shaking for ten seconds, optical measurement).

7.8 Quantification of free amino groups

Changes in the number of free amino groups were quantified for further characterisation of nHDL and hyp-OxHDL. Determination was carried out with fluorescamin that reacts with free amino groups to a fluorescent product. For this determination fluorescamin was dissolved in acetone (540µM in acetone). HDL were diluted to a concentration of 500µg/ml, thereafter borate buffer was added at a ratio of 1: 7.5 (HDL : borate buffer). Afterwards fluorescamin was added and amounted to fourth of the final volume. After this, readings at 390/475nm were performed.

7.9 Quantification of chloramines

Since N-Cl derivatives oxidize the sulfhydryl group of TNB^{2-} to disulphide (DTNB), this method facilitates photometrical detection of chloramines. Concentrations of TNB^{2-} were determined in a photometer at a wavelength of 412nm and these concentrations were calculated via the molar extinction coefficient.

100mM DTNB [5.5'-Dithio-bis(2-nitrobenzoic acid)] were hydrolysed in 0.1M NaOH for 5 minutes leading to TNB^{2-} formation. After centrifugation TNB^{2-} (in the supernatant) was diluted with PBS at a ratio of 1:50. At this point first measurement was car-

ried out. The second measurement was conducted after addition of the sample (blank value against buffer). There was a surplus of TNB^{2-} admixed and the difference between primarily added TNB^{2-} and the portion remaining after oxidation with N-Cl was calculated. Hence it was possible to quantify the number of chloramines.

Formula for calculating chloramines per HDL:

$$\frac{(TNB^{2-}\text{ primarily} - TNB^{2-}\text{ after oxidation with N-Cl})}{2 * 13600 * \text{mol HDL added}}$$

7.10 2-Chlorohexadecanal

Synthesis was carried out at our laboratory and was based on Van den Bergen (Van den Bergen *et al.* 1999). 2-ClHDA was synthesized in two steps by chlorination of n-hexadecanal dimethylacetal.

2-ClHDA was dissolved in dimethyl sulfoxide (DMSO). 23mg were diluted in 1ml DMSO. Pre-dilutions of 2-ClHDA were made and amounted to 0.1µg/µl. Different final concentrations (0µM, 0.5µM, 1µM, 2µM, 5µM, 10µM, 20µM) were prepared and final concentrations of DMSO had to be below 0.5%, because DMSO itself possibly leads to aggregation defects in platelets (Cetin *et al.* 2001). DMSO and PBS were used as control.

8 Results

8.1 Experiments with native HDL_2 and HDL_3

8.1.1 Effects of $nHDL_2$ and $nHDL_3$ on VASP-P

Phosphorylation state of VASP closely correlates with the inhibition of platelets and is therefore used as indicator for inhibitability of platelets (5.2.3.1) within the following experiments. Impact of $nHDL_2$ and $nHDL_3$ on PGE_1-induced VASP-P in GFP was determined by FACS analysis.

The first set of experiments was performed to clarify how $nHDL_2$ affect VASP-P and whether these effects are dose-dependent, or not. GFP were treated with specified concentrations of $nHDL_2$ (50µg/ml, 100µg/ml, 150µg/ml) and VASP-P was induced by different PGE_1 concentrations (6.7nM, 16.7nM, 33.3nM).

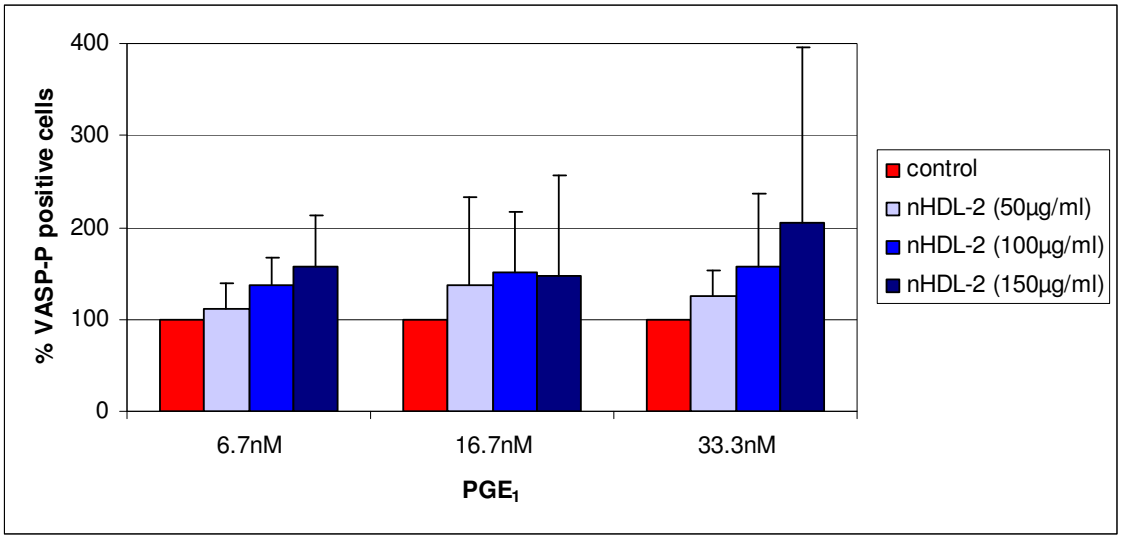

Figure 1: Effects of $nHDL_2$ (50µg/ml, 100µg/ml, 150µg/ml) on PGE_1-induced VASP-P in GFP (HDL from 4 donors, n=12).

	nHDL$_2$ (50µg/ml)	**nHDL$_2$** (100µg/ml)	**nHDL$_2$** (150µg/ml)
	Mean ± SD	*Mean ± SD*	*Mean ± SD*
6.7nM PGE_1	112.3 ± 26.4	137.2 ± 29.8	157.3 ± 55.8
16.7nM PGE_1	138.0 ± 94.8	150.3 ± 66.3	148.0 ± 109.6
33.3nM PGE_1	125.6 ± 28.5	157.8 ± 78.6	205.9 ± 190.7

Table 2: According to Figure 1 - mean values and standard deviations of results of PGE_1-induced VASP-P in GFP incubated with $nHDL_2$ (50µg/ml, 100µg/ml, 150µg/ml).

Figure 1 shows PGE_1-induced VASP-P in GFP without nHDL as 100%, which hence constitutes the control. Changes of VASP-P in GFP incubated with $nHDL_2$ are expressed in percent relative to control. Incubation of GFP with increasing concentrations of $nHDL_2$ leads to increasing VASP-P, which indicates their platelet-inhibiting properties. Dose-dependent effects of $nHDL_2$ on PGE_1-induced VASP-P are shown. Unfortunately standard deviations are quite high.

As for $nHDL_2$, effects of $nHDL_3$ on PGE_1-induced VASP-P were evaluated by quantifying VASP-P positive cells. GFP were treated with $nHDL_3$ at three different concentrations (50µg/ml, 100µg/ml, 150µg/ml) and VASP-P was induced by different concentrations of PGE_1 (6.7nM, 16.7nM, 33.3nM).

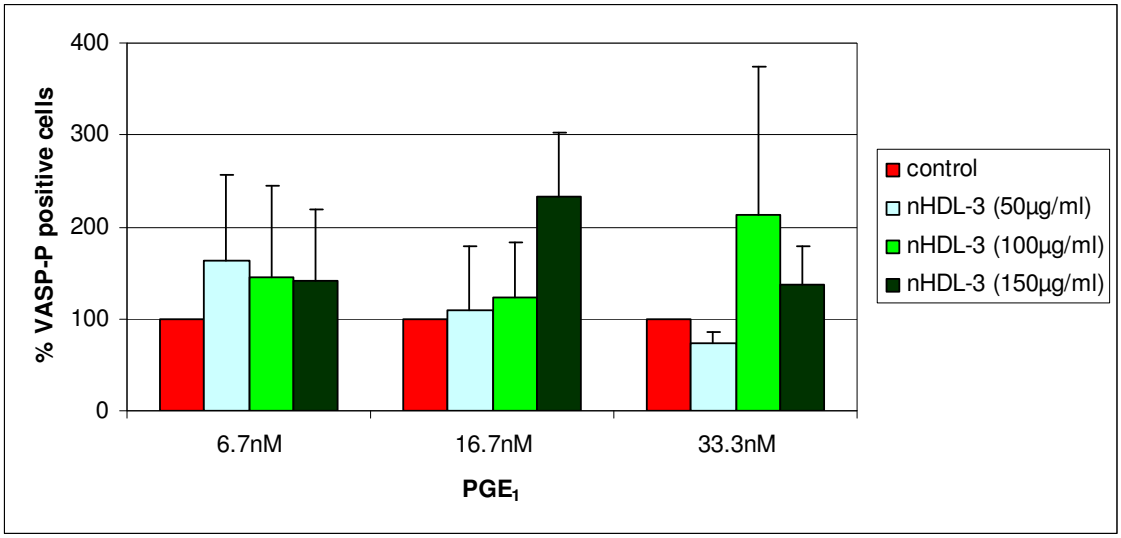

Figure 2: Effects of $nHDL_3$ (50µg/ml, 100µg/ml, 150µg/ml) on PGE_1-induced VASP-P in GFP (HDL from 4 donors, n=12).

	$nHDL_3$ (50µg/ml)	**$nHDL_3$** (100µg/ml)	**$nHDL_3$** (150µg/ml)
	Mean ± SD	*Mean ± SD*	*Mean ± SD*
6.7nM PGE_1	163.1 ± 94.0	145.3 ± 99.4	142.0 ± 77.8
16.7nM PGE_1	108.9 ± 71.0	123.7 ± 58.4	232.9 ± 70.5
33.3nM PGE_1	73.3 ± 12.9	212.3 ± 162.8	136.4 ± 42.6

Table 3: According to Figure 2 - mean values and standard deviations of results of PGE_1-induced VASP-P in GFP incubated with $nHDL_3$ (50µg/ml, 100µg/ml, 150µg/ml).

As shown in Figure 2, PGE_1-induced VASP-P in GFP without $nHDL_3$ is used as control and set 100%. VASP-P in GFP incubated with $nHDL_3$ is expressed in percent relative to control. $nHDL_3$ have the potential to increase VASP-P in GFP despite at a concentration of 50µg/ml at 33.3nM PGE_1, which is probably a maverick. This indicates platelet-inhibiting effects of $nHDL_3$. Moreover, a concentration dependency of $nHDL_3$ on 16.7nM PGE_1-induced VASP-P is shown. Due to high standard deviations a clear conclusion can not be made.

8.1.1.1 Calculation of effects of $nHDL_2$ and $nHDL_3$ (50µg/ml) on VASP-P

Obtained values from experiments, which indicated effects of $nHDL_2$ and $nHDL_3$ on PGE_1-induced VASP-P (Figure 1, Figure 2) were used as basis for calculation of influences of nHDL subclasses at a concentration of 50µg/ml on VASP-P induced by different concentrations of PGE_1. With these data a comparison between $nHDL_2$ and $nHDL_3$ is possible.

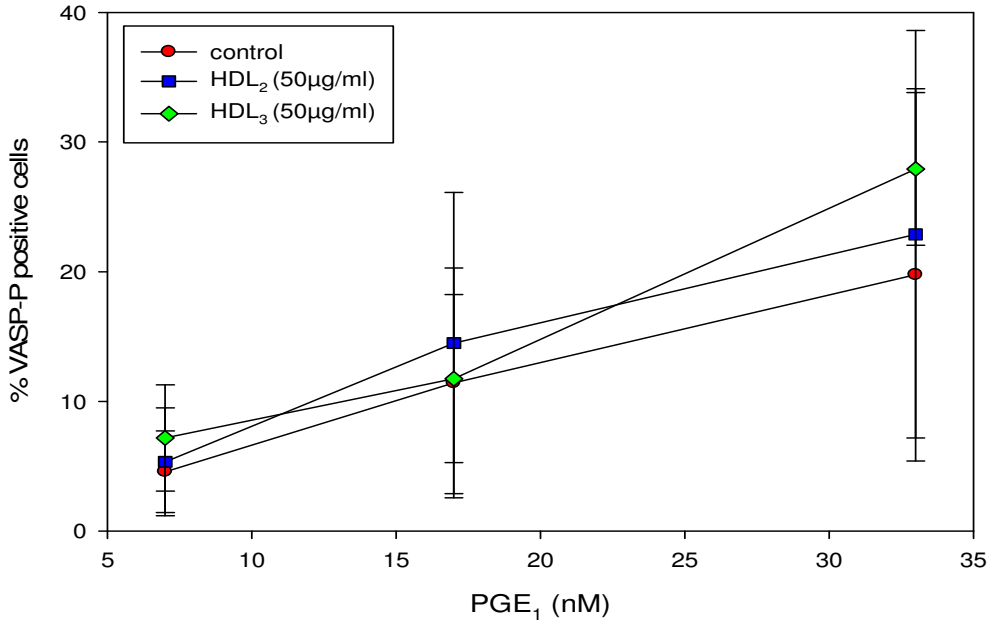

Figure 3: Effects of $nHDL_2$ and $nHDL_3$ (50µg/ml) on PGE_1-induced VASP-P in GFP (HDL from 4 donors, n=12).

	control	**$nHDL_2$** (50µg/ml)	**$nHDL_3$** (50µg/ml)
	Mean ± SD	*Mean ± SD*	*Mean ± SD*
6.7nM PGE_1	4.6 ± 3.1	5.3 ± 4.2	7.2 ± 4.1
16.7nM PGE_1	11.4 ± 8.9	14.5 ± 11.6	11.8 ± 6.5
33.3nM PGE_1	19.8 ± 14.3	22.9 ± 15.7	27.9 ± 5.9

Table 4: According to Figure 3 - mean values and standard deviations of results of PGE_1-induced VASP-P in GFP incubated with $nHDL_2$ and $nHDL_3$ (50µg/ml).

Figure 3 shows percentages of VASP-P positive cells. Both nHDL subclasses are associated with enhanced percentages of VASP-P positive cells compared to control. $nHDL_3$ increase VASP-P induced by 6.7nM and 33.3nM PGE_1 more effectively than $nHDL_2$, but at 16.7nM PGE_1 results are contrary. Differences between $nHDL_2$ and $nHDL_3$ are increasing with rising PGE_1 concentrations. Unfortunately results show no significance because of high standard deviations.

8.1.1.2 Effects of $nHDL_2$ and $nHDL_3$ (100µg/ml) on VASP-P

The following experiments were designed to show the effects of $nHDL_2$ and $nHDL_3$ at a concentration of 100µg/ml on PGE_1-induced VASP-P. GFP were treated with $nHDL_2$ and $nHDL_3$ (100µg/ml) and VASP-P was induced by different PGE_1 concentrations (6.7nM, 16.7nM, 33.3nM).

Figure 4 demonstrates a dot plot obtained by detecting VASP-P positive cells in PGE_1-stimulated GFP by means of flow cytometry.

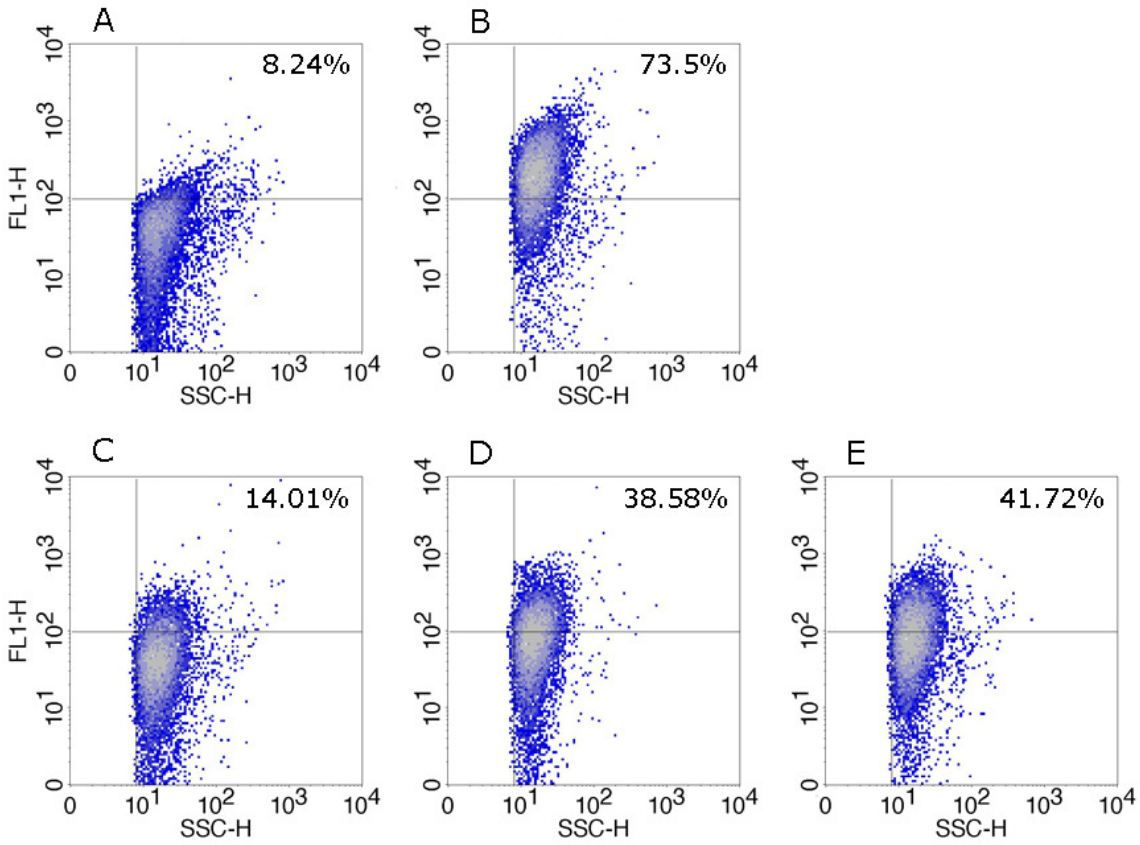

Figure 4: A typical result of an experiment. PGE_1-induced VASP-P in GFP incubated with $nHDL_2$ and $nHDL_3$ (100µg/ml).

A: basal

B: PGE_1 (33.3µM)

C: PGE_1 (33.3nM)

D: PGE_1 (33.3nM) + $nHDL_2$ (100µg/ml)

E: PGE_1 (33.3nM) + $nHDL_3$ (100µg/ml)

Percentages in the upper right indicate percentage of VASP-P positive cells.

As shown in Figure 4, $nHDL_2$ and $nHDL_3$ (D, E) show an enhancement in phosphorylation compared to GFP without nHDL (C). VASP-P of GFP without nHDL induced by a high concentration of PGE_1 is also shown (B). Furthermore, the result of this experiment demonstrates that $nHDL_3$ (E) are associated with a slightly higher impact on increasing VASP-P than $nHDL_2$ (D).

In Figure 5, results of 24 experiments are outlined to show significant data.

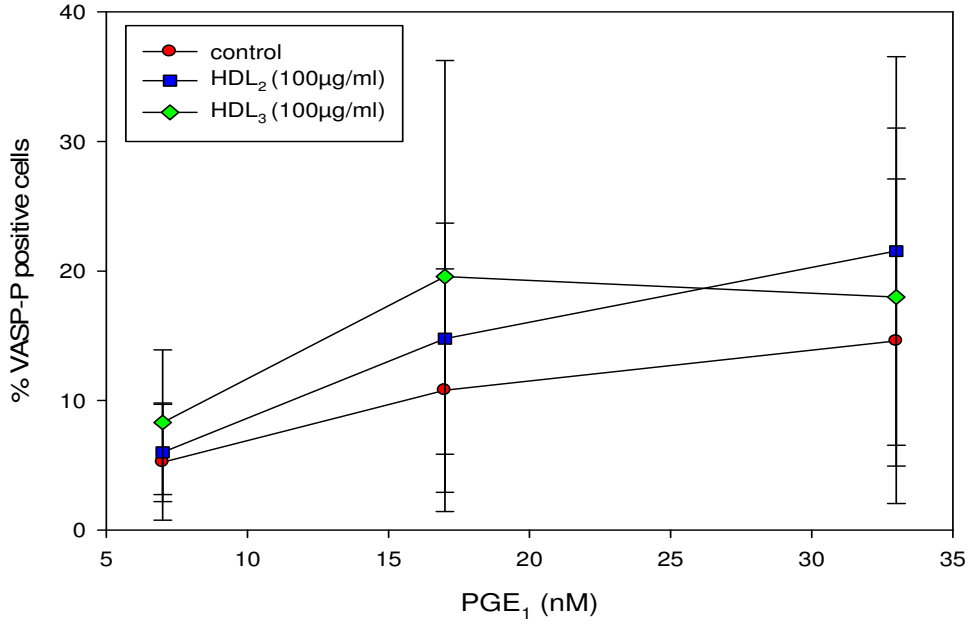

Figure 5: Effects of $nHDL_2$ and $nHDL_3$ (100µg/ml) on PGE_1-induced VASP-P in GFP (HDL from 11 donors, n=24).

	control	**$nHDL_2$** (100µg/ml)	**$nHDL_3$** (100µg/ml)
	Mean ± SD	*Mean ± SD*	*Mean ± SD*
6.7nM PGE_1	5.2 ± 4.5	6.0 ± 3.8	8.3 ± 5.6
16.7nM PGE_1	10.8 ± 9.4	14.8 ± 8.9	19.6 ± 16.7
33.3nM PGE_1	14.6 ± 12.5	21.5 ± 15.0	18.0 ± 13.0

Table 5: According to Figure 5 - mean values and standard deviations of results of PGE_1-induced VASP-P in GFP incubated with $nHDL_2$ and $nHDL_3$ (100µg/ml).

Figure 5 indicates VASP-P positive cells in percent. Both nHDL subclasses are able to increase PGE_1-induced VASP-P in GFP. $nHDL_3$ tend to show a higher percentage in VASP-P positive cells than $nHDL_2$ at concentrations of 6.7nM and 16.7nM PGE_1-induced VASP-P, but at 33.3nM PGE_1 results are inverse. Effects are not continuous throughout all concentrations of PGE_1. Because of high standard deviations, it is not possible to draw a clear conclusion.

8.1.1.3 Calculation of effects of nHDL$_2$ and nHDL$_3$ (150µg/ml) on VASP-P

Data of results of experiments dealing with the influence of nHDL$_2$ and nHDL$_3$ on PGE$_1$-induced VASP-P (Figure 1, Figure 2) were used as basis for calculation of effects of nHDL subclasses (150µg/ml) on VASP-P induced by different concentrations of PGE$_1$ to study differences between these nHDL subclasses.

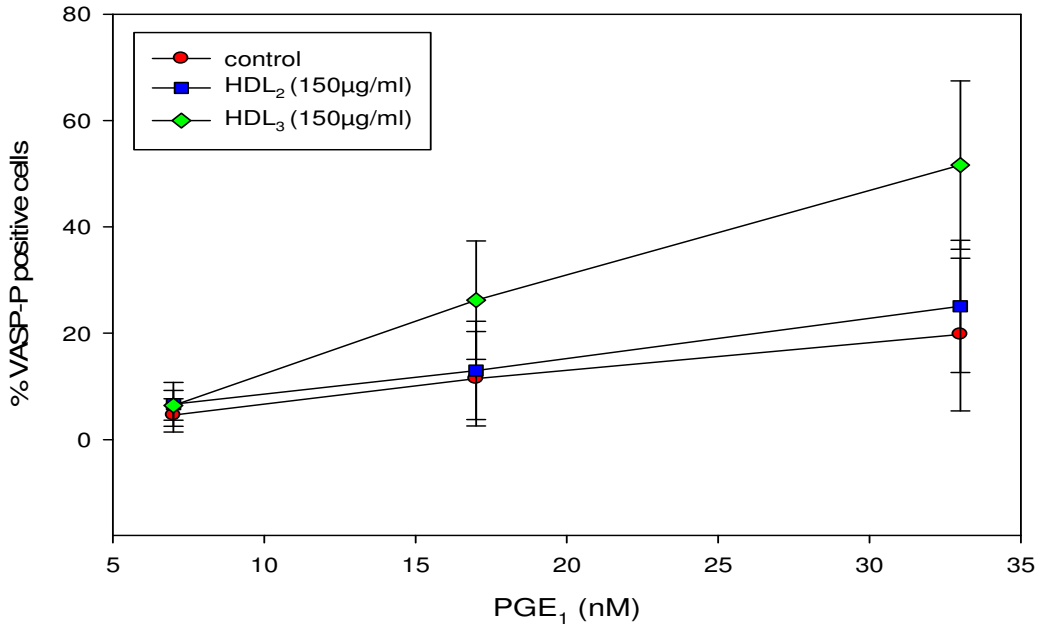

Figure 6: Effects of nHDL$_2$ and nHDL$_3$ (150µg/ml) on VASP-P in GFP induced by PGE$_1$ (HDL from 4 donors, n=12).

	control	**nHDL$_2$** (150µg/ml)	**nHDL$_3$** (150µg/ml)
	Mean ± SD	*Mean ± SD*	*Mean ± SD*
6.7nM PGE$_1$	4.6 ± 3.1	6.6 ± 4.1	6.4 ± 2.8
16.7nM PGE$_1$	11.4 ± 8.9	13.0 ± 9.2	26.2 ± 11.1
33.3nM PGE$_1$	19.8 ± 14.3	25.1 ± 12.4	51.6 ± 15.8

Table 6: According to Figure 6 - mean values and standard deviations of results of PGE$_1$-induced VASP-P in GFP incubated with nHDL$_2$ and nHDL$_3$ (150µg/ml).

Figure 6 demonstrates percentages of VASP-P positive cells. Both nHDL subclasses exhibit higher percentages of VASP-P positive cells than the control, but effects of $nHDL_3$ on PGE_1-induced VASP-P are more distinctive than effects of $nHDL_2$. Furthermore, the amount of these effects increases with enhanced concentrations of PGE_1 and also the differences between the nHDL subclasses get more intense with rising concentrations of PGE_1. Incubation of GFP with $nHDL_3$ leads to more potent effects on increasing PGE_1-induced VASP-P than $nHDL_2$ at least at 16.7 and 33.3nM PGE_1.

8.1.2 Influence of $nHDL_2$ and $nHDL_3$ on ADP-induced P-selectin surface expression

P-selectin is used as indicator for platelet degranulation and secretion. It is expressed on the surface of platelets after their activation, which can be induced by different agonists like ADP, thrombin, or collagen.

The following experiments were planned to investigate the influence of $nHDL_2$ and $nHDL_3$ on ADP-induced P-selectin expression. GFP were incubated with $nHDL_2$ and $nHDL_3$ (100µg/ml) and platelets were stimulated by different concentrations of ADP (1.7µM, 4.2µM, 8.3µM).

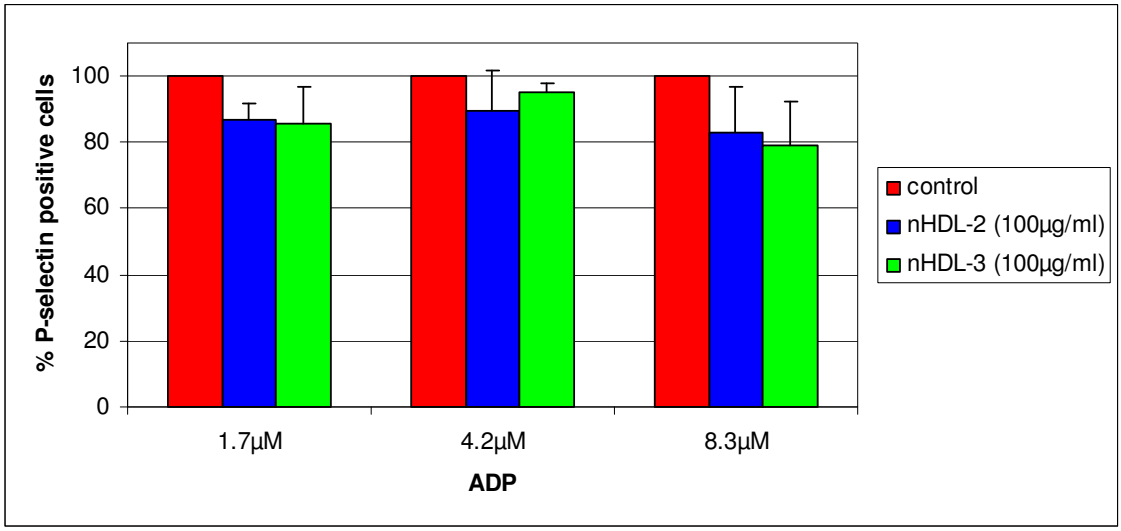

Figure 7: **Influence of $nHDL_2$ and $nHDL_3$ (100µg/ml) on ADP-induced expression of P-selectin in GFP (HDL from 17 donors, n=23).**

	$nHDL_2$ (100µg/ml)	**$nHDL_3$** (100µg/ml)
	Mean ± SD	*Mean ± SD*
1.7µM ADP	87.0 ± 5.0	85.9 ± 10.7
4.2µM ADP	89.4 ± 12.5	95.2 ± 2.6
8.3µM ADP	82.6 ± 14.3	79.0 ± 13.3

Table 7: According to Figure 7 - mean values and standard deviations of results of ADP-induced expression of P-selectin in GFP incubated with $nHDL_2$ and $nHDL_3$ (100µg/ml).

Figure 7 shows ADP-induced P-selectin expression in GFP without any nHDL as 100%. The amount of P-selectin expression in GFP with different HDL subclasses is shown in relation to control. $nHDL_2$ and $nHDL_3$ impair ADP-induced P-selectin expression in GFP, but there seems to be no definite finding whether $nHDL_2$ or $nHDL_3$ have more inhibiting effects on ADP-induced platelet degranulation of P-selectin in GFP. Results do not differ much and additionally standard deviations are quite high.

8.1.3 Influence of $nHDL_2$ and $nHDL_3$ on thrombin-induced P-selectin surface expression

The aim of these experiments was to gather information about the effects of $nHDL_2$ and $nHDL_3$ on thrombin-induced P-selectin expression. Experiments were arranged similar to trials dealing with the influence of nHDL subclasses on ADP-induced P-selectin expression in GFP. GFP were incubated with either $nHDL_2$ or $nHDL_3$ (100µg/ml) and platelet activation was induced by different concentrations of thrombin (6.7U/ml, 16.7U/ml, 33.3U/ml).

Figure 8 demonstrates a dot plot obtained by detecting P-selectin positive cells in thrombin-stimulated GFP by means of flow cytometry.

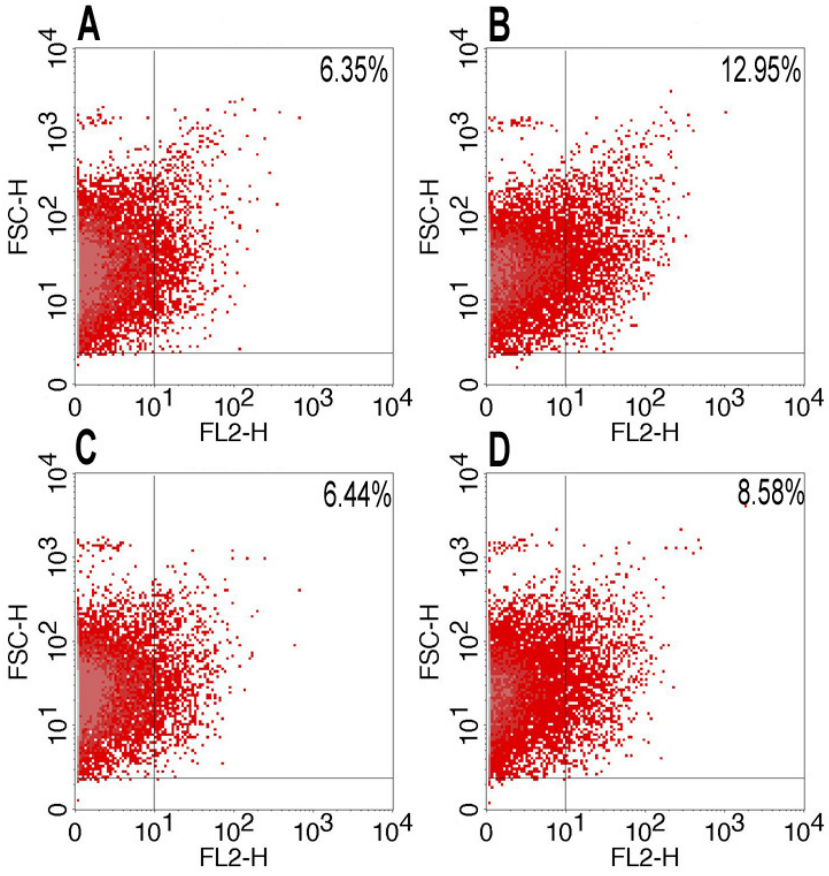

Figure 8: Result of an experiment. Thrombin-induced expression of P-selectin in GFP incubated with $nHDL_2$ and $nHDL_3$ (100µg/ml).

A: basal

B: thrombin (16.7U/ml)

C: thrombin (16.7U/ml) + $nHDL_2$ (100µg/ml)

D: thrombin (16.7U/ml) + $nHDL_3$ (100µg/ml)

Percentages in the upper right indicate percentage of P-selectin positive cells.

The result of an experiment, where the platelet response was quite prominent, is shown in Figure 8. Platelet response is dependent on activatability of the platelet itself and thus the experiments can differ in their magnitude of reaction. Amount of P-selectin positive cells in thrombin-stimulated GFP differs due to varying constituents added. Both nHDL subclasses impair thrombin-induced P-selectin expression (C, D) and it is shown that $nHDL_2$ (C) inhibit this P-selectin expression more potent than $nHDL_3$ (D). This is evident by comparing $nHDL_2$ (C) and $nHDL_3$ (D) with GFP without any nHDL (B).

Data of results of 30 experiments are outlined in Figure 9. It shows the effects of $nHDL_2$ and $nHDL_3$ on thrombin-induced P-selectin expression in GFP.

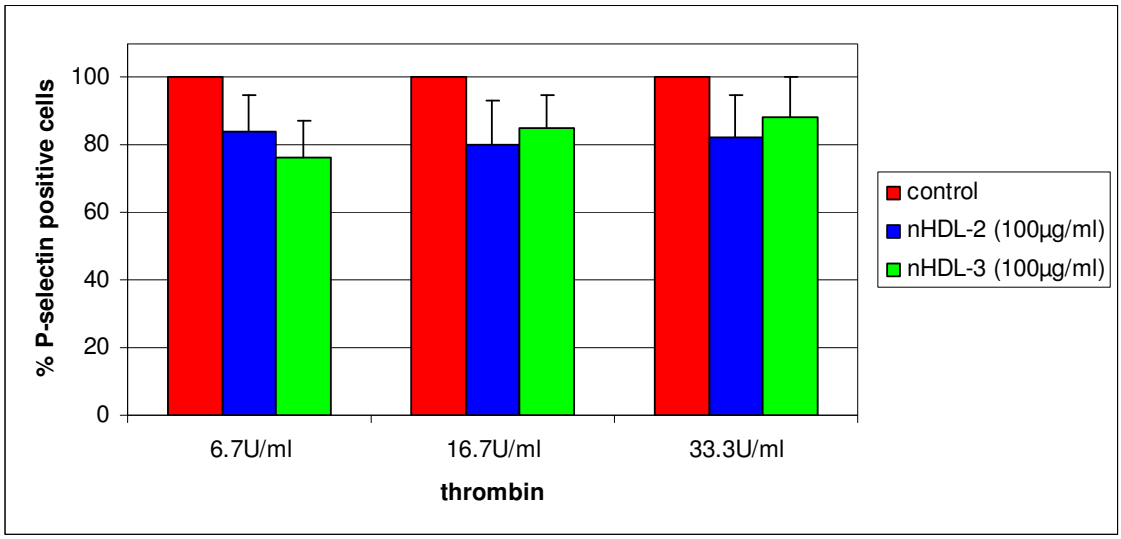

Figure 9: Influence of $nHDL_2$ and $nHDL_3$ (100µg/ml) on thrombin-induced expression of P-selectin in GFP (HDL from 19 donors, n=30).

	nHDL$_2$ (100µg/ml)	**nHDL$_3$** (100µg/ml)
	Mean ± SD	*Mean ± SD*
6.7U/ml thrombin	84.0 ± 10.7	76.2 ± 11.2
16.7U/ml thrombin	80.3 ± 12.9	85.2 ± 9.8
33.3U/ml thrombin	82.2 ± 12.6	88.5 ± 11.8

Table 8: According to Figure 9 - mean values and standard deviations of results of thrombin-induced expression of P-selectin in GFP incubated with nHDL$_2$ and nHDL$_3$ (100µg/ml).

In Figure 9, thrombin-induced expression of P-selectin in GFP without any nHDL is shown as control and set 100%. Changes of P-selectin expression in GFP with different nHDL subclasses are shown in relation to control. Both subclasses exhibit inhibiting effects on thrombin-induced P-selectin expression. nHDL$_2$ appear to have a stronger impact on decreasing P-selectin expression induced by 16.7U/ml and 33.3U/ml thrombin than nHDL$_3$. At a concentration of 6.7U/ml thrombin, nHDL$_3$ seem to inhibit thrombin-induced platelet activation more effectively than nHDL$_2$. Findings differ within diverse concentrations of thrombin.

8.1.4 Influence of nHDL$_2$ and nHDL$_3$ on collagen-induced P-selectin surface expression

The effects of nHDL$_2$ and nHDL$_3$ on collagen-induced P-selectin expression were examined within several experiments. An incubation of GFP with either nHDL$_2$ or nHDL$_3$ (100µg/ml) was performed. Subsequently P-selectin expression in GFP was induced by different concentrations of collagen (40µg/ml, 50µg/ml, 100µg/ml).

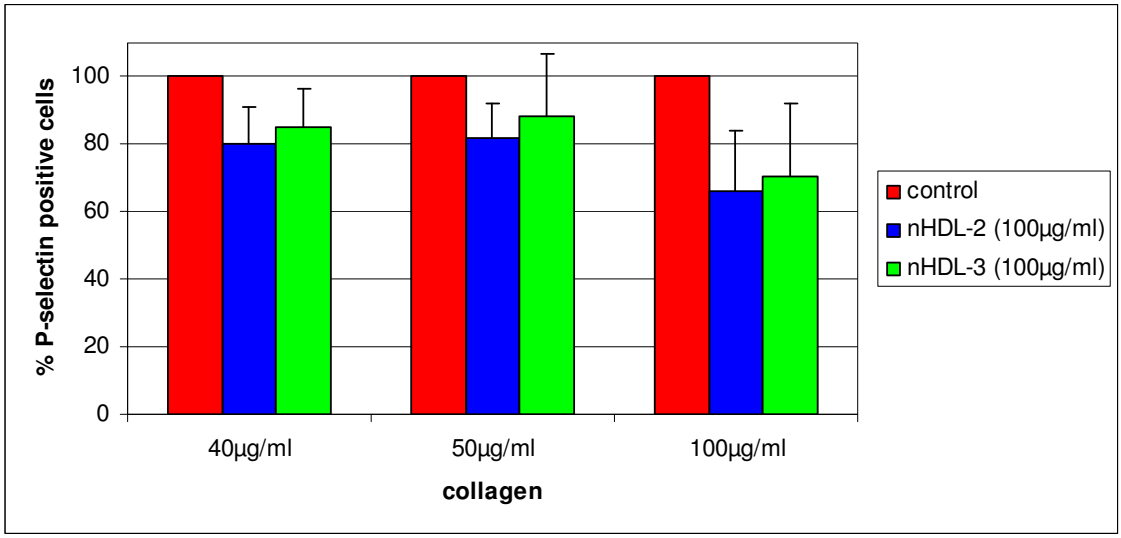

Figure 10: Influence of $nHDL_2$ and $nHDL_3$ (100µg/ml) on collagen-induced expression of P-selectin in GFP (HDL from 9 donors, n=15).

	$nHDL_2$ (100µg/ml)	**$nHDL_3$** (100µg/ml)
	Mean ± SD	*Mean ± SD*
40µg/ml collagen	80.4 ± 10.5	85.2 ± 11.2
50µg/ml collagen	81.6 ± 10.4	88.2 ± 18.3
100µg/ml collagen	65.9 ± 18.2	70.2 ± 21.9

Table 9: According to Figure 10 - mean values and standard deviations of results of collagen-induced expression of P-selectin in GFP incubated with $nHDL_2$ and $nHDL_3$ (100µg/ml).

As indicated in Figure 10, collagen-induced expression of P-selectin in GFP without any nHDL is again set 100%. Collagen-induced P-selectin expression in GFP in the presence of different HDL subclasses is shown in relation to control and was quantified to determine the degree of platelet activation. Both nHDL subclasses exhibit inhibiting properties on platelet activation. GFP incubated with $nHDL_2$ consistently impair collagen-induced P-selectin expression more effectively than GFP incubated with $nHDL_3$. During this study, the only platelet agonist that yielded continuous similar results at all concentrations added, was collagen.

8.2 Analytics of native and hypochlorite modified HDL_2 and HDL_3

8.2.1 Quantification of free amino groups

Number of free amino groups was determined by measurement of fluorescence of fluorescamin (7.8) to identify differences between native and hypochlorite modified HDL and to further characterize their subclasses.

We employed two strategies with regard to degree of hypochlorite modification of HDL. Within the first strategy, HDL_2 and HDL_3 were oxidized maximal (7.3), which implies that hypochlorite was added until optically evident bleaching of lipoproteins. This bleaching represents the disappearing of antioxidants in lipoproteins and the amount of antioxidants in the lipid phase of lipoproteins can vary. So the amount of hypochlorite added and therefore the modification of lipoproteins could differ from each other.

Within the second strategy, HDL_2 and HDL_3 were adjusted to identical protein concentrations. This adjustment was performed by addition of PBS. So HDL_2 and HDL_3 of the same donor were modified by the same excess of hypochlorite per mg protein. Hyp-OxHDL emerging from both hypochlorite modification strategies were used in experiments at a concentration of 100µg/ml.

	Mean ± SD
$nHDL_2$	100%
$nHDL_3$	100%
hyp-$OxHDL_2$	13.5% ± 5.4
hyp-$OxHDL_3$	12.5% ± 5.9
hyp-$OxHDL_2$ (ident. hyp.)	8.7% ± 1.1
hyp-$OxHDL_3$ (ident. hyp.)	3.6% ± 1.6

Table 10: Number of free amino groups in nHDL and hyp-OxHDL (HDL from 5 donors, n=7).

As shown in Table 10, nHDL are set 100% to see the decline in the number of free amino groups in hyp-OxHDL in comparison to nHDL. Number of free amino groups in hyp-OxHDL subclasses is much lower than in nHDL subclasses because of hypochlo-

rite modification. Number of free amino groups is almost the same in hyp-OxHDL$_2$ and in hyp-OxHDL$_3$. Moreover, results from experiments with hyp-OxHDL modified by identical excess of hypochlorite show that number of free amino groups in hyp-OxHDL$_2$ is again higher than in hyp-OxHDL$_3$.

8.2.2 Quantification of chloramines

Chloramines emerge from reaction of chlorine with amino groups. Differences between native and hypochlorite modified HDL$_2$ and HDL$_3$ were evaluated by photometrical detection of chloramines (7.9).

	HDL$_2$	**HDL$_3$**
	Mean ± SD	*Mean ± SD*
hyp-OxHDL	29.6 ± 6.5	35.3 ± 7.3
nHDL	0	0

Table 11: Content of chloramines per HDL (HDL from 5 donors, n=7).

Table 11 shows content of chloramines per nHDL and per hyp-OxHDL subclasses. Increase of chloramines in hyp-OxHDL$_3$ is higher than in hyp-OxHDL$_2$. Trials were also carried out with hyp-OxHDL (ident. hyp.) as used in 8.2.1 and show similar results (data not shown) as for hyp-OxHDL. Findings indicate an obvious increase of chloramines in hyp-OxHDL compared to nHDL.

8.3 Experiments with hypochlorite modified HDL$_2$ and HDL$_3$

Due to oxidative modification of lipoproteins in the body it is regarded as important to investigate the influences of modified HDL on platelet function. It is established that oxidized LDL show platelet-activating properties. Hypochlorite as a potent in vivo oxidant (5.3.8.1) was used for modification of HDL. ADP-, thrombin-, and collagen-induced P-selectin surface expression was quantified to determine level of platelet activation and further to verify loss of inhibiting functions of HDL in the course of oxida-

tive modification by hypochlorite (Assinger *et al.* 2008). Inhibiting properties of nHDL on platelet degranulation of P-selectin are shown in 8.1.2, 8.1.3 and 8.1.4.

8.3.1 Influence of hyp-OxHDL$_2$ and hyp-OxHDL$_3$ on ADP-induced expression of P-selectin

This set of experiments was carried out to detect differences in hypochlorite modified HDL subclasses with regard to their effects on ADP-induced P-selectin expression in GFP. Results of experiments with nHDL$_2$ and nHDL$_3$ show inhibiting effects on ADP-induced P-selectin expression (8.1.2) but reveal no great varieties between these subclasses. It is of importance to evaluate if results obtained by trials with nHDL are similar to results gained by hypochlorite modified HDL subclasses concerning their influences on ADP-induced P-selectin expression. Hyp-OxHDL$_2$ and hyp-OxHDL$_3$ (100µg/ml) were incubated with GFP and P-selectin expression was induced by different concentrations of ADP (1.7µM, 4.2µM, 8.3µM).

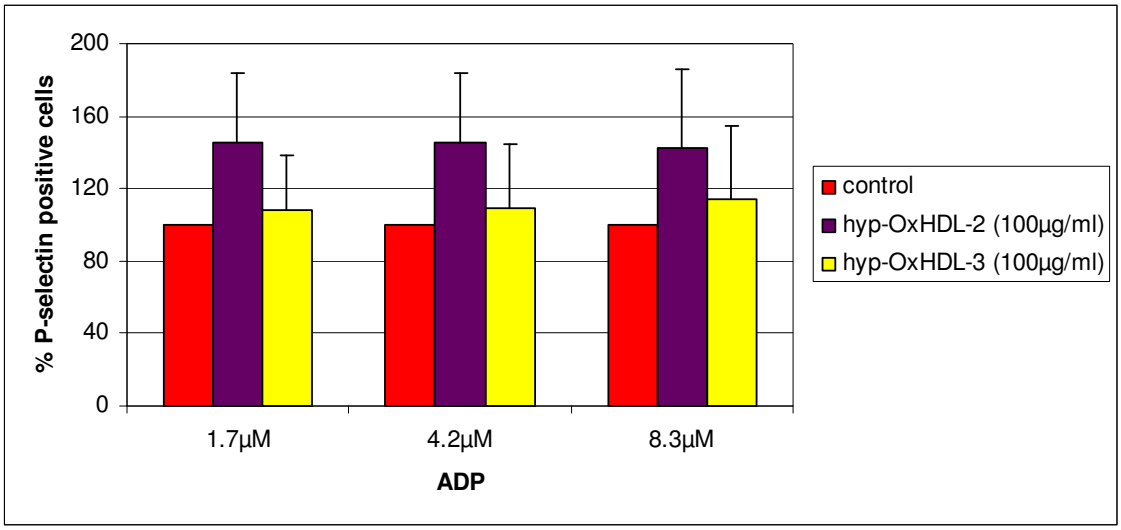

Figure 11: Influence of hyp-OxHDL$_2$ and hyp-OxHDL$_3$ (100µg/ml) on ADP-induced P-selectin expression in GFP (HDL from 9 donors, n=16).

	hyp-OxHDL$_2$ (100µg/ml)	**hyp-OxHDL$_3$** (100µg/ml)
	Mean ± SD	*Mean ± SD*
1.7µM ADP	145.8 ± 38.1	108.4 ± 30.3
4.2µM ADP	145.4 ± 38.7	109.2 ± 34.9
8.3µM ADP	142.8 ± 43.5	114.3 ± 39.8

Table 12: According to Figure 11 - mean values and standard deviations of results of ADP-induced expression of P-selectin in GFP incubated with hyp-OxHDL$_2$ and hyp-OxHDL$_3$ (100µg/ml).

Figure 11 shows ADP-induced P-selectin surface expression in GFP without any HDL as control and is set 100%. ADP-induced P-selectin expression in GFP incubated with hyp-OxHDL$_2$ or hyp-OxHDL$_3$ is expressed relative to control. Both hyp-OxHDL subclasses demonstrate platelet-activating effects. Hyp-OxHDL$_2$ indicate stronger inducing effects on platelet activation than hyp-OxHDL$_3$. The loss of inhibiting properties of hyp-OxHDL$_2$ and hyp-OxHDL$_3$ in contrast to nHDL$_2$ and nHDL$_3$ on platelet activation could be detected.

Two strategies of hypochlorite modification of HDL, which were already used in 8.2.1 and 8.2.2, were also adopted to the following experiments. These trials (Figure 12) were arranged with hyp-OxHDL subclasses (ident. hyp.), which were incubated with GFP and platelet activation was induced by different concentrations of ADP (1.7µM, 4.2µM, 8.3µM) to determine their influences on P-selectin expression.

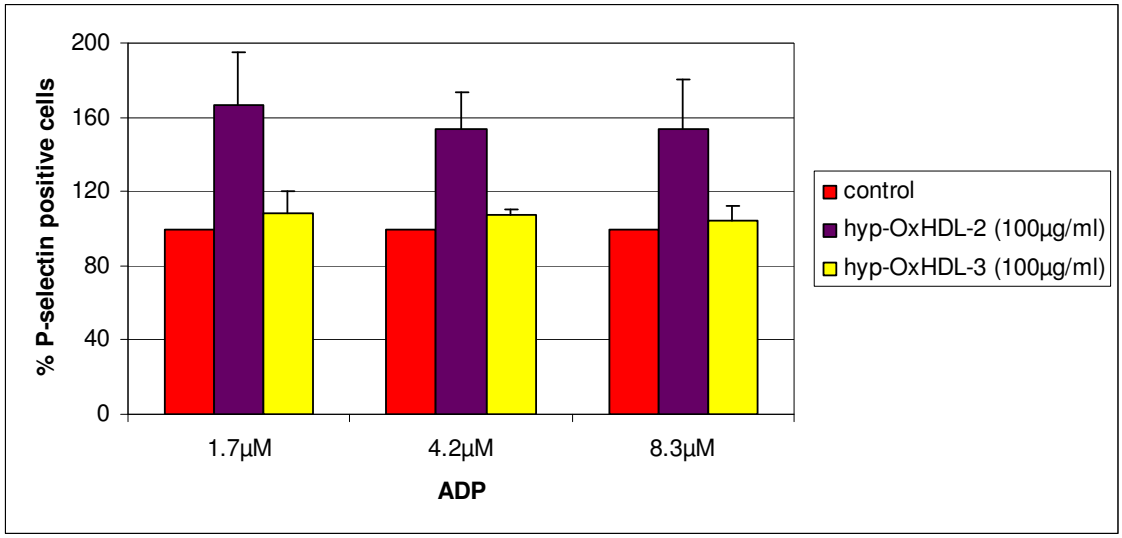

Figure 12: Influence of hyp-OxHDL$_2$ and hyp-OxHDL$_3$ (ident. hyp.) on ADP-induced P-selectin expression in GFP (HDL from 3 donors, n=3).

	hyp-OxHDL$_2$ (100µg/ml)	**hyp-OxHDL$_3$** (100µg/ml)
	Mean ± SD	*Mean ± SD*
1.7µM ADP	166.9 ± 28.1	108.5 ± 12.1
4.2µM ADP	154.1 ± 19.6	107.2 ± 2.8
8.3µM ADP	153.3 ± 26.9	104.8 ± 7.6

Table 13: According to Figure 12 - mean values and standard deviations of results of ADP-induced expression in GFP incubated with hyp-OxHDL$_2$ and hyp-OxHDL$_3$ (ident. hyp.).

Figure 12 indicates GFP without hyp-OxHDL (ident. hyp.) as 100%, which represent the control. GFP incubated with either hyp-OxHDL$_2$ or hyp-OxHDL$_3$ (ident. hyp.) are shown relative to control. nHDL acquire platelet-activating properties through oxidation process indicated by higher percentages of P-selectin positive cells in GFP incubated with hyp-OxHDL$_2$ and hyp-OxHDL$_3$ (ident. hyp.) compared to control. Hyp-OxHDL$_2$ (ident. hyp.) augment ADP-induced P-selectin expression more effectively than hyp-OxHDL$_3$ (ident. hyp.).

Figure 11 shows effects of hyp-OxHDL subclasses oxidized by the first strategy of hypochlorite modification that are comparable to effects of hyp-OxHDL subclasses (ident.

hyp.) from the second strategy (Figure 12) on ADP-induced P-selectin expression in GFP. Hyp-$OxHDL_2$ again increase ADP-induced platelet activation more effectively than hyp-$OxHDL_3$. Data are consistent at all three concentrations of ADP.

8.3.2 Influence of hyp-$OxHDL_2$ and hyp-$OxHDL_3$ on thrombin-induced expression of P-selectin

Experiments were aimed at investigating differences between the subclasses hyp-$OxHDL_2$ and hyp-$OxHDL_3$ in their effects on thrombin-induced P-selectin expression in GFP. GFP were provided with either hyp-$OxHDL_2$ or hyp-$OxHDL_3$ (100µg/ml) and platelet activation was induced by thrombin (6.7U/ml, 16.7U/ml, 33.3U/ml).

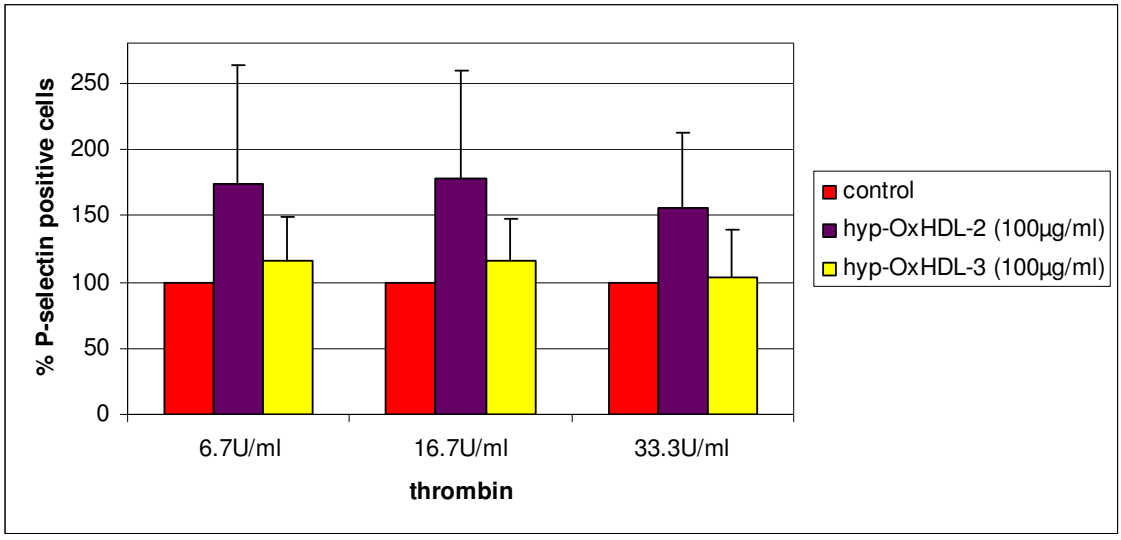

Figure 13: Influence of hyp-$OxHDL_2$ and hyp-$OxHDL_3$ (100µg/ml) on thrombin-induced P-selectin expression in GFP (HDL from 6 donors, n=11).

	hyp-OxHDL$_2$ (100µg/ml)	**hyp-OxHDL$_3$** (100µg/ml)
	Mean ± SD	*Mean ± SD*
6.7U/ml thrombin	174.4 ± 89.7	115.4 ± 33.4
16.7U/ml thrombin	178.2 ± 81.7	116.2 ± 32.0
33.3U/ml thrombin	156.2 ± 56.6	103.4 ± 35.7

Table 14: According to Figure 13- mean values and standard deviations of results of thrombin-induced expression in GFP incubated with hyp-OxHDL$_2$ and hyp-OxHDL$_3$ (100µg/ml).

As shown in Figure 13, GFP without hyp-OxHDL represent the control and therefore are set 100%. Thrombin-induced P-selectin surface expression in GFP coincubated with different hyp-OxHDL subclasses is shown in relation to control. Hyp-OxHDL$_2$ and hyp-OxHDL$_3$ exhibit platelet-activating properties indicated by higher percentages of P-selectin positive cells compared to control. Platelet-activating effects are again more pronounced with hyp-OxHDL$_2$. These findings were already identified by experiments dealing with the influence of hyp-OxHDL subclasses on ADP-induced expression of P-selectin.

8.3.3 Influence of hyp-OxHDL$_2$ and hyp-OxHDL$_3$ on collagen-induced expression of P-selectin

To determine differences in hyp-OxHDL subclasses, hyp-OxHDL$_2$ and hyp-OxHDL$_3$ were incubated with GFP and platelet activation was induced by different agonists to investigate the effects of hyp-OxHDL subclasses on ADP,- or thrombin-induced P-selectin expression (ADP: Figure 11 and Figure 12, thrombin: Figure 13). This time stimulation of GFP was arranged with collagen (40µg/ml, 50µg/ml, 100µg/ml) to evaluate influences of hyp-OxHDL subclasses (100µg/ml) on collagen-induced degranulation of P-selectin.

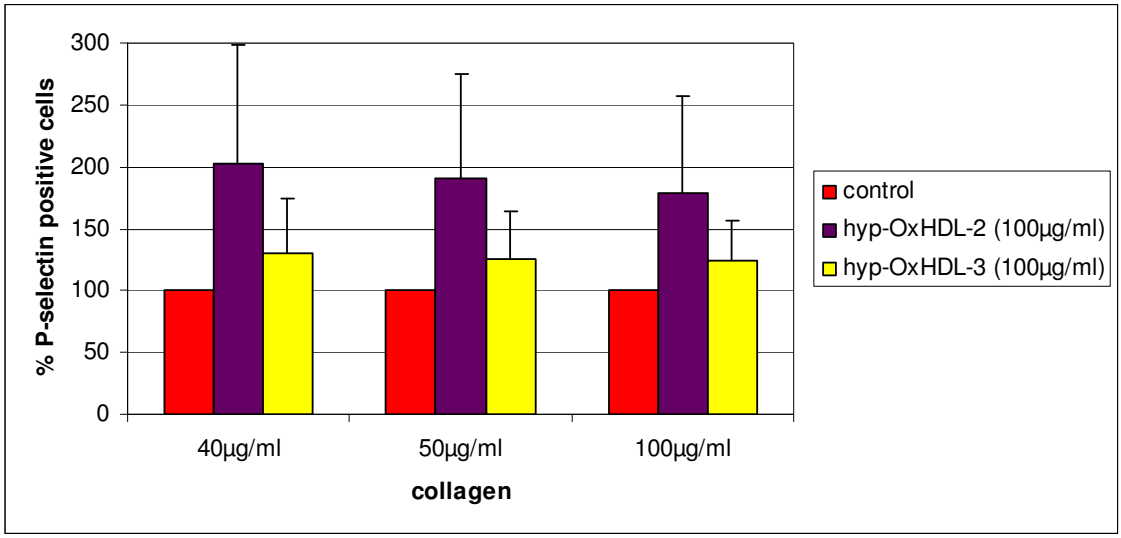

Figure 14: Influence of hyp-OxHDL$_2$ and hyp-OxHDL$_3$ (100µg/ml) on collagen-induced P-selectin expression in GFP (HDL from 6 donors, n=13).

	hyp-OxHDL$_2$ (100µg/ml)	**hyp-OxHDL$_3$** (100µg/ml)
	Mean ± SD	*Mean ± SD*
40µg/ml collagen	202.1 ± 96.2	129.7 ± 44.8
50µg/ml collagen	190.5 ± 84.5	126.1 ± 38.2
100µg/ml collagen	178.7 ± 77.9	123.6 ± 33.1

Table 15: According to Figure 14- mean values and standard deviations of results of collagen-induced expression in GFP incubated with hyp-OxHDL$_2$ and hyp-OxHDL$_3$ (100µg/ml).

As shown in Figure 14, collagen-induced P-selectin expression in GFP without any hyp-OxHDL is used as control and is set 100%. Percentages of P-selectin positive cells in GFP incubated with different hyp-OxHDL subclasses are shown relative to control. Hyp-OxHDL$_2$ as well as hyp-OxHDL$_3$ reveal platelet-activating characteristics. Moreover, hyp-OxHDL$_2$ show more potent activating effects on collagen-induced degranulation of P-selectin than hyp-OxHDL$_3$, which was also shown within experiments dealing with the influence of hyp-OxHDL subclasses on ADP- and thrombin-induced expression of P-selectin in GFP.

8.3.4 Different degrees of modification of hyp-OxHDL and their effects on VASP-P

The following experiments were intended to evaluate the effects of nHDL and of HDL modified by hypochlorite to different degrees on PGE_1-induced VASP-P in GFP. HDL were modified by hypochlorite to different degrees (7.3) and furthermore, max-OxHDL were treated with methionine (7.3) to obtain red-OxHDL. This methionine treatment results in elimination of lipoprotein associated chloramines and recovery of free amino groups. It is of interest, if biological effects of hyp-OxHDL can be reversed by elimination of chloramines. GFP were provided either with nHDL (100µg/ml) or with HDL modified by hypochlorite to different degrees and VASP-P was induced by PGE_1 (6.7nM, 16.7nM, 33.3nM).

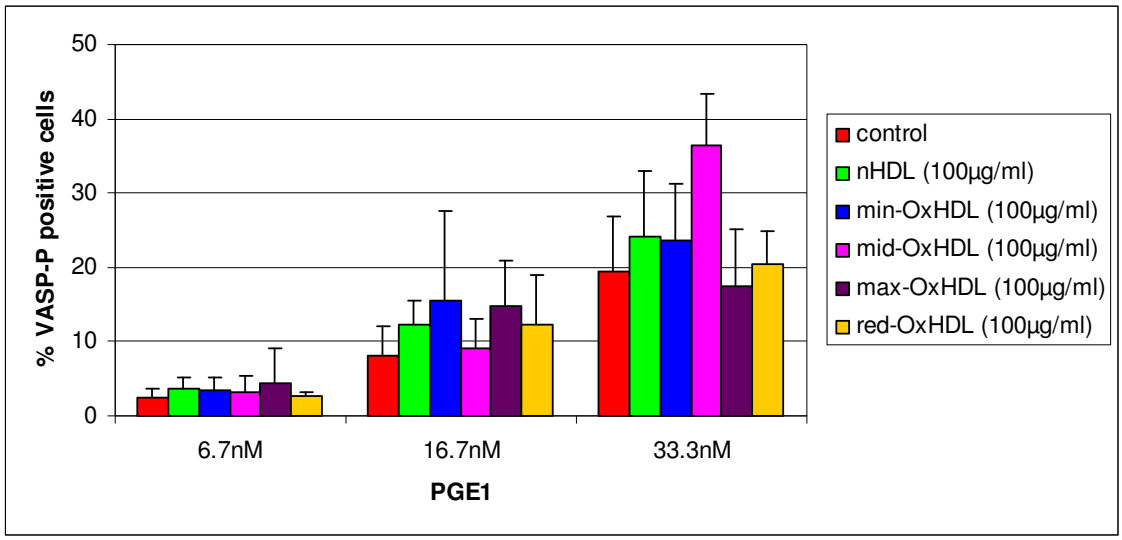

Figure 15: Influence of HDL modified by hypochlorite to different degrees on VASP-P (HDL from 3 donors, n=6).

	control	nHDL	min-OxHDL
	Mean ± SD	*Mean ± SD*	*Mean ± SD*
6.7nM PGE_1	2.6 ± 1.0	3.7 ± 1.6	3.5 ± 1.6
16.7nM PGE_1	8.1 ± 4.0	12.3 ± 3.3	15.6 ± 11.9
33.3nM PGE_1	19.5 ± 7.5	24.1 ± 8.8	23.5 ± 7.7
	mid-OxHDL	**max-OxHDL**	**red-OxHDL**
	Mean ± SD	*Mean ± SD*	*Mean ± SD*
6.7nM PGE_1	3.2 ± 2.1	4.5 ± 4.8	2.7 ± 0.6
16.7nM PGE_1	9.1 ± 4.0	14.8 ± 6.1	12.3 ± 6.6
33.3nM PGE_1	36.4 ± 6.9	17.4 ± 7.7	20.5 ± 4.5

Table 16: According to Figure 15 - mean values and standard deviations of results of PGE_1-induced VASP-P in GFP without hyp-OxHDL (control), incubated with nHDL (100µg/ml), min-OxHDL (100µg/ml), mid-OxHDL (100µg/ml), max-OxHDL (100µg/ml) and red-OxHDL (100µg/ml).

Figure 15 shows influences of HDL modified by hypochlorite to different degrees, nHDL and red-OxHDL on PGE_1-induced VASP-P in GFP. PGE_1-induced VASP-P in GFP without any HDL is used as control. It is not possible to find a correlation between level of hypochlorite modification of HDL and percentage of VASP-P and additionally no regularity within the different modification classes is identifiable. nHDL are supposed to show the highest percentage of VASP-P positive cells and max-OxHDL the lowest percentage of VASP-P positive cells. Nevertheless, max-OxHDL do not indicate significant effects and so one can not made significant conclusions about red-OxHDL, which was supposed to show a percentage of VASP-P positive cells as high as nHDL.

8.3.5 Influence of 2-Chlorohexadecanal on platelet function

2-ClHDA is an oxidation product of HDL associated plasmalogen and the oxidative cleavage of HDL is mediated by HOCl. A study shows that 2-ClHDA is able to decrease NO production in endothelial cells (Marsche *et al.* 2004) and another study demonstrates that 2-ClHDA takes part in neutrophil recruitment (Thukkani *et al.* 2002).

Due to its proven effects in endothelial cells we wanted to investigate the impact of 2-ClHDA on platelet function.

8.3.5.1 Influence of 2-ClHDA on platelet aggregation

Effects of 2-ClHDA on platelet aggregation using microplate reader were determined. We wanted to investigate if 2-ClHDA itself is able to trigger platelet aggregation and at what concentration, and how 2-ClHDA influences platelet aggregation in combination with the platelet agonist ADP. 2-ClHDA was added to GFP in two different concentrations (1µM, 2µM). GFP alone were used as control.

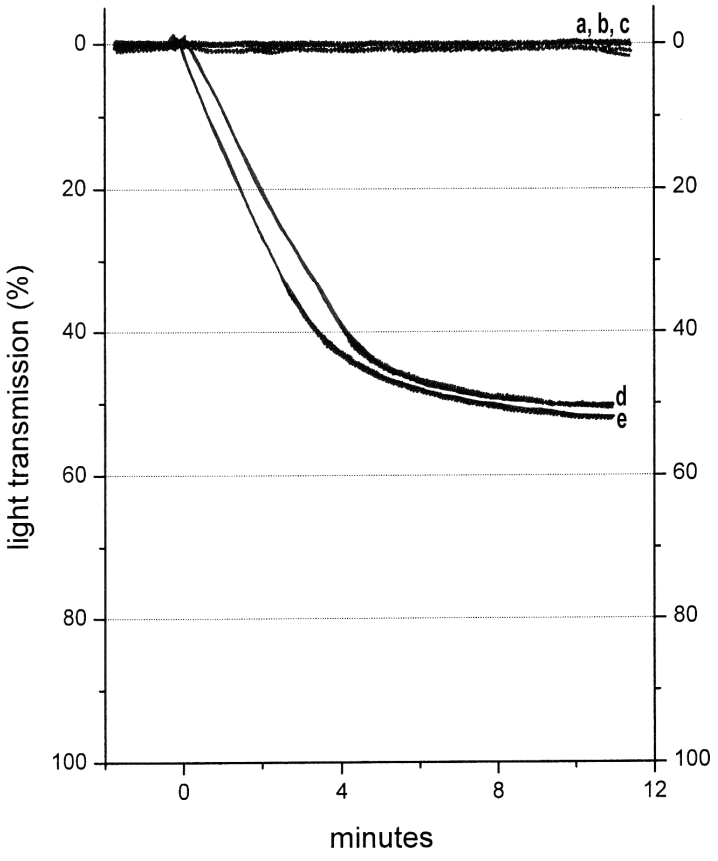

Figure 16: Influence of 2-ClHDA (1µM, 2µM) on platelet aggregation induced by ADP.

a: control

b: 2-ClHDA (f.c.: 1µM)

c: 2-ClHDA (f.c.: 2µM)

d: 2-ClHDA (f.c.: 1µM) + ADP (f.c.: 50µM)

e: ADP (f.c.: 50µM)

Figure 16 shows aggregation curves of platelets (a) after stimulation (b, c, d, e). 2-ClHDA was applied at two different concentrations (b, c) and does not trigger platelet aggregation itself. 2-ClHDA in combination with ADP (d) shows virtually identical aggregation response as ADP alone (e).

8.3.5.2 Influence of 2-ClHDA on ADP-induced expression of P-selectin in GFP

Experiments were aimed to determine the influence of 2-ClHDA on ADP-induced platelet degranulation of P-selectin and to detect eventual dose-dependent effects. GFP alone (control), incubated with increasing concentrations of 2-ClHDA (0.05µM, 0.1µM, 0.5µM, 1µM) and incubated with 0.004% DMSO were stimulated by different concentrations of ADP (1µM, 2.5µM, 5µM) to induce P-selectin expression.

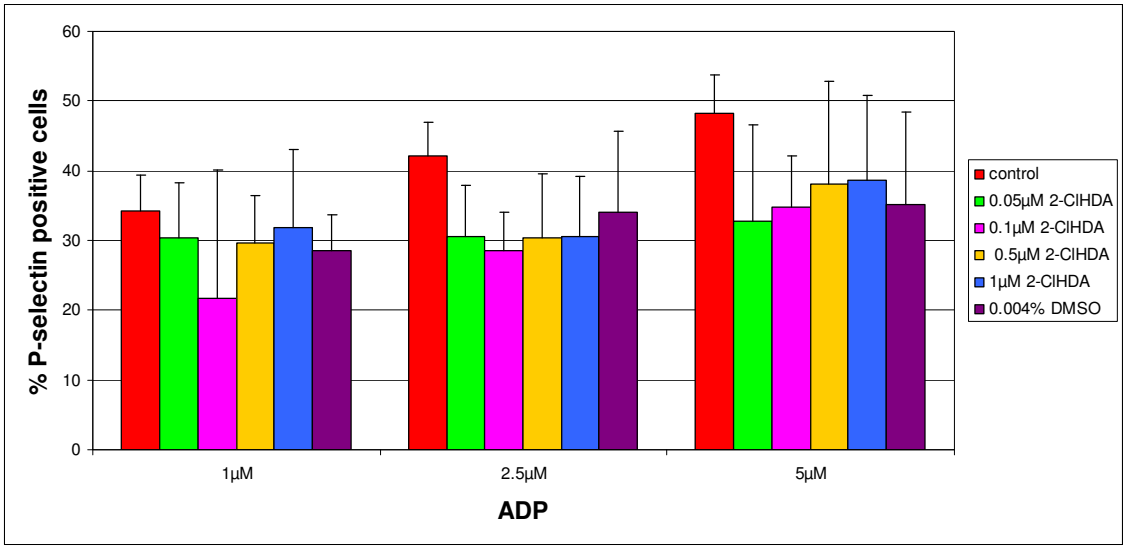

Figure 17: Influence of different concentrations of 2-ClHDA and 0.004% DMSO on ADP-induced expression of P-selectin in GFP (n=3).

	0.05µM 2-ClHDA	**0.1µM 2-ClHDA**	**0.5µM 2-ClHDA**
	Mean ± SD	*Mean ± SD*	*Mean ± SD*
1µM ADP	30.3 ± 8.1	21.7 ± 18.5	29.6 ± 6.8
2.5µM ADP	30.6 ± 7.4	28.5 ± 5.5	30.4 ± 9.0
5µM ADP	32.8 ± 13.7	34.7 ± 7.4	38.1 ± 14.7
	1µM 2-ClHDA	**0.004% DMSO**	
	Mean ± SD	*Mean ± SD*	
1µM ADP	31.8 ± 11.2	28.6 ± 5.0	
2.5µM ADP	30.5 ± 8.6	34.1 ± 11.6	
5µM ADP	38.7 ± 12.0	35.1 ± 13.3	

Table 17: According to Figure 17 - mean values and standard deviations of results of ADP-induced expression of P-selectin in GFP incubated with the indicated concentrations of 2-ClHDA and 0.004% DMSO.

Figure 17 demonstrates percentages of P-selectin positive cells in GFP incubated with different concentrations of 2-ClHDA. DMSO is also used as control, because 2-ClHDA was solubilised within this solvent (7.10). Incubation of GFP with 2-ClHDA at both concentrations results in weak effects on impairing ADP-induced P-selectin expression. There is a trend of 2-ClHDA towards a weak inhibition on ADP-induced P-selectin expression, but observed effects are mainly attributable to solvent DMSO. Taken together, 2-ClHDA is not supposed to be responsible for platelet-activating properties of hypOxHDL.

8.3.5.3 Influence of 2-ClHDA on PGE_1-induced VASP-P in GFP

The effects of 2-ClHDA on platelet activation were already identified in 8.3.5.2, so the following experiments were aimed at investigating the influences of 2-ClHDA on platelet inhibitability by determining state of VASP-P. Effects of 2-ClHDA on PGE_1-induced VASP-P in GFP were examined by quantification of VASP-P positive cells. GFP were treated with two different concentrations of 2-ClHDA (1µM, 2µM) and VASP-P was induced by different concentrations of PGE_1 (6.7nM, 16.7nM, 33.3nM), the same procedure for GFP without 2-ClHDA and GFP incubated with 0.04% DMSO.

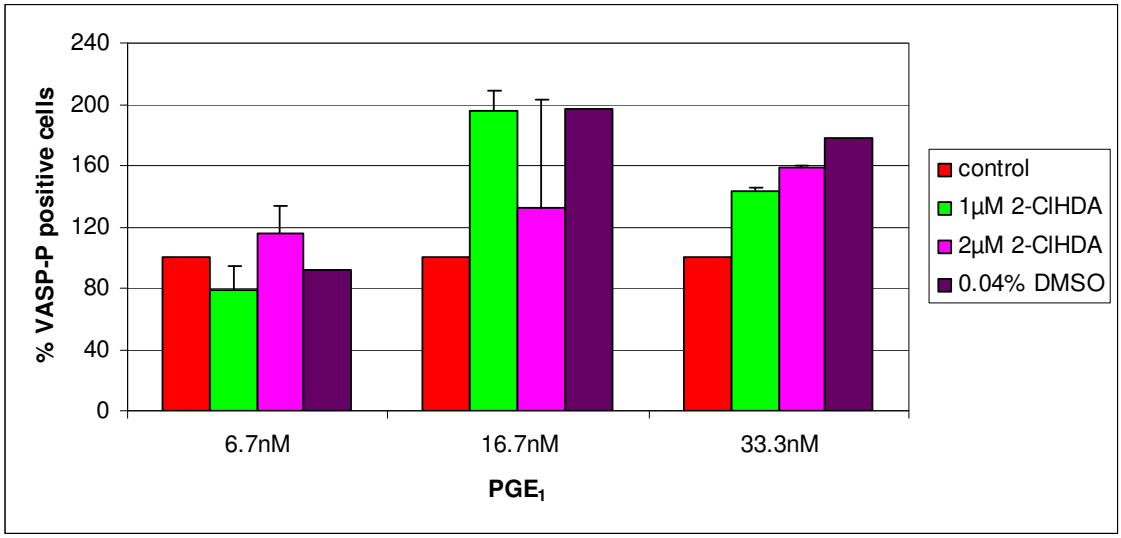

Figure 18: Influence of 2-ClHDA (1µM, 2µM) and 0.04% DMSO on PGE_1-induced VASP-P in GFP (n= 2 / for DMSO n=1).

	1µM 2-ClHDA	2µM 2-ClHDA	0.04% DMSO
	Mean ± SD	*Mean ± SD*	*Data*
6.7nM PGE_1	78.5 ± 16.3	116.1 ± 17.1	92.0
16.7nM PGE_1	195.6 ± 13.2	132.0 ± 71.0	197.1
33.3nM PGE_1	143.8 ± 1.8	159.2 ± 0.3	178.4

Table 18: According to Figure 18 - mean values and standard deviations of results of PGE_1-induced VASP-P in GFP incubated with 2-ClHDA (1µM, 2µM) and 0.04% DMSO.

PGE_1-induced VASP-P in GFP without any 2-ClHDA is set 100% and used as control. PGE_1-induced VASP-P in GFP with different concentrations of 2-ClHDA is set in relation to control. As shown in Figure 18, incubation of GFP with 2-ClHDA leads to a higher percentage of VASP-P positive cells than in the control, but observed effects are again mainly attributable to solvent DMSO. These effects do not occur at 1µM 2-ClHDA at a concentration of 6.7nM PGE_1, but sample size is too low to draw clear conclusions. There is a trend of 2-ClHDA towards a weak effect on increasing VASP-P.

8.3.5.4 Influence of nHDL subclasses pre-incubated with 2-ClHDA on ADP-induced expression of P-selectin in GFP

To determine effects of 2-ClHDA in combination and in interplay with nHDL on ADP-induced P-selectin expression, $nHDL_2$ and $nHDL_3$ were pre-incubated with 2-ClHDA. Additionally we incubated GFP with $nHDL_2$ and $nHDL_3$ but without 2-ClHDA. 2-ClHDA was applied at 2 different concentrations (1.27nM and 7.6nM 2-ClHDA) to GFP (7.6.2).

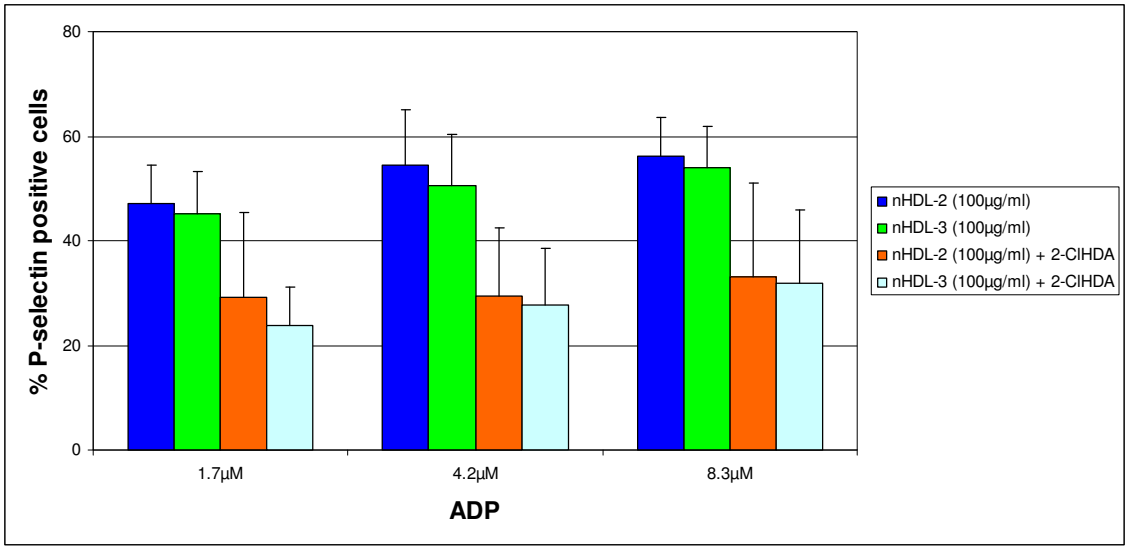

Figure 19: Influence of $nHDL_2$ and $nHDL_3$ (100µg/ml) pre-incubated with 2-ClHDA (1.27nM, 7.6nM) on ADP-induced P-selectin expression in GFP (n=2).

	$nHDL_2$ (100µg/ml)	**$nHDL_3$** (100µg/ml)
	Mean ± SD	*Mean ± SD*
1.7µM ADP	47.0 ± 7.3	45.3 ± 8.1
4.2µM ADP	54.4 ± 0.6	50.6 ± 9.7
8.3µM ADP	56.2 ± 7.4	54.1 ± 7.8
	$nHDL_2$ (100µg/ml) **+ 2-ClHDA**	**$nHDL_3$** (100µg/ml) **+ 2-ClHDA**
	Mean ± SD	*Mean ± SD*
1.7µM ADP	29.1 ± 16.2	23.8 ± 7.3
4.2µM ADP	29.5 ± 12.9	27.8 ± 10.8
8.3µM ADP	33.0 ± 18.0	31.9 ± 13.9

Table 19: According to Figure 19 - mean values and standard deviations of ADP-induced expression of P-selectin in GFP incubated with $nHDL_2$ (100µg/ml), $nHDL_3$ (100µg/ml), HDL_2 (100µg/ml) + 2-ClHDA and HDL_3 (100µg/ml) + 2-ClHDA. 2-ClHDA was added at two different final concentrations (1.27nM, 7.6nM).

Results of the experiments with 1.27nM and 7.6nM 2-ClHDA are at a similar level and are outlined in Figure 19, which shows percentages of P-selectin positive cells in GFP. $nHDL_3$ inhibit platelet activation more effectively than $nHDL_2$. $nHDL_2$ pre-incubated with 2-ClHDA shows similar effects on ADP-induced platelet activation as $nHDL_3$ pre-incubated with 2-ClHDA. Data for $nHDL_2$ incubated with 0.0015% DMSO (n=1) are 74.4 at 1.7µM ADP, 80.5 at 4.2µM ADP and 82.7 at 8.3µM ADP. Data for $nHDL_2$ incubated with 0.009% DMSO (n=1) are 66.5 at 1.7µM ADP, 77.0 at 4.2µM ADP and 72.4 at 8.3µM ADP. Experiments indicate a tendency of $nHDL_3$ in combination with 2-ClHDA to stronger inhibiting effects on ADP-induced P-selectin expression than $nHDL_2$ incubated with 2-ClHDA.

9 Discussion

Atherosclerosis is an inflammatory disease that causes a pro-oxidative environment. Therefore, lipoproteins in the vascular wall can be oxidized. In this scenario, HDL reacts as scavenger of the naturally occurring oxidant hypochlorite to protect other targets (e.g. LDL) from oxidative damage. The oxidative modification hypothesis says that oxidation of lipoproteins can be prevented by antioxidants (Jessup *et al.* 2004). Moreover, several studies and reviews confirm that HDL have atheroprotective effects and therefore diminish cardiovascular risk (Barter *et al.* 2003a;Buring *et al.* 1992;Chen and Mehta 1994;Mineo *et al.* 2006). Within this project two main strategies were pursued, effects of nHDL and hyp-OxHDL on platelet activation determined as P-selectin positive cells and their effects on inhibitability of platelets determined as VASP-P positive cells were defined. ADP-, thrombin-, and collagen-induced P-selectin surface expression as well as PGE_1-induced VASP-P in platelets were quantified. P-selectin represents an indicator for platelet degranulation and activation, because it is stored in α-granules and expressed on the surface of platelets after their activation. The phosphorylation of VASP is an indicator for inhibition of platelets and is induced by PGE_1 through an increase in cAMP dependent protein kinase (5.2.3.1). Thus these methods are appropriate to demonstrate influences of nHDL and hyp-OxHDL on platelet function. Investigations of $nHDL_2$ on PGE_1-induced VASP-P were carried out to compare these effects with the effects from $nHDL_3$. A dose dependency is necessary to evaluate, because then trials with dedicated concentrations of $nHDL_2$ and $nHDL_3$, whereat effects are known, can be carried out. Additionally, it is fundamental to know at which concentrations $nHDL_2$ and $nHDL_3$ have the lowest and the highest impact on PGE_1-induced VASP-P in GFP. Results of experiments show that increasing concentrations of $nHDL_2$ are associated with rising percentages of VASP-P positive cells. Similar investigations were made for $nHDL_3$, which fail to show dose-dependent effects on PGE_1-induced VASP-P. Data resulting from experiments with $nHDL_3$ on PGE_1-induced VASP-P were compared to data obtained by experiments with $nHDL_2$ at defined concentrations (50µg/ml, 100µg/ml, 150µg/ml). $nHDL_2$ and $nHDL_3$ do not vary much at concentrations of 50µg/ml and 100µg/ml in their effects on PGE_1-induced VASP-P. At a concentration of 150µg/ml, $nHDL_3$ increase VASP-P more effectively than $nHDL_2$. But due to high standard devia-

tions, data are not significant. The influence of nHDL subclasses on ADP-, thrombin-, and collagen-induced P-selectin expression was also determined. Simply at collagen-induced P-selectin expression in GFP, $nHDL_2$ have continually stronger inhibiting effects on P-selectin expression at all collagen concentrations. Both nHDL subclasses decrease agonist-induced P-selectin expression and therefore inhibit platelet activation. Moreover, other findings show that thrombin-induced platelet aggregation is decreased by HDL (Aviram and Brook 1983). Researchers suggest that most of the inhibiting effects of nHDL on platelet aggregation are situated in the HDL_2 fraction due to their content of apo-E (Desai *et al.* 1989). Further studies show that $nHDL_2$ inhibit collagen-, and thrombin-induced platelet aggregation more effectively than $nHDL_3$. Apo-E rich HDL from hyperalphalipoproteinemic subjects with plasma cholesteryl ester transfer protein deficiency exhibit more inhibiting effect on agonist-induced platelet aggregation than nHDL from normal donors (Higashihara *et al.* 1991b). Furthermore, researchers found that purified apo-E (10-50µg/ml), complexed with phospholipid vesicles (DMPC) diminish platelet aggregation induced by ADP, adrenaline, or collagen (Riddell *et al.* 1997). Thrombin-induced platelet aggregation is suppressed by apo-E DMPC in a dose dependent manner (Higashihara *et al.* 1991b). HDL-E, a minor subclass of HDL containing apo-E, have a potent anti-platelet effect. They represent a powerful inhibitor of agonist-induced platelet aggregation, obviously through interaction with saturable binding sites in the platelet surface membrane (Riddell *et al.* 1997;Riddell and Owen 1996). In contrast, findings demonstrate that the inhibitory potential of nHDL on platelet aggregation does not strictly depend on the presence of apo-E and that other factors probably play a role as well (Higashihara *et al.* 1991a). We have not determined the apolipoprotein composition of nHDL subclasses from our donors. The observed effects could probably be influenced by differing apolipoprotein contents.

Analytical measurements were conducted to identify differences in nHDL and hyp-OxHDL concerning their content of chloramines and the number of free amino groups. Content of free amino groups in hyp-OxHDL is decreased in comparison to nHDL, which is a result of hypochlorite modification. The first hypochlorite modification strategy was performed until optically evident bleaching of lipoproteins, which indicates the disappearing of antioxidants in lipoproteins. Due to varying amounts of antioxidants in

the lipid phase of lipoproteins, the amount of hypochlorite added was different. The second hypochlorite modification strategy was carried out with identical protein concentrations in HDL_2 and HDL_3 and so lipoproteins of the same donor were modified by the same excess of hypochlorite per mg protein. Results demonstrate that hyp-$OxHDL_2$ and hyp-$OxHDL_3$ do not vary much in their number of free amino groups. HDL subclasses modified by identical excess of hypochlorite show that the decrease in content of free amino groups is higher in hyp-$OxHDL_3$ (ident. hyp.) than in hyp-$OxHDL_2$ (ident. hyp.). Free amino groups are converted into chloramines through oxidation process. A reason for the greater decrease of free amino groups in hyp-$OxHDL_3$ could be that apoA-I is a major apolipoprotein component of HDL_3 and is a favored target for hypochlorite modification (Panzenboeck *et al.* 1997). The content of apoA-I and apoA-II is differing in HDL_2 and HDL_3 and we speculate that apoA-I and apoA-II are oxidized in a different way and speed. Another trial performed was the determination of chloramines in nHDL and hyp-OxHDL. Chloramines are increased in hyp-OxHDL compared to nHDL. The modification procedure by hypochlorite results in enhanced content of chloramines, which was also observed for LDL by Plass et al. (Plass *et al.* 2007). We are able to demonstrate that hypochlorite modification decreased content of free amino groups and increased content of chloramines in both hyp-OxHDL subclasses.

Oxidative modification of HDL_2 and HDL_3 was performed by sodium hypochlorite, which is formed from H_2O_2 by MPO in the presence of physiological chloride concentrations. HDL are a selective in vivo target for MPO catalyzed oxidation. MPO has in vivo relevance, which is confirmed by its occurrence in human lesion material and seems to be linked to HDL extracted from human atheroma (Malle *et al.* 2006b). Results of studies show that atheroprotective properties of nHDL may disappear as a result of their oxidative modification by oxidants generated by MPO (Nicholls *et al.* 2005). The loss of inhibiting properties of nHDL on platelet activation and aggregation in the course of oxidation by hypochlorite is already known. Furthermore, hyp-OxHDL are able to trigger platelet aggregation so that HDL are converted into an independent platelet activator (Assinger *et al.* 2008). The influence of hyp-$OxHDL_2$ and hyp-$OxHDL_3$ on ADP-, thrombin-, and collagen-induced P-selectin expression in GFP was also evaluated. Hyp-$OxHDL_2$ increase agonist-induced P-selectin expression more effectively

than hyp-OxHDL$_3$. Quantification of PGE$_1$-induced VASP-P in GFP incubated with HDL modified by hypochlorite to different degrees was also arranged. Surprisingly, VASP-P in GFP incubated with hyp-OxHDL increased. Freedman et al. detected that a large amount of NO is released shortly after activation to prevent further aggregation. The endothelial cell production of NO impedes partially adhesion of platelets to the vessel wall and NO inhibits platelet aggregation (Freedman *et al.* 1997). This can possibly be a cause for enhanced VASP-P by hyp-OxHDL.

Additionally, attempts to evaluate the impact of 2-ClHDA on platelet degranulation of P-selectin and VASP-P were made. HOCl mediated cleavage of plasmalogen, which is associated with HDL, results in formation of 2-ClHDA. The importance of 2-ClHDA is shown in studies, which suggest its potential to modulate and inhibit eNOS expression and activity in endothelial cells (Marsche *et al.* 2004). 2-ClHDA probably also plays a role in neutrophil recruitment, where it has inducing effects at physiologically relevant concentrations (Thukkani *et al.* 2002). It may have an impact on vascular injury, glomerulosclerosis, and atherosclerosis (Marsche *et al.* 2004). The hypothesis that 2-ClHDA might show an influence on platelet aggregation was tested by determining light transmission in microplate reader. GFP incubated with 2-ClHDA show unaltered ADP-induced aggregation response and 2-ClHDA does not trigger platelet aggregation itself. Trials dealing with the influence of 2-ClHDA on ADP-induced P-selectin expression in GFP show a tendency of 2-ClHDA to inhibiting effects on platelet activation, but sample size was too small to draw clear conclusions. However, 2-ClHDA is also able to increase PGE$_1$-induced VASP-P in GFP. 2-ClHDA in combination with nHDL$_3$ inhibit ADP-induced P-selectin expression more effectively than 2-ClHDA combined with nHDL$_2$.

9.1 Outlook

Results of experiments demonstrate that incubation of GFP with hyp-OxHDL leads to increased percentages of VASP-P (8.3.4). These results are contrary to findings that demonstrate platelet-activating properties of hyp-OxHDL (8.3.1, 8.3.2, 8.3.3). Hence we expected that hyp-OxHDL decrease PGE$_1$-induced VASP-P. One explanation for this

contrast could be that a large amount of NO is released shortly after activation to prevent further aggregation (Freedman *et al.* 1997). L-arginine is required for NO synthesis and through L-arginin analoga, L-NAME, L-NMMA, respectively it is possible to block this NO synthesis (Freedman *et al.* 1997). Future trials can thus be using such analoga, so that an increase of VASP-P due to release of NO as a result of platelet aggregation can not emerge. So falsification of results evoked by this mechanism will not be possible.

10 Summary

This diploma thesis was aimed at investigating the effects of native and modified high density lipoproteins (subclass 2 and 3) on platelet function such as activation state, determined as expression of P-selectin, and inhibitability of platelets, measured as intracellular degree of VASP phosphorylation.

Both nHDL subclasses exhibited inhibiting properties on agonist-induced P-selectin expression, but no significant differences between $nHDL_2$ and $nHDL_3$ were found. Only at collagen-induced P-selectin expression $nHDL_2$ impaired platelet activation more effectively than $nHDL_3$. Furthermore, nHDL were able to increase PGE_1-induced VASP-P, but again no significant varieties in nHDL subclasses were shown. However, $nHDL_3$ (150µg/ml) showed more platelet-inhibiting properties compared to $nHDL_2$ (150µg/ml). These findings affirm atheroprotective effects of nHDL.

Moreover, experiments with hypochlorite modified HDL were carried out. Results revealed that both hyp-OxHDL subclasses are able to enhance agonist-induced P-selectin expression in platelets. Thus it was shown that nHDL acquire platelet-activating properties through oxidation process. Hyp-$OxHDL_2$ showed more potent platelet-activating effects than hyp-$OxHDL_3$. HDL modified by hypochlorite to different degrees showed the trend towards increasing VASP-P.

A strong decrease in free amino groups as well as an increase in chloramines in hyp-OxHDL compared to nHDL was shown.

The influence of the oxidation product 2-ClHDA on platelet function was analyzed as well. 2-ClHDA did not trigger platelet aggregation itself and indicated weak effects with regard to an inhibition of P-selectin expression induced by ADP and to an enhancement in VASP-P induced by PGE_1.

11 Zusammenfassung

Ziel dieser Diplomarbeit war Einflüsse von nativen und modifizierten Lipoproteinen hoher Dichte (Unterklasse 2 und 3) auf den Aktivierungszustand von humanen Thrombozyten, gemessen als Expression von P-Selektin, und auf die Hemmbarkeit, gemessen als Phoshorylierung von VASP, zu untersuchen.

Native HDL Unterklassen zeigten dosisabhängige sowie hemmende Effekte auf die Thrombozytenaktivierung, die durch verschiedene Agonisten (ADP, Thrombin, Kollagen) induziert wurde, wobei keine signifikanten Unterschiede zwischen den HDL Unterklassen festgestellt werden konnten. Nur bei einer von Kollagen induzierten Thrombozytenaktivierung offenbarten $nHDL_2$ eine stärker hemmende Wirkung auf die Expression von P-Selektin als $nHDL_3$. Weiters führten beide nHDL Unterklassen zu einer Erhöhung der VASP-Phosphorylierung, wobei $nHDL_3$ bei einer Konzentration von 150µg/ml stärker steigernd wirkten als $nHDL_2$. Dadurch konnte eine erhöhte Hemmbarkeit der Thrombozyten gezeigt und die atheroprotektiven Effekte von nHDL bestätigt werden.

Zusätzlich wurden Versuche mit Hypochlorit modifizierten HDL durchgeführt, welche eine durch Agonisten induzierte Thrombozytenaktivierung verstärkten, wobei hyp-$OxHDL_2$ eine größere stimulierende Wirkung als hyp-$OxHDL_3$ zeigte. Hiermit konnte bestätigt werden, dass nHDL durch Oxidation Thrombozyten aktivierende Eigenschaften erlangen. Die VASP-Phosphorylierung wurde von unterschiedlich modifizierten HDL erhöht.

Weiters wurde ein starker Rückgang von freien Aminogruppen sowie ein Anstieg an Chloraminen in hyp-OxHDL verglichen mit nHDL festgestellt.

Darüber hinaus wurde der Einfluss von 2-Chlorohexadekanal auf die Thrombozytenfunktion untersucht. Zum einen wirkte es schwach inhibierend auf eine durch Agonisten induzierte Thrombozytenaktivierung und zum anderen führte es zu einer erhöhten Hemmung der Thrombozyten. Dieses Oxidationsprodukt hat weiters selbst keine Aggregation der Thrombozyten hervorgerufen und eine ADP-induzierte Agreggation nicht verändert.

12 Abbreviations

ABCA1:	ATP binding cassette A1
ADP:	adenosine diphosphate
cAMP:	cyclic adenosine monophosphate
CEPT:	cholesteryl ester transfer protein
CEs:	cholesteryl esters
CHD:	coronary heart disease
cGMP:	cyclic guanosine monophosphate
DMPC:	dimyristoylphoshatidylcholine
DMSO:	dimethyl sulfoxide
FACS:	fluorescent activated cell sorting
f.c.:	final concentration
GFP:	gel filtered platelets
GP:	glycoprotein
HDL:	high density lipoproteins
HDL_2:	high density lipoproteins subclass 2
HDL-2:	high density lipoproteins subclass 2
HDL_3:	high density lipoproteins subclass 3
HDL-3:	high density lipoproteins subclass 3
HOCl:	hypochlorous acid
hyp-OxHDL:	hypochlorite modified high density lipoprotein
ident. hyp.:	identical excess of hypochlorite added
kDa:	kilo dalton
LCAT:	lecithin cholesterol acyltransferase
LDL:	low density lipoproteins
MI:	myocardial infarction
MPO:	myeloperoxidase
NADPH:	nicotinamide adenine dinucleotide phosphate
NaOCl:	sodium hypochlorite
nHDL:	native high density lipoproteins

NIDDM:	non insulin dependent diabetes mellitus
NO:	nitric oxide
NOS:	nitric oxide synthase
OCl^-:	hypochlorite
PAR:	protease activated receptor
PGE_1:	prostaglandin E_1
PGE_2:	prostaglandin E_2
PGI_2:	prostaglandin I_2 = prostacyclin
PI3-kinase:	phosphoinositide kinase
PPARα:	peroxisome proliferator- activated receptor alpha
PRP:	platelet rich plasma
ROS:	reactive oxygen species
SR-B1:	scavenger receptor type B1
TG:	triglycerides
TXA_2:	thromboxane
VASP:	vasodilator-stimulated phosphoprotein
VASP-P:	phosphorylated VASP
VLDL:	very low density lipoproteins
vWf :	von Willebrand factor
2-ClHDA:	2-Chlorohexadecanal

13 References

Albert C J, Crowley J R, Hsu F F, Thukkani A K, and Ford D A 2001 Reactive chlorinating species produced by myeloperoxidase target the vinyl ether bond of plasmalogens: identification of 2-chlorohexadecanal; *J. Biol. Chem.* **276** 23733-23741

Arai T, Rinninger F, Varban L, Fairchild-Huntress V, Liang C P, Chen W, Seo T, Deckelbaum R, Huszar D, and Tall A R 1999 Decreased selective uptake of high density lipoprotein cholesteryl esters in apolipoprotein E knock-out mice; *Proc. Natl. Acad. Sci. U. S. A* **96** 12050-12055

Assinger A, Schmid W, Eder S, Schmid D, Koller E, and Volf I 2008 Oxidation by hypochlorite converts protective HDL into a potent platelet agonist; *Febs Letters* **582** 778-784

Assmann G and Schriewer H 1980 [HDL cholesterol: biochemical aspects (author's transl)]; *Klin. Wochenschr.* **58** 749-756

Aviram M and Brook J G 1983 Platelet interaction with high and low density lipoproteins; *Atherosclerosis* **46** 259-268

Bakogianni M C, Kalofoutis C A, Skenderi K I, and Kalofoutis A T 2001 Clinical evaluation of plasma high-density lipoprotein subfractions (HDL2, HDL3) in non-insulin-dependent diabetics with coronary artery disease; *J. Diabetes Complications* **15** 265-269

Barter P, Kastelein J, Nunn A, and Hobbs R 2003a High density lipoproteins (HDLs) and atherosclerosis; the unanswered questions; *Atherosclerosis* **168** 195-211

Barter P J, Brewer H B, Jr., Chapman M J, Hennekens C H, Rader D J, and Tall A R 2003b Cholesteryl ester transfer protein: a novel target for raising HDL and inhibiting atherosclerosis; *Arterioscler. Thromb. Vasc. Biol.* **23** 160-167

Bergt C, Reicher H, Malle E, and Sattler W 1999 Hypochlorite modification of high density lipoprotein: effects on cholesterol efflux from J774 macrophages; *FEBS Lett.* **452** 295-300

Best L C, Martin T J, Russell R G, and Preston F E 1977 Prostacyclin increases cyclic AMP levels and adenylate cyclase activity in platelets; *Nature* **267** 850-852

Buring J E, O'Connor G T, Goldhaber S Z, Rosner B, Herbert P N, Blum C B, Breslow J L, and Hennekens C H 1992 Decreased HDL2 and HDL3 cholesterol, Apo A-I and Apo A-II, and increased risk of myocardial infarction; *Circulation* **85** 22-29

Carnevale R, Pignatelli P, Lenti L, Buchetti B, Sanguigni V, Di S S, and Violi F 2007 LDL are oxidatively modified by platelets via GP91(phox) and accumulate in human monocytes; *FASEB J.* **21** 927-934

Castelli W P 1996 Lipids, risk factors and ischaemic heart disease; *Atherosclerosis* **124 Suppl** S1-S9

Cetin M, Eser B, Er O, Unal A, Kilic E, Patiroglu T, Coskun H S, Altinbas M, Arslan D, and Ilhan O 2001 Effects of DMSO on platelet functions and P-selectin expression during storage; *Transfus. Apher. Sci.* **24** 261-267

Chaer R A, Graham J A, and Mureebe L 2006 Platelet function and pharmacologic inhibition; *Vasc. Endovascular. Surg.* **40** 261-267

Chang Y N, Hsu H T, Hung M S, Chang C L, Li J G, and Wang D L 1990 Effects of PGE1 on platelet deformability; *Chin J. Physiol* **33** 31-40

Chen L Y and Mehta J L 1994 Inhibitory effect of high-density lipoprotein on platelet function is mediated by increase in nitric oxide synthase activity in platelets; *Life Sci.* **55** 1815-1821

Chisolm G M and Steinberg D 2000 The oxidative modification hypothesis of atherogenesis: an overview; *Free Radic. Biol. Med.* **28** 1815-1826

Chrysohoou C, Pitsavos C, Skoumas J, Masoura C, Katinioti A, Panagiotakos D, and Stefanadis C 2007 The emerging anti-inflammatory role of HDL-cholesterol, illustrated in cardiovascular disease free population; the ATTICA study; *Int. J. Cardiol.* **122** 29-33

Curtiss L K and Plow E F 1984 Interaction of plasma lipoproteins with human platelets; *Blood* **64** 365-374

Dardik R, Varon D, Tamarin I, Zivelin A, Salomon O, Shenkman B, and Savion N 2000 Homocysteine and oxidized low density lipoprotein enhanced platelet adhesion to endothelial cells under flow conditions: distinct mechanisms of thrombogenic modulation; *Thromb. Haemost.* **83** 338-344

Daugherty A, Dunn J L, Rateri D L, and Heinecke J W 1994 Myeloperoxidase, a catalyst for lipoprotein oxidation, is expressed in human atherosclerotic lesions; *J. Clin. Invest* **94** 437-444

Desai K, Bruckdorfer K R, Hutton R A, and Owen J S 1989 Binding of apoE-rich high density lipoprotein particles by saturable sites on human blood platelets inhibits agonist-induced platelet aggregation; *J. Lipid Res.* **30** 831-840

Drew B G, Fidge N H, Gallon-Beaumier G, Kemp B E, and Kingwell B A 2004 High-density lipoprotein and apolipoprotein Al increase endothelial NO synthase activity by protein association and multisite phosphorylation; *Proceedings of the National Academy of Sciences of the United States of America* **101** 6999-7004

Eigenthaler M, Lohmann S M, Walter U, and Pilz R B 1999 Signal transduction by cGMP-dependent protein kinases and their emerging roles in the regulation of cell adhesion and gene expression; *Rev. Physiol Biochem. Pharmacol.* **135** 173-209

Falk E, Shah P K, and Fuster V 1995 Coronary plaque disruption; *Circulation* **92** 657-671

Fan J, Unoki H, Iwasa S, and Watanabe T 2000 Role of endothelin-1 in atherosclerosis; *Ann. N. Y. Acad. Sci.* **902** 84-93

Fielding C J and Fielding P E 1995 Molecular physiology of reverse cholesterol transport; *J. Lipid Res.* **36** 211-228

Forte T M, Bielicki J K, Goth-Goldstein R, Selmek J, and McCall M R 1995 Recruitment of cell phospholipids and cholesterol by apolipoproteins A-II and A-I: formation of nascent apolipoprotein-specific HDL that differ in size, phospholipid composition, and reactivity with LCAT; *J. Lipid Res.* **36** 148-157

Freedman J E 2005 Molecular regulation of platelet-dependent thrombosis; *Circulation* **112** 2725-2734

Freedman J E, Loscalzo J, Barnard M R, Alpert C, Keaney J F, and Michelson A D 1997 Nitric oxide released from activated platelets inhibits platelet recruitment; *J. Clin. Invest* **100** 350-356

Gardner C D, Tribble D L, Young D R, Ahn D, and Fortmann S P 2000 Associations of HDL, HDL(2), and HDL(3) cholesterol and apolipoproteins A-I and B with lifestyle factors in healthy women and men: the Stanford Five City Project; *Prev. Med.* **31** 346-356

Garner B, Waldeck A R, Witting P K, Rye K A, and Stocker R 1998 Oxidation of high density lipoproteins. II. Evidence for direct reduction of lipid hydroperoxides by methionine residues of apolipoproteins AI and AII; *J. Biol. Chem.* **273** 6088-6095

Glass C K and Witztum J L 2001 Atherosclerosis. the road ahead; *Cell* **104** 503-516

Goto S, Tamura N, Ishida H, and Ruggeri Z M 2006 Dependence of platelet thrombus stability on sustained glycoprotein IIb/IIIa activation through adenosine 5'-diphosphate receptor stimulation and cyclic calcium signaling; *J. Am. Coll. Cardiol.* **47** 155-162

Griffin J H, Kojima K, Banka C L, Curtiss L K, and Fernandez J A 1999 High-density lipoprotein enhancement of anticoagulant activities of plasma protein S and activated protein C; *J. Clin. Invest* **103** 219-227

Halbrugge M, Friedrich C, Eigenthaler M, Schanzenbacher P, and Walter U 1990 Stoichiometric and reversible phosphorylation of a 46-kDa protein in human platelets in response to cGMP- and cAMP-elevating vasodilators; *J. Biol. Chem.* **265** 3088-3093

Harrison D, Griendling K K, Landmesser U, Hornig B, and Drexler H 2003 Role of oxidative stress in atherosclerosis; *Am. J. Cardiol.* **91** 7A-11A

Higashihara M, Kinoshita M, Kume S, Teramoto T, and Kurokawa K 1991a Inhibition of platelet function by high-density lipoprotein from a patient with apolipoprotein E deficiency; *Biochem. Biophys. Res. Commun.* **181** 1331-1336

Higashihara M, Kinoshita M, Teramoto T, Kume S, and Kurokawa K 1991b The role of apoE in inhibitory effects of apoE-rich HDL on platelet function; *FEBS Lett.* **282** 82-86

Honda H M, Wakamatsu B K, Goldhaber J I, Berliner J A, Navab M, and Weiss J N 1999 High-density lipoprotein increases intracellular calcium levels by releasing calcium from internal stores in human endothelial cells; *Atherosclerosis* **143** 299-306

Horstrup K, Jablonka B, Honig-Liedl P, Just M, Kochsiek K, and Walter U 1994 Phosphorylation of focal adhesion vasodilator-stimulated phosphoprotein at Ser157 in intact human platelets correlates with fibrinogen receptor inhibition; *Eur. J. Biochem.* **225** 21-27

Huang J M, Huang Z X, and Zhu W 1998 Mechanism of high-density lipoprotein subfractions inhibiting copper-catalyzed oxidation of low-density lipoprotein; *Clin. Biochem.* **31** 537-543

Ikewaki K, Zech L A, Kindt M, Brewer H B, Jr., and Rader D J 1995 Apolipoprotein A-II production rate is a major factor regulating the distribution of apolipoprotein A-I among HDL subclasses LpA-I and LpA-I:A-II in normolipidemic humans; *Arterioscler. Thromb. Vasc. Biol.* **15** 306-312

Jessup W, Kritharides L, and Stocker R 2004 Lipid oxidation in atherogenesis: an overview; *Biochem. Soc. Trans.* **32** 134-138

Jung S M and Moroi M 2000 Activation of the platelet collagen receptor integrin alpha(2)beta(1): its mechanism and participation in the physiological functions of platelets; *Trends Cardiovasc. Med.* **10** 285-292

Kleuser, B. Neue Therapiestrategien zur Stärkung von HDL. Pharmazeutische Zeitung 46. 2007.
Ref Type: Magazine Article

Knekt P, Ritz J, Pereira M A, O'Reilly E J, Augustsson K, Fraser G E, Goldbourt U, Heitmann B L, Hallmans G, Liu S, Pietinen P, Spiegelman D, Stevens J, Virtamo J, Willett W C, Rimm E B, and Ascherio A 2004 Antioxidant vitamins and coronary heart disease risk: a pooled analysis of 9 cohorts; *Am. J. Clin. Nutr.* **80** 1508-1520

Kontush A, Chantepie S, and Chapman M J 2003 Small, dense HDL particles exert potent protection of atherogenic LDL against oxidative stress; *Arterioscler. Thromb. Vasc. Biol.* **23** 1881-1888

Kontush A and Chapman M J 2006 Functionally defective high-density lipoprotein: a new therapeutic target at the crossroads of dyslipidemia, inflammation, and atherosclerosis; *Pharmacol. Rev.* **58** 342-374

Kovacsovics T J and Hartwig J H 1996 Thrombin-induced GPIb-IX centralization on the platelet surface requires actin assembly and myosin II activation; *Blood* **87** 618-629

Krauss R M 1982 Regulation of high density lipoprotein levels; *Med. Clin. North Am.* **66** 403-430

Kroll M H and Schafer A I 1989 Biochemical mechanisms of platelet activation; *Blood* **74** 1181-1195

Kullmer T and Kindermann W 1985 [Apolipoproteins and lipoproteins with differing physical activity and performance capability]; *Klin. Wochenschr.* **63** 1102-1109

Lamarche B, Moorjani S, Cantin B, Dagenais G R, Lupien P J, and Despres J P 1997 Associations of HDL2 and HDL3 subfractions with ischemic heart disease in men. Prospective results from the Quebec Cardiovascular Study; *Arterioscler. Thromb. Vasc. Biol.* **17** 1098-1105

Lawrence D W and Pryzwansky K B 2001 The vasodilator-stimulated phosphoprotein is regulated by cyclic GMP-dependent protein kinase during neutrophil spreading; *J. Immunol.* **166** 5550-5556

Libby P 2002 Inflammation in atherosclerosis; *Nature* **420** 868-874

Loscalzo J 2001 Nitric oxide insufficiency, platelet activation, and arterial thrombosis; *Circ. Res.* **88** 756-762

Mahley R W, Innerarity T L, Rall S C, Jr., and Weisgraber K H 1984 Plasma lipoproteins: apolipoprotein structure and function; *J. Lipid Res.* **25** 1277-1294

Malle E, Marsche G, Arnhold J, and Davies M J 2006a Modification of low-density lipoprotein by myeloperoxidase-derived oxidants and reagent hypochlorous acid; *Biochim. Biophys. Acta* **1761** 392-415

Malle E, Marsche G, Panzenboeck U, and Sattler W 2006b Myeloperoxidase-mediated oxidation of high-density lipoproteins: fingerprints of newly recognized potential proatherogenic lipoproteins; *Arch. Biochem. Biophys.* **445** 245-255

Marsche G, Furtmuller P G, Obinger C, Sattler W, and Malle E 2008 Hypochlorite-modified high-density lipoprotein acts as a sink for myeloperoxidase in vitro; *Cardiovasc. Res.* **79** 187-194

Marsche G, Heller R, Fauler G, Kovacevic A, Nuszkowski A, Graier W, Sattler W, and Malle E 2004 2-chlorohexadecanal derived from hypochlorite-modified high-density lipoprotein-associated plasmalogen is a natural inhibitor of endothelial nitric oxide biosynthesis; *Arterioscler. Thromb. Vasc. Biol.* **24** 2302-2306

Martin-Nizard F, Sqalli-Houssaini H, Walters-Laporte E, Boullier A, Fruchart J C, and Duriez P 1995 Oxidized high-density lipoproteins modulate endothelin secretion by adult bovine aortic endothelial cells; *J. Cardiovasc. Risk* **2** 263-267

McDonald J W and Stuart R K 1974 Interaction of prostaglandins E1 and E2 in regulation of cyclic-AMP and aggregation in human platelets: evidence for a common prostaglandin receptor; *J. Lab Clin. Med.* **84** 111-121

McPherson P A, Young I S, McKibben B, and McEneny J 2007 High density lipoprotein subfractions: isolation, composition, and their duplicitous role in oxidation; *J. Lipid Res.* **48** 86-95

Mendez A J, Oram J F, and Bierman E L 1991 Protein kinase C as a mediator of high density lipoprotein receptor-dependent efflux of intracellular cholesterol; *J. Biol. Chem.* **266** 10104-10111

Michalaki V, Koutroulis G, Syrigos K, Piperi C, and Kalofoutis A 2005 Evaluation of serum lipids and high-density lipoprotein subfractions (HDL2, HDL3) in postmenopausal patients with breast cancer; *Mol. Cell Biochem.* **268** 19-24

Mineo C, Deguchi H, Griffin J H, and Shaul P W 2006 Endothelial and antithrombotic actions of HDL; *Circ. Res.* **98** 1352-1364

Mowri H O, Patsch J R, Ritsch A, Foger B, Brown S, and Patsch W 1994 High density lipoproteins with differing apolipoproteins: relationships to postprandial lipemia, cholesteryl ester transfer protein, and activities of lipoprotein lipase, hepatic lipase, and lecithin: cholesterol acyltransferase; *J. Lipid Res.* **35** 291-300

Nakajima K, Nakano T, and Tanaka A 2006 The oxidative modification hypothesis of atherosclerosis: the comparison of atherogenic effects on oxidized LDL and remnant lipoproteins in plasma; *Clin. Chim. Acta* **367** 36-47

Navab M, Imes S S, Hama S Y, Hough G P, Ross L A, Bork R W, Valente A J, Berliner J A, Drinkwater D C, Laks H, and . 1991 Monocyte transmigration induced by modification of low density lipoprotein in cocultures of human aortic wall cells is due to induction of monocyte chemotactic protein 1 synthesis and is abolished by high density lipoprotein; *J. Clin. Invest* **88** 2039-2046

Nestel P J 1987 High-density lipoprotein turnover; *Am. Heart J.* **113** 518-521

Nicholls S J, Zheng L, and Hazen S L 2005 Formation of dysfunctional high-density lipoprotein by myeloperoxidase; *Trends Cardiovasc. Med.* **15** 212-219

Nofer J R, van der G M, Tolle M, Wolinska I, von Wnuck L K, Baba H A, Tietge U J, Godecke A, Ishii I, Kleuser B, Schafers M, Fobker M, Zidek W, Assmann G, Chun J, and Levkau B 2004 HDL induces NO-dependent vasorelaxation via the lysophospholipid receptor S1P3; *J. Clin. Invest* **113** 569-581

Noll G 1998 Pathogenesis of atherosclerosis: a possible relation to infection; *Atherosclerosis* **140 Suppl 1** S3-S9

O'Connell B J and Genest J, Jr. 2001 High-density lipoproteins and endothelial function; *Circulation* **104** 1978-1983

Okamura T, Hayakawa T, Kadowaki T, Kita Y, Okayama A, and Ueshima H 2006 The inverse relationship between serum high-density lipoprotein cholesterol level and all-cause mortality in a 9.6-year follow-up study in the Japanese general population; *Atherosclerosis* **184** 143-150

Olufadi R and Byrne C D 2006 Effects of VLDL and remnant particles on platelets; *Pathophysiol. Haemost. Thromb.* **35** 281-291

Oram J F 2003 HDL apolipoproteins and ABCA1: partners in the removal of excess cellular cholesterol; *Arterioscler. Thromb. Vasc. Biol.* **23** 720-727

Oram J F and Yokoyama S 1996 Apolipoprotein-mediated removal of cellular cholesterol and phospholipids; *J. Lipid Res.* **37** 2473-2491

Özüyaman B 2003 *Die endotheliale NO-Synthase spielt bei der Inhibition arterieller Thrombosen eine geringe Rolle,* Medizinischen Fakultät der Heinrich-Heine-Universität Düsseldorf

Panzenboeck U, Raitmayer S, Reicher H, Lindner H, Glatter O, Malle E, and Sattler W 1997 Effects of reagent and enzymatically generated hypochlorite on physicochemical and metabolic properties of high density lipoproteins; *J. Biol. Chem.* **272** 29711-29720

Park D H and Ransone J W 2003 Effects of submaximal exercise on high-density lipoprotein-cholesterol subfractions; *Int. J. Sports Med.* **24** 245-251

Plass C A, Schmid W, Holy E W, Kreatschitsch U, Laggner H, and Volf I 2007 Redox-sensitive impairment of porcine coronary artery vasodilation by hypochlorite-modified LDL; *Atherosclerosis* **190** 330-337

Riddell D R, Graham A, and Owen J S 1997 Apolipoprotein E inhibits platelet aggregation through the L-arginine:nitric oxide pathway. Implications for vascular disease; *J. Biol. Chem.* **272** 89-95

Riddell D R and Owen J S 1996 Inhibition of ADP-induced platelet aggregation by apoE is not mediated by membrane cholesterol depletion; *Thromb. Res.* **81** 597-606

Riddell D R and Owen J S 1999 Nitric oxide and platelet aggregation; *Vitam. Horm.* **57** 25-48

Ross R 1993 The pathogenesis of atherosclerosis: a perspective for the 1990s; *Nature* **362** 801-809

Ross R 1999 Atherosclerosis--an inflammatory disease; *N. Engl. J. Med.* **340** 115-126

Sakuma N, Saeki T, Yajima K, Hibino T, Yoshida T, Mizuno H, Mukai S, Sakata S, Kunimatsu M, and Kimura G 2005 Both HDL3 and HDL2 exert a powerful anti-oxidative and protective effect against acceleration of oxidative modification of LDL by ascorbic acid; *J. Nutr. Sci. Vitaminol. (Tokyo)* **51** 75-79

Sastre F G, Ribas V, and Blanco-Vaca F 2002 HDL: A molecular view of the «classic» antiatherogenic lipoprotein; *Contributions to Science* **2** 143-171

Saxena U, Ferguson E, and Bisgaier C L 1993 Apolipoprotein E modulates low density lipoprotein retention by lipoprotein lipase anchored to the subendothelial matrix; *J. Biol. Chem.* **268** 14812-14819

Schafer C, Parlesak A, Eckoldt J, Bode C, Bode J C, Marz W, and Winkler K 2007 Beyond HDL-cholesterol increase: phospholipid enrichment and shift from HDL3 to HDL2 in alcohol consumers; *J. Lipid Res.* **48** 1550-1558

Schiele F, De B D, Vincent-Viry M, Beisiegel U, Ehnholm C, Evans A, Kafatos A, Martins M C, Sans S, Sass C, Visvikis S, De B G, and Siest G 2000 Apolipoprotein E serum concentration and polymorphism in six European countries: the ApoEurope Project; *Atherosclerosis* **152** 475-488

Sillanaukee P, Koivula T, Jokela H, Pitkajarvi T, and Seppa K 2000 Alcohol consumption and its relation to lipid-based cardiovascular risk factors among middle-aged women: the role of HDL(3) cholesterol; *Atherosclerosis* **152** 503-510

Singh K, Chander R, and Kapoor N K 1997 High density lipoprotein subclasses inhibit low density lipoprotein oxidation; *Indian J. Biochem. Biophys.* **34** 313-318

Skinner E R 1994 High-density lipoprotein subclasses; *Curr. Opin. Lipidol.* **5** 241-247

Spieker L E, Sudano I, Hurlimann D, Lerch P G, Lang M G, Binggeli C, Corti R, Ruschitzka F, Luscher T F, and Noll G 2002 High-density lipoprotein restores endothelial function in hypercholesterolemic men; *Circulation* **105** 1399-1402

Srinivasan S R, Segrest J P, Elkasabany A M, and Berenson G S 2001 Distribution and correlates of lipoproteins and their subclasses in black and white young adults. The Bogalusa Heart Study; *Atherosclerosis* **159** 391-397

Steinberg D and Witztum J L 2002 Is the oxidative modification hypothesis relevant to human atherosclerosis? Do the antioxidant trials conducted to date refute the hypothesis?; *Circulation* **105** 2107-2111

Stocker R and Keaney J F, Jr. 2005 New insights on oxidative stress in the artery wall; *J. Thromb. Haemost.* **3** 1825-1834

Thukkani A K, Hsu F F, Crowley J R, Wysolmerski R B, Albert C J, and Ford D A 2002 Reactive chlorinating species produced during neutrophil activation target tissue plasmalogens: production of the chemoattractant, 2-chlorohexadecanal; *J. Biol. Chem.* **277** 3842-3849

Thukkani A K, Martinson B D, Albert C J, Vogler G A, and Ford D A 2005 Neutrophil-mediated accumulation of 2-ClHDA during myocardial infarction: 2-ClHDA-mediated myocardial injury; *Am. J. Physiol Heart Circ. Physiol* **288** H2955-H2964

Van den Bergen H, Daloze D, and Braekman J C 1999 Synthesis of analogues of 2-iodohexadecanal, a regulator of iodine metabolism in the tyroid gland; *J. Braz. Chem. Soc.* **10** 1-12

Vivekananthan D P, Penn M S, Sapp S K, Hsu A, and Topol E J 2003 Use of antioxidant vitamins for the prevention of cardiovascular disease: meta-analysis of randomised trials; *Lancet* **361** 2017-2023

Walter U, Eigenthaler M, Geiger J, and Reinhard M 1993 Role of cyclic nucleotide-dependent protein kinases and their common substrate VASP in the regulation of human platelets; *Adv. Exp. Med. Biol.* **344** 237-249

Watts G F and Burnett J R 2004 HDL Revisited: New Opportunities for Managing Dyslipoproteinaemia and Cardiovascular Disease; *Clin. Biochem. Rev.* **25** 7-18

Williams K J and Tabas I 1995 The response-to-retention hypothesis of early atherogenesis; *Arterioscler. Thromb. Vasc. Biol.* **15** 551-561

Willoughby S, Holmes A, and Loscalzo J 2002 Platelets and cardiovascular disease; *Eur. J. Cardiovasc. Nurs.* **1** 273-288

Zhang R, Brennan M L, Fu X, Aviles R J, Pearce G L, Penn M S, Topol E J, Sprecher D L, and Hazen S L 2001 Association between myeloperoxidase levels and risk of coronary artery disease; *JAMA* **286** 2136-2142

Zheng L, Settle M, Brubaker G, Schmitt D, Hazen S L, Smith J D, and Kinter M 2005 Localization of nitration and chlorination sites on apolipoprotein A-I catalyzed by myeloperoxidase in human atheroma and associated oxidative impairment in ABCA1-dependent cholesterol efflux from macrophages; *J. Biol. Chem.* **280** 38-47

14 Curriculum vitae

Name:	Sabine Ettinger
Date of birth:	31st of December 1984
Marital status:	single
Education:	October 2003 start Nutritional Sciences at University of Vienna
	1995-2003 private grammar school Kreuzschwestern Gmunden
	1991-1995 elementary school Gmunden
Work experience:	Internship at Interdisciplinary Research Center, Justus-Liebig
	University, Giessen, Germany (July, August 2007)
	Internship at Nestlé Linz (September 2006)
	Internship at AGES Linz (August 2006)
	Internship at FIAN Vienna (February 2006)

5th March 2009, Vienna