Université de Montréal

High-Density Lipoproteins (HDL) Functionality in Degenerative Cardiac Disease

Novel Cardioprotective Roles of HDL and Strategies to Target HDL Dysfunction

par

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Résumé

Introduction: Le récent échec des molécules destinées à faire augmenter les taux de lipoprotéines de haute densité (HDL) a mené à la conclusion que la fonctionnalité des HDL, plutôt que leur quantité, jouerait un rôle majeur dans les pathologies cardiovasculaires. L'augmentation de HDL fonctionnelles via l'administration de HDL reconstituées (rHDL) ou d'apolipoprotéine A-I (apoA-I) semble être une approche prometteuse dans les maladies coronariennes et la sténose de la valve aortique (SVA). Cependant, les biomarqueurs reflétant la fonctionnalité des HDL utilisables en clinique sont inexistants. De plus, peu d'information existe sur les mécanismes par lesquels les thérapies basées sur les HDL pourraient diminuer le risque cardiovasculaire, ainsi que sur la conversion possible de rHDL et de l'apoA-I en molécules dysfonctionnelles. Finalement, les données relatives aux effets secondaires possibles de telles thérapies sont rares. Ainsi, la présente étude a évalué l'utilité de l'estérification du cholestérol comme biomarqueur des effets cardioprotecteurs des HDL ; les effets bénéfiques des rHDL sur la réparation endothéliale ; les effets secondaires potentiels des thérapies basées sur les HDL ; et les stratégies visant la protection des HDL dans la SVA.

Méthodes et résultats: L'utilité de l'estérification du cholestérol lors de la maturation des particules de HDL, en tant que biomarqueur des effets cardioprotecteurs des HDL, a été analysée chez 267 patients atteints d'une maladie coronarienne stable. De plus, les effets des rHDL sur le mécanisme de réparation vasculaire ont été évalués chez 33 patients ayant récemment souffert d'un syndrome coronarien aigu (SCA), et la contribution de différentes protéases plasmatiques à la dégradation de l'apoA-I a été comparée chez des patients atteints d'une SVA, ainsi que dans un modèle de SVA chez le lapin. L'impact potentiel de l'administration d'un peptide mimétique de l'apoA-I sur les anomalies hémorragiques a également été évalué dans la même population de patients et le même modèle expérimental. L'estérification du cholestérol s'est révélée être un prédicteur puissant et indépendant de la charge athéroscléreuse chez les patients souffrant d'une maladie coronarienne. L'administration de rHDL a prévenu la réduction des cellules endothéliales progénitrices (EPC) chez les patients ayant

récemment souffert d'un SCA. Bien que l'administration d'apoA-I n'ait pas eu d'incidence négative sur les anomalies hémorragiques dans le cas de SVA, il a été trouvé que la protéase à cystéine cathepsine S est la protéase clé responsable de 70% de la dégradation de l'apoA-I chez les humains.

Conclusion : Les présentes données indiquent que l'estérification du cholestérol est un bon marqueur de la sévérité de l'artériosclérose. Ainsi, cibler l'estérification du cholestérol pourrait s'avérer une approche prometteuse pour réduire le risque cardiovasculaire. De plus, nos résultats montrent qu'une amélioration des mécanismes de réparation cardiovasculaire, suite à une lésion myocardique, pourrait faire partie des mécanismes par lesquels des HDL exogènes exercent leurs effets cardioprotecteurs. Cette dernière constitue également une stratégie particulièrement prometteuse pour le traitement de la SVA, et son efficacité pourrait être encore améliorée par l'inhibition de la protéase à cystéine cathepsine S.

Mots-clés : lipoprotéines de haute densité, apolipoprotéine A-I, lécithine cholestérol acyl transférase, maladie coronarienne, sténose valvulaire aortique, risque cardiovasculaire, cellules endothéliales progénitrices, coagulation

Abstract

Background: The recent failure of drugs targeted to high-density lipoprotein (HDL) cholesterol levels has led to the conclusion that HDL function rather than HDL quantity is the key player in cardiovascular pathologies. The augmentation of functional HDL through administration of reconstituted HDL (rHDL) or apolipoprotein A-I (apoA-I) seems a promising strategy to in coronary artery disease (CAD) and aortic valve stenosis (AVS). However, clinically applicable biomarkers reflecting functionality of HDL are lacking. In addition, little is known on the mechanisms governing the cardioprotective effects of HDL-based approaches as well as rHDL and apoA-I possible conversion into dysfunctional molecules. Finally, data regarding potential secondary effects of such therapy are sparse. Thus, the present project explored the value of cholesterol esterification as biomarker for cardioprotective HDL effects, assessed whether rHDL exerts beneficial effects in endothelial repair, and investigated potential side-effects of HDL-based therapies as well as HDL-preserving strategies in AVS.

Method and results: We explored the value of cholesterol esterification during maturation of HDL particles, as a biomarker for cardioprotective HDL effects in 267 patients with stable CAD. In addition, the effect of rHDL on vascular repair mechanism was assessed in 33 patients with a recent acute coronary syndrome (ACS), and the contribution of different plasma proteases to apoA-I degradation was compared in patients with AVS as well as in a rabbit model of AVS. Using the same population and experimental model, we also assessed possible impact of apoA-I administration on haemorrhagic disorders in patients with AVS. Cholesterol esterification was found to be a strong and independent predictor of atherosclerotic burden in patients with CAD. Administration of rHDL prevented endothelial progenitor cells decline in patients with a recent ACS. While apoA-I administration did not negatively affect haemorrhagic disorders in AVS, it was found that the cysteine protease cathepsin S is the key protease responsible for 70% of apoA-I degradation in humans.

Conclusion: The present data indicate that cholesterol esterification is a useful biomarker mirroring severity of atherosclerotic disease. Thus, targeting cholesterol esterification might be a promising therapeutic strategy to reduce cardiovascular

risk. Our data further indicate that an improvement of cardiovascular repair mechanisms following myocardial injury might be amongst the mechanisms by which exogenous HDL exerts its cardioprotective actions. The latter is also a particularly promising strategy in the treatment of AVS and its efficacy could be further enhanced by inhibition of the cysteine protease cathepsin S.

Keywords: High-density lipoproteins, apolipoprotein A-I, lecithin cholesterol acyltransferase, coronary artery disease, aortic valve stenosis, cardiovascular risk, endothelial progenitor cells, coagulation

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List of Abbreviations

ABCA1:	Adenosine triphosphate binding cassette A1
ABCG1:	Adenosine triphosphate binding cassette G1
ACE:	Angiotensin converting enzyme
ACEI:	ACE inhibitor
ACS:	Acute coronary syndrome
ADAMTS-13:	A disintegrin-like and metalloprotease with a thrombospondin type 1 motif, member 13
ADCY9:	Adenylate cyclase 9
ADMA:	Asymmetric dimethyl arginine
ANOVA:	Analysis of variance
AOI:	Area of interest
APC:	Activated protein C
apoB:	Apolipoprotein B
aPTT:	Activated partial thromboplastin time
ARB:	Angiotensin receptor blocker
ASE:	American Society of Echocardiography
ATP:	Adenosine triphosphate
AVA:	Aortic valve area
AVR:	aortic valve replacement
AVS:	Aortic valve stenosis
BET:	Bromodomain and extra-terminal protein
BMI:	Body mass index
BMS:	Bare-metal stent
BNP:	B-type natriuretic peptide
CABG:	Coronary artery bypass graft
CAD:	Coronary artery disease
CAIN:	Canadian Atherosclerosis Imaging Network
CER:	Cholesterol esterification rate
CETP:	Cholesteryl ester transfer protein
cIMT:	Carotid intima-media thickness

CRP:	C-reactive protein
CW:	Continuous wave
DES:	Drug-eluting stent
EAE:	European Association of Echocardiography
ELISA:	Enzyme-linked immunosorbent assay
eNOS:	Endothelial NO synthase
EPC:	Endothelial progenitor cells
Ex/Em:	Exitation/Emission
FC:	Free cholesterol
FER:	Fractional esterification rate
FFP:	Fresh-frozen plasma
GFR:	Glomerular filtration rate
γGT:	Gamma-glutamyl transferase
HDL:	High-density lipoproteins
HDL-C:	High-density lipoprotein cholesterol
HDL-FER:	Fractional esterification rate in apolipoproteinB depleted plasma
HDL-MER:	Molar esterification rate in apolipoproteinB depleted plasma
HDL-UC:	Unesterified cholesterol concentration in apoB depleted plasma
HMG-CoA:	3-hydroxy-3-methylglutaryl-coenzyme A
ICAM-1:	Intercellular adhesion molecule-1
INR:	International normalized ratio
IVUS:	Intravascular ultrasound imaging
KDR^{+}:	Kinase insert domain receptor
LCAT:	Lecithin cholesterol acyltransferase
LDL:	Low-density lipoprotein
LDL-C:	Low-density lipoprotein-cholesterol
LDLR:	Low-density lipoprotein receptor
LVEF:	Left ventricular ejection fraction
LVMMI:	Indexed left ventricular muscle mass
MCP-1:	Monocyte chemoattractant protein-1
MER:	Molar esterification rate
MHICC:	Montreal Health Innovations Coordinating Centre
MMP:	Matrix metalloproteinase

NO:	Nitric oxide
NSTEMI:	non-ST-elevation myocardial infarction
NYHA:	New York Heart Association
OxLDL:	oxidized LDL
PAF:	Platelet-activating factor
PAI-1:	Plasminogen activator inhibitor 1
PBMC:	Peripheral blood mononuclear cell
PCI:	Percutaneous coronary intervention
PCSK9:	Proprotein convertase subtilisin/kexin type 9
PGI2:	Prostacyclin
PI3K:	Phosphatidylinositol 3-kinase
Plasma-FER:	Fractional esterification rate in complete plasma
Plasma-MER:	Molar esterification rate in complete plasma
Plasma-UC:	Unesterified cholesterol concentration in complete plasma
PLTP:	Phospholipid Transfer Protein
PMSF:	Phenylmethylsulfonyl fluoride
PON-1:	Paraoxonase-1
PPAR-α:	Peroxisome proliferator receptor α
PT:	Prothrombin time
QCA:	Quantitative coronary angiography
RAM-11:	Monoclonal mouse anti-rabbit macrophage antibody
RCT:	Reverse cholesterol transport
RFU:	Relative fluorescence units
ROA:	Region of analysis
SDF-1:	Stromal cell-derived factor-1
rHDL:	Reconstituted HDL
STEMI:	ST-elevation myocardial infarction
rt-PA:	Recombinant tPA
RWT:	Relative wall thickness
SD:	Standard deviation
SE:	Standard error
SEM:	Standard error of the mean
siRNA:	small interfering RNA

SR-BI:	Scavenger receptor class B type 1
TF:	Tissue factor
TNF-α:	Tumor-necrosis factor
tPA:	Tissue plasminogen activator
UC:	Unesterified cholesterol
VAV:	transvalvular maximal velocity
VCAM-1:	Vascular cell adhesion molecule-1
VLVOT:	Trans left ventricular outflow tract velocity
vWF:	Von Willebrand factor
vWF:CB:	Collagen-binding activity

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1. Introduction

1.1 Residual Cardiovascular Risk and HDL Paradox

Cardiovascular disease is the leading global cause of death, accounting for 17.3 million deaths per year, a number that is expected to grow to more than 23.6 millions by 2030.[1] Even though the use of low-density lipoprotein (LDL)-lowering medications has led to a significant reduction of cardiovascular risk in both primary and secondary prevention, significant cardiovascular risk remains even after optimal LDL levels have been achieved. Indeed, the reduction in coronary events and cardiovascular mortality obtained with high doses of LDL-lowering medications such as 3-hydroxy-3-methylglutaryl-coenzyme (HMG-CoA) reductase inhibitors (known as statins) was only 30-35% in most trials,[2] indicating that there remains a high level of residual risk in susceptible individuals.[3, 4] Therefore, complementary therapy beyond statins is needed to further reduce the risk of coronary events.

On the basis of epidemiological analysis, cholesterol levels associated to high-density lipoproteins (HDL) has been identified as an inverse predictor of cardiovascular events and remained predictive even in patients fully treated with intense statin therapy.[4, 5] Accordingly, the vascular effects of HDL have attracted enormous interest and it was suggested that increasing HDL-cholesterol (HDL-C) might reduce residual cardiovascular risk. However, in contrast to epidemiological data, results from recent pharmacological trials with drugs that raise HDL-C, such as niacin and inhibitors of cholesteryl ester transfer protein (CETP), have been disappointing by not supporting a direct causal relation between HDL-C disorders and atherosclerosis per se.[6-10] These observations were supported by human genetic studies showing that genetic variants associated with higher HDL-C levels were not associated with a lower cardiovascular risk.[6] Thus, these studies have raised serious doubts regarding whether simply increasing HDL-C is atheroprotective. Importantly, two recent studies indicate that the inverse relationship of HDL-C with cardiovascular events is weakened in patients with coronary artery disease (CAD) because of modification of HDL, and have emphasized the important distinction between HDL function and plasma levels of

HDL-C in the assessment of cardiovascular risk.[10, 11] Thus, quantification of dynamic HDL function will be crucial in assessing the impact of future therapeutics aimed at enhancing HDL. Consequently, present studies investigate surrogate markers of HDL function, such as cholesterol efflux or HDL particle size, or more specific HDL increasing therapies such as apolipoproteinA-I (apoA-I)-containing infusions, and will hopefully provide a more complete picture of HDL-mediated cardiovascular risk reduction.

1.2 Association of HDL-C Levels with Coronary Artery Disease

Studies consistently support HDL-C as a significant, strong, and independent inverse predictor of cardiovascular risk, even when LDL-cholesterol (LDL-C) is reduced to very low levels by high dose statins.[5, 12-18] Thus, an increase in HDL-C by 1 mg/dl was estimated to be associated with a 2% and 3% risk reduction of CAD in men and women, respectively.[5] Importantly, in recent years, evidence has accumulated that even in patients with well controlled serum levels of LDL-C (<100 mg/dl), HDL-C serum concentrations remain a significant predictor of future cardiovascular events.[5, 19, 20] Amongst patients with CAD, patients presenting with an acute coronary syndrome (ACS) are the most vulnerable population. In fact, despite recent therapeutic advances, residual risk persists in those patients, which is mirrored by a high short-term risk for recurrent ischaemic events.[21] Importantly, population based studies indicate that a substantial proportion of patients with ACS presents with reduced or dysfunctional HDL,[22, 23] which, in turn, is associated with more extensive angiographic coronary disease, higher in-hospital mortality,[24] and higher risk of recurrent cardiovascular events.[25] Accordingly, a significant reduction in risk of recurrent events for an increment in baseline HDL-C has been reported in patients with ACS.[25] As a consequence, HDL-C has been suggested as an important target for modifying the high-risk state following ACS.

1.3 Association of HDL with Aortic Valve Disease

Aortic valve stenosis (AVS) is a major health problem facing aging societies. Indeed, one third of the elderly in Western populations show echocardiographic

evidence of calcific aortic valve sclerosis, a subclinical form of AVS.[26] Age, sex, tobacco use, hypercholesterolemia, hypertension, and type II diabetes mellitus all contribute to the risk of this disease. As severe symptomatic AVS usually leads to considerable morbidity and death in less than five years if left untreated, surgical or percutaneous valve replacement remains the primary management.[26] There are currently no medical treatments that can delay disease progression. The pathophysiology of AVS appears to share many similarities with the one of atherosclerosis. However, the biology of AVS is complex and involves a combination of inflammatory activation, increased oxidative stress, activation of valvular myofibroblasts and their osteoblastic transdifferentiation, as well as extracellular matrix remodelling and neovascularization.[27] One of the most important features of severe AVS is calcific changes. In fact, valvular calcification is a highly regulated molecular process characterized by the expression of osteogenic proteins and proteolytic enzymes, such as matrix metalloproteinases (MMPs) and cathepsins that are secreted by activated macrophages and myofibroblast-like cells.[28-30] The combination of these processes leads to a spectrum of abnormalities ranging from mild valve thickening to severe calcification with significantly impaired leaflet motion.[27] To date, the primary clinical marker for recommending aortic valve replacement to a patient with severe AVS is the development of symptoms. However, the challenge of accurately assessing whether or not symptoms are present in patients with AVS is widely known, [31] and it underlines the importance of accessible biomarkers for progression and severity of AVS. Several biomarkers for AVS severity and progression have been suggested, including B-type natriuretic peptide (BNP) levels at rest and during exercise,[32, 33] asymmetric dimethyl arginine (ADMA),[34] homocysteine,[35] tissue plasminogen activator (tPA),[36] leptin,[36] C-reactive protein (CRP),[37] markers of osteoblastic transdifferentiation such as fetuin A,[38] calcium-phosphorus product,[39] and osteopontin,[40] as well as gamma-glutamyl transferase (γGT).[41] Thus, as calcification and ossification are key pathogenetic mechanisms in AVS, any mediator interfering with these processes would be potentially advantageous in this disease. Given the antiatherogenic properties of HDL particles such as inhibition of vascular inflammation, enhancement of endothelial function, and antithrombotic actions, it is conceivable that HDL could play a protective role in

AVS. Indeed, while the role of LDL, the main target of the anti-inflammatory action of HDL, is still controversially discussed in AVS progression,[42] recent evidence suggests that apoA-I concentration is reduced in stenotic aortic valve tissue, and that the latter exerts an anticalcifying effect by inducing the secretion of osteoprotegerin and inhibition of tumor-necrosis factor (TNF)-α expression.[43] Accordingly, previous studies have reported decreased HDL-C levels in patients with AVS as well as an independent and negative prognostic value of HDL-C and HDL-associated enzymes for disease severity in AVS.[44-47] However, these changes were modest and were not validated in larger cohorts of patients. In addition, a prospective observational cohort study of 264 elderly high-risk but mildly dyslipidemic patients with calcified aortic valve disease failed to show any protective effects of HDL-C in the pathophysiology of AVS, but found that elevated HDL-C was the main negative risk factor for significant CAD in this population. [45] Finally, a recent study investigating the correlation of HDL-associated gene variants and HDL functional properties such as cholesterol efflux capacities with markers of AVS progression in 382 participants has challenged the prognostic value of HDL in AVS by showing that circulating HDL metabolism does not seem to predict the risk of AVS.[48]

1.4 HDL: Structure, Function and Metabolism

HDL are macromolecular pseudomicellar complexes, characterized by exhibiting the greatest density (1.063–1.21 g/mL) and smallest size (4–13 nm) among all lipoproteins. They are heterogenous particles differing by size, shape and composition of proteins, lipids, and microRNAs. HDL are synthesized and secreted from the liver and intestine as nascent pre-β-1 HDL or discoid HDL particles, conformed predominantly by apo A-I and phospholipids. These particles then arrive at peripheral tissues and remove free cholesterol (FC) as part of the reverse cholesterol transport (RCT). The protein component of HDL accounts for 55-60% of its mass and screening of this HDL 'proteome' by mass spectrometry technology has identified more than 100 HDL associated proteins including apolipoproteins, enzymes, and their cofactors.[49] The remaining HDL proportion consists of phospholipids and free cholesterol. Due to a continuous exchange of

apolipoproteins and lipids from the core, HDL particles show substantial heterogeneity with regard to their chemical composition, metabolism, and biologic activity.[49] Multiple HDL molecules may associate, forming a hydrophobic nucleus rich in cholesteryl esters (CE), covered by a layer of amphipathic lipids and proteins. HDL molecules also possess important quantities of CE on the lipoprotein surfaces for interaction with associated enzymes involved in HDL metabolism, such as hepatic lipase, cholesteryl ester transfer protein (CETP), Phospholipid Transfer Protein (PLTP), and Lecithin-Cholesterol Acyltransferase (LCAT).[50-52] The HDL proteome is mainly responsible for HDL's antiatherogenic properties including RCT, inhibition of monocyte migration and adherence to the endothelium, prevention of vascular thrombosis, stimulation of endothelial repair, and many others.[53] Accordingly, it is increasingly accepted that HDL composition and, thus, its biological activity rather than HDL-C serum concentration is the parameter determining cardiovascular risk.

1.5 Biological Activities of HDL

Even though RCT was first postulated to be a major contributor to the inverse association between low plasma HDL-C and CAD, there is growing evidence that HDL exert numerous other vaso- and cardioprotective effects (Figure 1, page 6). These include protective anti-inflammatory actions, inhibition of the oxidative modification of LDL, inhibition of vascular inflammation and enhancement of endothelial function by enhanced nitric oxide (NO) release, and angiogenesis.[54] Moreover, HDL has been reported to possess antithrombotic properties and to exert beneficial effects on glucose metabolism.[54] Taken together, these actions seem to result in regression of atherosclerosis. Outlined below are several of these atheroprotective HDL actions.

1.5.1 Anti-inflammatory Effects of HDL

Atherosclerosis is an inflammatory disease involving processes such as adhesion and migration of inflammatory cells into the vessel wall, as well as the action of inflammatory cytokines and chemokines. Therefore, targeting inflammation is a

major strategy in primary and secondary cardiovascular prevention. Interestingly, inflammation is associated with low serum levels of HDL-C and apoA-I, most likely due to increased catabolism and remodelling of HDL.[55] HDL have been shown to interfere with some of these inflammatory processes: HDL inhibit the expression of adhesion molecules such as vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1), and E-selectin, and reduce synthesis

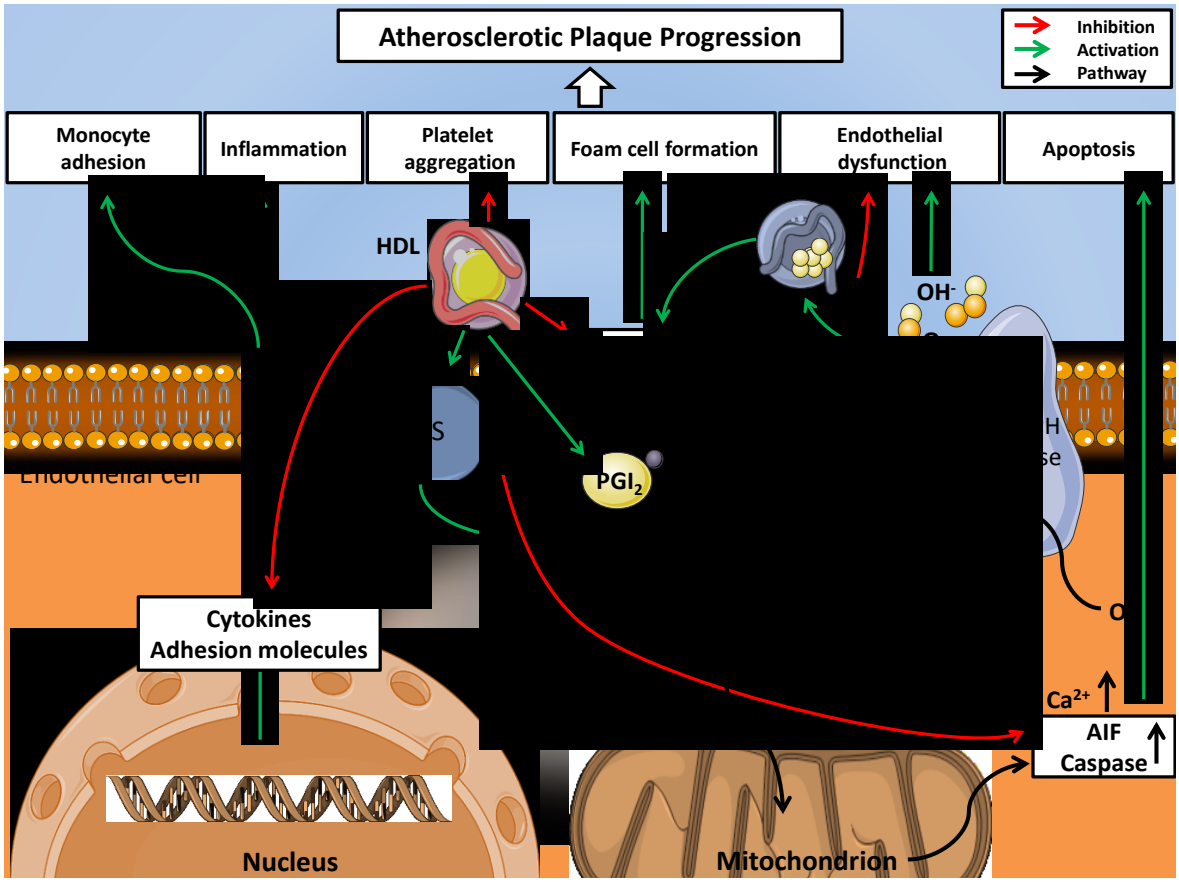

Figure 1: Atheroprotective effects of HDL. Important biological activities of HDL comprise anti-inflammatory (prevention of monocyte attraction and penetration through the vessel wall), immunomodulatory, anti-apoptotic, anti-oxidant (prevention of LDL oxidation), and anti-thrombotic (prevention of platelet activation by stimulation of NO and PGI2 release) actions. AIF, apoptosis inducing factor; NO, nitric oxide; eNOS, endothelial NO synthase; NADPH, nicotinamide adenine dinucleotide phosphate; PGI_2,

of monocyte chemoattractant protein-1 (MCP-1) in endothelial and vascular smooth muscle cells.[56-60] Accordingly, reconstituted HDL (rHDL) treatment inhibited leukocyte infiltration in the artery wall as well as VCAM-1, ICAM-1 and MCP-1 expression in a rabbit model of carotid artery stenosis.[61] Importantly, administration of apoA-I mimetic peptides enhanced the anti-inflammatory properties of HDL in experimental models, while vessel wall inflammation in

patients with CAD remained unaffected after administration of the CETP inhibitor dalcetrapib.[62, 63] Accordingly, C-reactive protein (CRP) levels in patients increased or remained unchanged following CETP inhibitor treatment.[8, 64]

1.5.2 Antioxidant and Anti-Apoptotic Effects of HDL

Oxidation of LDL in the arterial wall is a key mechanism involved in atherosclerosis initiation and progression.[65] Several antioxidant enzymes including PON-1, PAF-acetylhydrolase, reduced glutathione selenoperoxidase and LCAT have been shown to be associated with HDL. In support of the HDL functionality hypothesis, it has been demonstrated that HDL inhibit the formation of oxidized LDL (OxLDL) in healthy subjects, while HDL from patients with cardiovascular disease lack this ability.[66-69] OxLDL is a major activator of endothelial cell apoptosis, an important process in atherosclerosis. *In vitro* studies in endothelial cells have demonstrated that HDL and apoA-I prevent apoptosis induced by OxLDL, TNF-α or growth factor deprivation.[70-72] Of note, it has been demonstrated that small, dense, spherical HDL particles are more effective than larger ones in mediating the antioxidant, anti-inflammatory, antiapoptotic, and anti-infective properties of HDL.[53, 73]

1.5.3 Endothelial Repair and Improvement of Endothelial Function

The endothelium is a highly metabolically active organ that is involved in numerous homeostatic processes, such as the maintenance of a non-thrombotic surface, control of vasomotor tone, leukocyte adhesion, angiogenesis, and regulation of immune responses. In addition, endothelial cells produce a variety of thrombotic and antithrombotic mediators such as plasminogen activator inhibitor 1 (PAI-1), tissue factor (TF), von Willebrand factor (vWF), and P-selectin. HDL promote the production of the atheroprotective endothelial signalling molecule NO by upregulating and stimulating endothelial NO synthase (eNOS) expression via HDL receptor SR-BI.[74] HDL also protect endothelial cells from apoptosis and promotes endothelial cell migration via SR-BI–initiated signalling.[74] It is known that during post-ischemic reperfusion, endothelial progenitor cells (EPC), a subtype of

peripheral blood monocytes that express stem-cell-like antigens such as $CD34^+$, are mobilized. These cells are then rapidly recruited to sites of injury where they inhibit further platelet activation and trigger neovascularization, resulting in improved left ventricular function and a reduction of myocardial necrosis and scar area.[75-77] However, patients with an ACS fail to respond to reperfusion with early EPC mobilization.[78] Persistently low circulating EPC numbers and reduced functional capacity of EPCs following an ACS have a negative impact on event-free survival, and are associated with more significant left ventricular remodelling.[79-81] Thus, there is accumulating evidence suggesting a role for EPCs in the treatment of acute myocardial infarction, and recent studies indicate that beneficial effects of progenitor cell therapy may translate into reduced cardiovascular event rates.[82, 83] Accordingly, *in vivo* studies have reported that infusion of EPCs is associated with regression of atherosclerosis, and recent data indicate that EPC levels show an inverse association with cardiovascular risk factors. This supports the hypothesis that levels of circulating progenitor cells can be influenced therapeutically.[84, 85] Indeed, moderate-dose atorvastatin increased $CD34^+$ cells in patients with myocardial infarction, and systemic rHDL infusion improved the availability of $CD34^+$ cells in patients with type 2 diabetes.[86, 87] Experimental data indicate that rHDL may promote re-endothelialisation by improving EPC levels and functionality.[88] Similarly, low plasma HDL-C levels have been reported to be associated with a decreased number of EPCs.[89] Previous experimental studies have shown that HDL increase expression of eNOS in EPCs and enhance differentiation of human peripheral blood mononuclear cells (PBMCs) to EPCs via activation of the phosphatidylinositol 3-kinase (PI3K)/AKT pathway.[90, 91] Based on these data, it has been postulated that HDL inhibit EPC senescence by promoting telomerase activity via the PI3K/AKT pathway and by inducing NO synthesis.[40] Furthermore, HDL block apoptosis by inhibiting caspase-3 activation, promote EPC-mediated endothelial repair, and have been shown to enhance ischemia-induced angiogenesis.[90-92] Topical administration of HDL to murine vein grafts enhances incorporation of circulating progenitor cells in the endothelium and endothelial regeneration, and reduces atherosclerosis.[93] Although the exact mechanism by which HDL positively influence coronary heart disease has not been fully elucidated, beneficial effects on endothelial repair and function may

significantly contribute to the latter. The fact that heart failure due to ischemic heart disease remains a major cause of morbidity and mortality globally despite substantial advances in pharmacotherapy, device-based therapies, and cardiac transplantation, underlines the need for newer and more efficacious interventions. Whether infusions of rHDL would favourably influence EPCs in the setting of a recent ACS is unknown to our knowledge.

1.5.4 Effect of HDL on Haemostasis and Thrombosis

Antithrombotic and pro-fibrinolytic effects of HDL and apoA-I levels are well documented in numerous studies, indicating that an antithrombotic mechanism underlies the postulated beneficial effects of therapy targeted at HDL.[94] Indeed, elevated HDL-C levels were associated with improved fibrin clot permeability and lysis in healthy individuals.[95] In addition, vWF, a blood glycoprotein involved in platelet aggregation, varies inversely with HDL-C levels, suggesting that HDL exert inhibitory actions on vWF.[96] In support of this notion, preliminary data indicate that HDL and apoA-I interact with ultra-large vWF multimers thereby modifying their adhesive properties in vitro.[97] In addition, a negative association between ADAMTS-13 (a disintegrin-like and metalloprotease with a thrombospondin type 1 motif, member 13) and the risk of cardiovascular events observed in 466 patients with myocardial infarction was influenced by plasma lipid levels, in particular by HDL-C.[98] ADAMTS-13 is assumed to regulate the functional levels of vWF by cleaving ultra-large vWF multimers into smaller, less active particles, thereby reducing its platelet-tethering function. Deficiency of ADAMTS-13 promotes vWF-induced platelet aggregation, which can result in thrombotic complications and formation of early atherosclerosis in an experimental model.[98, 99] Consistent with its antithrombotic properties, atheroprotective effects of ADAMTS-13 have been described in a variety of cardiovascular conditions. [98, 100, 101] HDL also induce NO and prostaglandin I_2 (PGI2) release, both powerful mediators of thromboresistance at the platelet/vessel wall interface.[102-104] Similarly, high HDL-C levels in humans were associated with reduced *ex vivo* platelet thrombus deposition, and direct antithrombotic effects of apoA-I have been demonstrated in an experimental rat model of arterial thrombosis.[105, 106] Interestingly, experimental studies have shown

that these antithrombotic properties of HDL result from modulation of platelet signalling pathways as well as inhibition of intraplatelet cholesterol overload by HDL via SR-BI and ABCG4-dependent mechanism.[107] Of note, however, several observational studies have yielded conflicting results on the association of HDL with platelet reactivity and thrombus formation.[108, 109] These inconsistencies seem to arise from different effects of HDL subclasses on platelet function. Indeed, while HDL_3 exert only minimal antithrombotic effects, HDL_2 have been reported to be strong inhibitors of thrombin-induced platelet aggregation.[110] The antithrombotic properties of native HDL comprise not only inhibitory actions on platelet

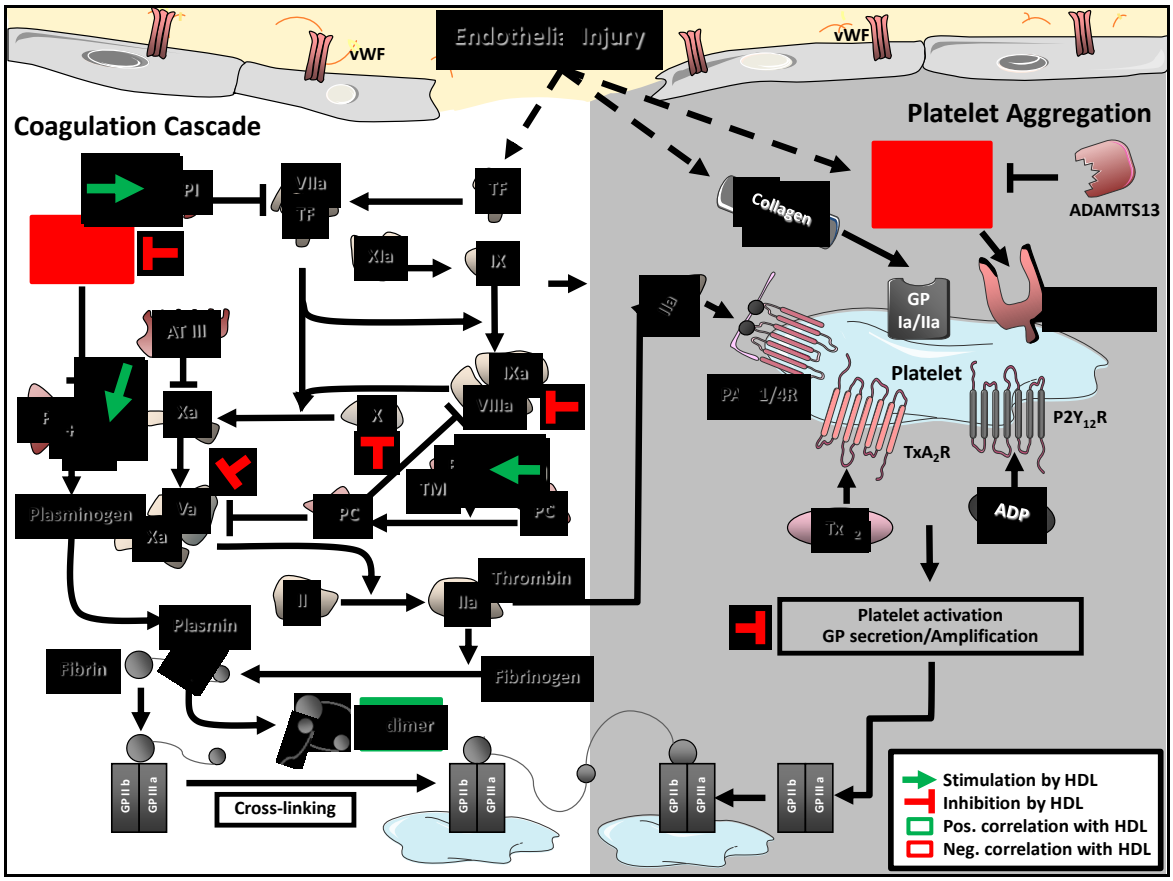

Figure 2: Modulation of the coagulation cascade and platelet aggregation by HDL. TF, tissue factor, vWF, von Willebrand factor, ADAMTS13, a disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13; HDL, high-density lipoproteins; TFPI, tissue factor pathway inhibitor; PAI-1, plasminogen activator inhibitor; µPA, urokinase-type plasminogen activator; tPA, tissue plasminogen activator; ATIII, antithrombin III; PC, protein C; APC, activated protein C; PS, protein S; TM, thrombomodulin; GP, glycopeptide; PAR, Proteinase-activated receptor; TXA_2, thromboxane; TXA_2R, thromboxane A_2 receptor; ADP, adenosine triphosphate.

Figure adapted from Gebhard et al. Ars Medici. 2010.

aggregation but also suppression of the coagulation cascade and stimulation of fibrinolysis. Indeed, HDL also favourably affect the balance between the activities of tissue factor and tissue factor pathway inhibitor, by suppressing the former and enhancing the latter.[111] Further, it has been reported that HDL inhibit coagulation factors Va, VIIIa and X, and an inverse association was found between HDL and prothrombin fragments cleaved from prothrombin during its conversion to thrombin.[112, 113] Further, purified HDL have been shown to enhance protein S-dependent cleavage of factor Va by activated protein C (APC) and rHDL administration enhanced thrombomodulin expression in atherosclerotic lesions resulting in activation of protein C and suppression of thrombin generation.[114, 115] In addition, HDL stimulates directly endothelial production of tPA and inhibits the expression of PAI-1 implying that HDL promotes plasmin generation and thus fibrinolysis.[116] Figure 2 (page 10) outlines the specific anticoagulant effects of HDL and apoA-I.

1.5.4.1 Effect of HDL on Haemostasis and Thrombosis - Distinctive Situation in AVS

An association between AVS and gastrointestinal bleeding (Heyde's syndrome) was first reported in 1958.[117] The haemorrhagic diathesis in those patients was explained by an acquired von Willebrand syndrome found in up to 70% of patients with severe AVS and characterized by hematologic abnormalities and bleeding complications due to a loss of ultra-large vWF multimers (Figure 3, page 12).[118-120] Shear stress-dependent cleavage of the largest vWF multimers by ADAMTS-13 has been claimed to be responsible for the loss of vWF multimers in AVS. Indeed, vWF abnormalities are directly related to the severity of AVS and are improved by surgical or transcutaneous valve replacement in the absence of prosthesis mismatch.[118, 121, 122] Recently, a decline in ADAMTS-13 following aortic valve replacement was observed in patients who underwent aortic valve replacement (AVR), suggesting that a portion of the hematologic benefit of AVR may be due to a postoperative decline in ADAMTS-13 rather than solely to relief of AVS.[123] Data on the effect of HDL on haemostatic parameters in AVS are lacking. However, evidence suggests that HDL and apoA-I may interact with vWF and ADAMTS-13

thereby exerting anti-thrombotic effects.[97, 98] Thus, given the known anti-thrombotic and fibrinolytic effects of HDL, a high dose HDL-based therapy might have the potential to aggravate hematologic abnormalities in patients with AVS.[95] To date, it is not known how anticoagulant effects of HDL and HDL-based therapies might affect haemostatic parameters in moderate or severe AVS.

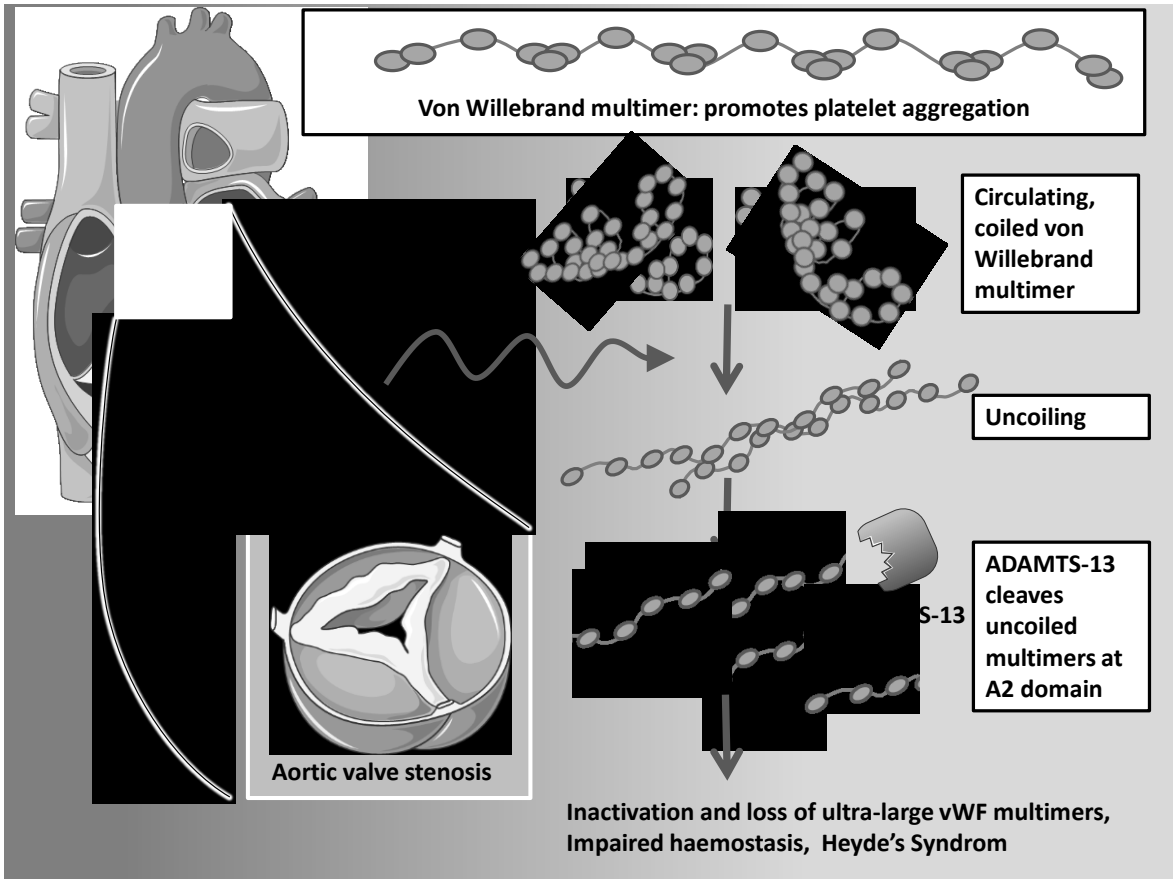

Figure 3: Acquired von Willebrand syndrome in aortic valve stenosis. ADAMTS-13, a disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13; vWF, von Willebrand factor.

1.6 Remodelling of the HDL Proteome in Disease States

Two decades ago, it was first described that HDL lose their inhibitory effect on LDL oxidation during an acute-phase response.[124] Since then, numerous studies have shown that HDL may lose their atheroprotective functions or even gain paradoxically proatherogenic functions in various disease states. In fact, 'dysfunctional' HDL has been observed in patients with diabetes mellitus, CAD and

chronic kidney disease. Under such condition HDL no longer stimulate endothelial nitric oxide (NO) release, and lose their anti-inflammatory and antiapoptotic effects, as well as their ability to inhibit adhesion of white blood cells to activated endothelial cells.[125-128] Oxidative modification of the HDL proteome and lipidome due to reduced levels of HDL-associated paraoxonase-1 (PON-1) has been suggested to account for HDL dysfunction.[125] In addition, posttranslational modification of apoA-I such as glycosylation, myeloperoxidase-mediated oxidation, or proteolysis have been described to lead to HDL dysfunction under various conditions.[129] Importantly, under certain conditions such as during acute phase response, dysfunctional HDL may not only lose their protective effects but may actually become proatherogenic, prooxidant, and proinflammatory.[124] Indeed, dysfunctional HDL are known to exert pro-inflammatory actions by promoting LDL-induced monocyte chemotactic activity. Accordingly, increased HDL-C has been associated with increased coronary events in patients with CAD.[130, 131] The molecular mechanisms involved in this 'HDL remodelling' as well as potential methods to measure HDL (dys-)function are detailed in the following sections.

1.6.1 Protein Targets and Measures of HDL (Dys-)function

1.6.1.1 HDL-C - an Inadequate Indicator of HDL Function

While the cholesterol component of HDL is one of the strongest biomarkers of cardiovascular risk to date, evidence is increasing that this association does not equal causation for HDL. Therefore, the pathogenic role and therapeutic suitability of HDL has increasingly been questioned. In fact, it seems that plasma HDL-C concentration may only represent a 'static snapshot'[132] of cholesterol metabolism and does not reflect any aspects of HDL function including reverse cholesterol transport (RCT). This underlines the importance of targeting HDL functionality for cardiovascular risk assessment and pharmacological intervention. HDL subclasses and the HDL proteome rise as promising candidates for the evaluation of cardiovascular risk. Therefore, reliable surrogate markers are needed to advance the development of novel HDL therapies. These 'surrogate markers' of HDL function may serve as proxies for HDL function and thus the antiatherogenic potential of HDL targeted therapies. However, controversies exist on how to

reliably monitor such functions. Therefore, assays to determine intrinsic HDL characteristics such as quantity, size, and composition are currently under evaluation since they generally benefit from greater ease and precision than methods to interrogate HDL function, facilitating use in large population studies and clinical trials.

1.6.1.2 Measure of HDL-Function: Reverse Cholesterol Transport

A key process in the formation of an atherosclerotic plaque is accumulation of cholesterol in macrophage foam cells. One of the mechanisms by which HDL is considered to reverse or prevent the formation of macrophage foam cells is the removal of excess free cholesterol from cells, and its transport to the liver for elimination in the bile, a process referred to as RCT (Figure 4, page 15). HDL are secreted by the liver and intestine as discoid particles containing lipid-poor apoA-I, and are rapidly loaded with free cholesterol and phospholipids from peripheral tissues through efflux of cholesterol from cells. Four different pathways have been described by which HDLs and apoA-I remove cholesterol from cells: (1) cells can release cholesterol toward HDLs by passive aqueous diffusion, (2) lipid-poor apoA-I can mediate cellular cholesterol efflux via adenosine triphosphate (ATP)–binding cassette A1 (ABCA1) thereby generating nascent pre-β discoid HDL particles, and (3) the latter may then serve as cholesterol acceptors via adenosine triphosphate (ATP)–binding cassette G1 (ABCG1) or (4) scavenger receptor class B type I (SR-BI).[133] Subsequently, lecithin cholesterol acyltransferase (LCAT) esterifies free cholesterol, thereby producing hydrophobic cholesteryl ester that are trapped in the HDL particle's core. Thus, more capacity for additional cholesterol uptake at HDL's surface is generated. The accumulation of cholesteryl esters in the core converts the lipid poor discoid pre-β particles to larger spherical HDL_2 and HDL_3 particles.[134] Cholesteryl ester from HDL_3 are then taken up by the liver either via hepatic SR-BI or via holoparticle endocytosis mediated by interaction of apoA-I with the ectopic *β*-chain of F_0F_1 ATPase and subsequent activation of the nuclear receptors. In addition, as an alternative pathway, cholesteryl esters can be transferred to apoB-containing lipoproteins, very-low-density lipoprotein (VLDL), and LDL by the plasma glycoprotein CETP, in

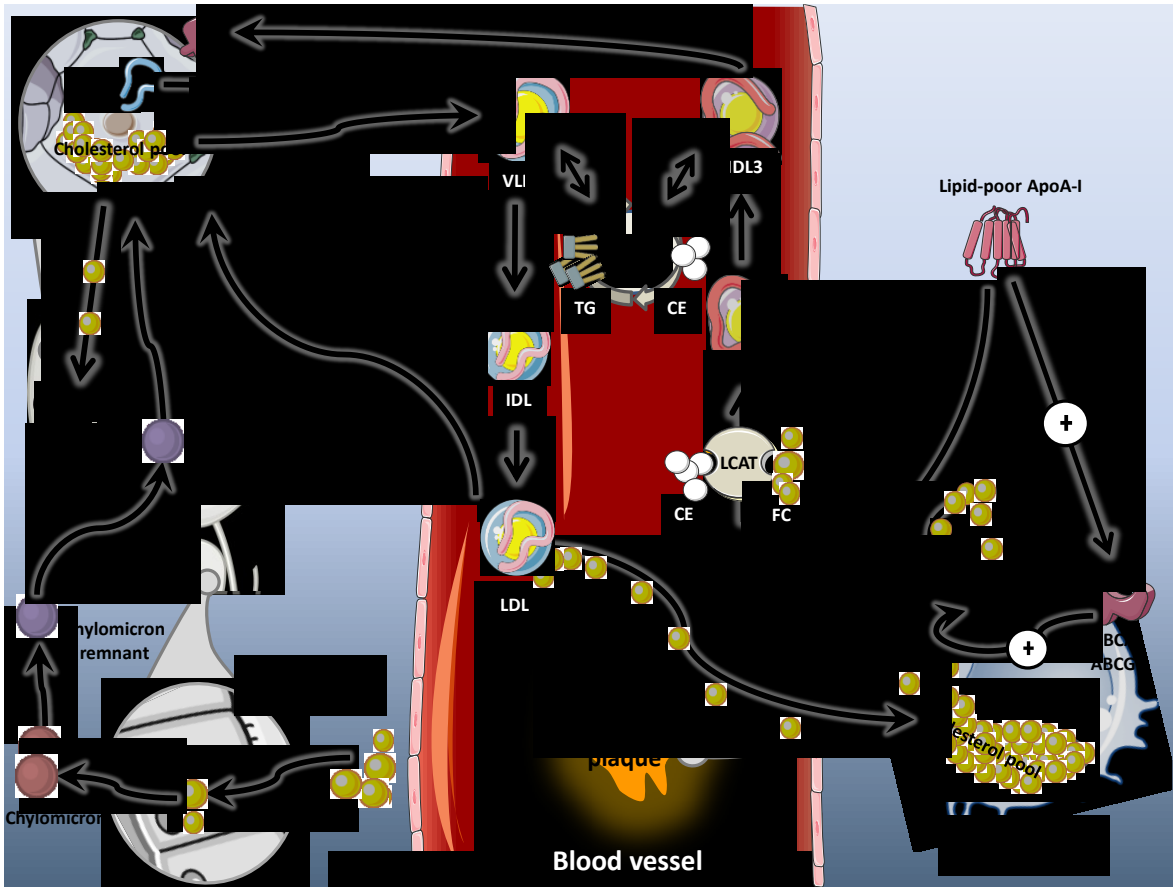

Figure 4: Reverse cholesterol transport (RCT) pathway. ApoA-I, apolipoprotein A-I; HDL, high-density lipoprotein; LDL, low-density lipoprotein, L, intermediate density lipoprotein; ABCA1, ATP-binding cassette transporter A1; ABCG1, ATP-binding cassette transporter G1; SR-BI, scavenger receptor class BI; HDLR, HDL receptor, CETP, Cholesteryl ester transfer protein; LCAT, Lecithin cholesterol acyltransferase; CE, cholesteryl ester; FC, free cholesterol.

exchange for triglycerides.[135] In addition, hepatic and endothelial lipases contribute to HDL remodelling thereby producing smaller, lipid-depleted particles. RCT is believed to play a pivotal role in the atheroprotective functions of HDL and thus, the capacity of the patient's plasma to promote cholesterol efflux from macrophages has been proposed as a measure of HDL function. Indeed, by preventing accumulation of cholesterol in foam cells located in the vessel wall, cholesterol efflux through the RCT pathway maintains cholesterol homeostasis.[132] Accordingly, a recent case-control study has shown that cholesterol efflux capacity of apolipoprotein B (apoB)-depleted serum is inversely associated with cardiovascular risk, independent of HDL levels,[136] thereby supporting pharmaceutical strategies targeting HDL-particle functionality, rather than HDL concentration alone.[137, 138] However, it should be noted that this view has been

challenged recently by a longitudinal cohort study showing that enhanced cholesterol efflux capacity was associated with an increased prospective risk of myocardial infarction, stroke and death.[139] The cause of these paradoxical findings is incompletely understood. While these results certainly support the concept that assessment of HDL function may prove informative in refining our understanding of HDL-mediated atheroprotection, they also underpin the need of additional studies to better understand HDL function.

1.6.1.3 HDL Proteome and HDL Subfractions: Mirror of HDL Functionality?

Plasma HDL are a heterogeneous group of complex macromolecules consisting of amphipathic lipids on the surface and non-polar lipids in the core that differ in structure and biological activity (Figure 5, page 17). HDL particles carry multiple proteins including apolipoproteins, hemopexin, haptoglobin, PON-1, and phospholipid transfer protein, which can all be subjected to posttranslational modification under certain conditions. ApoA-I is the most abundant apolipoprotein by representing up to 70% of the HDL protein mass, followed by apoA-II and other apolipoproteins including apoC, apoE, and apoA-IV.[140] Numerous studies have reported a strong and inverse relationship between apoA-I levels and cardiovascular risk.[141] In fact, apoA-I remained inversely and significantly associated with cardiovascular risk even after adjustment for HDL or apoB in two large cohort studies.[130] Besides apolipoproteins, HDL carry more than 100 different HDL-associated proteins, including complement factors, acute phase proteins, proteases and proteases inhibitors. Importantly, there is evidence indicating that composition of HDL-associated proteins depends on the HDL subclass. In addition, altered HDL composition and enzymatic activities have been named as the key mechanism behind the observation that HDL particles can be functionally defective and might therefore differ significantly in their antiatherogenic properties. Indeed, altered cholesterol content of HDL has been described in metabolic syndrome, diabetes mellitus and CAD.[142-144] Similarly, pharmacologic intervention with CETP inhibitors, niacin, fibrates and certain antidiabetic medications are

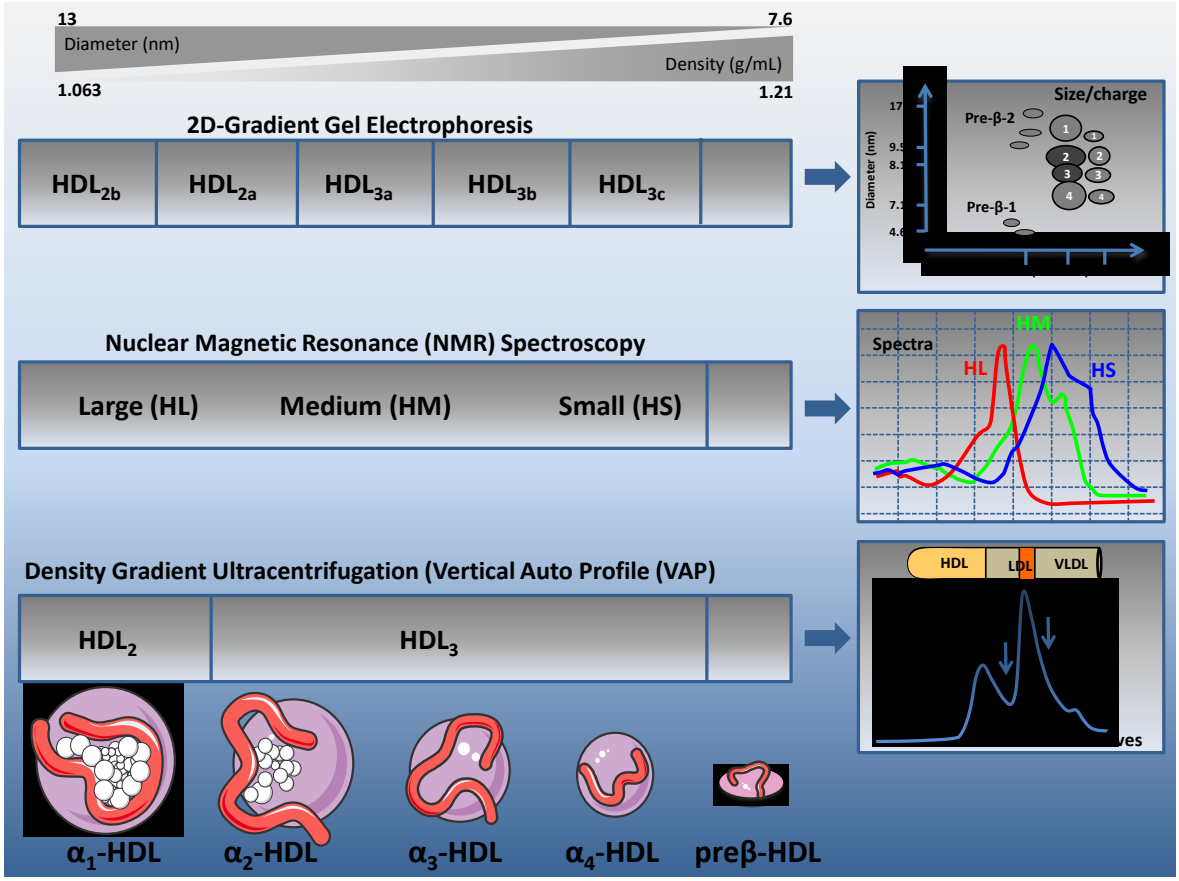

Figure 5: Comparison of lipoprotein fractionation methods. Upper panel: non-denaturing two-dimensional gradient gel electrophoresis can identify five major HDL particles (HDLα1–4 and pre-β1) by separating HDLs first by charge and then by size. Middle panel: nuclear magnetic resonance (NMR) spectroscopy distinguishes lipoproteins by analysing methyl group proton signals. Lower panel: single-spin density gradient ultracentrifugation (Vertical Auto Profile, VAP technique) separates lipoproteins by ultracentrifugation (vertical rotor), after which they are eluted to an autoanalyser and mixed with an enzymatic cholesterol reagent. A spectrophotometric tracing is obtained and mathematically decomposed into individual lipoproteins.

From Gebhard et al. Eur Heart J. 2014, with reprint permission from Oxford University press.

known to alter the distribution of cholesterol among HDL subclasses, indicating that total HDL plasma concentration may not reflect the number of HDL particles under such conditions.[145, 146] In support of this notion, it was recently shown that HDL-C is no longer predictive of cardiovascular risk after adjustment for HDL particle size, and two large epidemiological studies have reported an independent association between cardiovascular events and HDL particle size, suggesting that the latter may serve as a useful tool to assess HDL-directed pharmacotherapies.[147, 148] In addition, small pre-β1 HDL particles, the primary cholesterol acceptors in RCT, do not correlate well with total HDL-C level in

plasma.[149] Accordingly, there is growing evidence that small, dense HDL particles, such as pre-β1 HDL, exhibit more potent anti-atherogenic activity and promote more potently cholesterol efflux from lipid-loaded macrophages than larger HDL particles.[147, 150, 151] In addition antioxidant, anti-inflammatory, cytoprotective, antithrombotic, anti-infective, and endotoxin-neutralizing activities have been attributed to small, protein-enriched, cholesterol-depleted HDL particles but not to larger HDL particles.[53, 73]. Notably, CETP inhibitors increase the mean particle size of HDL, thereby leading to a rise in larger, less atheroprotective HDL particles.[152] The latter, however, have been shown to be associated with an increased risk of adverse cardiovascular events, a fact that might, at least in part, explain the lack of benefit from CETP inhibition in CAD.[14, 130] Even though data on HDL subclasses are encouraging, HDL subfraction analysis is still in its early years, and conclusions about HDL particles and associated proteins as a biomarker for disease are limited by the small number of published studies and the differences in analytical procedures that are far to be adapted to the clinical laboratory (Figure 5, page 17). Indeed, different methods such as nuclear magnetic resonance spectroscopy,[153] ion mobility,[154] as well as analytical methods based on electrophoretic mobility and apolipoprotein composition have been used to quantify HDL-subclasses (Figure 5, page 17). Thus, future studies will have to validate quantification of HDL particles by these methods. Nevertheless, quantification of the concentration of HDL particles may be useful to refine cardiovascular risk, and it will be important to extend the lessons learned from static HDL measures to functional assays that may provide important insights into the diverse antiatherogenic effects of HDL. Finally, studies incorporating plasma HDL-C concentrations, HDL particle number, HDL subpopulation heterogeneity and molecular composition, and the ability of HDL particles to mediate anti-atherogenic functions are needed to understand the association between HDL-P and cardiovascular outcomes.

1.6.1.4 Indicator of HDL Dysfunction: HDL Inflammatory Index

The HDL inflammatory index is a measure of HDL inflammatory/anti-inflammatory properties. It has been shown to be significantly increased in patients with atherosclerotic cardiovascular disease as compared to healthy control subjects.[131] It is quantified as the ratio of *in vitro* LDL oxidation of a fluorescein substrate incubated with and without a patient's HDL. Therefore, an HDL inflammatory index > 1 indicates that HDL in these patients has not only lost their anti-inflammatory activities, but are even exerting pro-inflammatory actions. Since the HDL inflammatory index has been demonstrated to better distinguish patients with atherosclerotic disease from healthy controls than HDL plasma levels, it has been suggested as an indicator of HDL functionality.[131]

1.6.1.5 CETP and LCAT – Features of Functional HDL?

CETP is a hydrophobic plasma glycoprotein that transfers cholesteryl esters from HDL to ApoB-containing lipoproteins in exchange for triglycerides, and therefore lowers plasma HDL-C levels.[155] Accordingly, CETP inhibition leads to accumulation of HDL particles and HDL-C in the plasma compartment, and it has been assumed that this may result in an enhanced RCT leading to atherosclerosis regression. However, studies of the effect of CETP gene transfer and CETP inhibitors on experimental atherosclerosis in animals, as well as studies in humans with hereditary disorders of CETP, have produced largely varying results.[156-161] Against this background of uncertainty, several CETP inhibitors including torcetrapib, dalcetrapib and evacetrapib, have been tested in clinical phase III studies. However, these studies were terminated prematurely because of an increase in cardiovascular events (torcetrapib) or because of lack of benefit (dalcetrapib and evacetrapib) even though HDL-C was significantly increased.[7, 8, 162] These disappointing results have led to the understanding that antiatherogenic therapies must address RCT for which functional features of the treatment-induced HDL particles, and not HDL-C quantity, are critical. This notion is supported by recent epidemiological findings, demonstrating that low CETP activity per se is likely to be detrimental independently of lipoprotein concentrations.[163-168] Taken together, it seems that low CETP activity might be accompanied by reduced rather

than enhanced RCT because of anti-inflammatory actions of CETP and its ability to generate atheroprotective pre-β HDL particles, the primary acceptor of cellular cholesterol.[169, 170] At present, one Phase III trial of the CETP inhibitor anacetrapib is still in progress and is due to complete by the end of 2017. Of note, inhibition of CETP by anacetrapib led to improved HDL particle function which is mirrored by enhanced cholesterol efflux and anti-inflammatory actions, making this compound a more promising candidate in atheroprotection than its precursors.[171]

Lecithin: cholesterol acyltransferase (LCAT), a 67-kDa glycoprotein of 416 amino acids, was first discovered in 1962.[172] LCAT is synthesized mainly in the liver and is one of the key players involved in HDL maturation and remodelling.[172-174] LCAT esterifies free cholesterol to cholesteryl esters in plasma by using apoA-I as a cofactor.[172] The LCAT reaction comprises two steps: fist, LCAT cleaves the fatty acid in the sn-2 position of phosphatidylcholine and transfers it onto a serine residue; then, the fatty acid is transesterified to the 3β-hydroxyl group on the A-ring of cholesterol to form cholesterol ester and lysolecithin.[175] Since cholesterol ester is more hydrophobic than free cholesterol, the latter drifts into the hydrophobic core of HDL. About 75% of plasma LCAT activity is associated with HDL. The remaining 25% of LCAT activity are bound on apoB containing lipoproteins such as LDL. Since LCAT plays a central role in HDL metabolism and RCT, the LCAT reaction is believed to explain HDL-mediated atheroprotection.

1.6.1.5.1 Controversial Role of LCAT and Cholesterol Esterification in Atherosclerosis

Proper LCAT function is crucial in maintaining normal plasma levels of HDL-C.[172] Therefore, it was suggested that LCAT promotes RCT and might play an important role in mediating atheroprotective effects of HDL. Accordingly, a defect in LCAT function was thus expected to enhance atherosclerosis by interfering with HDL maturation and RCT. However, despite the well-described role of LCAT in HDL metabolism and cholesterol trafficking, the effect of LCAT on atherogenesis remains controversial.

Indeed, previous studies have yielded conflicting results: genetic LCAT deficiency in humans resulted in very low HDL-C plasma levels, but is not

associated with premature atherosclerosis in affected individuals.[176-181] Thus, it was speculated that a defective LCAT enzyme may not preclude efficient RCT, and the paradoxical finding of complete HDL deficiency and absence of atherosclerosis in LCAT-deficient patients was used to reject the hypothesis that HDL is important in atheroprotection. In support of the former hypothesis, it was found that LCAT-deficient mice display a preserved ability to deliver peripherally derived cholesterol to the liver despite severely reduced HDL levels. Thus, it was suggested that high levels of pre-β HDL and enhanced ABCA1-mediated cholesterol efflux may preserve RCT under conditions of low HDL-C.[180, 182] Importantly, it was also shown that cholesterol can efficiently be transported to the liver without being esterified, and decreased LDL and apoB levels have also been put forward as an explanation for the lack of marked CAD in LCAT deficiency.[183] In addition, the relation of plasma LCAT mass or activity with atherosclerotic vascular disease or future cardiovascular events in subjects without genetic deficiency of this enzyme remains elusive to date since cross-sectional studies have yielded largely inconsistent results.[184-191] Similarly, investigations in various animal models overexpressing or lacking the LCAT gene have not provided clear answers.[192] In fact, overexpression of LCAT in rabbits resulted in reduced atherosclerosis, while the contrary was observed in mice.[193-195] The contradictory outcomes in previous studies are difficult to interpret and may result from population differences and the fact that no standardized method exist to measure LCAT activity. In addition, previous reports indicate that LCAT activity depends on HDL particle size and is interrelated with triglycerides and LDL metabolism.[196] Thus, if not controlled for in multivariate models, the latter might have confounded possible associations between angiographically assessed CAD and LCAT activity parameters in clinical studies.[197] In summary, even though loss of function LCAT mutations result in HDL deficiency, it is not clear whether LCAT is required for effective HDL function in macrophage cholesterol efflux and atheroprotection. In addition, it is unknown whether plasma LCAT activity can modify the association of HDL and cardiovascular risk. However, since promotion of cholesterol efflux through the RCT pathway may be a key atheroprotective function of HDL, it will be crucial to understand the biological underpinnings of the LCAT pathway to develop therapies targeting HDL. Currently, recombinant LCAT therapy in patients with familial LCAT

deficiency is in an advanced stage of development. Indeed, in a phase I study, recombinant LCAT administration was proven to be safe and well tolerated.[198, 199] Thus, it is anticipated that enzyme replacement studies will help to clarify the divergent role of LCAT in atherosclerosis.

1.6.1.5.2 Potentially Beneficial Functions of LCAT

Besides its role in cholesterol remodelling and maturation, LCAT exerts various effects that might affect the development of atherosclerosis. In fact, LCAT also transesterifies and hydrolyses platelet-activating factor (PAF) and oxidized phospholipids with long chains in the *sn*-2 position.[200-202] Therefore, it has been suggested that LCAT contributes to the antioxidant and anti-inflammatory actions of HDL.[68] In support of this hypothesis, carriers of LCAT mutations present with a markedly reduced potential to inhibit LDL oxidation, an important factor in atherosclerotic plaque progression.[203] However, considering that the HDL proteome consists of more than 50 different proteins, it remains unclear to date if the reduced antioxidant properties of HDL associated with LCAT deficiency are a direct effect of impaired LCAT function, or related to concomitant anomalies of the HDL proteome.[134] In addition, adrenals from LCAT deficient mice have been shown to be severely depleted of cholesterol, a key component in the production of glucocorticoids. Since LCAT is not expressed in adrenal tissue, severe depletion of plasma HDL-C and subsequent reduction of adrenal lipid delivery might account for the reduced adrenal cholesterol content in LCAT deficient animal models.[204] The latter might lead to a severe reduction of adrenal glucocorticoid production which is known to exert vascular anti-inflammatory actions. Thus, altered glucocorticoid production due to LCAT deficiency may result in enhanced vessel wall inflammation, an initial step in the development of atherosclerosis. In humans, LCAT activity was positively associated with obesity and insulin resistance, important risk factors for CAD.[205-207] Further, thrombin binding and thus platelet activation seems to be altered in platelets from LCAT deficient patients.[208] However, data are derived from small populations and further research is warranted to study the association of LCAT and platelet aggregation. In summary, in addition to its role in RCT, LCAT interferes with several

physiological and pathophysiological processes that might alter the development of atherosclerosis. Further studies investigating the contribution of LCAT activity in atheroprotection are warranted.

1.6.2 Role of Proteases in HDL Dysfunction

There is accumulating evidence that several proteases proteolytically modify HDL thereby affecting its function (Figure 6, page 27). Proteases fall into five main groups, depending on the nature of their active site: metallo-, serine, cysteine, threonine, and aspartic acid proteases. Amongst those, metallo-, serine, and cysteine proteases have previously been shown to impair HDL function.

1.6.2.1 Metalloproteinases

MMPs, a family of at least 16 proteases specialized in degrading various components of extracellular matrix, are secreted by macrophages which are highly abundant in atherosclerotic plaques and diseased aortic valve tissue.[209-211] Even though MMPs play important roles in vascular remodelling and repair, dysregulation of their activity has been shown to promote atherosclerotic plaque growth and plaque destabilization.[212, 213] The MMP family includes MMPs-1, -2, -3, -7, -9, and -12 [214-218] of which MMP-3 (stromelysin-1), MMP-7 (matrilysin), MMP-8 (neutrophil collagenase), and MMP-12 (human metalloelastase) have previously been shown to truncate the carboxyl terminus of apoA-I, thereby reducing its capacity to promote reverse cholesterol efflux from macrophages (Figure 6, page 27).[219, 220] Indeed, the carboxyl-terminal domain of apoA-I is especially important for promoting cholesterol efflux from cholesterol-loaded macrophages, and incubation of HDL_3 with MMPs led to a 70% loss of its cholesterol efflux promoting ability.[219] The latter has led to the hypothesis that C-terminally truncated apoA-I may be a potential novel biomarker for atherosclerosis. MMP-2 is the main MMP secreted by mesenchymal cells, whereas MMP-3, MMP-9, MMP-7 and MMP-12 are synthesized by inflammatory cells.[221] Extracellular matrix remodelling, including collagen synthesis and elastin degradation by MMPs, contributes to aortic valve leaflet stiffening and calcification.[27] Osteogenic differentiation of

myofibroblasts and smooth muscle cells represents an essential part in the pathogenesis of AVS and evidence suggests that this process is triggered by elastin derived peptides which are released due to stimulation or cleavage by macrophage-derived MMPs.[222-225] Accordingly, MMP-3 and MMP-9 activity was found to be significantly increased in stenotic aortic valves.[226] In addition, MMP-1 has recently been shown to be expressed on tissue macrophages in calcific aortic stenosis thereby inducing transmigration in an artificial valve model.[227] Further, MMP-3 seems to mediate vascular smooth muscle cell migration and neointima formation in mice,[228] while a selective MMP-12 inhibitor inhibited monocyte/macrophage invasion and reduced macrophage accumulation in an experimental atherosclerosis model.[229] Accordingly, MMP-7 and MMP-12 have recently been found to be independently associated with more severe atherosclerosis and an increased incidence of acute coronary events.[230] In addition, MMPs and their tissue inhibitors (TIMPs) are expressed in pressure-overloaded human myocardium during heart failure progression, underlining their value as a marker of severe disease and decompensation.[231, 232] Further, circulating MMP-3 has been suggested as a marker of ventricular arrhythmia, a life threatening complication, in adolescent patients with hypertrophic cardiomyopathy.[233] Of note, elevated circulating MMP levels have been measured in patients with hypertensive heart disease and correlate directly with an increase in myocardial MMP expression.[234] MMP inhibitors are commercially available and their safety profile has previously been tested in clinical trials. [235]

1.6.2.2 Serine Proteases

Amongst serine proteases, the mast cell proteases chymase,[236-238] chymotrypsine and tryptase,[239] as well as plasmin, subtilisin, kallikrein, and Staphylococcus V8 protease[240-243] have all been shown to degrade lipid-free apoA-I or to deplete the minor subpopulation of preβ-HDL, and so impair the high-affinity efflux of cholesterol from macrophage foam cells that is promoted by HDL_3.[236, 244-246] Chymase, a chymotrypsin-like serine protease that is primarily found in mast cells and in basophil granulocytes, is involved in a variety of functions, including immune responses to parasite antigens, and conversion of angiotensin I to

angiotensin II. Mast cells accumulate in the adventitia of human atherosclerotic plaques and may contribute to plaque progression and instability.[247] Accordingly, mast cell chymase inhibition reduces atherosclerotic plaque progression and improves plaque stability in apoE-/- mice.[248] In addition, mast cells can be found in high amounts in stenotic aortic valves and may accelerate the progression of aortic valve stenosis by altering the balance between angiogenic and antiangiogenic factors.[249] Moreover, human genetic studies have associated mutations in the chymase gene with left ventricular mass in male patients with symptomatic aortic stenosis.[250] Accordingly, mast cell chymase release has been demonstrated to be involved in adverse cardiac remodelling in an experimental model of left ventricular pressure overload,[251] with the latter being frequently observed in patients with severe AVS.[252] Similar to angiotensin-converting enzyme (ACE), chymase converts angiotensin I to angiotensin II, which in turn is a powerful mediator of inflammation and fibrosis and is known to promote hypertrophy of cardiac myocytes and hyperplasia of cardiac fibroblasts.[253] Importantly, both chymase and ACE are upregulated in stenotic aortic valves, indicating that therapeutic interventions aiming at inhibiting these processes may slow AVS progression.[254] In this regard, the specific chymase inhibitor RO5066852® has recently been introduced and has shown promising results in vitro and ex vivo.[248] Chymase degrades apoA-I by truncating its amino- or carboxy terminus at tyrosine 18, phenylalanine 33 or phenylalanine 225, respectively (Figure 6, page 27).[238, 255] Moreover, tissue-specific activation of mast cells with ensuing release of chymase also seems to proteolytically inactivate apoA-I in the environment of activated mast cells, thereby locally impairing macrophage reverse cholesterol transport in vivo.[256]

1.6.2.3 Cysteine Proteases

Several cysteine proteases including cathepsin B, cathepsin F, cathepsin S and endoproteinase-Arg-C (Clostripain) can degrade apoA-I and HDL_3 thereby reducing the efficiency of these particles as acceptors of macrophage cholesterol.[257, 258] The cysteine protease cathepsin S is found in lysosomal/endosomal compartments of antigen presenting cells, such as B cells, macrophages and

dendritic cells, and can also be induced in adipocytes and vascular smooth muscle cells. It cleaves apoA-I at its carboxy-terminal end, thereby blocking its capacity to induce cholesterol efflux (Figure 6, page 27).[257, 259] Cathepsin S plays an important role in antigen presentation and is constitutively secreted into the extracellular space by monocyte-derived macrophages. It also contributes significantly to proteolysis of elastic laminae.[259, 260] During progression of vascular and valvular heart disease, a disruption of the balance between cathepsin and its inhibitor cystatin C occurs and previous studies have shown that cathepsin S-induced elastolysis contributes to the pathogenesis of atherosclerosis and accelerates aortic valve calcification.[259, 261, 262] Similar to MMPs, cathepsins degrade elastin, thereby generating soluble peptides.[222, 263] These elastin-derived peptides serve as biologically active molecules that are highly chemotactic for macrophages and trigger the release of bone-regulating proteins from vascular smooth muscle cells and valvular myofibroblasts, thus leading to plaque growth and calcification. [224, 225, 264, 265] Importantly, human genetic studies link mutations in the elastin gene to calcific AVS.[28, 266] Accordingly, the contribution of cathepsin S to the pathogenesis of atherosclerosis [259, 261] and AVS[267] has previously been established. Of note, also cultured vascular smooth muscle cells, when stimulated with Interleukin-1b or Interferone-c, secrete catalytically active cathepsins, and a possible role for mast cell derived cathepsins in adverse valve remodelling and AVS progression was recently suggested.[260, 268] Thus, given its involvement in several key pathways triggering inflammation, fibrosis and calcification, cathepsin S may represent an important link between inflammatory, pro-fibrotic and pro-osteogenic molecular mechanism contributing to atherosclerotic vascular disease as well as AVS progression. In addition, given its role in antigen presentation, its inhibition is expected to result in immunosuppression, making this enzyme an attractive target to potentially treat autoimmune and inflammatory diseases. Indeed, a previous study demonstrated that cathepsin S is significantly elevated in synovial fluid of patients with rheumatoid arthritis[269] and the efficacy of the orally taken cathepsin S inhibitor RO5459072 is currently being evaluated in patients with Sjogren's syndrome (NCT02701985).

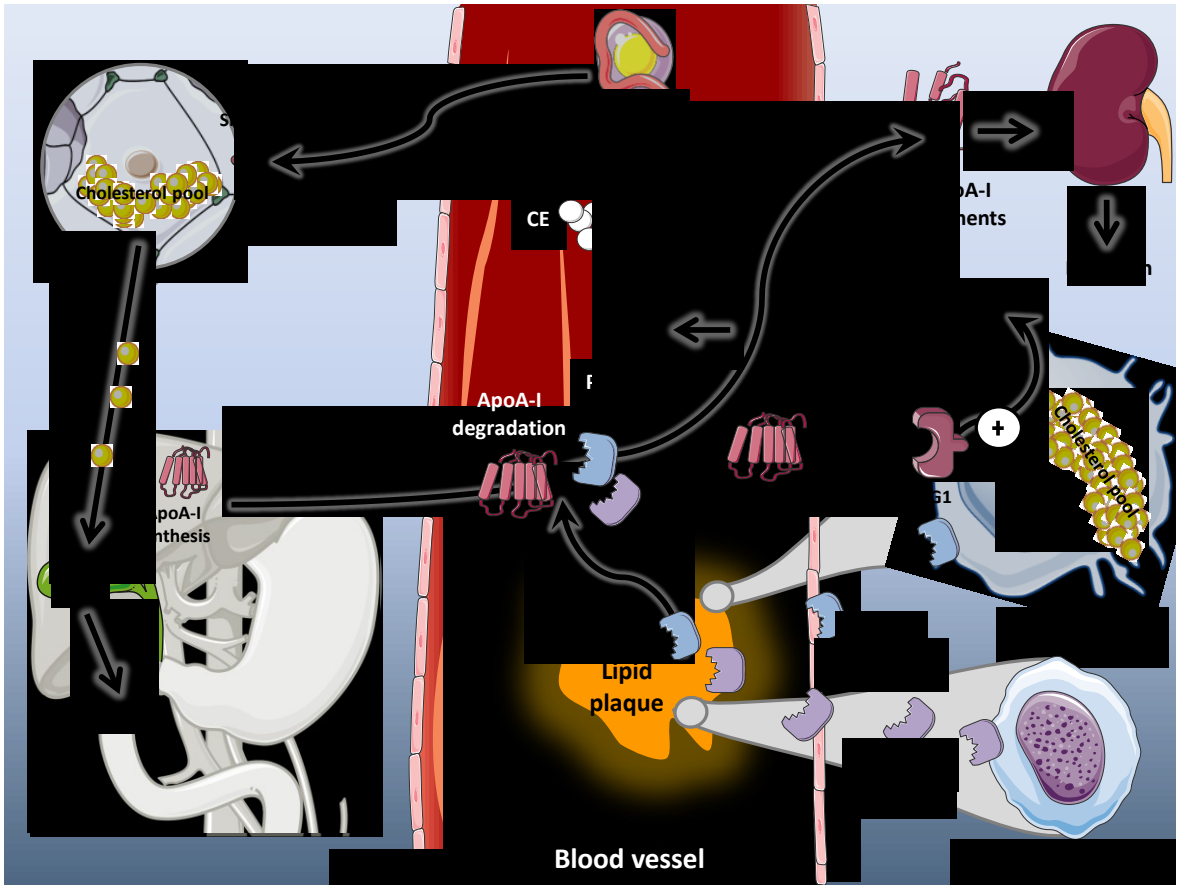

Figure 6: ApoA-I degradation by proteases secreted by activated mast cells and macrophages/foam cells in atherosclerotic plaques. MMP, metalloproteinase; ApoA-I, apolipoproteinA-I; HDL, high-density lipoprotein; ABCA1, ATP-binding cassette transporter A1; ABCG1, ATP-binding cassette transporter G1; SR-BI, scavenger receptor class B; HDLR, HDL receptor; LCAT, Lecithin cholesterol acyltransferase; CE, cholesteryl ester; FC, free cholesterol.

1.6.2.4 Clinical Importance of Proteolytic ApoA-I Degradation

Elucidation of the effects of proteases on apoA-I and its cholesterol efflux promoting and other properties remains an important challenge for future studies. The proteolytic modifications of apoA-I may turn out to be of major importance in blocking the physiological functions of apoA-I in the arterial intima, such as mediation of the initial steps of RCT from human atherosclerotic lesions consisting of numerous macrophage-derived foam cells (Figure 6, page 27). In addition, apoA-I breakdown by circulating recombinant t-PA (rt-PA) has been described in patients with ACS following thrombolytic treatment, thereby providing a potential mechanism for the apparent reduction of benefit of facilitated versus nonfacilitated percutaneous coronary intervention (PCI).[270] Of note, since truncated ApoA-I can

be found in minimal amounts in normal human serum, its possible application as biomarker for progression of atherosclerosis has recently been suggested.[238]

1.7 Outcome Parameters Used in Clinical Studies Assessing HDL

Clinical cardiovascular endpoints such as fatal or non-fatal myocardial infarction, cardiac death and vessel revascularisation provide the most conclusive and complete information on the efficacy and safety of novel anti-atherosclerotic treatment strategies. However, surrogate cardiovascular endpoints are increasingly used as a replacement for these morbidity and mortality endpoints in clinical trials. Even though these surrogate endpoints may not be the true predictor of a clinical outcome and may not yield a quantitative measure of clinical benefit that can be weighed directly against adverse, they offer the advantage of a smaller sample size and the ability to bring effective therapies quickly to clinical practice. Surrogate endpoints frequently used to evaluate HDL-targeted therapies include plaque size, plaque composition, plaque vulnerability and stability, as well as arterial remodelling. Vascular imaging modalities characterizing these plaque properties are developing rapidly and include both more traditional approaches such as quantitative coronary angiography, intravascular ultrasound, and carotid intima-media thickness (cIMT), as well as newer optical methodologies. cIMT depicts the distance between blood-intima and media-adventitia layers of the carotid artery wall, and is one of the best-established and most commonly used surrogate markers of atherosclerosis.[271] Indeed, it correlates with many cardiovascular risk factors and with the severity of CAD, and it has been demonstrated to predict clinical events.[272] In addition, cholesterol efflux capacity from macrophages was shown to have a strong inverse relation with cIMT, independently of the HDL cholesterol level.[136] Historically, quantitative coronary analysis has been the reference method for the angiographic assessment of CAD severity and progression.[273] However, intravascular ultrasound imaging (IVUS) has emerged as a more sensitive tool for the assessment of plaque severity and morphology compared to quantitative coronary angiography (QCA) or cIMT.[274] In contrast to QCA, IVUS allows to directly visualize the arterial wall and to assess artery remodelling. Therefore, IVUS has become the gold standard for studying

plaque development and assessing the effectiveness of pharmacological treatments on plaque's evolution. Interestingly, endothelial function assessment has recently been shown to predict clinical outcomes in patients treated with the CETP inhibitor dalcetrapib, indicating that changes in flow-mediated vasodilation are a reliable surrogate endpoint in predicting treatment effects in high-risk patients.[275]

1.8 Current HDL-targeted Therapeutic Approaches

1.8.1 HDL- and ApoA-I-derived Peptides

Because of their ability to enhance cholesterol efflux from the arterial wall and their anti-inflammatory properties, HDL theoretically hold the potential to be effective in patients with CAD as a substantial proportion of these patients present with low levels of HDL-C (Figure 7, page 32).[276] In addition, apoA-I, which comprises 70% of HDL protein content, has been shown to be antiatherogenic. Indeed, many intervention studies in experimental atherosclerosis have proven the atheroprotective potential of apoA-I and HDL-based treatments.[277-286] Importantly, changes in atherosclerotic plaque characteristics observed in these studies occurred quickly and usually only after one high dose of treatment.[287] However, studies assessing the effect of rHDL or apoA-I on atherosclerotic plaque burden in humans have yielded mixing results thereby underlining the complex biology of HDL function: The **E**ffect of **r**HDL on **A**therosclerosis **S**afety and **E**fficacy (ERASE) trial demonstrated changes in plaque characterization indices, as quantified by IVUS, following 4 weekly infusions of rHDL in patients with recent ACS.[288] Further refinements of this apoA-I derived formulation (CSL112) have been tested in phase II trials that demonstrated its safety and tolerability in patients with CAD as well as enhanced cholesterol efflux without major adverse effects. [289, 290] In a recent study in post-ACS patients, 7 weekly infusions of the patient's own delipidated HDL resulted in a decrease in total atheroma volume measured by IVUS.[291] Since IVUS-derived measures and plaque characteristics have been reported to be predictive of future adverse cardiovascular events,[292, 293] these data indicate that HDL-based therapeutic approaches may have the potential to improve plaque stability and subsequent clinical outcomes in ACS patients. In

addition, rHDL or apoA-I infusion resulted in improvements of plaque volume and endothelial function in patients with CAD[288, 294, 295] and beneficial effects of direct apoA-I infusions on coronary atherosclerosis in patients with ACS have been demonstrated.[295] In contrast, human rHDL infusion did not improve vascular function, markers of vascular inflammation, and oxidative stress in patients with ACS on optimal background therapy.[296] Further, a randomized trial using the synthetic HDL mimetic CER-001 (Cerenis Therapeutics, Toulouse, France) failed to show atherosclerosis regression in 507 patients with ACS, thereby supporting the hypothesis that atheroprotective actions of HDL may be influenced or modified under certain conditions.[297] A second trial, Modifying Orphan Disease Evaluation (MODE), tested CER-001 in patients with homozygous familial hypercholesterolemia and showed a reduction of atherogenic changes in the carotid artery while no significant changes in HDL-C or LDL-C were observed. [298]

Of note, although there is a benefit with rHDL-based therapy in some studies, the clinical cost of such therapy would be very high. Indeed, rHDL are difficult to produce and cumbersome to administer. Therefore, the search for cheaper alternatives has generated several apoA-I mimetic peptides. These 18-22 amino acid amphipathic peptides differ in their primary amino acid sequence structure from apoA-I (which contains 243 amino acids) but mimic the secondary helix-like structure of apoA-I. They are structurally diverse, allowing the selection of peptides with greater potency and better pharmacokinetic profiles than apoA-I itself. While many of these peptides (ETC-462, D4F, L4F, 5A, and ATI-5261) have shown great promise in various experimental models of disease,[62, 299, 300] only two studies have assessed their use in humans. In patients with CAD, apoA-I mimetics significantly improved the HDL inflammatory index, a measure of HDL function.[301] However, failure of a subsequent clinical study[302] and the finding that high-doses of apoA-I mimetic have to be administered to achieve sufficient peptide levels in the small intestine[303] point out that more refined strategies are needed for HDL-based therapeutic approaches. In this regard, RVX-208, a bromodomain and extra-terminal (BET) protein inhibitor that selectively simulates apoA-I production has been developed. However, while this novel compound enhanced cholesterol efflux capacity in serum from patients treated with RVX-208, a recent trial failed to show any effect of RVX-208 on coronary artery disease progression in patients with

symptomatic CAD and low HDL-C.[304, 305] Currently, other emerging HDL-based therapies including P_2Y_{13} receptor agonists (CER-209), selective liver X receptor agonists, and antisense oligonucleotides targeted to several nodes in the HDL pathway are in early development stage and might overcome the shortcomings of other compounds.[306] Shown in Figure 7 (page 32) are the different drug targets to increase HDL levels.

1.8.2 Niacin, Fibrates, Statins

Amongst the currently available lipid-modifying agents, niacin has the greatest HDL-C-raising effect. Despite adherence problems due to side effects of niacin, such as flushing and gastrointestinal irritation, the efficacy of niacin in reducing cardiovascular events in secondary prevention has been proven by many randomized trials.[307-312] However, many of these trials have been conducted prior to the routine use of intensive statin therapy. Indeed, results of the more recent AIM-HIGH (Atherothrombosis Intervention in Metabolic Syndrome with Low HDL/High Triglycerides: Impact on Global Health Outcomes) trial have questioned the effectiveness of adding niacin in statin-treated patients.[313] In fact, although niacin therapy significantly increased HDL-C levels and lowered triglycerides and LDL levels (Figure 7, page 32), there was no further risk reduction from the addition of niacin to statin therapy, and the trial was stopped prematurely because of lack of efficacy.[313] Similarly, the HPS2-THRIVE (Heart Protection Study 2: Treatment of HDL to Reduce the Incidence of Vascular Events) study was stopped because of an increased incidence of myopathy rates in the extended release niacin/simvastatin arm.[314]

Fibrates act on the peroxisome proliferator receptor alpha (PPAR-α), thereby lowering plasma triglycerides and LDL, and raising HDL-C by 6% - 20% (Figure 7, page 32).[315] However, the efficacy of fibrates in reducing cardiovascular risk has been controversially discussed. While the Helsinki Heart Study and the Veterans Affairs HDL Intervention Trial (VA-HIT) demonstrated a cardiovascular risk reduction in patients treated with fibrates,[316, 317] the Bezafibrate Infarction Prevention (BIP) and the Fenofibrate Intervention and Event Lowering in Diabetes (FIELD) studies failed to show any cardiovascular risk reduction in the fibrate

treatment arm.[318, 319] However, a meta-analysis comprising 18 studies showed a modest (10%) but significant risk reduction of cardiovascular events in patients treated with fibrates.[320] In addition, fenofibrate has been shown to be the only lipid-lowering agent capable of improving microvascular complications such as diabetic retinopathy in diabetic patients.[321, 322] The Action to Control Cardiovascular Risk in Diabetes (ACCORD) trial was designed to test whether a combination therapy comprising statin and fibrate leads to a greater risk reduction than statins alone in diabetic patients. However, no significant difference was observed in the fibrate group as compared to placebo.[323]

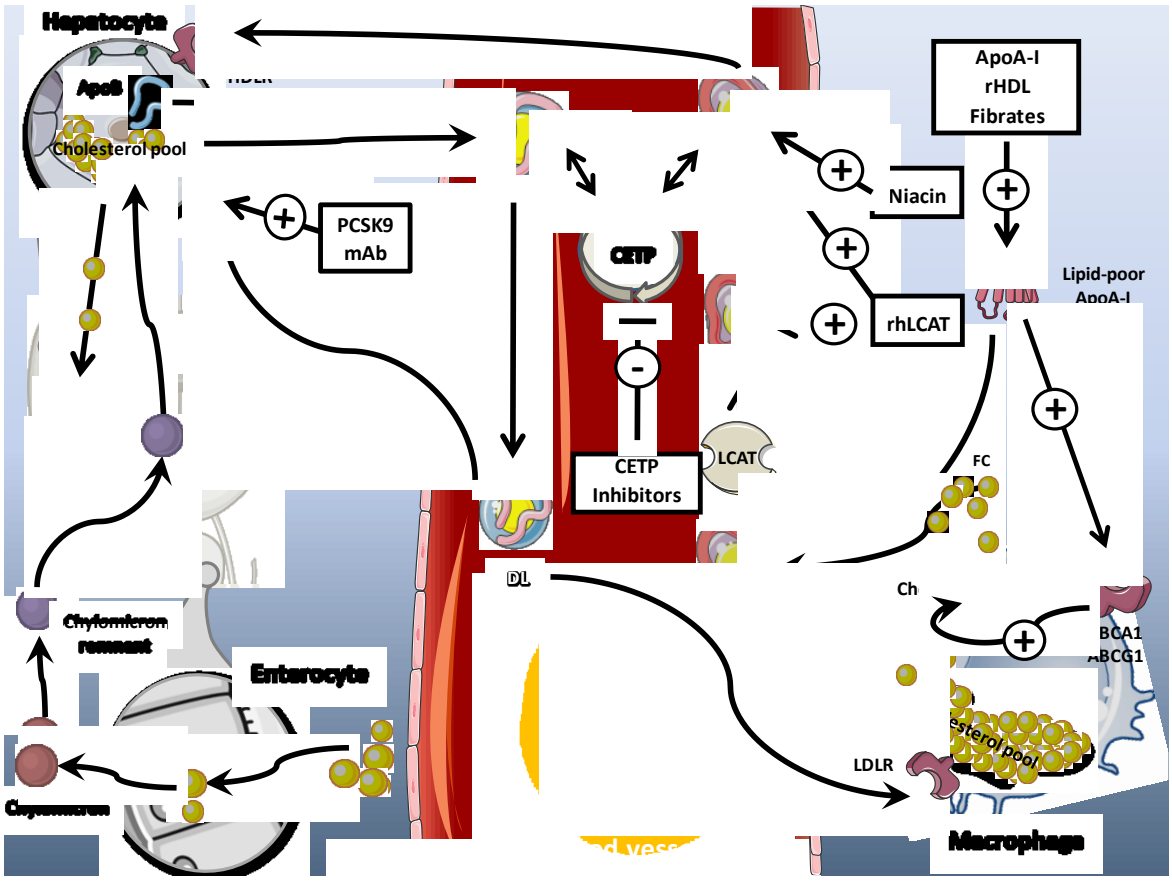

Figure 7: Different HDL-raising therapeutic approaches and their effect on reverse cholesterol transport (RCT) pathway. ApoA-I, apolipoprotein A-I; HDL, high-density lipoproteins; LDL, low-density lipoproteins, L, intermediate density lipoproteins; ABCA1, ATP-binding cassette transporter A1; ABCG1, ATP-binding cassette transporter G1; CE, cholesteryl ester; CETP, Cholesteryl ester transfer protein; FC, free cholesterol; HDLR, HDL receptor; LCAT, Lecithin cholesterol acyltransferase; PCSK9:Proprotein convertase subtilisin/kexin type 9; SR-BI, scavenger receptor class BI.

Although fibrates seem to reduce cardiovascular risk, in particular in subjects with mixed dyslipidaemia, it is currently unknown if this risk reduction is a

consequence of HDL-C increase, triglycerides reduction or due to a pleiotropic effect of the drug.

1.8.3 Cholesteryl Ester Transfer Protein (CETP) Inhibitors

As previously discussed, CETP redistributes cholesterol esters from HDL particles onto proatherogenic VLDL and LDL particles in exchange for triglycerides, and therefore lowers plasma HDL-C levels (Figure 7, page 32). Naturally, CETP-deficient rodents are fairly resistant to diet-induced atherosclerosis, while rabbits, which have high levels of CETP, develop rapidly atherosclerotic lesions under high-fat diet. Accordingly, inhibition of CETP in rabbits resulted in atherosclerosis regression.[159, 324] However, humans with genetic CETP deficiency have been reported to be either protected or at increased risk for atherosclerotic disease despite markedly elevated levels of HDL-C.[325] A large meta-analysis showed only a moderate (5%) risk reduction in patients having mutations leading to a modest reduction in CETP function.[326] Similarly, a genome-wide association study revealed that common CETP polymorphisms are not associated with differences in cardiovascular risk, despite an association with variation in HDL-C levels, while a single nucleotide polymorphism at the CETP locus was associated with a significant 24% risk reduction for future cardiovascular events in the Women's Genome Health Study.[327, 328] Although the first clinical experience with CETP inhibitors was promising by demonstrating a 60-100% increase in HDL-C, randomised trials testing safety and efficacy of the CETP inhibitor torcetrapib reported either an unexpected increase in cardiovascular events most likely due to off-target effects of the drug, or no overall effect on atherosclerosis regression at all, despite substantial increases in HDL-C.[7, 329-331] Similarly, dalcetrapib did not show any benefit in the studies of the dal-HEART program and has even been shown to increase inflammation as revealed by a raised plasma level of high-sensitivity CRP.[8, 63, 332] Interestingly, a pharmacogenomics-based response to dalcetrapib has recently been described in the dal-PLAQUE-2 trial, indicating that polymorphisms in the adenylate cyclase 9 (*ADCY9*) gene influence dalcetrapib's effects on cardiovascular outcomes.[333] In fact, patients with the AA genotype at rs1967309 had a relative cardiovascular risk reduction in response to dalcetrapib

treatment of 39% while the GG genotype showed a 27% increase in risk. Heterozygotes presented a neutral result.[333] Concordant results were obtained for atherosclerosis regression, inflammatory status (high-sensitivity CRP), and cholesterol efflux.[333, 334] Currently, the Dal-GenE randomized trial (NCT02525939) is recruiting patients with a recent ACS bearing the AA genotype at rs1967309 in the *ADCY9* gene in order to confirm the effects of dalcetrapib on cardiovascular outcomes.[335]

While a promising safety and efficacy profile was attributed to evacetrapib in preclinical and phase I trials, the phase III ACCELERATE (Assessment of Clinical Effects of Cholesteryl Ester Transfer Protein Inhibition With Evacetrapib in Patients at a High Risk for Vascular Outcomes) study which evaluated the efficacy and safety of evacetrapib in participants with high-risk atherosclerotic cardiovascular disease was terminated in October 2015 because of lack of benefit.[162, 336] As a consequence, development of evacetrapib for the treatment of atherosclerotic cardiovascular disease was discontinued by Eli Lilly and Company. Besides dalcetrapib (Dal-GenE trial), three CETP inhibitors, BAY 60-5521, TA-8995 and anacetrapib, are currently remaining in studies. Treatment with anacetrapib for one year in patients with familial hypercholesterolemia was well tolerated and resulted in substantial reductions in LDL-C concentration, pointing towards a role of CETP inhibitors as a supplementary treatment in patients where low LDL-C concentrations cannot be achieved with statins alone.[337] In addition, Anacetrapib is associated with much greater HDL-C increases as compared to its precursors while lacking the off-target effects of torcetrapib. Anacetrapib is currently being evaluated in the **R**andomized **Ev**aluation of the **E**ffects of **A**nacetrapib Through **L**ipid-Modification (REVEAL) trial (NCT01252953.). Results of this ongoing trial are expected in 2017. Of note, however, given the significant LDL-C-lowering effects of anacetrapib, it might be difficult to differentiate whether its potentially beneficial cardiovascular effects are derived from raising HDL-C levels or lowering LDL-C. BAY 60-5521 has been shown to be clinically safe and well tolerated in healthy humans and exerted a clear pharmacodynamic effect on CETP inhibition and HDL-C.[338] TA-8995 exerted beneficial effects on LDL-C, HDL-C and Lp(a) in patients with mild dyslipidaemia.[339] The compound is now being tested in phase III studies. Antisense oligonucleotide inhibitors of CETP are novel compounds

associated with reductions in CETP mRNA thereby inducing an enhanced effect on macrophage RCT and reductions of aortic plaques in experimental studies. Given their distinct features, these class of inhibitors might provide a unique therapeutic profile when applied in patients.[340]

In summary, although low baseline levels of HDL-C are an independent predictor of cardiovascular risk, the results of the CETP trials emphasize the poor information that is provided by HDL-C plasma levels. Even if anacetrapib still remains in study and the precision medicine approach of the Dal-GenE study is still underway, it seems that aggressive increasing of HDL-C is not sufficient to reduce cardiovascular risk. In other words, the pharmacological elevation of HDL-C does not translate into an improvement of HDL metabolism and cardiovascular events. Accordingly, normal plasma levels of HDL-C do not protect from clinical events. Controversy exists as to whether HDL-C continues to predict risk in the setting of effective statin therapy. Moreover, it remains unclear whether therapeutic increases in HDL-C provide incremental benefit beyond statins alone.

1.8.4 Proprotein convertase subtilisin/kexin type 9 (PCSK9) Inhibitors

Proprotein convertase subtilisin/kexin type 9 (PCSK9) is a circulating serine protease and the ninth member of a family of proteases denoted as the proprotein convertases. In 2003, the discovery of gain-of-function mutations in the PCSK9 gene causing familial hypercholesterolemia led to the development of therapeutic antagonism of PCSK9.[341] PCSK9 binds to the hepatic LDL-receptor (LDLR) and targets it for destruction in the lysosome (Figure 7, page 32). Through inhibition of PCSK9, the degradation of LDLR is prevented thereby improving the hepatic absorption LDL-C subsequently leading to lower LDL-C plasma concentrations. Since its discovery, studies assessing loss-of-function mutations in PCSK9 have demonstrated a significant reduction in LDL-C which, in turn, was associated with a lower risk of cardiovascular disease.[342] Since then, two monoclonal antibodies to PCSK9, evolocumab and alirocumab, have been developed and have recently been approved by the European Medicine Agency and the US Food and Drug Administration for the treatment of elevated plasma LDL-C.[343-345] The development of another monoclonal PCSK-9 inhibitor, bococizumab, however, was recently

stopped due to auto-antibodies formation against the compound that significantly reduced its LDL-C-lowering capacity. [346, 347] While approaches using monoclonal PCSK9 antibodies are the farthest along in clinical development, adnectins, vaccines, single domain antibodies, antisense oligonucleotides, small interfering RNAs (siRNA), and mimetic peptides have also been developed.[348-350] Amongst those, siRNA molecules and vaccines targeting PCSK9 have recently shown promising results.[348, 349]

Importantly, besides its effects on LDL-C, PCSK9 also seems to play a role in HDL-C metabolism. Indeed, administration of antisense oligonucleotides or monoclonal PCSK9 antibodies in mice has been shown to significantly reduce HDL-C. [351, 352] Accordingly, PCSK9 null mice exhibited reduced levels of HDL-C, ApoE and ApoA-I which, in turn, was associated with a significant impairment of cholesterol efflux capacity of sera derived from PCSK9 null mice.[353] However, while several epidemiological and genetic studies reported positive relationship between plasma PCSK9 and HDL-C levels,[354-356] pharmacological inhibition of PCSK9 increased plasma HDL-C levels by 4.6-7.0% in clinical trials.[343, 344] Degradation of other lipoprotein receptors by PCSK9 such as the ApoE receptor 2, the VLDL receptor, and the LDLR related protein 1 might be responsible for the positive association between PCSK9 and HDL-C/ApoA-I levels, likely by reducing the uptake of the ApoE-containing HDL particles.[357] Further, the increase in HDL-C following pharmacological inhibition of PCSK9 has been suggested to occur due to a reduced ability of cholesterol to be transferred from HDL to LDL rather than due to a direct effect of PCSK9 inhibitors on HDL.[358] The conflicting results of experimental and clinical studies have been attributed to the differences in lipid metabolism between rodents and humans. Indeed, the former lack CETP and carry most of their plasma cholesterol in ApoE-rich HDL.[359] Thus, it seems likely that therapies affecting LDL-R expression in rodents might reduce plasma HDL-C.[358]

Taken together, although inhibition of PCSK9 holds tremendous promise, the physiological role of PCSK9 and its interactions with other lipoproteins including HDL still needs to be elucidated. In addition, the long-term efficacy and safety of PCSK9 inhibitors is unknown to date and ongoing large clinical outcomes trials such as the FOURIER (NCT01764633) and the ODYSSEY OUTCOMES trials

(NCT01663402) will determine the fate of this new pharmacological approach. Finally, the costs for PCSK9 inhibitors are significantly higher than for statin therapy and may limit their practical use.

1.8.5 LCAT Replacement Therapy

Therapeutic upregulation of LCAT activity has recently gained interest as a potential antiatherogenic treatment because of the ability of LCAT to improving or accelerating the maturation of smaller HDL particles (Figure 7, page 32). Different strategies have been tested so far in animal models including recombinant LCAT protein administration, viral expression of LCAT, and small-molecule activators of LCAT with all leading to a marked increase in HDL-C levels.[134] Following adenoviral LCAT overexpression in rabbits, a decreased intima/media ratio as well as enhanced cholesterol uploading of atherosclerotic lesions were observed, indicating that LCAT overexpression slowed the progression of atherosclerosis.[360] Recombinant LCAT (ACP-501) has recently been administered to subjects with low LCAT activity or CAD. It was shown that in both groups LCAT infusions led to a rise in HDL-C. [198, 199, 361] In addition, ACP-501 infusions improved plasma lipids and anaemia in patients with familial LCAT deficiency.[362] Thus, the results of these studies seem promising with respect to efficacy and antiatherogenic profile of these compounds. However, given the complex interactions of LCAT with different lipoproteins, studies in larger populations are needed to evaluate the applicability of these therapies. In addition, the short half-life of LCAT must be taken into consideration, as well as the high concentrations that are needed to increase HDL-C in the presence of CETP. Table I (page 38) summarizes relevant HDL-raising therapeutic strategies and their therapeutic effects.

Agent	Substance class	Development status	Reference
Torcetrapib	CETP-Inhibitor	Failed in phase III owing to excess of mortality in treatment group	Barter PJ et al. 2007
Dalcetrapib	CETP-Inhibitor	Failed in phase III owing to lack of efficacy	Schwartz GG et al. 2012
Evacetrapib	CETP-Inhibitor	Failed in phase III owing to lack of efficacy	Nicholls SJ et al. 2015
Anacetrapib	CETP-Inhibitor	Currently in phase III	Cannon CP et al. 2010
RVX 208	ApoA-I transcriptional regulator	Phase II completed	Nicholls SJ et al. 2015
CER-001	HDL mimetic	Currently phase III	Tardif JC et al. 2014
CSL111	HDL mimetic	discontinued	Tardif JC et al. 2007
CSL112	HDL mimetic	Supersedes CSL111, currently in phase IIa	Tricoci P et al. 2015
Recombinant apoA-I Milano ETC-216 (now MDCO-216)	Naturally occuring variant of apoA-I variant	No current development	Nissen SE et al. 2003 Nicholls SJ et al. 2006
D-4F (also APP018), 5A, 6F, L-4F, ATI-5261	Oral/intravenous apoA-I mimetics, improves cholesterol efflux, antioxidant and anti-infammatory properties	No current development	Bloedon LT et al. 2008 Watson CE et al. 2011
Delipidated HDL	Lipid-poor HDL produced by selective delipidaton of HDL	No current development	Waksman R et al. 2010
ACP-501	Recombinant human LCAT	Phase I	Shamburek RD et al. 2015
LXR-623, T0901317, GW3965, GW6340, ATI-111, AZ876	LXR agonists, upregulates ABCA1 and ABCG1 expression	Phase I (LXR-623)	Katz A et al 2009
FXR-450	FXR agonists, accelerates cholesterol excretion via HDL	Preclinical development	Hambruch E et al. 2012
Endothelial lipase inhibitors	Inhibition of HDL lipid hydrolysis	Preclinical development	Weijun J et al. 2003
Antagonists of miR-33	MicroRNA antagonists, upregulation of ABCA1 and ABCG1 expression	Preclinical development	Rayer KJ et al. 2011
Antisense oligonucleotides targeting CETP and APOC3	Inhibitors of CETP and APOC3 gene expression	Phase II (APOC3) Preclinical development (CETP)	Gaudet D et al. 2015 Bell TA et al. 2013

Table I: List of relevant HDL-raising therapeutic strategies and their development status. HDL, high-density lipoproteins; ApoA-I, apolipoprotein A-I; CETP; cholesteryl ester transfer protein; LCAT, lecithin–cholesterol acyltransferase; LXR, liver X receptor; FXR, farnesoid X receptor; ABCA1, ATP-binding cassette transporter A1; ABCG1, ATP-binding cassette transporter G1.

1.8.6 HDL-based Therapeutic Approaches in Aortic Valve Disease – Challenges

While statins have been reported to reduce ischemic cardiovascular events in AVS patients, they have thus far failed to halt or regress AVS progression.[363, 364]. In contrast, HDL-based therapies such as reconstituted HDL or apoA-I mimetic

peptides resulted in a significant regression of AVS in experimental models due to a decrease in aortic valve lesion extent and less calcifications.[365-367] Improved endothelial function and integrity, antioxidant and/or anti-inflammatory effects of HDL and better leaflet repair by increased incorporation of bone marrow-derived progenitor cells have been suggested to account for these findings. However, even though these results are encouraging, the current effective dose of apoA-I used experimentally is high and renders such therapy less applicable in humans. Recent studies indicate that this suboptimal therapeutic efficiency may be due to apoA-I degradation by plasma or tissue proteases localized in stenotic aortic valves and hypertrophic left ventricles. Indeed, MMP-3 (stromelysin-1), MMP-7 (matrilysin), MMP-8 (neutrophil collagenase) and MMP-12 (human metalloelastase) have been shown to truncate the carboxyl terminus of apoA-I in HDL_3 *in vitro*, thereby dramatically reducing its capacity to induce cholesterol efflux from human macrophage foam cells.[219, 220] In addition, several other proteases found in the vessel wall can degrade apoA-I and HDL_3, and reduce the efficiency of these particles as acceptors of macrophage cholesterol. In fact, the mast cell proteases chymase[236-238] and tryptase, [239] as well as plasmin, kallikrein[240] and cathepsin F and S[257] specifically degrade lipid-free apoA-I or deplete the minor subpopulation of preβ-HDL, and so impair the high-affinity efflux of cholesterol from macrophage foam cells that is promoted by HDL_3.[236, 244-246] Finally, apoA-I can be degraded in vitro by chymotrypsin, subtilisin, arginine C endopeptidase, and Staphylococcus V8 protease.[241-243] Importantly, some of these apoA-I degrading proteases have been shown to be present in stenotic aortic valves, and are abundant in plasma of patients suffering from AVS.[226, 227, 231, 267, 268] However, proteases are not only present in diseased valve tissue but also in hypertrophic left ventricles.[231, 232, 251] Left ventricular hypertrophy develops due to pressure overload in patients with aortic stenosis,[252] and is associated with increased mortality and morbidity before and after aortic valve surgery.[368] It is currently unclear, however, which of these proteases has the most deleterious effect on apoA-I function. Since protease activity is not only important for normal cellular processes but also involved in many pathologies such as cancer, atherosclerosis, and neurodegenerative disorders, there is an increasing interest in developing

methods to measure and image protease activity *in vitro*, in live cells, and in whole organisms.[369] Fluorescent probes are widely used and an excellent tool to assess the biology of proteases in different specimen such as serum, plasma or other

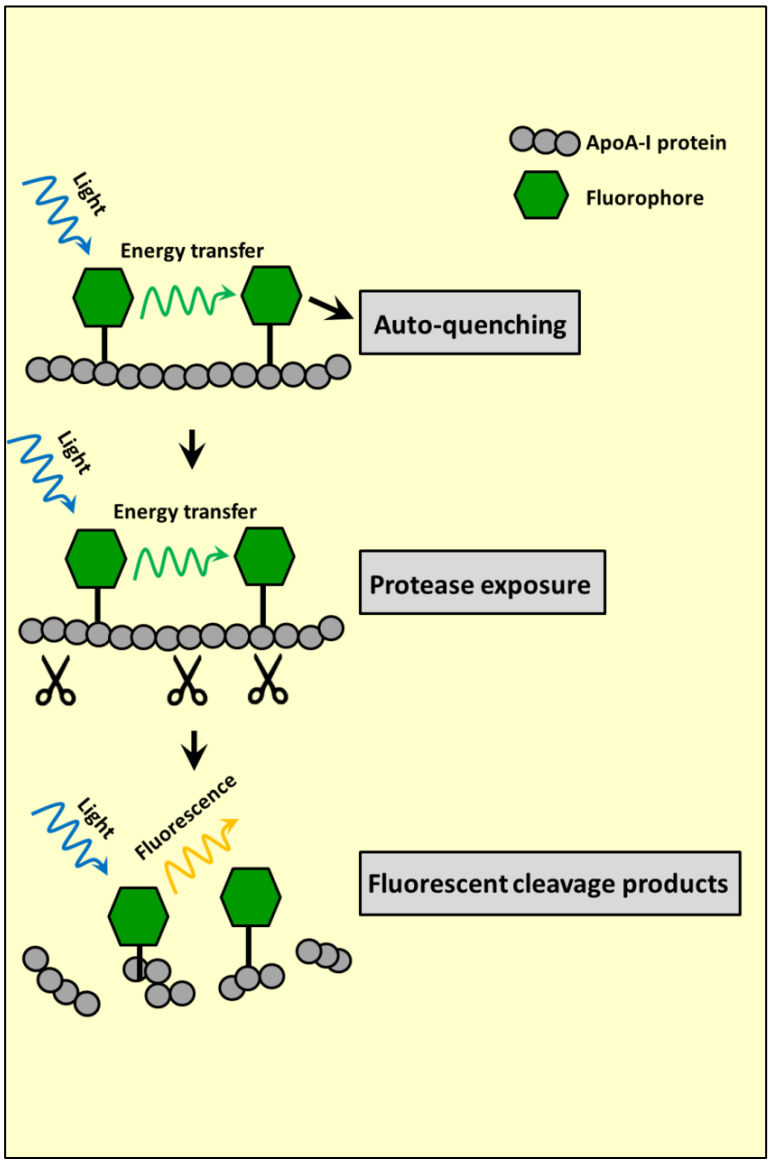

Figure 8: Principle of auto-quenching: Full-length apolipoproteinA-I (apoA-I) fluorescence probe. Due to the high labeling, each fluorophore acts as a quencher: in the presence of a quencher (=another fluorophore), the excited fluorophore returns to the ground state by transferring its energy to the quencher, without the emission of light, while the quencher is promoted to its excited state. If the quenched probe is exposed to proteases, quenching is relieved by protease cleavage of the apoA-I protein. Fluorescence is then generated when a substance absorbs light energy at a short (higher energy) wavelength and then emits light energy at a longer (lower energy) wavelength. By using this apoA-I probe, it was demonstrated that the fluorescence signal time-dependently increases following incubation with proteases.

body fluids, and to screen for their pharmacologic modulators as they report not the mere expression but rather the activity of the target enzyme. In the herein investigation, an activatable probe using a fluorescently quenched full-length apoA-I protein was generated to specifically target apoA-I-degrading protease activity in plasma derived from a rabbit model of AVS as well as in human plasma (Figure 8, page 40).[370] Such probes also provide a valuable tool to accurately measure and visualize apoA-I degradation *in vivo* and to evaluate novel strategies to preserve or enhance HDL function. The rabbit model AVS used in the present

investigation is an established experimental model in rabbits where vitamin D hypervitaminosis treatment has been shown to result in proliferation of valvular interstitial cells, valvular calcification and significant hemodynamic AVS changes (Figure 9, page 41).

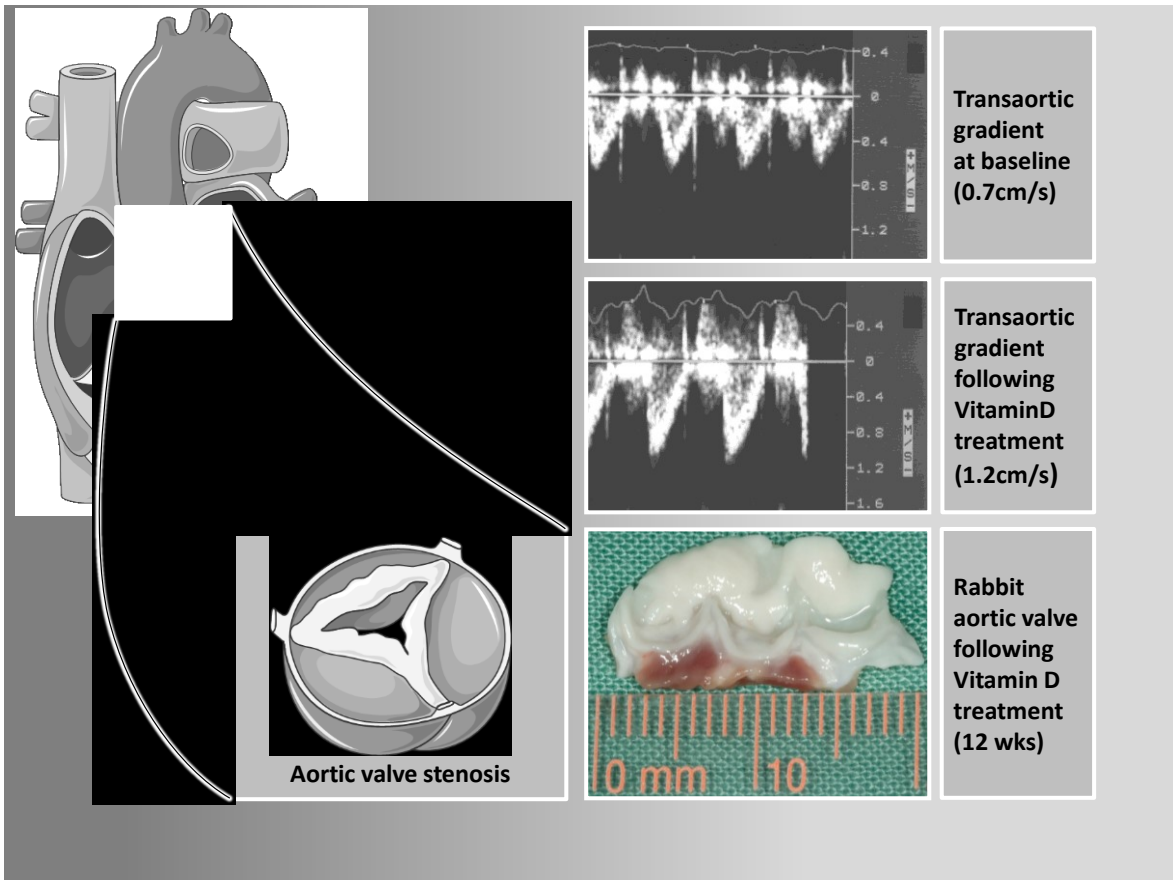

Figure 9: Rabbit model of aortic valve stenosis. New Zealand white rabbits are fed a cholesterol-enriched diet with high-dose vitamin D2 for 12 weeks. After 12 weeks, animals exhibit significant aortic valve stenosis (upper and middle panel) due to proliferation of valvular interstitial cells and calcification of the aortic valves (lower panel).

2. Summary and Aims

Recent clinical trials have brought into question the validity of the HDL cholesterol hypothesis—whether increasing HDL-C will reduce the risk of cardiovascular disease. However, the results of long-standing epidemiological studies and animal models demonstrating atheroprotective effects of HDL are undeniable. Therefore, it has been suggested that HDL function rather than HDL-C quantity is the key player in the assessment of cardiovascular risk.

Indeed, numerous studies have shown that HDL loses its atheroprotective functions or even gains proatherogenic functions under certain conditions ('dysfunctional HDL'). Since the population of patients suffering from conditions associated with dysfunctional HDL is steadily increasing, quantification of dynamic HDL (dys) function will be crucial in assessing the impact of future therapeutics. However, it is uncertain to date what surrogate markers of HDL function might provide the most complete information on HDL mediated cardiovascular risk reduction in clinical routine. In addition, while many beneficial actions of functional HDL have been described, mechanisms governing the cardioprotective effects of HDL-based therapeutic approaches are incompletely understood at the present time. Similarly, strategies to prevent HDL from converting into dysfunctional molecules *in situ* and approaches to improve the efficacy of HDL-based therapies have not yet been explored. Given the epidemic proportions of HDL dysfunction and the high costs of HDL-based therapies, the development of strategies to preserve HDL function under pathologic conditions is crucial. Finally, secondary or pleiotropic effects of therapeutic approaches involving HDL or HDL-associated molecules are sparse and need to be explored.

To address these problems, the following specific aims were developed:

(1) To explore the value of cholesterol esterification and LCAT as biomarkers for cardioprotective HDL effects

(2) To assess whether rHDL exerts beneficial effects in endothelial repair

(3) To explore strategies that protect HDL-associated proteins from protease degradation

(4) To assess whether apoA-I administration deteriorates haemorrhagic disorders under conditions of increased shear-stress, as it is observed in patients with AVS

To achieve these aims we will analyse

(1) The relationship between cholesterol esterification by plasma LCAT and atherosclerosis plaque burden, as assessed by IVUS in a prospective, multi-centre imaging study.

(2) The short-term effects of rHDL on circulating endothelial progenitor cells in patients with a recent ACS.

(3) The contribution of plasma protease activity to apoA-I degradation in a rabbit model of AVS and in plasma collected from patients with severe AVS undergoing surgical valve replacement.

(4) The effect of HDL on ADAMTS-13 activity and vWF in a rabbit model of AVS and in plasma collected from patients with severe AVS undergoing surgical valve replacement.

3. Manuscripts

3.1 Manuscript #1: Elevated Level of Lecithin: Cholesterol Acyltransferase (LCAT) are Associated with Reduced Coronary Atheroma Burden

3.1.1 Foreword to Manuscript #1:

As outlined in the introduction, it is crucial that we identify clinically applicable diagnostic biomarkers of HDL function that show close correlations with disease status in different cardiovascular conditions. The HDL-associated protein LCAT is the key enzyme responsible for esterification of free cholesterol (FC) to cholesteryl esters (CE) and seems to be a promising candidate to mirror HDL functionality in cardiovascular conditions. Indeed, LCAT maintains a concentration gradient of unesterified cholesterol (UC) from cells to HDL thereby facilitating reverse cholesterol transport. To determine the ability of LCAT to serve as a marker of HDL function in cardiovascular risk prediction, we investigated the relationship between circulating LCAT and atherosclerosis plaque burden in a prospective, multi-centre imaging study. In detail, by using intravascular ultrasound (IVUS) imaging in patients with CAD, manuscript #1 assesses the prognostic value of LCAT protein mass in cardiovascular risk prediction.

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Author contributions:
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3.1.2 Abstract

Background: Lecithin:cholesterol acyltransferase (LCAT), a key enzyme in high-density lipoprotein (HDL) metabolism and reverse cholesterol transport (RCT), has been associated with atheroprotection. However, this link has not been confirmed to date. We determined the relationship between plasma LCAT mass concentration and plaque burden, as assessed by intravascular ultrasonography (IVUS) in a multi-centre imaging study.

Method and results: Two hundred sixty-seven patients with angiographically proven coronary artery disease (CAD) underwent IVUS imaging. Ninety-six patients without CAD served as controls for biochemistry assessments. Plasma LCAT mass concentration was higher in CAD patients as compared to controls (8.94±2.51 µg/mL vs. 7.89±2.99 µg/ml, p=0.003) and correlated positively with LDL cholesterol (r=0.17, p=0.005), triglycerides (r=0.30, p<0.0001), and oxLDL (r=0.24, p<0.0001). Plasma LCAT mass was inversely correlated with percent atheroma volume in the 5-mm subsegment with largest plaque burden (r=-0.17, p=0.005). Plasma level of LCAT mass was found to be a significant predictor of percent atheroma volume at that site (Beta coefficient -0.8, p=0.002) when tested in a stepwise linear regression model against known CAD risk factors as predictor variables. Accordingly, patients with LCAT mass in the highest quartile had significant less atheroma burden than those in the lower quartiles (p=0.013for highest vs. lowest quartile).

Conclusion: Plasma LCAT mass concentration is upregulated in CAD patients and appears to exert atheroprotective effects. Further studies assessing LCAT as a therapeutic target in cardiovascular disease are warranted.

3.1.3 Introduction

Despite intensive statin therapy the risk of cardiovascular events in patients with CAD remains high. This residual risk has a large impact on individuals and global healthcare costs and therefore, complementary therapies are eagerly sought.[371] During the last decade, high-density-lipoprotein (HDL)-cholesterol increasing therapies were at the centre of research efforts. However, recent data from large scale intervention trials and Mendelian randomization studies have challenged the widely held contention that HDL particles exert cardioprotective properties. These studies have underscored the importance of understanding the multiple biological actions of HDL underlying its potential atheroprotective functions, including promotion of cholesterol efflux and reverse cholesterol transport.[7, 8, 313, 314] Lecithin:cholesterol acyltransferase (LCAT), a 63 kDa glycoprotein synthesized in the liver, catalyses the esterification of free cholesterol in plasma lipoproteins. Esterification by LCAT maintains the gradient of free cholesterol between the cellular membrane and the surface of the HDL particle, thereby generating a continuous flow of cholesterol from the cell to lipoproteins. The latter is considered the most important pathway in HDL mediated reverse cholesterol transport, an atheroprotective process responsible for removal of excess cholesterol from peripheral tissue.[172, 173]

Despite the fact that loss-of-function LCAT mutations invariably result in profound HDL deficiency, [372-374] the effect of LCAT on atherogenesis remains poorly understood. Indeed, investigations in various animal models overexpressing or lacking LCAT have not provided clear answers and have led to the conclusion that the effects of LCAT on lipoprotein metabolism largely depend on the presence of additional proteins involved in reverse cholesterol transport, such as cholesteryl ester transfer protein (CETP) and the low-density lipoprotein (LDL) receptor.[193, 375-378] In addition, studies of human LCAT gene mutation carriers as well as subjects without genetic deficiency of this enzyme have yielded largely controversial results that are difficult to interpret and most likely result from differences in populations studied and in methods used to assess LCAT activity.[177, 178, 180, 181, 190, 191, 379] .Thus, a clear role of LCAT activity in predicting cardiovascular risk in different populations could not be established to date.[179, 184-187, 380] Importantly, studies assessing the predictive value of LCAT mass in patients with coronary artery

disease (CAD) are lacking. In fact, only one study reported that low LCAT plasma concentration was not associated with increased cardiovascular risk in a healthy population.[185] Furthermore, previous studies were limited by the assessment of atheroma burden by carotid angiography or ultrasound in small populations. In contrast, intravascular ultrasonography (IVUS) typically requires smaller numbers of patients and takes into account vessel remodelling characteristics. Thus, to explore the role of LCAT in human atherosclerosis, we compared plasma LCAT mass concentration in subjects with and without CAD and assessed the extent to which this parameter is associated with coronary plaque burden on IVUS imaging.

3.1.4 Method

Study Design and Population

The study population consisted of 267 patients who had been included at 50 Canadian sites participating in the Canadian Atherosclerosis Imaging Network (CAIN)-3 study and accepted to participate in the biomarkers substudy, and 96 control subjects who were recruited using the CAIN-3 enrolment criteria (Figure 10, page 56). Eligible patients were women (without childbearing potential) and men over the age of 18 years who were scheduled for clinically indicated coronary angiography. Patients had to have angiographic evidence of CAD as defined by at least one lesion in any of the three major native coronary arteries that had >20% reduction in lumen diameter by angiographic visual estimation or prior history of PCI. Patients enrolled in this study were required to have one target artery for IVUS examination that was not influenced by prior or present percutaneous coronary intervention (PCI) and that had not been the cause of a recent myocardial infarction. The proximal 4 cm of the target artery in which IVUS examination was performed had to have a stenosis < 50% of the lumen diameter by visual assessment of the angiogram, a reference diameter ≥ 2.5 mm, and had to be free of any anatomical characteristic that would impede IVUS interrogation. Patients with greater than 50% stenosis in the left main coronary artery, previous coronary artery bypass graft (CABG) surgery, symptomatic congestive heart failure (CHF) (New York Heart Association [NYHA] Class III or IV), clinically significant valvular heart disease, presence of severe liver disease, a GFR < 45

ml/min, or a history of malignancy during the 3 years prior to the screening were excluded. Control subjects also needed to be above 18 years of age, have no history of CAD or structural heart disease, no history of stroke or transient ischemic attack, and have undergone coronary angiography that showed no luminal narrowing (<20%) within the last 3 years. Following written informed consent, patients underwent medical history, physical examination, and ECG; haematology, clinical biochemistry and lipid profiles were also collected. The study complied with the Declaration of Helsinki and was approved by the Ethics Committee of the Montreal Heart Institute and by institutional ethics committees at all Canadian study centres.

Coronary Intravascular Ultrasonography Examinations and Core Laboratory Analysis

VUS examinations were performed using 40 or 45 MHz catheters after intracoronary nitroglycerin (0.10-0.30 mg) had been administered. The IVUS catheter was advanced into the target vessel ≥40 mm beyond the coronary artery ostium, to a recognizable landmark. The IVUS transducer was then pulled back automatically at a speed of 0.5 mm/sec up to the guiding catheter using a motorized device and IVUS images were recorded.

IVUS recordings were analysed in a blinded fashion by the Montreal Heart Institute IVUS core laboratory as previously reported.[288] Image interpretation was uniformly performed in the core laboratory by experienced technicians supervised by a cardiologist, according to published standards.[381] For each IVUS cross-section, the operator performed manual planimetry to trace the leading edges of the luminal and external elastic membrane borders. Atheroma area was calculated as external elastic membrane (EEM) area minus luminal area. Total atheroma volume was calculated through the summation of plaque areas of all traced cross-sections over the length of the segment, and results were then indexed to a 30-mm segment to compensate for differences in segment length between subjects. Percent atheroma volume was computed by dividing atheroma volume by elastic membrane volume and then multiplying by 100%.

Biochemical analyses

Blood was collected after an overnight fast. Plasma samples were kept at –80°C prior to analysis by the Montreal Heart Institute clinical biochemistry core lab. Plasma total cholesterol, LDL-C, HDL-C, and triglyceride levels were determined with certified enzymatic methods. Plasma apoA-I and apoB levels were measured with the Nephelometric BNII System (Dade Behring). High-sensitivity C-reactive protein (hs-CRP) levels were measured by the Dade Behring method. Plasma LCAT mass concentration was measured in duplicate by an immunoenzymatic assay as previously described.[382]

Statistical analyses

Continuous variables were summarized as mean ± SD and categorical variables were summarized as percentages. Clinical and anthropometric variables were compared between groups by unpaired Students' *t* -test or Chi-square test, as appropriate. The relationships between biochemistry parameters and IVUS measurements were tested by Spearman correlations. ANOVA was used to analyse differences between LCAT quartiles. Comparisons adjusted for potential confounders (age, sex, body mass index (BMI), smoking status, hypertension, diabetes, and LDL cholesterol) were performed by ANCOVA. Multivariate linear regression analysis was applied to assess the association of plaque burden with LCAT related parameters. For the IVUS parameters showing strongest correlations with LCAT concentration (total atheroma volume, percent atheroma volume, and percent atheroma volume over the 5 mm subsegment with largest plaque burden), univariate and multivariate models were used in order to identify independent predictors. Several predictive models were tested to assess the changes in effect size by adding known risk predictors for plaque burden including age, sex, body mass index (BMI), smoking status, hypertension, diabetes, and LDL cholesterol. All tests were two-sided, and *P* values below 0.05 were considered significant. All analyses were performed using the SAS statistical package (version 9.1 or higher, SAS Institute Inc., Cary, NC).

3.1.5 Results

Baseline characteristics of study participants

A total of 363 subjects, 147 women and 216 men, were enrolled in the study: 267 patients suffered from CAD, while 96 control patients were free of cardiovascular disease (Figure 10, page 56). There were slightly more men (71.1% vs 58.3%, p=0.02) and more current smokers (16.1% vs 5.2%, p=0.006) in the CAD group than in controls, and patients in the CAD group had more often dyslipidaemia (86.1% vs 42.7%, p=0.0001), hypertension (71.2 vs 43.2%, p=0.0001), and type 2 diabetes(24.3 vs 12.5%, p=0.01). Baseline characteristics and medications of all subjects are indicated in Table II (page 58).

Plasma lipid levels in CAD patients and controls

Total cholesterol, LDL-C, triglycerides and apolipoprotein B (apoB) were all significantly lower in CAD patients as compared with controls ($p<0.05$, Figure 11, page 56 and Table III, page 59). Significantly more patients in the CAD group were treated with statins (86.9 % vs 41.7%, $p<0.001$) as compared with patients in the control group (Table II, page 58). HDL cholesterol and apolipoproteinA-I (apoA-I) were also both lower in the CAD group than in controls (1.10±0.35 mM vs 1.31±0.43 mM and 1.33±0.26 g/L vs 1.49±0.26 g/L, respectively, $p<0.0001$) (Table III, page 59). Oxidized low-density lipoprotein mass concentration (oxLDL) was significantly lower in CAD patients as compared to controls (55.2±17.9 vs 63.5±21.8, p=0.0003). No differences in other laboratory parameters including glucose, hs-CRP, bilirubin and creatinine levels were observed between groups (Table III, page 59).

Plasma LCAT concentration in CAD patients and controls

Plasma LCAT concentration was significantly higher in CAD patients compared to controls (8.94±2.51 vs 7.89±2.99 µg/mL p=0.003, Table III, page 59 and Figure 11, page 56). Significantly higher LCAT concentration was measured in female CAD patients as compared to males (9.6±2.3 vs 8.6±2.5 µg/mL, p=0.002, data not shown). In CAD subjects, plasma LCAT concentration correlated positively with

LDL-C (r=0.17, p=0.005), triglycerides (r=0.30, p<0.0001), oxLDL (r=0.24, p<0.0001) and apoA-I (r=0.15, p=0.014, Table IV, page 59). No significant correlation was found between LCAT mass and HDL-C (p=0.08). Furthermore, LCAT concentration was positively correlated with BMI (r=0.22, p<0.0001), while no correlation was found between age and LCAT concentration (p=NS). Patients with higher LCAT concentration (highest quartile) were more likely to have a history of dyslipidaemia (p=0.026), were more likely to be obese (BMI p=0.031), had higher levels of oxLDL (p<0.0001), and had higher triglyceride and apoB levels (p=0.03 and p=0.005, respectively, Table V, page 60).

LCAT mass concentration and atheroma burden in CAD patients

Mean total atheroma volume and percent atheroma volume were 152.7±66.6mm^3 and 36.8±9.0%, respectively. Percent atheroma volume over the 5 mm subsegment with the largest plaque burden was 43.5±10.3%. Percent atheroma volume over the 5 mm subsegment with the largest plaque burden and total atheroma volume were inversely correlated with plasma LCAT mass concentration (r=-0.17, p=0.005; and r=-0.14, p=0.027, respectively, Figure 12B and 12C, page 57) in CAD patients. The observed correlations were similar for both sexes (ANOVA, p=NS, data not shown). Accordingly, patients with LCAT mass concentration in the highest quartile had significantly less percent atheroma volume over the 5 mm subsegment with the largest plaque burden than patients in the lower quartiles (p=0.013 for highest vs. lowest quartile, Figure 12A, page 57).

Predictors of atheroma burden in CAD patients

When LCAT mass was tested in a stepwise linear regression analysis with IVUS, percent atheroma volume over the 5 mm subsegment with the largest plaque burden being the dependent variable, and age, sex, BMI, hypertension, diabetes, smoking, and LDL-C as predictor variables, three significant variables were identified (LCAT concentration, diabetes and sex). The probability was best explained by LCAT concentration (Beta coefficient -0.8, p=0.002, model 1), followed by diabetes (Beta coefficient 3.7, p=0.01, model 2), and male sex (B coefficient 2.8, p=0.042, model 3) (Table VI, upper panel, page 61). Similarly,

LCAT concentration was a significant negative predictor for total atheroma volume (model 1=sex: Beta coefficient 31.3, p<0.0001; model 2=sex+BMI: Beta coefficient 2.1, p=0.001; model 3=sex+BMI+LCAT concentration: B coefficient -3.2, p=0.045, Table VI, lower panel, page 61). LCAT concentration remained a significant negative predictor variable for both percent atheroma volume over the 5 mm subsegment with the largest plaque burden and total atheroma volume, even when HDL-C was added as a predictor variable to these three models (model 3=sex+BMI+LCAT concentration: B coefficient -3.2, p=0.045 and model 1=LCAT concentration: B coefficient -0.8, p=0.002, respectively).

3.1.6 Discussion

This analysis demonstrates that plasma LCAT protein concentration is upregulated in patients with CAD compared to controls and correlates inversely with coronary atheroma burden as assessed by IVUS imaging. The increase in LCAT concentration might therefore exert atheroprotective functions in CAD patients.

The effect of LCAT on atherogenesis and cardiovascular risk is controversial despite previous carotid ultrasound or MRI studies. A recent study comprising five families suffering from heterozygote LCAT deficiency showed that carotid intima-media thickness was greater in LCAT deficient patients compared with unaffected family members.[177] In contrast, LCAT activity was associated with increased carotid? atherosclerosis in patients with metabolic syndrome[190] and women[191], whereas an opposite nonsignificant trend was observed in men.[191] Accordingly, elevated LCAT concentration in plasma has been shown to be associated with increased cardiovascular risk in women but not in men, [185, 186] raising the question of whether the opposite trends in men and women underlie the divergent findings in the mixed gender studies. Indeed, unaltered, decreased or increased LCAT levels have been reported in patients with stable CAD, acute coronary syndrome or moderate hyperlipidaemia.[185, 187, 188, 379, 380, 383]

Overall, these seemingly contradictory results are likely to be explained by extensive differences in study populations and laboratory methods used to determine LCAT activity or mass levels. Previous study populations have comprised families with genetic deficiency where LCAT concentration is greatly

reduced, stable CAD patients with only small variations of LCAT levels, patients with ACS, and patients suffering from a variety of metabolic disorders. This heterogeneity is mirrored by wide discrepancies in lipid levels in those studies. There is strong evidence that plasma lipid metabolism and LCAT are interrelated and that LCAT levels might be influenced by BMI, insulin sensitivity and lipid-lowering drugs.[384],[385, 386],[190] Previous studies have also been hampered by the paucity of clinical events in small numbers of individuals, as well as by the fact that other parameters of the reverse cholesterol transport pathway were not studied.

Rosuvastatin treatment has recently been found to decrease LCAT activity in patients with metabolic syndrome, while simvastatin markedly increased LCAT activity in ApoE-/- mice fed a high-fat diet. [387, 388] In our study, 87% of CAD patients were treated with statins resulting in an average LDL-cholesterol level of 1.94±0.70 mmol/L, and LCAT levels were similar irrespective of lipid-lowering drug use. Because LCAT mass concentration reflects LCAT activity assessed by the exogenous method[185, 382, 389, 390] and fractional cholesterol esterification rate is determined by LCAT mass and HDL particle size,[391] analysis of LCAT concentration may permit identification of substrate or cofactor abnormalities. Interestingly, there was no correlation between LCAT mass level and HDL-cholesterol in our study, an observation that is in agreement with several previous reports [185, 389, 390] although two cross-sectional studies have reported positive correlations.[385, 392] These conflicting findings may indicate that the LCAT reaction may not be the rate-limiting step in HDL genesis.[185] In addition, the lack of association between LCAT concentration and HDL-C in CAD patients may also be indicative of dysfunctional HDL in our high-risk population. Indeed, a strong association of LCAT concentration with the small dense HDL_3 subfraction has previously been reported, while no correlations were found with the larger HDL_2 particles.[385] Although HDL_3 has been attributed more powerful actions against LDL oxidation than HDL_2 as well as potent anti-inflammatory and cytoprotective effects,[393, 394] it also appears to be more vulnerable to detrimental effects and more likely to lose its normal biological function.[395-398]

Consistent with previous reports, both LDL-cholesterol and triglyceride levels were positively correlated with LCAT mass levels in the present study.[185, 374] Combined with the positive correlation between body-mass index and LCAT mass

in our and previous reports, these data may indicate that LCAT plays a role as a salvage pathway against dyslipidaemia in metabolic syndrome.[185, 190] Similarly, the higher LCAT mass concentration measured in CAD patients as compared to controls and the inverse correlation between LCAT mass and coronary atheroma burden in the former together suggest that upregulated LCAT synthesis could represent a defence mechanism n CAD. Since LCAT is activated by an optimal conformation of a complex formed by apoA-I and the phospholipid-cholesterol bilayer, higher plasma LCAT concentrations measured in CAD patients might also compensate for suboptimal presentation of its lipid substrate due to lower apoA-I level in this population.

OxLDL has been attributed a key role in the development of atherosclerosis and cardiovascular risk prediction.[399, 400] However, its role in HDL metabolism is less clear.[401] Given that LCAT has been shown to trans-esterify and hydrolyse oxidized phospholipids,[202] thereby contributing to the antioxidant properties of HDL,[61, 402] the positive correlation between LCAT mass and oxLDL observed in the present study may indicate a stimulatory effect of the latter on LCAT synthesis, which in turn may initiate a counterregulatory antioxidant defence mechanism.

In line with a previous report, we observed significantly higher LCAT concentrations in women as compared to men.[185] Another study showed that both fractional and molar LCAT rates were positively correlated with obesity in women but not men,[205] indicating that there are gender-specific regulations of LCAT mass and activity that might be triggered by dyslipidemic conditions or metabolic imbalance. However, those opposite trends in men and women did not translate into different gender-specific correlations of LCAT mass with atherosclerosis in our study. Since the current study was not powered for a gender-specific analysis, it remains unclear if higher levels of LCAT in women would protect against CAD. This will have to be assessed by a prospective gender-specific analysis.

Although we adjusted for multiple factors, residual confounding by imperfectly measured or unmeasured confounders cannot be excluded as a potential limitation. In conclusion, this study indicates that LCAT is upregulated in patients with CAD and might have atheroprotective effects. Newly developed LCAT activators and recombinant LCAT infusions will provide valuable information

to establish whether targeting LCAT in patients is a promising therapeutic strategy to reduce cardiovascular risk.

3.1.7 Figures and Tables

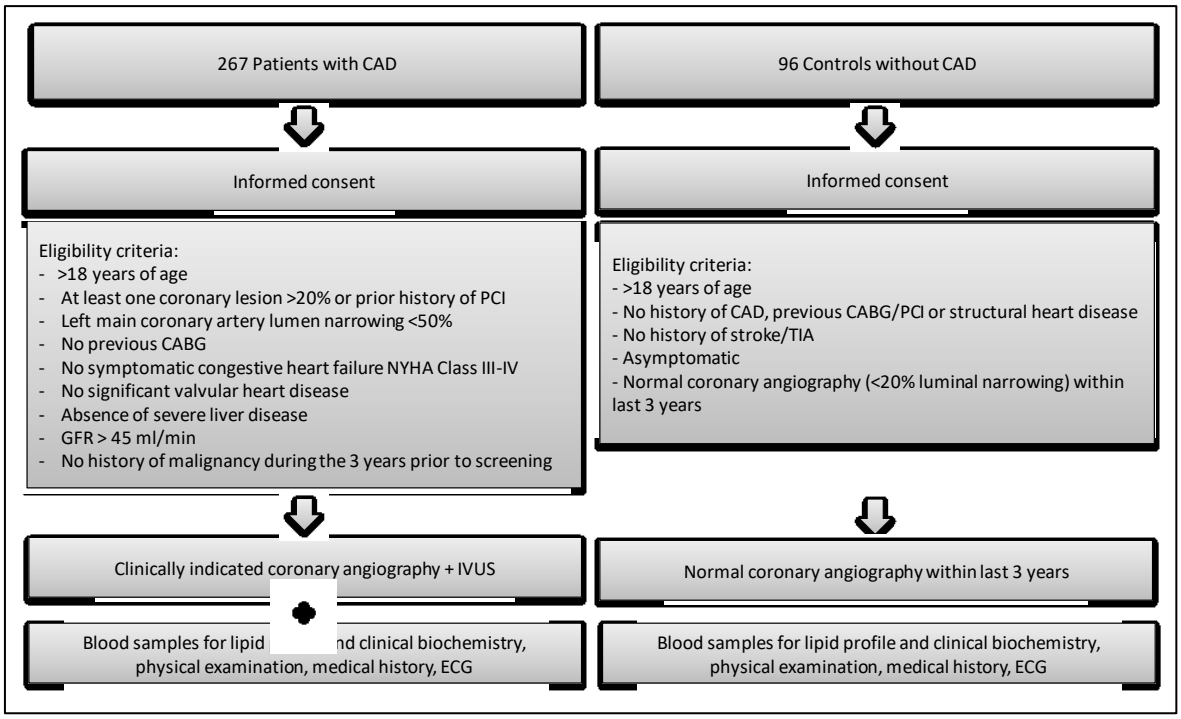

Figure 10: Flow chart of patient recruitment and follow-up in patients

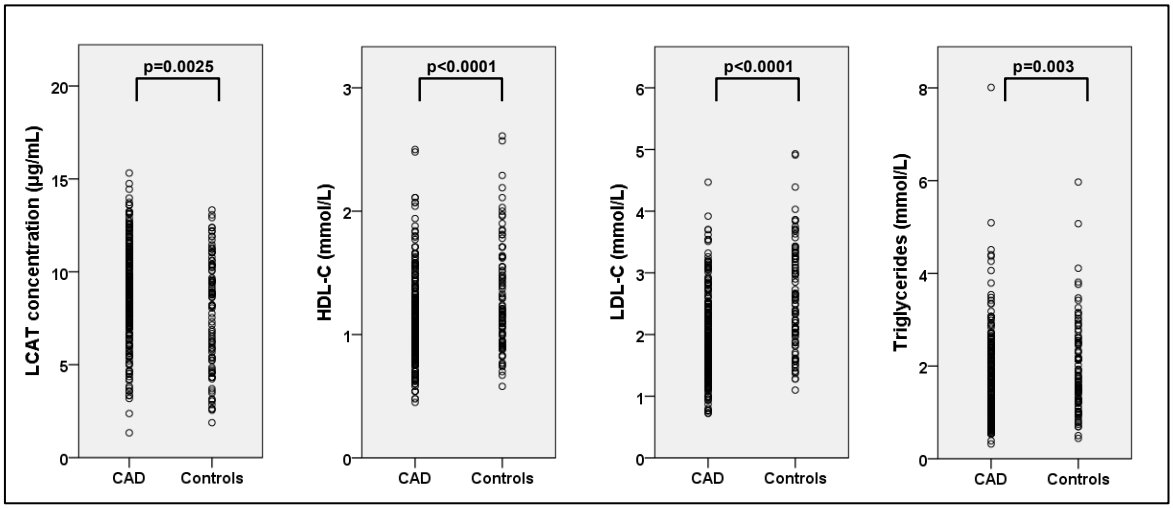

Figure 11: Comparison of LCAT concentration, HDL-C, LDL-C and triglycerides in cases and controls. LCAT, Lecithin: cholesterol acyltransferase; HDL, high density lipoproteins; LDL, low density lipoproteins.

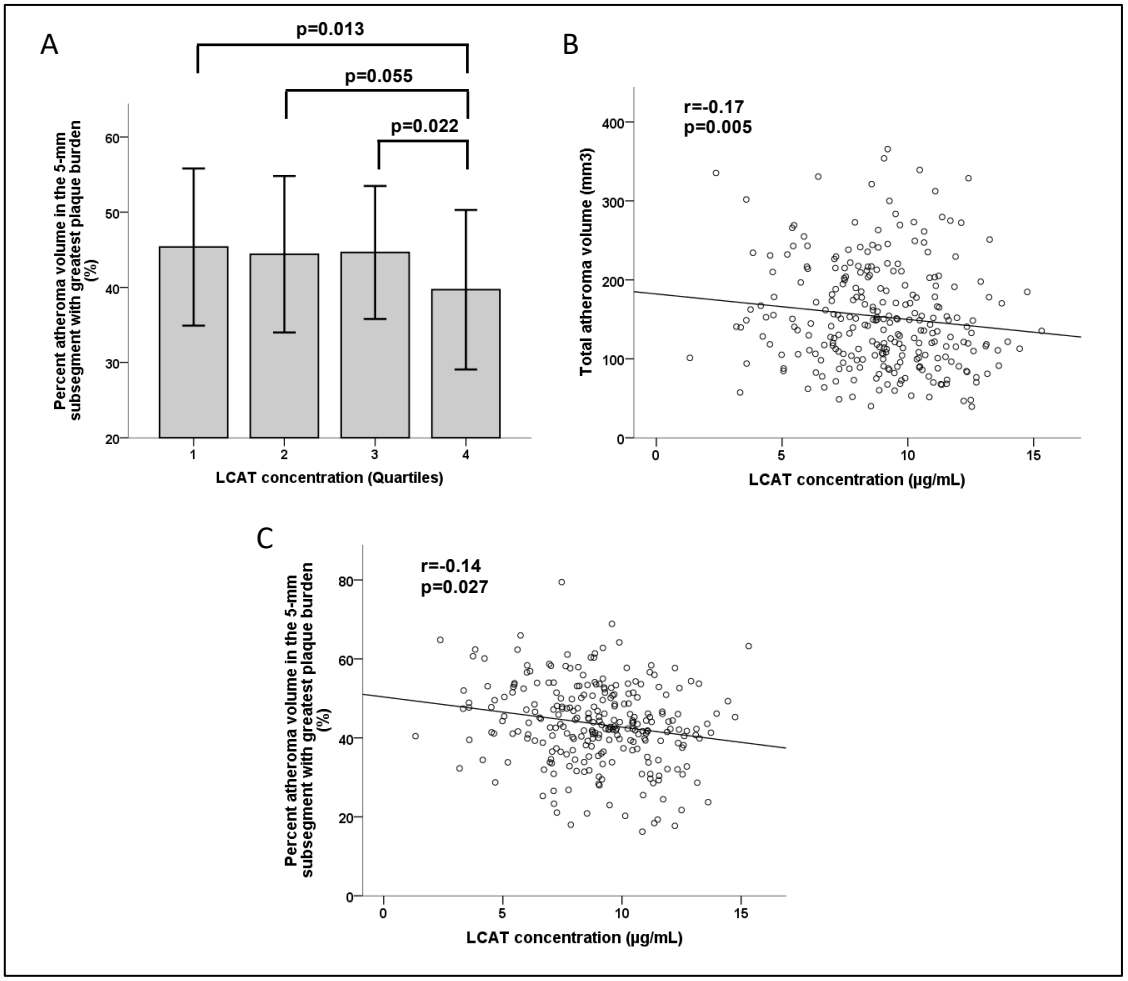

Figure 12: Plasma lecithin: cholesterol acyltransferase concentration and atheroma burden in CAD patients. A. Atheroma burden indicated as percent atheroma volume in the5-mm subsegment with greatest plaque burden by IVUS in patients with CAD, stratified by LCAT quartiles. **B.** Inverse correlation of percent atheroma volume over the 5-mm subsegment with greatest plaque burden and plasma LCAT concentration in CAD patients. Spearman r and p-values are indicated. **C.** Inverse correlation of total coronary atheroma volume and plasma LCAT concentration in CAD patients. Spearman r and p-values are indicated.

Baseline characteristics	CAD (n=267)	Controls (n=96)	P-value
Age, years (mean ± SD)	61.1 ± 9.0	60.2 ± 10.5	NS
Men, n (%)	190 (71.1)	56 (58.3)	0.0211
BMI, kg/m^2 (mean ± SD)	28.1 ± 5.9	30.7 ± 6.1	0.0004
Dyslipidemia, n (%)	230 (86.1)	41 (42.7)	0.0001
Hypertension, n (%)	190 (71.1)	41 (43.2)	0.0001
Type 2 diabetes, n (%)	65 (24.3)	12 (12.5)	0.0149
Current smoking, n (%)	43 (16.1)	5 (5.2)	0.0066

Medications	CAD (n=267)	Controls (n=96)	P-value
Statins, n (%)	232 (86.9)	40 (41.7)	<0.0001
Fibrates, n (%)	10 (3.8)	1 (1.0)	ns
ACEI or ARB, n (%)	177 (66.3)	40 (41.7)	<0.0001
Beta blockers, n (%)	181 (67.8)	51 (53.1)	0.010
Calcium channel blockers, n (%)	104 (39.0)	19 (19.8)	<0.0007
Insulin therapy, n (%)	14 (5.2)	2 (2.1)	ns
Metformin, n (%)	48 (18.0)	8 (8.3)	0.025
PPARγ agonists, n (%)	6 (2.2)	0	ns
Sulfonylureas, n (%)	20 (7.5)	4 (4.2)	ns
Aspirin, n (%)	248 (92.9)	54 (56.3)	<0.0001
Antiplatelet agents, n (%)*	180 (67.4)	2 (2.1)	<0.0001

Table II: Baseline characteristics and medication. Upper panel: baseline characteristics. **Lower panel:** medication. Data are mean±SD or n (%). BMI, body mass index. *mainly Simvastatin, Pravastatin, and Atorvastatin. ACEI, ACE inhibitor; ARB, angiotensin receptor blocker; PPAR, peroxisome proliferator-activated receptor.

Biomarkers	CAD (n=267)	Controls (n=96)	P-value
Total cholesterol, mM	3.68±0.88	4.72±1.04	<0.0001
LDL-cholesterol, mM	1.93±0.66	2.60±0.83	<0.0001
HDL-cholesterol, mM	1.10±0.35	1.31±0.43	<0.0001
Triglycerides, mM	1.65±1.07	1.93±0.97	0.0031
Apolipoprotein A-I, g/L	1.33±0.26	1.49±0.26	<0.0001
Apolipoprotein B, g/L	0.69±0.10	0.85±0.23	<0.0001
Lipoprotein (a), g/L	0.32±0.34	0.24±0.30	0.0146
OxLDL mass, U/L	55.17±17.68	63.48±21.79	0.0003
Hs-CRP, mg/L	3.79±6.06	3.20±8.87	NS
Glucose, mM	6.55±2.30	6.31±2.49	NS
Bilirubin, µM	8.83±5.05	9.46±5.48	NS
Creatinine, µM	85.45±17.07	90.85±23.51	NS
LCAT concentration, µg/mL	8.94±2.51	7.89±2.99	0.0025

Table III: Plasma lipid levels and biochemistry parameters in cases and control subjects. Data are mean±SD. ApoA-I, apolipoproteinA-I; ApoB, apolipoproteinB; oxLDL, oxidized low density lipoprotein mass concentration; LDL, low density lipoproteins; HDL, high density lipoproteins; LCAT, lecithin: cholesterol acyltransferase.

Correlations between LCAT concentration and biochemistry parameters in CAD patients (n=267)		
	Spearman r	P-value
Total cholesterol, mM	0.23	0.0002
LDL-cholesterol, mM	0.17	0.005
HDL-cholesterol, mM	0.11	0.08
Triglycerides, mM	0.30	<0.0001
OxLDL, U/L	0.25	<0.0001
ApoA-I	0.15	0.014

Table IV: Univariate correlations of plasma lecithin: cholesterol acyltransferase activity with plasma lipoproteins in patients with coronary artery disease (CAD). Spearman correlation coefficients and p-values are shown. oxLDL, oxidized low density lipoprotein mass concentration; LDL, low density lipoproteins; HDL, high density lipoproteins.

Biochemistry assessment and risk factors according to LCAT concentration in CAD patients (n=267)					
	LCAT conc. Quartile 1	LCAT conc. Quartile 2	LCAT conc. Quartile 3	LCAT conc. Quartile 4	p-value ANOVA/ Chi-square
LCAT concentration, µg/mL	<7.3	7.3-8.9	9.0-10.7	>10.7	<0.0001
LDL-cholesterol, mM (mean ± SD)	1.88±0.72	1.82±0.72	1.96±0.61	2.11±0.64	0.06
HDL-cholesterol, mM (mean ± SD)	1.03±0.34	1.14±0.39	1.08±0.34	1.13±0.32	0.21
Triglycerides, mM (mean ± SD)	1.40±1.04	1.63±1.38	1.67±0.81	1.95±0.92	0.03
Apolipoprotein A-I, g/L (mean ± SD)	1.27±0.30	1.35±0.26	1.32±0.24	1.38±0.20	0.09
Apolipoprotein B, g/L (mean ± SD)	0.67±0.22	0.65±0.18	0.70±0.18	0.76±0.19	0.005
OxLDL, U/L (mean ± SD)	51.56±15.77	49.80±14.14	57.30±18.34	62.83±19.37	<0.0001
Male sex, n (%)	52 (78.8)	50 (76.9)	46 (69.7)	39 (59.1)	0.053
Age, years (mean ± SD)	61.3±8.8	60.5±9.0	62.5±8.0	59.8±10.0	0.332
BMI, kg/m^2 (mean ± SD)	29.4±5.6	29.9±6.6	31.5±5.8	32.3±6.2	0.031
Dyslipidemia, n (%)	52 (78.8)	52 (80.0)	63 (95.5)	59 (89.4)	0.016
Hypertension, n (%)	42 (63.6)	49 (75.4)	48 (72.7)	49 (74.2)	0.43
Type 2 diabetes, n (%)	13 (19.7)	18 (27.7)	19 (28.8)	15 (22.7)	0.59
Current smoking, n (%)	44 (33.3)	47 (27.7)	40 (39.4)	43 (34.8)	0.60
Statin treatment, n (%)	45 (68.2)	45 (69.2)	48 (72.7)	55 (83.3)	0.18

Table V: Characteristics and lipid levels of CAD patients stratified by plasma LCAT quartiles. Data are mean±SD or n (%). BMI, body mass index; LDL, low density lipoproteins; HDL, high density lipoproteins; LCAT, lecithin: cholesterol acyltransferase.

Stepwise linear regression model for percent atheroma volume in the 5-mm subsegment with the greatest plaque burden in CAD patients (n=267)					
	R^2	F	b coefficient	SE	p-value
Model 1	0.036	9.61	50.3		<0.0001
LCAT conc.			-0.772	0.25	0.002
Model 2	0.06	8.2	49.8		<0.0001
LCAT conc.			-0.812	0.25	0.001
Diabetes			3.66	1.4	0.011
Model 3	0.075	6.9	46.8		<0.0001
LCAT conc.			-0.714	0.25	0.005
Diabetes			3.86	1.4	0.007
Sex			2.83	1.4	0.042
Excluded variables: Age, Hypertension, BMI, Smoking, LDL-cholesterol					

Stepwise linear regression model for total atheroma volume in CAD patients (n=267)					
	R^2	F	b coefficient	SE	p-value
Model 1	0.047	12.7	130.6	7.4	<0.0001
Sex			31.4	8.8	<0.0001
Model 2	0.085	11.8	21.7	3.0	<0.0001
Sex			34.4	8.7	<0.0001
BMI			2.1	0.6	0.001
Model 3	0.099	9.4	24.9	3.6	<0.0001
Sex			31.1	8.8	<0.0001
BMI			2.3	0.6	<0.0001
LCAT conc.			-3.2	1.6	0.045
Excluded variables: Age, Hypertension, Diabetes, Smoking, LDL-cholesterol					

Table VI: Predictor models for atheroma volume assessed by intravascular ultrasound. Upper panel. Model obtained by a stepwise selection procedure for percent atheroma volume over the 5-mm subsegment with the greatest plaque burden. Initial set of predictor variables was BMI, age, sex, hypertension, diabetes, smoking, LDL-cholesterol, and LCAT mass. **Lower panel.** Model obtained by a stepwise selection procedure for total atheroma volume. Initial set of predictor variables was BMI, age, sex, hypertension, diabetes, smoking, LDL-cholesterol, and LCAT mass. SE, standard error; BMI, body mass index, LDL, low-density lipoproteins, LCAT, Lecithin:cholesterol acyltransferase.

3.2 Manuscript #2: Unesterified Cholesterol and Cholesterol Esterification Rate in Apolipoprotein B-depleted Plasma are Powerful Negative Predictors of Atherosclerosis

3.2.1 Foreword to Manuscript #2:

In manuscript #1 we demonstrated a strong and inverse association of LCAT protein mass with atherosclerotic lesion development in humans indicating that LCAT is an important negative predictor of CAD. However, previous studies have reported conflicting data on the role of LCAT in cardiovascular risk prediction. These inconsistent data seem to result from differences in the methods that were applied to measure LCAT protein mass and activity. These methods can be divided into those estimating LCAT mass (protein), LCAT activity using an exogenous substrate method, and those measuring CER using the subject's own plasma or plasma depleted of apoB-containing lipoproteins (endogenous method). In order to address these previous inconsistencies, we aimed to compare these different methods in manuscript #2. In addition, different imaging modalities and image derived scores are currently in use to determine atherosclerotic plaque burden including quantitative coronary angiography (QCA), coronary IVUS, and carotid artery ultrasound. Previous studies were unable to achieve consensus on the impact of LCAT related parameters on coronary and carotid artery plaque burden which might also be due to differences in measurement variability of these imaging systems. Thus, the aim of manuscript #2 was to create a methodological report comparing the predictive value of different methods to measure LCAT and their association with outcome variables of atherosclerosis determined by different imaging modalities.

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3.2.2 Abstract

Background: Cholesterol esterification by lecithin: cholesterol acyltransferase (LCAT) plays a central role in reverse cholesterol transport, a major mechanism by which high-density-lipoprotein (HDL) exerts its antiatherogenic effect. The role of cholesterol esterification in the prevention of atherosclerosis remains controversial.

Method and Results: We determined the association of unesterified cholesterol (UC) concentration and molar cholesterol esterification rate (MER) in apolipoprotein B (apoB)-depleted plasma (HDL-MER) with atherosclerotic plaque burden defined by quantitative coronary angiography (QCA), coronary intravascular ultrasound imaging (IVUS), and carotid artery ultrasound in 267 patients with known coronary artery disease (CAD) and 96 controls. UC concentration and MER in HDL-plasma were significantly lower in plasma of CAD patients as compared to controls (189.5 ± 57.8 µM vs. 236.4 ± 67.0 µM, $p<0.0001$ and 101.5 ± 24.2 µM vs. 115.1 ± 23.8 µM, $p<0.0001$). Both parameters correlated inversely with global plaque area score measured by QCA ($r=-0.23$, $p=0.0002$ and $r=-0.2$, $p=0.002$), and total atheroma volume over a 30 mm IVUS pullback ($r=-0.18$, $p=0.003$ and $r=-0.14$, $p=0.024$), while only UC was negatively associated with common carotid intima-media thickness (cIMT, $r=-0.18$, $p=0.005$). Accordingly, HDL-MER was found to be a significant negative predictor of global plaque area score by QCA (beta coefficient=-0.13, $p=0.039$), total atheroma volume by IVUS (beta coefficient=-0.4, $p=0.029$), and cIMT (beta coefficient=-0.001, $p=0.031$) when tested in a stepwise linear regression analysis against known CAD risk factors as predictor variables. Similarly, HDL-UC was selected as a negative predictor for global plaque are score by QCA (beta coefficient -0.07, $p=0.006$) and cIMT (beta coefficient -0.0001, $p=0.024$).

Conclusion: Cholesterol esterification is downregulated in patients with atherosclerotic disease and exerts anti-atherogenic effects.

3.2.3 Introduction

The large residual burden of disease in optimally treated patients with cardiovascular disease necessitates the search for complementary therapeutics.[371] Thus, metabolism and vascular effects of high-density lipoprotein (HDL) have attracted enormous interest. HDL has a critical role in atheroprotection through reverse cholesterol transport. However, recent studies showing that high HDL-cholesterol (HDL-C) levels are not always protective have led to a shift in paradigm stressing the importance of HDL functionality rather than quantitative measurement of HDL in the assessment of cardiovascular risk.[7, 8, 313, 403]

LCAT is the key enzyme responsible for esterification of free cholesterol to cholesteryl esters, thereby maintaining a concentration gradient of unesterified cholesterol (UC) from cells to HDL and facilitating reverse cholesterol transport.[134, 172] Although the role of cholesterol esterification in the reverse cholesterol pathway has been clearly established, the influence of variation in plasma cholesterol esterification on human atherosclerosis development is still poorly understood. Indeed, investigations in carriers of LCAT deficiency[176, 177, 180, 404] and studies in various animal models overexpressing or lacking LCAT have been largely contradictory and inconclusive.[156, 193, 195, 376, 405-408] Similarly, increased, reduced and unchanged cholesterol esterification rate (CER) have been reported in subjects with cardiovascular disease, and both, negative and positive correlation between LCAT activity and cardiovascular risk have been described.[184, 185, 187, 188, 190] These inconsistent results might partly be due to the imprecision of quantifying cardiovascular risk in a small group of individuals but are also likely to be explained by differences in the laboratory methods used to measure cholesterol esterification rate in plasma: the current methods can be divided into those estimating LCAT mass (protein), LCAT activity using an exogenous substrate method, and those measuring CER using the subjects own plasma or plasma depleted of apoB-containing lipoproteins (endogenous method).[187, 189, 379, 380] Of note, LCAT activity, assayed with the exogenous substrate method closely mirrors its mass concentration.[390, 409] In addition, it is important to realize that the esterification reaction in complete or apolipoproteinB (apoB)-depleted plasma reflects the effects of various lipoprotein particles on the reaction rate and depends on the availability of UC and its transfer from different compartments of the plasma

pool. However, despite these limitations, measurement of CER in human plasma, a natural substrate containing both the subject's enzyme and lipoprotein particles, seems to better reflect the process in vivo than an assay using an artificial substrate. Indeed, it has been proposed that cholesterol esterification in plasma could be anti- or proatherogenic depending on the metabolic context.[410]

Thus, the present study was initiated to compare plasma levels of UC as well as plasma CER, expressed as molar esterification rate (MER), in a cohort of CAD patients and to explore their predictive value for the development of atherosclerotic disease as assessed by IVUS, QCA and carotid intima-media thickness (cIMT). To separate the effects of apoB-containing lipoproteins and HDL, and in particular to avoid the flow of UC from LDL, we have analysed CER rate in both apoB-depleted (HDL-) and complete plasma of CAD patients and controls.

3.2.4 Method

Study Design and Population

267 patients were randomized at 50 Canadian sites participating in the Canadian Atherosclerosis Imaging Network (CAIN) and 96 control subjects who were recruited at the Montreal Heart Institute (Figure 13, page 80). CAIN is a prospective, multi-centre imaging study designed to investigate the natural history, progression, and regression of atherosclerosis.[411] The present CAIN-substudy included patients scheduled for clinically indicated coronary angiography. All angiograms were analysed at the Montreal Heart Institute quantitative coronary angiography core laboratory using the clinical measurements solutions system (MEDIS, Leiden, the Netherlands). Patients enrolled in this study were required to have one target artery for IVUS examination that was not influenced by prior or present percutaneous coronary intervention (PCI), that is not a candidate to undergo PCI in the next 24 months, and that has not been the cause of a recent myocardial infarction. The proximal 4 cm of the target artery in which IVUS examination was performed had to have a diameter stenosis < 50% lumen diameter by visual assessment of the angiogram, a reference diameter > 2.5 mm, and had to be free of any anatomical characteristic that would impede IVUS

interrogation. Eligible patients were women (without childbearing potential) and men over the age of 18 years. Patients had to have angiographic evidence of coronary artery disease as defined by at least one lesion in any of the three major native coronary arteries that has >20% reduction in lumen diameter by angiographic visual estimation or prior history of PCI. Patients with greater than 50% stenosis in the left main coronary artery, previous coronary artery bypass graft (CABG) surgery or probable need for CABG in the next 24 months, symptomatic congestive heart failure (CHF) (New York Heart Association [NYHA] Class III or IV), clinically significant valvular heart disease, presence of severe liver disease, a GFR < 45 ml/min prior to baseline imaging procedures, a life expectancy of less than 2 years, or a history of malignancy during the 3 years prior to the screening were excluded. Following providing written informed consent, patients underwent physical examination, and medical history, ECG, haematology, and clinical biochemistry and lipid profiles were collected. Examination by IVUS and carotid ultrasound were then performed. The study complies with the Declaration of Helsinki and was approved by the Ethics Committee of the Montreal Heart Institute and by institutional ethics committees at all Canadian study centres.

Coronary Angiography and Intravascular Ultrasound Procedures

Intracoronary nitroglycerin (0.1-0.3 mg) was administered into each coronary artery before angiographic examination. The segments of interest were visualized in multiple transverse and sagittal views. Following coronary angiography, the operator selected a single major epicardial vessel for IVUS interrogation based on the following criteria: the vessel has not been influenced by prior or present PCI and has not been the cause of a recent myocardial infarction. The proximal 4 cm of the target artery in which IVUS examination was performed had to have a diameter stenosis < 50% lumen diameter by visual assessment of the angiogram, a reference diameter > 2.5 mm, and had to be free of any anatomical characteristic that would impede IVUS interrogation. A 40 or 45 MHz, 2.6 F (0.87 mm) IVUS catheter was advanced into the target vessel ≥40 mm beyond the coronary artery ostium, to a recognizable landmark. The IVUS transducer was

then withdrawn by a motor drive at a speed of 0.5 mm/sec up to the guiding catheter. IVUS images were obtained at 30 frames/s and recorded.

Quantitative Coronary Angiography Core Laboratory Analysis

All coronary angiograms were analysed at the Montreal Heart Institute QCA Core Laboratory by means of the Clinical Measurements Solutions system (QCA-CMS, Version 5.1; MEDIS Imaging Systems, Leiden, The Netherlands). Comparison of the diameter of the angiographic catheter tip with its known dimension was used to calibrate the system. QCA was performed by experienced technicians supervised by an expert physician. For each lesion, an end-diastolic frame was selected that best showed the stenosis at its most severe degree with minimal foreshortening and branch overlap. Computer software automatically calculated the minimum lumen diameter, reference diameter of the non-diseased arterial segment, percentage diameter stenosis and stenosis length. Percent diameter stenosis was defined as the difference between reference vessel diameter and minimal lumen diameter divided by reference vessel diameter times100%. The QCA variables obtained included the cumulative coronary stenosis score and the global plaque area score. The cumulative coronary stenosis score was calculated by adding all percent diameter stenoses in SI units (50% = 0.50). The global plaque area score was calculated by averaging all of the plaque area values in the segments studied. The global plaque area by QCA is an index of the anatomic extension and severity of CAD and is represented by the area between the estimated interpolated reference and luminal contours within the obstruction boundaries.

Intravascular Ultrasound Core Laboratory Analysis

IVUS pullbacks were analysed off-line in a blinded fashion by the core laboratory as previously reported.[288] Image interpretation was uniformly performed in accordance with the standards of the American College of Cardiology by experienced technicians supervised by a cardiologist.[412] The IVUS examination was screened for image quality by the core laboratory and only patients meeting prespecified image quality requirements were eligible for randomization. For each IVUS cross-section, the operator performed manual planimetry to trace the leading edges of the luminal and external elastic membrane borders. The maximum

atheroma thicknesses were also directly measured. Atheroma area was calculated as external elastic membrane (EEM) area minus luminal area. Since image cross-sections were obtained at 0.5-mm intervals, the total atheroma volume could be calculated as mean atheroma area multiplied by pullback length in millimetres. The percent atheroma volume was computed as: (∑atheroma areas/∑EEM areas)*100.

Measurement of carotid intima-media thickness (cIMT)

For carotid artery image acquisition, high-resolution carotid ultrasound scanning was performed with ultrasound instruments optimized for carotid imaging. Images were acquired to assess cIMT. The common carotid artery and the internal carotid artery were bilaterally scanned over a length of 10 mm. Images were saved and the IMT of the far wall of the respective segments were measured. cIMT measurements were performed in a strictly standardized fashion to ensure that the carotid arteries are imaged in a reproducible way and that appropriate comparisons between baseline and follow-up IMT measurements could be made. Data are given as maximal common (only common carotid artery measured) cIMT.

Biochemical analyses

Blood was collected after an overnight fast. Plasma samples were kept at –80°C prior to analysis. Plasma total cholesterol (total-C), HDL-C, and triglyceride levels were determined with certified enzymatic methods. LDL-cholesterol (LDL-C) was calculated by the equation of Friedewald. Plasma apolipoproteinA-I (apoA-I) and apoB were measured with the nephelometric BN Prospec II System (Dade Behring). High-sensitivity C-reactive protein (hs-CRP) levels were measured by the Dade Behring method. UC content and CER were measured in plasma by using enzymatic/colorimetric assay according to manufacturer's instructions (Wako Free Cholesterol E, #435-35801, Wako Chemicals, Richmond, VA). Samples were incubated at 4°C and 37°C during 2 hrs and the amount of UC lost during 2 hours of incubation was measured by reading the absorbance of the blue colour with a Synergy H1 Hybrid Reader (Biotech). Samples kept at 4°C and non-incubated samples were used as negative controls. No difference was found between the UC content in samples kept at 4°C and assayed at once or incubated for 2 hrs. To separate the effects of the apoB-containing lipoproteins, and in particular to avoid

the flow of unesterified cholesterol from LDL, CER was measured in HDL-enriched plasma (referred to as HDL-plasma) after precipitating apoB-containing particles, as well as in complete plasma. In the absence of VLDL and LDL, the other plasma proteins (particularly albumin) facilitate the diffusion of UC among lipoprotein particles and also bind lysolecithin, thus preventing product inhibition of the reaction. CER in HDL-plasma was reported as µM cholesterol esterified per 2hrs (HDL-MER) as well as % UC esterified per 2hrs (HDL-FER). Similarly, CER in normal plasma were reported as µmol/L cholesterol esterified per 3hrs (plasma-MER) as well as % UC esterified per 3hrs (plasma-FER). Plasma LCAT mass concentration was measured in duplicate by an immunoenzymatic assay from Alpco Diagnostics as previously described.[382]

Statistical analyses

Numerical variables were summarized as means ± SD. Categorical variables were summarized as percentages. Clinical and anthropometric variables were compared between groups by unpaired Student's *t* -test or Chi-square test, as appropriate. The relationship between biochemistry parameters and IVUS measurements were tested by Spearman correlations. Multivariate linear regression analysis was applied to assess the association of plaque burden with CER and UC. For IVUS, QCA and cIMT parameters showing strongest correlations with CER and UC, multivariate models were used in order to identify independent predictors. Using multivariate models, the predictive value of CER and UC on atheroma burden was tested after stepwise selection (criterion: probability-of-F-to-enter <=0.05) by adding known risk predictors for plaque burden including age, sex, body mass index (BMI), smoking status, hypertension, diabetes, HDL-C and LDL-C. All tests were two-sided, and *P* values below 0.05 were regarded as significant. All analyses were performed using the SAS statistical package (SAS Institute Inc., Cary, NC) version 9 and IBM SPSS statistics v23.0.

3.2.5 Results

Baseline characteristics of study participants

The study population comprised 363 subjects (246 males), including 267 participants with CAD and 96 controls free of cardiovascular disease. Patients in the CAD group were more often hypertensive (71.2 vs. 43.2%, p=0.0001), male (71.1% vs. 58.3%, p=0.02), diabetic (24.3% vs. 12.5%, p=0.014), and current smoker (16.1% vs. 5.2%, p=0.007). Moreover, patients in the CAD group had more often dyslipidaemia (86.1% vs. 42.7%, p=0.0001) and were more often treated with statins (86.9% vs. 41.7%, p<0.0001). The characteristics of the study cohort are indicated in Table VII (page 82).

Biochemistry and lipid parameters in CAD patients and controls

Total-C, LDL-C, HDL-C, triglycerides, apoB, apoA-I, and oxidized low density lipoprotein mass concentration (oxLDL) were all significantly lower in CAD patients as compared to controls (at least p<0.05, Table VIII, page 83). Only lipoprotein (a) and LCAT mass plasma concentrations were significantly higher in CAD patients compared with controls (p=0.02 and p=0.003, respectively). No differences in other laboratory parameters including glucose levels, hs-CRP, bilirubin, and creatinine were observed between groups (data not shown). HDL-MER and plasma-MER were significantly lower in CAD patients (101.5 ± 24.2 vs. 115.1 ± 23.8 and 253.6 ± 83.9 vs 315.3 ± 115.0, respectively, p<0.0001, Table VIII, page 83), while HDL-FER was higher in CAD patients (55.4±11.2 vs. 51.2±13.0, p=0.0028, Table VIII, page 83). HDL-UC and plasma-UC were significantly lower in CAD patients as compared to controls (189.5 ± 57.8 vs. 236.4 ± 67.0 and 985.3 ± 189.2 vs. 1249.5 ± 217.4, p<0.0001, Table VIII, page 83). Women had significantly higher levels of UC and a higher MER than men in both apoB depleted plasma and complete plasma (p<0.0001 for HDL-UC, p<0.0001 for HDL-MER, p<0.0001 for plasma-UC, and p=0.012 for plasma-MER, data not shown).

Correlation of unesterified cholesterol and cholesterol esterification rate with other lipid markers and risk factors

HDL-UC was positively correlated with HDL-MER (r=0.7, p<0.0001), triglycerides (r=0.3, p<0.0001), apoA-I (r=0.8, p<0.0001), and total-C (r=0.4, p<0.0001). HDL-

MER correlated significantly with triglycerides (r=0.2, p<0.05), HDL-C (r=0.5, p<0.0001), apoA-I (r=0.7, p<0.0001), and total-C (r=0.5, p<0.0001, Table IX, page 83). HDL-MER, plasma-UC and plasma-MER, all correlated positively and significantly with LCAT mass concentration (p<0.05) in CAD, while no correlation was found between HDL-UC and LCAT mass (p=NS, Table IX, page 83). The correlation between HDL-MER and LCAT mass was significantly stronger in controls as compared to CAD patients (r=0.45 in controls vs. r=0.18 in CAD patients, p=0.006, ANOVA, Figure 15, page 82). When characteristics of participants were stratified by quartiles of HDL-MER, patients with higher HDL-MER (4th quartile) were less likely to have a history of dyslipidaemia (p=0.026), were less often on statins, and were more likely to be female than patients with lower HDL-MER (1st and 2nd quartiles, p<0.05, ANOVA, data not shown). Patients with higher HDL-UC (4th quartile) were more likely to be female and were older than patients with lower HDL-UC (1st and 2nd quartiles, p<0.05, ANOVA, data not shown). No significant differences between HDL-MER or HDL-UC quartiles were found for body mass index (BMI), oxidized LDL (oxLDL), hypertension, diabetes mellitus, and smoking (p=NS, ANOVA).

Correlation of cholesterol esterification with coronary plaque burden in CAD patients

IVUS study – HDL-CER and HDL-UC correlated significantly with plaque burden as assessed by IVUS, while no correlations were found between cholesterol esterification and plaque burden when complete plasma was assayed. In detail, HDL-UC was inversely correlated with the following IVUS parameters: total (r=-0.18, p=0.003, Table X, page 84, Figure 14A, page 81) and percent (r=-0.14, p=0.03) coronary atheroma volume, total vessel volume (r=-0.15, p=0.02), total lumen volume (r=-0.12, p=0.049), total atheroma volume over 5 mm with most plaque burden (r=-0.2, p=0.002), total vessel volume over 5 mm with most plaque burden (r=-0.18, p=0.003), and total lumen volume over 5 mm most plaque burden (r=-0.12, p=0.048, Table X, page 84). HDL-MER correlated inversely with total (r=-0.14, p=0.02, Table X, page 84 and Figure 14B, page 81) and percent (r=-0.14, p=0.03) coronary atheroma volume, total atheroma volume over 5 mm with most

plaque burden (r=-0.16, p=0.008), percent atheroma volume over 5 mm with most plaque burden (r=-0.13, p=0.03), total vessel volume over 5 mm with most plaque burden (r=-0.12, p=0.04), and percent atheroma volume over 5 mm with least plaque burden (r=-0.12, p=0.047, Table X, page 84). Accordingly, patients with HDL-UC ranging in the highest quartile had significant less total atheroma volume than patients in the lower quartiles (p=0.023, ANOVA). Similarly, patients with HDL-MER ranging in the highest quartile had significant less total atheroma volume than patients in the lower quartiles (p=0.031, ANOVA). Notably, no significant correlation was found between HDL-FER and IVUS plaque burden.

QCA study – HDL-MER and HDL-UC correlated significantly with plaque burden as assessed by QCA, while only weak correlations or no correlations were found for MER and UC in complete plasma. Of note, no correlations were found between FER in HDL and plaque burden as assessed by QCA. HDL-UC was inversely correlated with cumulative coronary stenosis score (r=-0.16, p=0.01, Table X, page 84) and global plaque area score (r=-0.23, p=0.0002). Similarly, HDL-MER correlated inversely with cumulative coronary stenosis score (r=-0.14, p=0.02, Table X, page 84) and global plaque area score (r=-0.2, p=0.002, Table X, page 84). Accordingly, patients with HDL-UC ranging in the highest quartile had a lower cumulative coronary stenosis score and lower global plaque area score than patients in the lower quartiles (p=0.041 and p=0.001, respectively, data not shown). Similarly, patients with HDL-MER ranging in the highest quartile had a lower cumulative coronary stenosis score and lower global plaque area score than patients in the lower quartiles (p=0.029 and p=0.014, respectively, data not shown).

cIMT study – HDL-UC correlated significantly and negatively with mean and maximal cIMT as assessed by B-mode ultrasound (r=-0.16, p=0.009 and r=-0.18, p=0.005, respectively, data not shown). A weaker positive correlation was seen between HDL-FER and mean and maximal cIMT (r=0.12, p=0.047 and r=0.137, p=0.03, data not shown) and no correlation with carotid artery plaque burden were found for HDL-MER and when normal plasma was assayed. Patients with HDL-UC ranging in the highest quartile had significant less maximal cIMT than patients in the lower quartiles (p=0.02, ANOVA, data not shown).

Predictors of atheroma burden in CAD patients

When HDL-MER was tested in a forward stepwise linear regression analysis with total atheroma volume (IVUS) being the dependant variable and BMI, age, gender, hypertension, diabetes, smoking, and LDL-C as predictor variables, three significant variables were found: gender, BMI and HDL-MER. The probability was best explained by gender (beta coefficient 31.1, $p<0.0001$, model 1), followed by BMI (beta coefficient 2.1, $p=0.001$, model 2), and HDL-MER (beta coefficient -0.38, $p=0.029$, model 3) (Table XI, upper panel, page 85). When all lipid parameters including apoA-I, apoB, apoB/ApoA ratio, oxLDL, MER, FER, UC, triglycerides, Lp(a), LDL-C, HDL-C, total C, and LCAT mass were set as predictor variables, gender, BMI and HDL-MER remained significant predictor variables (data not shown). Similarly, HDL-MER was a significant predictor for total atheroma volume over 5 mm with least plaque burden (IVUS) (model 1 = BMI: beta coefficient 0.29, $p=0.008$; model 2 = BMI+HDL-MER: beta coefficient -0.06, $p=0.047$, Table XI, lower panel, page 85) and global plaque area score as assessed by QCA (model 1 = gender: beta coefficient 14.6, $p<0.0001$; model 2 = gender+HDL-MER: beta coefficient -0.13, $p=0.039$, Table XII, upper panel, page 86). When HDL-UC was included in the set of predictors with global plaque area score as assessed by QCA being the dependant variable, two significant variables were found including gender and HDL-UC (model 1 = gender: beta coefficient 14.6, $p<0.0001$; model 2 = gender+HDL-UC: beta coefficient -0.07, $p=0.006$, Table XII, lower panel, page 86). Both, HDL-UC and HDL-MER were significant predictors of maximal cIMT as assessed by carotid artery ultrasound when tested in similar models ($p=0.008$ and $p=0.031$, respectively, Table XIII left and right panel, page 87). However, if both parameters were entered into predictor models of atheroma burden, HDL-UC was selected over HDL-MER by the model (data not shown).

3.2.6 Discussion

This study demonstrates that HDL-MER and HDL-UC are downregulated in patients with CAD and that both parameters are significant negative predictors of

carotid artery and coronary plaque burden when tested in a stepwise linear regression model.

The value of CER in cardiovascular risk prediction has been controversially discussed in the past and has remained elusive. The conflicting results seen in previous studies seem to mirror the variety of methods used to measure cholesterol esterification in small and heterogeneous populations: consistent with our data, an earlier study reported lower LCAT activity in 90 patients with angiographically proven CAD as compared to controls by using an endogenous substrate method.[187, 413] Likewise, Sethi and co-workers report lower LCAT activity in 53 patients with ischemic heart disease by using a proteoliposome cholesterol esterification assay.[188] Other investigators described reduced LCAT activity in 90 patients with CAD[414] and in 60 patients with acute myocardial infarction.[174, 391] In contrast, high CER, as assessed by an endogenous substrate method, was associated with an increased risk of CAD and sudden cardiac death in Japanese women.[184] By using an exogenous substrate method, Dullart and colleagues found an elevated CER rate in 74 patients with metabolic syndrome which was positively associated with atherosclerosis.[190] Although, most of these studies lack a prospective design, these seemingly contradictory results from heterogeneous populations may support the concept that CER depends on the metabolic context, differs between men and women and may vary depending on the method (exogenous vs. endogenous substrate) used to assess CER. Given the effects of various plasma lipoprotein particles on CER, we applied an endogenous substrate method in the current study. This assay measures the difference in plasma concentration of UC before and after incubation at 37°C and was chosen for CER analysis since it seems to reflect best the process in vivo. In contrast, LCAT activity assessed by the exogenous method is associated with LCAT mass concentration and might not permit to detect substrate or cofactor abnormalities.[185, 382, 389, 390]

Importantly, in our study, we observed only weak correlations between CER and atherosclerosis development when complete plasma was assayed, while strong correlations were found in HDL-enriched plasma. Importantly, in the absence of VLDL and LDL, other plasma proteins facilitate the diffusion of UC among lipoprotein particles.[415] Thus, it has been suggested that CER in apoB

depleted plasma reflects the ability of an individual's HDL-LCAT complex to esterify UC that has been transported by diffusion from surfaces of either lipoproteins. Our study is the first comparing the value of these different parameters in predicting atherosclerotic plaque burden and our data indicate that only MER and UC measured in apoB depleted plasma but not in complete plasma are significant predictors of plaque burden when assessed in a stepwise liner regression model.

Despite the well documented association of HDL-FER with coronary disease,[197, 312, 416-418 419] we did not detect any correlation between HDL-FER and coronary plaque burden or cIMT in our study. HDL-FER has been suggested to be an index of HDL composition, and has been proposed to have potential clinical value in the assessment of cardiovascular risk.[416, 420] However, FER expresses only the percentage of decrease of UC per hour of incubation, while MER takes into account the subjects actual concentration of UC, directly reflecting LCAT activity.[415] Thus, the strong inverse association we observed between HDL-UC and lesion development in CAD patients might be the trigger for a concomitant significant relation between MER and plaque burden, which, in turn, is reflected by a strong positive correlation (r=0.7) between MER and UC. Thus, our results highlight the stimulatory role of UC availability and its mobilization from various compartments of the plasma pool in the cholesterol esterification reaction.

High amounts of UC-rich lipid particles have been detected in atherosclerotic lesions and subendothelial accumulation of UC has therefore been suggested as an early marker in atherosclerotic lesion development.[421, 422] By creating a concentration gradient, the esterification of UC by LCAT generates a flux of cholesterol from cell membranes into the plasma compartment, thereby facilitating reverse cholesterol transport. Thus, cholesterol efflux from cell membranes is the major mechanism for the removal of cellular UC and is critical in preventing foam cell formation and atherosclerotic lesion development. However, cell membranes seem only be a minor donor of UC to the LCAT reaction and most UC has been shown to originate from apoB containing lipoproteins such as LDL and VLDL given their function as a reservoir to store UC.[423, 424] The fact that LDL and not cell membranes is the major donor of UC, however, would not support the presumed important role of cholesterol esterification in reverse cholesterol

transport. In this regard, it has been reported that UC in LDL originates from peripheral cells and that an ongoing transfer of cellular UC to LDL and an ongoing redistribution from LDL to preβ-HDL and α-HDL occurs.[424] In line with this notion, Sprandela and colleagues have recently demonstrated that a high rate of UC transfer to HDL in the plasma is protective against CAD.[425] In agreement with our results, these authors found a diminished UC content in plasma as well as reduced UC transfer to HDL in CAD patients[425] indicating that the amount of UC in plasma might be a useful new marker for atherosclerotic lesion development. Of note, since HDL is an extracellular acceptor of unesterified cholesterol, intracellular hydrolysis of stored cholesteryl esters by macrophage CEH is a limiting factor in the mobilization of cholesterol esters in addition to efflux from the cell surface.

In accordance with ample previous evidence, CER correlated with LCAT protein concentration in the present study.[172] Interestingly, this correlation was significantly stronger in controls as compared to CAD patients indicating the influence of a cofactor, the presence of inhibitors or binding of LCAT to lipoproteins not permitting cholesterol esterification. Indeed, LCAT inhibition by interference with metabolites such as L-lactate or fatty acids, limited availability of cholesterol acceptors, reduced levels of cholesteryl ester transfer protein (CETP) or oxidative modifications of apoA-I and LCAT have been suggested to account for a downregulation of CER in CAD patients.[187, 426-429] However, considering the significantly lower concentration of UC measured in plasma of CAD patients as compared to controls, it seems that a defect in substrate availability rather than a reduced coactivation or oxidative modification of LCAT might be responsible for reduced cholesterol esterification seen in CAD patients in our study. Given the strong negative predictive value of UC for atheroma burden and considering the fact that LCAT mass concentration was found to be significantly higher in CAD patients as compared to controls, we hypothesize that the decrease in CER observed in CAD patients may most likely reflect a reduced availability and activity of substrate in this population rather than a reduced enzyme concentration.

Carotid IMT is one of the best established and most commonly used surrogate markers of human atherosclerosis. In addition, it correlates well with the severity of CAD and is a risk predictor of cardiovascular events in prospective

studies.[272, 430] However, previous carotid ultrasound studies in carriers of LCAT mutations were unable to achieve consensus on the impact of CER and LCAT on carotid atherosclerosis. Indeed, Hovingh et al. showed that cIMT was significantly increased in carriers of LCAT gene mutations, while Calabresi et al. showed the opposite.[177, 180] Further, in patients with either angiographically documented coronary artery disease or acute myocardial infarction, both decreased and increased LCAT activity have been observed.[187, 379, 380] This apparent discrepancy may be explained by differences in the populations (homozygote vs. heterozygote carriers of LCAT mutations vs. general population). Nevertheless, our data support the concept that decreased CER in the general population is associated with accelerated atherogenesis. Importantly, our results complement recent findings showing that cIMT is negatively determined by plasma LCAT activity and cholesterol efflux capacity from macrophages.[136, 178] By using a stepwise linear regression model, HDL-MER and HDL-UC were both selected as significant negative predictors of maximal cIMT in our study. Interestingly, the strongest correlation was found between HDL-UC and cIMT while the association between HDL-MER and cIMT did not reach statistical significance. The latter most likely pertains to the lack of power due to the higher measurement variability of ultrasound cIMT, as attested to by the significant QCA and IVUS findings for both parameters in our study. Indeed, significant negative correlations were found for CER, UC and coronary atherogenesis by means of QCA and IVUS. In addition, HDL-MER and HDL-UC were selected as significant negative predictors for global plaque area score by QCA in a stepwise linear regression model.

There are several limitations to our study. Previous studies have revealed that CER in plasma is positively associated with triglycerides, while oxLDL or increasing HDL-C inhibit the LCAT reaction.[205, 431-434] In line with this possibility, it has been hypothesized that a cardioprotective role of cholesterol esterification depends on the quality of HDL particles.[410] Accordingly, the HDL_2 subfraction has been suggested to inhibit CER, whereas HDL_3 and smaller HDLs may serve as substrates facilitating cholesterol esterification.[435] Importantly, statins have been shown to increase the large α_1-HDL subpopulation which, in turn, suppresses the esterification rate of cholesterol.[436] In our study, significantly more patients in the CAD group were treated with statins. In addition, triglycerides, oxidized LDL and

HDL-C levels were significantly lower in CAD patients as compared to controls. However, all three parameters showed a strong and positive correlation with CER making an inhibitory effect on the LCAT reaction unlikely. Nevertheless, we did not assess HDL subfractions in the present study. Thus, we cannot exclude an inhibitory effect of HDL subpopulations on CER in CAD patients. Further, in the current study we found higher CER and UC concentrations in women as compared to men, thereby confirming previous reports.[184] In addition, a positive association of UC with increasing age was found in CAD patients. At this point, however, we have no explanation for this effect and our study was not powered to assess whether CER and UC in women and men exert opposite trends in CAD prediction. This will have to be investigated in a sufficiently powered, gender- and age-specific prospective analysis. Finally, no conclusion is allowed as to whether plasma CER predicts progression of subclinical atherosclerosis, inherent to the cross-sectional design of our study.

To the best of our knowledge, this is the first study to determine the relation of plasma CER as a measure of LCAT activity with prevalent atherosclerosis by using three different imaging modalities including IVUS. IVUS imaging provides more details on plaque biology and geometry than conventional QCA and permits the assessment of vessel remodelling characteristics. Combined, our findings point to accelerated atherogenesis in both coronary and carotid vessels in individuals with reduced HDL-UC and CER. Thus, measuring CER and UC in apoB depleted plasma using the endogenous substrate method is useful in predicting atherosclerosis development. Finally, based on the present data, it is tempting to speculate that increasing LCAT activity is an interesting target to reduce cardiovascular risk.

3.2.7 Figures and Tables

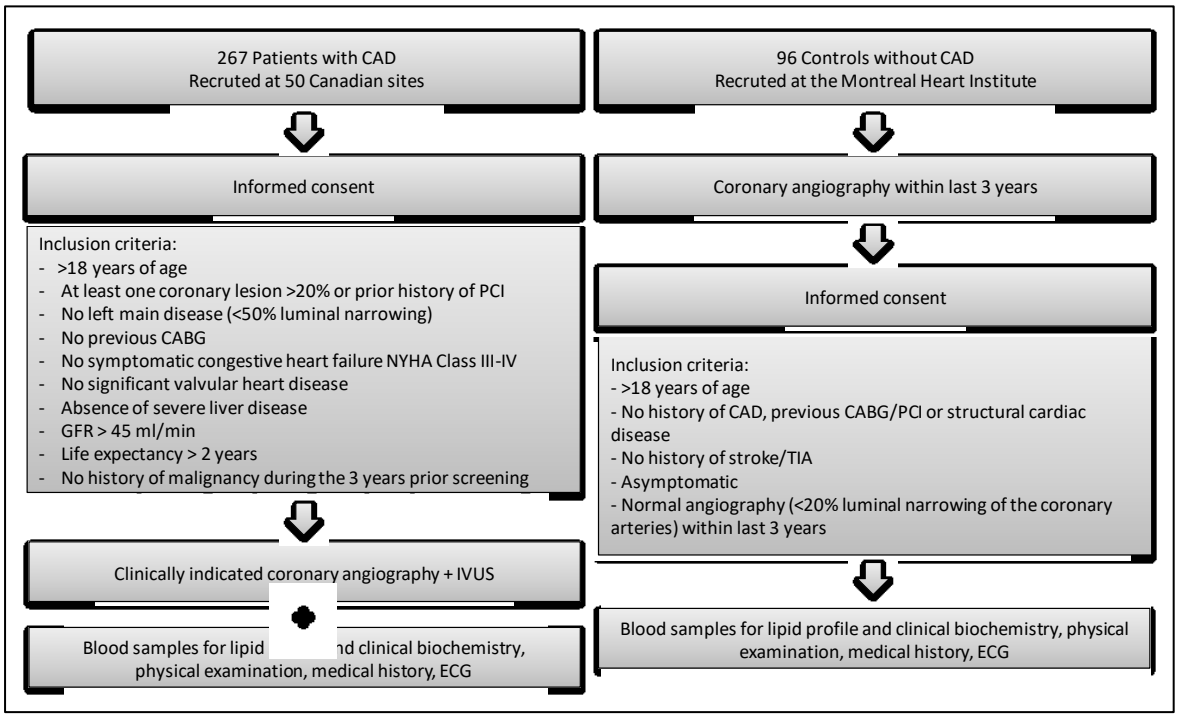

Figure 13: Flow chart of patient recruitment and follow-up in patients

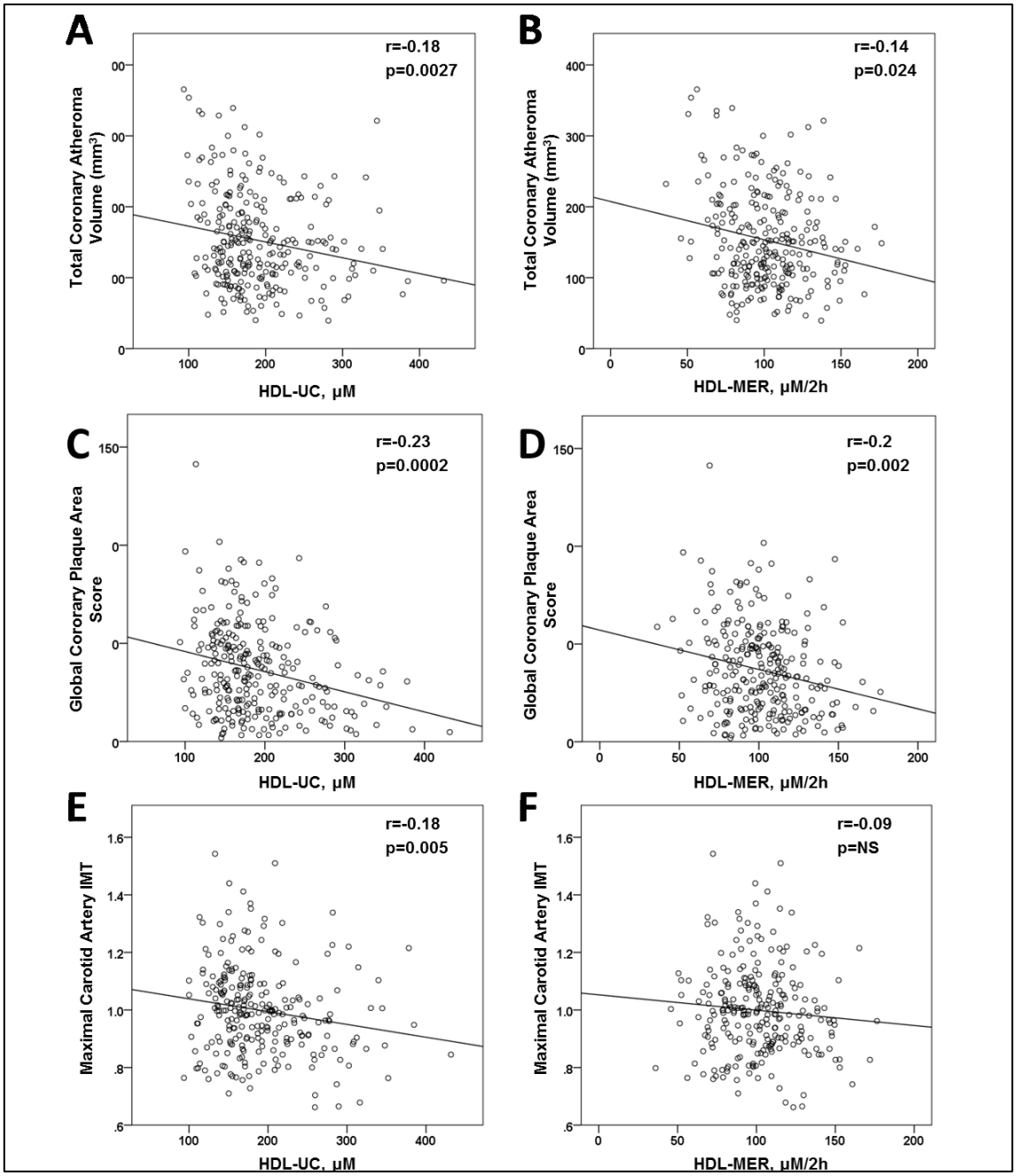

Figure 14: Unesterified cholesterol concentration, molar esterification rate and atheroma burden in CAD patients. A. Inverse correlation of unesterified cholesterol concentration in apoB depleted plasma and total atheroma volume as assessed by intravascular ultrasound. **B.** Inverse correlation of molar esterification rate in apoB depleted plasma and total atheroma volume as assessed by intravascular ultrasound. **C.** Inverse correlation of unesterified cholesterol concentration in apoB depleted plasma and global plaque area score as assessed by quantitative coronary angiography. **D.** Inverse correlation of molar esterification rate in apoB depleted plasma and global plaque area score as assessed by quantitative coronary angiography. **E.** Inverse correlation of unesterified cholesterol concentration in apoB depleted plasma and carotid intima media thickness (IMT) as assessed by B-mode ultrasound. Pearson r and p-values are indicated. HDL-UC, unesterified cholesterol concentration in apoB depleted plasma; HDL-MER, molar esterification rate in apoB depleted plasma

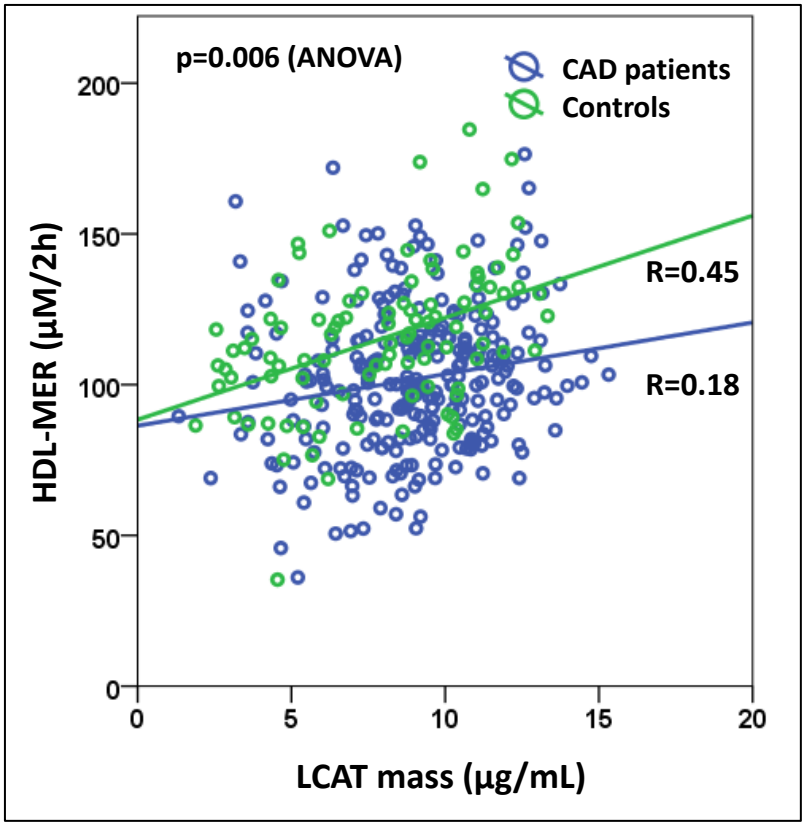

Figure 15: Correlation of LCAT mass with molar esterification rate in ApoB depleted plasma (HDL-MER). Comparison between patients (blue) and controls (green). Pearson r and p-values are indicated.

Baseline characteristics	CAD (n=267)	Control (n=96)	P-value
Age, years (mean ± SD)	61.1 ± 9.0	60.2 ± 10.5	NS
Men, n (%)	190 (71.1)	56 (58.3)	0.02
BMI, kg/m^2 (mean ± SD)	28.1 ± 5.9	30.7 ± 6.1	0.0004
Dyslipidemia, n (%)	230 (86.1)	41 (42.7)	0.0001
Hypertension, n (%)	190 (71.2)	41 (42.7)	0.0001
Type 2 diabetes, n (%)	65 (24.3)	12 (12.5)	0.02
Current smoker, n (%)	43 (16.1)	5 (5.2)	0.007
Statins*, n (%)	232 (86.9)	40 (41.7)	<0.0001
Fibrates, n (%)	10 (3.75)	1 (1.0)	NS

Table VII: Baseline characteristics and lipid lowering medication of study population. Data are mean±SD or n (%). BMI, body mass index. *mainly Simvastatin, Pravastatin, and Atorvastatin.

Biomarker	CAD (n=267)	Control (n=96)	P-value
Total cholesterol, mM	3.7 ± 0.9	4.7 ± 1.0	<0.0001
LDL-cholesterol, mM	1.9 ± 0.7	2.6 ± 0.8	<0.0001
HDL-cholesterol, mM	1.1 ± 0.4	1.3 ± 0.4	<0.0001
Triglycerides, mM	1.7 ± 1.1	1.9 ± 1.0	0.003
Apolipoprotein A-I, g/L	1.3 ± 0.3	1.5 ± 0.3	<0.0001
Apolipoprotein B, g/L	0.7 ± 0.2	0.9 ± 0.2	<0.0001
Lipoprotein (a), g/L	0.3 ± 0.3	0.2 ± 0.3	0.02
OxLDL mass, U/L	55.2 ± 17.7	63.5 ± 21.8	0.0003
LCAT mass, µg/mL	8.9 ± 2.5	7.9 ± 3.0	0.003
Plasma-UC, µM	985.3 ± 189.2	1249.5 ± 217.4	<0.0001
Plasma-MER, µM/2h	253.6 ± 83.9	315.3 ± 115.0	<0.0001
Plasma-FER, %	25.8 ± 7.4	25.0 ± 7.0	NS
HDL-UC, µM	189.5 ± 57.8	236.4 ± 67.0	<0.0001
HDL-MER, µM/2h	101.5 ± 24.2	115.1 ± 23.8	<0.0001
HDL-FER, %	55.4 ± 11.2	51.2 ± 13.0	0.0028
Ratio HDL-UC/Plasma-UC	0.2 ± 0.06	0.2 ± 0.06	NS

Table VIII: Lipid related parameters in cases and control subjects. Data are mean±SD. oxLDL, oxidized low density lipoprotein mass concentration; LDL, low density lipoproteins; HDL, high density lipoproteins; FER, fractional esterification rate; MER, molar esterification rate; UC, unesterified cholesterol, LCAT, lecithin: cholesterol acyltransferase. HDL-UC, unesterified cholesterol concentration in apoB depleted plasma; HDL-MER, molar esterification rate in apoB depleted plasma; HDL-FER, fractional esterification rate in apoB depleted plasma; plasma-UC, unesterified cholesterol concentration in complete plasma; plasma-MER, molar esterification rate in complete plasma; plasma-FER, fractional esterification rate in complete plasma.

Correlation of Esterification Rate and Lipid Parameters in CAD patients (n=267)										
	Plasma-UC	HDL- UC	TG	HDL-C	LDL-C	ApoA-I	ApoB	Total-C	Plasma-UC/ Total-C	LCAT mass
Plasma-UC	-	-	0.36*	0.22*	0.76*	0.35*	0.79*	0.86*		0.18*
Plasma-MER	0.51*	-	0.36*	0.09	0.23*	0.23*	0.32*	0.31*	0.16*	0.15*
HDL-UC	-	-	0.33*	0.91*	-	0.89*	-	0.41*		-
HDL-MER	-	0.67*	0.15*	0.53*	0.27*	0.65*	-	0.46*		0.18*

Table IX: Univariate correlations of cholesterol esterification rate and traditional lipid parameters in patients with coronary artery disease (CAD). Pearson correlation coefficients and p-values are shown. oxLDL, oxidized low density lipoprotein mass concentration; LDL, low density lipoproteins; HDL, high density lipoproteins; HDL-UC, unesterified cholesterol concentration in apoB depleted plasma; HDL-MER, molar esterification rate in apoB depleted plasma; plasma-UC, unesterified cholesterol concentration in complete plasma; plasma-MER, molar esterification rate in complete plasma; apoA-I, apolipoproteinA-I; apoB, LCAT, Lecithin: cholesterol acyltransferase; TG, triglycerides; total-C, total cholesterol; *p<0.05.

Correlation between HDL-CER and plaque burden as assessed by QCA and IVUS in CAD patients (n=267)						
	HDL-UC		HDL-MER		HDL-FER	
	r	P-value	r	P-value	r	P-value
QCA						
Minimal lumen diameter	0.03	0.6	0.09	0.17	0.03	0.6
Cumulative coronary stenosis score	-0.16	0.01*	-0.14	0.02*	0.09	0.2
Global plaque area score	-0.23	0.0002*	-0.2	0.002*	0.1	0.1
IVUS						
Total atheroma volume (30 mm)	-0.18	0.003*	-0.14	0.02*	0.05	0.4
Percent atheroma volume (30 mm)	-0.14	0.03*	-0.14	0.03*	0.05	0.4
Total vessel volume (30 mm)	-0.15	0.02*	-0.1	0.1	0.04	0.5
Total lumen volume (30 mm)	-0.12	0.049*	-0.06	0.4	0.04	0.5
Total atheroma volume over 5 mm with most plaque burden	-0.2	0.002*	-0.16	0.008*	0.04	0.5
Percent atheroma volume over 5 mm with most plaque burden	-0.1	0.1	-0.13	0.03*	-0.01	0.8
Total vessel volume over 5 mm with most plaque burden	-0.18	0.003*	-0.12	0.04*	0.06	0.3
Total lumen volume over 5 mm with most plaque burden	-0.12	0.048*	-0.07	0.3	0.06	0.3
Total atheroma volume over 5 mm with least plaque burden	-0.12	0.05	-0.08	0.2	0.03	0.6
Percent atheroma volume over 5 mm with least plaque burden	-0.1	0.08	-0.12	0.047*	-0.002	0.9
Total vessel volume over 5 mm with least plaque burden	-0.09	0.2	-0.02	0.7	0.04	0.5
Total lumen volume over 5 mm with least plaque burden	-0.07	0.3	-0.01	0.9	-0.03	0.6

Table X: Correlation of cholesterol esterification rate (CER), unesterified cholesterol concentration (UC) and plaque burden. Pearson correlation coefficients and p-values are shown. QCA, quantitative coronary angiography, IVUS, intravascular ultrasound; HDL-UC, unesterified cholesterol concentration in apoB depleted plasma; HDL-MER, molar esterification rate in apoB depleted plasma; HDL-FER, fractional esterification rate in apoB depleted plasma.

Stepwise linear regression model for total atheroma volume (30 mm) in CAD patients (n=267)					
	R^2	F	b coefficient	SE	p-value
Model 1	0.047	12.7			<0.0001
Gender			31.1	8.7	<0.0001
Model 2	0.085	12.0			0.004
Gender			34.1	8.6	<0.0001
BMI			2.1	-0.64	0.001
Model 3	0.102	9.8			<0.0001
Gender			26.5	9.2	0.004
BMI			2.2	0.64	0.001
HDL-MER			-0.38	0.17	0.029
Excluded variables: age, LDL-C. HDL-C, diabetes, smoking, hypertension					

Stepwise linear regression model for total atheroma volume over 5 mm with least plaque burden in CAD patients (n=267)					
	R^2	F	b coefficient	SE	p-value
Model 1	0.03	7.3			0.009
BMI			0.29	0.11	0.008
Model 2	0.042	5.7			0.001
BMI			0.32	0.11	0.004
HDL-MER			-0.06	0.03	0.047
Excluded variables: age, gender, LDL-C. HDL-C, diabetes, smoking, hypertension					

Table XI: Predictor model for plaque volume as assessed by intravascular ultrasound (IVUS) found by stepwise selection procedure. Upper panel: total atheroma volume. **Lower panel:** total atheroma volume over 5 mm with least plaque burden. Initial set of predictor variables: BMI, age, gender, hypertension, diabetes, smoking, LDL cholesterol, and molar esterification rate in apoB depleted plasma (HDL-MER). SE, standard error; BMI, body mass index, LDL, low density lipoproteins.

Stepwise linear regression model for global plaque area score (QCA) in CAD patients (n=267)					
	R^2	F	b coefficient	SE	p-value
Model 1	0.083	23.8			<0.0001
Gender			14.6	2.99	<0.0001
Model 2	0.098	14.2			<0.0001
Gender			12.0	3.2	<0.0001
HDL-MER			-0.13	0.06	0.039
Excluded variables: age, hypertension, diabetes, smoking, LDL-C, BMI					

Stepwise linear regression model for global plaque area score (QCA) in CAD patients (n=267)					
	R^2	F	b coefficient	SE	p-value
Model 1	0.083	23.8			<0.0001
Gender			14.6	3.0	<0.0001
Model 2	0.110	16.1			<0.0001
Gender			11.6	3.2	0.001
HDL-UC			-0.07	0.03	0.006
Excluded variables: age, hypertension, diabetes, smoking, LDL-C, BMI					

Table XII: Model found by stepwise forward selection procedure for global plaque area score assessed by quantitative coronary angiography (QCA). Upper panel: Initial set of predictor variables: BMI, age, gender, hypertension, diabetes, smoking, LDL cholesterol, and molar esterification rate in apoB depleted plasma (HDL-MER). **Lower panel:** Initial set of predictor variables: BMI, age, gender, hypertension, diabetes, smoking, LDL cholesterol, and unesterified cholesterol in apoB depleted plasma (HDL-UC). SE, standard error; BMI, body mass index, LDL, low density lipoproteins.

Stepwise linear regression model for maximal common cIMT in CAD patients (n=267)

	R^2	F	b coefficient	SE	p-value
Model 1	0.13	37.7			<0.0001
Age			0.007	0.001	<0.0001
Model 2	0.17	25.5			<0.0001
Age			0.008	0.001	<0.0001
LDL-C			0.058	0.017	0.001
Model 3	0.19	19.8			<0.0001
Age			0.008	0.001	<0.0001
LDL-C			0.054	0.016	<0.0001
BMI			0.005	0.002	0.008
Model 4	0.2	16.2			0.002
Age			0.008	0.001	<0.0001
LDL-C			0.064	0.017	<0.0001
BMI			0.005	0.002	0.024
HDL-MER			-0.001	0.0001	0.031

Excluded variables: gender, diabetes, smoking, hypertension

Stepwise linear regression model for maximal common cIMT in CAD patients (n=267)

	R^2	F	b coefficient	SE	p-value
Model 1	0.13	37.7			<0.0001
Age			0.007	0.001	<0.0001
Model 2	0.17	25.5			<0.0001
Age			0.008	0.001	<0.0001
LDL-C			0.056	0.017	0.001
Model 3	0.19	19.8			<0.0001
Age			0.008	0.001	<0.0001
LDL-C			0.062	0.016	<0.0001
HDL-UC			-0.001	0.0001	0.008
Model 4	0.2	16.4			0.002
Age			0.008	0.001	<0.0001
LDL-C			0.059	0.016	<0.0001
HDL-UC			-0.0001	0.0001	0.024
BMI			0.004	0.002	0.025

Excluded variables: gender, diabetes, smoking, hypertension

nd by stepwise forward selection procedure for maximal common carotid artery intima media
: panel: Initial set of predictor variables: BMI, age, gender, hypertension, diabetes, smoking, LDL esterification rate in apoB depleted plasma (HDL-MER). **Right panel:** Initial set of predictor variables: ertension, diabetes, smoking, LDL cholesterol, and unesterified cholesterol in apoB depleted plasma d error; BMI, body mass index, LDL, low density lipoproteins.

3.3 Manuscript #3: Beneficial Effects of Reconstituted High-density Lipoprotein (rHDL) on Circulating $CD34^+$ Cells in Patients after an Acute Coronary Syndrome

3.3.1 Foreword to Manuscript #3

Manuscript #1 and #2 have demonstrated that LCAT related parameters show close correlations with disease status. Based on our data, these parameters reflecting HDL functionality seem to be novel, useful markers of severity of atherosclerotic disease. LCAT activators and recombinant LCAT infusions in experimental and clinical studies will provide valuable data to establish whether targeting of LCAT is a promising therapeutic strategy to reduce cardiovascular risk. However, while the identification of biomarkers of HDL function will be essential to establish novel HDL-based treatment approaches, many atheroprotective activities of HDL remain to be elucidated. Indeed, mechanisms by which HDL-based therapeutic approaches such as rHDL or apoA-I administration exert their beneficial effects in cardiovascular diseases are incompletely understood. Thus, aim 2 of the present investigation was to assess whether rHDL affects vascular repair mechanisms following ischemic myocardial injury. Therefore, manuscript #3 reports the short-term effects of rHDL on circulating endothelial progenitor cells, which are key mediators of vascular repair, in thirty-three patients with a recent ACS.

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Author contributions:
Conceptual design of the study: ER, CB, PLL, JCT
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Acquisition and analysis of data: CG, CB
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Statistical analysis: CG, ER, MCB
Drafting of the manuscript: CG, ER
Critical review of data integrity: MCB, MCG
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3.3.2 Abstract

Background: High-density lipoproteins (HDL) favourably affect endothelial progenitor cells (EPC). Circulating progenitor cell level and function are impaired in patients with acute coronary syndrome (ACS). This study investigates the short-term effects of reconstituted HDL (rHDL) on circulating progenitor cells in patients with ACS.

Methods and Findings: The study population consisted of 33 patients with recent ACS: 20 patients from the ERASE trial (randomized to receive 4 weekly intravenous infusions of CSL-111 40 mg/kg or placebo) and 13 additional patients recruited as controls using the same enrolment criteria. Blood was collected from 16 rHDL (CSL-111)-treated patients and 17 controls at baseline and at 6-7 weeks (i.e. 2-3 weeks after the fourth infusion of CSL-111 in ERASE). $CD34^+$ and $CD34^+$/kinase insert domain receptor (KDR^+) progenitor cell counts were analysed by flow cytometry. We found preserved $CD34^+$ cell counts in CSL-111-treated subjects at follow-up (change of 1.6%), while the number of $CD34^+$ cells was reduced (-32.9%) in controls ($p = 0.017$ between groups). The level of circulating SDF-1 (stromal cell-derived factor-1), a chemokine involved in progenitor cell recruitment, increased significantly (change of 21.5%) in controls, while it remained unchanged in CSL-111-treated patients ($p = 0.031$ between groups). *In vitro* exposure to CSL-111 of early EPC isolated from healthy volunteers significantly increased $CD34^+$ cells, reduced early EPC apoptosis and enhanced their migration capacity towards SDF-1.

Conclusions: The relative increase in circulating $CD34^+$ cells and the low SDF-1 levels observed following rHDL infusions in ACS patients point towards a role of rHDL in cardiovascular repair mechanisms.

3.3.3 Introduction

Several studies have consistently supported high-density lipoprotein (HDL)-cholesterol as a significant, strong, and independent inverse predictor of cardiovascular risk, even when low-density lipoprotein cholesterol (LDL-C) is reduced to very low levels by high dose statins.[437] While the inverse association between HDL-C and cardiovascular outcomes has been proven to be very robust, recent high profile pharmacological intervention studies and a Mendelian randomization analysis have challenged the concept that raising endogenous plasma HDL-C will uniformly translate into improved cardiovascular outcomes.[6, 438] These recent studies have caused growing awareness that the effects of HDL may vary in different clinical settings and that an increase of dysfunctional HDL particles could also be detrimental, a phenomenon referred as 'HDL dysfunction'. Indeed, population-based studies indicate that a substantial proportion of patients with ACS present with reduced or dysfunctional HDL which, in turn, is associated with a higher risk of early recurrent cardiovascular events.[21, 23, 25] As a consequence, exogenous HDL has been suggested as a treatment option for modifying the high-risk state following ACS and beneficial effects on coronary atherosclerosis in patients with ACS have been suggested after infusions of reconstituted HDL (rHDL).[295, 439]

While the anti-atherosclerotic action of HDL is believed to be mostly related to its role in reverse cholesterol transport, experimental data indicate that rHDL may promote re-endothelialisation by improving endothelial progenitor cell (EPC) levels and functionality.[88] Accordingly, low plasma HDL-C levels have been reported to be associated with a decreased number of EPCs.[89] Progenitor cell based therapies might also reduce short- and long-term recurrent cardiovascular events in patients with ACS,[83] and *in vivo* data indicate that vascular repair by EPCs might be one of the underlying mechanisms.[440, 441] Following percutaneous coronary intervention (PCI), bone marrow-derived stem and vascular progenitor cells that express stem-cell-like antigens such as CD34 are mobilized, rapidly recruited to sites of injury thereby inhibiting further platelet activation and leading to neovascularization, improved left ventricular function and reduced myocardial lesion area.[75, 76] However, several populations, including patients with ACS, seem

to fail to respond to PCI with progenitor cell mobilization, resulting in increased mortality and more significant left ventricular remodelling.[78, 81, 442, 443]

An epidemiologic study showed an association of statin use with higher $CD34^+$ progenitor cell counts, thereby supporting the hypothesis that levels of EPCs may be influenced therapeutically[85]. Indeed, moderate-dose atorvastatin increased $CD34^+$ cells in patients with myocardial infarction, and systemic rHDL infusion can improve the availability of $CD34^+$ cells in patients with type 2 diabetes[86]. However, whether infusions of rHDL can favourably influence EPCs or $CD34^+$ progenitor cells in the setting of recent ACS is not known.

Given that 1- endogenous HDL and progenitor cell functions are impaired in ACS patients, a population characterized by a high short-term risk for recurrent ischaemic events, 2- EPCs, $CD34^+$ progenitors and rHDL may exert rapid beneficial effects on some atherosclerotic plaque characteristics, and 3- rHDL increases EPC levels *in vivo* in patients with diabetes, we hypothesized that some of the beneficial effects of rHDL infusions may be mediated via an improvement of circulating EPC or $CD34^+$ progenitor levels and function in patients with ACS.

3.3.4 Methods

Subjects

The study population consisted of 33 patients with recent ACS: 20 patients from the ERASE trial (16 CSL-111-treated and 4 placebo-treated patients) and 13 additional patients who were recruited as controls using the ERASE enrolment criteria[439]. Further, twenty-six patients without ACS and with normal coronary arteries who underwent coronary angiography for different reasons served as controls for baseline EPC measurements. Details of the ERASE trial were previously published.[439] Briefly, ERASE was a randomized, double-blind, placebo-controlled, multicentre trial which evaluated the effects of the rHDL CSL-111 (CSL Ltd, Parkville, Australia) on plaque burden as assessed by intravascular ultrasonography in patients who were recruited within 2 weeks of an ACS, defined as unstable angina, non-ST-segment elevation myocardial infarction (MI) or ST-segment elevation MI. Patients with significant left main coronary artery disease (≥ 50% stenosis), renal insufficiency, liver disease, active cholecystitis,

uncontrolled diabetes mellitus, New York Heart Association (NYHA) class III or IV heart failure, known soybean allergy, history of alcohol or drug abuse, planned anticoagulation treatment, or previous or planned coronary bypass graft surgery were excluded from study participation. Patients were randomized to receive 4 weekly intravenous infusions of placebo or CSL-111 40 mg/kg. Blood collection was performed at baseline (prior to the first infusion) and then at 6 to 7 weeks (2 to 3 weeks after the fourth study infusion). For the 13 additional control subjects with ACS recruited, blood was also collected at baseline and at 6 to 7 weeks (similar to the subjects of the ERASE trial).

To evaluate the effects of CSL-111 on cell adhesion, growth, apoptosis and migration, we performed *in vitro* experiments on blood collected from 10 additional healthy subjects. The study complied with the declaration of Helsinki and was approved by the Institutional Review Board of the Montreal Heart Institute, with all subjects providing written informed consent.

Blood Sampling and Circulating Progenitor Quantification by Flow Cytometry

Venous blood was collected in the recumbent position (35 mL in potassium-EDTA-containing tubes and 5 mL in tubes without anticoagulant for separation of serum). The blood samples were immediately transported to laboratories for processing. EPCs were quantified in triplicate using previously reported guidelines for progenitor cell enumeration[444] with some modifications. Briefly, 100 µL of blood was immunostained for 10 minutes at room temperature with anti-human KDR antibody[445, 446] (Abcam, Cambridge, MA; conjugated using Zenon® Alexa Fluor®647-RPE labelling kit, Invitrogen, Burlington, ON). Then, Stem-Kit™ (Beckman Coulter, Brea, CA) monoclonal antibodies (mAbs) were added using a fluorescein isothiocyanate (FITC)-conjugated anti-human CD45 antibody and a phycoerythrin (PE)-conjugated anti-CD34 antibody for 20 minutes at room temperature. Isotype-identical antibodies were used as controls. Following incubation, erythrocytes were lysed using 1x NH_4Cl lysing solution provided with the Stem-Kit for 10 minutes at room temperature prior to cytometry analysis. Stem-count fluorospheres were added to samples to determine absolute EPC counts by flow cytometry (Beckman Coulter EPICS® XL™ flow cytometer).

We used a modification of the Stem-Kit protocol which itself is based on the ISHAGE guidelines for $CD34^+$ cell determination by flow cytometry.[447] As indicated in a representative example (Figure 16, page 103), histogram 1 displays all events except the fluorospheres (shown on histogram 7, R8). The region R1 is positioned to include all $CD45^+$ events. This region will exclude CD45 negative events (i.e. red blood cells, platelets and cell debris). The region R6 represents lymphocytes (bright CD45, low scatter). Histogram 2 displays events from region R1. The region R2 is adjusted to include $CD34^+$ cells with low Side Scatter. Histogram 3 is showing the events from regions R1 and R2. The region R3 is placed to include the low Side Scatter and low to intermediate CD45 staining. Histogram 4 represents all events from regions R1, R2 and R3 displayed on a FSC vs SSC dot plot to confirm that the selected events fall into a lymph-blast region (R4). $CD34^+$ cells number is counted in the region R4 (events meeting all the fluorescence and light scatter criteria of ISHAGE Guidelines for $CD34^+$ cells). $CD34^+$ number determination was performed in triplicate for each patient and the mean $CD34^+$ value was used. An appropriate isotype control was used as a control. Histogram 5 displays the events included in regions R1, R2, R3 and R4. A quadrant was positioned to separate the positive and the negative cells for VEGFR2 staining. An appropriate isotype control was used to adequately place the quadrant. Region R5 represents the total EPCs ($CD34^+$/$VEGFR2^+$ cells). Histogram 6 shows events from region R6. This region is used to set the region R4 (histogram 4) to include events no smaller than lymphocytes. Histogram 7 represents all events. This histogram is useful to establish the lower limit of CD45 expression for the $CD34^+$ events. The region R8 is placed in the right top of the histogram to count all Stem-count fluorospheres accumulated for each sample for absolute quantification. Histogram 8 shows events from region R8. This region includes the Stem-count fluorospheres singlet population. It is used to verify that fluorospheres accumulate constantly over time. Absolute numbers of $CD34^+$ and $CD34^+$/KDR^+ cells per µL of blood were determined and results were expressed as relative changes from the respective baseline values.

ELISA Assays

Serum levels of VEGF and stromal cell-derived factor-1 (SDF-1) were quantified by ELISA (Quantikine kits, R&D Systems).

***In Vitro* Experiments with CSL-111**

One hundred mL of venous blood was collected on sodium citrate anticoagulant from each of the 7 healthy volunteers for *in vitro* experiments (exposure of early EPC (eEPC) to CSL-111). Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation (Ficoll-Paque™ PLUS medium, GE Healthcare, QC), plated at a density of 1.5 x $10^6/cm^2$ on four fibronectin-coated plates (BD Biosciences, Mississauga, ON), and cultured under 5% CO_2 (37°C) in endothelial growth media (EGM®-2, Lonza, QC), which was supplemented with 20% embryonic stem cell-qualified foetal-bovine serum (Invitrogen, Burlington, ON). For each of the dishes, cells were exposed to 1 mg/mL of CSL-111 for either days 0 to 4, days 4 to 7, days 0 to 7, or unexposed. Non-adherent cells were removed by washing after 4 days in culture, for eEPC culture. On day 7, adherent cells were harvested and stained with antibodies against CD34 (FITC-conjugated) and KDR (APC-conjugated). Cells were incubated for 30 minutes at 4°C before the addition of 50 000 SpheroTM AccuCount fluorescent beads (Spherotech Inc.) and their analysis on a BD LSRII flow cytometer. Apoptosis of the treated and untreated eEPCs was quantified in the following fashion: After 7 days in culture, eEPCs were washed, harvested by mild treatment with dispase (0.5 mg/mL), stained with Annexin V conjugated to Alexa Fluor 350 and with propidium iodide (BD Biosciences, Mississauga, ON) and then analysed by flow cytometry.

Transwell Migration Assay

eEPC migration was assessed using modified Boyden chambers. Briefly, eEPCs from healthy volunteers (n = 3) were cultured in presence or absence of CSL-111 as above and then harvested and resuspended in DMEM containing 10% (v/v) FBS. A polycarbonate filter membrane (5-µm-pore-diameter; Neuroprobe) was placed on the top of the lower wells, the latter filled with the medium mentioned above and supplemented with SDF-1 (100 ng/mL) as a chemoattractant. The chamber was tightened and cell suspensions (4 x 10^5 cells/cm^2) were added to the

upper wells. After allowing cell migration for 16 hours, cells were scraped from the upper side of membranes using Kimwipes/cotton swab. The membrane with migrated cells was fixed and stained using Diff-quick (Thermo Fisher Scientific) staining kit. Stained cells were then counted directly under the microscope using 40X objective. Each experiment was assayed in quadruplicate and 3 randomly selected high-power fields for each well were counted to determine the number of cells that had migrated. Migration is presented as the chemotactic index, obtained by division of the number of migrating cells in the treated groups by the number of migrating cells in the corresponding control wells.

Statistical Analyses

Statistical analyses were conducted at the Montreal Heart Institute Coordinating Centre (MHICC) using SAS (version 9.1 or higher, SAS Institute, Cary, NC). Categorical data were expressed as frequencies and percentages. For continuous variables, depending on the distribution of the data, results are expressed as mean ± standard deviation or median (Q1;Q3). Baseline characteristics were compared between groups using Student t-test or chi-square test where appropriate. Comparisons of experimental data between the CSL-111-treated group *vs* control were made by the Mann-Whitney test. Between-group comparisons of the time lapses between ACS and baseline blood collection as well as between baseline and follow-up were assessed using the Student t-test or the Mann-Whitney test, as appropriate. For the parameters measured during the CSL-111 *in vitro* treatments, the overall effect of exposure to CSL-111 (days 0 to 4, days 4 to 7, days 0 to 7, or unexposed) was tested using the Friedman test and, if significant, Wilcoxon signed-rank tests were used to compare paired types of exposure. A mixed model analysis of variance (ANOVA) with terms for block (to account for the fact that patients contribute to data in quadruplicate), exposure to CSL-111, SDF-1 (yes/no) and interaction exposure to CSL-111 x SDF-1 was used to compare the EPC among the combinations of exposure to CSL-111/SDF-1 in the migration assay. The appropriate pairwise comparisons followed if global F tests were significant. Statistical significance was defined as $p < 0.05$.

3.3.5 Results

The baseline characteristics of the groups of CSL-111-treated patients and control patients were similar (Table XIV, page 108). Also, there was no significant difference between groups in terms of days from presentation with ACS to the time of baseline blood collection for progenitor cells (3.3+/-2.8 and 2.6+/-1.4 days after myocardial infarction (MI) for controls and CSL-111-treated patients, respectively) and in the median number of days from ACS event to the follow-up sampling (43 [40-46] and 39 [39-41] days after MI for controls and CSL-111-treated patients, respectively). A slightly higher BMI was found in the 4 placebo-treated patients as compared to the 13 additional patients who were recruited as controls using the ERASE enrolment criteria ($p = 0.04$).

We first measured the levels of circulating progenitor cells in ACS patients and normal controls at baseline. As compared to patients with normal coronary arteries (n=26), patients with ACS (n=29) had significantly higher levels of both, $CD34^+$ and $CD34^+/KDR^+$ progenitor cells ($p<0.0001$ and $p=0.001$, respectively, Figure 17, page 104). The follow-up samples for the CSL-111-treated ACS patients were obtained 16 ± 4 days following completion of the 4 weekly rHDL infusions. The median relative changes in $CD34^+$ progenitor cells, as quantified by flow cytometry from blood samples collected at baseline and in the follow-up, were -32.9% and 1.6% for the control group and CSL-111-treated group, respectively ($p = 0.017$, Figure 18A, page 105). These significant differences between control group and CSL-111-treated group persisted when changes in $CD34^+$ progenitor cells in relation to total number of leucocytes ($CD45^+$ cells) were analysed ($p = 0.03$, data not shown). In contrast, the median relative changes in $CD34^+/KDR^+$ endothelial progenitor cells were -11.7% (control group, *n* = 16) and -14.2% (CSL-111, *n* = 13) without any significant difference between groups (Figure 18C, page 105, $p = 0.98$). Similarly, no changes in total peripheral leucocyte count ($CD45^+$) were observed following rHDL treatment as compared to controls (data not shown). Changes of absolute $CD34^+$ and $CD34^+/KDR^+$ endothelial progenitor cell count before and after treatment are indicated in Figure 18B and 18D (page 105).

We next investigated whether the serum concentrations of cytokines known to be involved in the mobilisation of vascular progenitor cells such as VEGF and

SDF-1 were affected by the CSL-111 treatment. Although we did not observe differences between the groups in the serum concentrations of VEGF (data not shown), we found that the median relative change in serum SDF-1 from baseline was 21.5% in controls and 0.9% in the CSL-111-treated group (Figure 19, page 106, p = 0.031).

To further analyse the effects of CSL-111, we next isolated PBMCs from healthy subjects and assessed the effect of exposing them to CSL-111 during culture and differentiation of eEPC. Early EPCs were non-treated or treated with CSL-111 (1 mg/mL) either from day 0 to 7, day 0 to 4, or day 4 to 7. Day 4 is the day when non-adherent cells were washed out and media was changed in the procedure for eEPC culture. We obtained a median number of total fibronectin-adherent eEPC that was 1.9-fold higher when cells were incubated for 7 days in presence of CSL-111 than when cells were not exposed to CSL-111 (Figure 20A, page 107, p = 0.031). A similar increase in eEPC was seen when cells were incubated with CSL-111 from day 0 to 4 (p = 0.016 versus untreated cells) whereas eEPC exposure to CSL-111 from day 4 to 7 did not have a significant effect on the total number of eEPCs when compared to untreated cells. Similarly, we observed an increase in the median number of $CD34^+$ eEPC when cells were exposed to CSL-111 during days 0 to 7 (1.5-fold higher) and 0 to 4 (2.1-fold higher) (Figure 20B, page 107, p-values 0.031 and 0.016, respectively). We also observed a decrease in the median values of the percentage of eEPC apoptosis when cells were treated with CSL-111 from days 0 to 7 or 0 to 4 compared to untreated cells (Figure 20C, page 107, p-values 0.016 for both treatment types).

We also characterized the effect of CSL-111 on eEPC migration toward SDF-1 as a chemotactic agent. We observed that cells that were treated with CSL-111 during days 0 to 4 or 0 to 7 had a higher migration capacity toward SDF-1 (Figure 21, page 108, p = 0.0003 and 0.0135, respectively) compared to controls, whereas exposure of eEPC from day 4 to 7 did not result in an increase of migration capacity of the cells compared to control (not exposed to CSL-111) eEPCs. Western blot analysis of the expression of SDF-1 receptor CXCR4 indicates that CSL-111 increased protein expression of CXCR4 in eEPCs when it was present from day 0 to 4 or 0 to 7 (data not shown), similar to migration data.

3.3.6 Discussion

This is the first clinical study exploring short-term effects of intravenous rHDL infusions on circulating progenitor cell number and function in patients with recent ACS. In line with previous reports we observed that the number of circulating progenitor cells including $CD34^+$ and $CD34^+/KDR^+$ cells increases acutely following an ACS.[448, 449] An early decline of EPC levels or failure to mobilize EPCs from the bone marrow have been described in ACS patients, which in turn is associated with increased mortality.[448, 450, 451] We report here that the decline in progenitor cells can be prevented by four weekly infusions of rHDL (CSL-111), while a significant drop of 33% in $CD34^+$ cell count is observed in untreated control patients.

Low progenitor cell numbers following PCI for ACS or stable coronary artery disease are associated with an increased risk of mortality and recurrent major adverse cardiac events (MACE), with the risk of MACE being highest during the first six months following the primary event.[21, 80, 442] Remarkably, changes in $CD34^+$ progenitor counts in our study population were observed after only a short duration of treatment underlining the rapid action of rHDL on progenitor cell levels. Similarly, beneficial changes in some plaque characteristics were observed in the ERASE study after only four weeks of rHDL treatment.[439] Thus, the present study supports the hypothesis that HDL-raising strategies may exert at least part of their potentially beneficial effects on plaque morphology via an improvement of circulating bone marrow-derived progenitor levels.[439]

One of the major limitations in studying EPCs is the lack of consensus on the identity of 'true' EPCs, which in turn limits the translation of EPC research into clinical studies. The surface marker profile of progenitor cells changes during the process of mobilization and maturation; as they mature, EPCs, a subtype of peripheral blood monocytes that express stem-cell-like antigens such as CD34, lose the CD133 marker and acquire vascular endothelial growth factor (VEGF) receptor-2, also known as KDR.[452] Thus, $CD34^+$ cells form a more generic population of 'early' progenitor cells, while $CD34^+KDR^+$ cells are committed to the endothelial lineage.[453] In our study, we detected preserved numbers of $CD34^+$ cells in patients receiving rHDL infusions, while there was no significant difference

in $CD34^+/KDR^+$ cells in patients treated with rHDL compared to controls. However, there is conflicting evidence regarding the predictive power of $CD34^+/KDR^+$ cells in patients with CAD; while some studies have shown that the number of circulating $CD34^+/KDR^+$ cells predicts outcome in healthy individuals and patients with CAD,[454, 455] a comparative analysis in patients with ACS reported that the $CD34^+/CD133^+$ phenotype, but not the $CD34^+/KDR^+$ or the $CD133^+/KDR^+$ phenotype, is predictive of recurrent ACS or MACE.[456] A pooled analysis from four longitudinal studies, however, demonstrated that both $CD34^+$ cells and $CDR34^+/KDR^+$ cells showed consistent results, suggesting that there is no clear evidence that one phenotype is superior to the other in terms of risk prediction.[80] These apparent discrepancies could be attributed to the very low number of $CD34^+/KDR^+$ EPCs in blood samples and the consequential high interobserver variability in assessing their quantity, the different methods and time points used to assess EPC in humans, and the relatively small number of patients in heterogeneous populations assessed.[457] Furthermore, previous data suggest that $CD34^+$ cell level is more stable over time than $CD34^+/KDR^+$ cell level, which may be more influenced by pharmacological treatment.[80, 458] Indeed, in patients with ACS, a reduced number of $CD34^+$ rather than $CD34^+/KDR^+$ EPCs has been shown to be predictive of recurrent ACS.[456, 459-461] In light of these studies and our results, one might conclude that $CD34^+$ cells play an important role in the vascular repair process, particularly in the setting of an acute ischemic event. However, the mechanisms explaining how rHDL exerts different effects on the two progenitor populations are unclear and await further studies.

It has been shown previously that the percentage of apoptotic $CD34^+$ progenitor cells is significantly increased in patients with ACS as compared to healthy subjects and is associated with the extent of coronary stenosis by angiography.[462] Thus, functional impairment of progenitor cells through enhanced apoptosis may underlie atherogenesis and cardiovascular events, while improving survival seems to be vital for neovascularization and arterial injury repair.[463] In our study, additional experiments using eEPCs isolated from healthy subjects demonstrated favourable effects of early administration of rHDL on eEPC apoptosis. These findings are consistent with the known anti-apoptotic effects of rHDL in another setting[90] and raise the possibility that early administration would

be relevant for maximizing the therapeutic benefits of rHDL infusions in ACS patients. We speculate that a reduction in apoptosis might be one possible mechanism for the relative preservation of $CD34^+$ cell counts following rHDL infusions. The reduction in apoptosis, seen in our *in vitro* study, was paralleled by a higher migration capacity towards SDF-1 in eEPC cell cultures treated with CSL-111. A reduction in EPC migratory and proliferation capacity was previously observed in patients with ACS[464] and correlates with increased atherosclerotic load in humans.[465] Therefore, our findings with eEPCs raise the hypothesis that an increase in progenitor cells level and migration capability could contribute to favourable effects of rHDL in patients with ACS.

We also measured the circulating concentration of chemokines known to be involved in EPC recruitment, such as stromal cell-derived factor-1 (SDF-1; also known as CXCL12) and vascular endothelial growth factor (VEGF). We observed that CSL-111-treated patients had lower SDF-1 circulating levels than controls; however, this finding is counterbalanced by our observation of enhanced SDF-1-mediated migration following CSL-111 treatment *in vitro*. Further, there were no differences in VEGF levels between the treatment and control groups. This is in line with previous observations where no correlation between levels of SDF-1 or VEGF and numbers of vascular progenitors in patients with ACS were observed.[76, 78] Indeed, chemokine levels have been shown to undergo rapid changes in experimental models where SDF-1 levels increase sharply 3 days post-MI but go back to normal levels after 1 week.[466] Thus, our findings could be explained by a reduction of SDF-1 levels that may have occurred through negative feedback-mechanism as a consequence of better cardiovascular tissue repair due to improved adhesion of progenitor cells to damaged tissues. Furthermore, the improved migration capacity observed in cultured eEPC following rHDL treatment may explain why patients treated with rHDL managed to maintain higher levels of $CD34^+$ progenitor cells despite lower SDF-1 levels. Interestingly, experimental work suggests that SDF-1 signalling could even be detrimental for infarct size and left ventricular function in an ischemia–reperfusion injury model, due to the recruitment of inflammatory cells and fibrocytes.[467] Thus, based on these conflicting data and the high individual variability of SDF-1 levels observed in clinical studies, it has been proposed that for progenitor cell homing the local

expression of SDF-1 in the heart is more important than SDF-1 blood levels.[76] Further studies are necessary to clarify and definitively assess the role of SDF-1 signalling in EPC mobilization during ischemia.

There are limitations to our study. Our finding of a potential beneficial effect of rHDL on progenitor levels and function is limited by a small sample size which led us to enrol additional control subjects. In addition, our small study population precluded further subgroup analyses pertaining to the metabolic syndrome and diabetes which are known to be associated with reduced EPC counts and function.[86, 126, 468] Prospective studies are required to specifically evaluate the therapeutic potential of HDL infusions in these subpopulations in the clinical setting of ACS.

In conclusion, we have found that in patients suffering from ACS, rHDL administration preserves circulating $CD34^+$ levels, possibly via beneficial effects on improved migration and reduced apoptosis of progenitor cells. Prospective clinical trials are needed to evaluate whether $CD34^+$ cell count may be a useful biomarker in the evaluation of novel HDL-raising therapies, particularly those where rHDL or mimetics are involved.

3.3.7 Figures and Tables

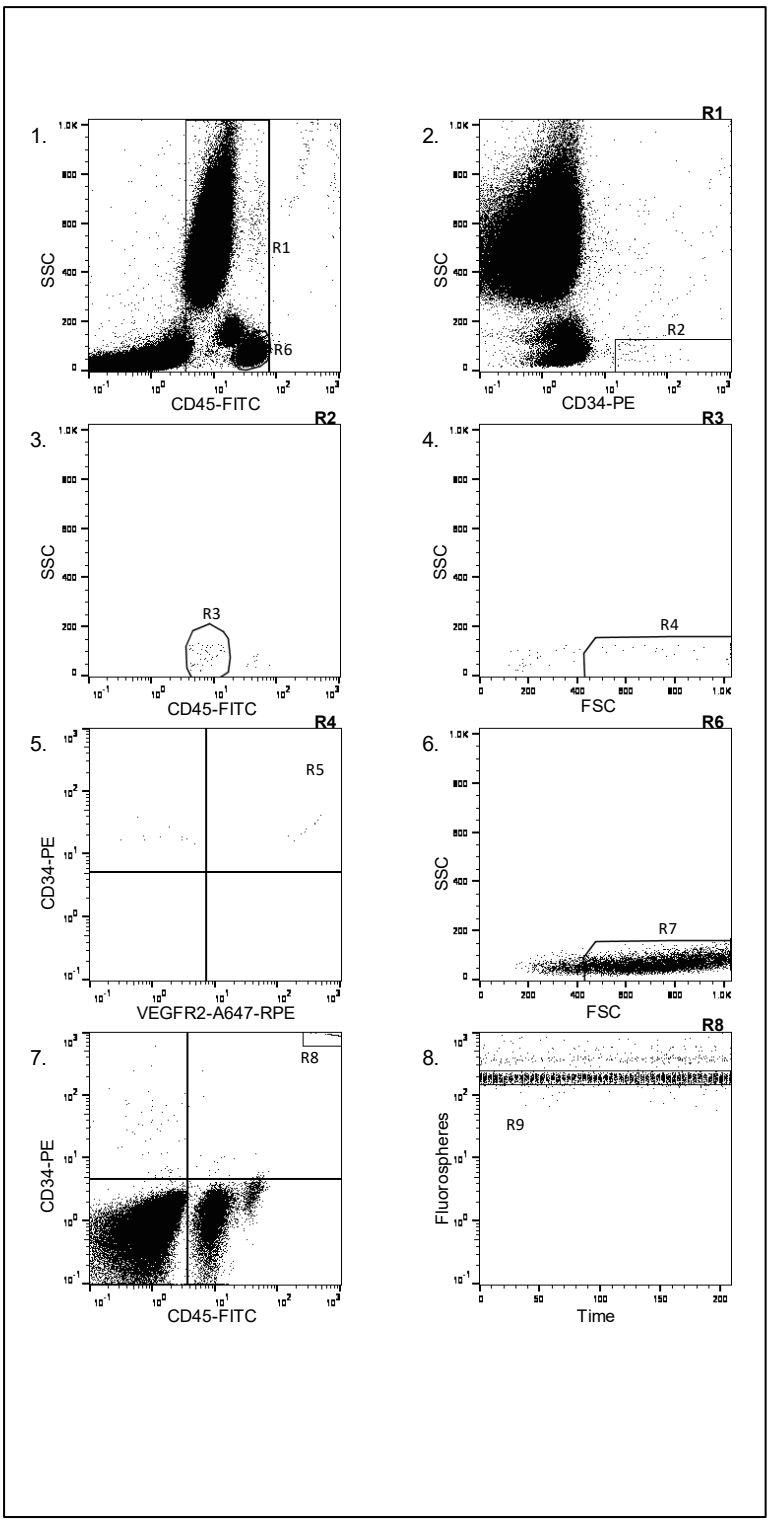

Figure 16: Repesentative example of sequential gating strategy for flow cytometric analysis of endothelial progenitor cells. A modified ISHAGE strategy was applied for EPC quantification. 1) Representative sample stained with CD45-FITC. Region R6 represents lymphocytes. 2) Anti-CD34-PE staining of cells from R1. Region R2 represents $CD34^+$ cells. 3) Region R3 is placed to include the low Side Scatter and low to intermediate CD45 staining. 4) R4 represents all events from regions R1, R2 and R3 displayed on a FSC vs SSC dot plot to confirm that the selected events fall into a lymph-blast region. 5) Displays the events included in regions R1, R2, R3 and R4. A quadrant was positioned to separate the positive and the negative cells for VEGFR2 staining. An appropriate isotype control was used to adequately place the quadrant. Region R5 represents the total EPCs (CD34+/VEGFR2+ cells). 6) Events from region R6. This region is used to set the region R4. 7) All events. This histogram is useful to establish the lower limit of CD45 expression for the $CD34^+$ events. The region R8 is placed in the right top of the histogram to count all Stem-count fluorospheres accumulated for each sample for absolute quantification. 8) Events from region R8. This region includes the Stem-count fluorospheres singlet population.

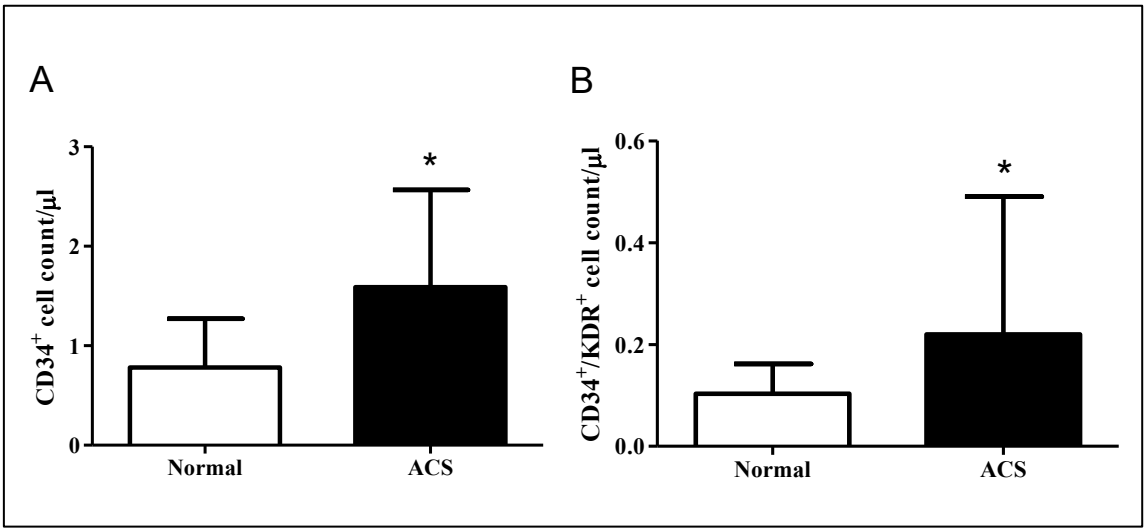

Figure 17: CD34+ (Left) and CD34+/KDR+ cells in patients with acute coronary syndrome. $CD34^+$ (Left) and $CD34^+/KDR^+$ (Right) endothelial progenitor cell counts at baseline in patients with acute coronary syndrome (ACS) and patients with normal coronary arteries (normal) as assessed by coronary angiography. *p<0.05 vs normal.

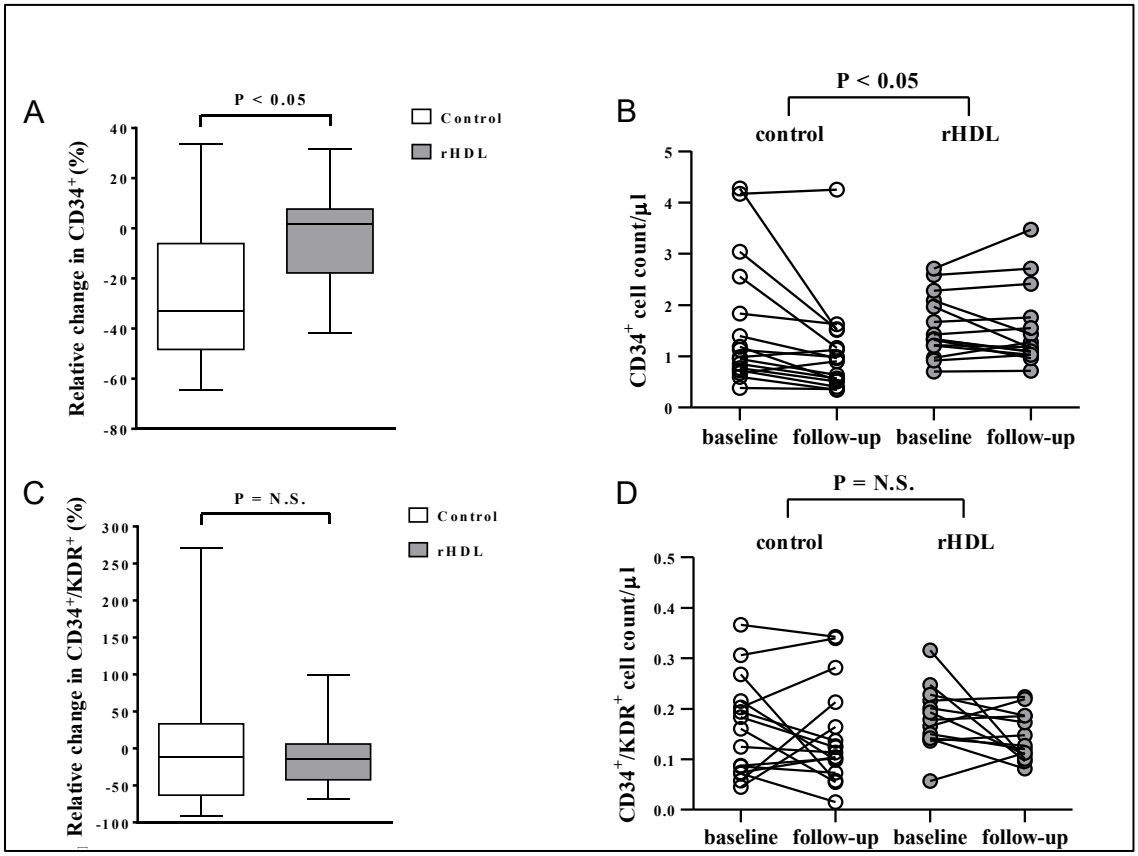

Figure 18: Relative preservation of CD34+ cells in patients with acute coronary syndrome following treatment with reconstituted high-density lipoprotein (rHDL) compared to controls. The $CD34^+$ progenitor cells (**A and B**) and $CD34^+/KDR^+$ endothelial progenitor cells (**C and D**) were quantified in blood samples collected at baseline and at follow-up. The follow-up samples for the CSL-111-treated group were obtained 16 ± 4 days following completion of the 4 weekly rHDL infusions. Each box plot in **A** and **C** shows the median, the interquartile range, the maximum and the minimum of the relative change. **B** and **D** show absolute numbers of $CD34^+$ **(B)** and $CD34^+/KDR^+$ **(D)** endothelial progenitor cell count at baseline and at follow-up. $p < 0.05$ between groups **(A and B)**, p = N.S. **(C and D)** from Mann-Whitney tests.* one outlier (baseline cell count 1.6/µl, follow-up 0.1/µl) not presented in figure due to axis limits.

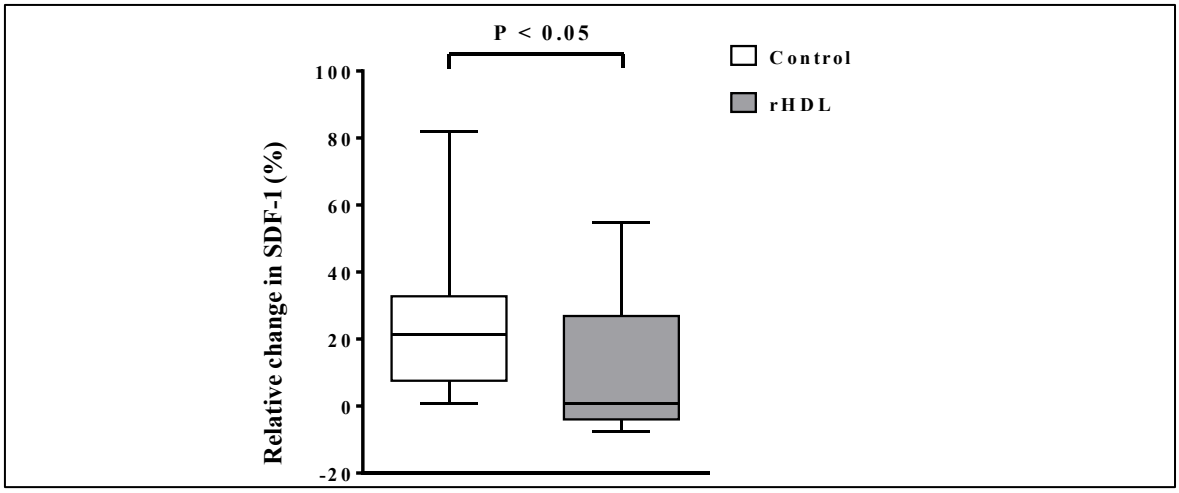

Figure 19: Decreased levels of serum stromal cell-derived factor-1 (SDF-1) in patients with acute coronary syndrome following treatment with reconstituted high-density lipoprotein (rHDL) compared to controls. Relative changes from baseline in serum SDF-1 in the control group and in the rHDL-treated group. Each box plot shows the median, the interquartile range, the maximum and the minimum. $P < 0.05$ between groups from Mann-Whitney test.

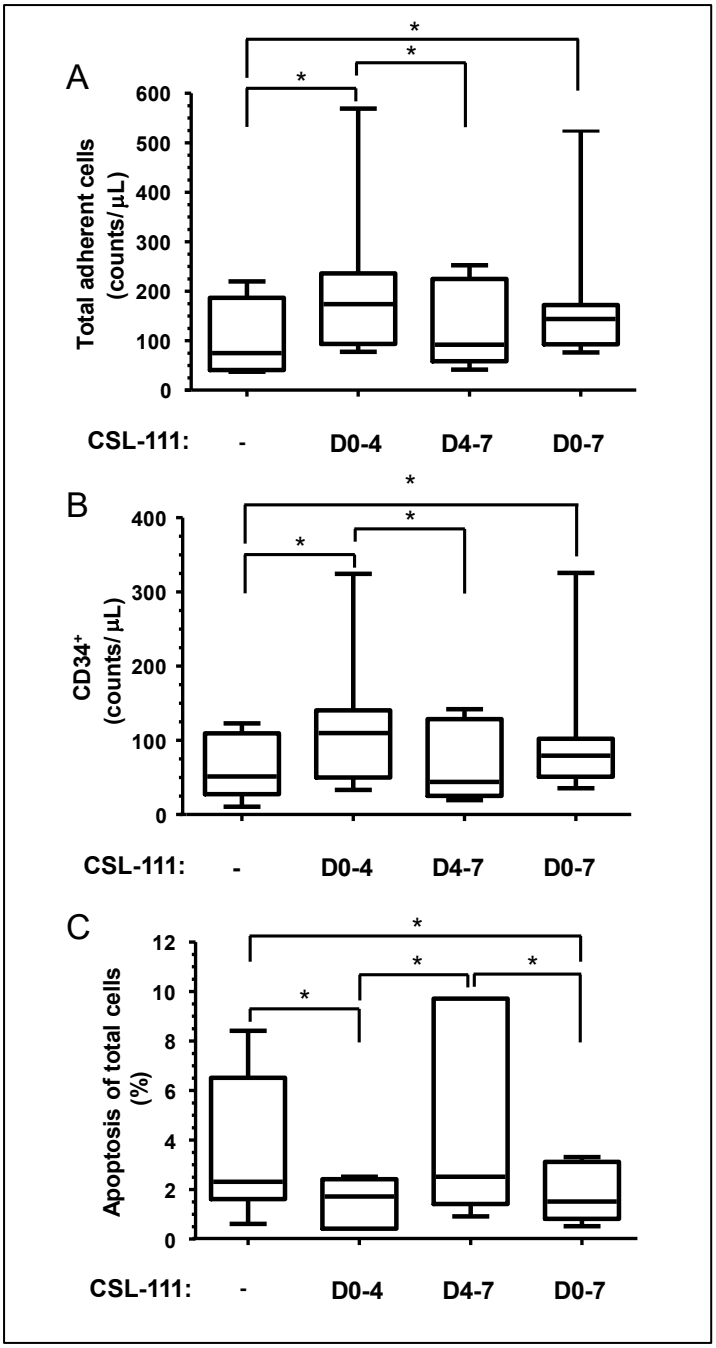

Figure 20: In vitro exposure of circulating progenitor cells to CSL-111. Peripheral blood mononuclear cells (PBMCs) were isolated from healthy donors (n = 7) and plated on fibronectin-coated plates in the absence or presence of CSL-111 (1 mg/mL) from day 0 to day 4 (D0-4), 4 to 7 (D4-7) or 0 to 7 (D0-7). After 7 days of culture, adherent cells were harvested and analyzed by flow cytometry. (A) All adherent cells were quantified by flow cytometry using cell counting beads for enumeration. (B) CSL-111 treatment increases the total number of CD34+ cells when added to cell culture media at D0-4 and D0-7; CD34+ cells were quantified by flow cytometry. (C) CSL-111 treatment reduces basal apoptosis in eEPCs when added to cell culture media at D0-4 and D0-7. Apoptosis was measured by flow cytometry using Annexin V labeling. Each box plot shows the median, the interquartile range, the maximum and the minimum of the relative change. * indicates $p < 0.05$ between groups from Wilcoxon signed-rank tests.

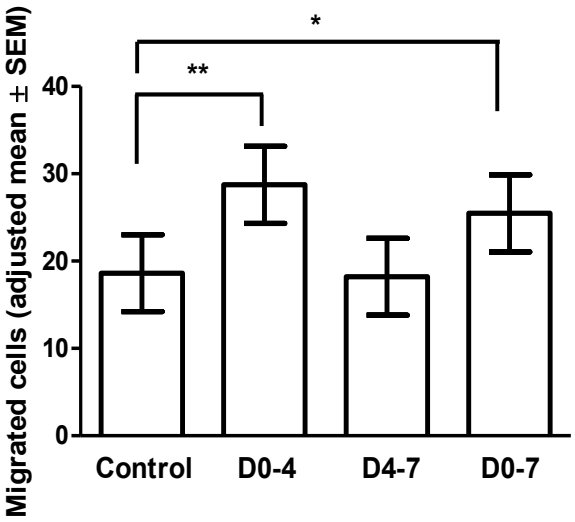

Figure 21: In vitro studies on the effect of CSL-111 on migratory capacity of eEPC. Peripheral blood mononuclear cells (PBMCs) were isolated from healthy donors (n = 3) and plated on fibronectin-coated plates in the absence or presence of CSL-111 (1 mg/mL) from day 0 to day 4 (D0-4), 4 to 7 (D4-7) or 0 to 7 (D0-7). On day 7 of culture, adherent cells were harvested and assayed in a modified Boyden chamber for their capacity to migrate along an SDF-1 gradient. Significantly increased migration was observed among cells treated with CSL-111 for day 0 to day 4 (**; p = 0.0003) and 0 to 7 (*; p = 0.0135) compared to controls. Figure shows adjusted mean±standard error of the mean (SEM). p-values are reported from mixed model ANOVA.

	Control (n = 17)	CSL-111 (n = 16)
Age (years)	55±11	57±9
Male (n (%))	14 (82)	16 (100)
Weight (kg)	86.5±22.4	90.3±17.5
BMI (kg/m^2)	29.4±6.5	29.2±5.0
Diabetes (n (%))	2 (12)	1 (6)
Hypertension (n (%))	11 (65)	11 (69)
Current tobacco use (n (%))	6 (35)	4 (25)
Total cholesterol (mmol/L)	4.7±1.4	4.6±1.0
LDL cholesterol (mmol/L)	2.7±1.1	2.6±0.9
HDL cholesterol (mmol/L)	1.2±0.3	1.2±0.3
Triglycerides (mmol/L)	1.9±0.9	1.8±0.9
Use of lipid lowering medication (n (%))	15 (88)	15 (94)
Use of inhibitors of the renin-angiotensin system (n (%))	12 (71)	11 (69)
Prior PCI (n (%))	10 (59)	13 (81)
Unstable Angina (n (%))	13 (76)	12 (75)
NSTEMI (n (%))	2 (12)	3 (19)
STEMI (n (%))	2 (12)	1 (6)

Table XIV: Baseline characteristics of the subjects. Values shown are mean±SD for continuous variables or frequencies and percentages for categorical variables. No statistically significant difference between groups for all the parameters listed. BMI, body mass index; HDL, high-density lipoprotein; LDL, low-density lipoprotein; PCI, percutaneous coronary intervention; NSTEMI, non ST-elevation myocardial infarction; STEMI, ST-elevation myocardial infarction.

3.4 Manuscript #4: Apolipoprotein A-I Proteolysis in Aortic Valve Stenosis - Species and Sex Differences

3.4.1 Foreword to Manuscript #4

Our observation (manuscript #3) that the decline in endothelial progenitor cells in patients suffering from an ACS can be prevented by rHDL attributes functional HDL an important role in endothelial repair mechanisms after ischemic injury. Our data indicate that infusion of rHDL may be a valuable therapy in the vulnerable phase following the occurrence of an ACS. Unfortunately, a major drawback of such therapies is their high cost. Indeed, high concentrations of rHDL have to be administered in order to reach sufficient bioavailability. Protease degradation of HDL has been suggested to contribute to the reduced bioavailability and impaired function of both, exogenous and endogenous HDL, and, thus, strategies to prevent HDL proteolysis are being explored. Indeed, given the epidemic proportions of HDL dysfunction, the development of strategies to prevent HDL from converting into dysfunctional molecules in situ and approaches to improve the efficacy of HDL-based therapies to preserve HDL function under pathologic conditions is crucial. To explore approaches that protect HDL from degradation, manuscript #4 assesses the contribution of different plasma proteases to apoA-I degradation in plasma collected from patients with severe AVS undergoing surgical valve replacement as well as in a rabbit model of AVS. This experimental model was chosen since previous work from our laboratory had demonstrated beneficial effects of apoA-I infusions on AVS regression in rabbits and mice. However, a high effective dose of apoA-I was used experimentally and we assume that this suboptimal therapeutic efficiency may be due to apoA-I degradation by plasma or tissue proteases originating from stenotic aortic valves. Thus, this experimental model, together with a fluorescently quenched full-length apoA-I probe designed in our laboratory, offered us the possibility to study the contribution of different proteases to apoA-I degradation and, ideally, to identify the protease exerting the most deleterious effects on apoA-I integrity.

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Current status of study:
project finalized, manuscript is being prepared for submission

Author contributions:
Conceptual design of the study: CG, ER, JCT
Patient recruitment: ABOM
Acquisition and analysis of data: CG, BES, ABOM, AEK; VL, MM, TMA, DB
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3.4.2 Abstract

Background: Aortic valve stenosis (AVS) is the most common valvular heart disease in the western world. Therapy based on apolipoprotein A-I (apoA-I), the major protein component of high-density lipoproteins (HDL), results in AVS regression in experimental models. Despite promising preclinical results, there is evidence indicating that apoA-I degradation by proteases might lead to suboptimal efficiency of such therapy.

Methods and results: An activatable probe using a fluorescently quenched full-length apoA-I protein was generated to assess apoA-I-degrading protease activity in plasma derived from a rabbit model of AVS as well as from sixty-four patients with severe AVS (age 65.0±10.4 years, 44 males). In human and rabbit AVS plasma, apoA-I-degrading protease activity was significantly higher than in controls ($p<0.05$). Through the use of protease inhibitors, we identified metalloproteinases (MMP), in particular MMP-7 and MMP-12, to exert the most potent proteolytic effect on apoA-I in AVS rabbits (67%, $p<0.05$ versus control), while the cysteine protease cathepsin S accounted for 54.2% of apoA-I degradation in human plasma ($p<0.05$ versus control), with the maximum effect seen in women (68.8%) ($p<0.05$ vs males). Accordingly, cathepsin S activity correlated significantly with mean transvalvular aortic gradient in women ($r=0.5$, $p=0.04$) but not in men ($r=-0.09$, $p=0.6$), and was a significant positive predictor of disease severity in women ($p=0.001$), when tested in a stepwise linear regression analysis.

Conclusion: ApoA-I proteolysis is increased in AVS. Significant species and sex differences exist with regard to protease activity involved in apoA-I degradation. Approaches targeting cathepsin S or HDL-based therapies that are less susceptible to cathepsin S degradation may lead to new therapies in human aortic valve disease.

3.4.3 Introduction

Aortic valve stenosis (AVS) is the most common valvular heart disease in the western world and its prevalence increases with age.[26] As severe symptomatic AVS usually leads to considerable morbidity and death in less than 5 years if left untreated, surgical or percutaneous valve replacement remains the primary management.[26] The pathophysiology of AVS appears to share many similarities with atherosclerosis.[469] However, the biology of AVS is complex and involves a combination of lipoprotein deposition, inflammatory activation, increased oxidative stress, extracellular matrix remodelling and neovascularization, as well osteoblastic transdifferentiation of valvular myofibroblasts and subsequent valvular calcification.[27] The latter is a highly regulated molecular process characterized by the expression of osteogenic proteins and proteolytic enzymes, such as matrix metalloproteinases (MMPs) and cathepsins that are secreted by activated macrophages and myofibroblast-like cells.[28-30] The combination of these processes leads to a spectrum of abnormalities ranging from mild valve thickening to severe calcification with significantly impaired leaflet motion.[27] While statins have thus far failed to halt or regress AVS progression, HDL-based therapies such as reconstituted HDL or apolipoprotein A-I (apoA-I) mimetic peptides resulted in significant regression of AVS in experimental models, most likely via improved endothelial function, as well as antioxidant and anti-inflammatory effects of apoA-I.[365-367] Even though these results are encouraging, the current effective dose of apoA-I used experimentally is high and renders such therapy less applicable in humans. Recent studies indicate that this suboptimal therapeutic efficiency may be due to proteolytic degradation of apoA-I. Indeed, MMP-3 (stromelysin-1), MMP-7 (matrilysin), MMP-8 (neutrophil collagenase) and MMP-12 (human metalloelastase) have been shown to truncate the carboxyl terminus of apoA-I in HDL_3, thereby dramatically reducing its capacity to induce cholesterol efflux from human macrophage foam cells.[219, 220] In addition, several other proteases found in the vessel wall can degrade apoA-I and HDL_3 and reduce the efficiency of these particles as acceptors of macrophage cholesterol. In fact, the mast cell proteases chymase and tryptase, as well as plasmin, kallikrein and cathepsin F and S specifically degrade lipid-free apoA-I or deplete the minor, yet critical,

subpopulation of preβ-HDL, and so impair the high-affinity efflux of cholesterol from macrophage foam cells that is promoted by HDL_3.[236-240, 244-246, 257] Importantly, some of these apoA-I-degrading proteases have been shown to be present in stenotic aortic valves and pressure overloaded left ventricles and are abundant in plasma of patients suffering from AVS.[226, 227, 231, 267, 268] Accordingly, a proteomic analysis of plasma revealed a reduced expression of protease inhibitors in patients with AVS as compared to controls.[470] It is currently unclear, however, which of these proteases exert the most deleterious effect on apoA-I function. Given that apoA-I-targeting proteolytic activity in AVS may result in decreased efficacy of HDL-based therapies, we aimed to investigate the relative contribution of different plasma proteases to apoA-I degradation in a rabbit model of AVS as well as in patients with severe AVS. We further intended to identify culprit proteases that are responsible for most of the apoA-I-degrading activity. In addition, the value of these culprit proteases to delineate severity and course of AVS was investigated.

3.4.4 Method

Experimental Aortic Valve Stenosis in Rabbits

A previously characterized rabbit model of AVS was used in the present study.[367, 471] This model involves the administration of high-dose vitamin D (up to 50000 U per day during 10-18 weeks) and induces AVS in >70% of animals.[366] At 12-13 weeks of age, 30 male New Zealand White rabbits (weight 2.9-3.0 kg) were divided into three groups: 1) normal diet (n=8), 2) cholesterol-enriched diet (0.5% cholesterol (Harlan, Indianapolis, Indiana; n=8), and 3) cholesterol-enriched diet plus 50 000 IU/day vitamin D2 (Sigma; Markham, Ontario, Canada) in drinking water (n=14). Animals in group 3 were fed vitamin D2 until significant AVS, as defined by a >10% decrease in AVA was detected by echocardiography (usually after 10-14 weeks of treatment). Echocardiograms were performed every 3–4 days. Following the final echocardiogram, animals were killed by cardiac puncture under anaesthesia. The aortic valves were removed for histological analyses. Blood samples were obtained through the marginal vein of the ear before death. Total cholesterol, HDL cholesterol, triglycerides and calcium levels were measured with an automated filter photometer system (Dimension RxL Max; Dade Behring,

Deerfield, IL, USA). Animal care and procedures complied with the Canadian Council on Animal Care guidelines and were approved by the institutional ethics committee for animal research.

Echocardiography in rabbits

For echocardiographic assessment, animals were sedated with intramuscular injections of ketamine (45 mg/kg) and midazolam (0.75 mg/kg). Ultrasound images were obtained using a phased-array probe 10S (4.5~11.5 Megahertz) and a Vivid 7 Dimension system (GE Healthcare Ultrasound, Horten, Norway). The aortic valve area (AVA) was measured by the standard continuity equation. Trans left ventricular outflow tract velocity (VLVOT) was obtained with pulsed-wave Doppler sampled proximally to the aortic valve in the apical 5-chamber view. Continuous wave (CW) Doppler interrogation across the aortic valve was used to obtain transvalvular maximal velocity (VAV) in the same view. VLVOT/VAV ratio was calculated to determine AVS development. The average of three consecutive cardiac cycles was used for each measurement. Animals were weighted at every echocardiogram.

Histology and morphometry of rabbit aortic valve tissue

After sacrifice, the ascending aortic section containing the aortic valve was harvested, opened longitudinally and the three valvular cusps were separated. Two cusps were frozen in an embedding medium and stored at -80°C, one cusp was immersion-fixed in 10% buffered formalin and embedded in paraffin. Stained or immunohistochemically-labelled tissue sections obtained from the central third of each cusp were analysed with a computer-based digitizing image system (Image Pro Plus, version 5.1). The region of analysis (ROA) was composed of 1000 μm of the Valsalva sinus from the leaflet base and 500 μm of the leaflet from the leaflet base, as previously described.[367] Lesion area and leaflet lesion length were also measured. For lipid infiltration analysis, tissue sections were stained with Oil Red O. Haematoxylin-phloxin-saffron, von Kossa and Sirius red stained sections were prepared for general morphology, tissue calcification and collagen studies, respectively. For immunohistochemistry evaluation, all sections were preincubated with mouse immunoglobulin G2a monoclonal antibody against rabbit

smooth muscle cell α-actin (Dako, Mississauga, Ontario, Canada). Smooth muscle cells and calcification areas were quantified in the ROA on digital images acquired at x40 magnification. Images from each section were digitally captured with the same illumination settings, and automatic computer-based analysis was performed with the same colour threshold for all specimens. Data are expressed as % labelled area in the ROA.

Patients

For the present study, sixty-four consecutive patients (20 females) with isolated AVS scheduled for aortic valve replacement were recruited at the Montreal Heart Institute between October 2008 and May 2013. Normal plasma samples from 20 apparently healthy donors free of cardiovascular risk factors and without signs of cardiovascular disease were obtained from BioreclamationIVT (NY, United States). Participating AVS patients needed to be at least 18 years of age, and had indications for non-emergency aortic valve replacement surgery with an aortic valve area smaller than 1 cm^2 and an aortic transvalvular mean gradient higher than 35 mmHg. Patients with significant systemic illness (e.g. inflammatory bowel disease, cancer), severe renal disease (creatinine > 120 μmol/L), previous valve replacement or repair, history of complicated diabetes mellitus, atrial fibrillation/flutter or any irregular heart rhythm at the time of investigation, history of clinical instability (including cardiogenic shock, congestive heart failure, suspected acute myocarditis, endocarditis, or cardiac tamponade, hemodynamically significant hypertrophic cardiomyopathy, restrictive cardiomyopathy or congenital heart disease, suspected dissecting aortic aneurysm), known previous medical condition yielding expected survival less than 18 months, and pregnancy were excluded. Plasma from all 64 patients with AVS was collected within 7 days before patients underwent surgical valve replacement. Plasma was incubated at 37°C with the apoA-I-Cy5.5 probe and fluorescence signal was recorded. Lipid profiles including LDL-cholesterol, HDL-cholesterol, total cholesterol, and triglycerides were analysed, as well as glucose, hs-C-reactive protein (CRP), and calcium concentrations. The study has been approved by the Montreal Heart Institute Ethics Committee and every patient gave written informed consent. Each of the 64 patients with AVS had undergone an M-mode, 2D, and Doppler echocardiographic

study using commercially available equipment (GE Vivid 7, GE Healthcare, Wauwatosa, WI, USA). Image acquisition was obtained in accordance with the recommendations of the American Society of Echocardiography (ASE) and the European Association of Echocardiography (EAE).[472] Severity of AVS was defined based on peak jet velocity, mean transvalvular gradient, aortic valve area (AVA) calculated by the continuity equation, and indexed AVA. Relative wall thickness (RWT) was calculated by dividing two times the left ventricular posterior wall thickness by the left ventricular internal dimensions, and indexed left ventricular muscle mass (LVMMI) was assessed by the Cube formula.[473] Left ventricular concentric remodelling was defined as a RWT > 0.42 with a normal LVMMI (≤ 95 g/m^2 for women, ≤ 115 g/m^2 for men). Left ventricular ejection fraction (LVEF) was measured by the biplane Simpson's method,[474] and left ventricular diastolic function assessed according to current guidelines.[475]

Bioactivatable near-infrared apoA-I-Cy5.5 fluorescent probe

A quenched apoA-I probe was generated using 50-fold molar excess fluorescent dye labelling of 2 mg/mL human purified apoA-I (Biomedical Technologies Inc) in phosphate buffer with the cyanine fluorochrome Cy5.5 NHS ester (GE Healthcare Life Sciences, Canada). Unreacted excess free dye was removed by centrifugal filtration (Amicon) and Sephadex G25 filtration. This resulted in self-quenching of the dye that was relieved by protease cleavage of the apoA-I protein thereby leading to signal amplification (excitation/emission 675/720 nm). *In vitro* validation studies indicated that this apoA-I conjugated quenched probe allows the quantification of proteolytic activities from a wide range of proteases including serine (chymase), cysteine (cathepsin S) and metallo-proteinases (MMP-12).[370]

Effect of recombinant proteases on apoA-I integrity

To compare apoA-I proteolytic activity of different proteases, each protease was incubated at 0.1 µg/mL concentration with the bioactivatable near-infrared apoA-I-Cy5.5 fluorescent probe (5µg/mL) for 5 hours. For protease inhibitor studies, proteases were incubated with inhibitors 1 hour at 37°C before the apoA-I-Cy5.5 probe was added. Fluorescence readings were performed for 5 hours (Interval 60 sec, Ex/Em 675/720 nm, Synergy H1 Hybrid Multi-Mode microplate reader, Biotek,

Winooski, VT). Data were analysed with Gen-5 Biotek software. Human recombinant (rh) MMP-1, MMP-2, MMP-3, MMP-7, MMP-8, MMP-9, MMP-12, and MMP-13 were purchased from R&D. All MMPs, except MMP-3, were activated by incubation with 1 mM p-aminophenylmercuric acetate (APMA) in activation buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10 µM $CaCl_2$, 0.05% Triton X-100) for MMP-12 or reaction buffer (50 mM Tris-HCl, pH 7.5, 10 µM $CaCl_2$, 150 mM NaCl, 0.05% Triton X-100, 5 µM $ZnCl_2$) for all other MMPs at 37°C for 1h (MMP-2, MMP-7, MMP-8), 2 hrs (MMP-1, MMP-13) or 24 hrs (MMP-9, MMP-12). MMP-3 was activated by incubation with 5 µg/mL chymotrypsin at 37°C for 30 min. MMP-3 activation was stopped with 2 mM phenylmethylsulfonyl fluoride (PMSF, Calbiochem). MMPs were incubated with the apoA-I probe during 5 hrs at 37°C in reaction buffer (50 mM Tris-HCl, pH 7.5, 10 µM $CaCl_2$, 150 mM NaCl, 0.05% Triton X-100, 5 µM $ZnCl_2$).

Fluorescence activity was continuously measured every 60 secs during 5 hrs. Recombinant human chymase (rh-CMA-1) was purchased from Sigma-Aldrich (# C8118) and diluted to 0.1 ug/mL in reaction buffer (20 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, pH 7.4). Recombinant human cathepsin S (rh-Cathepsin S) was purchased from R&D (#118-CY) and activated at 100 µg/mL in activation buffer (50 mM sodium acetate, 5 mM DTT, 250 mM NaCl, pH 4.5) for 2 hrs at room temperature. For measurement of apoA-I proteolysis, cathepsin S was diluted to 0.1 ug/mL in reaction buffer (50 mM sodium acetate, 5 mM DTT, 250 mM NaCl, pH 6.5). Recombinant human ADAMTS13 was purchased from R&D (#6165-AD) and diluted in reaction buffer (50 mM Tris-HCl, 2 mM $CaCl_2$, 0.01% Brij-35, pH 8.5). α-chymotrypsin from bovine pancreas was purchased from Sigma-Aldrich (#C3142) and diluted in assay buffer (100 mM Tris-HCl, 10 mM $CaCl_2$). Recombinant human tryptase β-2 (rh-TPSB2) was purchased from R&D and activated by incubation with 0.1 µg/mL thermolysin (15 min at room temperature) in maturation buffer (50 mM Tris, 10 mM $CaCl_2$, 150 mM NaCl, 0.05% Triton X-100, pH 7.5), followed by 2 hrs of incubation at room temperature in heparin buffer (100 µg/mL heparin, 50 mM 2-ethanesulfonic acid/MES, pH 5.5). For measurement of apoA-I proteolysis, activated tryptase was diluted in assay buffer (50 mM Tris-HCl, pH 8.5). Plasminogen was purchased from R&D (#1939-SE) and converted to plasmin by incubation with 4 µg/mL µ-plasminogen activator

(μPA/Urokinase, R&D, #1310-SE) in activation buffer (50 mM Tris-HCl, 0.01% Tween-20, pH 8.5) for 15 min at 37°C. For measurement of apoA-I proteolysis, plasmin was diluted in reaction buffer (0.1 M Tris, 0.1 M NaCl, pH 7.5).

Measurement of protease and apoA-I-degrading activity in human and rabbit plasma

To identify the most potent apoA-I-degrading protease(s) in plasma, rabbit and human (citrated) plasma samples were centrifuged at 20 000 rpm at 4°C for 20 min in order to remove excess lipids. Plasma was then incubated with bioactivatable apoA-I-Cy5.5 probe. In pilot experiments, reaction velocity was found to be the parameter that correlated best with protease activity. Reaction velocity was obtained by determining the slope of a line fit to the initial linear portion of the data plot (average duration of linear portion of data plot=5.3±1.2 min, range 2.2 – 15.8 min). The fluorescence signal was reported as average reaction velocity in RFU (relative fluorescence units) versus time. Fluorescence readings (Ex/Em 675/720 nm) were taken every 60 seconds for 12 hours in a Biotek synergy H1 hybrid multimode microplate reader and analysed with Gen-5 Biotek software. Identification of the culprit protease was performed by incubation of the plasma with different broad-spectrum and protease-specific inhibitors in the presence of the apoA-I-Cy5.5 probe. Data were reported as % remaining protease activity (= [reaction velocity inhibitor/reaction velocity control] x 100). The following inhibitors were used: a serine proteases inhibitor cocktail containing PMSF 1 mM, and 100 μg/mL of trypsin-chymotrypsin inhibitor-soybean (Sigma-Aldrich #T9777), a cysteine proteases inhibitor cocktail containing 20 μM E64 (Calbiochem #324890), 0.25 mM Cathepsin Inhibitor I (Calbiochem #219415), an MMP inhibitor cocktail containing 10 mM ethylenediaminetetraacetic acid (EDTA, Sigma-Aldrich #E9884) and 2 mM phenanthroline (Sigma-Aldrich #131377), and the unspecific MMP inhibitor Marimastat® (5 nM final concentration, Calbiochem #444289). The specific Cathepsin S inhibitor RO5461111® (50 nM final concentration), and the specific chymase inhibitor RO5066852 (10 mM final concentration) were obtained from Roche AG, Basel, Switzerland. *In vitro* enzyme inhibition and cross-reactivity studies showed that both, the chymase inhibitor RO5066852® and the cathepsin S

inhibitor RO5461111® are selective inhibitors of human chymase and cathepsin S and displaying IC50 values in the nanomolar (cathepsin S) and millimolar (chymase) range and being relatively inert for other proteases of the same family (data not shown). The chymase inhibitor had previously been used *in vivo* and *in vitro.*[248] A specific urokinase-type plasminogen activator (µPA) inhibitor was purchased from Santa Cruz (#sc-356184, 1.5 µM final concentration). The inhibitory potential of all inhibitors was validated and their IC50 values were calculated using recombinant proteases tested with the respective commercially available specific fluorogenic peptide substrate probes and the apoA-I-Cy5.5 probe. The following fluorogenic peptide substrates were used: For MMPs (except MMP-3): MCA-Lys-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH_2 (final concentration 10 µM, Ex/Em 320/405, R&D, #ES010), for MMP-3: MCA-Arg-Pro-Lys-Pro-Val-Glu-NVal-Trp-Arg-Lys(DNP)-NH_2 (final concentration 10 µM, Ex/Em 320/405 nm, R&D, #ES002), for cathepsin S: MCA-Gly-Arg-Trp-Pro-Met-Gly-Leu-Pro-Trp-Glu-Lys-D-Arg-NH_2 (final concentration 10 µM, Ex/Em 340/405 nm, Calbiochem, #219498), for chymase: Suc-Ala-Ala-Pro-Phe-AMC (final concentration 200 µM, Ex/Em 380/460 nm, Calbiochem, #230914), for plasmin: Suc-Ala-Phe-Lys-AMC (final concentration 100 µM, Ex/Em 380/460 nm, Bachem, #I-1325), for tryptase: MCA-Arg-Pro-Lys-Pro-Val-Glu-NVal-Trp-Arg-Lys(Dnp)-NH_2 (final concentration 10 µM, Ex/Em 320/ 405 nm, R&D, #ES002), for ADAMTS13: Asp-Arg-Glu-A_2pr(Nma)-Ala-Pro-Asn-Leu-Val-Tyr-Met-Val-Thr-Gly-A_2pr(Dnp)-Pro-Ala-Ser-Asp-Glu-Ile-Lys-Arg-Leu-Pro-Gly-Asp-Ile-Gln-Val-Val-Pro-Ile-Gly-Val-Gly-Pro-Asn-Ala-Asn-Val-Gln-Glu-Leu-Glu-Arg-Ile-Gly-Trp-Pro-Asn-Ala-Pro-Ile-Leu-Ile-Gln-Asp-Phe-Glu-Thr-Leu-Pro-Arg-Glu-Ala-Pro-Asp-Leu-Val-Leu-Gln-Arg (FRETS-vWF73, final concentration 2 µM, Ex/Em 340/450 nm, Peptide Institute, #3224-s).

Statistical analysis

If not otherwise specified, data are presented as mean±standard deviation (SD) or median (Q1; Q3) for continuous variables and frequency and percentage for categorical variables. Prior to analyses, basic assumptions were checked. The rabbit groups were compared using an analysis of variance (ANOVA) or the Kruskal–Wallis test. In the patients' population, the comparisons of males vs. females and healthy control vs. AVS patients in continuous variables were tested

using the Student's t-test or the Mann-Whitney test. The chi-square test was used for comparisons of categorical variables. The mean apoA-I-degrading activity and the mean remaining apoA-I proteolysis for each inhibitor were tested against 100, which represents the no inhibitor value, using one *s*ample t-tests. The apoA-I-degrading activity measured with the different recombinant proteases and the protease activity were analysed using repeated measures ANOVA. Adjustment for multiple comparisons was used. Relationships between protease activity and histomorphometrical or echocardiographic variables were evaluated using Pearson or Spearman's correlation coefficient. The predictive value of cathepsin S activity on transvalvular aortic valve gradient was assessed using a stepwise selection model including known cardiovascular risk predictors (criterion: probability-of-F-to-enter <=0.05, remove=0.10). Statistical significance was set at $p<0.05$. Statistical analyses were performed with IBM SPSS statistics version 23.0 and SAS version 9.4.

3.4.5 Results

ApoA-I digestion by different proteases

When the apoA-I Cy5.5 probe was exposed to proteases, which have previously been shown to degrade apoA-I, at 0.1 µg/mL, the highest initial reaction velocity was measured for α-chymotrypsin (1.2±0.2 RFU/sec, $p<0.05$ vs proteases with only minimal apoA-I-degrading activity including rh-MMP-1=0.015±0.02 RFU/sec, rh-MMP-2=0.03±0.04 RFU/sec, rh-MMP-3=0.03±0.04 RFU/sec, rh-MMP-8=0.02±0.05 RFU/sec, rh-MMP-9=0.03±0.04 RFU/sec, rh-ADAMTS-13=0.003±0.002 RFU/sec, rh-tryptase-0.003±0.01 RFU/sec and plasmin=0.01±0.02 RFU/sec) followed by rh-CMA-1 (0.72±0.02 RFU/sec, n=4), rh-MMP-12 (0.31±0.03 RFU/sec), rhMMP-13 (0.26±0.01 RFU/sec), rh-Cathepsin S (0.19±0.11 RFU/sec), and rh-MMP-7 (0.14±0.01 RFU/sec, n=4, Figure 22, page 130).

Echocardiographic, histomorphological and biochemistry parameters in rabbits

All rabbits were fed a cholesterol-enriched diet containing vitamin D2 for a mean duration of 12.7±3.2 weeks. Following treatment, all animals presented aortic valve lesions (Table XV upper panel, page 136). AVA decreased from 22.9±3.4 mm^2 at baseline to 18.6±2.5 mm^2 following treatment (18.8% decrease, $p<0.05$ vs baseline). Histological analysis revealed significant aortic valve lesions, valve calcifications and lipid infiltrations (Table XV upper panel, page 136). Significant differences existed between the three experimental groups regarding lipid parameters and serum calcium concentration (Table XV lower panel, page 136).

Augmented apoA-I-degrading activity in AVS rabbit plasma

Plasma from all groups was incubated at 37°C with the apoA-I-Cy5.5 probe and fluorescence signal was recorded. The apoA-I-Cy5.5 probe in the absence of plasma served as negative control. Figure 23A, page 131 and Table XV lower panel (page 136, column 2) show the increase in the initial rate of fluorescence of the quenched apoA-I-Cy5.5 probe following exposure to rabbit plasma. The highest apoA-I proteolytic activity was measured in rabbits with AVS (cholesterol-enriched diet+vitamin D2, Figure 23A, page 131). Rabbits fed a cholesterol-enriched diet (without vitamin D2) showed lower apoA-I proteolytic activity than the AVS group, however, this difference did not reach statistical significance (Figure 23A, page 131, p=NS).

MMPs account for the majority of apoA-I-degrading proteolytic activity in rabbit plasma

The strongest inhibiting effect on apoA-I proteolysis was seen when AVS rabbit plasma was incubated with an MMP inhibitor cocktail or with the MMP inhibitor Marimastat® (Figure 23B, page 131), indicating that one or more MMPs might be amongst the main culprit proteases. In our pilot experiments, Marimastat®, a broad class MMP inhibitor that had previously been used in experimental and clinical trials,[476] inhibited MMP-7, MMP-12, and MMP-13 activities by >80% (n=3, Figure 24A, page 132). As mentioned earlier, those three MMPs were found to contribute for >95% of MMP-dependent apoA-I proteolysis (Figure 22, page 130).

Accordingly, incubation of plasma with Marimastat® resulted in inhibition of apoA-I proteolysis activity by 67% (Figure 23B, page 131), indicating that MMP-7 and/or MMP-12 and/or MMP-13 are/is the culprit protease(s) responsible for most of apoA-I degradation in plasma from AVS rabbits. When protease activity was measured in plasma from AVS rabbits by using different, commercially available specific peptide probes, highest signal was found for MMPs (7.8±2.5 RFU/sec), while lower fluorescence signal was detected for rh-cathepsin S (0.38±0.2 RFU/sec), rh-CMA1 (0.04±0.01 RFU/sec) and plasmin (0.03±0.02 RFU/sec) (Figure 24B, page 132, *$p<0.05$ vs rh-cathepsin S, rh-CMA1 and plasmin, **$p<0.05$ vs rh-CMA1and plasmin). Plasma MMP activity was highest in rabbits with AVS as compared to controls (normal diet) ($p<0.05$ vs control, Figure 24C, page 132).

Enhanced apoA-I-degrading proteolytic activity in plasma of AVS rabbits is associated with total lesion area on diseased aortic valve leaflets

In rabbits with AVS (cholesterol-enriched diet+vitamin D2), a significant and positive correlation was found between apoA-I proteolytic activity in plasma and the area of atherosclerotic-like plaque lesion on diseased aortic valve leaflets (Spearman $r=0.7$, $p<0.05$, Figure 25, page 133), whereas no significant correlation was found between apoA-I proteolytic activity in plasma and valve calcification, lipid infiltration, and leaflet thickness (p=NS, data not shown).

Patient study-baseline characteristics

Baseline characteristics of the study population are summarized in Table 2. Out of 64 patients with severe AVS (mean AVA 0.8±0.2 cm^2), 31% were women, mean age was 65.0±10.4 years and mean body mass index (BMI) was 31.2±6.3 kg/m^2. Patients in the control group were significantly younger (mean age 43.1±12.8 years, $p<0.05$ vs AVS group) than patients in the AVS group and had no cardiovascular risk factors. In contrast, prevalence of cardiovascular risk factors was high in the AVS group, with 62.5% of patients being hypertensive, 60.7% of patients suffering from dyslipidaemia, 19.6% of patients being current smokers, and 19.6% of patients being diabetic. 53.1% of patients in the AVS group were on statin therapy. No differences in risk factors, statin therapy, BMI and age were

found between men and women. None of the patients had significant coronary artery disease. Mean and peak aortic valve gradient in the AVS group were 52.2±14.8 mmHg and 83.1±20.9 mmHg, respectively (p=NS for male vs females). 55% of patients in the AVS group had bicuspid aortic valve stenosis. Left ventricular mass index was 123.3±32.9 g/m^2 in males and 100.5±23.6 g/m^2 in females (p=0.0204), while left ventricular ejection fraction was 60%[57;65] in males and 60%[60;65] in women (p=NS). Severe aortic regurgitation was not observed. Baseline echocardiographic characteristics are summarized in Table XVI, page 137. Lipid parameters, serum calcium, hs-C-reactive peptide (CRP) and glucose levels did not differ significantly between sexes (p=NS, Table XVII, page 137).

Augmented apoA-I-degrading proteolytic activity in plasma from AVS patients

A significantly higher apoA-I proteolytic activity was measured in patients with AVS as compared to healthy volunteers (0.04±0.001 vs. 0.02±0.005, p<0.0001, Figure 26A, page 133). When protease activity was measured in plasma from AVS patients by using commercially available peptide probes, a significantly higher cathepsin S activity was observed in AVS patients as compared to healthy volunteers (0.05[0.01;0.14] vs 0.01[0.006;0.02], p<0.05, Figure 26B, page 133). No differences were found between AVS patients and controls for chymase activity and MMP activity (p=NS, Figure 26C and 26D, page 133, respectively).

Cathepsin S activity is the main culprit for apoA-I proteolysis in human AVS plasma

The quenched apoA-I-Cy5.5 probe was incubated with plasma from 64 AVS patients in the presence and absence of protease inhibitors cocktails as well as specific protease inhibitors. In contrast to the observations made in rabbits, the strongest inhibiting effect on apoA-I proteolysis in humans was seen when plasma was incubated with a cysteine protease inhibitor cocktail (53.5% inhibition, p<0.05 vs no inhibitor, Figure 27A, page 134). A similar inhibitory effect on apoA-I proteolysis was observed with the cathepsin S specific inhibitor RO5461111® (54.2% inhibition, p<0.05 vs no inhibitor, Figure 27A, page 134) indicating that the

cysteine protease cathepsin S accounts for most of the apoA-I degradation in plasma. A weaker (25.2%) but significant inhibition of apoA-I proteolysis was observed when plasma was incubated with a serine protease inhibitor cocktail ($p<0.05$ vs no inhibitor). Similarly, inhibition by the specific chymase inhibitor RO5066852® resulted in 74% remaining apoA-I proteolysis ($p<0.05$ vs no inhibitor), indicating that the serine protease chymase contributes by 26% to apoA-I proteolysis in plasma from AVS patients. We then compared females and males in terms of the effects of the different inhibitors on apoA-I-degrading activity. In females, apoA-I proteolysis was inhibited by 69% and 71% by the cysteine protease inhibitor cocktail and the cathepsin S inhibitor RO5461111®, respectively ($p<0.05$ vs no inhibitor, Figure 27B, page 134). A much weaker inhibition (16%) of apoA-I proteolysis in plasma of female AVS patients was obtained by adding serine protease inhibitors ($p<0.05$ vs no inhibitor, Figure 27B, page 134) or MMP inhibitors (p=NS, Figure 27B, page 134). In contrast, in males, apoA-I proteolysis was inhibited by only 47% by the cysteine protease inhibitor cocktail and the cathepsin S inhibitor RO5461111® (Figure 27C, page 134), while the serine protease inhibitor and the specific chymase inhibitor reduced apoA-I proteolysis by 30% and 31%, respectively ($p<0.05$, Figure 27C, page 134). A weaker but significant inhibition of apoA-I proteolysis in males was observed with the MMP inhibitor cocktail and Marimastat® (17% and 15% inhibition, respectively, $p<0.05$ vs no inhibitor, Figure 27C, page 134).

Plasma cathepsin S activity is associated with echocardiographic characteristics of AVS in human females but not in males

A significant and positive correlation was found between plasma cathepsin S activity and aortic valve peak (Spearman r=0.5, p=0.035, Figure 28A, page 135) and mean gradients (Spearman r=0.5, p=0.04, Figure 7B) in females but not in males (p=NS, Figure 28A and 28B, page 135). Accordingly, cathepsin S activity was found to be a significant positive predictor of mean aortic valve gradient in women (standardized beta coefficient 0.832, $p<0.001$, Table XVIII, page 138), when tested in a stepwise linear regression analysis against known cardiovascular risk factors including age, BMI, diabetes, LDL-C, smoking and hypertension as

candidate predictor variables. No significant correlation was seen between apoA-I proteolysis and echocardiographic AVS characteristics (p=NS, data not shown).

3.4.6 Discussion

The present study demonstrates that apoA-I proteolysis is augmented in plasma from patients with severe AVS as well as in a rabbit model of AVS. MMP-7, MMP-12 and MMP-13 (or a subset of those MMPs) were found to account for most of the apoA-I degradation in rabbit plasma, while cathepsin S was the main culprit protease in humans, with the greatest cathepsin S-dependent apoA-I proteolysis seen in women. The latter correlated significantly and positively with AVS severity in women, as assessed by echocardiographic transvalvular gradients. In addition, plasma apoA-I proteolytic activity was found to be significantly associated with atherosclerotic-like lesions of diseased aortic valves in rabbits.

Our study is the first comparing directly the apoA-I-degrading proteolytic potential of different groups of recombinant proteases under comparable experimental conditions. In line with previous reports, apoA-I was highly susceptible to proteolysis from members of the MMP family, in particular MMP-7 and MMP-12. MMPs, a family of at least 26 proteases specialized in degrading various components of extracellular matrix are secreted mostly by macrophages which are highly abundant in diseased aortic valve tissue. These include MMPs-1, -2, -3, -7, - 9, and -12 of which MMP-3, MMP-7, MMP-8, and MMP-12 have previously been shown to truncate the carboxyl terminus of apoA-I, thereby reducing its capacity to promote reverse cholesterol efflux from macrophages.[219, 220] Indeed, the carboxyl-terminal domain of apoA-I is especially important for promoting cholesterol efflux from cholesterol-loaded macrophages and incubation of HDL_3 with MMPs led to a 70% loss of its cholesterol efflux-promoting ability.[219] Of note, MMP concentration in inflamed tissues such as synovial fluids has been described to range between 1 to 110 µg/ml, a concentration that is up to 1 000x higher than the recombinant MMP concentration (0.1 µg/mL) used in the present study and substantial evidence implicates MMPs in plaque rupture and fatal thrombosis.[477-479]

We found a significant and positive association between plasma apoA-I proteolytic activity and the extent of aortic valve lesions on diseased aortic valve leaflets and sinus of Valsalva in rabbits, thereby suggesting a link between apoA-I degradation in plasma, inflammatory injury and lesion extent on diseased valve tissue. Importantly, lipid-poor apoA-I is highly susceptible to proteolytic degradation and the vast majority of apoA-I in atherosclerosis-laden tissues is lipid-poor, while only a minor part of lipid-poor apoA-I can be found in the circulation.[480] Thus, the observed correlation between tissue injury and plasma apoA-I degradation observed in the present study may point towards an even more pronounced deleterious effect of proteases on apoA-I function in the diseased vessel wall. In addition, apoA-I might be prone to proteolytic degradation during a process called HDL-apoA-I exchange (HAE). The latter occurs due to the dynamic ability of HDL to undergo remodelling and has recently been suggested to significantly contribute to serum HDL efflux capacity.[481] Thus, proteolysis of apoA-I during this important and potentially rate-limiting aspect of RCT has the potential to substantially impact cholesterol mobilization from the vascular wall.

No previous reports are available on the association of apoA-I proteolysis and AVS progression, however, recent studies have detected protease-digested apoA-I fragments or low apoA-I levels in serum of patients with acute coronary syndrome, inflammatory diseases or AVS.[55, 270, 470] Since apoA-I proteolysis in rabbit plasma was mainly driven by MMP-7, MMP-12 and/or MMP-13, our data indicate a functional role for these proteases in AVS progression in this rabbit model. Indeed, MMP-7 and MMP-12 have recently been found to be independently associated with more severe atherosclerosis, an increased incidence of coronary events, and calcific aortic valve stenosis, while a selective MMP-12 inhibitor reduced macrophage invasion and accumulation in an experimental atherosclerosis model.[229, 230, 482]

Mast cells and basophil granulocytes are filled with cytoplasmic secretory granules that contain the two serine proteases, tryptase and chymase, a chymotryptic endopeptidase. Both, tryptase and chymase have previously been shown to degrade apoA-I in HDL_3. [238, 239, 255] However, under our conditions, we detected only minimal apoA-I degradation by recombinant tryptase, while, amongst all proteases tested, recombinant chymase and α-chymotrypsine were

found to exert the most detrimental activity against apoA-I. Chymase is involved in a variety of functions, including immune responses to parasite antigens, and conversion of angiotensin I to angiotensin II. Importantly, although a strong apoA-I proteolytic potential was attributed to recombinant chymase in our *in vitro* assay, using chymase inhibitors, we found only limited contribution of circulating chymase to apoA-I degradation in human or rabbit plasma, with the upmost chymase-dependent apoA-I proteolysis seen in male humans (30%). These results seem to indicate that apoA-I proteolysis by chymase might be a rather local process limited to the environment of activated mast cells within inflamed tissue. [256] Indeed, inhibitor inactivation of chymase in extracellular fluids soon after exocytosis might explain its low apoA-I-degrading potential in plasma. [483, 484]

Cathepsin S, one of the most potent mammalian elastases abundantly expressed by macrophages and vascular smooth muscle cells, cleaves apoA-I at its carboxy terminal end, thereby removing its capacity to induce cholesterol efflux.[257, 259] Cathepsin S is constitutively secreted into the extracellular space by monocyte-derived macrophages and mast cells and contributes significantly to proteolysis of elastic laminae. During progression of vascular and valvular disease, a disruption of the balance between cathepsin S and its inhibitor cystatin C occurs, and previous studies have shown that cathepsin S induced elastolysis accelerates adverse valve remodelling and valve calcification.[262, 268] In our study, cathepsin S activity was found to be responsible for up to 70% of apoA-I degradation in human plasma and was positively associated with AVS severity, as assessed by echocardiographic transvalvular gradient, in women. This association remained significant after adjusting for other potential contributors of AVS severity such as age, diabetes, BMI and LDL-cholesterol. In addition, cathepsin S activity was found to be a significant positive predictor of mean aortic valve gradient in women when tested in a stepwise linear regression analysis against known cardiovascular risk factors. These observations provide clinical support to previous experimental studies demonstrating a role for cathepsin S in the promotion of AVS and suggest that cathepsin S might be a potential biomarker reflecting severity of AVS in women. Indeed, patients with AVS are currently evaluated solely based on clinical data to determine the need to initiate therapy. Although several biomarkers for AVS severity have been suggested, the actual value of those biomarkers remains

unclear at the current time. Thus, as the prevalence of AVS increases and treatment options expand, developing better ways to risk-stratify patients is becoming increasingly important to determine optimal timing for valve replacement, and whether valve replacement is potentially futile. Given its involvement in several key pathways triggering fibrosis and calcification in AVS, cathepsin S represents an important link between inflammatory, pro-fibrotic and pro-osteogenic mechanism contributing to AVS progression. These characteristics, along with its high abundancy and activity in human plasma, make plasma cathepsin S activity a valuable tool to connect changes in circulation with changes that occur in diseased valve tissue thereby possibly improving patient selection for therapeutic intervention.[485] Of note, diagnostic approaches capable of measuring circulating levels and activity of cathepsin S in human serum have recently been introduced and the development of selective inhibitors of cathepsin S is currently pursued by several pharmaceutical companies.[485]

In the present study, we were unable to demonstrate an association between circulating apoA-I-degrading proteolytic activity and severity of AVS in humans, even though cathepsin S, as the probable main culprit apoA-I-degrading protease in human plasma, correlated significantly with the latter. Importantly, the correlation between cathepsin S and AVS severity was only seen in women but not in men. One possible explanation for these discrepancies could be the relative low number of subjects studied (n=65). However, sex-specific differences in protease levels and activity have recently come into light. Indeed, an inhibitory effect of oestrogen on mast cell chymase secretion in pressure-overloaded myocardium has lately been described,[251] and opposite effects on MMP and chymase activity have been shown for testosterone and oestrogen.[486, 487] Importantly, male patients had a significantly higher LVMMI in our study. Given the pivotal role of mast cell chymase and MMPs in chronic pressure overload–induced LV hypertrophy,[250, 251] it is conceivable that sex differences in apoA-I-degrading protease activity may mirror different pattern of adverse LV remodelling during AVS progression in males and females. Further, the complex process of left ventricular remodelling in aortic valve disease as well as the multiple comorbid conditions usually observed in an elderly population with AVS may also account for the species differences observed in the present study. Indeed, while treatment

with the MMP inhibitor Marimastat® inhibited apoA-I cleavage in rabbit plasma by 70%, it only had limited effect in human plasma. Considerable species differences in protease activity and HDL metabolism exist [488], thus, animal models may be limited in their ability to mimic the pathophysiology of apoA-I-degrading proteolytic activity in AVS and should be interpreted carefully.

There are some limitations of the present study that should be considered. The observational nature of our study does not allow for conclusions to be made regarding the functional relevance of apoA-I proteolysis in AVS, such as the loss of cholesterol efflux promoting capacity. However, our observations of associations between plasma protease activity and morphological characteristics of AVS are well in line with previous studies, implicating proteolytic activity of tissue MMPs and cathepsin S in the development of AVS.[226, 262, 267] In addition, the high abundance of apoA-I-degrading proteases in diseased aortic valve tissue raises the question whether local tissue proteases or circulating proteases play a primary role in apoA-I degradation. We did not measure the activities of apoA-I-degrading proteases in human or rabbit aortic valves, thus our study cannot answer this question. However, considering that reconstituted HDL therapy is usually administered intravenously in human, apoA-I degradation of such lipid-poor particles by circulating proteases might significantly reduce the bioavailability of similar HDL-based therapy before it reaches its site of action. As mentioned earlier, a wide variety of tissues, including pressure overloaded myocardium and arterial walls, produce apoA-I-degrading proteases. Even though apoA-I on large HDL is known to be resistant to proteolysis, HAE is a property reflecting functionality and may, in conditions of high apoA-I-degrading proteolytic activity, be associated with significant degradation.[481] It was shown that chemical modification of apoA-I may impair its exchangeability. Thus, the most active fraction of apoA-I in relation to cholesterol efflux, such as that on pre-beta HDL, lipid-poor apoA-I and exchangeable apoA-I, may be the most susceptible in circulation, even though the major fraction of apoA-I is associated with the bigger HDL and consequently, would be expected to be more protease-resistant. Thus, both circulating protease activity and local protease expression in inflamed aortic valves may determine apoA-I degradation and its pathophysiological significance. [231, 232] In addition, increased age and impaired renal function in an elderly

population with AVS could further enhance circulating protease levels and thus apoA-I degradation in plasma. Further, we used apparently healthy volunteers as a control sample for comparison of apoA-I proteolysis. Since AVS is associated with a number of comorbid conditions, we cannot exclude an influence of these cofactors on apoA-I degradation. Finally, about 25% of apoA-I proteolytic activity persisted even after use of broad class protease inhibitors in human and rabbit plasma indicating that our analysis was not exhaustive. Considering the composition of inhibitors used in the present study, it is conceivable that threonine or aspartic acid proteases are causative of the remaining apoA-I proteolysis. Alternatively, post-translational protease modifications could hamper the selectivity and efficacy of the protease inhibitors used in the present study.

Collectively, our findings suggest that plasma apoA-I proteolysis is augmented in AVS and that cathepsin S exerts the most deleterious effects on apoA-I integrity in humans. Notably, peptide sequences cleaved by cathepsin S (e.g. Arg-Phe) are not only present in full-length apoA-I but also in some apoA-I mimetic peptides such as ETC-642, which are increasingly used in experimental and clinical studies. [302, 489] Thus, the design of HDL surrogates containing peptides that mimic apoA-I functions and are resistant to adverse modification by cathepsin S may lead to new therapies in human aortic valve disease.

3.4.7 Figures and Tables

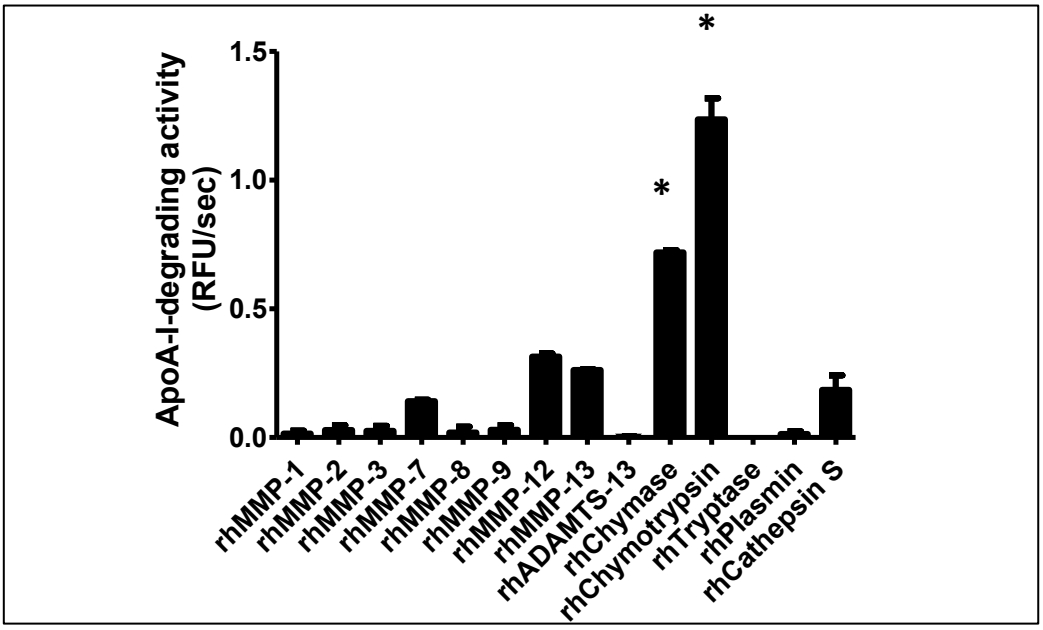


Figure 22: Exposure of apoA-I-Cy5.5 probe to different recombinant proteases at 0.1 µg/mL concentration. ApoA-I proteolysis is estimated from the increase in relative fluorescence units (RFU)/sec. MMP, matrix metalloproteinase; rh; recombinant human; rm, recombinant mouse; ADAMTS, a disintegrin and metalloproteinase with thrombospondin motifs.*p<0.05 vs all other inhibitors.

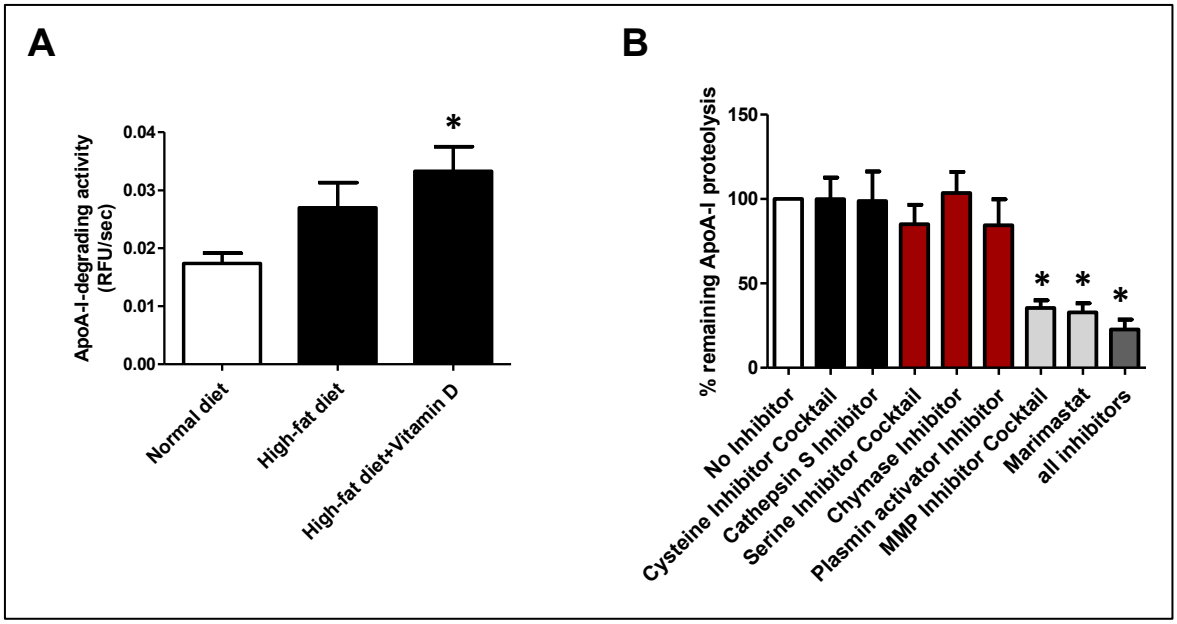


Figure 23: ApoA-I degradation in rabbit plasma with and without protease inhibition. A. ApoA-I digestion in rabbit plasma. ApoA-I proteolysis is estimated from the increase in relative fluorescence units (RFU)/sec. *p<0.05 vs rabbits fed a normal diet (n=8 for normal diet controls, n=8 for high-fat diet, n=14 for AVS). **B.** Remaining apoA-I proteolysis in plasma of rabbits fed a cholesterol-enriched diet and vitamin D following protease inhibition with different protease inhibitors. Data are reported as percentage of remaining protease activity (=increase in relative fluorescence units per sec with inhibitor/ increase in relative fluorescence units per sec without inhibitor x 100). *p<0.05 versus control (no inhibitor).

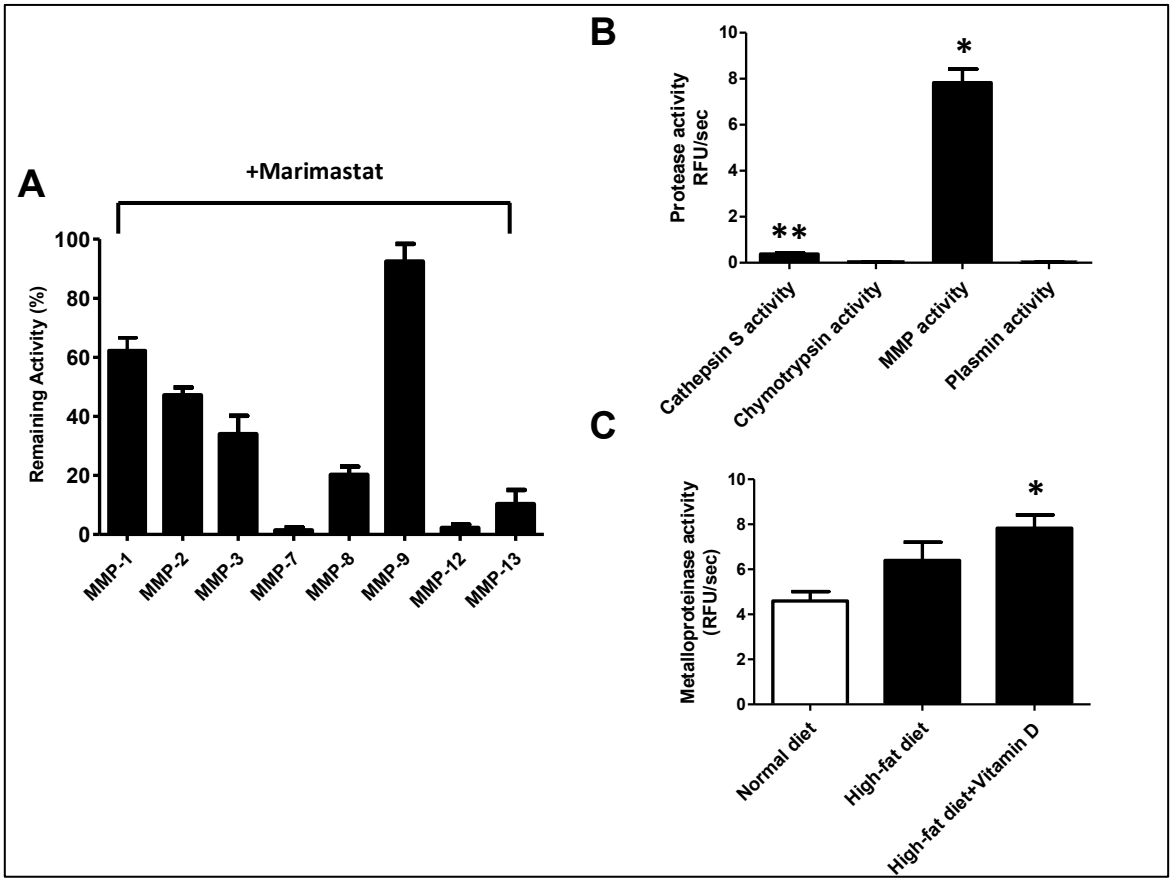


Figure 24: Protease activity in rabbit plasma. A. Inhibitory potential of Marimastat® on different matrix metalloproteinases. Data are reported as percentage of remaining protease activity (=increase in relative fluorescence units per sec with inhibitor/ increase in relative fluorescence units per sec without inhibitor x 100). **B.** Measurement of cathepsin S, chymase, matrix metalloproteinase, and plasmin activity in plasma from AVS rabbits fed a cholesterol-enriched diet and vitamin D2; protease activity is indicated as increase in relative fluorescence units (RFU)/sec *p<0.05 vs all other proteases (n=14). **C.** Plasma matrix metalloproteinase activity in all experimental groups. Data are indicated as increase in relative fluorescence units (RFU)/sec. *p<0.05 versus control (normal diet). MMP, matrix metalloproteinase.

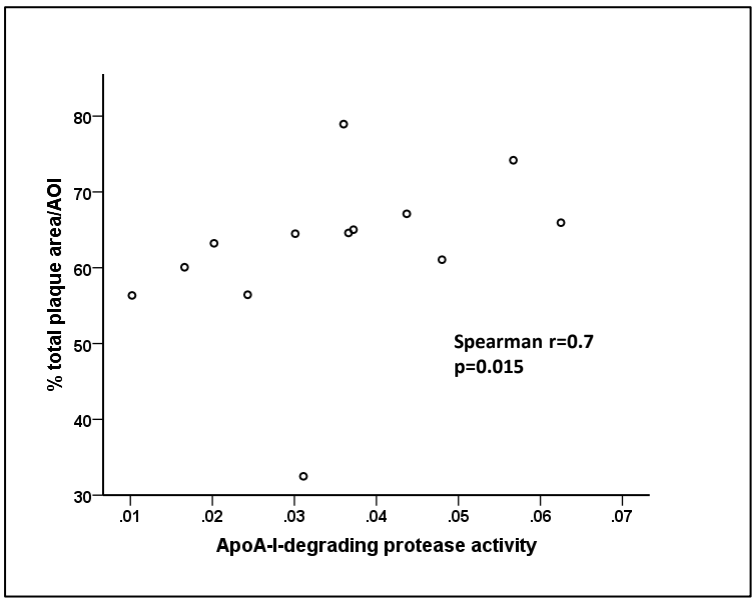


Figure 25: Correlation between apoA-I-degrading protease activity in plasma and percentage of lesion extend on aortic valve leaflets in AVS rabbits fed a cholesterol-enriched diet and vitamin D. (n=13)**.** AVS, aortic valve stenosis.

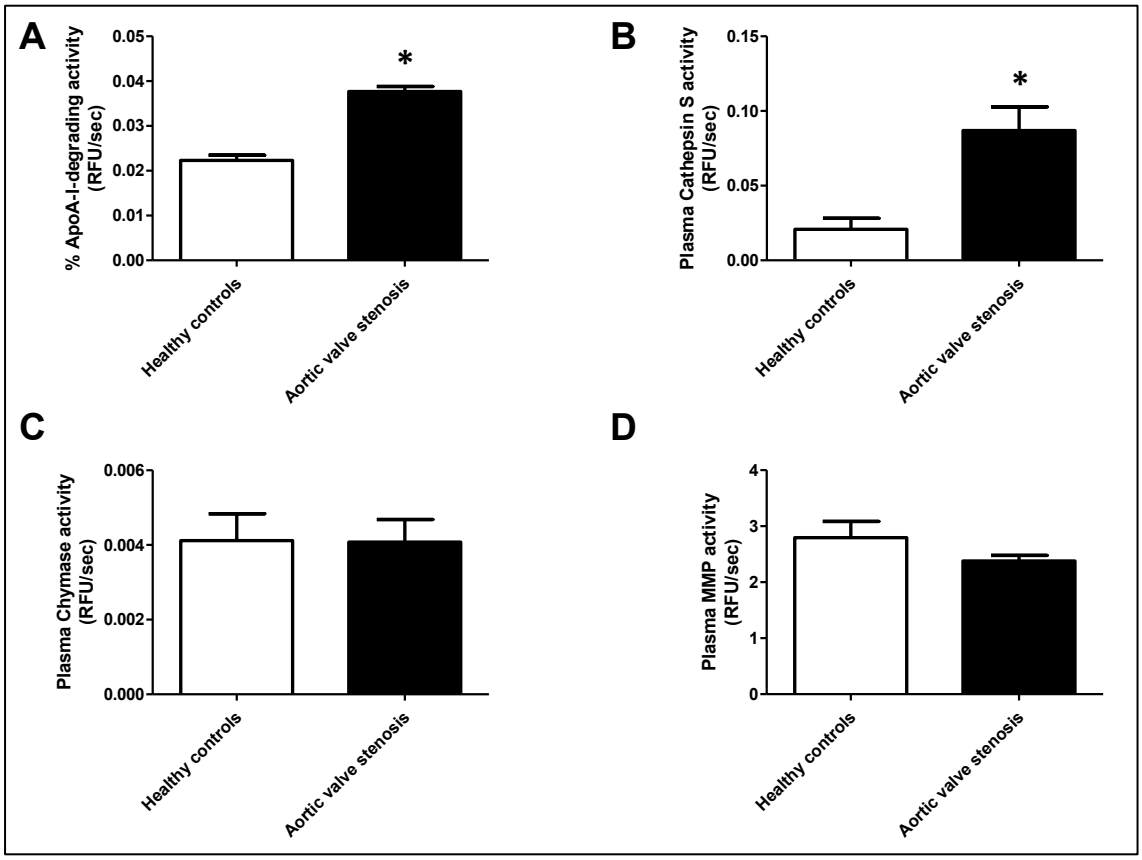
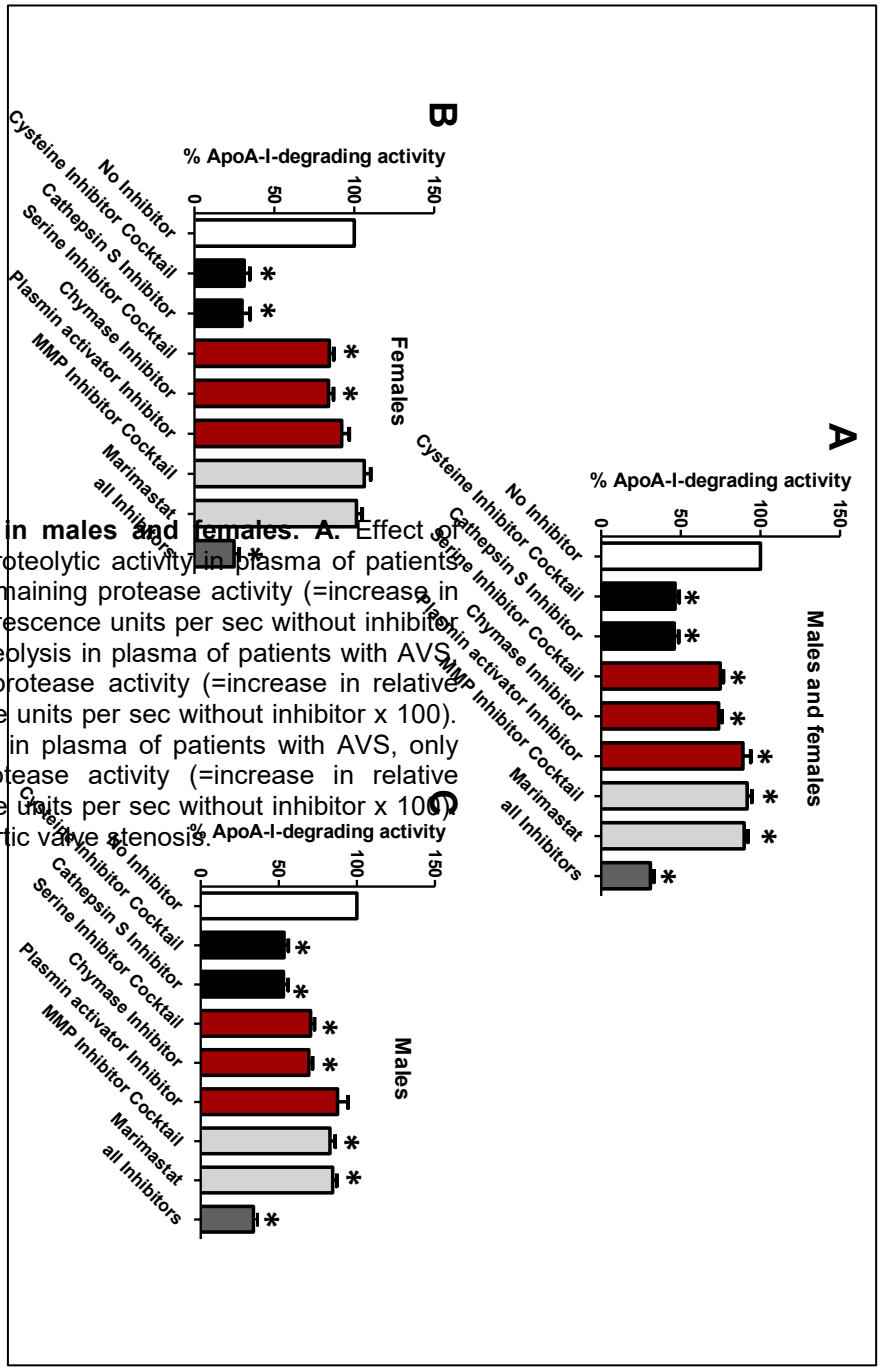


Figure 26: Protease activity in patient plasma. A. ApoA-I proteolytic activity in plasma from AVS patients and healthy controls. Data are indicated as increase in relative fluorescence units (RFU)/sec. *p<0.05 versus healthy controls. **B**. Cathepsin S activity in plasma from AVS patients patients and healthy controls. Data are indicated as increase in relative fluorescence units (RFU)/sec. *p<0.05 versus healthy controls. **C**. Chymase activity in plasma from AVS patients and healthy controls. Data are indicated as increase in relative fluorescence units (RFU)/sec. **D.** Matrix metalloproteinase activity in plasma from AVS patients and healthy controls. Data are indicated as increase in relative fluorescence units (RFU)/sec. MMP, matrix metalloproteinase; AVS, aortic valve stenosis.



of protease inhibitors on Apo-A-I degradation in males and females. A. Effect of and their associated remaining apoA-I-degrading proteolytic activity in plasma of patients ders (n=64). Data are reported as percentage of remaining protease activity (=increase in e units per sec with inhibitor/increase in relative fluorescence units per sec without inhibitor sus control (no inhibitor). **B.** Remaining apoA-I proteolysis in plasma of patients with AVS,). Data are reported as percentage of remaining protease activity (=increase in relative er sec with inhibitor/increase in relative fluorescence units per sec without inhibitor x 100). trol (no inhibitor). **C.** Remaining apoA-I proteolysis in plasma of patients with AVS, only a are reported as percentage of remaining protease activity (=increase in relative er sec with inhibitor/increase in relative fluorescence units per sec without inhibitor x 100). rol (no inhibitor). AVS, aortic valve stenosis.AVS, aortic valve stenosis.

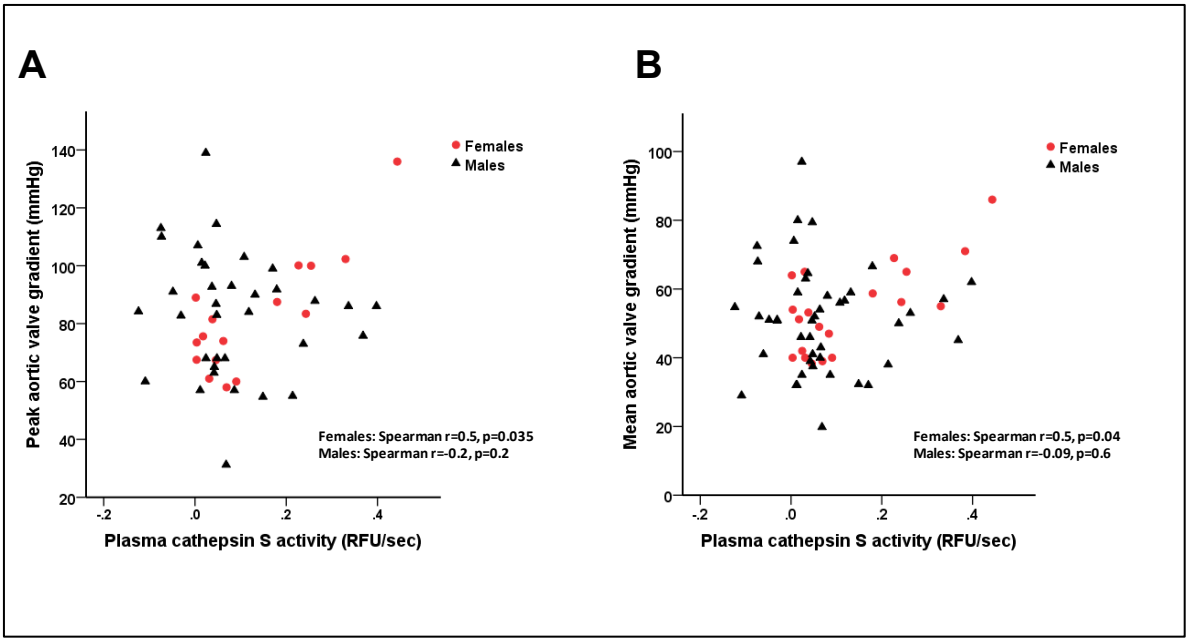


Figure 28: Correlation between cathepsin S activity and severity of aortic valve stenosis. **A**. Correlation between cathepsin S activity in plasma of male and female AVS patients and aortic valve peak gradient assessed by echocardiography. RFU, relative fluorescence units. Spearman r and p-value are indicated. (n=64, females=20) **B.** Correlation between cathepsin S activity in plasma of male and female AVS patients and aortic valve mean gradient assessed by echocardiography. RFU, relative fluorescence units; AVS, aortic valve stenosis. Spearman r and p-value are indicated. (n=64, females=20). AVS, aortic valve stenosis.

Aortic valve and aorta characteristics	weight (kg)	% sinus calcification/total sinus and leaflet area	% plaque area/total sinus and leaflet area	% Oil Red O positive surface/total aortic arch area	Aortic leaflet thickness (mm)
Normal diet (control, n=8)	3.0±0.2	-	-	-	-
High-fat diet (ATX, n=8)	2.9±0.3	-	-	-	-
High-fat diet+Vit D (AVS, n=14)	2.9±0.2	5.4±3.8	62.3±10.9	74.6±17.6	0.7±0.2

Biochemistry parameters	ApoA-I-degrading protease activity (RFU/sec)	Total cholesterol (mmol/l)	HDL-cholesterol (mmol/l)	Triglycerides (mmol/l)	Calcium (mmol/l)
Normal diet (control, n=8)	0.017±0.005	0.5±0.2	0.3±0.1	0.4[0.3;0.6]	3.0[2.9;3.2]
High-fat diet (ATX, n=8)	0.027±0.01	20.6±3.9	0.8±0.1	1.6[0.8;3.3]	2.9[2.8;3.0]
High-fat diet+Vit D (AVS, n=14)	0.033±0.02	15.2±9.3	0.4±0.1	0.5[0.3;1.2]	3.2[3.1;3.3]
p	0.03	<0.0001	<0.0001	0.003	0.02

Table XV: Rabbit echocardiographic, histomorphological and biochemistry parameters. **Upper panel:** Echocardiographic and histomorphological aortic valve parameters in rabbits. **Lower panel:** Biochemistry parameters in rabbits. ATX, high-fat diet; AVS, aortic valve stenosis (cholesterol-enriched diet and vitamin D2 treatment); AVA, aortic valve area.

Baseline characteristics	AVA (cm^2)	Peak gradient (mmHg)	Mean gradient (mmHg)	BMI (kg/m^2)	Age (years)	Bicuspid valve (n,%)	LVMMI (g/m^2)	LVEF (n,%)	Significant CAD (n)	Dyslipidemia (n,%)	Smoking (n,%)	Diabetes mellitus (n,%)	Hypertension (n,%)	Statin treatment (n,%)
Aortic valve stenosis (n=64)	0.8±0.2	83.1±20.9	52.2±14.8	31.2±6.3	65.0±10.4	35 (55)	116.1±31.9	60[60;65]	0 (0)	34 (60.7)	11 (19.6)	11 (19.6)	35 (62.5)	34 (53.1)
Aortic valve stenosis Females (n=20)	0.8±0.2	82.3±20.3	54.2±13.0	31.0±7.9	65.3±11.3	11 (55)	100.5±23.6	60[60;65]	0 (0)	8(50)	5(25)	3(19)	8(50)	9(45)
Aortic valve stenosis Males (n=44)	0.8±0.2	83.5±21.5	51.3±15.6	31.3±5.6	64.8±10.0	24 (54.5)	118.3±32.9	60[57;65]	0 (0)	26(65)	6(15)	8(20)	27(68)	25(57)
Healthy controls (n=20, females n=10)	-	-	-	-	43.1±12.8	-	-	-	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
p (male vs female AVS)	NS	NS	NS	NS	NS	NS	0.02	NS	NS	NS	NS	NS	NS	NS

VI: Baseline characteristics of study population. AVA, aortic valve area, BMI, body mass index, LVMMI, left ventricular muscle ma
t ventricular ejection fraction; AVS, aortic valve stenosis. p value given for male vs females.

Biochemistry parameters	Total cholesterol (mmol/l)	HDL cholesterol (mmol/l)	LDL cholesterol (mmol/l)	Triglycerides (mmol/l)	Calcium (mmol/l)	Glucose (mmol/l)	CRP (mmol/l)
Aortic valve stenosis (n=64)	3.6[3.1;4.2]	1.1[0.8;1.3]	2.1[1.8;2.7]	1.4[1.1;1.8]	2.1±0.1	5.6[5.1;6.4]	1.7[0.8;5.5]
Aortic valve stenosis Females (n=20)	3.7[3.0;4.4]	1.1[0.9;1.3]	2.2[1.7;3.0]	1.5[1.1;2.1]	2.1±0.1	5.7[5.1;6.3]	1.6[1.0;5.1]
Aortic valve stenosis Males (n=44)	3.4[3.1;4.0]	1.0[0.8;1.3]	2.0[1.8;2.6]	1.3[1.1;1.7]	2.1±0.1	5.6[5.1;6.4]	1.9[0.8;5.6]
P (males vs females)	NS	NS	NS	NS	NS	NS	NS

Stepwise linear regression model for mean aortic valve gradient (mmHg) in women (n=16)				
Model 1	R^2	Standardized B coefficient	t	p-value
Cathepsin S activity	0.7	0.832	5.62	<0.001
Excluded variables: BMI, age, LDL-cholesterol, diabetes, smoking, hypertension				

Table XVIII: Model found by stepwise selection procedure for mean aortic valve gradient as assessed by echocardiography. Initial set of predictor variables: Cathepsin S activity in plasma, BMI, age, hypertension, diabetes, smoking, LDL cholesterol. SE, standard error; BMI,

Table XVII: Biochemistry parameters of study population. HDL, high-density lipoprotein; LDL, low-density lipoprotein; CRP, C-reactive protein. p value given for male vs females.

3.5 Manuscript #5: Beneficial Effects of High-density Lipoprotein on Acquired von Willebrand Syndrome in Aortic Valve Stenosis

3.5.1 Foreword to Manuscript #5

The preceding investigations provide strong support for the beneficial effect of HDL-based therapies including rHDL and apoA-I in CAD and AVS. They further introduce strategies to render such therapy more efficient. However, little is known about secondary or pleiotropic effects of therapeutic approaches involving HDL or HDL-associated molecules. In fact, HDL is known to exert its vasoprotective actions by mediating antithrombotic and anticoagulant effects. The latter, however, might potentially deteriorate pre-existing haemorrhagic disorders observed in patients with AVS. Indeed, hematologic abnormalities are frequently observed in patients with aortic valve disease and most likely due to a direct effect of shear stress on the von Willebrand factor (vWF) - cleaving protease ADAMTS-13. In addition, given the high peri-operative bleeding risk of these elderly patients, there is a critical need to explore strategies to improve hematologic disorders associated with AVS. Thus, the following study explores the effects of HDL on ADAMTS-13 in a rabbit model of AVS and in plasma collected from patients with severe AVS undergoing surgical valve replacement.

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Current status of study:
project finalized, manuscript is being prepared for submission

Author contributions:
Conceptual design of the study: CG, ER, JCT
Patient recruitment: ABOM, LP
Acquisition and analysis of data: CG, BES, CEG, ABOM, MM, TMA; VL, AEK, YS
Performing experiments: CG, FM, AB, WN
Statistical analysis: CG, MCB
Drafting of the manuscript: CG
Critical review of data integrity: MCB, ER
Review of manuscript for intellectual content: AB, DR, DB, ER, JCT

3.5.2 Abstract

Background: Infusions of apolipoprotein A-I (apoA-I), the major protein component of high-density lipoproteins (HDL), results in aortic valve stenosis (AVS) regression in experimental models. Severe AVS can be complicated by acquired von Willebrand syndrome, a haemorrhagic disorder associated with loss of ultra-large von Willebrand factor (vWF) multimers, the latter being a consequence of increased shear stress and enhanced vWF-cleaving protease (ADAMTS-13) activity. Antithrombotic actions of HDL have been described; however, the effects of HDL on ADAMTS-13 and vWF in AVS are unknown.

Methods and Results: ADAMTS-13 activity was assessed in plasma derived from a rabbit model of AVS (n=29) as well as in plasma collected from sixty-four patients with severe AVS (age 65.0±10.4 years, 44 males) undergoing aortic valve replacement (AVR). In both, human and rabbit AVS plasma, ADAMTS-13 activity was higher than in controls ($p<0.05$). Accordingly, AVS patients had less ultra-large vWF multimers than controls (66.3±27.2 vs 97.2±24.1, $p<0.0001$) and both, ADAMTS-13 activity and ultra-large vWF multimers correlated significantly with aortic transvalvular gradients, thereby showing opposing correlations ($r=0.3$, $p=0.018$ and $r=-0.4$, $p=0.003$, respectively). Administration of an apoA-I mimetic peptide reduced ADAMTS-13 activity in AVS rabbits as compared to placebo treated AVS rabbits (2.0±0.5 RFU/sec vs 3.8±0.4 RFU/sec, $p<0.05$). Similarly, a significant negative correlation was found between ADAMTS-13 activity and HDL cholesterol levels in patients with AVS ($r=-0.3$, $p=0.045$).

Conclusion: HDL are associated with reduced ADAMTS-13 activity and increased vWF multimers. HDL-based therapies may reduce the hematologic abnormalities of the acquired von Willebrand syndrome in AVS.

3.5.3 Introduction

Aortic valve stenosis (AVS) is the most common valvular heart disease in western countries and has reached endemic proportions given the aging of the population.[26] As severe symptomatic AVS usually leads to considerable morbidity and death if left untreated, surgical or percutaneous aortic valve replacement (AVR) remains the primary management.[26] While statins have thus far failed to halt or regress AVS progression, [490, 491] HDL-based therapies, such as reconstituted HDL (rHDL) or apolipoprotein A-I (apoA-I) mimetic peptides resulted in a significant regression of AVS in experimental models, at least partly via improved endothelial function, as well as antioxidant and anti-inflammatory effects of apoA-I.[365-367] HDL may have a significant role in preventing fibrocalcific remodelling of the aortic valve and HDL cholesterol (HDL-C) levels and HDL-bound antioxidant enzymes have a negative predictive value for disease severity in AVS.[44-46]

Von Willebrand factor (vWF) is a blood glycoprotein playing a major role in physiological haemostasis and thrombotic disease.[492] Activity of vWF is largely modulated by the protease ADAMTS-13 (a disintegrin-like and metalloprotease with a thrombospondin type 1 motif, member 13). The latter is assumed to regulate the pro-haemostatic properties of vWF by cleaving ultra-large vWF multimers into smaller, less active particles, thereby reducing its platelet-tethering function.[493] Deficiency of ADAMTS-13 promotes vWF-induced platelet aggregation, which can result in thrombotic complications and formation of early atherosclerosis in experimental models.[98, 99] Consistent with its antithrombotic properties, atheroprotective effects of ADAMTS-13 have been described in cardiovascular disease states and the potential therapeutic benefit of restoration of ADAMTS-13 is currently being discussed.[101, 493] However, enhanced vWF cleavage has been described in the context of AVS where the latter is directly related to the severity of AVS and improved by aortic valve replacement (AVR).[118, 121, 122] This acquired von Willebrand syndrome is found in up to 70% of patients with severe AVS and is characterized by haematological abnormalities and bleeding complications due to loss of ultra-large vWF multimers.[118, 119, 494] Shear stress-dependent cleavage of

ultra-large vWF multimers by ADAMTS-13 has been claimed to be responsible for the latter.[495, 496]

Recently, antithrombotic and fibrinolytic effects of HDL and apoA-I, such as downregulation of tissue factor, selectin, and thrombin generation, have been described, indicating that anti-coagulant mechanisms contribute to the postulated beneficial effects of therapy targeted at HDL.[74, 95] In addition, preliminary data indicate that HDL and apoA-I may interact with ultra-large vWF multimers thereby modifying their adhesive properties *in vitro*.[497, 498] Further, a negative association between ADAMTS-13 and the risk of cardiovascular events seems to be influenced by plasma HDL-C levels.[98] Although no data exist on direct interactions between lipid parameters and ADAMTS-13, these studies suggest that regulation of ADAMTS-13 and vWF may be another mechanism by which HDL exert antithrombotic and vasoprotective effects. However, given the enhanced haemorrhagic risk associated with an acquired von Willebrand syndrome in AVS, a potential inhibitory effect of an AVS-targeted HDL-based therapy on platelet adhesion could hamper beneficial effects of such therapy by increasing bleeding complications. The association between ADAMTS-13 and HDL in AVS has not been studied, yet. Thus, the aim of this study was to characterize the effect of apoA-I treatment on ADAMTS-13 activity in a rabbit model of AVS and to assess the correlation between ADAMTS-13 activity, vWF multimers and HDL-C in patients with severe AVS undergoing surgical AVR.

3.5.4 Methods

Experimental Aortic Valve Stenosis in Rabbits

Normal-diet, untreated rabbits as well as a rabbit model of AVS were used in the present study.[367, 471] Briefly, at 12-13 weeks of age, twenty-nine male New Zealand White rabbits (weight 2.6-3.1 kg) received a cholesterol-enriched diet (0.5% cholesterol, Harlan, Indianapolis, Indiana), plus up to 50000 U/day vitamin D2 (Sigma; Markham, Ontario, Canada) in drinking water. After 12.5±3.4 weeks, significant AVS, defined as a >10% decrease in AVA, was detected by echocardiography. Animals then returned to standard diet and received either saline (n=18) or an apoA-I mimetic peptide (25 mg/kg body weight) for one (n=6)

or two weeks (n=5). The apoA-I mimetic peptide (H-Pro-Val-Leu-Asp-Leu-Phe-Arg-Glu-Leu-Leu-Asn-Glu-Leu-Leu-Glu-Ala-Leu-Lys-Gln-Lys-Leu-Lys-OH, Polypeptide Laboratories, Torrance, CA, USA) was complexed with egg sphingomyelin and 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (both from Avanti Polar Lipids, Alabaster, AL, USA) in a 1:1:1 mass ratio, as previously described.[367] Either saline (control group) or apoA-I mimetic peptide were given intravenously through the marginal ear vein three times per week. Echocardiograms were performed every 3–4 days. Two days following the last infusion, animals underwent a final echocardiogram and were euthanized under anaesthesia by cardiac puncture. The aortic valves were excised for histological analyses. Blood samples were obtained through the marginal ear vein at baseline, prior to treatment and before euthanasia. Total cholesterol, HDL cholesterol, triglycerides and calcium levels were measured with an automated filter photometer system (Dimension RxL Max; Dade Behring, Deerfield, IL, USA). Animal care and procedures complied with the Canadian Council on Animal Care guidelines and were approved by the institutional ethics committee for animal research.

Echocardiography in rabbits

For echocardiographic assessment, animals were sedated with intramuscular injections of ketamine (45 mg/kg) and midazolam (0.75 mg/kg). Ultrasound images were obtained with a 10S probe connected to a Vivid 7 imaging system (GE Healthcare Ultrasound, Horten, Norway). The aortic valve area (AVA) was measured by the standard continuity equation. Left ventricular outflow tract velocity (VLVOT) was obtained with pulsed-wave Doppler sampled proximally to the aortic valve in the apical 5-chamber view. Continuous wave (CW) Doppler interrogation across the aortic valve was used to obtain transvalvular maximal velocity (VAV) in the same view. VLVOT/VAV ratio was calculated to determine AVS development. The average of three consecutive cardiac cycles was used for each measurement. Animals were weighted at every echocardiogram.

Histology and morphometry of rabbit aortic valve tissue

Histomorphological assessment of aortic valve tissue was performed as previously described.[367] Briefly, after sacrifice, the ascending aortic section containing the aortic valve was resected, opened longitudinally and the three valvular cusps were separated. Two cusps were frozen in an embedding medium and stored at -80°C, one cusp was immersion-fixed in 10% buffered formalin and embedded in paraffin. Stained or immunohistochemically labelled tissue sections obtained from the central third of each cusp were analysed with a computer-based digitizing image system (Image Pro Plus, version 5.1). The region of analysis (ROA) was composed of 1000 mm of the Valsalva sinus from the leaflet base and 500 mm of the leaflet from the leaflet base. Lesion area and leaflet lesion length were also measured. For lipid infiltration analysis, tissue sections were stained with Oil Red O. Haematoxylin-phloxin-safran, von Kossa and Sirius red stained sections were prepared for general morphology, tissue calcification and collagen studies, respectively. For immunohistochemistry evaluation, all sections were preincubated with either mouse immunoglobulin G2a monoclonal antibody against rabbit macrophage (RAM11) or rabbit smooth muscle cell α-actin (Dako, Mississauga, Ontario, Canada). Smooth muscle cells, macrophages and calcification areas were quantified in the ROA on digital images acquired at x40 magnification. Images from each section were digitally captured with the same illumination settings, and automatic computer-based analysis was performed with the same colour threshold for all specimens. Data are expressed as % labelled area in the ROA.

Patients

For the present study, sixty-four consecutive patients (20 females) with isolated AVS scheduled for AVR replacement and 35 patients (4 females) with severe coronary artery disease (CAD) undergoing coronary artery bypass graft surgery (CABG) were recruited at the Montreal Heart Institute between October 2008 and May 2013. Participating patients needed to be at least 18 years of age, and had indications for non-emergency CABG or AVR surgery with an aortic valve area smaller than 1 cm^2 and an aortic transvalvular mean gradient higher than 35 mmHg. Patients with significant systemic illness (e.g. inflammatory bowel disease, cancer), severe renal disease (creatinine > 120 μmol/L), previous valve

replacement or repair, history of complicated diabetes mellitus, atrial fibrillation/flutter or any irregular heart rhythm at the time of investigation, history of clinical instability (including cardiogenic shock, congestive heart failure, suspected acute myocarditis, endocarditis, cardiac tamponade, hemodynamically significant hypertrophic cardiomyopathy, restrictive cardiomyopathy or congenital heart disease, suspected dissecting aortic aneurysm), known previous medical condition yielding expected survival less than 18 months, and pregnancy were excluded. Plasma samples were obtained from each subject before surgery. Glucose levels, calcium-concentration, LDL-cholesterol, HDL-cholesterol and triglycerides were determined by spectrophotometric methods using a multianalyzer Dimension RxL Max (Dade Behring Diagnostics, Marburg, Germany), C-reactive protein (CRP) was quantified using the Dade Behring N High Sensitivity assay (Dade Behring Diagnostics, Marburg, Germany) on the BN ProSpec Nephelometer. The study has been approved by the Montréal Heart Institute Ethics Committee and every patient gave written informed consent. Each of the sixty-four patients with AVS had undergone an M-mode, 2D, and Doppler echocardiographic study using commercially available equipment (GE Vivid 9, GE Healthcare, Wauwatosa, WI, USA). Image acquisition was obtained in accordance with the recommendations of the American Society of Echocardiography (ASE) and the European Association of Echocardiography (EAE).[472] Severity of AVS was defined based on peak jet velocity, mean transvalvular gradient, aortic valve area (AVA) calculated by the continuity equation, and indexed AVA. Relative wall thickness (RWT) was calculated by dividing two times the left ventricular posterior wall thickness by the left ventricular internal dimensions, and indexed left ventricular muscle mass (LVMMI) was assessed by the cube formula.[473] Left ventricular concentric remodelling was defined as a RWT >0.42 mm with a normal LVMMI (≤95 g/m^2 for women, ≤115 g/m^2 for men). Left ventricular ejection fraction (LVEF) was measured by the biplane Simpson's method,[474] and left ventricular diastolic function was assessed according to current guidelines.[475]

Perioperative period

Anticoagulation during cardiopulmonary bypass was performed by heparin to maintain activated clotting time of 400 s. Antifibrinolytic therapy with a 10 mg/kg

body weight bolus of tranexamic acid was administered to every subject at the beginning of surgery. Erythrocyte concentrates and haemostatic products including fresh-frozen plasma (FFP), platelet concentrates, and fibrinogen concentrate were administered at the discretion of direct care providers. During the immediate postoperative course, blood loss from mediastinal drainage after arrival on the ICU, 12h after surgery, was assessed and prospectively recorded by the caregivers on the ICU. Transfusions of erythrocyte concentrates and haemostatic products during surgery and hospital stay were assessed.

Haematological assays

Haematological data were analysed blinded to patient information. Citrated plasma was obtained by centrifugation of the blood at 1600 g for 20 min, and plasma was then centrifuged for 5 min at 1300 g and stored at -80 °C. Haemoglobin level, leukocyte count, platelet count, prothrombin time (PT), activated partial thromboplastin time (aPTT), and fibrinogen were assayed by routine laboratory techniques. The proteolytic activity of ADAMTS13 was measured using a fluorogenic synthetic VWF peptide (FRETS-VWF73; Peptides Institute, Osaka, Japan), according to the manufacturer's instructions. Briefly, rabbit or human plasma was incubated with the FRETS-VWF73 peptide and the increase in fluorescence signal following exposure to plasma was measured during 2 h using a Synergy 2 Biotek Multi-Mode microplate reader (Ex/Em 340/450 nm) and analysed with Gen-5 Biotek software. The fluorescence signal was reported as reaction velocity in RFU (relative fluorescence units) versus time for each sample. Reaction velocity was obtained by determining the slope of a line fit to the initial linear portion of the data plot. Plasma ADAMTS-13 protein mass was measured by enzyme-linked immunosorbent assay (Technozym ADAMTS-13 Antigen ELISA, Technoclone GmbH, Vienna, Austria). To measure plasmin activity, citrated plasma was incubated with a fluorogenic peptide substrate (Suc-Ala-Phe-Lys-AMC, Bachem Americas Inc., Torrance, CA). Fluorescence readings were performed for 2 hours (Interval 60 sec, Ex/Em 380/460 nm, Synergy H1 Hybrid Multi-Mode microplate reader, Biotek, Winooski, VT). Data were analysed with Gen-5 Biotek software. For multimer analysis, vWF multimers were separated on a 1.4% agarose gels, transferred onto nitrocellulose membranes using a semi-dry

high-speed transfer apparatus (Trans-Blot® Turbo™ Bio-Rad), and detected using a horseradish peroxidase-conjugated rabbit anti-human vWF polyclonal antibody (Dako, Glostrup, Denmark) and 4-chloro-1-naphthol as substrate (Sigma-Aldrich). The amount of ultra-large vWF multimers (more than 14-mers) was determined after densitometric scanning of the membranes and the ratio of ultra-large vWF multimers to total vWF multimers was calculated. This ratio was then normalized to a reference sample obtained from a pool of normal plasmas. Plasma VWF antigen levels (vWF:Ag) were measured with an immune-turbidimetric assay (STA-LIATEST #00518, STAGO, Asnière, France). vWF activity (vWF:Ac) was determined using an ELISA assay (abp vWF activity test kit, Corgenix, Broomfield, CO) Collagen-binding activity of vWF (vWF:CB) was measured using the REAADS® CBA ELISA kit (Corgenix)

Statistical analysis

Data are presented as mean ± standard deviation (SD) or median (Q1; Q3) for continuous variables and frequency and percentage for categorical variables. Prior to analyses, basic assumptions were checked and triglycerides and hs-CRP were log transformed. Student's t-test, Mann-Whitney test, analysis of variance (ANOVA) or Kruskal-Wallis test was used for group comparisons of continuous variables. For categorical variables, chi-square tests were used. To test the change over time in AVA, paired t-tests were used. Relationships between haematological parameters and histomorphometrical or echocardiographic variables were evaluated using Pearson or Spearman's correlation coefficient, as appropriate. Using multivariate models, the predictive value of HDL on coagulation parameters as well as the predictive value of ADAMTS-13 on severity of AVS was tested after forward selection (criterion: probability-of-F-to-enter <=0.05; Probability-of-F-to-remove>=0.1) by adding known risk predictors. Statistical tests were two-sided and significance was set at $p<0.05$. Statistical analyses were performed with IBM SPSS statistics v23.0, GraphPad Prism (v4.0, GraphPad Software, San Diego, CA), and SAS version 9.4 (SAS Institute Inc., Cary, NC, USA).

3.5.5 Results

Baseline characteristics and echocardiography data in patients

Baseline characteristics of the study population are summarized in Table XIX (page 162). Out of 64 patients with severe AVS (mean AVA 0.8±0.2 cm^2), 31.2% were women, mean age was 65.0±10.4 years, mean body mass index (BMI) was 31.2±6.3 kg/m^2. Mean age in the CABG group was 62.6±8.9 years with 11.4% being females. Prevalence of cardiovascular risk factors was high in both groups (Table XIX, page 162). Except for dyslipidaemia, which was more often detected in the CABG group (p=0.03 vs AVR), no differences in cardiovascular risk factors were found between both populations. Within the AVR group, 55% of patients had bicuspid aortic valve stenosis. Mean and peak aortic valve gradients were 52.2±14.8 mmHg and 83.1±20.9 mmHg, respectively. Left ventricular mass index was 116±32 g/m^2 in the AVR group and 117±31 g/m^2 in the CABG group (p=NS), while left ventricular ejection fraction (LVEF) was slightly higher in the AVR group (60 [60;65] vs 60 [54;60], p=0.007). Severe aortic regurgitation was not observed. As expected, patients in the CABG group received more often treatment with P_2Y_{12} inhibitors (11.4% vs 1.8%, p=0.049), aspirin (85.7% vs 59.6%, p=0.01 vs AVR), or beta-blockers (54.3% vs 19.3%, p=0.0006) as compared to AVR patients (Table XX, page 162). While no differences between groups were observed for triglycerides, patients undergoing AVR had higher levels of total cholesterol (3.8±1.0versus 3.0±0.6 mmol/L, p<0.0001) and LDL cholesterol (2.3±0.8 versus 1.8±0.5 mmol/L, p<0.0001) than patients undergoing CABG (Table XXI, page 163). Similarly, HDL cholesterol was found to be higher in the AVR group as compared to the CABG group (1.1±0.3 versus 1.0±0.2 mmol/L, p=0.022, Table 3). Serum calcium, hs-CRP and glucose levels did not differ significantly between groups (p=NS, Table XXI, page 163).

Echocardiographic, aortic valve histomorphological and biochemistry parameters in AVS rabbits

All animals developed aortic valve lesions and more than 90% of animals showed significant AVS (AVA=19.2±3.1 mm^2 versus 23.8±4.0 mm^2 at baseline, p<0.0001, n=29) following 12.5±3.4 weeks of treatment with a cholesterol-enriched diet

containing vitamin D2. Amongst those, 55.2% of animals (n=16) developed an AVA of ≤19 mm^2. ApoA-I mimetic treatment for 14 days resulted in a significant improvement of AVA (23.6±2.9 mm^2 vs 18.6±2.1 mm^2 before treatment, p=0.0005, n=5), while a one-week apoA-I treatment did not significantly change AVA (18.5±3.4 mm^2 after treatment vs 18.5±3.0 mm^2 before treatment, p=NS, n=6). AVS rabbits developed significant aortic valve lesions located at aortic valve leaflets and sinus of Valsalva (62.0±12.7% total lesion area/total leaflet and sinus area). Similarly, histological analysis revealed significant sinus of Valsalva calcification as assessed by von Kossa staining (5.7±3.7% sinus calcification/total sinus area) and lipid infiltration (70.3%±4.5 Oil Red O positive surface/total sinus area, n=6), on aortic valve leaflets. Treatment with apoA-I mimetic peptide for two weeks led to a significant reduction of total lesion area (40.8±10.2%, p=0.01 vs placebo), sinus of Valsalva's calcification (1.5±2.2%, p=0.01 vs placebo), and lipid infiltration (50.8%±14.4, p=0.01 vs placebo). No significant changes in total aortic valve lesion area and sinus of Valsalva's calcification were observed following apoA-I mimetic treatment for one week (p=NS). No significant differences existed between the three AVS groups with regard to lipid parameters and serum calcium concentration at the end of the treatment (p=NS). As compared to normal control animals (n=11), AVS-Placebo rabbits (n=18) had significantly higher levels of total cholesterol (14.8±8.6 mmol/L vs 0.5±0.2 mmol/L, p<0.05 for AVS-Placebo vs normal controls), while no significant differences between AVS rabbits (n=18) and normal controls (n=11) were found with regard to serum calcium and triglyceride levels (p=NS for AVS-Placebo vs normal controls). Echocardiographic and biochemistry parameters in rabbits are presented in Table XXII (page 164).

Normalization of increased ADAMTS-13 activity in AVS rabbits treated with apoA-I mimetic peptide

The highest ADAMTS-13 activity was measured in AVS-placebo rabbits with a moderate AVS, defined as an AVA≤19 mm^2 (3.8±0.4 RFU/sec), while normal control rabbits and AVS rabbits treated with apoA-I mimetic peptide for one or two weeks had significantly lower ADAMTS-13 activity (2.3±0.7 RFU/sec, 2.1±0.7 RFU/sec, and 2.0±0.5 RFU/sec, respectively, p<0.05 vs moderate AVS, Figure 29A, page 158). On the other hand, plasma plasmin activity was significantly

increased in AVS-apoA-I rabbits as compared to AVS-Placebo rabbits (0.047±0.005 RFU/sec for two weeks apoA-I mimetic treatment, 0.054±0.021 RFU/sec for one week apoA-I mimetic treatment, and 0.020±0.003 RFU/sec for AVS-Placebo rabbits with moderate AVS, $p<0.05$ vs placebo treated animals with moderate AVS, Figure 29B, page 158). Accordingly, in a pooled analysis of all AVS rabbits (n=27), a significant negative correlation was found between plasma plasmin activity and plasma ADAMTS-13 activity (r=-0.46; Pearson p=0.02).

Enhanced ADAMTS-13 activity and reduced ultra-large vWF multimers in patients with severe AVS

ADAMTS-13 activity was assessed in plasma collected from 64 patients with severe AVS undergoing surgical AVR as well as from 35 patients with CAD, free of structural heart disease, undergoing CABG. ADAMTS-13 activity was found to be significantly higher in patients undergoing AVR as compared to patients undergoing CABG (3.7±1.2 RFU/s versus 2.9±0.7 RFU/s, p=0.0003, Figure 2), while no significant differences were observed in ADAMTS-13 protein mass between both groups (1.1 [0.9;1.3] µg/mL in AVR versus 1.1 [0.9;1.3] µg/mL in CABG, p=NS, Table XXI, page 163). Of the 99 subjects included in the study, 74.7% showed altered vWF multimer structure evident by the loss of the largest vWF multimers. The proportion of subjects with altered vWF structure was significantly higher in the AVR group than in the CABG group (57 [89.1 %] vs 17 [48.6%], $p<0.001$). Patients with altered vWF multimer structure also had lower values for vWF:CB (0.9±0.4 U/mL vs 1.1±0.4 U/mL, p=0.036), for vWF:Ag (1.3±0.4 U/mL vs 1.7±0.6 U/mL, p=0.001), and for vWF:Ac (0.9±0.4 U/mL vs 1.2±0.4 U/mL, p=0.01) than patients with normal vWF multimers structure. In the entire cohort, vWF multimer structure showed a strong and negative correlation with ADAMTS-13 activity (r=-0.4, $p<0.001$, data not shown). Accordingly, a significant correlation was seen between ADAMTS-13 antigen and ADAMTS-13 activity (r=0.31, p=0.002, data not shown).

Hematologic parameters and postoperative bleeding

Median pre-operative INR values were 1.0 [1.0;1.1] in the AVR group and 1.0 [1.0;1.1] in the CABG group (p=NS, Table XXI, page 163). Pre-operative platelet

count, haemoglobin, and PT values did not differ between AVR and CABG groups (p=NS, Table XXI, page 163) and were within the normal range in all subjects with the altered vWF multimer structure. When all patients were stratified according to ADAMTS-13 activity quartiles, no significant differences were found for platelet count, haemoglobin, INR, and PT between quartiles (p=NS). 12 h following surgery, cumulative loss from mediastinal drainage was 568±313 mL (range 150–1600 mL) and 416±166 mL (range 90–710 mL) in the group with reduced and normal multimers, respectively (p=0.03). Major bleeding, defined as >1000 mL blood loss within 12 h following surgery, was observed in 9.6% of patients with reduced ultra-large vWF multimers, while no major bleeding complication occurred in patients with normal vWF multimers (p=NS). A significant positive association was found between ADAMTS-13 activity and cumulative mediastinal blood loss 12 h following surgery (r=0.24, p=0.02, data not shown). No correlation was detected between ADAMTS-13 protein mass and postoperative mediastinal blood loss (p=NS).

Negative association between plasma HDL-cholesterol level and ADAMTS-13 activity in AVS

A significant and negative correlation was found between plasma ADAMTS-13 activity and HDL-cholesterol in patients undergoing AVR (r=-0.3, p=0.045, Figure 31A, page 159), while no such correlation was seen for LDL-cholesterol, total cholesterol and triglycerides (p=NS, data not shown). The negative correlation between HDL-cholesterol and ADAMTS-13 in the AVR group was even more pronounced in patients treated with statins (r=-0.5, p=0.004 for ADAMTS-13 activity, Figure 31B, page 159, and r=-0.34, p=0.047 for ADAMTS-13 protein mass, data not shown). Accordingly, HDL-cholesterol level was found to be a significant negative predictor of ADAMTS-13 activity when tested in a multivariate regression analysis against hs-CRP, LDL-cholesterol, platelet count, and vWF multimer structure as predictor variables (B-coefficient -1.1, 95% CI (-1.9; -0.3), p=0.008). No correlation between HDL-cholesterol and ADAMTS-13 activity was seen in patients with CAD (p=NS).

Association of ADAMTS-13 and ultra-large vWF multimers with disease severity of AVS

In humans, a significant and positive correlation was found between plasma ADAMTS-13 activity and aortic transvalvular pressure gradients (r=0.3, p=0.018 for peak aortic valve gradient, Figure 32A, page 160 and r=0.4, p=0.004 for mean aortic valve gradient, data not shown) in AVS patients. Similarly, ADAMTS-13 protein mass correlated significantly with peak aortic valve gradients in AVS patients (r=0.4, p=0.008, Figure 32B, page 160). Accordingly, ADAMTS-13 activity was found to be a significant positive predictor of mean aortic valve gradient (B coefficient 3.6, 95% CI (0.2; 7.1), p=0.04), when tested in a multivariate model against known risk factors for AVS including hypertension, dyslipidaemia, diabetes, male gender, smoking, and age as predictor variables. In addition, a significant and negative correlation was found between ultra-large vWF multimers and aortic transvalvular pressure gradients (r=-0.4, p=0.003 for aortic valve mean gradient, Figure 32C, page 160) as well as between vWF:CB and aortic valve peak acceleration time in AVS patients (r=-0.3, p=0.04, data not shown).

ADAMTS-13 activity is associated with aortic valve lesion area and plasma markers of inflammation in AVS

A significant correlation was detected between ADAMTS-13 activity and total lesion area on diseased aortic valve leaflets and sinus of Valsalva (r=0.66, p<0.001, Figure 33A, page 161) in all three groups of AVS rabbits, whereas no significant correlation was found between ADAMTS-13 activity and valve calcification, lipid infiltration, leaflet thickness, and lipid content of the aortic arch and descending aorta (p=NS). In patients with AVS, a significant positive association was observed between plasma ADAMTS-13 activity and plasma hs-CRP levels (r=0.3, p=0.03, Figure 33B, page 161) while plasma hs-CRP levels were not associated with vWF multimer structure, vWF:Ac , vWF:CB or echocardiographic parameters of AVS severity (p=NS).

3.5.6 Discussion

The present study demonstrates that ADAMTS-13 activity is markedly increased in plasma from patients with severe AVS as well as in a rabbit model of AVS. Treatment with an apoA-I mimetic peptide resulted in reduction of enhanced ADAMTS-13 activity in AVS rabbits. Accordingly, a significant and negative association was observed between HDL cholesterol and ADAMTS-13 activity in patients with AVS.

ADAMTS-13 represents an important link between intra-vascular shear stress and the ability of vWF to bind platelets. The latter is compromised in up to 74% of patients with severe AVS leading to an increased risk for cutaneous or mucosal bleeding.[118] AVS is estimated to be prevalent in up to 7% of the population over the age of 65. These elderly patients are known for an increased bleeding-risk due to comorbidities, decreased clotting ability, and lower clearance kinetics of anticoagulant drugs.[499, 500] Therefore, factors influencing ADAMTS-13 activity might have the potential to trigger such haemorrhagic tendency in this fragile population. Given evidence for an inhibiting effect of HDL particles on vWF induced platelet aggregation,[501, 502] we hypothesized that high-doses of HDL-based therapies, as they have been used to induce AVS regression in experimental models, could be potentially disadvantageous by enhancing haemorrhagic risk in this disease. To test our hypothesis, we used a rabbit model of AVS. This experimental model was chosen because apoA-I mimetic treatment had resulted in attenuation of valvular calcification and regression of AVS in these animals.[367] In addition, in contrast to other species, rabbit plasma has recently been shown to cleave human recombinant vWF as efficient as human plasma, and ADAMTS-13 activity levels in rabbits are similar to those in humans.[503]

Intriguingly, contrary to our initial hypothesis, in our study, we observed a significant reduction of ADAMTS-13 activity in AVS rabbits treated with apoA-I mimetic peptide as compared to placebo controls. Accordingly, in AVS patients, a significant and negative correlation was seen between HDL-cholesterol and ADAMTS-13 activity which confirms one previous report stating a similar correlation between ADAMTS-13 and HDL-cholesterol levels in plasma of patients with myocardial infarction.[98] Our results were unexpected given the known

antithrombotic effects of apoA-I and ADAMTS-13. Indeed, ADAMTS-13 has been shown to reduce the adhesiveness of hyperactive ultra-large vWF multimers and to limit thrombus growth at the site of an ongoing thrombus generation process,[100, 504] while deficiency of ADAMTS-13 can lead to severe thrombotic microangiopathy.[101] A decreased ADAMTS-13 activity has been observed in patients after AVR indicating that interventions aimed at eliminating valvular obstruction exert an inhibiting effect on ADAMTS-13.[123] In line with these observations, treatment with apoA-I for 14 days resulted in both, AVS regression and reduction of ADAMTS-13 activity in our experimental AVS model. However, shorter treatment duration of 7 days had no effect on aortic valvular obstruction but did result in a significant inhibition of ADAMTS-13 activity, indicating that a portion of the hematologic benefit of apoA-I may be due to a direct effect of apoA-I on ADAMTS-13 rather than solely due to regression of AVS.

Interestingly, inactivation of ADAMTS-13 by serine proteases such as thrombin and plasmin has recently been described, and an association of enhanced thrombin formation with deficiency in vWF multimers was found in patients with AVS.[505, 506] In our study, plasmin activity was markedly increased in apoA-I-treated rabbits, which confirms previous studies reporting a plasmin promoting effect of HDL in experimental models and humans.[116, 507, 508] Accordingly, a significant negative correlation was found between plasma plasmin activity and plasma ADAMTS-13 activity in all AVS rabbits. Thus, an increase in plasmin activity might have accounted, at least in part, for the observed reduction in ADAMTS-13 activity in apoA-I mimetic-treated animals. In line with a previous observation, we observed a positive association between systemic inflammation and ADAMTS-13 activity in patients with AVS,[509] indicating that apoA-I might downregulate ADAMTS-13 activity by provoking an anti-inflammatory response. This hypothesis is further supported by the fact that a significant and positive correlation was found between aortic valve lesion area and ADAMTS-13 activity in AVS rabbits, while no association was detected between ADAMTS-13 activity and valve calcification, valvular lipid infiltration or fibrosis. Indeed, inflammatory cells are the predominant cell type in early aortic valve lesions and the aortic valve area occupied by inflammatory cells is about 35% in our experimental model.[367] Finally, given the protective effect that apoA-I exerts on the vascular endothelium by

stimulating endothelial nitric oxide (NO) production and prostacyclin synthesis, [510-512] it is tempting to hypothesize that apoA-I may act indirectly on ADAMTS-13 activation via effects on endothelial cells leading to reduction of shear stress and inhibition of platelet activation.

While numerous studies describe vWF regulation in AVS, data on ADAMTS-13 activity in patients with AVS are sparse and only one study has reported a postoperative decline in ADAMTS-13 activity following AVR.[123] The augmented ADAMTS-13 activity in AVS along with the positive association between ADAMTS-13 activity and the magnitude of valve gradient observed in the present study supports the view that abnormally high shear stress created by AVS might promote increased ADAMTS-13 activity in these patients. However, as yet, there is no direct proof that excessive shear stress is the only cause of increased ADAMTS-13 activity in AVS. Indeed, we observed a positive and significant correlation between hs-CRP levels and ADAMTS-13 activity in patients with AVS suggesting that the increase in ADAMTS-13 activity could partly be caused by an inflammatory process. In addition, ADAMTS-13 activity in rabbits correlated significantly with the extent of aortic valve lesions on diseased aortic valve leaflets and Valsalva sinus in our study, thereby further supporting this hypothesis. As previously discussed, systemic inflammation is present in patients with AVS, and higher CRP levels have been shown to be associated with faster progression of this disease.[37, 513]

Along this line, the systemic inflammatory status in AVS may also account for the fact that the negative correlation between HDL and ADAMTS-13 was most pronounced in patients treated with statins. Indeed, the clinical benefits of these drugs are not only related to their cholesterol-lowering effects but also to an improvement of endothelial dysfunction, and anti-inflammatory and immunomodulatory actions.[514] Given that both, hs-CRP levels and HDL, were associated with ADAMTS-13 activity in our study thereby showing opposing trends, it is reasonable to assume that the anti-inflammatory effects of statin treatment may have unmasked a true association between ADAMTS-13 and HDL. In support of this hypothesis, a recent study demonstrated that Simvastatin® can reverse the effect of inflammatory cytokines on ADAMTS-13 mRNA expression *in vitro*,[515] indicating that a linkage exists between inflammation and haemostasis.

Future studies of ADAMTS-13 may therefore need to take into account inflammatory markers when assessing its association with other variables. In addition, confounding effects of higher LDL-levels in non-statin users on ADAMTS-13 activity cannot be excluded. However, since LDL-cholesterol was not associated with ADAMTS-13 activity in our study, this explanation seems less likely. Finally, numerous pleiotropic properties of statins have been described in recent years, thus, direct effects of statins might as well account for our observations. Indeed, a significant upregulation of ADAMTS-13 expression in visceral epithelial cells treated with Simvastatin® has been reported,[515] even though the mechanism by which Simvastatin® regulates ADAMTS-13 in these cells remains to be studied.

Our study has several limitations. First, we did not collect data on diet and eating habits in our patient cohort. Since diets high in monounsaturated fat have been shown to result in a decrease of plasma vWF, this could have influenced our data.[516] Second, our study has the inherent limitations of any small series. Third, all variables were analysed at a single time point before the corrective surgery and haematological assays were not performed serially following the valvular intervention, thus, we cannot draw conclusions about the middle- and long-term effects of AVR on ADAMTS-13 and its association with lipid parameters. Forth, only patients with severe AVS were included in our study, thus, our findings might not be extrapolated to subjects with a lower mean transvalvular gradient. Finally, patients with bicuspid aortic valve disease were not analysed separately in our study due to the low number of patients included. Since this population is somewhat different than the older population with tricuspid AVS, further studies are required to clarify the link between lipid parameters and coagulation in these patients.

In summary, this study adds to a growing literature identifying a role of HDL in haemostasis and supports the view that an increase in ADAMTS-13 activity contributes to the hematologic abnormalities seen in AVS. Our results indicate that HDL-based therapies may counterbalance haemorrhagic tendencies in AVS. Additional studies are needed to clarify the mechanisms responsible for the beneficial effect of HDL on haemostasis in AVS and to explore the impact of lipid modifying drugs on the acquired von Willebrand syndrome of AVS.

3.5.7 Figures and Tables

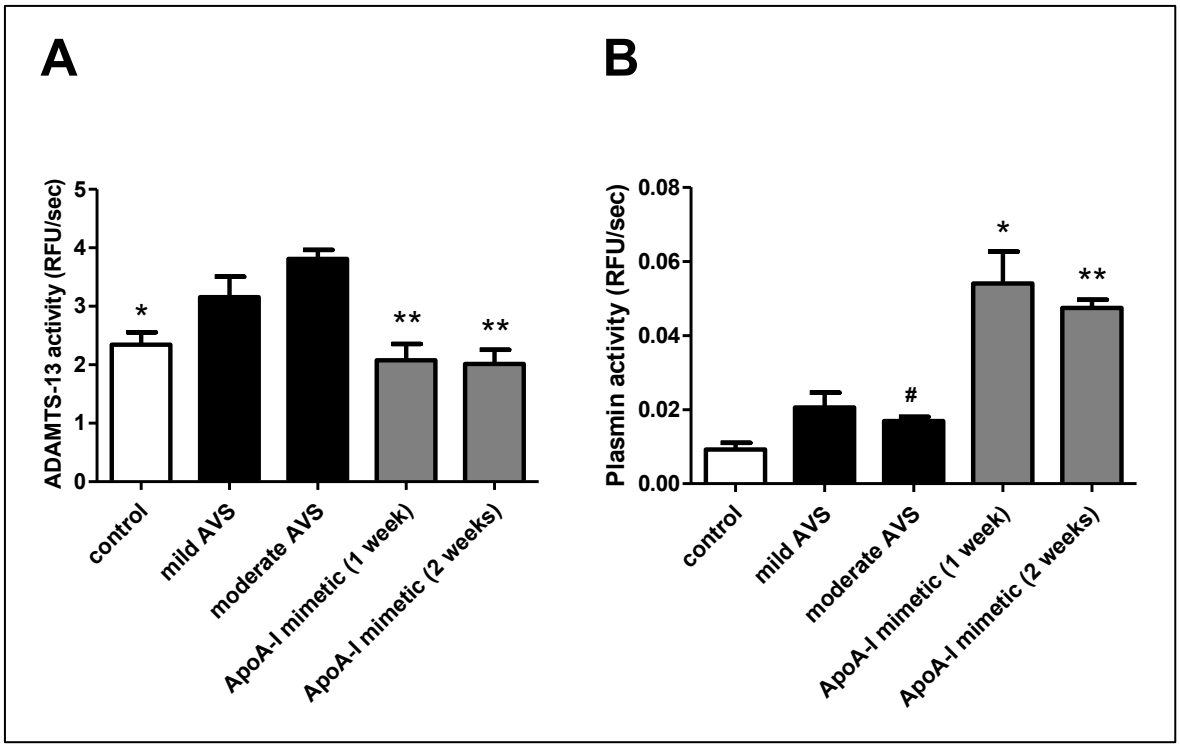

Figure 29: ADAMTS-13 and plasmin activity in rabbit plasma. A. ADAMTS-13 activity in rabbit plasma. ADAMTS-13 activity is indicated as increase in relative fluorescence units (RFU)/sec. * adjusted (Tukey-Kramer) $p<0.05$ vs moderate AVS. **B.** Plasmin activity in rabbit plasma. Plasmin activity is indicated as increase in relative fluorescence units (RFU)/sec. #adjusted (Tukey-Kramer) $p<0.05$ vs normal controls; *adjusted (Tukey-Kramer) $p<0.05$ vs placebo treated moderate AVS and normal controls; **adjusted (Tukey-Kramer) $p<0.05$ vs placebo treated (mild and moderate) AVS and normal controls. AVS, aortic valve stenosis; ApoA-I, Apolipoprotein A-I; ADAMTS-13, a disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13.

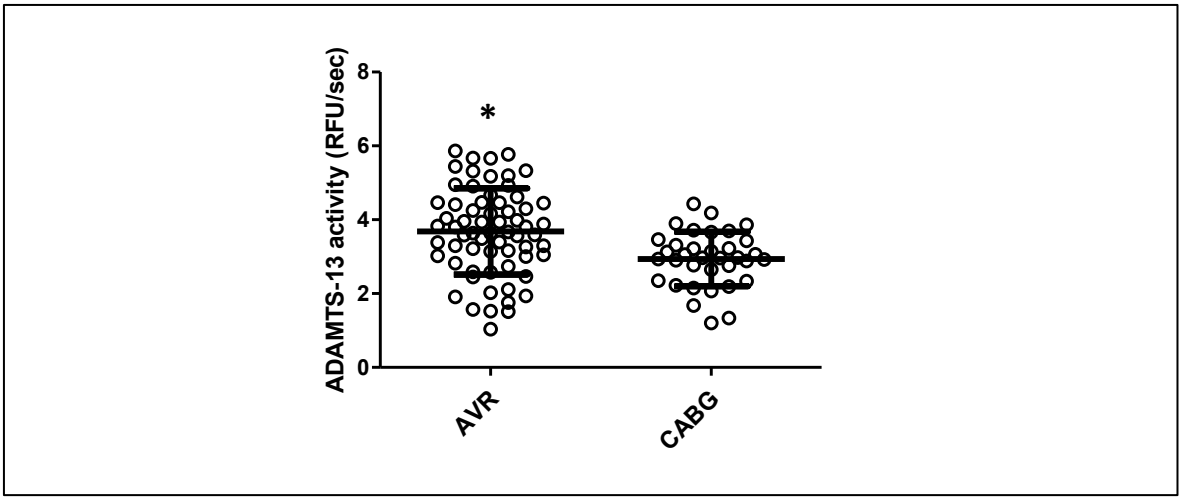

Figure 30: ADAMTS-13 activity in human plasma. ADAMTS-13 activity is indicated as increase in relative fluorescence units (RFU)/sec. AVR, aortic valve replacement; CABG, coronary artery bypass grafting. *p<0.05 vs CABG

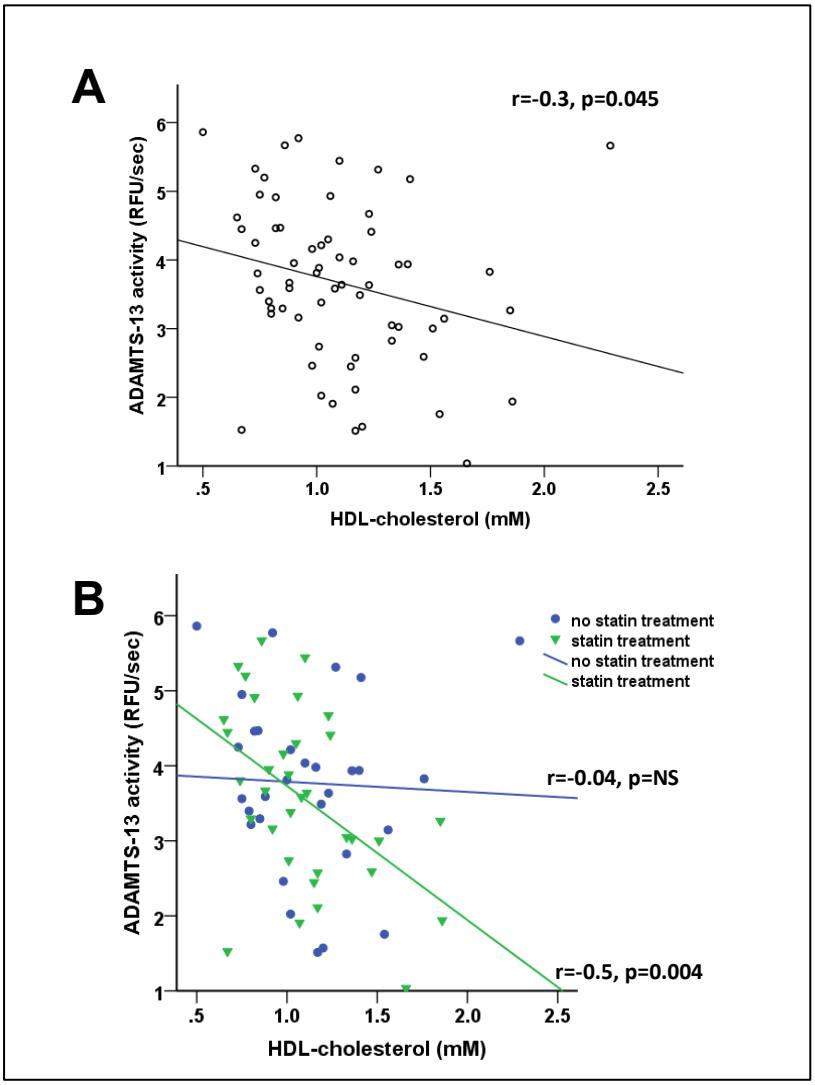

Figure 31: Correlation between ADAMTS-13 activity and plasma HDL-cholesterol in patients with aortic valve stenosis. A. Correlation between ADAMTS-13 activity and plasma HDL-cholesterol in patients with aortic valve stenosis, entire study population. **B.** Correlation between ADAMTS-13 activity and plasma HDL-cholesterol according to statin treatment. ADAMTS-13 activity is indicated as increase in relative fluorescence units (RFU)/sec. HDL, high-density lipoprotein; ADAMTS-13, a disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13.

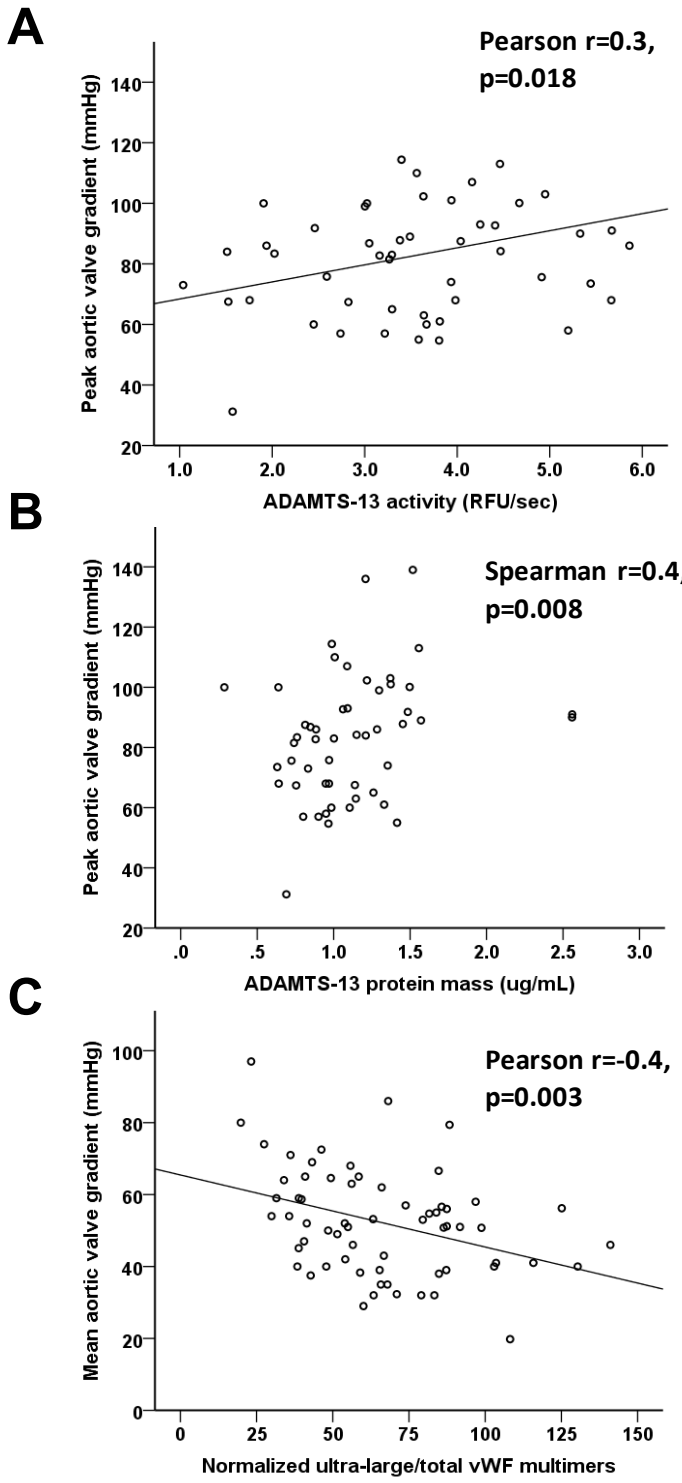

Figure 32: Association between ADAMTS-13 or vWF multimer size and severity of aortic valve stenosis in patients. **A**. Correlation of peak aortic valve gradient and ADAMTS-13 activity. **B**. Correlation of peak aortic valve gradient and ADAMTS-13 protein mass. **C.** Correlation of mean aortic valve gradient and normalized ratio of ultra-large vWF multimers to total vWF multimers. ADAMTS-13 activity is indicated as increase in relative fluorescence units (RFU)/sec. ADAMTS-13, a disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13; vWF, von Willebrand factor.

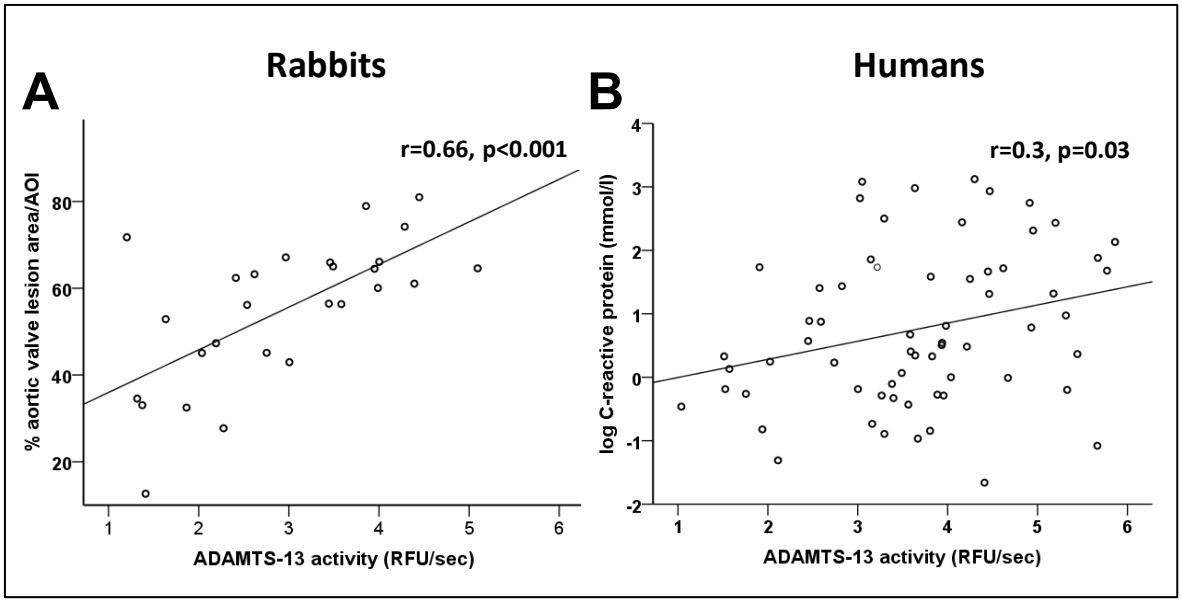

Figure 33: Association between ADAMTS-13 and markers of inflammation in rabbits and humans. A. Rabbits: Association between ADAMTS-13 and total lesion area on aortic valve leaflets and sinus Valsalva in AVS rabbits fed a cholesterol-enriched diet and vitamin D. **B.** Correlation between plasma CRP levels and ADAMTS-13 activity in patients with aortic valve stenosis. ADAMTS-13 activity is indicated as increase in relative fluorescence units (RFU)/sec. ADAMTS-13, a disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13.

Baseline characteristics	AVR (n=64)	CABG (n=35)	P-value
Males (n,%)	44 (68.8)	31 (88.6)	0.03
Age (years, mean±SD)	65.0±10.4	62.6±8.9	NS
BMI (kg/m^2, mean±SD)	31.2±6.3	28.6±3.7	0.01
AVA (cm^2, mean±SD)	0.8±0.2	2.7±1.1*	0.02
Peak gradient (mmHg, mean±SD)	83.1±20.9	5.1±1.5**	<0.001
Mean gradient (mmHg, mean±SD)	52.2±14.8	3.7±1.3$^{\#}$	<0.001
LVMMI (g/m^2, mean±SD)	116±32	117±31$^{\#\#}$	NS
LVEF (%, median[Q1;Q3])	60[60;65]	60[54;60]	0.007
Bicuspid valve (n,%)	35 (54.7)	-	-
Significant (>20% luminal narrowing) CAD (n,%)	0 (0)	35 (100)	<0.001
Dyslipidemia (n,%)	34 (60.7)	29 (82.9)	0.03
Smoking (n,%)	11 (19.6)	5 (14.3)	NS
Diabetes mellitus (n,%)	11 (19.6)	12 (34.3)	NS
Hypertension (n,%)	35 (62.5)	27 (77.1)	NS

Table XIX: Baseline characteristics of patient populations. AVR, aortic valve replacement; CABG, coronary artery bypass grafting; AVA, aortic valve area; BMI, body mass index; LVMMI, left ventricular muscle mass index; LVEF, left ventricular ejection fraction; AVS, aortic valve stenosis, CAD, coronary artery disease. *n=5, **n=8; $^{\#}$n=3, $^{\#\#}$n=10 in CABG group.

Medication	AVR (n=57)	CABG (n=35)	P-value
Betablocker (n, %)	11 (19.3)	19 (54.3)	0.0006
ACE-inhibitors /ARBs (n, %)	26 (46.0)	18 (51.4)	NS
Statins (n, %)	34 (59.6)	25 (71.4)	NS
Calcium antagonists (n, %)	8 (14.0)	0 (0)	0.019
Diuretics (n, %)	16 (28.1)	5 (14.3)	NS
Warfarin (n, %)	6 (10.5)	2 (5.7)	NS
P_2Y_{12}-inhibitors (n, %)	1 (1.8)	4 (11.4)	0.049
Aspirin (n, %)	34 (59.6)	30 (85.7)	0.01

Table XX: Medication of study population. AVR, aortic valve replacement; CABG, coronary artery bypass grafting; ACE-inhibitor, angiotensin-converting-enzyme inhibitor.

Biochemistry parameters	AVR (n=64)	CABG (n=35)	P-value
Total cholesterol (mmol/L, mean±SD)	3.8±1.0	3.0±0.6	<0.0001
LDL cholesterol (mmol/L, mean±SD)	2.3±0.8	1.8±0.5	<0.0001
HDL cholesterol (mmol/L, mean±SD)	1.1±0.3	1.0±0.2	0.022
Triglycerides (mmol/L, median [Q1;Q3])*	1.4 [1.1;1.8]	1.5[1.1;1.8]	NS
Calcium (mmol/L, mean±SD)	2.1±0.1	2.1±0.1	NS
Glucose (mmol/L, median [Q1;Q3])	5.6[5.1;6.4]	5.5[5.0;6.5]	NS
CRP (mmol/L, median [Q1;Q3])*	1.7[0.8;5.5]	2.4[1.6;7.1]	NS
ADAMTS-13 activity (RFU/s, mean±SD)	3.7±1.2	2.9±0.7	0.0003
ADAMTS-13 antigen (µg/mL, median [Q1;Q3])	1.1[0.9;1.3]	1.1[0.9;1.3]	NS
vWF:CB (U/mL, mean±SD)	0.9±0.3	1.1±0.5	0.024
vWF activity (U/mL, mean±SD)	0.9±0.3	1.2±0.5	0.008
Normalized ultra-large/total vWF multimers (mean±SD)	66.3±27.2	97.2±24.1	<0.0001
vWF:Ag (U/mL, mean±SD)	1.3±0.4	1.6±0.6	0.005
Platelets (x1000/mL, mean±SD)	212.2±53.7	219±48.0	NS
Hemoglobin (g/L, mean±SD)	138.8±12.5	139.7±19.2	NS
Leucocytes (x1000/mL, mean±SD)	6.9±1.6	7.4±2.0	NS
INR (ratio, median [Q1;Q3])	1.0[1.0;1.1]	1.0[1.0;1.1]	NS
PT (sec, median [Q1;Q3])	11.4[10.6;11.9]	11.8[10.8;12.2]	NS
Fibrinogen (g/L, mean±SD)	3.7±0.9	4.0±0.9	NS

Table XXI: Biochemistry parameters of study population. AVR, aortic valve replacement; CABG, coronary artery bypass grafting; ADAMTS-13, a disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13; vWF, von Willebrand factor; vWF:CB, von Willebrand Collagen Binding Activity; Ag, antigen; INR, international normalized ratio; PT, prothrombin time; LDL, low-density lipoprotein; HDL, high-density lipoprotein; CRP, C-reactive protein.* t-test performed by using log-transformed data

Characteristics	AVS+Placebo (n=18)	AVS+apoA-I mimetic (7 days) (n=6)	AVS+apoA-I mimetic (14 days) (n=5)	Normal controls (n=11)	p-value (ANOVA)
AVA (mm^2, mean±SD) (baseline)	23.8±3.8	23.6±4.9	24.4±4.7	-	NS
AVA (mm^2, mean±SD) (before treatment)	19.6±3.4	18.5±3.0	18.6±2.1	-	NS
AVA (mm^2, mean±SD) (after Placebo/ApoA-I-treatment)	21.1±3.1	18.5±3.4	23.6±2.9	-	<0.05
Weight (kg, mean±SD)	2.9±0.2	2.9±0.1	3.0±0.1	3.0±0.2	NS
Total cholesterol (mmol/l, mean±SD)	14.8±8.6	12.9±5.3	13.0±7.1	0.5±0.2	<0.0001
HDL-cholesterol (mmol/l, mean±SD)	0.4±0.1	0.5±0.1	0.4±0.1	0.3±0.1	0.02
Triglycerides (mmol/l, median[Q1;Q3]	0.5[0.3;1.0]	0.6[0.3;0.7]	0.5[0.5;0.6]	0.4[0.3;0.6]	NS
Calcium (mmol/l, mean±SD)	3.2±0.2	3.3±0.2	3.2±0.1	3.1±0.2	NS

Table XXII: Echocardiographic aortic valve parameters and biochemistry in rabbits. AVS, aortic valve stenosis; AVA, aortic valve area; ApoA-I, apolipoproteinA-I; HDL, high-density lipoprotein. p-values indicated for ANOVA.

4. Discussion

Despite five decades of intense research, HDL has not been successfully targeted for the prevention or cure of cardiovascular disease. This failure originates mainly from directing novel drugs to HDL-C levels, which reflect neither the functionality of HDL particles nor the activity of reverse cholesterol transport. As a consequence, it has been emphasized that it is crucial to identify the most relevant atheroprotective biological activities of HDLs, their mediating molecules within HDL, as well as clinically applicable diagnostic biomarkers of HDL function in different disease conditions. Such an approach would allow to monitor patients at increased risk for cardiovascular disease, and to establish HDL-based, personalized treatment approaches. Biomarkers that show close correlations with disease status are the most interesting candidates for subsequent validation in animal and patient studies. To date, however, it is unclear whether such biomarkers will be native proteins, lipids or other cargo molecules of HDL.

The present investigation is the first to demonstrate a strong and inverse association of the HDL-associated protein LCAT with atherosclerotic lesion development in humans. By using three different imaging modalities and by assessing a variety of LCAT related parameters, the level of unesterified cholesterol, CER, as well as LCAT mass concentration were identified as significant, negative predictors of coronary and carotid artery plaque burden. Thus, based on our data, these parameters reflecting HDL functionality seem to be novel, useful markers of severity of atherosclerotic disease. In future studies, newly developed LCAT activators and recombinant LCAT infusions in humans will provide valuable data to establish whether targeting of LCAT is a promising therapeutic strategy to reduce cardiovascular risk.

One aspect of our study, however, merits further discussion: While we observed enhanced plasma LCAT mass concentrations in CAD patients, CER was downregulated in this population. Nevertheless, both parameters were found to be strong, independent negative predictors for atheroma burden in patients with CAD, indicating their anti-atherogenic effects. Given evidence from previous studies showing that LCAT mass concentration reflects CER/LCAT activity, our findings seem conflicting.[185, 382, 389, 390] However, it is known that CER is not only

determined by LCAT mass concentration but also by HDL particle size[391] and its composition.[517] Therefore, substrate and cofactor abnormalities may influence the association between LCAT mass concentration and CER in certain populations. Indeed, in our study, we did not observe a correlation between LCAT mass level and HDL cholesterol, which could be indicative of dysfunctional HDL in our high-risk population. In addition, LCAT is activated by an optimal conformation of a complex formed by apoA-I and the phospholipid-cholesterol bilayer; therefore, higher plasma LCAT mass concentrations measured in CAD patients might be a compensatory mechanism to account for a suboptimal presentation of its lipid substrate due to lower apoA-I level measured in this population. Furthermore, the fact that the correlation between LCAT mass concentration and CER was significantly stronger in controls as compared to CAD patients supports this hypothesis and might also imply the influence of a cofactor, the presence of inhibitors, apoA-I modifications (either oxidative or proteolytic) or binding of LCAT to lipoproteins inhibiting cholesterol esterification. However, a significantly lower concentration of unesterified cholesterol was measured in plasma of CAD patients as compared to controls, indicating that a defect in substrate availability and presentation, rather than the influence of other factors, is the most likely mechanism responsible for our observations.

While the identification of biomarkers of HDL function will be essential to establish novel HDL-based treatment approaches, many atheroprotective activities of HDL remain to be elucidated. Our study extends previous research on biological activities of HDL by demonstrating a favourable effect of HDL treatment on endothelial regeneration and vascular repair in high-risk patients with ACS. In addition, we found that exogenous HDL-based treatment normalized hematologic abnormalities, which are frequently observed in patients with aortic valve disease and most likely due to a direct effect on the coagulation protease ADAMTS-13. AVS is the most frequent acquired valvular pathology in the Western world and is present in more than 12% of elderly individuals.[518] Thus, with the aging of the population, the prevalence of AVS will further increase in the upcoming years. Given the high peri-operative bleeding risk of these elderly patients, there is a critical need to explore strategies to improve hematologic disorders associated with AVS. Our data provide novel insights into the function of HDL and apoA-I with

regard to regulation of ADAMTS-13 and vWF dependent haemostasis. In fact, our study adds to a growing literature identifying a role of HDL in coagulation responses, and it supports the view that an increase in ADAMTS-13 activity contributes to the hematologic abnormalities seen in AVS, and that HDL-based therapies may counterbalance such haemorrhagic tendencies. However, while our data suggest that an apoA-I-dependent effect on the serine protease plasmin or anti-inflammatory actions of HDL on endothelial cells might account for the observed reduction in ADAMTS-13 activity in AVS, the exact mechanisms underlying this clinically highly relevant phenomenon have yet to be determined. Laboratory studies focusing on the chemical composition of various subclasses of HDL particles may pinpoint specific components that mediate its effects on ADAMTS-13 activity and may, thus, identify more specific analytes for diagnostic testing or for consideration as therapeutic agents.

Our observation that the decline in endothelial progenitor cells in patients suffering from an ACS can be prevented by rHDL attributes functional HDL an important role in endothelial repair mechanisms after ischemic injury. Our data indicate that infusion of rHDL, which rapidly raise HDL, may be a valuable therapy in the vulnerable phase following the occurrence of an ACS. Indeed, there is solid evidence supporting beneficial effects of EPC treatment on morphological and clinical endpoints in ACS.[75-77, 82, 83] Therefore, the strategy to enhance endothelial regenerative capacity by increasing $CD34^+$ mobilization may help to stabilize vulnerable plaques and may, thus, have the potential to reduce the risk of recurrent events. Of note, more than 90% of PCIs involve stent placement. Drug-eluting stents (DES) improve the efficacy of PCI by preventing neointimal proliferation, thereby reducing restenosis by 70% across broad patient subsets. However, the antiproliferative agents used to prevent smooth muscle cell proliferation also delay re-endothelialisation in the stented segment and impair endothelial function, which is linked to stent thrombosis, an infrequent but major complication of PCI resulting in myocardial infarction or death.[519] In patients who received DES, the number of $CD34^+$ cells has been shown to be significantly less after PCI compared with those who received bare-metal stents (BMS), suggesting that the antiproliferative stent drug also attenuates EPC mobilization [468]. Despite initial optimism, therapeutic approaches with bioengineered EPC-capture stents in

patients undergoing PCI have failed.[520] Thus, the search for targeted pharmacological therapies to promote reparative progenitor cell responses after DES PCI is on-going, and the effects of rHDL on $CD34^+$ counts and SDF-1 observed in the present study may represent a promising therapeutic approach to improve arterial healing and inhibit smooth muscle cell proliferation after stent implantation. Therefore, given the known prognostic value of changes in $CD34^+$ levels following ACS and the beneficial effects of rHDL on plaque stability, it will be critical to investigate the influence of rHDL therapy on clinical endpoints. In addition, prospective clinical trials are needed to evaluate whether $CD34^+$ cell count may be a useful biomarker in the evaluation of novel HDL-raising therapies.

Unfortunately, a major drawback of such therapies is their high cost. Indeed, high concentrations of rHDL have to be administered in order to reach sufficient bioavailability. Protease degradation of HDL has been suggested to contribute to the reduced bioavailability and impaired function of both exogenous and endogenous HDL and, thus, strategies to prevent HDL proteolysis are being explored. Our study is the first identifying proteases that exert the most deleterious effect on apoA-I and HDL integrity in cardiac disease. In fact, we demonstrated that apoA-I proteolysis is augmented in plasma from patients with severe AVS as well as in a rabbit model of AVS. While MMPs were found to account for most of the apoA-I degradation in rabbit plasma, cathepsin S was the culprit protease in humans, with the greatest cathepsin S dependent apoA-I proteolysis seen in women. Therefore, specific Cathepsin S protease inhibitors may have the potential to prevent HDL dysfunction of endogenous HDL, as well as to enhance the efficacy of HDL-based therapeutic strategies in AVS and could render such therapy more applicable and economically feasible. Given the great interest in the development of strategies to increase bioavailable and functional HDL in humans, it is hoped that, in the near future, drugs improving HDL function will enter clinical practice. Our data support the notion that ‘hybrid’ strategies involving an HDL-based treatment and compounds preserving HDL function may result in effective and functional plasma HDL levels in patients. However, as such efforts proceed, caution is warranted when findings regarding HDL function are extrapolated from animal models to the clinical setting. Indeed, we have demonstrated that significant differences exist between species with regard to HDL-degrading

protease activity: despite the fact that rabbits closely resemble humans in their lipoprotein metabolism, Cathepsin S was found to be the culprit protease in humans, while MMPs were responsible for most of the HDL-degrading activity in rabbit plasma. Of note, considerable species differences in protease activity and HDL metabolism have been described previously.[488] Therefore, animal models may be limited in their ability to mimic the pathophysiology of apoA-I proteolysis in AVS and should be interpreted carefully. In addition, sex differences in apoA-I proteolytic activity observed in our study merits further investigation. Likewise, we observed significantly higher LCAT concentrations and CER in women as compared to men, thereby confirming previous reports,[184] and a positive association of UC with increasing age was detected in CAD patients. Importantly, those opposite trends in males and females did not translate into different, sex-specific correlations of LCAT with atherosclerosis in our study. Thus, at this point, we have no explanation for this effect and our investigations were not powered to assess sex and age dependent differences in HDL function and metabolism. However, our data further emphasize the need to explore sex-specific preventive and therapeutic approaches in cardiovascular disease. Of note, the established rabbit model of AVS used in the present study involves administration of high-dose vitamin D.[367, 471] Vascular calcium metabolism has been shown to be sex and age dependent and thus, the generalization of this experimental model may be limited.[521]

Besides the unknown generalizability to women and different age groups, several other methodological aspects of our investigation need to be considered: no conclusion is allowed as to whether plasma LCAT activity predicts progression of subclinical atherosclerosis, or whether HDL may affect postoperative ADAMTS-13 activity, inherent to single occasion measurements and the cross-sectional design of our study. In addition, HDL subfractions were not assessed in our studies; thus, we cannot exclude differential effects of apoA-I degrading proteases on HDL subpopulations. Further, we enrolled relatively small numbers of patients in our study, and our assay methods to assess LCAT activity and concentration should be evaluated in a larger number of patients for verification of its clinical utility. Finally, the small study population precluded further subgroup analyses

pertaining to the metabolic syndrome, diabetes, and DES implantation which are known to be associated with reduced endothelial repair mechanisms.[86, 126, 468].

In conclusion, given the known prognostic value of changes in endothelial progenitor cells in patients with ACS, our study provides a strong rationale to further investigate the influence of rHDL therapy on clinical endpoints. Prospective clinical trials are needed to evaluate whether endothelial progenitor cell count may be a useful biomarker in the evaluation of novel HDL-raising therapies. In addition, the present investigation provides evidence that the HDL associated protein LCAT is a strong, independent predictor of atherosclerotic disease severity, and should be further assessed in larger populations with regard to its clinical applicability as a marker of HDL function and cardiovascular risk prediction, as well as an efficacy-indicator of HDL-based therapeutic approaches. Importantly, the association of LCAT mass with reduced atheroma burden observed in our study should also prompt further research work assessing the suitability of LCAT as a therapeutic tool. In fact, administration of ACP-501, a recombinant human LCAT, has recently been shown to be effective and safe in patients with familial LCAT deficiency or CAD[361, 362] and ACP-501 is currently being further developed by MedImmune (AstraZeneca). In addition, an agonistic LCAT monoclonal antibody (27C3) has been introduced in 2015,[522] thus, this field of research promises to be very active in the upcoming years. In light of recent results from phase I and II studies and the data obtained by our investigation, it might be a promising approach to assess the effect of LCAT in other cardiovascular conditions, such as in AVS. Indeed, AVS and atherosclerosis share a common pathologic background and administration of rHDL or apoA-I mimetic peptides resulted in a significant regression of AVS in experimental models.[365-367] As well, the present work demonstrates beneficial effect of apoA-I mimetic administration on haemostatic parameters in experimental AVS, thereby further supporting the effectiveness and safety of an HDL-based therapeutic approach in AVS. Given that the apoA-I mimetic peptide ETC 642 has also been shown to promote LCAT activation,[523] subsequent experimental studies might test recombinant LCAT against ETC 642 in an experimental AVS model. Of note, as previously mentioned, ETC 642 exhibits peptide sequences matching the cleavage sites of MMP-12 and Cathepsin S.[489] Thus, in light of our study demonstrating a high susceptibility of apoA-I to MMP-12 (rabbits) and Cathepsin S

(humans) degradation, co-administration of a protease inhibitor should be considered in order to improve the efficacy of this compound. Indeed, MMP inhibitors such as Marimastat® as well as Cathepsin S inhibitors are commercially available and Marimastat® has previously been tested in clinical trials[235] while the Cathepsin S inhibitor RO5459072 is currently being evaluated in patients with Sjogren's syndrome (NCT02701985). In addition, the involvement of MMP-12 and Cathepsin S in several key pathways triggering the development of atherosclerosis and AVS has been established. Therefore, inhibition of these proteases may exert additional beneficial effect on AVS/atherosclerosis progression.[259, 261, 262, 267] Nevertheless, with regard to future clinical applications of protease-resistant apoA-I therapies in AVS and/or atherosclerotic disease, potential side effects of protease inhibitors also need to be taken into account. In fact, given their role in antigen presentation (Cathepsin S) and vascular remodelling (MMPs), inhibition of these proteases is expected to result in immunosuppression, inhibition of angiogenesis and vascular repair mechanism. Thus, the development of an apoA-I mimetic peptide resistant to proteolysis by Cathepsin S and MMP-12 might represent a safe alternative to a 'hybrid' strategy involving apoA-I plus protease inhibitor. Therefore, future studies will have to assess whether or not different HDL/apoA-I sources hold varying resistance to degradation by Cathepsin S and MMP-12. The use and further development of the apoA-I conjugated molecular imaging probe applied in the herein studies will provide a valuable tool to accurately measure and visualize apoA-I degradation *in vivo,* and to design and evaluate novel, protease-resistant therapeutic approaches. As well, efforts to render HDL-based therapies resistant to proteolysis should be complemented by studies assessing alternative treatment strategies aimed at improving HDL functionality in vivo. In this regard, the recent advances in the field of PCSK9 inhibitors also need to be considered. Indeed, besides lowering LDL-C, pharmacological inhibition of PCSK9 resulted in increased plasma HDL-C levels in clinical trials.[343, 344] In addition, a recent observational study in 103 083 individuals showed that the PCSK9 R46L loss-of-function mutation was associated with a reduced risk of developing AVS, thereby indicating that PCSK9 inhibitors may have a role in the treatment of patients with AVS.[524] Given the tremendous promise that PCSK9 inhibitors hold, future experimental projects should ideally incorporate a treatment arm testing a PCSK9

inhibitor in order to compare the efficacy of optimized apoA-I based therapies in AVS with this new compound.

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