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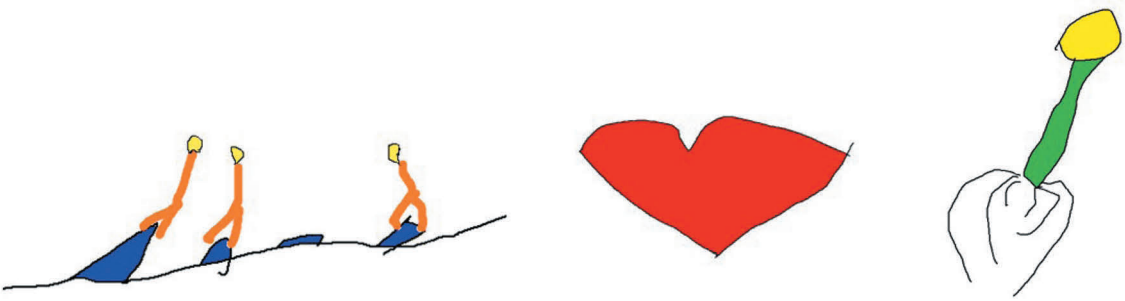

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“Foresight is not about predicting the future, it’s about minimizing surprise”
-Karl Schroeder

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PRIYANKA NEGI: Phage display based approach for identifying novel HDL particle binders: Recombinant HDL antibodies as novel diagnostic tool for risk assessment and monitoring of coronary artery disease
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ABSTRACT

Cholesterol content of high density lipoprotein (HDL-C) has been a well established cardiac risk marker for coronary artery disease (CAD), a major cause of mortality worldwide. Various epidemiological and experimental animal studies have shown the inverse correlation between concentration of HDL-C and CAD. However, recent pharmacological studies and genetic studies have demonstrated lack of protection from CAD despite of elevated HDL-C. HDLs are a heterogenous group of particles with varying protein/lipid composition and anti-atherogenic (atheroprotective) function. Atheroprotective HDL particles could turn dysfunctional and thereby attenuate their atheroprotective properties under particular circumstances such as among subjects with metabolic syndrome. Therefore, it is hypothesised that the risk assessment and clinical management of atherosclerotic CAD (ATCAD) should target these dysfunctional HDL particles rather than focusing on HDL-C.

The aim of this thesis was to develop antibodies against HDL particles by using phage display based antibody library and implement them for development of immunoassay for diagnosis of CAD and its risk estimation in symptomatic and asymptomatic individuals. To that end in publication I, a large set of HDL antibodies were isolated against the HDL derived from CAD patients. These antibodies were characterized against different HDL preparations, most abundant proteins of HDL (i.e. apoA-I and apoA-II) and plasma. A variety of binders were identified. In publication II, three distinct pairs of HDL antibodies which were mainly recognizing the apoA-I were employed to develop three different phage based two-site apoA-I immunoassays identified as assay 022-454, assay 109-121 and assay 110-525. Assays 109-121 and 110-525 showed promise to further improve the diagnostic and predictive value for cardiac conditions. In publication III, simplified versions of two of the phage based two-site immunoassays (109-121 and 110-525) is presented and clinically assessed with a cohort of cardiac patients. Higher level of apoA-I measured with the assay 110-525 showed clear association with ATCAD especially in patients not using lipid lowering medication. In light of these observations, it can be said that that a large number of antibodies were obtained against the intact HDL molecules with phage display approach. The results from testing clinical specimens with the new combinations of HDL binders suggest a different and improved performance over existing test technologies in estimating risk of ATCAD. However, further evaluation of these HDL binders with larger cohorts is clearly warranted. In addition, these antibodies could also serve as analytical tools for unravelling the etiology and the pathological process of atherosclerosis in relation to HDL.

KEYWORDS: HDL, CAD, phage display, apoA-I, antibody

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TIIVISTELMÄ

Sepelvaltimotauti on merkittävä kuolinsyy maailmalaajuisesti. Suuritiheyksisen lipoproteiinin kolesterolipitoisuus (high density lipoprotein cholesterol, HDL-C) on laajalti tunnustettu sepelvaltimotaudin riskitekijä. Monet epidemiologiset sekä kokeelliset eläintutkimukset ovat osoittaneet, että HDL-C on käänteisesti yhteydessä sepelvaltimotautiin. Viimeaikaisissa farmakologisissa ja geneettisissä tutkimuksissa ei ole kuitenkaan pystytty osoittamaan, että koholla oleva HDL-C suojelisi sepelvaltimotaudilta. HDL-partikkelit ovat heterogeeninen ryhmä partikkeleja, joiden proteiini- ja lipidikoostumus sekä ateroskleroosilta suojelevat ominaisuudet vaihtelevat. Tietyissä yhteyksissä, kuten esimerkiksi metabolista oireyhtymää sairastavilla, ateroskleroosilta suojaavat HDL-partikkelit voivat muuntua toiminnaltaan, jolloin niiden ateroskleroosilta suojaavat ominaisuudet voivat heikentyä. Tästä syystä on esitetty, että ateroskleroosiin liittyvän sepelvaltimotaudin riskinarvio ja hoito pitäisi perustua toiminnaltaan heikentyneisiin HDL-partikkeleihin HDL-C:n sijaan.

Tämän väitöskirjan tavoitteena oli löytää vasta-aineita HDL-partikkeleille faaginäyttötekniikkaan perustuvalla vasta-ainekirjastolla sekä kehittää niitä käyttäen immunomäärityksiä sepelvaltimotaudin diagnostiikkaan ja riskinarviointiin. Näiden tavoitteiden puitteissa julkaisussa I eristettiin suuri ryhmä HDL-vasta-aineita käyttämällä kohteena sepelvaltimotautipotilaiden HDL-partikkeleita. Vasta-aineet karakterisoitiin käyttämällä hyväksi erilaisia HDL-valmisteita, HDL-partikkelien yleisimpiä proteiineja (apoA-I ja apoA-II) ja plasmaa. Eristettyjen vasta-aineiden joukosta tunnistettiin erilaisia vasta-aineita. Julkaisussa II kehitettiin kolme apoA-I-proteiinia tunnistavaa kaksipuolista immunomääritystä (määritys 022-454, määritys 109-121 ja määritys 110-525), joissa hyödynnettiin kolmea keskenään erilaista, pääosin apoA-I -proteiinia tunnistavaa vasta-aineparia. Määritykset 109-121 ja 110-525 näyttivät lupaavilta sen suhteen, että ne voisivat pystyä parantamaan sydänsairauksia diagnostiikkaa ja ennustettavuutta. Käsikirjoituksessa III esitetään yksinkertaistetut versiot kahdesta faagipohjaisesta kaksipuolisesta immunomäärityksestä (109-122 ja 110-525) ja arvioidaan niiden kliininen toiminta sydänpotilasryhmän kanssa. Määrityksellä 110-525 mitattu korkeampi apoA-I pitoisuus oli selvästi yhteydessä ateroskleroosiin liittyvään sepelvaltimotautiin erityisesti niillä potilailla, joilla ei ollut lipidejä laskevaa lääkitystä. Näiden havaintojen valossa voidaan todeta, että tutkimuksessa löydettiin faaginäyttötekniikalla useita HDL-molekyylejä tunnistavia vasta-aineita. Kliinisillä näytteillä saatujen tulosten perusteella näiden uusien sitojien kombinaatiot näyttävät ennustavan ateroskleroosiin liittyvää sepelvaltimotautia eri tavalla ja paremmin kuin nykyään käytössä olevat testimenetelmät. Tulokset pitää kuitenkin vahvistaa laajemmilla potilasjoukoilla. Tutkimuksessa löydetyt vasta-aineet voisivat lisäksi olla hyödyllisiä analyyttisinä työkaluina tutkittaessa ateroskleroosin etiologiaa ja patologisia prosesseja suhteessa HDL-partikkeleihin.

ASIASANAT: HDL, CAD, Faaginäyttöön, apoA-I, vasta-aineet

Table of Contents

Abbreviations

ABCA1	ATP-binding cassette transporter A1
ACC	American College of Cardiology
AHA	American Heart Association
ACE	Angiotensin I-converting enzyme
ACS	Acute coronary syndrome
AMI	Acute myocardial infarction
ANP	Atrial natriuretic peptide
AP	Alkaline phosphatase
Apo	Apolipoprotein
ATCAD	Atherosclerotic coronary artery disease
ATCVD	Atherosclerotic cardiovascular disease
ATP	Adenosine triphosphate
AUC	Area under curve
BITC	Bi-isothiocyanate
BNP	Brain natriuretic peptide
BSA	Bovine serum albumin
CABG	Coronary artery bypass grafting
CAC	Coronary artery calcium
CAD	Coronary artery disease
CCTA	Coronary CT angiography
CDR	Complementary-determining regions
CEC	Cholesterol efflux capacity
CERT	Ceramide-based Coronary Event Risk Test
CETP	Cholesteryl-ester transfer protein
CH	Constant heavy chain
CHD	Coronary heart disease
CK	Creatinine kinase
CK-MB	Creatinine kinase-myocardial band
CL	Constant light chain
CRP	C-reactive protein
CT	Computerized tomography

CV	Coefficient of variation
CVD	Cardiovascular disease
DNA	Deoxyribonucleic acid test
DTPA	Diethylenetriamine pentaacetate
DTT	Dithiothreitol
EAS	European Atherosclerosis Society
ECG	Electrocardiogram/electrocardiograph
EDTA	Ethylenediaminetetraacetic acid
EFI	Europium fluorescence intensifier
EIA	Enzyme immunoassay
EL	Endothelial lipase
ELISA	Enzyme linked immunosorbent assay
eNOS	Nitric oxide synthase in vascular endothelial cells
ESC	European Society of Cardiology
ESI	Electrospray ionization
FC	Free cholesterol
FGF	Fibroblast growth factor
FIA	Fluoroimmunoassay
FRS	Framingham Risk Score
GRACE	Global registry of acute coronary events
HDL-C	HDL-cholesterol
HL	Hepatic lipase
hs	High sensitivity
ICA	Invasive coronary angiography
ICAMs	Intercellular adhesion molecules
IDL	Intermediate-density lipoprotein
Igs	Immunoglobulins
IL	Interleukin
IMAC	Immobilized metal affinity chromatography
IPTG	Isopropyl-β-D-thio-galactoside
Li	Lithium
LCAT	Lecithin–cholesterol acyltransferase
LC-ESI-MS/MS	Liquid chromatography-electrospray ionization-tandem mass spectrometry
LDL	Low density lipoprotein
LPDS	Lipoprotein deficient serum
LPL	Lipoprotein lipase
Lp-PLA 2	Lipoprotein-associated phospholipase A2
Mab	Monoclonal antibody
MALDI	Matrix-assisted laser desorption/ionization

MBF	Myocardial blood flow
MI	Myocardial infarction
miRNA	micro RNA
MPO	Myeloperoxidase
MRI	Magnetic resonance imaging
MRM-MS	Multiple-reaction monitoring mass spectrometry
MS/MS	Tandem-mass spectrometry
MUFA	Monounsaturated fatty acid
MPO	Myeloperoxidase
NaCl	Sodium chloride
NMR	Nuclear magnetic resonance
NO	Nitric oxide
Non-MI	Non myocardial infarction
NSTEACS	Non ST elevated ACS
NSTEMI	Non-ST elevated MI
NT-pro-BNP	N-terminal prohormone of brain natriuretic peptide
OD	Optical density
OR	Odds ratio
Pab	Polyclonal antibody
PAF-AH	Platelet activating factor acetyl hydrolase
PBS	Phosphate buffer saline
PC	Phosphatidyl choline
PCI	Percutaneous coronary intervention
PCR	Polymerase chain reaction
PCSK-9	Proprotein convertase subtilisin/kexin type-9
PEG	Polyethylene glycol
PL	Phospholipids
PON	Paraoxonase
PROCAM	Prospective cardiovascular münster
PUFA	Polyunsaturated fatty acid
RCT	Reverse cholesterol transport
rHDL	Reconstituted HDL
RIA	Radioimmunoassay
RT	Room temperature
SBP	Systolic blood pressure
SB	Super broth
SBTI	Soyabean trypsin inhibitor
SCD	Sudden cardiac death
ScFvs	Single chain variable fragments
SCORE	Systematic coronary risk evaluation

SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SELDI	Surface enhanced laser desorption/ionization
STEMI	ST-elevated (a particular pattern on an electrocardiogram) MI
TC	Total cholesterol
TG	Triglyceride
TMI	Thrombolysis in Myocardial Infarction score
Tn	Troponin
TNF	Tumor necrosis factor
TOF	Time-of-flight
t-Pa	Tissue plasminogen activator
TRF	Time-resolved fluorescence
TRFIA	Time-resolved fluorescence immunoassay
UA	Unstable angina
VAP	Vertical auto profile
VCAM	Vascular cell adhesion molecule
VH	Variable heavy chain
VL	Variable light chain
VLDL	Very low density lipoprotein
GWAS	Genome wide association studies
2D-PAGE	Two-dimensional agarose-PAGE

List of Original Publications

This thesis is based on the following original publications, which are referred in text by their Roman numerals:

I Priyanka Negi, Janita Lövgren, Päivi Malmi, Nina Sirkka, Jari Metso, Tuomas Huovinen, Eeva-Christine Brockmann, Kim Pettersson, Matti Jauhiainen, Urpo Lamminmäki. Identification and analysis of anti-HDL scFv-antibodies obtained from phage display based synthetic antibody library. Clin Biochem, 2016, 49: doi: 10.1016/j.clinbiochem.2015.11.020

II Priyanka Negi, Taina Heikkilä, Terhi Tallgren, Päivi Malmi, Juha Lund, Juha Sinisalo, Jari Metso, Matti Jauhiainen, Kim Pettersson, Urpo Lamminmäki, Janita Lövgren. Three two-site apoA-I immunoassays using phage expressed detector antibodies - Preliminary clinical evaluation with cardiac patients. J Pharm Biomed Anal., 2021, 194: doi: 10.1016/j.jpba.2020.113772

III Priyanka Negi, Taina Heikkilä, Karoliina Vuorenpää, Emilia Tuunainen, Wail Nammas, Teemu Maaniitty, Juhani Knuuti, Jari Metso, Janita Lövgren, Matti Jauhiainen, Urpo Lamminmäki, Kim Pettersson, Antti Saraste. Time-resolved fluorescence based direct two-site apoA-I immunoassays and their clinical application in patients with suspected obstructive coronary artery disease. Front. Cardiovasc. Med., 2022, 9: doi: 10.3389/fcvm.2022.912578.

1 Introduction

Coronary artery disease (CAD) belongs to the category of cardiovascular diseases (CVDs) and it usually refers to atherosclerosis, i.e. narrowing of coronary artery due to subendothelial plaque formation via lipid, primarily cholesterol accumulation. It remains the leading cause of death worldwide (1)(2), despite several advances in prevention, diagnosis and clinical management over the past few decades. The disease remains silent in many cases and unfortunately, sometimes sudden cardiac death (SCD) is the first manifestation of CAD (1). The burden of the disease translates into huge number of deaths and enormous medical cost to the society, which could be brought down if better models for atherosclerotic CAD (ATCAD) risk prediction and preventive approaches are developed.

HDL is a dynamic pool of a heterogeneous subpopulation of particles differing in their lipid and protein components as well as in their particle size and charge (3). HDL has several anti-atherogenic properties such as participation in reverse cholesterol transport (RCT) i.e. ability to remove cholesterol from macrophage-foam cells in the arterial wall intima, inhibition of oxidation of low density lipoprotein (LDL), promotion of endothelial repair, improvement of endothelial function, anti-thrombotic and anti-inflammatory properties, and inhibition of the binding of monocytes to the endothelium (3). Apolipoprotein (apo) A-I is the major structural protein of HDL and is very closely associated with the functionality of HDL (4–6). HDL-cholesterol (HDL-C) is a well-established risk marker and is used in clinical routine for risk estimation of ATCVD or ATCAD. However, HDL has many atheroprotective properties (mentioned above) which are unrelated to its cholesterol content but are related to CVDs. Under pathologic conditions such as infection, inflammation, diabetes or other cardiometabolic derangements, HDL or apoA-I lose their atheroprotective functions and become dysfunctional which may actually promote ATCVD (7,8). Therefore, it is assumed that risk assessment and treatment strategies of ATCAD must specifically target dysfunctional HDL particles rather than the concentration of HDL-C (9). Monoclonal antibodies which can discriminate the dysfunctional HDL particles from the atheroprotective functional HDL forms are considered as one of the specific tools for evaluation and clinical risk stratification of ATCAD (10).

Phage display is a well-established method for the generation of recombinant antibodies against various types of targets (11). It has several other advantages over conventional hybridoma technique of antibody production such as speed and possibility to freely adjust the reaction conditions for antibody generation (12,13). Time-resolved fluorescence immunoassay (TRFIA) uses lanthanide-chelates as the detection label. These labels provide high sensitivity (14) to TRFIA because of their distinct fluorescent properties (15–19).

The aim of the present thesis was to generate HDL specific antibodies by making use of single chain variable fragments (scFvs) based phage display library and implement them to develop TRFIA for diagnosis and risk estimation of CAD. To that end, these HDL antibodies were generated against HDL particles derived from CAD-patients (CAD HDL) and their performance was evaluated with cohorts of cardiac patients.

2 Review of Literature

2.1 Coronary artery disease (CAD)

2.1.1 Epidemiology

CAD is one of the leading cause of death in the world and is responsible for approximately one-third of all the deaths in the individuals older than 35 years (1). According to World health organization (WHO), in year 2019 32% (17.9 million people) of global deaths were due to CVDs (20). Mortality rate of CVD is shown in Figure 1. European Society of Cardiology (ESC) has categorized Finland into Moderate-risk countries for CVD death, representing 100 to <150 CVD deaths (age and sex standardized) per 100,000 person (21). Approximately half of all middle-aged men and one-third of all middle-aged women in the United States develop some manifestations of CAD (1). However, while mortality rate is decreasing slowly but progressively across the world, the death rates remain higher in developing nations (22). Improvement in treatment of cardiovascular risk factors and improvement in health care systems/policies are considered to certainly contribute in reducing the mortality worldwide.

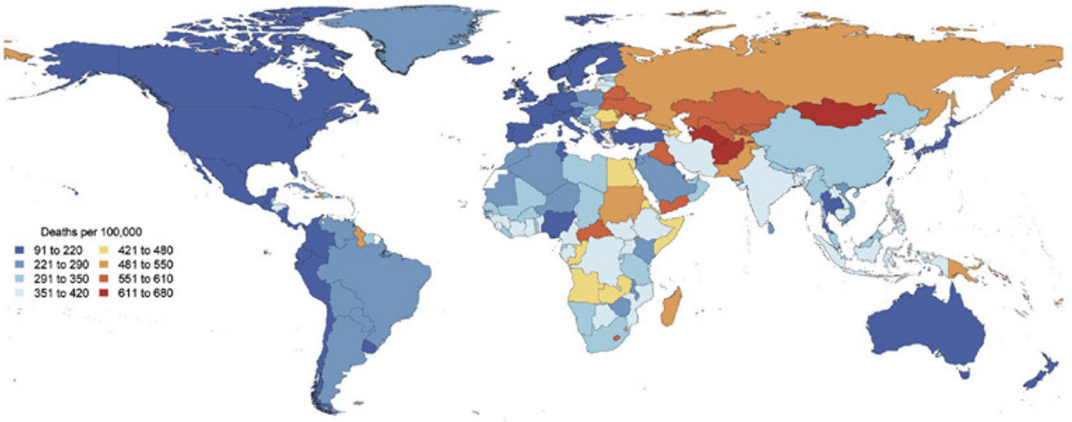


Figure 1. Map showing the estimated age-standardized mortality rate of total CVD in 2015 for each country. Modified with permission of Roth GA et al. (23). Copyright © 1969, Elsevier.

2.1.2 Pathophysiology and clinical characteristics

The pathologic mechanism of CAD is a gradual process which usually evolves for decades prior to any clinical manifestation. The beginning of CAD is generally attributed to a chronic inflammatory process, starting from the earliest formation of subendothelial fatty streak to the final formation of advanced atherosclerotic plaques. Atherosclerotic plaque development is the hallmark of the pathophysiology of CAD. The plaques are defined as the regions of thickened intima (inner layer of coronary artery wall) and are composed of various mixtures of fibrous tissue, smooth muscle cells, inflammatory cells such as macrophage-foam cells filled with lipid (24).

The atherosclerotic process is associated with endothelial dysfunction, a process identified as impaired vasodilation and endothelial permeability. Endothelium is a very thin cell lining of vascular wall which forms an interface between the circulating blood in the lumen and the subendothelial intimal layer (25). Its dysfunction would develop due to cardiovascular risk factors such as smoking, aging, hypertension, hyperglycemia, family history of premature atherosclerotic disease, chronic infection/inflammation, hypercholesterolemia, oxidation injury due to free radicals and genetic alterations (25–29). Early signs of atherosclerosis include intimal inflammation and the formation of fatty streaks which are formed by subendothelial deposition of lipid-laden macrophages, i.e. macrophage-foam cells (24,30). Continuous inflammation then leads to local elevation of oxidation, plaque formation and causes thickening of inner layer (intima) of coronary artery wall. This may over time slowly narrow the lumen of the artery up to various degrees and cause ischemic symptoms as a result of myocardial under-perfusion. Approximately 50 % narrowing of luminal diameter (75 % luminal area) is required to affect the blood flow (31). However, this slow progression of the atherosclerosis process might be interrupted by thrombus formation due to plaque disruption/ erosion or calcified nodulation or plaque haemorrhage (32).

Clinical presentation of CAD has a wide spectrum ranging from asymptomatic, stable angina (SA), and acute coronary syndrome (ACS) which includes unstable angina (UA), non-ST elevated MI (NSTEMI), and ST-elevated MI (STEMI) to sudden cardiac death (SCD) (32). Chest pain or angina pectoris is considered as the clinical hallmark of myocardial ischemia and occurs due to CAD. Angina is defined as stable when the symptoms develop with stress (physical or emotional), usually stays for less than five minutes and disappears with rest. However, in UA the symptoms appear even at rest, unexpectedly and last longer. Most of the symptomatic acute coronary events unstable angina (UA), acute myocardial infarction (AMI), and SCD occur as a result of rupture/ erosion of plaque, calcified nodulation of plaques and subsequent thrombus formation causing partial or complete occlusion of the artery (33,34).

2.1.3 Diagnosis and risk evaluation

The standard test for diagnosing CAD is cardiac catheterization, but it is not used first line because of its high cost, radiation exposure and invasive nature. The evaluation of patients suspected of CAD is based on physical examination, medical history, biochemical tests and diagnostic test for example ECG, stress test and cardiac enzymes such as creatinine kinase (CK) and troponin (Tn) in patients who present with chest pain. Basic investigation includes ECG, X- ray, biochemical tests /blood tests which includes blood count, thyroid function test, renal function test and lipid profile test. Lipid profile test includes determination of concentration of total cholesterol (TC), HDL-C, low density lipoprotein cholesterol (LDL-C), lipoprotein (a), triglyceride (TG) and glucose. Chest pain or shortness of breath are typically the symptoms leading to the suspicion of ACS. As per the current guidelines of the European Society of Cardiology (ESC), an ACS diagnosis is based on a rule-in rule-out algorithm based on triage of symptoms, ECG and serially measured troponin (Tn) values (35,36), Figure 2. Additionally, in chest pain patients with normal or near normal ECG results and negative cardiac Tn additional functional testing or anatomic testing is done (37). Functional investigations include exercise ECG, stress echocardiography, nuclear perfusion imaging, cardiac magnetic resonance imaging (MRI) while anatomic testing might include invasive coronary angiography (ICA), coronary artery calcium (CAC) scoring, coronary CT angiography (CCTA). A negative result of these investigations further reduces the possibility of ischemia as the cause of chest pain and hence ACS (38).

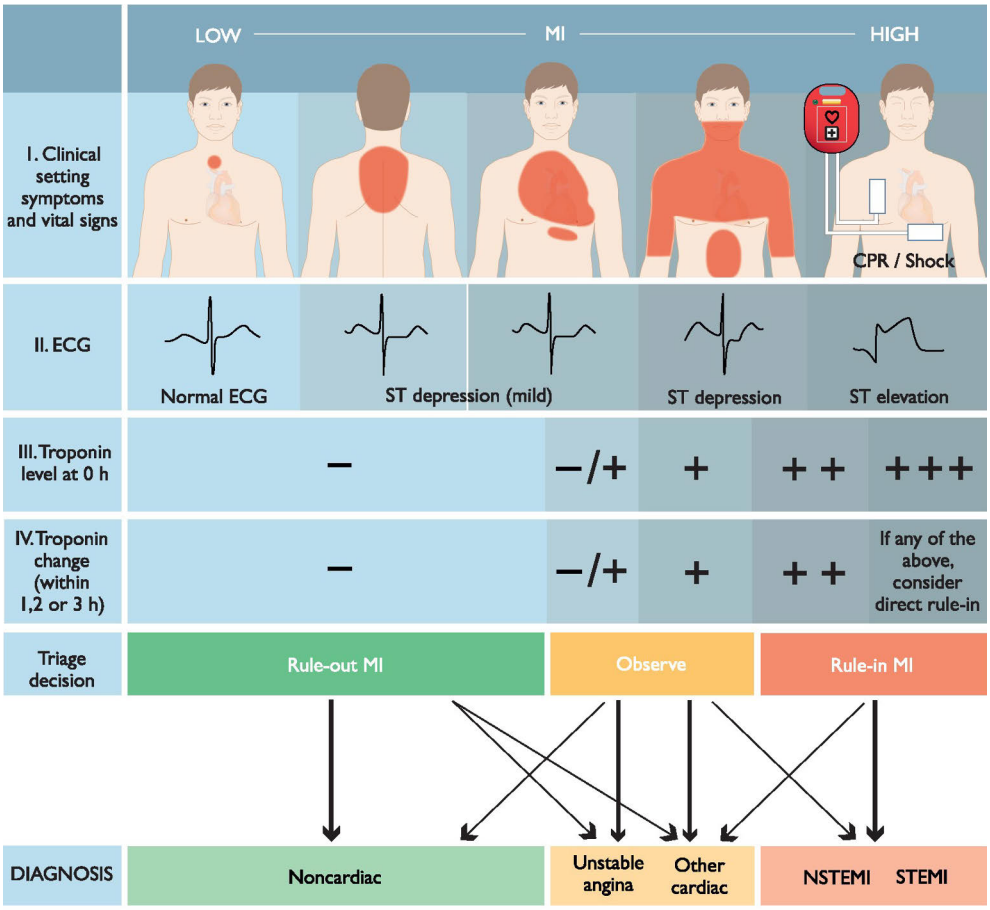


Figure 2. Diagnostic algorithm and triage in acute coronary syndrome (36). The initial assessment is based on the integration of low likelihood and/or high likelihood features derived from the clinical setting (i.e. symptoms, vital signs), the 12-lead ECG, and the cardiac troponin concentration determined at presentation to the emergency department and serially thereafter. 'Other cardiac' includes – among others – myocarditis, Takotsubo syndrome, or congestive heart failure. 'Non-cardiac' refers to thoracic diseases such as pneumonia or pneumothorax. Cardiac troponin and its change during serial sampling should be interpreted as a quantitative marker: the higher the 0 h level or the absolute change during serial sampling, the higher the likelihood for the presence of MI. In patients presenting with cardiac arrest or haemodynamic instability of presumed cardiovascular origin, echocardiography should be performed/interpreted by trained physicians immediately following a 12-lead ECG. If the initial evaluation suggests aortic dissection or pulmonary embolism, D-dimers and CCTA angiography are recommended according to dedicated algorithms. CPR = cardiopulmonary resuscitation; ECG = electrocardiogram/electrocardiography; MI = myocardial infarction; NSTEMI = non-ST-segment elevation myocardial infarction; STEMI = ST-segment elevation myocardial infarction.

In addition to all the diagnostic tests mentioned beforehand, various scoring systems have been developed to help in determining the future risk of all-cause mortality or

the combined risk of all-cause mortality or MI or CVD. Some of the commonly used scoring systems are Global registry of acute coronary events (GRACE) (39)(40), Systematic Coronary Risk Estimation (SCORE) (23), Framingham risk score (43)(42), and The Thrombolysis in Myocardial Infarction score (TMI) (44)(45). Also, American College of Cardiology (ACC)/American Heart Association (AHA) (46) and European Society of Cardiology (ESC)/European Atherosclerosis Society (EAS) (47) guidelines are followed for risk evaluation of CVDs. These scoring systems in general include age, anginal symptoms, CAD associated risk factors (hypertension, smoking, hypercholesteremia and diabetes), pulse rate, ST change, elevated level of cardiac biomarkers such as CK-myocardial band (MB) fraction and cardiac specific Tn (39,40,42,44,45).

While there are updated guidelines available based on the vast array of diagnostic methods, clinicians are left to apply clinical acumen to decide which test to use and when. Finding a balance between the diagnostic clarity and good clinical acumen, as well as managing patient, remains a challenge in diagnosis of CAD. Although, early diagnosis of CAD is essential, particularly prior to MI or SCD, unfortunately early diagnosis of coronary atherosclerosis is currently not feasible in the general population. Given the fact that approximately one third of patients with coronary atherosclerosis/ CAD die suddenly, its early estimation must be emphasized.

2.1.4 Treatment and risk management

Treatment of CAD is guided by the symptoms and the clinical presentation of the patient. Moreover, prevention also plays an important role in risk management of CAD. The main goal of treatment is: (1) prevention of MI and decrease in mortality and (2) to decrease symptoms and incidence of CAD to ensure better quality of life. Several methods are available to achieve this goal, for example disease prevention/treatment can be delivered by adopting a healthy life style (e.g. healthy diet, physical activity, smoking cessation) at individual and population level, use of therapeutic drugs, and surgical and interventional therapies (1,46,48–51)(20,52). In relation to drug therapy, antiplatelet agents, lipid-lowering drugs particularly statins and its combination with newer drugs such as proprotein convertase subtilisin/kexin type 9 (PCSK-9) inhibitors and inhibitors of cholesterol absorption, beta-blockers after AMI, and angiotensin I-converting enzyme (ACE) inhibitors help to decrease the incidence of MI and increase patient survival (1,53,54). Recently, angiopoietin-like 3 (ANGPTL3) has emerged as a promising molecular target to reduce CHD risk due to its ability to regulate all of the three major lipid traits: LDL-C, HDL-C, and triglycerides (TG) (55). On the other hand, nitrates, calcium-channel blockers, and trimetazidine decrease the symptoms and episodes of myocardial ischemia, and improve the patient's quality of life. In patients with STEMI, reperfusion on an

emergent basis, with primary percutaneous coronary intervention (PCI) is preferred at presentation, which is complemented with adjunctive medical therapy (e.g. antiplatelet/anticoagulant therapy) for disease stabilization (35,56). If there is no immediate access to PCI, then thrombolytic therapy with streptokinase and tissue plasminogen activators i.e. t-PAs is initiated. In NSTEACS (non ST elevated ACS), appropriate therapy is selected based on risk stratification using risk score. For patients who present with stable angina, treatment is provided primarily to relieve the symptoms of the disease and prevent complications associated with CAD (32). All patients are given medical therapy (e.g. anti-ischemia, antiplatelet/ anticoagulant, lipid lowering medication) to provide relief of ischemia and to prevent the recurrence of ischemic events, and high-risk patients are additionally considered for revascularization by PCI or coronary artery bypass grafting (CABG) (1,33)(37,49,56). In this respect, Finnish current-care guidelines are very well aligned with those described by European society of cardiology (ESC) (35)(36,49,52).

2.2 Cardiac risk markers

A continuously improving knowledge of atherosclerosis has led to the identification of several cardiac biomarkers that could be used in clinical practice. According to National Institute of Health Consortium, biomarker is defined as a "characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention" (57). A biomarker could be measured from biosamples (such as blood, urine or tissue), it could be a recording from an individual (such as blood pressure, ECG), or it could be an imaging test (such as CT and MRI scans). In context of CVD, biomarkers provide a powerful tool to understand the spectrum of the disease with applications in areas including screening, diagnosis, prognostication, prediction of disease recurrence and therapeutic monitoring (58). Technical advances in functional genomics, proteomics, lipidomics, metabolomics, bioinformatics and molecular imaging have revealed innumerable putative cardiac risk markers (58). However, assessment of risk markers has been afflicted by confounding factors/ comorbidities, methodological limitations and statistical-clinical irrelevance or dilemmas (59). Some of the cardiovascular biomarkers (60) are listed in Table 1.

Table 1. Clinical CVD biomarkers.

Basis of Classification	Biomarker
Disease specificity	
Heart Failure	BNP, NT-proBNP, ANP, ST-2 etc. (60,61).
Atherosclerotic coronary disease	Tn T or I, creatinine phosphokinase-MB.
Use to monitor disease stage	
Acute Changes	Copeptin, hs troponin, galectin-3, ST2 (60).
Chronic stage of CVD to estimate prognosis	Coronary calcium (60)
Pathologic process	
Inflammation and thrombosis	hsCRP, IL-6, fibrinogen, monocyte chemotactic protein-1, TNF alpha, MPO etc. (60,62).
Oxidative stress	Isoprostanes
Metabolic	Lipoprotein (a), LDL, HDL, apoB-100, TG-rich particle remnants, Lp-PLA 2, homocysteine, vitamin D, FGF 23, adiponectin, glycated hemoglobin, haptoglobin etc. (60).
Lipid based risk markers	
Ceramides • Cardiovascular outcomes • Future CVD events • Risk stratifiers for primary and secondary prevention of atherosclerotic CVD • Cardiovascular mortality, and overall mortality in CVD patients	CERT 1 (CERT) 1 and Phosphatidylcholine based CERT 2 (63–65).
MUFA and PUFA • MUFA positively associated with risk of CVDs • PUFA inversely associated with the risk of CVD	(66–68)
TG species • Adverse CVD outcome • Recurrent CVD incidence and CVD mortality • Increased risk of CVDs	For example, TG 48:1, TG 48:2, TG 48:3, TG 50:3 and TG 50:4 (68–71).
Long non-coding RNA	
Risk and severity of CHD	For example, H19, ANRIL, lincRNA-P21 (72).
Biochemical risk markers used in ESC guidelines • Establish the risk profile of CAD suspected patients • Ascertain the need of treatment	Lipid profiling including total cholesterol, HDL-C, LDL-C, and triglycerides (49,52,62). Fasting plasma glucose and glycated haemoglobin (HbA1c) (75).

Despite the availability of myriad of CVD biomarkers (few mentioned in Table 1), there is constant need to discover novel risk markers which may allow for even accurate risk stratification, with the ultimate aim of directing management, treatments or intervention only to those individuals who will benefit from them. To that end, known theories and related facts have been revised for example for the relevance of HDL-C as a biomarker of atherosclerosis. Traditionally HDL-C level is used as one of the biomarkers for CVD risk prediction. However, the recent findings (detailed in section 2.2.4) have questioned its importance to evaluate CVD risk and have enforced the researchers to rethink/ relook for better markers associated with circulating HDL particles to predict the risk of CVDs.

2.2.1 HDL and its biogenesis

HDLs were first identified in the 1950s as the smallest and densest of all the lipoprotein classes (76)(77)(78). Their biogenesis is a complex and multistep process (Figure 3) which involves several membrane bound and plasma proteins (79). In the early step of HDL biogenesis, apoA-I is secreted by liver and intestine (80). The secreted apoA-I interacts with the lipid transporter ATP-binding cassette A1 (ABCA1) which is located on the plasma membrane of hepatocytes and enterocytes. ABCA1 transfers cellular phospholipids (PL) and cholesterol and forms lipid-poor apoA-I. Then, the lipid poor apoA-I undergoes a series of steps and gets converted into discoidal HDL particle (81). The esterification of free cholesterol by the enzyme lecithin/ cholesterol acyltransferase (LCAT) (82) converts the discoidal HDL particle to mature spherical HDL particles. The metabolism of HDL involves the transfer of cholesteryl-esters to very low density lipoprotein (VLDL)/LDL via the function of plasma cholesteryl-ester transfer protein (CETP), hydrolysis of PLs and residual triglycerides (TG) by the various lipases [lipoprotein lipase (LpL), hepatic lipase (HL), and endothelial lipase (EL)], and the transfer of PLs from post-lipolytic VLDL particles to HDL by the phospholipid transfer protein (PLTP) (83)(84). In addition to apoA-I, apoE and apoA-IV can also synthesize HDL-like particles by following a similar pathway (85,86). However, HDL particles containing only apoE or apoA-IV without apoA-I are rare and comprise only a small proportion of the total HDL pool in circulation.

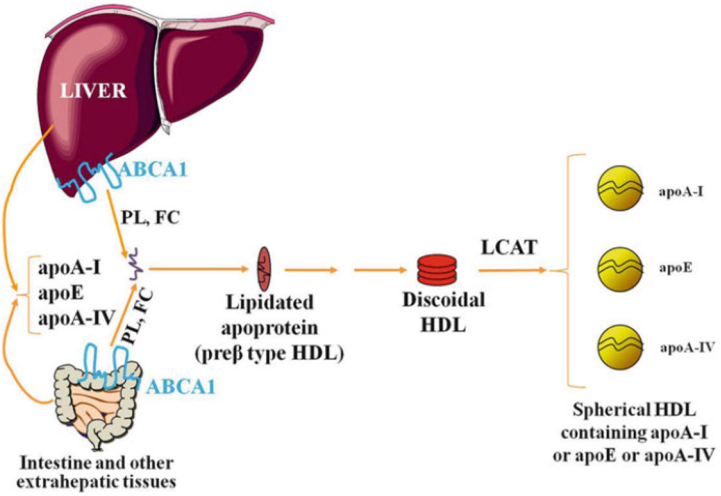


Figure 3. Schematic representation of biogenesis of HDL containing apoA-I, apoE and apoA-IV. Copyright © (87) 2015. ABCA1, ATP-binding cassette A1; PL, phospholipid; FC, free cholesterol; apo, apolipoprotein; HDL, high density lipoprotein; LCAT, acyltransferase.

2.2.2 Molecular components, structure and function of HDL

HDL particles are composed of several lipids (over 200 lipids, mainly polar and some nonpolar) and proteins (over 85) (88)(89,90). In addition, these particles also serve as cargo for vitamins, hormones, and microRNA (miRNA) (91). The structure of an HDL particle is depicted in Figure 4. HDL particle consists of a central core of neutral lipids mainly cholesterol esters (CE) and some TG, surrounded by a monolayer of phospholipids (PL), free cholesterol (FC), and apolipoproteins (apo), predominantly apoA-I and apoA-II (88,92). The particles are present in different shapes, sizes, charges and densities throughout HDL metabolism cascades (90,93).

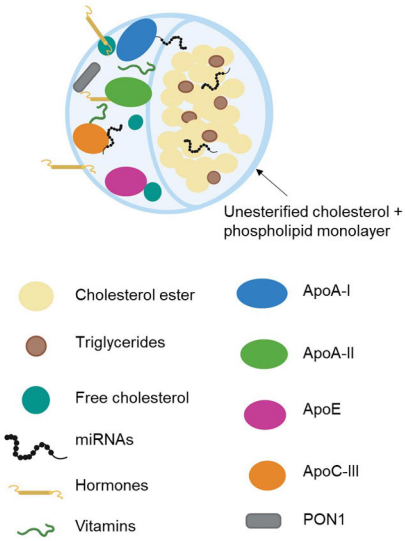


Figure 4. Structure of HDL particle. Apo, apolipoprotein; PON, paraoxonase; miRNA, micro RNA. Figure is generated with Biorender.com.

Structure of HDL is continuously modified as a function of its metabolic cascades and is largely affected by its apolipoproteins as well as lipid components. The structural classes of HDL are discoidal lipid-poor HDL and spherical lipid-rich HDLs (94). Majority of HDL particles exist in spherical form in human circulation. Discoidal HDL forms are very unstable and are present at very low concentration of approximately 5-8 % of total HDL pool in circulation. These forms are also generated in-vitro for structural studies by incubating apolipoproteins with lipids and referred to as reconstituted HDLs (rHDLs) (95). Recently novel mAbs were developed which could monitor structural transition of apoA-I upon HDL formation (96).

The proteins present in HDL can be classified mainly to apolipoproteins, enzymes, lipid transfer proteins, acute-phase response proteins, complement components, proteinase inhibitors and other protein components (94). Among these, apolipoproteins are the major protein components. Apolipoprotein A-I (apoA-I) constitutes 70% of total protein mass and is present in almost all of the circulating HDL particles (4,5). It serves as the major structural and functional component of HDL. The other 15-20% protein contribution is of apoA-II which is present on about two-thirds of HDL particles in humans (9,97). ApoA-I has conformational dynamic domain which can undergo changes depending on the size/ composition of HDL particle and serve as a domain of interaction with lipid-modifying proteins such as LCAT, CETP and PLTP (95). Depending upon the size of HDL particles 3–5 apoA-I molecules per HDL particle are present. Its major functions involve interaction with

cellular receptors such as ABCA1, activation of LCAT and launching multiple anti-atherogenic functions in HDL. The remaining 10–15% of protein mass is mainly composed of minor proteins, including apoCs, apoE, apoD, apoM, apoL1, apoH, apoJ and apoA-IV, enzymes [PON 1, platelet activating factor acetyl hydrolase (PAF-AH)], lipid transfer proteins such as LCAT, CETP and PLTP, acute phase protein and proteinase inhibitors etc. (95). HDL proteins are non-uniformly distributed across the HDL subpopulations and the protein-to-lipid ratio may range from 1:2 in large HDL2 to 10:1 in preβ-HDLs (98). These proteins often, exist in multiple isoforms due to co- and posttranslational modification such as glycosylations, truncations and phosphorylation (99,100). Eventually these diverse compositional traits of HDL lead to highly heterogeneous structural, chemical and biological properties of HDL particles.

Lipidome constitutes an important part of HDL (over 200 lipid species) (101). They are classified to hydrophobic neutral lipids (30–40% CE and 2–88% TG present in the HDL core), and amphipathic lipids (5–10% FC or 35–50% PLs mainly located on the HDL surface) (97,102). In this context, PLs (phosphatidylcholine and -serine) are associated with the cellular cholesterol efflux process, antioxidative, antiapoptotic and anti-inflammatory actions, and in modulating platelet activity (103–105). In addition, both core lipids in HDL and surface lipids can impact on surface fluidity and, in turn, antioxidative and many other functions (103,106). Many studies have shown association of HDL lipidome with CVDs (104,105,107–110).

Lipid-protein interaction (90,93) and protein-protein (88) interaction also drive the formation of individual HDL particle species and lipoprotein complexes such as overall compositional and structural features translate into expression of highly defined biological functions. HDL has a number of biological activities related to anti-atherogenic and vasoprotective activities. These includes: cellular cholesterol efflux capacity (CEC) also referred as reverse cholesterol transport (RCT) process, i.e removal of cholesterol from the arterial wall macrophage-foam cells and HDL-facilitated cholesterol transport to liver for excretion, anti-inflammatory function, anti-oxidative function, vasodilatory and anti-thrombotic activities (111). HDL also has a role in glucose metabolism and in anti-apoptotic processes (94,112–114).

2.2.3 Epidemiology of HDL-C in context of CVDs

The heritability of CAD has been estimated to be between 40% to 50% on the basis of twin and family studies (115). Evidence derived from large scale epidemiological studies, clinical data and intervention trials have shown that low circulating levels of HDL-C constitute a strong, significant, and independent, predictor of CVDs (116–118). According to simultaneous analysis of four American prospective studies (Framingham Heart Study, Lipid Research Clinics Prevalence Mortality Follow-up Study, Coronary

Primary Prevention Trial and Multiple Risk Factor Intervention Trial), a 1 mg/dl decrease in plasma levels of HDL-C was associated with a 2–3% increase in the risk of coronary heart disease (CHD) (117). Later, this inverse association between HDL-C and cardiovascular risk was confirmed in multivariate-adjusted meta-analyses performed among individuals with different ethnicities (119–124).

As a result of shown clinical relevance between HDL-C and CVDs, HDL is used as a common CVD risk marker. It is implemented in ACC/AHA (46) and ESC/EAS guidelines (47) and is used in many algorithms to estimate risk of CVD (as stated in section 2.1.3), for example the Framingham risk prediction tool (42,125), the prospective cardiovascular münster (PROCAM) score (126) and the systematic coronary risk evaluation (SCORE) approach (121). However, HDL-C is not used as an independent risk marker but is used together with other risk factors such as gender, age, TC, TGs, apoB-100, SBP, smoking status, diabetes, hypertensive treatment, or TC/HDL-C ratio and LDL-C.

2.2.4 HDL-C paradox and HDL quality

With recent findings, the view of inverse relation between HDL-C and CVDs has been challenged. Mendelian randomization study (83)(129) and some other genome wide association studies (128,130) have not supported the causal relation between HDL-C levels and the atherosclerotic disease process. Furthermore, several randomized controlled clinical trials of HDL-C raising interventions such as use of CETP inhibitors have failed to demonstrate the reduction in risk of major adverse cardiac events or were stopped during the study because of the associated pathological (off-target) finding or lack of efficacy (131–135). In addition, it has been reported that there is a significant difference in HDL subpopulations of healthy individuals and CHD patients (136). This difference is independent/ less associated with HDL-C levels (137,138). Moreover, a U-shaped relationship between HDL-C and total mortality was demonstrated in the report from over one million US veterans (139), and similar observations were obtained in Copenhagen cohorts (140) and other cohorts (141).

These studies over time have implied that the relationship between HDL-C and CHD is complex, and cholesterol content of HDL (HDL-C) may not solely be an appropriate metrics of the impact of this lipoprotein fraction on CVD risk. These observations introduced/reinforced the principle of 'HDL quality' in atherosclerosis rather than HDL-C alone. The idea is a shift from HDL-cholesterol to HDL-function where HDL quality refers to the functionality of HDL particles (section 2.2.2), which in turn is defined by its protein and lipid content (134). The major atheroprotective function of HDL is connected to its ability to remove cholesterol from macrophage-foam cells, i.e. CEC or RCT which measures *in vitro* the potential of individual's

HDL pool to promote cholesterol removal from cholesterol donor cells typically as macrophages. However, molecular determinants of CEC, and, the effects of various diseases and therapeutic interventions on CEC have not been clarified in detail yet. Therefore, it becomes very beneficial to identify a biomarker that could reflect the HDL-associated function (HDL quality) in context of atherosclerosis and is measurable with standardized assays. However, it also needs to be determined if HDL-quality related biomarkers could be used as independent biomarkers or in association with other CVD related risk factors.

2.2.5 HDL analyses

HDL measurement in clinical practice is performed by quantitating the total HDL-C by using standardized precipitation based techniques followed by cholesterol estimation by enzymatic method. This precipitation method is not much in use at the time being and the so called direct HDL methods are widely used. Briefly, in direct HDL-C assay, cholesterol in non-HDL particles (i.e. apoB-containing lipoproteins) is “masked” with antibodies (anti-apoB antibody) and HDL-C can be quantified. Other methods to avoid the effect of apoB-lipoproteins is to use polymers (such as polyethylene glycol) or combination of polyanions, typically such as heparin–$MnCl_2$, dextran sulfate–$MgCl_2$ or phosphotungstate–$MgCl_2$, and a divalent cation, such as magnesium, heparin–manganese, or calcium that cause apoB-particles to precipitate and HDL-C can be analyzed from the supernatant after centrifugation by an enzymatic reaction (142,143). As said, direct HDL methods are part of modern lipidology in clinical chemistry laboratories connected to hospitals.

HDL particles harbor many pleiotropic properties that are unrelated to their cholesterol content or cholesterol transport in circulation but, however, are related to CVDs. These properties could be measured by HDL functional assays (Table 2). Physicochemical traits are considered to provide some insight into HDL functionality (101,144) and are therefore also used to measure HDL. Techniques are available for separating HDL particles based on particle composition/ physicochemical characteristics (Table 2). Standardized immunoassays (ELISA-based, nephelometric or turbidimetric methods) for serum apoA-I measurement are also available (145). Detailed HDL protein/lipid composition (physicochemical properties) and function add to the further challenges in HDL research and investigation of cardiovascular field and the accumulating data comparison is rather difficult (146). Limited proof of concept (of relation between functional properties of HDL and CVDs) and other disadvantages such as complicated, difficult-to-standardize functional test procedures, need of specialized laboratory and experts for carrying out these assays make their wide application/certification quite challenging at the time being (147,148).

Table 2. Methods for HDL measurement or analysis.

Method	Purpose
Precipitation based direct method	Quantification of HDL-C
HDL functional assays	Measure the functions of HDL
Cholesterol efflux assay (143,149)	• to quantify the rate of cholesterol efflux from cultured cells to an acceptor particle or to plasma
LCAT assay (150,151)	• fractional esterification rate
HDL anti-inflammatory potential • MCP1 production: inflammatory index (160,161) • Monocyte chemotactic assay (154)	In vivo analysis of ability of HDL to inhibit LDL oxidation and monocyte chemoattractant protein 1 (MCP1) expression.
HDL antioxidant capacity assays • Cell free assay (155,156) • PON1 and MPO assays (143,157,158)	• examines the effect of HDL on the production of reactive oxygen species • to measure activity of antioxidant enzymes
Endothelial assays • Vascular endothelial eNOS assay(159,160) • Endothelial ICAM/VCAM assay (159)	• measure the ability of HDL to modulate eNOS expression and NO production. • to quantify the expression of ICAMs and VCAM-1 derived from vascular endothelial cell which are modulated by HDL
Proteomic	To identify diversity of HDL associated proteins and peptides
Shotgun • LC-MS based MALDI-TOF (156) • LC-MS/MS based SELDI-TOF (161) Laser desorption ionization approaches • LC-MS/MS ESI (162)	• effect of protein heterogeneity on the functionality of HDL particles
Lipidomic	To identify lipid diversity
Shotgun: direct infusion • LC–ESI-MS (163) • ESI-MS/MS (164) Indirect infusion • MALDI (QIT)-TOF-MS (163,165) • Triple quadrupole-MRM-MS (166)	• evaluation of effect of different lipid species on the risk of atherosclerotic CVD and on HDL functionality
Based on particle composition/ physicochemical characteristics of HDL (143) • Ultracentrifugation (density-gradient and vertical auto profile (VAP) • Two-dimensional electrophoresis (2D-PAGE) • Nuclear magnetic resonance (NMR) • Ion mobility • Capillary electrophoresis, immuno-affinity chromatography and size-exclusion chromatographic methods	To isolate different HDL subpopulation

2.3 Antibodies and their development

Antibodies are a class of proteins which have a very important role as a tool of biomedical research and *in vitro* diagnostics. It is the unique binding properties of antibodies i.e. ability to specifically and strongly bind to a particular antigen, that makes them ideal reagents for various biomedical applications.

2.3.1 Conventional antibodies

Antibodies also known as immunoglobulins (Igs) are glycoproteins produced by plasma cells (B cells) in response to foreign antigenic molecules known as immunogens or antigens. There are five types of Igs in humans: IgD, IgE, IgM, IgA, and, IgG. Their basic Y-shaped structural unit is made up of four polypeptide chains with two identical heavy chains having molecular weight (MW) of 50 kDa and two identical light chains with MW of 25 kDa (167,168) (Figure 5). The light chain consists of a variable (VL) domain and a single constant (CL) domain (169), whereas the heavy chain consists of a variable (VH) domain and three or four constant (CH) domains (170,171). Among the Ig classes, IgG is the most abundantly found in human serum (75% of all Ig) (illustrated in Figure 5). IgGs are also by far most commonly used in the immunodiagnostic applications. As consisting of one Y-shaped unit and containing some conserved glycans, the total MW of an IgG is ~160 kDa. Each IgG contains two antigen-binding fragments (Fab) in addition to the tail part termed as fragment crystallizable (Fc) (172). Six regions distributed between the VH (CDRH1, 2 and 3) and VL (CDRL1, 2 and 3) which are referred to as complementary-determining regions (CDR) are primarily responsible for binding to the antigen (173,174). Fc region on the other hand is composed of constant domains from the heavy chains and is responsible for modulating immunological activities (175).

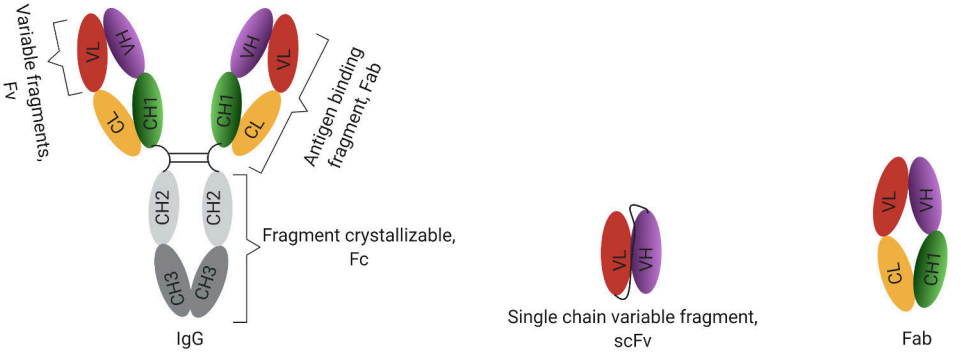


Figure 5. Schematic representation of a full-length antibody (IgG), and different antibody fragments derived from it. In the figure, CH, constant heavy chain; CL, constant light chain; IgG, immunoglobulin; Fab, antigen binding fragment; scFv, single chain variable fragment; VH, variable heavy chain; VL, variable light chain. Figure is generated by using Biorender.com.

2.3.2 Recombinant antibodies

The modular nature of the antibodies allow the generation of smaller antigen binding fragments such as the single chain fragment variable (scFv) and fragment of antigen binding (Fab) (Figure 5) by using genetic engineering tools or enzymatic methods (176,177). Recombinant antibodies are produced by expressing antibody coding genes in a recombinant host. Due to the relatively simple molecular structure of the antibody fragments also bacterial host can be used for that purpose. The fragments could be used as such (178–181) or as fusion to other proteins such as other antibody fragments/domains (182–184) or bacterial alkaline phosphatase (AP) (185–188). Fusion to these proteins generate binders with improved avidity, multi-specificity or additional enzymatic activity, which help in improving the performance of immunoassays e.g., through faster kinetics (178–181,185,186).

The scFv was first described by Bird et al. in 1988. It is 25-27 kDa in size and is composed of a VL and a VH domain (Figure 5) linked by glycine- and serine-rich flexible peptide of 15-20 amino acids (176,189–192). ScFv fragments have been utilized in many antibody development campaigns to generate binders against various targets, and also applied in the development of immunoassays for diagnostic use (178,179,185,188,193–198). There are variants of scFvs available such as tandem scFvs (199–201) and diabodies (202) which provide benefits in terms of avidity or bi-specificity (ability to bind two different antigen).

Fabs are another commonly used antibody fragments which are studied for diagnostic purposes(179–181), therapeutic (203) and clinical studies (204). They have molecular mass of ~50 kDa and are composed of a light chain (VL and CL domains), and, VH and CH1 domains of the heavy chain (Figure 5). Compared to

scFvs, Fabs typically show higher stability (205,206), which is due to the non-covalent interaction between CL and CH1 and the inter-chain disulfide bond between them (176,190).

2.3.3 Phage display and antibody libraries

Monoclonal, single epitope recognising antibodies i.e. mAbs are routinely developed through hybridoma technology (207) or by biopanning of recombinant antibody libraries using display techniques (208,209). There are many display techniques available such as phage display (210), yeast display and display on bacterial surfaces(211). Filamentous phage display was first demonstrated by George P. Smith (212). It was the first and is still the most popular *in vitro* antibody selection technique in use because of its robustness and simplicity (213,214). Several advantages of *in vitro* display techniques have made them an attractive choice for antibody generation over conventionally used hybridoma technique. Unlike the hybridoma technique, *in vitro* display technique in combination of a recombinant antibody library is independent of animal immunization. This provides better control of target antigen (over antigen proteolysis and clearance etc.) (11) and the selection conditions such as buffer, pH, temperature, and competitor proteins (12). *In vitro* display technologies allows generation of antibodies against challenging targets such as different strains of the pathogens, toxic molecules and highly conserved or non-immunogenic targets (11,204,215). In *in vitro* display technology, counter selection against undesired epitopes/ antigens is possible with biopanning approach (Figure 6) by using various conformation states or homologues of antigens to direct the antibody selection (216,217). Moreover, it is possible to obtain a variety of binders against an antigen (218,219) and to carry out further affinity maturation to obtain binders with good affinities (ranging from picomolar to up to femtomolar levels) (220–224). The readily accessible antibody gene sequence enables the genetic tailoring (via multimerization, addition of enzymes, fluorescent proteins or tags) of antibody constructs for improved properties such as improved binding and possibility for direct detection. Furthermore, since *in vitro* display methods are primarily based on microbial system, antibody generation is comparatively cost-effective, less time consuming and have potential for high-throughput processes via automation (12,13). Although there are very many advantages of *in vitro* display technique, the method also has a few drawbacks. The antibodies discovered by in vitro display techniques might have unfavorable biophysical properties, for example they could be prone to aggregation and polyreactivity (225–227). Therefore, it is important to screen, eliminate or improve antibody candidates at earlier stages.

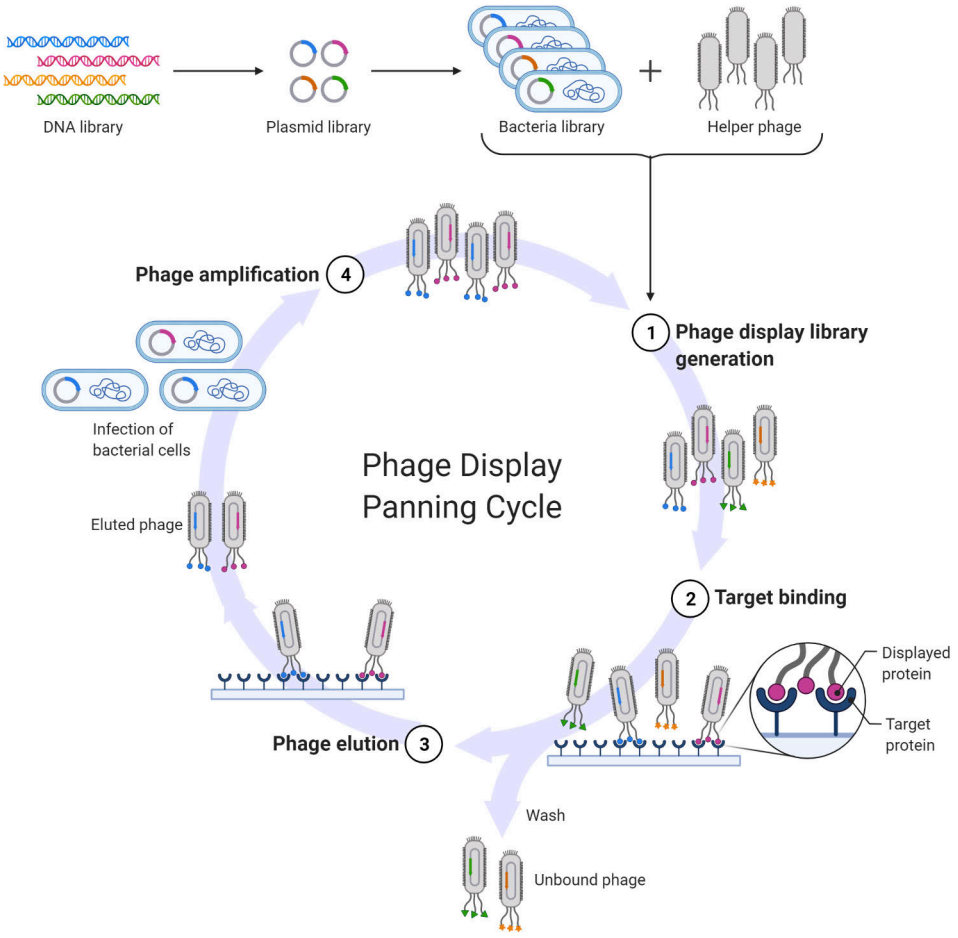


Figure 6. Phage display library generation and biopanning process for selection of target specific antibodies. In the biopanning procedure, the target molecule of interest is immobilized on a solid support and incubated with the phage library to allow phage binding (2). Unbound phages are washed away, while bound phages are eluted (3). The eluted phages are then amplified by infecting the bacterial cell with the eluted phage (4), and applied to the next selection round. Depending on target complexity, this procedure is repeated 3-6 times. This figure is created by using Biorender.com.

In order to construct a phage displayed antibody library (Figure 6), a repertoire of antibody fragment encoding genes is fused to the gene of phage coat protein, typically protein III (pIII) (228). Transformation of *E. coli* cells with plasmids containing such gene construct results in production of phage particles that display the antibody fragments on phage. The display can be either mono- or multivalent. In monovalent display in average a single copy of an antibody per phage, whereas in multivalent display 3-5 copies of the antibody are displayed as a fusion to pIII on phage. The valency of the display can be controlled by using different phage display

vectors such as phagemid vs. phage vectors, and different helper phages such hyper-phage for multivalent display on pIII (229).

The antibody libraries are termed differently depending on the source from where the immunoglobulin genes are obtained from. The established library is called natural antibody library if the source is an immunized or non-immunized donor (immune or naïve library) (208,209) or synthetic library (230) if the genes of interest are artificially designed.

The binders are isolated from the established library using affinity selection process called biopanning (Figure 6). For biopanning, the phage displayed library of antibodies is incubated with an immobilized target (antigen), followed by repeated washing to remove unbound/unspecific phage. Binders are then eluted using acid, high salt or protease treatment and then propagated in the appropriate host cells (*E. coli* in the case of filamentous phage display) (231). This method has been widely used in many laboratories to produce a tremendous numbers of antibodies (228).

2.4 Immunoassays

Immunoassay can be defined as an analytical technique which detects the presence of or measures the concentration of a target molecule (referred as analyte) in solution by making use of specific antibody (usually) or antigen (sometimes) as recognition agent. Immunoassays are used commonly to analyse the analytes, primarily different proteins but also low-molecular-weight compounds, in biological fluids. The first immunoassay developed was for insulin by Solomon Berson and Rosalyn S. Yalow in 1959 (232).

2.4.1 Basic assay configurations

In general there are four basic immunoassay configurations (schematic representation shown in Figure 7), depending on whether the immunoreaction takes place on a solid phase (heterogeneous) or in solution (homogeneous) and whether the antibody–antigen binding is monitored directly (non-competitive) or via displacement of a labeled antigen or antibody (competitive) (232,233). Common solid phases used in heterogeneous immunoassay are microtitre plates on which the antigen is either bound directly or captured by the use of an appropriate antibody (Figure 7 A (i-iii)). The presence of the antigen is then determined through the binding of an appropriately labeled antibody and measuring, e.g., the fluorescence signal. In non-competitive assays, the signal is directly proportional to the amount of antigen in the sample. The non-competitive immunoassay can be further categorized into single-site immunoassay (234) if the antibody binds to single epitope (Figure 7 (i)) and into two-site immunoassay (235) if two different epitopes

(spatially well separated) are targeted by two different antibodies (Figure 7 A (ii)), respectively. In competitive assays (232), the labeled antibody competes with the unlabeled antibody present in the sample for the binding to the target antigen or in other case labeled antigen could also be used for competing with unlabeled antigen for binding to the antibody (Figure 7 A (iii)). The competition thereby leads to the reduced signal which is inversely proportional to the amount of antibody or antigen present in the sample.

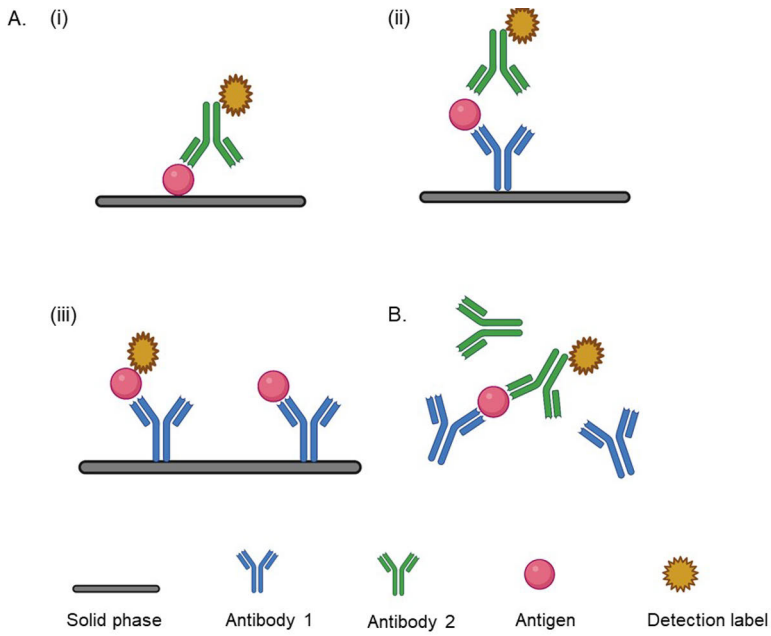


Figure 7. Standard configurations of immunoassay. (A) Heterogeneous immunoassays: (i) Single-site immunoassay, (ii) Two-site immunoassay, and (iii) Competitive immunoassay, and (B) Homogeneous immunoassay. The signal is generated by the labelled antibody in each assay type. Figure is generated using Biorender.com.

Based on the detectable label (reporters) used in the immunoassays the assays are named as radioimmunoassay (RIA), enzyme immunoassay (EIA), fluoroimmunoassay (FIA) and time-resolved fluorescence immunoassay (TRFIA) when the labels used are radioactive molecules, enzymes, fluorescent molecules and lanthanide chelates, respectively. Labels with high specific activity are one of the key factors for reaching high assay signal and high sensitivity in immunoassays. Among the stated labels, radioactive labels have very low specific activity, for example in case of Iodine ^{125}, only one detectable event occurs per 7.5 x 10^6 molecules. Due to various other disadvantages such as health hazards, need of special handling of the reagents and short half-life time, radiolabels are rarely used

in immunoassays presently (233) compared to non-radioactive labels. Enzymes are most commonly used labels for immunoassays (EIA) because of the possibility of signal amplification that is, production of multiple detectable molecules by each bound antibody–enzyme conjugate. However, the enzymatic assays have drawbacks related to limited linear range, the need of additional reagent and time required for signal development (233). Some of these problems are circumvented in FIAs where fluorescence signal produced by antibody-fluorophore conjugates complex can be measured directly on the surface or in solution thus avoiding the need of additional reagent and time. Fluorophores, in general absorb light at one wavelength and emit light at a longer wavelength. Fluorescent labels have high specific activity and are capable of delivering many detectable events/signals per label molecule (236). The use of fluorescent labels in immunoassay, however, can be limited by disturbing high background signaling which arises due to fluorescence and scattering from the sample itself (serum, serum components and plasma) or employed assay compartments. These factors may severely restrict the practical assay sensitivity (237,238). These interferences can be effectively overcome by using lanthanide ion based chelates as labels in the immunoassays as described in the chapter 2.4.1.1.

2.4.1.1 Time-resolved fluorescence immunoassay (TRFIA)

TRFIA is lanthanide reporter based immunoassay where fluorescence emission is measured after a certain delay of time following pulsed excitation (Figure 8) (237). Lanthanides are a group of metals with atomic numbers ranging from 57 to 71. The most common lanthanide ion used in the TRFIA is europium (Eu^{3+}). Other lanthanides used in TRFIA based clinical investigations include samarium (Sm^{3+}), dysprosium (Dy^{3+}) and terbium (Tb^{3+}). TRFIA is highly sensitive (to up to 10^{-14} mol/L) (14), and has wide dynamic range. This is offered by the enhanced fluorescent properties of the lanthanides when chelated with some ligands (239) which helps to transfer the excitation energy to the lanthanide ion for fluorescence emission. Following are the properties of lanthanide chelates:

(1) Long fluorescence lifetime (from microseconds to milliseconds) (15,16) enables the fluorescent emission to be read at a time well after the background fluorescence (lifetime in nanosecond scale) (240) has diminished, providing a greater dynamic range to the assay.

(2) Wide excitation spectrum as well as narrow and sharp emission spectrum (241,242), enables easier identification of different lanthanides from one another and contribute to improved signal/background ratio (S/B).

(3) A large difference (200–300 nm) between absorption and emission spectra (known as Stokes shift) (17–19), with improved spatial resolution minimizes

interference from fluorescent molecules in the sample (e.g., serum proteins, NADH, bilirubin etc.) and from light scattering due to proteins or colloids.

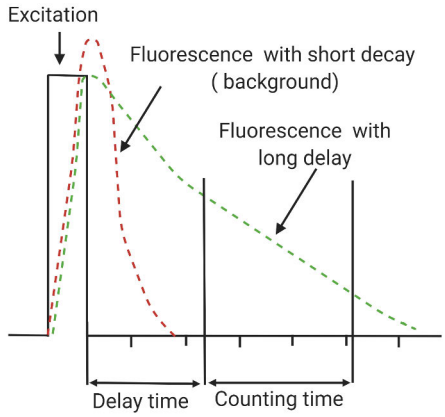


Figure 8. Principle of time-resolved fluorescence (TRF) measurement. In TRF measurements the sample is first excited with a short UV-light pulse and fluorescence is measured after a delay time during which the background fluorescence reduces to almost zero. Figure is created using Biorender.com.

Dissociation-enhanced lanthanide fluorescence immunoassay (DELFIA) is a TRF immunoassay format which employs two different chelate systems, one chelate for labelling purpose and another for developing and enhancing the fluorescence. In this assay format, the lanthanide chelate coupled to the tracer antibody is not fluorescent and in a final step, an enhancement solution (ES) is added (239). ES has acidic pH which enables the release of the lanthanide ion from the antibody bound chelate. ES also contains high concentration of light harvesting chelating compound which helps to transfer the excitation energy to the lanthanide ion for TRF emission.

2.4.2 Optimization and standardization of the assay

Immunoassay development typically involves optimization of the conditions for all the steps within the test protocol. Optimization of an immunoassay can be described as a process or methodology of making a design as fully perfect or functional as possible by establishing ideal concentration for each assay reagent and for each step (243). A poorly optimized assay could lead to method-related causes of standardization differences.

Standardization of immunoassay is a process which ensures that the assay performed for a particular analyte at different times and locations give the same result (244). It requires, (i) value assignment to unknown samples using a standard, (ii) a standard that is identical to the analyte in the test samples, (iii) absence of assay

interference, (iv) a reference method, (v) demonstration of inter-method agreement (245). Attention to the aspects of assay design can significantly reduce method-related bias, and sample interferences. Therefore, standardization depends on exactly defined and optimized assay reagents and procedures, for example composition of buffers, formulation of antibodies or antigens whether labeled, unlabeled or immobilized, and the mode of separation and signal measurement (245). Use of separation matrix (the solid phase) with proper and efficient washing steps aid to minimize the assay interference. Immunoassays critically rely on the ability of the antibody to bind the analyte in question. Lack of specificity and adequate affinity in the presence of other potentially disturbing or cross-reacting substances are the root cause of immunoassay bias and variation. Assay sensitivities of both competitive and non-competitive assays are dependent on antibody affinity. The sensitivity of competitive assays is directly limited by the affinity of the antibody while in non-competitive assays it is critically dependent on the degree of nonspecific binding of the labelled antibody (236). Detection labels are one of the important contributors to the performance of the immunoassay in terms of signal strength and sensitivity. From optimization point of view, the labelling of antigen or antibody should be as lenient as possible to minimize effects on the antibody antigen interaction. The buffer composition and other assay conditions may have an influence on the conformation of proteins, which in turn could affect antibody binding to its epitope. Hence, careful selection of buffer ingredients, optimization of pH, assay temperature, ionic strength and other active constituents can help to minimize methodological bias and secure a standardized, robust performance of immunoassays.

3 Aim of the study

The main goal of this study was to develop novel HDL detection based immunoassay tools for improved risk assessment and monitoring of CAD. A phage display based strategy was used for the antibody generation to identify binders that could bind differently to CAD and non-CAD HDL. In context of risk assessment, HDL-antibodies were sought to recognize the same epitope present on HDL of CAD-patient derived samples, as well as in samples of asymptomatic individuals at an increased risk of CAD. To this end, the isolated antibody fragments were produced in different genetically modified formats and evaluated through implementation into different immunoassay designs. The specific goals of the thesis were as follows:

1. To isolate HDL antibodies from phage display based antibody library and characterize them to identify various CAD specific HDL antibodies.
2. To engineer and produce the recombinant anti-HDL antibodies of different formats (scFv-AP, scFv-phage) to enable incorporation into different immunoassay formats.
3. To develop novel two-site apoA-I/HDL TRF-immunoassays using phage expressed detector antibodies and to enable a first clinical validation of their capability to identify adverse cardiac events using longitudinal samples from a chest pain cohort.
4. To further simplify and develop novel apoA-I/HDL TRF-immunoassays using a direct two-site assay format based on Eu-labelled scFv-APs as detector antibodies. Evaluation of their diagnostic performance with a cohort of well characterized samples from CAD suspected patients.

4 Materials and Methods

A summary of material and methods used in this study is provided here. Main steps of the thesis are shown in Figure 9. The detailed information is available in the original publications I-III

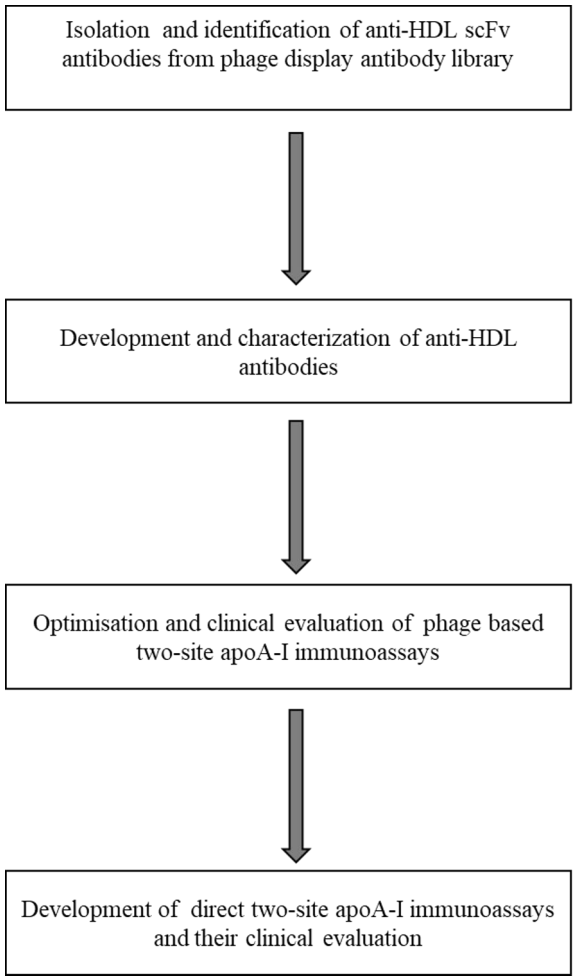


Figure 9. Flowchart showing the main steps of the thesis study.

4.1.1 Samples

The samples used in this study (Publication I-III) are listed in Table 3. Samples from cohort-I had patients with (i) ACS where patients had UA, STEMI or NSTEMI, n = 12, (ii) patients with stable CAD (n = 12), and, (iii) patients without CAD (no-CAD, n=12). Samples of cohort-II were from individuals who visited the hospital due to chest pain. Samples were collected from individuals at the time of admission to the hospital (n = 195) and from individuals (n=134) selected for hospital stay for at least 24 hrs at the time of discharge. The patients were divided into group of individuals who did not have myocardial infarction (non-MI: admission samples, n = 109 and discharge samples, n = 48) and did have myocardial infarction (MI: admission samples, n = 86 and discharge samples, n = 86). Cohort-III (n = 252) was consisted of symptomatic patients referred for angiography due to suspected obstructive CAD (Figure 10). Informed consent was obtained from the participants prior to sample collection. All the samples were collected according to normal laboratory routines and were generally stored at -70 °C for long-term preservation. The study protocol was approved by the Ethics Committee of the Hospital District of Southwest Finland and the study complies with the Declaration of Helsinki as revised in 2013. All participants gave an informed consent.

Table 3. Description of the samples used in the study.

Collection place	Number of samples used	Study population	Sample matrix	Publication
Central Hospital Kristianstad (Sweden)	10	Routine patients with apoA-I concentration between 97 – 257mg/dL	Serum	II
Helsinki University Hospital (HUS)	10	Routine patients referred to investigate lipid parameters; HDL-C concentration between 0.81 – 2.74 mmol/L	Serum	I, II, III
Department of Biotechnology /University of Turku (DBUT)	10	Non-cardiac and healthy individuals	Serum, Li-heparin plasma and EDTA plasma	II, III
Turku University Hospital	10	Patients referred to investigate lipid parameters; HDL-C concentration between 0.78 -2.58 mmol/L	Li-heparin plasma	I, II
Corogene (Cohort-I)	36	Cardiac patients assigned to angiogram. (i) 12 ACS patients (unstable angina or STEMI or NSTEMI, (ii) 12 patients with stable CAD, and, (iii) 12 patients without CAD	Serum	II
Turku University Hospital (Cohort-II)	Leftover samples, 195	Cardiac patients visiting emergency department. Samples collected from 195 patients at admission (non-MI 109, MI 86) to hospital and from 134 patients during discharge (non-MI 48, MI 86) from hospital.	EDTA-plasma	II
Turku University Hospital (Cohort-III)	252	Cardiac patients assigned to angiogram. 197 samples analyzed; 116 patients with atherosclerosis and 86 patients were without atherosclerosis.	Serum	III

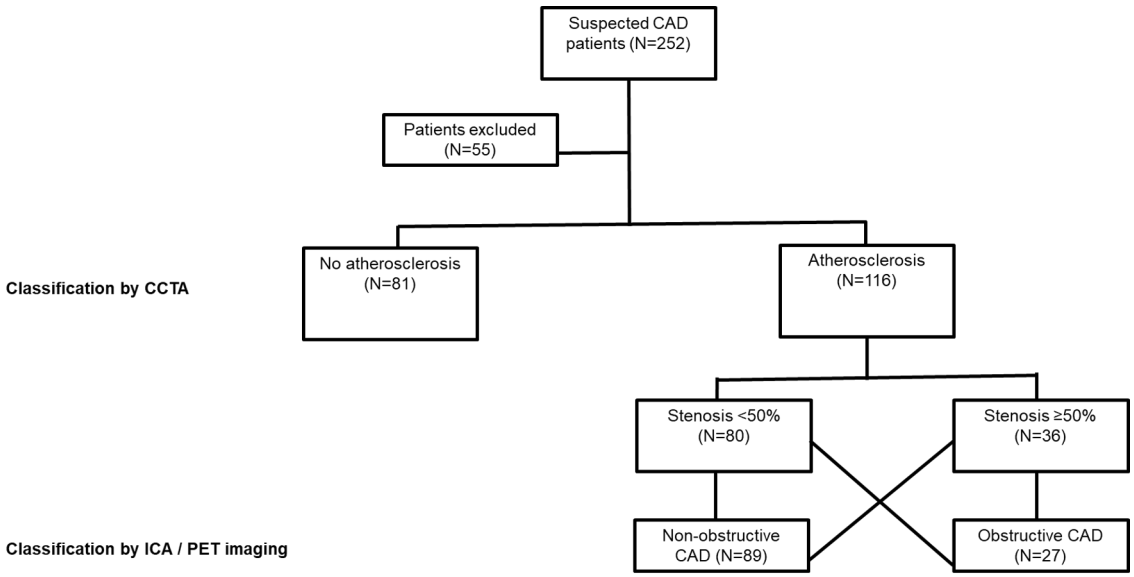


Figure 10. Study population. Obstructive CAD is defined as coronary atherosclerosis associated with abnormal stress myocardial blood flow (MBF) on CCTA or >70% coronary stenosis on ICA. N,number of individuals; CAD, coronary artery disease; CCTA, coronary computed tomography angiography (CCTA); PET, positron emission tomography; ICA, invasive coronary angiography. Adapted from publication III.

4.1.1.1 Measurement of biochemical parameters

Several biochemical parameters were measured from the patient samples used in this study. ApoA-I concentration was determined by using polyclonal anti-apoA-I with immunoturbidimetric method (Abott Architect, Sweden) (Publication II) and by ELISA (Publication II and III) as described by Siggins et al. (246). Concentration of HDL-C was measured using enzymatic method (Publication II) as described by Laaksonen et al. (65) or by phosphotungstate-$MgCl_2$ – precipitation method (Publication II and III) (247). Details regarding measurement of other parameters: apoB-100 concentrations (Publication II), TC and TG (Publication II and III), LDL-C (Publication II and III), CETP (Publication II), PLTP (Publication II and III), PON-1 activity (Publication III), free glycerol (Publication III), serum PL (Publication III) concentration could be found in original publications.

4.1.2 Antibodies

Conventional mono- and polyclonal antibodies used in this study are listed in Table 4.

Table 4. List of research antibodies used in the study.

Antibody	Specificity/ Antigen	Molecular form	Manufacturer	Publication
Anti-phage 9E7	M13 phage major coat protein	Mab	Department of Biotechnology, University of Turku (DBUT) (Finland)	I,II
Anti-*E. coli* AP	AP	Pab	Life Span Biosciences (USA)	I, II
Anti-apoA-I	Human apoA-I	Mab	Medix Biochemica (Finland)	I, II
Anti-human ApoA-I	Human apoA-I	Pab	Academy Bio-Medical Company (USA)	I
Anti-apoB	Human apoB	Pab	Fizgerald (USA)	I

4.1.3 Isolation and preparation of lipoproteins

Total HDL (=HDL_2 + HDL_3 subclasses) (Publication I, II and III), LDL (Publication I), very low-density lipoprotein (VLDL) (Publication II) and intermediate-density lipoprotein (IDL) (Publication II) were isolated using ultracentrifugation from serum of healthy individual/s. HDL was isolated from 25 individuals who had CAD and did not had CAD using ultracentrifugation and pooled; pooled preparations were used as CAD HDL and ctrl HDL (Publication I).

Reconstituted HDL particles (rHDL) containing apoA-I were prepared by the modified cholate dialysis method (Publication I) (248). Different rHDL complexes were prepared with the following apoA-I:phosphtidyl choline (PC):sodium cholate (C) molar ratios: rHDL, 1:30:12.5; rHDL1, 1:30:7; rHDL2, 1:140:7; rHDL3; 1:140:12.5, and rHDL4, 1:250:30.

4.1.4 Other reagents

ApoA-I was from Yo Proteins AB, Sweden and apoA-II (Publication I) was from Athens Research & Technology, USA. Hyperphage was from PROGEN Biotechnik GmbH, Germany and VCS M13 helper phage was from Stratagene.

Assay buffer (AB) (Publication I), wash buffer (Publication I-III), streptavidin coated plates (regular type, Publication I and II, and, low fluorescence type, Publication III) and europium fluorescence intensifier (EFI) (Publication I-III) were from Kaivogen (Finland). HDL AB contained 50 mM Tris–HCl pH 7.75, 150 mM NaCl, 0.05% Na-azide, 20 μM DTPA, 20 μg/ml cherry red, 0.05% bovine γ-globulin and bovine serum albumin (BSA) (0.5%, Publication I or 2.3%, Publication II and Publication III or 4% in Publication II and Publication III). The para-nitrophenylphosphate (pNPP) substrate buffer (Publication I) contained 5 mM

pNPP, 500 mM Tris–HCl pH 9, 200 mM NaCl and 10mMMgCl_2. Lipoprotein deficient serum (LPDS) (Publication II) and Intralipid (20 % emulsion) (Publication II) were from Merck, Germany.

HisPur Ni-NTA (Publication I-III) Spin Columns were bought from Thermo Scientific (USA), Lysozyme-Chicken Egg white was from Sigma Aldrich (USA), and Benzonase was from Merck (Germany). Reagents for SDS-PAGE and Bradford assay were purchased from Bio-Rad (USA).

4.1.5 HDL antibody fragment selection and production

4.1.5.1 Selection of CAD HDL antibodies from synthetic antibody libraries

Phage-displayed scFv antibodies were isolated from the synthetic antibody phage libraries scFvP (249) and scFvM (250) as described in (249), only exception being the use of Hyperphage for phage generation to obtain multivalent display (Publication I). The selection of the library was done against isolated HDL or HDL3 pooled from 24 CAD patients (CAD HDL) by using thirteen different panning reactions. For removal of antibodies against undesired proteins subtractive panning was done with HDL pooled from 24 healthy individuals (ctrl HDL) or with apoA-I or apoA-II (Publication II).

The pannings were performed in six different buffer types using paramagnetic beads saturated either with the CAD HDL, ctrl HDL, apoA-I or apoA-II (Publication III). In the first panning cycle, 1E13 scFv-phage were used for initial negative selection and incubated with ctrl HDL, apoA-I or apoA-II coated beads. The unbound phage were then let to react with the beads coated with target antigen CAD HDL. Additionally, in panning D (Supplementary Table 1 in Publication I) ctrl HDL was used as a soluble blocker and the reactions were done at 4 °C to minimize component exchange between the HDL particles. Then the beads were washed with panning buffer to remove weakly bound unspecific phage and the remaining bound phage were eluted with trypsin or with DTT. Trypsin activity was blocked by adding 5× molar excess of SBTI. After the first panning round, the scFv-DNA was recovered from the eluated phage using PCR based amplification. The amplified PCR product was purified with a PCR purification kit (Qiagen, Germany) and digested with SfiI (Thermo Scientific, Waltham, USA) followed by ligation into the pEB32x vector (250). Later, the ligation mixture was transformed into SS320 cells by electroporation and the cells were then infected with VCS M13 (Stratagene, California) for phage production (251). The subsequent rounds of enrichment were performed with 10-100 times (1E12 or 1E11) lesser amount of phage and antigen coated beads , and phage were obtained by infection of XL1-Blue *E. coli* (250).

4.1.5.2 Cloning of anti-HDL antibody fragments as scFv-AP and scFv-phage

ScFv fragments of HDL antibodies were cloned into scFv-AP format by inserting the scFv-DNA into sfiI digested vector pLK06H (Publication I-III) (250,251). HDL antibody clones were already available as scFv-phage after the later rounds of panning (section 4.1.5.1).

4.1.5.3 Production and purification of anti-HDL scFv-AP fragments

The HDL binding clones (anti-HDL scFv) were produced as scFv-AP fusion protein with a 6× histidine tag in *E. coli* XL1-Blue cells in 96-well culture plates (Publication I) or in 200 ml (Publication I-III) shake flask into medium containing SB with 0.05% glucose, tetracycline and ampicillin. The bacteria were cultivated at 37 °C (Publication I-III) and induced with 200 µM IPTG followed by overnight production at 26 °C. Produced cultures were centrifugated for 20 min, 4000 ×g, +4 °C and pellets were stored at -70 °C before purification. Cells in the pellet were lysed by freeze-thaw and lysate was used for screening assay (Publication I) or the scFv-AP antibodies were purified with immobilized metal affinity chromatography (IMAC) using Ni-NTA spin columns (Thermo Scientific, USA) following manufacturer's instructions. The purified protein was stored in PBS (containing 500 mM imidazole due to the IMAC elution), pH 7.4 at +4 °C. Protein purity was analyzed with SDS-PAGE and protein quantification was done with Bradford assay.

4.1.5.4 Production of anti-HDL scFv-phage

Anti-HDL scFv-phage were produced either in monovalent (Publication I) or multivalent (Publication II) form using 200 µl (Publication I) and 20 ml (Publication II) culture volumes, respectively. In brief, cells were grown in SB medium containing 0.05% glucose or 1% glucose and appropriate antibiotics (10 µg/ml tetracycline and 25 µg/ml chloramphenicol) at 37 °C with shaking. Subsequently, for monovalent phage production the cells were infected with VCS M13 helper phage and induced with IPTG (100 µM) followed by overnight culturing at 26 °C (Publication I). The supernatant was collected from 200 µl culture and used for screening assay (section 4.1.7.2) (Publication I). For multivalent phage production, the cells were infected with hyperphage (Publication II). After overnight incubation at 26 ºC, the cells were pelleted by centrifugation and the pellets were dissolved in fresh SB medium containing 0.05 % glucose with appropriate antibiotics as before. The cells were grown at 30 ºC with shaking for 1.5 hr. Then, 50 µg/mL kanamycin and isopropyl-β-D-thio-galactoside (IPTG) (100 µM) were added, and subsequently the cells were grown overnight at 26 °C with appropriate shaking. Phage were

purified from culture supernatants by two precipitations with PEG/NaCl using a standard protocol. The phage titers were determined by measuring absorbance at 265 nm.

4.1.6 Labeling with lanthanide chelate and biotin

Isolated HDL particles: CAD HDL and ctrl HDL and rHDL1-4 were biotinylated with 30-fold molar excess of EZ-Link-NHS-SS-PEG_4 biotin (Thermo Scientific, USA) according to the manufacturer's instructions (Publication I). LDL, apoA-I, apoA-II and rabbit anti-AP Pab (publication I) were biotinylated with 30-fold molar excess biotin isothiocyanate (BITC) (from Department of Biotechnology, University of Turku) as described by Eriksson et al. (252).

ScFv-APs (sc 022, sc 109 and sc 110) were biotinylated with 20-fold molar excess of EZ-Link-NHS-SS-PEG_4-biotin from Thermo Scientific (USA) according to the manufacturer's instructions (Publication II and III) using PBS (pH 7.4).

Anti-human apoA-I Mab 2002 (Publication I and II) and anti-human apoA-I Pab (Publication I and II), anti-apoB100 Pab (Publication I), anti-phage antibody 9E7 Mab (Publication I and II), rabbit anti-*E. coli* AP Pab (Publication I and II) were labeled with 100-150 fold molar excess of Europium (Eu^{+3}) chelate of N1-(4-isothiocyanatobenzyl) diethylenetriamine-N1,N2,N3,N4-tetrakis(acetic acid) (N1-Eu) (obtained from DBUT) as described by Eriksson et al. (252).

ScFv-APs, sc 121 and sc 525 were labeled with 50- and 25-fold molar excess of Eu^{+3} chelate of tetra-tert-butyl 2,2',2'',2'''-(((((4-((4- aminophenyl)ethynyl)-pyridine-2,6-diyl)bis(methylene))bis((2-(tert-butoxy)-2-oxoethyl) azanediyl))bis(ethane-2,1-diyl))bis(azanetriyl))-tetraacetate (Eu^{+3}-WN) (253); Publication III), respectively, as described by (252), with the exception that the reaction was done in PBS (pH 7.4) at 4 °C, overnight on shaking.

The labelled products were separated from free biotin or Eu^{+3}-chelate by gel filtration using Tris-buffered saline with azide (TSA) (50 mmol/L Tris-HCl, pH 7.75, 150 mmol/L NaCl and 0.5 g/L NaN3) for elution except for scFv-APs where PBS (pH 7.4) was used. All europium labeled and biotinylated reagents except lipoprotein preparations were stabilized with BSA (1 g/L), additionally 8% glycerol was used for scFv-APs.

4.1.7 Immunoassays

Several kind of immunoassays were used in this study to monitor the enrichment of binders in phage display selections (Publication I), to screen individual antibody clones (Publication I), to characterize the antigen specificity of the antibodies

(Publication I and II) and for selecting the antibody pairs for two-site immunoassays (Publication II).

4.1.7.1 Monitoring the enrichment of HDL binding phage in pannings

Enrichment of anti-HDL antibody clones (later also referred as HDL binders) in the phage display selection (Publication I) was monitored using phage immunoassay (Figure 11 A). Briefly, for the assay, biotinylated analytes i.e. CAD HDL, ctrl HDL and apoA-I were captured on 96-well streptavidin plates and incubated with shaking at RT. The wells were washed twice. Thousand-fold dilutions of the phage stocks (obtained after each panning round) in AB were added to antigen coated wells and incubated with shaking at RT. The plate was washed twice and N1-Eu anti-phage Mab 9E7 in AB was added. The plate was incubated with shaking at RT and washed four times. 200 µl of EFI was added into the wells, and shaked for 10 min at RT. Finally, time-resolved fluorescence (TRF) for europium was measured with Victor plate reader (PerkinElmer, USA).

4.1.7.2 Screening of individual HDL antibody clones

After enrichment of the anti-HDL scFv antibodies with successive rounds of pannings, the individual antibody clones were screened for binding against HDL using biotinylated CAD HDL, ctrl HDL, apoA-I or rHDL (apoA-I:PC:C, mol/mol ratio; 1:30:12.5) with an immunoassay (Publication I). The screening was done either with scFv-phage (Figure 11. A) or scFv-alkaline phosphatase fusion protein (scFv-AP) (Figure 11 B, C) constructs of antibodies.

For the immunoassay, biotinylated antigens diluted in AB were added to streptavidin wells and incubated. Wells were washed twice. Then culture supernatant (scFv-phage) (see section 4.1.5.4) or lysate (scFv-AP) (see section 4.1.5.3) diluted in AB was added, incubated and washed twice. The bound scFv-phage were detected similarly as described for phage immunoassay in section 4.1.7.1 (Figure 11 A). Detection of bound scFv-AP was done by using pNPP substrate buffer (Figure 11 B) or by using N1-Eu labeled rabbit anti-AP Pab (Figure 11 C). In the former assay, pNPP was added, incubated and absorption was measured at 405 nm (Figure 11 B). In the latter assay, for detection of bound scFv-AP, N1-Eu labeled rabbit anti-AP Pab was added (Figure 11 C) and incubated at RT with shaking. Then, wells were washed four times and Eu TRF was measured as in section 4.1.7.1.

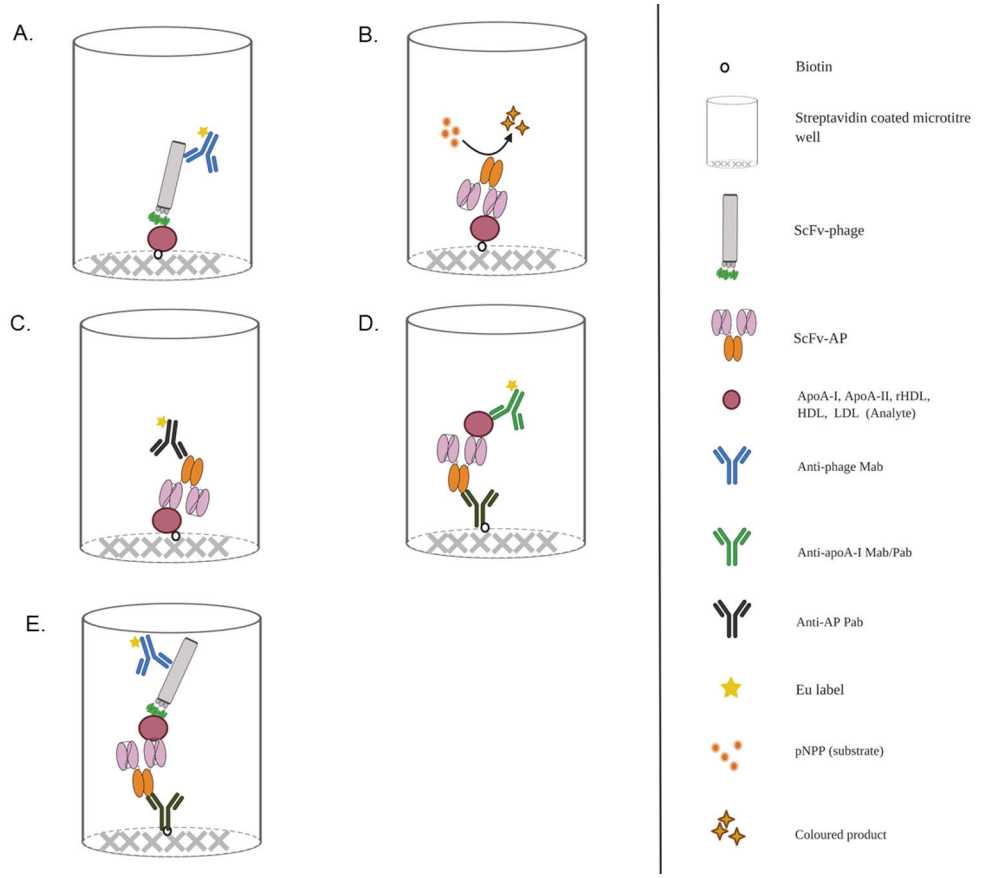


Figure 11. Diagrammatic representation of different immunoassays used for screening and characterisation of the anti-HDL scFv antibodies in this study (Publication I and II). Figure is created by using Biorender.com.

4.1.7.3 Characterisation of antibody fragments against HDL related targets

The purified antibody clones were tested for binding against biotinylated CAD HDL, ctrl HDL, apoA-I, apoA-II, rHDL of different sizes and LDL by immunoassay (Figure 11 C), and, against plasma HDL (section 4.1.7.4.2) in a separate immunoassay (Figure 11 D) (Publication I). In the former assay, the antigens diluted in HDL AB were immobilized on casein blocked streptavidin coated microtiter wells by incubating for 1 h at RT with shaking. The wells were washed twice, purified scFv-APs diluted in HDL AB (with 0.5% BSA) were added and incubated at RT on shaking. Then, the wells were washed twice. Detection of bound scFv-AP was done using N1-Eu labeled rabbit anti-AP Pab as in Section 4.1.7.1.

4.1.7.4 Immunoreactivity of anti-HDL scFv antibodies

4.1.7.4.1 Competitive assay

Antibody clones were tested with competitive assay (Figure 12) (Publication II) by using following steps. Biotinylated HDL diluted in HDL AB (supplemented with 0.5% BSA) was added to streptavidin-coated microtiter plates, incubated at RT on shaking and washed twice. Preincubated (at 4 °C for 3 hrs, on slow shaking) mixture of scFv-AP antibody clone, sample dilution of plasma pool (25x, 50x and 100x) from ten routine samples (Turku University Hospital) and N1-Eu labeled anti-AP Pab was prepared in HDL AB (supplemented with BSA 0.5%) and added into the microtiter wells and incubated on slow shaking at + 4 °C. The wells were washed twice. TRF was measured as stated earlier (section 4.1.7.1).

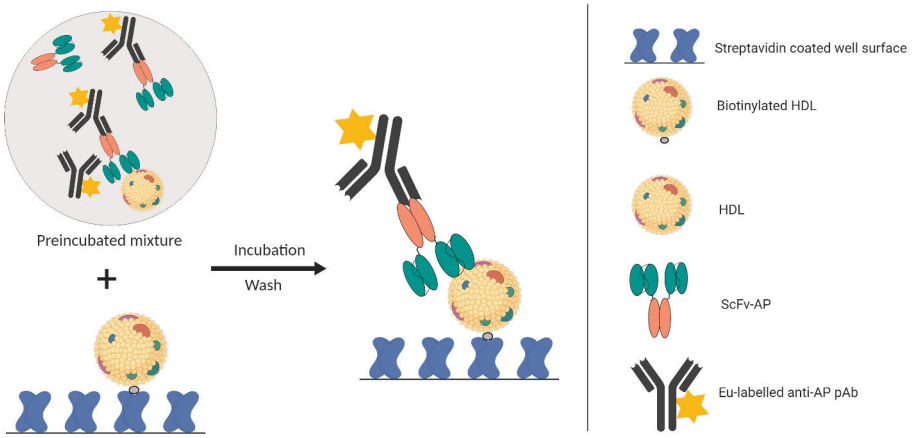


Figure 12. Diagrammatic representation of competitive assay (Publication II). Figure is created by using Biorender.com.

4.1.7.4.2 Sandwich-type immunoassay

ScFv-AP HDL antibodies were individually tested for their immunoreactivity towards (1) plasma pool (Publication I) and (2) serum samples with different apoA-I concentrations using small subset of routine serum samples (Central Hospital Kristianstad, Sweden) in sandwich-type immunoassays (Figure 11 D) (Publication II). In the assays, biotinylated rabbit anti-AP Pab (per well) diluted in HDL AB (with 0.5% BSA) was immobilized on casein blocked streptavidin coated microtiter wells. The wells were washed twice. Purified scFv-AP was added and incubated at RT with shaking followed by two washes. Next, plasma pool diluted 25- and 100- fold in HDL AB (with 0.5% BSA) was added and incubated at RT on shaking. The wells were

washed twice and then N1-Eu anti-apoA-I Mab 2002 or N1-Eu anti-apoA-I Pab diluted in HDL AB (0.5% BSA) was added and incubated with shaking for 1 h at RT. The wells were washed twice and Eu TRF was measured as in section 4.1.7.1. The latter assay with a small subset of serum samples (2) was done similarly except that the sample dilution of 100x and N1-Eu-anti-apoA-I Mab as detection antibody were used.

ScFv combinations (scFv-AP and scFv phage) were tested with plasma pool of ten samples (routine clinical laboratory samples from Turku University Hospital) for identification of suitable HDL antibody pair by using a sandwich-type immunoassay (Figure 11 E). The assay was performed similarly as above where subset of serum samples were tested with the exception of using scFv-AP (100 ng/50 µl/well) as a capture antibody (biotinylated or unbiotinylated), one additional incubation step with scFv-phage (5E8 /50 µl/well) as detection antibody and use of N1-Eu labeled anti-phage Mab as tracer (100 ng/50 µl/well). In case of using biotinylated scFv-AP, biotinylated anti-alkaline phosphatase Pab was not used. Each step included incubation for 1 h at RT on shaking followed by two washes.

4.1.7.5 The final optimized immunoassays for HDL measurement

4.1.7.5.1 Phage based two-site apoA-I assays

Schematic representation of the final optimized phage-based two-site apoA-I assay is shown in Figure 13. Three different phage-based two-site apoA-I immunoassays: assay 022-454, 109-121 and 110-525 were optimized using phage displayed HDL recognising apoA-I antibodies (Publication II). Each assay employed two different apoA-I antibodies: the capture antibody as scFv-AP being either sc 022, sc 109 or sc 110 and the detection antibody (as scFv-phage) being either sc 454, sc 121 or sc 525, respectively.

The assays 022-454 and 109-121 were performed on casein-blocked streptavidin-coated plates using HDL AB supplemented with 4% BSA (in assays 022-454 and 109-121) as diluent. Assay 110-525 was performed with HDL AB containing 2.3 % BSA on BSA blocked streptavidin plates. Total HDL isolated from serum of a healthy individual (section 4.1.3) was used as a calibrator or standard in the assays.

For the assays, biotinylated apoA-I antibodies sc 022, sc 109 and sc 110 (100 ng/50 µl/well) diluted in HDL AB were immobilized on separate streptavidin-coated microtiter wells, incubated for 60 min at RT on shaking followed by two washes. Calibrator and samples diluted in HDL AB, were added into the microtiter wells in separate wells in four replicates and incubated at RT on shaking for 60 min in assay 022-454 and 109-121, and, for 30 min in assay 110-525. After washing the wells twice, corresponding detection antibodies sc 454, sc 121 and sc 525 (2.5E8/50

µl/well) diluted in HDL AB were added in the respective assays and incubated for 1hr at RT with shaking. Afterwards, wells were aspirated and N1-Eu labeled anti-phage Mab (50ng/50 µl/well) diluted in HDL AB was added to them, and incubated for 1 hr at RT on shaking. Finally, wells were washed four times. EFI (200 µl) was added into each well and TRF was measured as described in section 4.1.7.1.

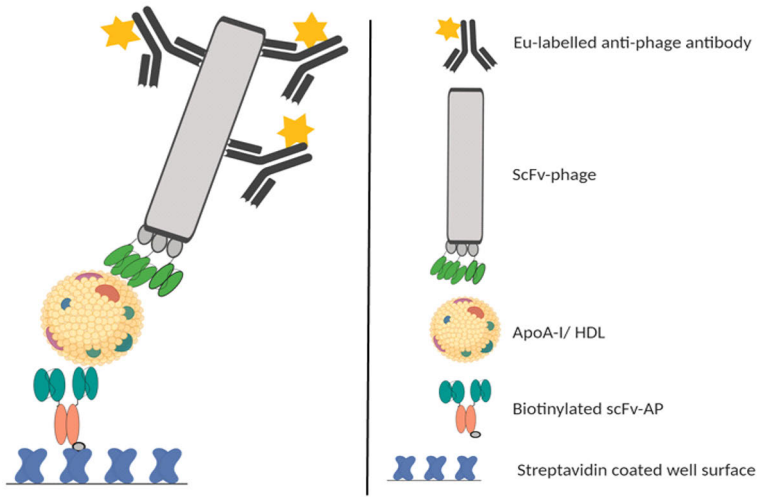


Figure 13. Schematic representation of the principle of the phage based two-site apoA-I immunoassay (adapted from publication II). First the biotinylated scFv-AP (capture antibody) was immobilized on streptavidin-coated plates, then the analyte (HDL / apoA-I) was added and subsequently scFv-phage (detection antibody) was added. The whole complex of scFv-AP- HDL–scFv-phage was then detected by the Eu-labeled anti-phage antibody. Adapted from Publication II.

4.1.7.5.1 Direct two-site apoA-I assays

Direct two-site apoA-I immunoassays were designed using apoA-I antibody pairs sc 109 - sc 121 and sc 110 – sc 525 (Publication III), also used in phage-based two-site apoA-I assays (section 4.1.7.5.1). Principle of these direct two-site apoA-I assays is presented in Figure 14.

Each assay used two different apoA-I antibodies: capture antibody (scFv-AP) sc 109 and sc 110, and, corresponding Eu^{+3}-WN labeled detection antibody (scFv-AP) sc 121 and sc 525, respectively. The assays were performed on streptavidin-coated microtiter plates (low florescence), and, using HDL AB supplemented with 4 % BSA (in assay 109-121) or with 2.3 % BSA (in assay 110-525). Total HDL obtained from serum of a healthy individual (section 4.1.3) was used as standard. Biotinylated capture antibodies sc 109 and sc 110 (100 ng/50 µl/well) diluted in HDL AB were immobilized on separate streptavidin coated microtiter wells and incubated for 1h at

RT on shaking followed by two washes. Calibrator and sample diluted in HDL AB were added in separate wells in five replicates and incubated for 1h at RT with shaking in both the assays. After two washes, Eu^{+3}-WN labelled detection antibodies sc 121 (100ng/50 µl/well) and sc 525 (200ng/50 µl/well) diluted in HDL AB were added in the respective assays and incubated for 1h at RT with shaking. Finally, wells were washed four times and EFI (200 µl) was added into each well. TRF for europium was measured with Victor plate reader as described in section 4.1.7.1.

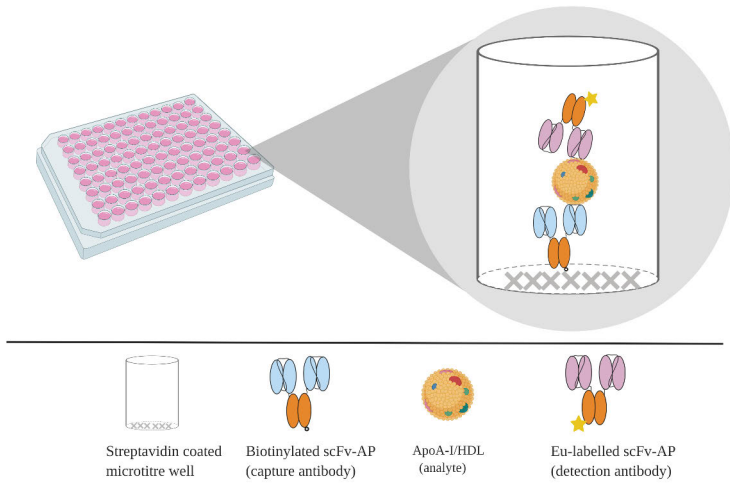


Figure 14. Schematic representation of the principle of the direct two-site apoA-I immunoassay (adapted from publication III). The detection is based on time-resolved fluorescence of europium attached to single chain variable fragment-alkaline phosphatase fusion protein (scFv-AP). Biotinylated scFv-AP was immobilized on the streptavidin coated plate, , subsequently analyte was added. The complex of scFv-AP-HDL is detected by the Eu-labeled ScFv-AP.

4.1.8 Statistical analysis

The analyses were performed with JMP (II, III), SPSS (III) and Origin (Publication II and III). In all statistical tests, two-sided P values <0.05 were considered statistically significant. The used statistical analysis methods and tests are specified with detail in the original publication II and III. In brief, clinical groups were compared using two sample t-test (II, III), Kruskal-Wallis test (II), one-way analysis of variance (one-way ANOVA) (II, III) or Kruskal-Wallis test (II). Correlation between the variable was done by Spearman's correlation or Pearson's correlation (II, III). Categorical variables were compared by Chi-squared test or Fisher's exact test (III). Survival curves were plotted with Kaplan–Meier (K–M) method (II). Logistic regression analysis was performed to estimate odds ratios (III). Area under receiving-operating characteristic (ROC) curve analysis was also done (III).

5 Results and Discussion

This chapter provides a summary and discussion of the results which are available in detail in the original publications (I-III). In the first publication (I), a study where anti-HDL scFv-antibodies were isolated and identified from the phage display based synthetic antibody library is reported. The second publication (II) describes the implementation of anti-HDL antibodies in scFv-AP and scFv-phage format for development of TRF based phage immunoassays and their clinical evaluation with cardiac disease related cohorts. The third publication presents (III) a direct immunoassay approach utilizing anti-HDL antibodies in scFv-AP format and their clinical evaluation with samples of patients suspected of obstructive CAD.

5.1 Isolation and identification of anti-HDL scFv-antibodies from phage display based synthetic antibody library

Synthetic antibody libraries, scFvP and scFvM, were employed to isolate antibodies recognizing HDL (Publication I). For enrichment of antibodies, several rounds of selection were performed using different antigens (ctrl HDl, apoA-I and apoA-II). The primary interest was to obtain binders recognizing specifically HDL of CAD patients (CAD HDL), and therefore, subtractive pre-selection was done with HDL of healthy individuals (ctrl HDL). The other motive was to enrich the binders against the rare proteins of HDL (i.e. proteins other than the most abundant proteins of HDL namely apoA-I and apoA-II). Accordingly, negative selections were done with apoA-I and apoA-II. In addition of using different target antigens, the reaction conditions were also varied by using different panning buffers and blocker proteins (Publication I). Enrichment of the HDL binding phage could be observed after the 2nd round of selection. Minimal difference in the enrichment of ctrl HDL binders and CAD HDL binders was observed despite of performing the subtractive pre-selection with ctrl HDL (Figure 15. A). However, the enrichment of apoA-I binders was efficiently suppressed by performing the subtractive selection with apoA-I on every selection round (Figure 15. B).

In all the pannings approximately 15–20% and 85–95% HDL binding active clones were identified after the 2nd and 3rd round of panning, respectively

(Publication I). Out of the total 3000 screened clones there were 1200 clones showing capability to bind to HDL. Sequencing of 337 anti-HDL clones disclosed that 264 of these were unique. This shows the capability of phage-display to isolate antibodies against a complex molecule like HDL. This is mainly due to the possibility and flexibility of choosing different panning conditions which includes use of different reaction set-ups and antigens for negative selection.

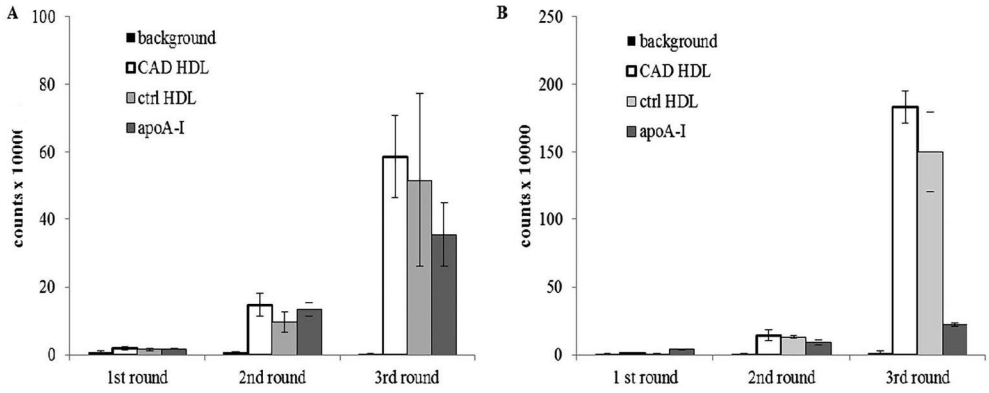


Figure 15. Enrichment of HDL binding scFv-phage in the panning reaction against CAD HDL monitored by phage immunoassay. Results are shown for two of the pannings performed. (A) Panning D having subtractive pre-selection against ctrl HDL on rounds 1 and 2, and against apoA-I and apoA-II on the third round. (B) Panning E where subtractive pre-selection against apoA-I was carried out on every round. Description of reaction condition can be found in supplementary Table 1 of Publication I. Figure is adapted from publication I.

5.2 Anti-HDL antibody production and characterization

5.2.1 Expression and Purification of anti-HDL antibodies as scFv-AP

Sixty one anti-HDL antibody clones, which displayed difference in binding between CAD HDL and ctrl HDL or showed no or minimal binding to apoA-I or rHDL were selected for shake flask culture expression as scFv-APs in *E.coli* and were affinity purified. An example of the purified anti-HDL scFv-AP antibody clone is shown in Figure 15. Enrichment of HDL binding scFv-phage in the panning reaction against CAD HDL monitored by phage immunoassay. Results are shown for two of the pannings performed. (A) Panning D having subtractive pre-selection against ctrl HDL on rounds 1 and 2, and against apoA-I and apoA-II on the third round. (B) Panning E where subtractive pre-selection against apoA-I was carried out on every

round. Description of reaction condition can be found in supplementary Table 1 of Publication I. Figure is adapted from publication I.

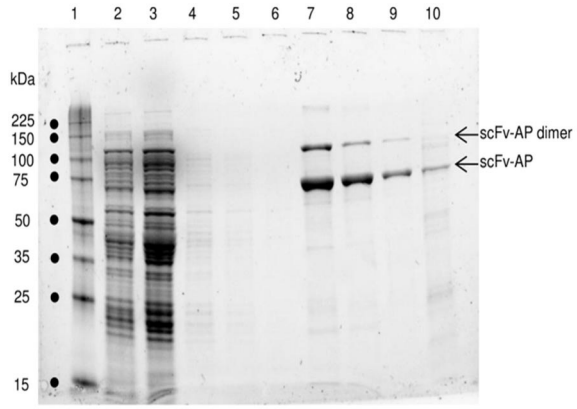


Figure 16. Reducing SDS-PAGE analysis of purified anti-HDL scFv-AP antibody. Lane 1: protein marker with molecular weight on left, Lane 2: bacterial lysate, Lane 3: flowthrough, Lanes 4–6: wash fractions 1–3, Lanes 7–9: eluate fractions 1–3, Lane 10: resin after elution. Black arrows on right denote scFv-AP monomer and dimer. Adapted from publication I.

5.2.2 Specificity profile of HDL binders

The purified anti-HDL scFv-AP antibodies were tested for binding to several antigens using a TRF immunoassay (Figure 11 C) based approach (Publication I). The antigens which were tested included different HDL preparations including spherical HDL isolated from healthy individuals (ctrl HDL) and CAD patients (CAD HDL), lipid free form of most abundant proteins of HDL (apoA-I and apoA-II), apoA-I lipidated with varying amount of lipids (rHDL1-4) and LDL (Figure 17). All the 61 purified antibodies were able to bind the isolated spherical HDLs, showed variable binding to rHDL subforms and purified apoA-I and apoA-II (Figure 17). None of these HDL binders demonstrated cross reactivity with LDL. Previous studies performed elsewhere have demonstrated that apoA-I has a very dynamic conformation reflecting the nature and concentrations of its lipid cargo. Therefore its conformation is different when in its lipid free form or as part of HDL (254). Few previous studies have reported that affinity of monoclonal apoA-I antibodies depend on lipid induced effect on protein conformation and on the size of HDL particle (254)(255)(256). In this study it was observed that, the HDL binding antibodies were predominantly recognizing apoA-I associated epitopes in a lipid governed environment as altogether 55 clones (out of 61) recognized lipidated apoA-I (rHDL1), but only about half of the clones (n = 36) were able to recognize the purified lipid-free apoA-I, and, most of these clones did not recognize well the rHDL

subforms 2, 3 or 4 having higher lipid content than that of rHDL1 (Figure 18 A). None of the binders bound exclusively to lipid free apoA-I. Antibody clone 110 had a unique specificity profile as it was binding to all the tested forms of lipidated apoA-I (rHDL 1-4), in addition to lipid free apoA-I (Figure 18 B). There were 17 antibody clones, which did not bind to lipid free apoA-I (specific signal < background + 2 × SD), but were efficiently recognizing rHDL1 (Figure 18 C).

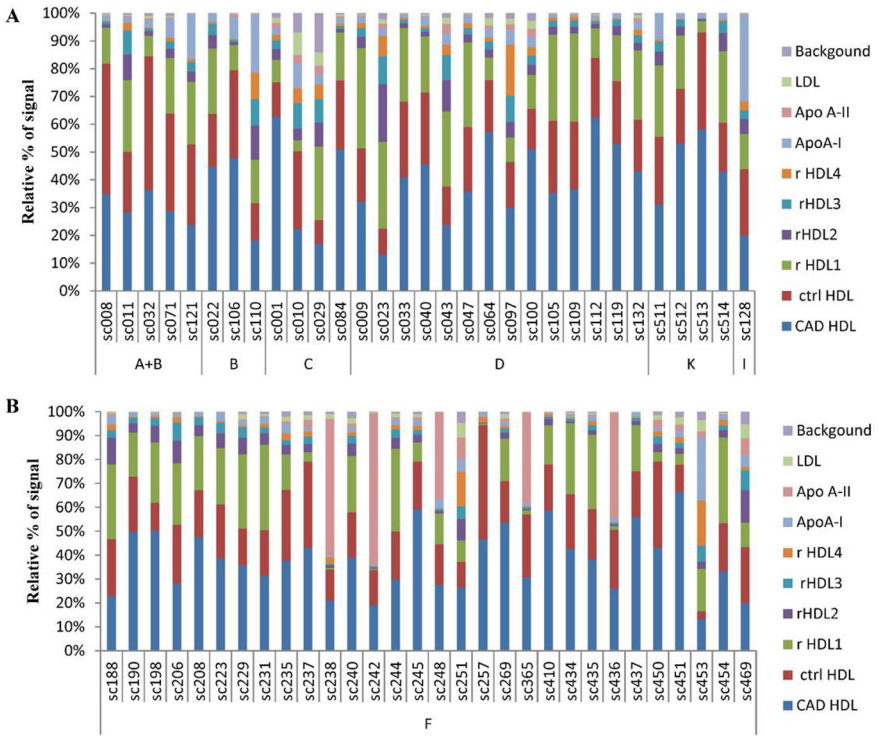


Figure 17. Binding of 61 HDL antibodies (scFv-AP) to different targets. ScFv-AP tested with different biotinylated antigens; CAD HDL, control HDL (ctrlHDL), reconstituted HDL1-4, apoA-I, apo A-II and LDL. For clarity of the data presentation, clones are displayed in two panels A and B. In each panel X-axis represents clone names and panning type (explained in Supplementary Table 1 of publication I), and Y-axis displays relative percentage of signals. Figure is adapted from publication I.

Importantly, 39 out of the 61 tested clones demonstrated differential binding to CAD HDL and ctrl HDL with a signal difference of >50% (examples shown in Figure 18 C, E and F), three of these antibody clones did not bind strongly to apoA-I or apoA-II (an example in Figure 18 F). In addition, 7 clones were identified as apoA-II binders (an example in Figure 18 D). While phage display offers advantages in generating diverse antibodies towards a complex molecule like HDL, there is also a risk that during the selection process enrichment of binders against the abundant

protein is favored compared to the more scarce proteins. Apo-I and apoA-II contributes to 70% and 20% of total protein fraction of HDL and are the most abundant proteins of HDL (257), hence, other proteins are found in very less proportion (257) in HDL. In this study, despite of performing subtractive selections with apoA-I and apoA-II, we obtained only three binders not recognizing epitopes within apoA-I (lipid free or lipid associated) or apoA-II suggesting that they are potential binders against the less abundant proteins of HDL. In future the enrichment of binders against the rare proteins could possibly be augmented by elimination of lipid-dependent apoA-I binders via rHDL1 guided subtractive selections.

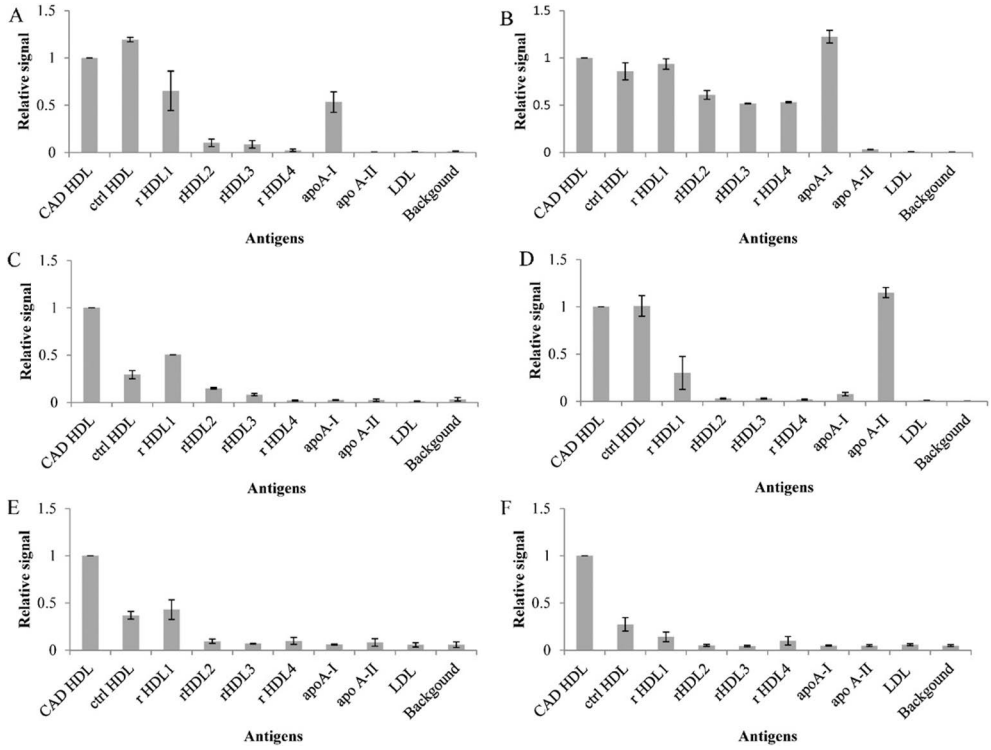


Figure 18. Binding profiles of six selected antibody clones. The clones were tested for binding against biotinylated antigens. (A) sc121 obtained from pannings A + B is an apoA-I binder recognizing rHDL1 but rHDL 2–4 to lesser extent, (B) sc110 from panning B is an apoA-I binder recognizing all forms of rHDLs, (C) sc198 from panning F is a rHDL1 binder not recognizing purified apoA-I, (D) sc365 from panning F is an apoA-II binder, (E) sc132 from panning D is showing difference between CAD and ctrl HDL, (F) sc451 from panning F is an antibody not recognizing or giving low signals with rHDL1–4, apoA-I, and apoA-II. Value associated with each bar is an average of relative signal values from the assays performed on different days. Panning description is available in Supplementary Table 1 of publication I. Adapted from publication I.

Most of these anti-HDL scFv-AP antibodies (53 in total) were able to recognize HDL in plasma samples (Publication I, Figure 6) in a sandwich immunoassay with anti-HDL scFv-AP clones as capture antibodies and Eu-labeled commercial anti-apoA-I Mab or Pab as detection antibody (Figure 11 D). This demonstrated that these binders were capable to bind to the native form of HDL present in circulation.

5.2.3 Identification of antibody pair to be used in two-site assays

Anti-HDL scFv antibody clones were preselected to identify antibody pair for two-site immunoassay based on their different reactivity profiles (Publication II). Thirty-two functional scFv antibody pairs were identified. Eventually, based on the technical performance, three anti-HDL antibody pairs, were selected. These antibody pairs sc 022 - sc 454, sc109 - sc121 and sc110 - sc 525 (in each pair, the former binder as scFv-APs and the latter as scFv-phage), all of them recognized as apoA-I binders (section 5.2.2), were used for optimizing the two-site apoA-I immunoassay.

5.3 Two-site apoA-I immunoassays using phage displayed detector antibodies

5.3.1 Characteristics and validation of the assays

The two-site apoA-I assays were set up and optimized using biotinylated scFv-APs sc 022, sc 109 and sc 110 as capture antibodies and scFv-phage sc 454, sc 121 and sc 525 as detection antibodies, respectively (Publication II). In the assays, total HDL (= HDL 2 + HDL3 subclass) was used as the calibrator because of the unknown epitopes of these antibodies. Different parameters were optimized including buffer composition, incubation time of the sample, wash steps, amount and incubation time of antibodies (data not shown). Conditions displaying comparatively good signal-to-background ratio and better precision (<20% CV) were selected to be used in the final assay procedure. The optimized assays are referred to as “phage based two-site apoA-I assays”.

Calibration curves of the optimized assays are shown in Figure 19. Sample dilutions of upto 1500-fold, 1000-fold, and, 1000-fold were found suitable for assays 022-454, 109-121 and 110-525, respectively. The inter-assay (within 8 days) coefficient of variation (CV%) for the calibrator (HDL, n= 32) was 1.6-17%, 1.9-9.3% and 1.4-7.3% in the assays 022-454, 109-121 and 110-525, respectively. In the serum samples (n=32) the corresponding values were 6-14%, 5.3-8.1% and 12-18.8% (Publication II, Supplementary Table S1). The intra-assay (within assay) variation was < 20%.

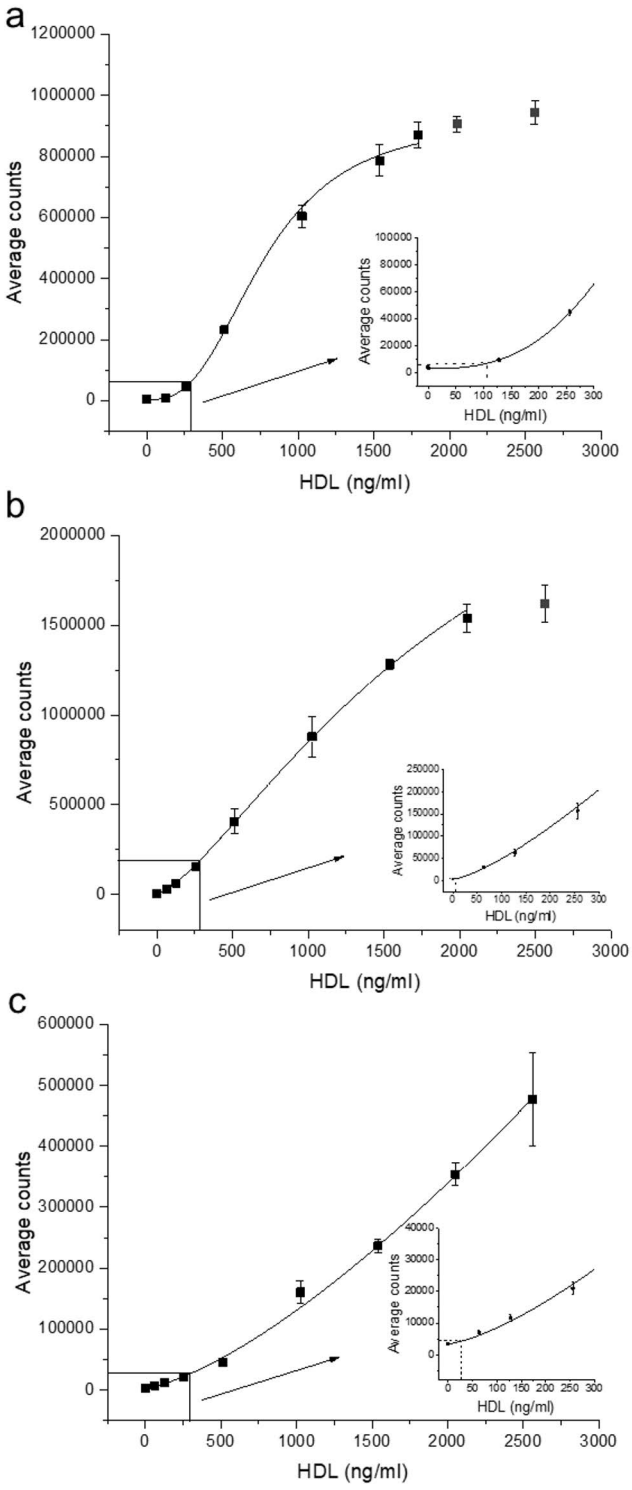


Figure 19. Standard curves of the two-site apoA-I assays: (a) assay 022-454, (b) assay 109-121 and (c) assay 110-525. The curve was fitted with four-parametric logistic function. Signals of the assays are represented as average counts of eight replicate measurements. The error bars represent the standard deviation (SD) of the average counts. The analytical sensitivity (background + 3xSD) of the assay 022-454 (114 ng/ml), 109-121 (7 ng/ml) and 110-525 (25 ng/ml) is illustrated as vertical dash-line (---). The assays were done with sc 022, sc 109, and sc 110 as capture antibody and sc 454, sc 121 and sc 525 as detection antibody. Total HDL was used as calibrator. Adapted from publication II.

The assays were not severely affected by the type of sample matrix (Lithium-heparin plasma, EDTA plasma and serum) used. However, the assays were found to be affected by freeze-thaw treatment (up to ten cycles tested) of samples. The assay 110-525 being the most sensitive in this respect while the assay 022-454 and assay 109-121 were more resistant to such effects and showed recovery between 93-119% and 77-121% (after three freeze-thaw cycles), respectively (Figure 20). This effect could be because of the fact that HDL particles are sensitive to repeated freeze-thaw treatment (258).

Figure 20. Effect of sample matrices and freeze-thawing cycles on the performance of the two-site apoA-I assays. The graph represents the relative recoveries of the samples after freeze-thawing cycles with the two site apoA-I-assays: (a) assay 022-454, (b) assay 109-121, (c) assay 110-525. The three sample matrices; Li-heparin (LiH) plasma, EDTA plasma, and serum from three individuals were tested. The samples were measured after 1x, 2x, 3x, 5x and 10x freeze-thaw cycles at -70 °C and RT. Adapted from publication II.

The effect of lipids (especially TGs) on the assays was tested by supplementing TG rich formulations (VLDL-IDL and intralipid emulsion containing 2mg/ml of TG) in LPDS and in up to eight serum samples (Publication II, Supplementary Table S2). Assay 022-454 and assay 109-121 were able to reliably measure samples in presence of moderately elevated level of lipids. However, assay 110-525 seemed to be slightly affected due to moderately elevated lipids, as recoveries with TG-enriched VLDL-IDL and intralipid emulsion were between 69-140% and 60-126%, respectively.

5.3.2 Clinical evaluation of the assays

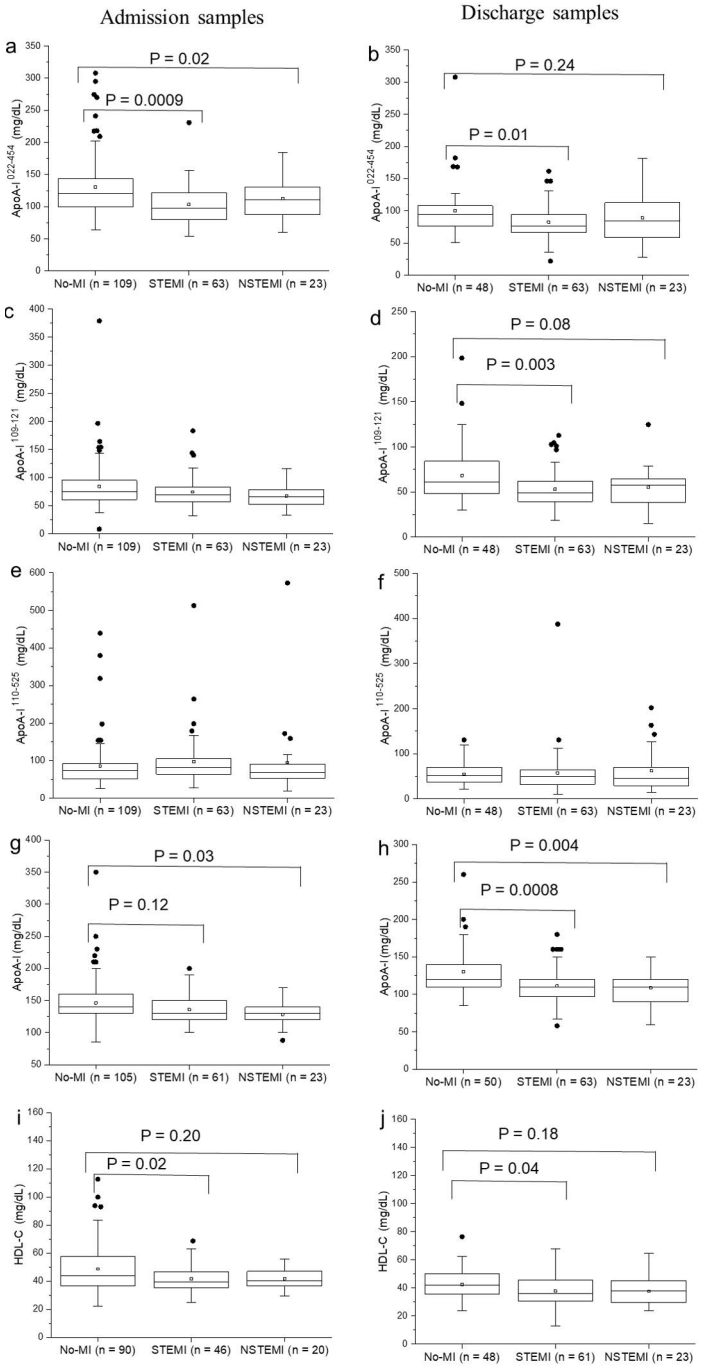
To explore the clinical value of these novel anti-HDL/apoA-I scFv binder based assays and their association with cardiac conditions, the assays were tested with cardiac samples from two different cohorts. First, the evaluation was done with small number of samples (cohort of 36 individuals, referred as cohort-I) derived from a subset of cardiac patients from Corogene study (259) and then with a larger cohort of 195 patients (referred as cohort-II) who had primary symptom of chest pain (260) (Publication II). The clinical performance of the two-site apoA-I assays was compared with polyclonal anti-apoA-I based conventional routine apoA-I assay (immunoturbidometry/ELISA method) and HDL-C assay (precipitation based direct method).

5.3.2.1 Comparison between clinical groups of patients

Previous reports have suggested that profile of HDL changes or evolves during the ACS process (261) and dysfunctional alteration in HDL is more pronounced in ACS patients than in individuals with stable CAD (262). It is also reported that the antiatherogenic functions of HDL such as cholesterol efflux and anti-inflammatory properties are markedly impaired in STEMI patients than in non-cardiac or NSTEMI patients (263). The cohort-I in this study had patients with ACS, stable CAD and no CAD, and, cohort-II had MI (STEMI, NSTEMI) and non-MI patients (detail in section 4.1.1). The detailed background of these study groups could be found in Publication II.

In cohort-I, the level of apoA-I determined with two-site phage assay was significantly different between the clinical groups (Publication II). Particularly the apoA-I concentration was lower in ACS patients than in those without CAD (assay 110-525, Steel Dwass test, $P = 0.03$) (Publication II, Table 1, Figure 4 c). Similarly, the concentration of conventionally determined apoA-I was significantly lower in ACS cases (Steel Dwass test, $P = 0.03$) (Publication II, Table 1, Figure 4 d) but there was no significant difference in the concentration of HDL-C (Publication II, Table 1, Figure 4 e). In cohort-II, particularly, the difference between no-MI vs STEMI

patients was much evident than the difference between no-MI vs NSTEMI patients (◄ Figure **21**). The significant difference between no-MI vs STEMI patients was found with assay 022-454 (in admission samples, P = 0.0009 and in discharge samples, P = 0.01), assay 109-121 (in discharge samples, P = 0.003), conventional ELISA apoA-I assay (in discharge samples, P = 0.008) and with HDL-C (in admission and discharge samples, P = 0.02 and 0.04). Additionally, there was significant difference between no-MI and NSTEMI patients with assay 022-454 (in admission samples, P = 0.02) and conventional apoA-I assay (in admission samples, P = 0.03 and in discharge samples, P = 0.004).


Admission samples
Discharge samples
a
P = 0.02
P = 0.0009
ApoA-I 022-454 (mg/dL)
No-MI (n = 109)
STEMI (n = 63)
NSTEMI (n = 23)
b
P = 0.24
P = 0.01
ApoA-I 022-454 (mg/dL)
No-MI (n = 48)
STEMI (n = 63)
NSTEMI (n = 23)
c
ApoA-I 109-121 (mg/dL)
No-MI (n = 109)
STEMI (n = 63)
NSTEMI (n = 23)
d
P = 0.08
P = 0.003
ApoA-I 109-121 (mg/dL)
No-MI (n = 48)
STEMI (n = 63)
NSTEMI (n = 23)
e
ApoA-I 110-525 (mg/dL)
No-MI (n = 109)
STEMI (n = 63)
NSTEMI (n = 23)
f
ApoA-I 110-525 (mg/dL)
No-MI (n = 48)
STEMI (n = 63)
NSTEMI (n = 23)
g
P = 0.03
P = 0.12
ApoA-I (mg/dL)
No-MI (n = 105)
STEMI (n = 61)
NSTEMI (n = 23)
h
P = 0.004
P = 0.0008
ApoA-I (mg/dL)
No-MI (n = 50)
STEMI (n = 63)
NSTEMI (n = 23)
i
P = 0.20
P = 0.02
HDL-C (mg/dL)
No-MI (n = 90)
STEMI (n = 46)
NSTEMI (n = 20)
j
P = 0.18
P = 0.04
HDL-C (mg/dL)
No-MI (n = 48)
STEMI (n = 61)
NSTEMI (n = 23)

◄ **Figure 21.** Comparison between individuals with and without myocardial infarction based on apoA-I concentration measured with the two-site apoA-I assays and conventional apoA-I assay, and concentration of HDL-C in admission and discharge samples (cohort-II). (a-b) Assay 022-454 (apoA-I $^{022-454}$) (admission and discharge samples; ANOVA P = 0.0004 and 0.01), (c-d) assay 109-121(apoA-I $^{109-121}$) (admission and discharge samples; ANOVA P = 0.07 and 0.004), (e-f) assay 110-525 (apoA-I $^{110-525}$) (admission samples; ANOVA P = 0.16, discharge samples; Kruskal-Wallis test P= 0.84), (g-h) apoA-I (admission and discharge samples; ANOVA P = 0.07 and 0.0003) (i-j) HDL-C (admission samples; Kruskal-Wallis test P = 0.01, discharge samples; ANOVA P = 0.04). Y-axis represents the apoA-I values obtained with two-site apoA-I assays, conventional apoA-I assay, and, values of HDL-C. Overall statistical difference between the groups was tested by one way analysis of variance (ANOVA) or Kruskal-Wallis test and if significant, pairwise comparison p-values between groups is corrected with Tukey-Kramer method or Steel-Dwass method (marked in the figure). Mean is marked as a square (□) inside the box and the solid circles (●) outside the box represents the outliers. The whiskers represent the 5th and 95th percentile. ApoA-I, apolipoprotein A-I; MI, myocardial infarction; STEMI, ST elevated MI; NSTEMI, non-ST elevated MI. Adapted from Publication II.

5.3.2.2 Clinical outcome during follow-up time of 1.5 years and survival analysis

In few studies, higher apoA-I levels have been found associated with lower risk of chronic heart disease (CHD) (264), MI, MI or CV death (265)(266). In this study, during the follow-up of 1.5 years (cohort-II) 27 patients died and 28 patients encountered MI patients (Figure 22).

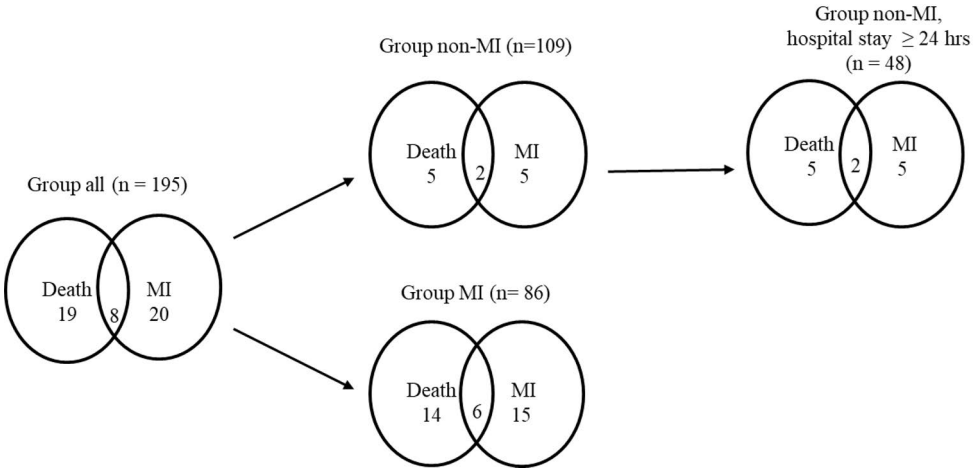


Figure 22. Events (death and MI) that occurred during 1.5 yrs follow-up time in cohort-II. The figure represents the death and MI which took place in whole cohort and in patients divided into MI and non-MI group, and, in non-MI group who had hospital stay ≥ 24 hrs. In the figure, n= number of individuals, MI= myocardial infarction. Adapted from publication II.

For Kaplan-Miere (K-M) survival analysis of these follow-up events of death and MI, all the patients were categorized into tertiles (1st tertile had the lowest

concentration) based on the concentration of apoA-I obtained from the two-site apoA-I assays and conventional apoA-I, and the concentration of HDL-C of admission and discharge samples (Publication II). K-M survival analysis for MI and death was done from admission and discharge samples of all the patients altogether and separately in MI and non-MI groups. Survival analysis for MI within follow-up of 1.5 yrs did not show any significant difference in MI free survival with measured admission and discharge samples in all the patients altogether, and, in MI and non-MI patients separately under different tertiles of HDL-C and, apoA-I obtained either with the two-site apoA-I assays or conventional apoA-I assay (Publication II). It was noticeable that 19.4% individuals (38 out of 195) of this cohort had a history of CAD prior to admission to hospital, therefore, possibly HDL in those patients was already modified which might have affected the results. In context of mortality, a recent study has shown U-shaped association between HDL-C and mortality (141) and in contrast some of the previous studies have also failed to show the relation between increased level of HDL-C and mortality (133)(267)(268). In this study cohort, HDL-C tertiles of admission (Figure 23 e) and discharge samples (Figure 25 e) did not show any significant difference in the survival of patients. However, in contrast, according to tertiles of apoA-I (two-site apoA-I assay 109-121, Figure 23 b and assay 110-525, Figure 25 c), and conventional apoA-I assay (Figure 23 d) there was difference in survival during 1.5 yrs in individuals when analysed altogether and separately in subgroup of MI patients (assay 109-121, Figure 23 b). This observation is in agreement to the reports where apoA-I and mortality (266)(269) have been found to be associated. It is noteworthy, that in patients without MI, assay 110-525 was able to significantly discriminate between the tertiles of individuals who died (Figure 24), where 6 deaths out of the 7 deaths were in the lowest tertile. The survival during 6 months follow-up was significantly different according to the tertiles of two-site apoA-I assay 109-121 (all patient and MI patients, Figure 23 b) and assay 110-525 (all patient, Figure 25 c) but not according to conventional apoA-I assay (Figure 23 d and Figure 25 d).

Based on these clinical evaluation and observations (section 5.3.2.1 and 5.3.2.2), it can be anticipated that the corresponding CAD specific recombinant apoA-I antibodies (sc109 + sc 121 and sc 110 + sc 525) could be helpful in diagnosing the presence of CAD (MI or ACS) and in predicting the risk of mortality in chest pain/ symptomatic patients within short period of six months and 1.5yrs. From a clinical point of view these assays may provide a tool for improved prognosis of CVD patients and in managing lipid lowering and other therapies. However, these propositions need further extensive evaluation. The phage-based assay format described and investigated here (Publication II) in general is rather complex and deviates from routinely used enzyme-immunoassays and immunoturbidimetric assays. This calls for further simplification of these phage based two-site apoA-I assays. Therefore, antibody pair sc109 + sc 121 and sc 110 + sc 525 were used further to develop a simplified assay format and were clinically evaluated.

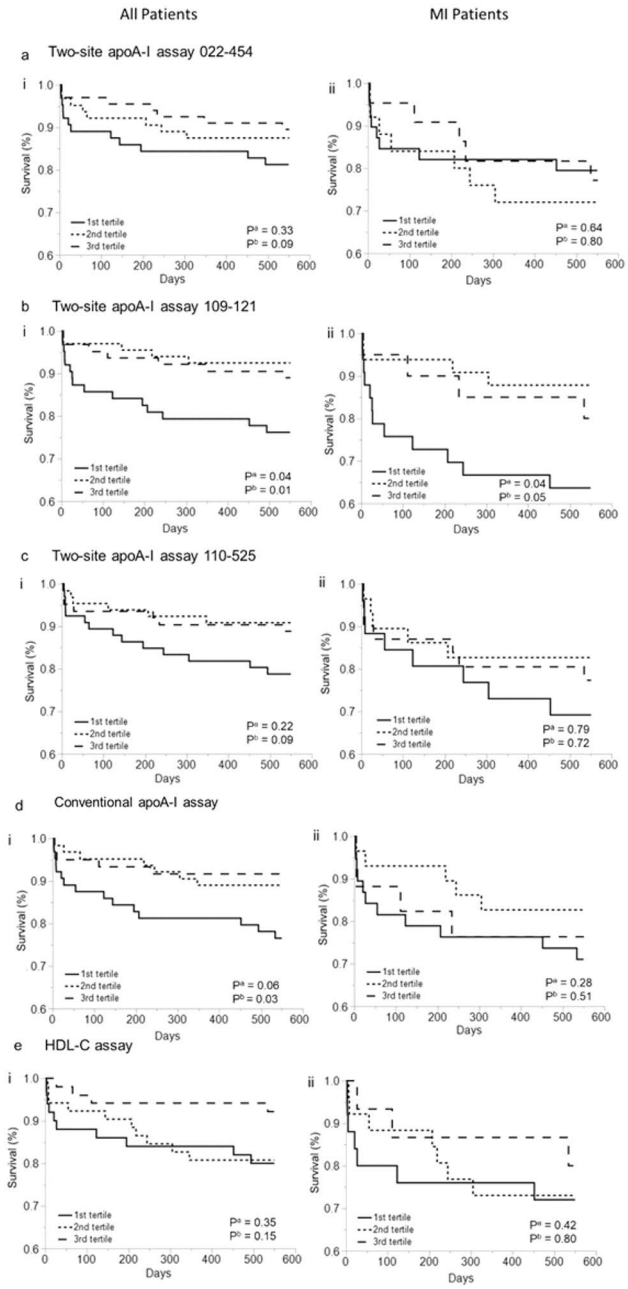


Figure 23. Kaplan-Meier survival curves for mortality (within 1.5yrs) based on admission samples measured from all the cardiac patients (n = 195) and the patients categorized into MI (n = 86) (cohort-II). Patients were stratified according to apoA-I concentration obtained from two-site apoA-I assay 022-454 (a), 109-121 (b), 110-525 (c), conventional apoA-I assay (d), and, the HDL-c (e) measured from samples collected at the time of admission to hospital. Statistical testing is done with log-rank test; P <0.05 is significant. P^a and P^b represents the P values obtained within follow-up period of 6 months and 1.5 years. Adapted from publication II.

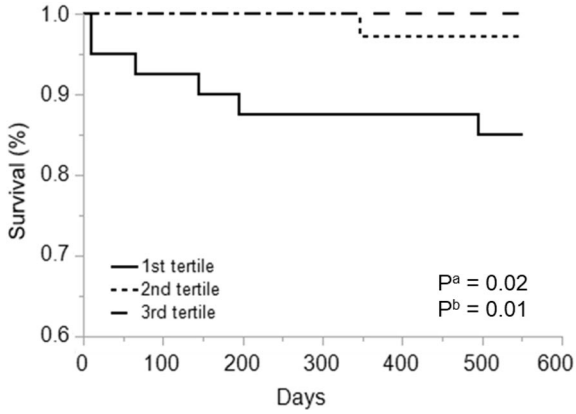


Figure 24. Kaplan-Meier survival curves for mortality (within 1.5yrs) based on admission samples measured from the patients without MI (cohort-II, n = 109). Patients were stratified according to apoA-I concentration obtained from two-site apoA-I assay 110-525 measured from samples collected at the time of admission to hospital. Statistical testing is done with log-rank test; P <0.05 is significant. P^a and P^b represents the P values obtained within follow-up period of 6 months and 1.5 years, respectively Adapted from publication II.

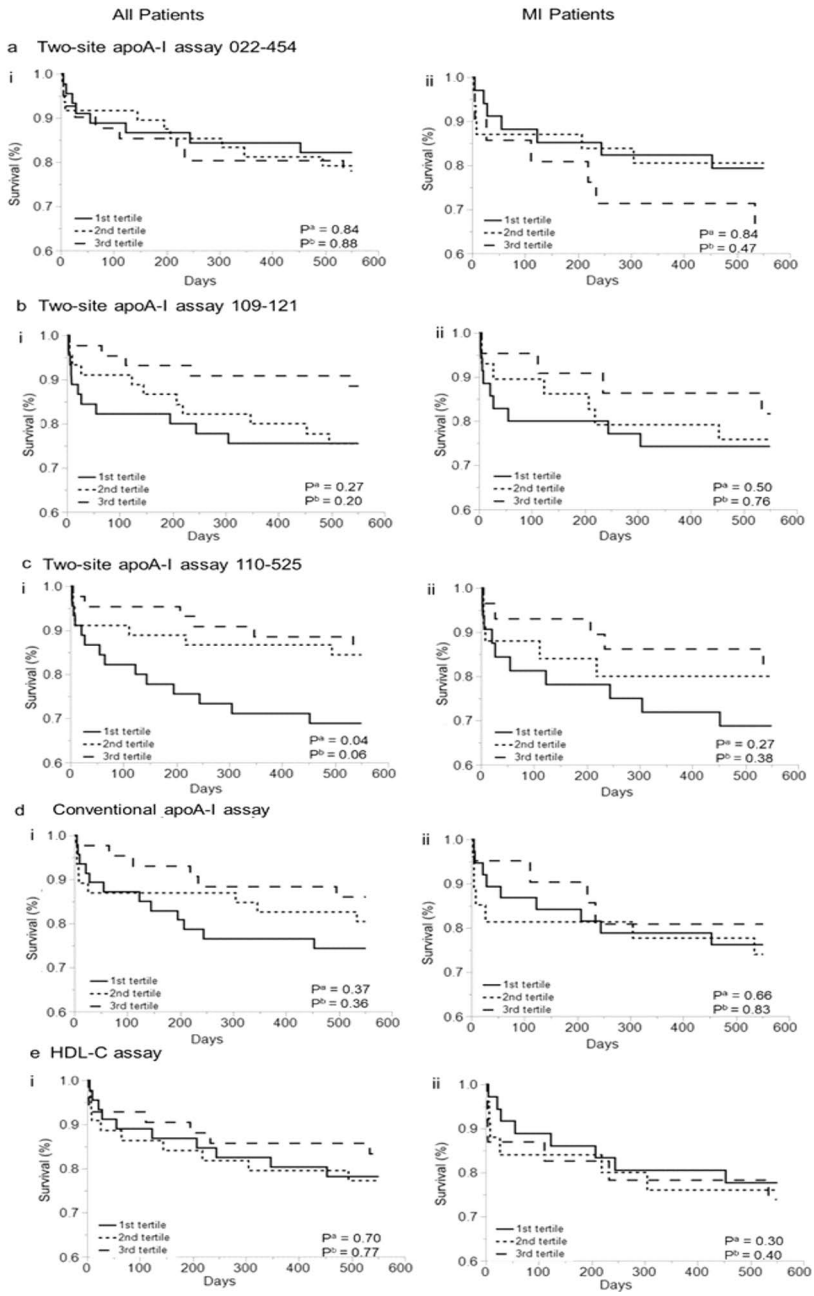


Figure 25. Kaplan-Meier survival for mortality (within 1.5yrs) based on discharge samples measured from all the cardiac patients (n = 134) and the patients categorized into MI (n = 86) (cohort-II). Patients were stratified according to apoA-I concentration obtained from two-site apoA-I assay 022-454 (a), 109-121 (b), 110-525 (c), conventional apoA-I assay (d), and, the HDL-c (e) measured from samples collected at the time of discharge from hospital. Statistical testing is done with log-rank test; $P < 0.05$ is significant. P^a and P^b represents the P values obtained within follow-up period of 6 months and 1.5 years. Adapted from publication II.

5.4 Direct two-site apoA-I immunoassays

5.4.1 Assay validation

The previously optimized phage based two-site apoA-I assays which were used to evaluate recombinant apoA-I antibody pair (section 5.3) were rather complex in terms of configuration (Figure 13) and procedure mainly due to intervening wash and incubation steps. Two of the assays, assay 109-121 and 110-525 were continued for simplification as they had shown ability to diagnose MI/predict mortality in chest pain patients (section 5.3.2). In order to further simplify the assays, the capture as well as the detection antibodies (previously in scFv-phage format, section 5.3) were used in scFv-AP format. The scFv-APs which were used in the assays as the capture antibodies (sc 109 and sc 110) were biotinylated and the detection antibodies (sc 121 and sc 525) were directly labeled with Eu^{+3} chelate (Figure 14). The assays are referred to as "direct two-site apoA-I assay". The concentration of apoA-I obtained with direct two-site apoA-I assay 109-121 and 110-525 is referred as apoA-$I^{109\text{-}121}$ and apoA-$I^{110\text{-}525}$, respectively. The calibration curves of the assays are shown in Figure 26, the measurement range of the assay 109-121 and assay 110-525 were between 128-2564 ng/ml (r^2 = 0.98) and 6.8-2564 ng/ml (r^2 = 0.99), respectively. The inter-assay variation (within three days) with direct two-site assay 109-121 was between 2-9 % and 4-16 % in standards and 8 samples (n = 12); the inter-assay variation with direct two-site apoA-I assay 110-525 in standards and samples (n = 12) was 3-22 % and 7-26 %, respectively (Publication III). The intra-assay variation (n = 4) was below 10% and 20% with the direct two-site assay 109-121 and 110-525, respectively. Sample dilution 62.5 – 1000-fold (R^2 = 0.99) and 250 – 1000- fold (R^2 = 0.99) was found appropriate for the direct two-site assay 109-121 and 110-525, respectively.

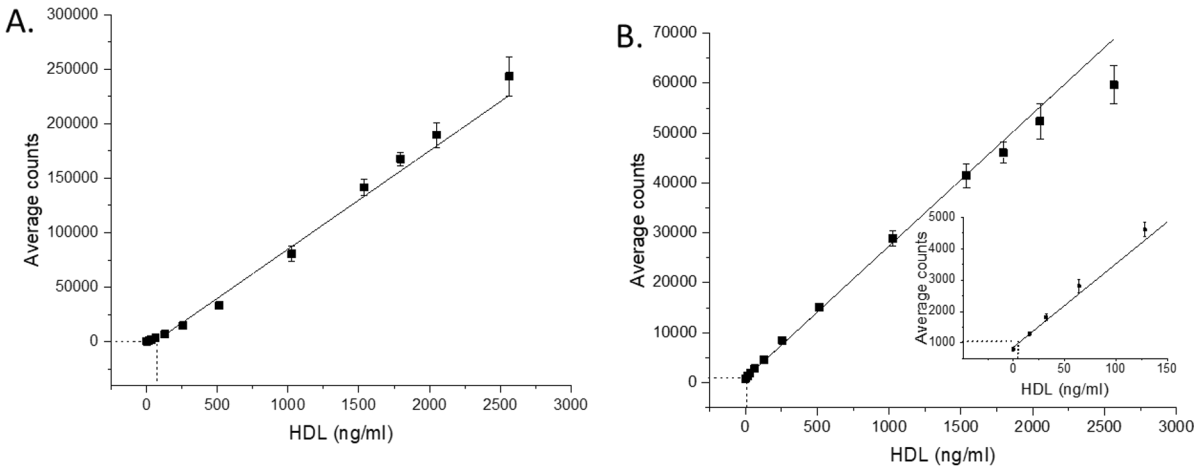


Figure 26. Standard curves of the direct two-site apoA-I immunoassays: (A) assay 109-121 and (B) assay 110-525. X axis represents the concentration of the standard (HDL), and Y axis-represents the average of the counts obtained from the replicates of the standard. The error bars represent the standard deviation (SD) of the average counts. The curve was fitted with a linear fitting function. The analytical sensitivity (background + 5*SD) of the assay 109-121 (63.8 ng/ml) (B) and 110-525 (6.8 ng/ml) (C) is illustrated as dash-line (---). ApoA-I: apolipoprotein A-I; HDL: high density lipoprotein. Adapted from Publication III.

5.4.2 Clinical evaluation of assays

The direct two-site apoA-I assay 109-121 and 110-525 were clinically evaluated with cohort of patients suspected of obstructive CAD (n = 197) (Figure 10). Approximately 1/3rd population (72 out of 197 analysed patients) was under lipid lowering medication (LLM); detailed characteristic of the patients can be found in publication III). Statins or LLM are commonly used for lipid management (LDL-C, TG and HDL-C) and for avoiding the risk of CVDs (270) (271). Because these drugs have effect on HDL metabolism (272,273), therefore to evaluate these HDL derived apoA-I antibodies the data was analysed separately in patients who were not using and who were using the LLM (statins), in addition to the whole cohort.

Clinical performance of the direct two-site apoA-I assays was compared to performance of HDL-C, conventional apoA-I ELISA and Framingham Risk Score (FRS). The FRS for CHD risk estimation, uses age, diabetes and smoking status, blood pressure, total cholesterol, HDL-C and low-density lipoprotein cholesterol (LDL-C) as predictors (43).

5.4.2.1 Prediction of coronary atherosclerosis and obstructive CAD

Previous studies have demonstrated that low levels of serum apoA-I is associated with cardiac risk or CHD (274) (266). In addition, increased level of modified apoA-I forms (such as chlorinated and oxidized forms) are identified in CAD/ACS patients. These molecular modified form of apoA-I are found inefficient for cholesterol efflux, which is an important atheroprotective mechanism (275)(276).

In this objective of the study, significant difference in apoA-I levels (determined with direct apoA-I assays) of patients with and without atherosclerosis was observed depending upon if the patients were using LLM or not. The level of apoA-I$^{110-525}$ was significantly higher in patients with coronary atherosclerosis compared to patients without coronary atherosclerosis (P = 0.01) among the individuals not using LLM, but not in the overall population (P = 0.29). That is apparently because the apoA-I$^{110-525}$ tended to be lower in the presence of atherosclerosis with borderline significance (P = 0.05) among the patients using LLM (Table 5). This different pattern of apoA-I$^{110-525}$ level could have been because statins (LLM) are found to affect the HDL metabolism and increase the level of apoA-I (272)(273). ApoA-I$^{110-525}$ also showed significant association with atherosclerosis in multivariate analysis where age, gender [Model 1, OR (95% CI): 3.02(1.30-7.04); P = 0.010] and other CAD risk factors were considered [Model 2, OR (95% CI): 3.89(1.39-10.9); P = 0.009] in patients not using LLM (Table 6). The ROC analysis (Figure 27) of apoA-I $^{110-525}$ combined with age and sex (Model 1) provided slightly better but not statistically significantly better prediction for coronary atherosclerosis than FRS (P > 0.05) in non-LLM users [(95% CI): 0.75 (0.66-0.84) vs 0.71 (0.62-0.81)]. This proposes that

possibly a simpler model based on CAD HDL derived apoA-I antibodies (sc 110-525) could be employed for risk estimation of atherosclerosis particularly in individuals not using LLM. Since statin (LLM) seemed to possibly hinder or expose the reactive epitopes of these apoA-I antibodies, therefore use of these antibodies should be limited to individuals not using LLM. ApoA-I$^{109-121}$ level was significantly higher in patients without any coronary atherosclerosis compared to those with coronary atherosclerosis among LLM users (P = 0.03), but not in patients who were not using LLM (P = 0.88) or in overall patient cohort (P = 0.16) (Table 5). However, in multivariate analysis apoA-I$^{109-121}$ in presence of risk factors (Model 1 and Model 2) did not show any association with atherosclerosis (Publication III). ApoA-I determined with conventional method could not discriminate between the patients with and without atherosclerosis (Table 5). On the other hand, FRS was significantly higher in the patients with atherosclerosis than in the patients without atherosclerosis in whole cohort (P < 0.0001) but not separately in LLM users and non-LLM users (Table 5). A recent study by Jia C et al. (277) has shown that the anti-inflammatory capacity of HDL is inversely associated with the CV events and could improve the their risk estimation, independent of HDL-C level. In this study, HDL-C did not show any significant difference between the group of individuals with and without atherosclerosis (Table 5), and showed no association with atherosclerosis (Table 6). This finding is in line with the theory that the quantity of cholesterol content of HDL (HDL-C) (278)(9)(279) alone is inefficient of estimating the risk of atherosclerosis.

While being able to differentiate between patients with atherosclerosis and without atherosclerosis, these apoA-I antibodies were not able to identify the individuals with obstructive CAD (abnormal stress myocardial blood flow (MBF) or stenosis >70%) (Publication III). But this ambiguity in analyisng obstructive CAD could also arise because of very small number of patients with obstructive CAD in this study (in whole population, n= 27, separately in LLM users, n = 11 and in no-LLM users = 16).

Table 5. Comparison of the direct two-site apoA-I assays (assay 109-121 and 110-525), biochemical parameters and FRS CHD in patients with and without atherosclerosis. Adapted from publication III.

Parameters	ALL PATIENTS (NON-LLM AND LLM USERS)			Non-LLM users			LLM users		
	No atherosclerosis (N=81)	Atherosclerosis (N=116)	P	No atherosclerosis (N=64)	Atherosclerosis (N=61)	P	No atherosclerosis (N=17)	Atherosclerosis (N=55)	P
ApoA-I$^{109-121}$ (mg/dL)	40.5 (30.42-53.2)	37.38 (27.87-47.45)	0.16	39.52 (29.34-54.38)	38.78 (28.7-48.5)	0.88	46.17 (33.86-53.2)	33.66 (27.74-44.21)	0.027
ApoA-I$^{110-525}$ (mg/dL))	18.23 (14.32-26.24)	19.91 (14.66-25.99)	0.29	17.66 (13.38-24.48)	21.88 (15.89-27.44)	0.01	22.58 (17.97-31.21)	18.06 (14.4-23.51)	0.052
HDL-C (mmol/L)	0.94 (0.78-1.01)	0.91 (0.78-1.1)	0.29	0.91 (0.77-1.1)	0.88 (0.78-1.02)	0.58	0.96 (0.87-1.23)	0.94 (0.76-1.1)	0.178
ApoA-I (mg/dL)	123 (109.5-150)	131 (110-144.75)	0.81	122.5 (106.75-150.5)	123 (110-145)	0.76	125 (112.5-151.5)	132 (110-145)	0.882
FRS CHD	15 (11-22)	22 (14-32)	<0.0001	15 (11-24)	22 (16-32)	0.22	17 (13-21.5)	17 (11-27)	0.24

In the table, clinical groups (atherosclerosis and no atherosclerosis) were compared in a whole patient population (LLM users and non-LLM users) and separately in those patients who were using LLM (LLM users) or not using LLM (non-LLM users). Data is shown as a median (25-75th percentile). Natural log transformed values of the all the parameters except FRS CHD were used for statistical testing. Statistical testing was done using a t-test. P values less than 0.05 were considered statistically significant. N, number of patients; ApoA-I$^{109-121}$, apoA-I measured with direct two-site assay 109-121; ApoA-I$^{110-525}$, apoA-I measured with direct two-site apoA-I assay 110-525; LLM, lipid lowering medication; apoA-I, apolipoprotein A-I; HDL-C, high density lipoprotein cholesterol; FRS CHD, Framingham risk score for coronary heart disease in 10 years.

Table 6. Multivariate logistic regression analysis of the direct two-site apoA-I assay 110-525 for the presence of atherosclerosis in all the patients and separately in patients undertaking LLM (LLM users) and not taking LLM (non-LLM users). Adapted from publication III.

Characteristics	Model 1						Model 2					
	All patients (Non-LLM and LLM users) OR (95% CI)	**P**	**Non-LLM users OR (95% CI)**	**P**	**LLM users OR (95% CI)**	**P**	**All patients (Non-LLM and LLM users) OR (95% CI)**	**P**	**Non-LLM users OR (95% CI)**	**P**	**LLM users OR (95% CI)**	**P**
Age (years)	1.07 (1.04-1.11)	<0.0001	1.04 (0.97-1.11)	<0.0001	1.04 (0.97-1.11)	0.21	1.08 (1.04-1.12)	0.0003	1.08 (1.03-1.13)	0.004	1.03 (0.94-1.13)	0.59
Male	3.08 (0.33-6.25)	0.001	4.19 (1.69-10.39)	0.001	1.83 (0.48-6.93)	0.36	2.93 (1.27-6.74)	0.011	4.94 (1.51-16.23)	0.008	2.4 (0.5-11.7)	0.27
ApoA-I$^{110-525}$ (> 19 mg/dL)	1.57 (0.83-2.99)	0.161	3.02 (1.30-7.04)	0.01	0.41 (0.12-1.39)	0.15	1.63 (0.77-3.46)	0.209	3.89 (1.39-10.9)	0.009	0.49 (0.11-2.27)	0.35
Diabetes							2.35 (0.74-7.47)	0.148	6.09 (0.92-40.37)	0.061	1.02 (0.18-6.04)	0.98
Hypertension							1.33 (0.62-2.84)	0.47	1.49 (0.51-4.36)	0.472	1.33 (0.29-6.14)	0.71
Smoking							1.14 (0.42-3.11)	0.805	1.59 (0.41-6.15)	0.505	0.36 (0.05-2.73)	0.32
LDL-C (> 3.65 mmol/L)							0.7 (0.33-1.49)	0.346	1 (0.34-2.97)	0.991	1.11 (0.23-5.48)	0.89
HDL-C (< 0.91 mmol/L)							1.52 (0.72-3.2)	0.280	1.78 (0.64-4.97)	0.277	3.48 (0.55-22.3)	0.189

Model 1 includes age, sex and apoA-I measured with direct two site apoA-I assay 110-525 (apoA-I$^{110-525}$). Model 2 includes parameters included in model 1, and, diabetes, hypertension, smoking, LDL-C and HDL-C. Regression analysis with Model 2 was not possible due to very low number of obstructive perfusion defect positive cases and the considerable parameters. In the analysis the decided cut-off values for apoA-I$^{110-525}$, HDL-C and LDL-C were the median values which were used as categorical variable. LLM, lipid lowering medicine; CI, confidence interval, ApoA-I $^{110-525}$, apoA-I measured with direct two-site apoA-I assay 110-525; OR, Odds ratio; LDL-C, low density lipoprotein cholesterol; HDL-C, high density lipoprotein cholesterol; apoA-I, apolipoprotein A-I.

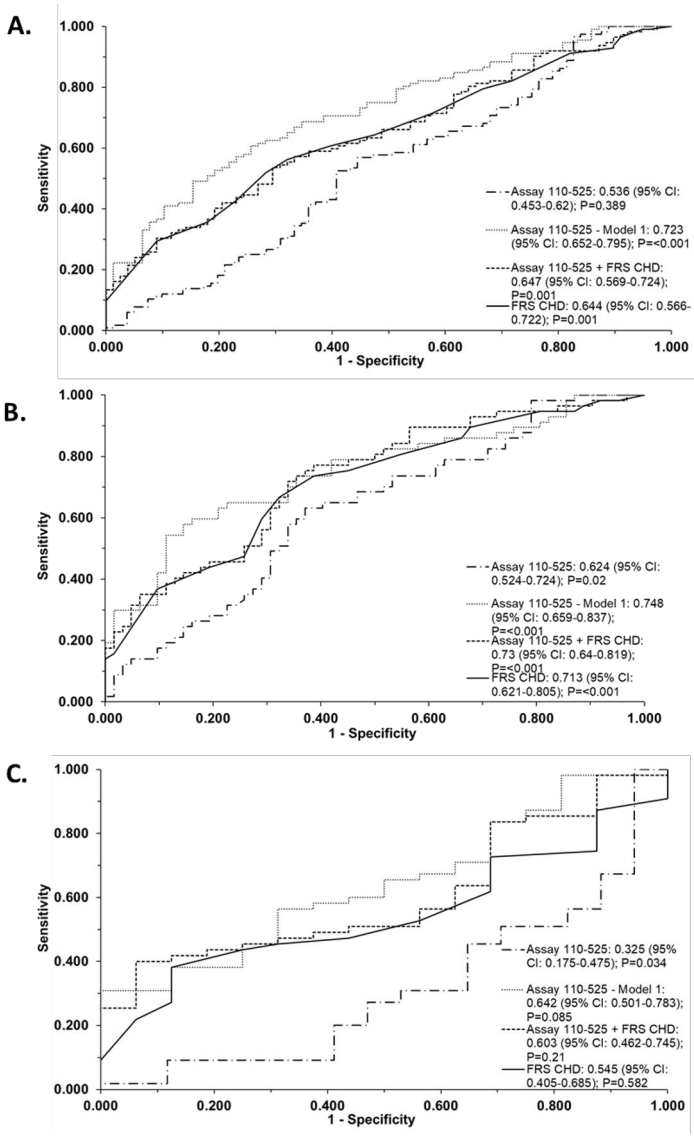


Figure 27. Direct two-site apoA-I assay 110-525 as predictors of coronary atherosclerosis. ROC curve of apoA-I assay 110-525, apoA-I assay 110-525 combined with age and sex (Model 1), apoA-I assay 110-525 combined with 10-year coronary heart disease risk by Framingham Risk Score (FRS CHD), and FRS CHD alone for detection of coronary atherosclerosis in the whole population (A), in patients without LLM (B) and in patients on LLM (C). The area under curve (AUC) (95% CI) and the P values are represented in the bottom right-hand corner of the figure. The assay 110-525 Model-1 used age and gender as the covariates. FRS CHD estimation included age, gender, smoking, hypertension, diabetes, HDL-C and LDL-C. ROC, receiver operator characteristic; LLM, lipid lowering medication; HDL-C, high density lipoprotein cholesterol; LDL-C, low density lipoprotein cholesterol. Adapted from publication III.

6 Summary/Conclusions

There has been considerable interest to develop diagnostic tools for early risk assessment and diagnosis of CAD due to the overwhelmed burden of this disease around the globe. In that context, HDL has been in the focus as a marker of CAD risk estimation since last many decades. However, generation of antibodies to develop assays for relevant forms of HDL has been considered challenging. The challenge arises due to the complex structure, heterogeneous nature and differing atheroprotective properties related to different HDL-particle type. Nowadays, genetic engineering of antibodies and phage display technology provide a platform to target complex antigens such as HDL and generate antibodies against the epitopes present at interface of the large intact biomolecular complexes.

In this thesis development of HDL/apoA-I antibodies against the intact HDL particles isolated from plasma of CAD subjects is described. Three pairs of isolated apoA-I antibodies (sc 022+454, sc 109+121 and sc 110+525) were implemented in development of two-site assays in two different TRF-based immunoassay formats. First assay format was “phage based approach”. The assay utilises single chain variable fragment fused to bacterial alkaline phosphatase (scFv-AP) and to filamentous phage (scFv-phage) as capture and detection antibodies, respectively, to target apoA-I on the surface of HDL particle. The second direct approach used scFv-APs both for capture and identification of target apoA-I on HDL. The designed immunoassays were evaluated with different sets of relevant clinical cohorts. Based on the published results, outcome of this thesis work could be summarized as follows:

I. It is possible to derive a large number of functional antibodies against HDL from synthetic antibody library using phage display. Access to a wide panel of antibodies can help in finding functional antibody pairs for immunoassay development. Several of antibodies obtained in this study showed capability to differentially recognize HDL particles derived from CAD patients and healthy subjects. The results showed that a variety of antibodies can be obtained using different selection strategies, however, it was also found that, in general, the enrichment of antibodies against the

most abundant proteins of HDL (i.e. apoA-I and apoA-II) predominated compared to more scarce proteins on HDL.

II. Three novel phage based two-site apoA-I assays (assay 022-454, 109-121 and 110-525) were developed by implementing the generated recombinant apoA-I antibodies. The assays did not appear to suffer from matrix interference whereas repeated freezing and thawing of the samples affected the results. Based on limited archival sample cohorts, these antibodies (recognizing apoA-I on the surface of a variety of HDL subpopulations) demonstrated clear indication of association with adverse cardiac outcome of atherosclerosis. The assays 109-121 and 110-525 seemed to have the possibility to add to the diagnostic and predictive value for cardiac conditions over polyclonal apo A-I and HDL-C.

III. A simplified version of two-site apoA-I assay 109-121 and 110-525 was presented by using shorter assay procedure and simpler antibody format (scFv-APs). The assay performance was found to be influenced with the use of statins or lipid lowering medication (LLM) by study patients. Clinical evaluation with patients suspected of obstructive CAD particularly in those not using LLM showed a clear association between the high level of apoA-I identified with the antibody pair 110-525 and the presence of coronary atherosclerosis. It can be suggested that the assays targeting heterogeneity in apoA-I /HDL could provide a potential and simpler tool to improve the identification of patients at risk of atherosclerosis particularly in individuals not under the use of LLM.

In conclusion, the phage display based technique seems to be efficient in identifying the antibodies against variable epitopes on the HDL particle (not in the soluble proteins). In potential future studies, use of next generation sequencing (NGS) methods could further facilitate the identification of apparently rare clones binding to less abundant proteins of HDL. It can be suggested that the immunoassays based on the HDL or apoA-I antibodies targeting the heterogeneity of HDL could be considered as an additional tool to follow-up the subjects with dyslipidemia and possibly linked to estimation of CAD risk. Hence, such assays/antibodies could be useful in clinical management of the individuals at risk of atherosclerosis. In addition, the antibodies generated in this study could serve as an interesting tools for studying the functional aspects of HDL to understand the atherosclerosis pathology. There are some limitations of the present study which must be taken into account. For example, the lack of epitope characterization on apoA-I when using these specific recombinant apoA-I in immunoassays. It is also notified that the clinical validation in this study is based on the limited number and type of clinical samples. Hence, there is need of substantially expanded testing with (i) both non-acute and

chest pain type cohorts to be correlated with both mid- and long-term adverse cardiac events, and, (ii) longitudinal study samples, where follow-up would indicate later cardiac incidents and if the actual risk could be recognized early, (iii) extensive and clinically well characterized cardiac cohort in order to investigate the ability to monitor CAD. Altogether, this thesis provides a novel approach of isolating HDL antibodies by employing phage display based synthetic library, and demonstrate design and optimization of two-site assays suggestive of improved clinical performance for CAD risk estimation together with already used existing reference biomarkers.

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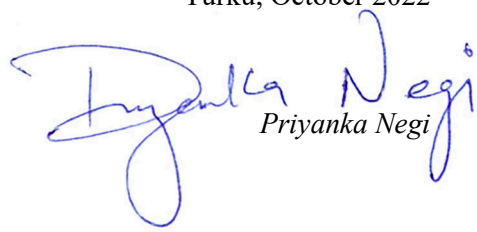

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