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# Three two-site apoA-I immunoassays using phage expressed detector antibodies – Preliminary clinical evaluation with cardiac patients

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ABSTRACT

High density lipoproteins (HDL) are a heterogenous group of subpopulations differing in protein/lipid composition and in their anti-atherogenic function. There is a lack of specific and robust assays which can target the functionality of HDL with respect to atherosclerosis. With recently generated CAD HDL targeted, single chain recombinant antibodies (scFvs) we set out to design and optimize apo A-I tests to compare it with conventional HDL-C and apo A-I analyses for diagnosis and risk assessment of coronary artery disease (CAD) and its outcome. Three highly sensitive two-site apo A-I assays: 022−454, 109−121 and 110−525 were optimized. A preliminary clinical evaluation of these assays, after proper sample dilution procedure, was performed using samples derived from 195 chest pain patients (myocardial infarction (MI), n = 86 and non-MI, n = 109), collected at the time of admission and at discharge from hospital (hospital stay ≤ 24 h). The clinical performance of the assays was compared with apo A-I measured with polyclonal anti-apo A-I antibody using conventional ELISA. Apo A-I data was in addition compared with HDL-C concentration of the samples. The concentration of apo A-I was significantly lower in MI patients than in non-MI individuals with assay 022−454 (admission and discharge samples, P < 0.0001 and = 0.004); assay 109−121 (admission and discharge samples, P = 0.04 and 0.0009), and, ELISA based apo A-I test (admission and discharge samples, P = 0.008 and < 0.0001). HDL-C (admission and discharge samples, P = 0.002 and P = 0.01) was also significantly lower in MI patients. In Kaplan- Meier analysis, two-site assay 109−121 assay predicted mortality from admission samples at 1.5 yrs (whole cohort, P = 0.01 and in MI patients, P = 0.05) and at 6 months (whole cohort, P = 0.04). Assay 110−525 predicted mortality at 1.5 yrs from admission samples of non-MI patients (P = 0.01) and at 6 months from whole discharge sample cohort (P = 0.04). Polyclonal anti-apo A-I based conventional assay predicted mortality at 1.5 yrs from admission samples of whole cohort (P = 0.03). Two-site apo A-I assay 022−454 and HDL-C provided no capability of predicting mortality in the whole cohort or any sub-group. In conclusion, two of the tested recombinant apo A-I antibody combinations (sc 109−121 and sc 110-525) display promising outcome to improve diagnosis and prediction of future cardiac events in cardiac patients over polyclonal apo A-I ELISA and HDL-C assays. The noted differences, while interesting, are preliminary and need however to be verified in extensive cohorts of pathological cardiac conditions and healthy controls.



*Abbreviations:* ScFv, single chain variable fragment; AP, alkaline phosphatase; CAD HDL, high density lipoproteins isolated from coronary artery disease patients; ctrl HDL, HDL isolated from control healthy individuals; rHDL, reconstituted HDL; EFI, europium fluorescence intensifier; TRF, time-resolved fluorescence; HDL-C, high density lipoprotein cholesterol; LDL-C, low density lipoprotein cholesterol; MI, myocardial infarction; TC, total cholesterol TG, triglyceride, apo A-I, apolipoprotein A-I, apoB apolipoprotein B-100; CETP, cholesterol ester transfer protein; PLTP, phospholipid transfer protein.

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## 1. Introduction

Atherosclerotic changes in coronary arteries are initiated already at early age to reach advanced stages of atherosclerotic coronary artery disease (ATCAD) by the time symptoms appear in adult life. Many of the changes in developing ATCAD turn out to be deleterious causing numerous premature deaths. An ideal approach to prevent premature ATCAD dependent deaths calls for its early risk estimation which demands the invention of novel and specific diagnostic tools.

High density lipoprotein (HDL) and its major structural protein apolipoprotein A-I (apo A-I) is known for its atheroprotective role. 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 [1]. Former studies have shown that apo A-I, cholesteryl ester/triglyceride (TG) and lipid transfer proteins cholesteryl ester transfer protein (CETP) and phospholipid transfer protein (PLTP) play vital roles in HDL remodelling [2]. In diagnostic practice, the plasma level of HDL cholesterol (HDL-C) is considered an established marker when evaluating the risk of ATCVD together with several other parameters such as low density lipoprotein cholesterol (LDL-C), non-HDL-C, apolipoprotein B (apoB), apo A-I, ratio of apoB/apo A-I and ratio of HDL-C/LDL-C, total cholesterol (T-C)/HDL-C. Many epidemiological and experimental animal studies have shown inverse correlation between HDL-C and ATCAD risk [3,4]. On the other hand, pharmacological studies and meta-regression analyses have reported conflicting results demonstrating lack of protection from CAD events despite of elevated HDL-C [5]. Further, Mendelian randomization studies have demonstrated that HDL-C levels are not causally related to coronary heart disease risk [6]. This has raised questions whether HDL-C levels can be considered as a relevant marker for CAD diagnosis. Under particular circumstances (such as diabetes and metabolic syndromes), HDL or apo A-I lose the atheroprotective function and may actually promote ATCAD [7]. It is assumed that ATCAD risk assessment and treatment strategies must specifically target dysfunctional HDL particles rather than assessing the absolute concentration of HDL-C [8]. In other words, this suggests a move from HDL-C hypothesis toward considering functional qualities of HDL.

Currently available methods for human plasma HDL assessment are based on direct measurement of HDL associated cholesterol (HDL-C) or apo A-I measurements mainly using conventional immunoassay techniques such as immunoturbidimetry or enzyme-linked immunosorbent assays (ELISA). The direct measurement of HDL-C or apo A-I do not offer any information about the heterogeneity of HDL particles. On the other hand, more advanced and complex techniques such as ultracentrifugation and electrophoresis, high performance liquid chromatography (HPLC), precipitation-based methods, and, nuclear magnetic resonance (NMR) - while potentially providing detailed information on HDL particle heterogeneity have not been adopted for routine clinical purposes [9,10]. At present, there are some HDL functional assays to specifically evaluate early steps in reverse cholesterol transport process [11,12] but they either lack reproducibility or need further validation with larger number of clinical samples [10,13]. Despite the availability of methods for HDL quantitation and HDL functionality, there is still an urgent need for robust, selective methods essentially evaluating functional HDL characteristics and which can be made available for routine clinical use. Monoclonal antibodies that can differentiate the dysfunctional HDL particles from the protective functional HDL forms are considered as one of the specific tools for evaluation and clinical risk stratification of HDL association with ATCAD [13]. The HDL functionality is closely related to its most abundant protein, apo A-I [14]. Exploring different ways of measuring molecular aspects of apo A-I could be relevant for capturing changes in HDL functionality related to CAD.

Aim of this study was to design and optimize two-site or sandwich-type, time-resolved fluorometry (TRF) based immunoassays using recombinant phage-displayed single chain (scFv) anti-HDL antibodies (apo A-I antibodies) and to perform a preliminary evaluation on cohort of chest pain patients, with samples obtained at hospital admission and discharge, against mortality and myocardial infarction follow-up. The recombinant apo A-I antibodies used in these assays were carefully isolated from an extensive synthetic antibody library with phage display as described in our previous work [15]. In an attempt to consider the highly dynamic and diverse, environment-dependent nature of HDL particles, these antibodies were intentionally selected to recognize HDL particles isolated from CAD affected individuals (CAD HDL). The two-site assays established with these antibodies were clinically evaluated with samples from cardiac patients to reflect the presence of ATCAD and to predict the risk of future cardiac events and all-cause mortality. The clinical performance of these HDL or apo A-I subform targeting assays was compared with conventional apo A-I and HDL-C analytes.

## 2. Materials and methods

### 2.1. Clinical samples

Routinely analysed serum samples (n = 10) from Central Hospital Kristianstad (Sweden) were used in analyzing the differences in specificity of scFv-APs (see details below). Plasma samples (n = 10; HDL-C in the range of 30.16–99.76 mg/dL) from individuals referred for HDL-C measurement at Turku University Hospital (Finland) were collected and pooled. The plasma pool was used in testing the competitive profile of the antibody clones and in identifying the functional antibody pair for two-site phage immunoassay. Serum samples (n = 200) from Helsinki University Hospital (Finland) which were routinely analyzed for investigating the lipid parameters were collected and used as controls for optimization and validation of assays. Samples (n = 6) (serum, Li- heparin plasma and EDTA plasma) from apparently healthy volunteers were collected at Department of Biochemistry/Biotechnology, University of Turku (Finland) and were used in assays as controls, and for studying assay characteristics. The assays were clinically evaluated with subset of serum/plasma samples of cardiac patients from Corogene study (cohort-I) [16] and cardiac patients with chest pain attending the emergency department of Turku University Hospital (referred as cohort-II) [17]. Cohort-I was comprised of 36 individuals who were categorized into three categories; (i) acute coronary syndrome (ACS) patients (patients had unstable angina, ST elevated myocardial infarction (STEMI) or non-STEMI (NSTEMI), n = 12), (ii) patients with stable CAD (n = 12), and, (iii) patients without CAD (no-CAD, n = 12). The subjects from these groups were age-sex matched. Samples in cohort-II 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 h 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 (admission samples, n = 86 and discharge samples, n = 86). The MI patients were subjects with NSTEMI (admission and discharge samples, n = 23) and STEMI (admission and discharge samples, n = 63). In these patients, during follow-up of 1.5 years death was confronted by 27 (13.8 %) individuals and 28 (14.3 %) individuals encountered MI. The follow-up events of these patients are represented in Fig. 1. Samples used in this study were stored at -70 or -80 °C prior to use. The samples were used after obtaining ethical permission and informed consent of the subjects.

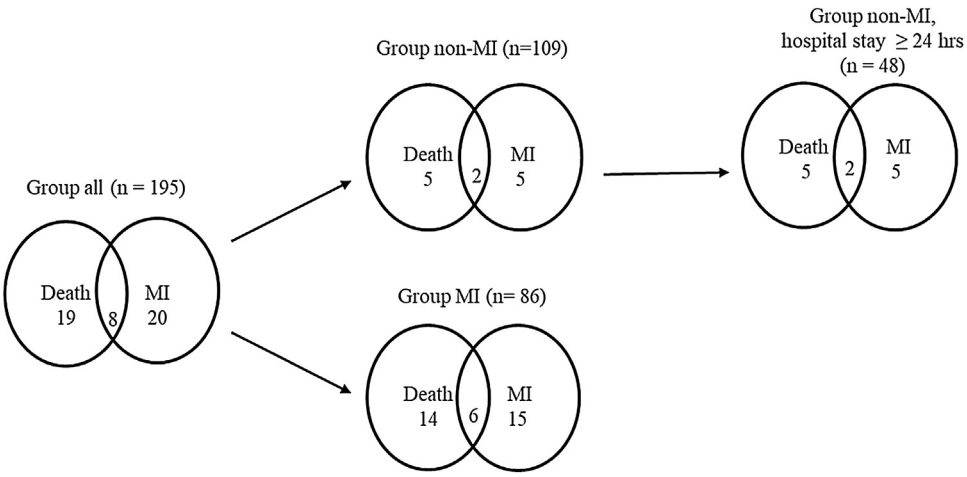


**Fig. 1.** Events (death and MI) that occurred during 1.5 yrs follow-up time in cohort-II. The figure represents the death and MI which took place in whole cohort and in patients divided into MI and non-MI group, and, in non-MI group who had hospital stay ≥ 24 h. In the figure, n = number of individuals, MI = myocardial infarction.

### 2.2. Reagents

Affinity purified scFv-AP were biotinylated with EZ-Link-NHS-PEG4-biotin from Thermo Scientific (USA) according to the manufacturer's instructions. Anti-phage 9E7 monoclonal antibody (Mab) was produced in our laboratory at Department of Biochemistry/Biotechnology, University of Turku. Rabbit anti-*Escherichia coli* alkaline phosphatase (AP) polyclonal antibody (Pab) was from LifeSpan Biosciences (USA). Anti-apoA1 monoclonal antibody (Mab) were from Medix Biochemica (Espoo, Finland). Mabs were labeled with europium (III) chelate of N1-(4-isothiocyanatobenzyl) diethylenetriamine-N1,N2,N3,N4-tetrakis (acetic acid) (N1-Eu) [18]. Hyper phage was purchased from PROGEN Biotechnik GmbH, Germany.

Wash buffer, streptavidin coated plates (bovine serum albumin (BSA) or casein blocked) and europium fluorescence intensifier (EFI) were from Kaivogen (Finland). HDL assay buffer 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 0.5 or 2.3 or 4% BSA.

Lipoprotein deficient serum (LPDS) and Intralipid (20 % emulsion) were from Merck, Germany.

### 2.3. Isolation of lipoproteins

Isolation of total HDL (=$HDL_2$ + $HDL_3$ subclasses), very low-density lipoprotein (VLDL) and intermediate-density (IDL) lipoproteins is done by sequential ultracentrifugation using KBr for density adjustment as described previously [15]. In this study human plasma obtained from healthy volunteers (Finnish Red Cross Blood Service, Helsinki, Finland) was used for isolation of VLDL and IDL, and, human serum sample obtained from healthy individual was used for isolation of total HDL. Table-top ultracentrifuge (Beckmann Optima TL, USA) and solid KBr for density adjustment were used in this procedure. Plasma (for isolation of VLDL and IDL in this study) or serum (in case of isolating total HDL) samples were first adjusted to the density (d) of 1.019 g/mL and the centrifuge tube filled with a d =1.019 g/mL KBr solution to the total volume of 3 mL. The samples were centrifuged at 5 °C for 2−3 h at 100,000 rpm (corresponding to relative centrifugal force of 500,000×*g*). After centrifugation, very low and intermediate density lipoproteins (VLDL + IDL) were recovered in the top 1 mL fraction and dialyzed against PBS, pH 7.4 and stored at -80 °C for further analyses. The bottom fraction was adjusted to d =1.063 g/mL using solid KBr, filled to 3 mL with d =1.063 g/mL KBr solution and centrifuged (5 °C, 3 h, 500,000 ×*g*). The top 1 mL fraction of LDL was removed. To get the total HDL the bottom fraction was adjusted with solid KBr to d =1.21 g/mL and centrifuged (5 °C, 18 h, 500,000 ×*g*). HDL was obtained in top 1 mL fraction. The isolated HDL was dialyzed against PBS, pH 7.4 and stored at −80 °C. HDL subfractions HDL2 and HDL3 were isolated at their densities 1.063–1.125 g/mL and 1.125–1.21 g/mL as above (5 °C, 18 h, 500,000 ×*g*).

### 2.4. Cloning, production, purification and immunoreactivity of recombinant HDL antibodies; scFv-AP and scFv-phage

Cloning, production and purification of the scFv-alkaline phosphatase fusion protein (scFv-AP) and their immunoreactivity towards different antigens is described in detail previously [15]. For cloning into scFv-phage format, the scFv-DNA of HDL antibodies were inserted at the SfiI digested chloramphenicol-resistant pEB32x phagemid and electroporated into *E. coli* XL-1 blue cells. ScFv fragments were then displayed multivalently on M13 filamentous phage by superinfection with hyper-phage and produced [19]. Purification of the phage was done by PEG/NaCl precipitation according to standard protocol. The purified phages were stored at 4 °C in TBS containing 0.02 % $NaN_3$ and 1% BSA.

### 2.5. Antibody selection for two-site apo A-I assays

Purified antibody clones (HDL antibodies) were tested as scFv-phage or scFv-AP in several assays to select the antibodies which could be further used in optimization of two-site immunoassays.

Antibodies which were selected based on their reactivity profile to different antigens [15] were tested with a competitive assay performed using total HDL fraction, and, pre-incubated mixture of scFv-AP, plasma dilution and N1-Eu anti-AP Pab; the TRF for europium was measured with Victor plate reader (PerkinElmer, USA) (Supplementary information, Methods). ScFv-AP HDL antibodies were then shortlisted based on this data and further tested for their immunoreactivity with small subset of routine serum samples (Central Hospital Kristianstad, Sweden) with known apo A-I concentrations by performing sandwich-type immunoassay with commercial apo A-I (Mab) as described earlier [15]. Shortly, the sandwich-type immunoassays was done with immobilized biotinylated anti-alkaline phosphatase Pab, scFv-AP antibodies as a capture antibody, sample, and, N1-Eu-anti-apo A-I Mab as detection antibody. Out of the tested antibodies, those antibodies with different binding profiles to samples were selected.

Several combinations of the selected antibodies (in format of scFv-AP and scFv-phage) were tested with plasma pool that was made from routine plasma samples in a sandwich-type immunoassay (Supplementary information, method section) to identify the suitable antibody pair. Briefly, the assay was done with either immobilized biotinylated anti-alkaline phosphatase Pab or scFv-AP antibodies (biotinylated or unbiotinylated) as a capture antibody, sample, scFv-phage as detection antibody and N1-Eu-anti-phage as a tracer.

### 2.6. Final optimized two-site apo A-I assays

Three different phage based two-site apo A-I immunoassays; assay 022−454, 109−121 and 110−525 were optimized. Diagrammatic representation of the principle of these two-site apo A-I assays is shown in Fig. 2. Each assay used two different apo A-I antibodies; capture antibody (scFv-AP) sc 022, sc 109 and sc 110 and corresponding detection antibody (scFv-phage) sc 454, sc 121 and sc 525, respectively. Assay 022−454 and 109−121 were done with HDL-assay buffer supplemented with 4 % BSA on casein blocked streptavidin plates and assay 110−525 was performed with HDL-assay buffer containing 2.3 % BSA on BSA blocked streptavidin plates. Total HDL isolated from serum of a healthy individual was used as a calibrator.

Biotinylated capture antibodies sc 022, sc 109 and sc 110 (100 ng/50 μl/well) were immobilized on separate streptavidin coated microtiter wells and incubated for 60 min at RT on shaking followed by two washes. Calibrator and sample (50 μl/well) were added into the microtiter 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. The wells were then washed twice and then detection antibodies sc 454, sc 121 and sc 525 (2.5E8/50 μl/well) were added in the respective assays and incubated for 1 h at RT on shaking. Wells were aspirated and N1-Eu labeled anti-phage Mab (50 ng/50 μl/well) was added with further incubation for 1 h at RT on shaking. Finally, wells were washed four times. EFI was added into the well, incubated for 10 min. on shaking and TRF measurement was done with Victor plate reader (PerkinElmer, USA).

### 2.7. Assay validation

The optimized assays were tested with sample dilution of 1:1000 and 1:1500; 1:750 and 1:1000; 1:750−1:1500 using six serum samples (Helsinki University Hospital, Finland) for assay 022−454, 109−121and 110−525, respectively. Inter-assay variation was assessed by repeating the assays eight times during different days using four replicates of calibrator (HDL, n = 32) and four replicates of three serum samples (HDL-C concentration = 41.37–81.2 mg/dL, n = 32). Effects of sample matrix and sample stability on these apo A-I - assays were studied by analyzing 3–5 matched plasma (Li-heparin and EDTA) and serum samples of healthy volunteers. Sample stability was investigated by measuring the samples stored at RT for 0−4 h and after freezing-thawing the samples up to ten times. Effect of elevated lipid levels on the assays was studied by supplementing the lipoprotein deficient serum (LPDS) and up to eight serum samples (HDL-C range was 31.32–105.95 mg/dL) with 2 mg/mL of TG (normal concentration of human plasma TG is <1.7 mmol/L which is about 1.5 mg/mL) incorporated in VLDL-IDL and in Intralipid emulsion, and, comparing the assay results performed with unsupplemented LPDS and serum samples.

### 2.8. Measurement of Biochemical parameters in clinical samples (cohort-I and cohort-II)

Biochemical parameters were estimated using standard methods as described in Supplementary information, Method section. In sample from cohort-I, biochemical parameters including concentration of HDL-C, LDL-C, apo A-I, triglycerides (TG), and total cholesterol (TC), cholesterol ester transfer protein (CETP) and phospholipid transfer protein (PLTP) activities were measured. In cohort-II all the above mentioned parameters (except CETP and PLTP activity) in addition to apoB and free fatty acid (FFA) were measured.

### 2.9. Clinical evaluation of two-site apo A-I assays

The clinical performance of the optimized two-site apo A-I immunoassays was evaluated using samples from two different set of panels of patients with cardiac related disease, cohort-I and cohort-II (described in section 2.1). In the patients of cohort-I, concentration of apo A-I determined with two-site apo A-I assays were compared between (i) ACS patients (n = 12), (ii) patients with stable CAD (n = 12) and (iii) patients without CAD (no-CAD, n = 12). In the patients from cohort-II, the concentration of apo A-I obtained with two site apo A-I - assays in admission and discharge samples was compared between MI (admission and discharge samples, n = 86) and non-MI group (admission samples, n = 109 and discharge samples, n = 48). For survival analysis of death and myocardial infarction, the patients were divided into tertiles based on apo A-I concentration in admission and discharge samples obtained with two-site apo A-I -immunoassays (assay 022−454, 109−121 and 110−525). Then, the survival analysis was done in all the patients (whole cohort), and, the patients categorized into MI and non-MI groups separately. The clinical performance of these apo A-I antibodies of two site apo A-I assays were compared with the clinically relevant CAD risk biomarkers apo A-I and HDL-C whose concentrations are estimated via conventional routine methods.

### 2.10. Statistical analysis

The statistical analysis was done using Origin 2015 (OriginLab Corporation, Wellesley Hills, USA) and JMP Pro 14 (SAS Institute Inc., Cary, NC; USA); tests results were considered significant for two tailed P value <0.05.

In smaller cohort (cohort-I), level of lipid parameters, PLTP activity, CETP activity and level of apo A-I measured with two-site apo A-I assays was compared between the clinical groups using Kruskal-Wallis test and if significant pairwise comparision was corrected with Steel-Dwass method; the data was expressed as median (25th percentile – 75th percentile). Age was also expressed as median (25th percentile – 75th percentile). Categorical variable is presented as numbers and percentage. Spearman's correlation (r) between concentration of apo A-I estimated with two-site apo A-I assays and with conventional apo A-I test was calculated. In larger panel (cohort-II), normality distribution assumption was checked visually together with Shapiro-Wilks test and Q—Q plot. To enable normal distribution, lipid parameters and apo A-I measured with two-site apo A-I assays were naturally log transformed and were compared between MI and non-MI group with two sample *t*-test; their data was back-transformed to report the geometric mean (95 % CI). Comparison between non-MI, STEMI and NSTEMI individuals was done using one-way analysis of variance (one-way ANOVA) or Kruskal-Wallis test and if significant pairwise comparison was done with Tukey-Kramer method or Steel-Dwass method. Categorical variables are compared between MI and non-MI group using Fisher's exact test or Chi-square test as appropriate and reported as numbers and percentage. Pearson's correlation (r) between con-

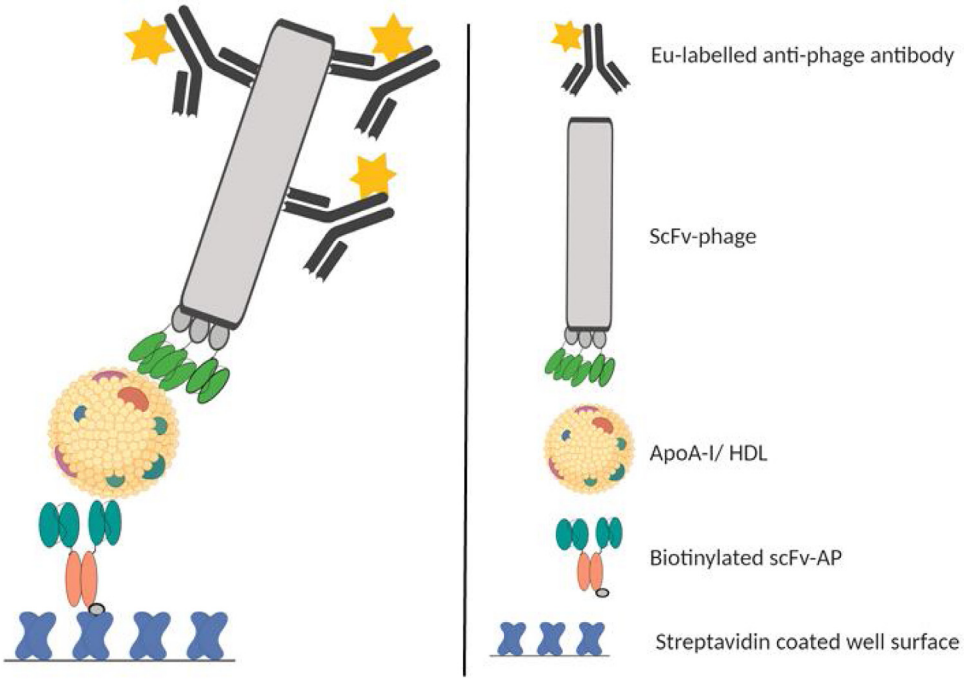


**Fig. 2.** Schematic representation of the principle of the used two-site apoA-I immunoassay. The detection is based on time-resolved fluorescence of europium attached to anti-phage antibody.

centration of apo A-I estimated with two-site apo A-I assays and with conventional apo A-I test was calculated; correlation among the two-site apo A-I assays was also determined. Age of individuals in MI and non-MI group was compared using two sample *t*-test and presented as median (25th percentile – 75th percentile). Survival curves of tertiles obtained from two-site apo A-I assays, HDL-C, and conventional apo A-I assay were plotted with Kaplan–Meier (K–M) method and compared using log-rank test.

## 3. Results

### 3.1. Recombinant HDL antibodies and their immunoreactivity

Twenty six scFv antibodies, which in the previous report [15] showed distinguished profiles and good signal levels in binding to different HDL antigens were successfully produced and purified both as fusions to bacterial alkaline phosphatase (scFv-APs) and as displayed on filamentous phage (scFv-phage). An additional antibody clone sc 525 (isolated later from the antibody library) was also produced and purified for further tests. These antibodies were first tested in scFv-AP format for binding to HDL using a competitive immunoassay design and different dilutions of human plasma HDL as competing analyte. The antibody clones which showed distinct responses to different plasma dilutions were considered for further testing. Based on the results of the assay (as examples, responses of the of the clones eventually selected for the two-site apo A-I sandwich combinations are shown in Figure S1), thirteen out of 26 antibody clones displaying good signal-to-background ratios and variable competitive profiles were chosen for further studies. The chosen antibodies were tested against 10 plasma samples with variable apo A-I concentrations in a sandwich immunoassay using scFv-AP clones as capture antibody and europium-labeled commercial anti-apo A-I mAb as tracer. Antibodies showed quite variable reaction pattern with individual samples, and one example of three scFv-APs antibodies (used in two-site apoA-I immunoassays later) is shown in supplementary Figure S2. In the next phase, two hundred combinations of the above mentioned antibody clones were tested in a sandwich-type immunoassay using scFv-AP as capture antibody and scFv-phage as detection antibody with pooled plasma. Thirty-two functional scFv antibody pairs (specific signal > background + 2 × SD) were found. Based on technical performance qualities, eventually six unique antibodies in three two-site combinations were selected for the development of further optimized assay; three apo A-I antibody pairs (sc 022- sc 454, sc109 - sc121 and sc110 – sc 525 (the former as scFv-APs and the latter as scFv-phage) were selected for further experiments.

### 3.2. Assessment of two-site apo A-I immunoassays

Three two-site apo A-I -immunoassays: assay 022−454, 109−121 and 110−525 were optimized using biotinylated scFv-APs (022, 109 and 110) as capture antibodies and scFv-phages (454, 121 and 525) as detection antibodies. Briefly, the optimized parameters included assay buffer composition, incubation time of samples, wash steps, amount and incubation time of antibodies. Generally, conditions displaying higher signal-to-background ratio and better precision were the criteria of selection for the final immunoassay.

#### 3.2.1. Characteristics of two-site apo A-I assays

The calibration curves of the optimized apo A-I assays using HDL (= $HDL_2$ +$HDL_3$ subclasses) as calibrator are shown in Fig. 3. In assays 022−454, 109−121 and 110−525, saturation of signals was not observed with HDL (calibrator) protein concentration of up to 1800 ng/mL, 2000 ng/mL and 2500 ng/mL, respectively. The analytical sensitivity was calculated by adding three times the standard deviation to the average counts (n = 8) of the blank sample (HDL =0 ng/mL); analytical sensitivities for assay 022−454, 109−121 and 110−525 were 114 ng/mL, 7 ng/mL and 25 ng/mL, respectively. The inter-assay coefficient of variation (CV%) in the calibrator (HDL, n = 32) in the assays 022−454, 109−121 and 110−525 analyzed was 1.6–17 %, 1.9–9.3 % and 1.4–7.3 %. In the serum samples (n = 32) the inter-assay variation was 6–14 %, 5.3–8.1 % and 12–18.8 % with assay 022−454, 109−121 and 110−525, respectively (Supple-

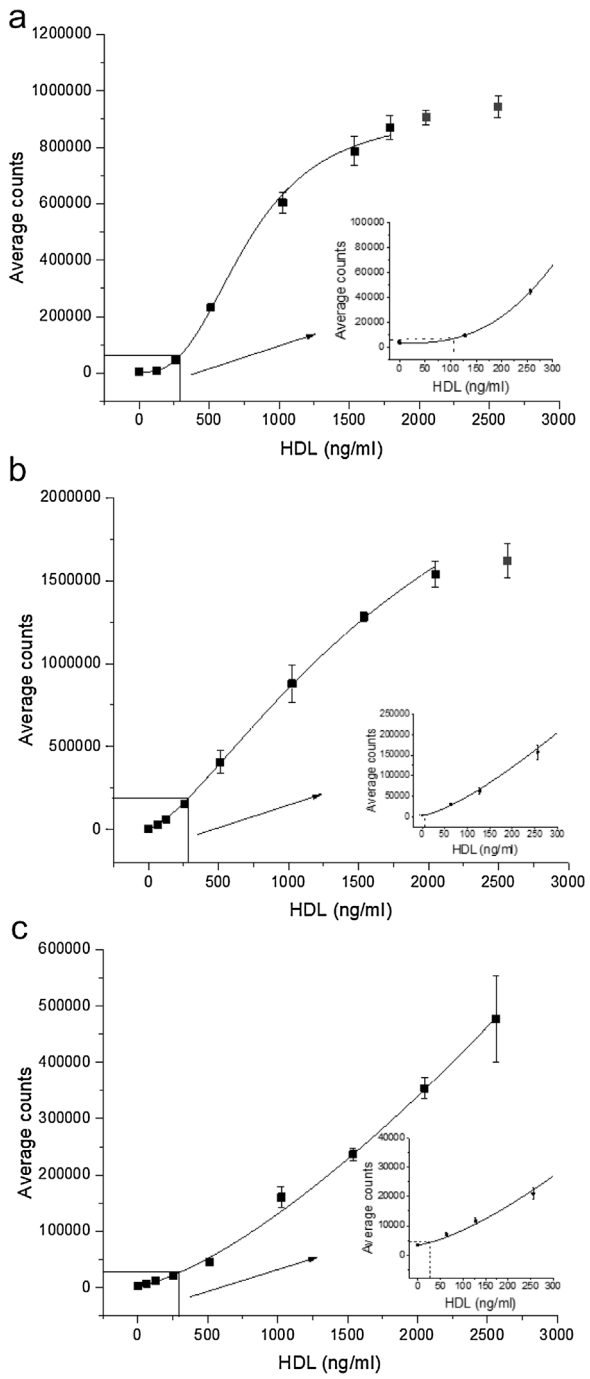


**Fig. 3.** Standard curves of the two-site apoA-I assays: (a) assay 022-454, (b) assay 109-121 and (c) assay 110-525. The curve was fitted with four-parametric logistic function. Signals of the assays are represented as average counts of eight replicate measurements. The error bars represent the standard deviation (SD) of the average counts. The analytical sensitivity (background + 3*SD) 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 (—).

mentary Table S 1). The intra-assay variation was < 20 % in these assays.

### 3.2.2. Assay validation

The analytical recovery of calibrator studied in different matrices (Lithium-heparin plasma, EDTA plasma and serum) of five individuals were in range of 95–121 % with assay 022−454, 77–101 % with assay 109−121 and 89–121 % with assay 110−525 (Supplementary Figure S3). Freeze-thawing cycles of three samples in different matrices (Lithium-heparin plasma, EDTA plasma and serum) showed that the assay 022−454 was minimally affected with up to ten freeze-thawing cycles (recovery range was 93–119 %). Assay 109−121 tolerated up to three freeze-thawing cycles (recovery range was 77–121 %), however, assay 110−525 was very sensitive to this treatment (Supplementary Figure S4).

Sample dilutions of 1000- and 1500-fold, 750- and 1000-fold, and, 500-, 750- and 1000-fold were found suitable for assays 022−454, 109−121 and 110−525, respectively.

The effect of moderate level of lipids (especially TGs, 2 mg/mL) on the assays was tested (Supplementary Table S2). Whereas assay 022−454 and assay 109−121 were found to be unaffected, assay 110−525 seemed to be slightly affected, with recoveries of TG-enriched VLDL-IDL and intralipid emulsion varying between 69–140 % and 60–126 %, respectively.

### 3.3. Clinical evaluation of two-site apo A-I assays

The three optimized phage based two-site apo A-I assays were evaluated using samples derived from subset of cardiac patients from Corogene study (cohort-I) [16] and chest pain patients who visited Emergency unit of Turku University hospital (cohort-II) [17]. The two-site apo A-I assay results were compared with polyclonal anti-apo A-I based conventional routine apo A-I assay (immunoturbidometry/ELISA method) and HDL-C assay (precipitation based direct method).

#### 3.3.1. Cardiac patients from cohort-I

Cohort-I was categorized into groups of individuals not suffering from CAD (no CAD, n = 12), patients with stable CAD (stable CAD, n = 12) and patients with ACS (ACS, n = 12) (Table 1). The patients in these groups were age and sex (all males) matched and therefore these parameters were not compared among the three groups. All the three two-site apo A-I assays, i.e. immunoassays 022−454, 109−121 and 110−525 had significantly positive correlation (Spearman's r, P value) with apo A-I determined with conventional routine method (immunoturbidimetric) (0.68, P < 0.0001; 0.48, P = 0.002, and 0.54, P = 0.0007, respectively). Two-site apo A-I assay 110–525 (Fig. 4 c and Table 1) and conventional apo A-I assay (Fig. 4 d and Table 1) were able to differentiate between the clinical groups (Kruskal Wallis test P = 0.03 and 0.01) and apo A-I was significantly lower in ACS patients compared to individuals without CAD (assay 110–525 and conventional apo A-I test, Steel Dwass test, P = 0.03, Fig. 4 c and 0.03, Fig. 4 d). However, either of these assays could not differentiate between the patients with stable CAD and without CAD (Table 1). On the other hand, assay 022−454, 109−121 and HDL-C test could not differentiate between the clinical groups (Kruskal Wallis test P = 0.08, 0.06 and 0.07, respectively) (Fig. 4 and Table1). One striking feature observed in this panel was that the apparent apo A-I values obtained with two-site apo A-I assay 110−525 were several folds higher in comparison to the other two-site apo A-I assays and the conventional apo A-I assay (Table 1 and Fig. 4).

#### 3.3.2. Cardiac patients from cohort-II

*3.3.2.1. Baseline characteristics.* The background information of this cohort (all patients and patients categorized into MI and non-MI group) is shown in Table 2A. There was significantly higher number of individuals with hypercholesterolemia (P = 0.001), hypertension (P = 0.001), previous CAD (P < 0.0001) and with family history of CAD (P = 0.02) in the MI group than in non-MI group. There was no significant difference between the age (P = 0.21), in the proportion of males (P = 0.376), smokers (P = 0.30) and diabetic (P = 0.30) individuals present in these groups. In MI group, higher number of individuals died (P = 0.0007) and suffered MI (P = 0.0003) within 1.5 years.

**Table 1**
Baseline characteristics of cardiac patients from cohort-I.

| | No CAD (n = 12) | ACS (n = 12) | Stable CAD (n = 12) | P | All (n = 36) |
|---|---|---|---|---|---|
| **Background information** | | | | | |
| Men [a] | 12 (100) | 12 (100) | 12 (100) | # | 36 (100) |
| Age [b] | 71(55.75–73.75) | 72(55.75–73.75) | 72(55.7–73.7) | # | 72 (55.7; 73) |
| **Lipid parameters** | | | | | |
| ApoA–I [b], mg/dL | 125.5(108.2–145.2) | 98.5(72.75–108) * | 121(108.2–127.2) | 0.01 | 112 (98.2–127.5) |
| HDL-C [b], mg/dL | 39.5(31.2–46.3) | 27.4(20.6–36.875) | 36.1(32.2–40.15) | 0.07 | 33.5 (26.8–41.1) |
| TC [b], mg/dL | 154(146.2–170.2) | 127.5(111.5–162.2) | 142.5(117.25–173.5) | 0.39 | 149 (122.2–168.7) |
| TG [b], mg/dL | 109.5(71.2–126.5) | 89(76.5–106.5) | 89.5(71.75–138.25) | 0.86 | 91.5 (72.5–114.7) |
| LDL-C [b], mg/dL | 94.6(83.7–103.85) | 84.6(64.4–103.8) | 86.75(55.45–117) | 0.64 | 93.2 (71.8–106.6) |
| **Phospholipid mobilizing activity** | | | | | |
| PLTP [b], nmol/mL/h | 5615(3800–7712.7) | 5611(3871.5–7047.7) | 7550.5(6017.5–8605) | 0.10 | 6147 (4886.2–7752.5) |
| CETP [b], nmol/mL/h | 29.1(23.5–34.1) | 30.05(25.4–31.4) | 31.8(27.175–35.55) | 0.49 | 30.1(24.5–34.1) |
| **Two-site apoA–I assays (apoA–I)** | | | | | |
| Assay 022–454 [b], mg/dL | 77.3 (68.0–109.2) | 65.39 (50.7–101.2) | 93.9 (78.0–120) | 0.08 | 79.2 (64.0–112.3) |
| Assay 109–121 [b], mg/dL | 42.3 (26.4–48.2) | 32.9 (19.4–54.1) | 56.8 (40.3–85.2) | 0.06 | 43.2(26.4–55.5) |
| Assay 110–525 [b], mg/dL | 364.1 (275–665.3) | 206.4 (155.1–282.7) * | 391.0 (230.1–464.1) | 0.03 | 288.4 (211.5–448.7) |

Overall comparison between clinical groups was done using Kruskal-Wallis test (P values is shown in the table) and if significant pairwise comparision was done by Steel-Dwass method. # no comparision was done between the groups; * P < 0.05 for comparison between no CAD vs ACS ¤ P < 0.05 for comparison between no CAD vs stable CAD. CAD, coronary artery disease; No CAD, individuals without CAD; ACS, acute coronary syndrome ; stable CAD, individuals with stable CAD; TC, Total cholesterol; TG, triglyceride; FFA, free Fatty acid; HDL-C, high density lipoprotein cholesterol; LDL-C, low density lipoprotein cholesterol; ApoA–I, apolipoprotein A–I; CETP, cholesteryl ester transfer protein; PLTP, phospholipid transfer protein.

[a] N (%); [b] Median (Quantiles25 - Quantiles75).

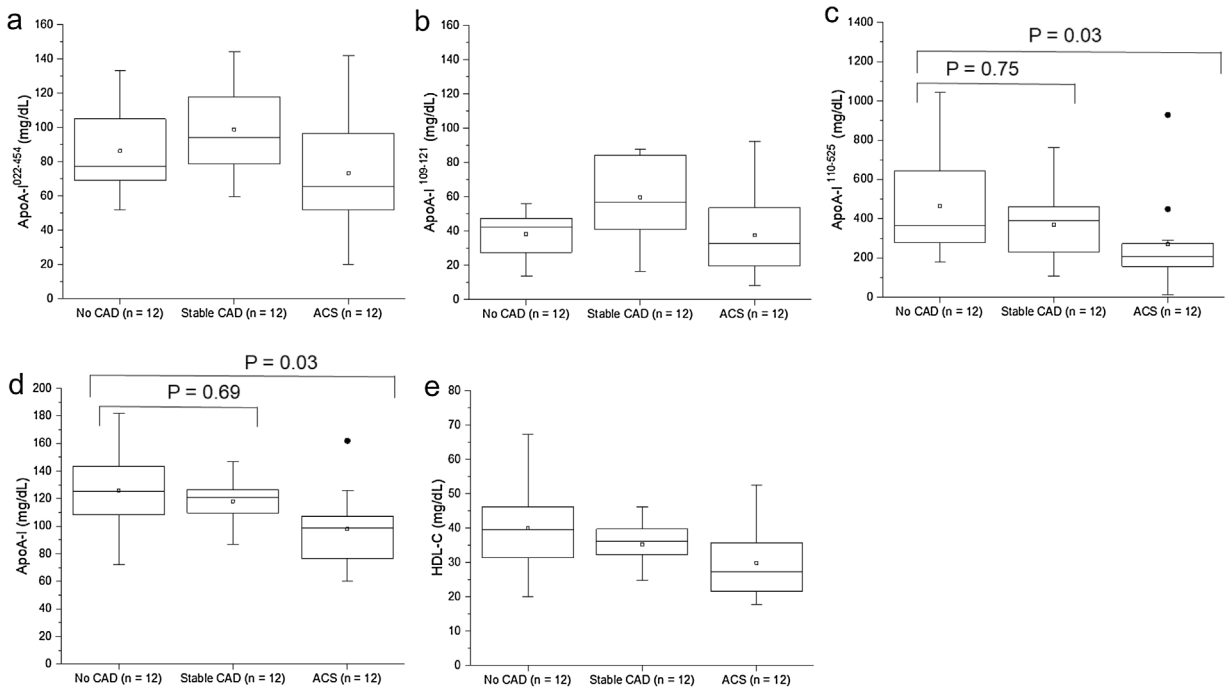


**Fig. 4.** Comparison between individuals with ACS, stable CAD and without CAD (no CAD) based on apoA-I concentration measured with the two-site apoA-I assays (a-c), apoA-I measured with conventional immunoturbidimetric assay (d) and HDL-C (e). Assay 022-454 (apoA-I $^{022-454}$) (Kruskal-Wallis test, P = 0.08), (b) assay 109-121 (Kruskal-Wallis test, P = 0.06), (c) assay 110-525 (Kruskal-Wallis test, P = 0.03), (d) apoA-I (Kruskal-Wallis test, P = 0.01) (e) HDL-C (Kruskal-Wallis test, P = 0.07). Y-axis indicates the apoA-I values obtained with two-site apoA-I assays, conventional apoA-I assay, and, values of HDL-C. Statistical testing is done by Kruskal-Wallis test, and if significant, pairwise comparison between groups is done with Steel-Dwass method and P values are calculated (presented in figure). The whiskers represent the 5th and 95th percentile, the horizontal line in the box represents the median. Mean is marked as a square (□) inside the box and the solid circles (●) outside the box represents the outliers. Description of the respective panel (cohort-I) can be found in Table 1. ApoA-I; apolipoprotein A-I; ACS, acute coronary syndrome; CAD, coronary artery disease.

The levels of biochemical parameters (in admission and discharge samples) including TC, TG, FFA, HDL-C, LDL-C, apoB, apo A-I (determined with conventional routine ELISA method) and apo A-I measured with two-site apo A-I assays are shown in Table 2B. In admission samples, there was no significant difference in levels of TC (P = 0.39), TG (P = 0.47) and apoB (P = 0.09) and LDL-C (P = 0.98). In discharge samples, the geometric mean concentration of TC (P = 0.0006), TG (P = 0.002), apoB (P = 0.02) and LDL-C (P = 0.02) were significantly higher in non-MI group as compared to MI group. In admission samples, geometric mean concentration of FFA (P < 0.0001) was significantly higher in MI individuals than in individuals without MI, but there was no significant difference between the groups using the discharge samples (P = 0.23). In admission and discharge samples, geometric mean concentration of apo A-I measured with the two-site apo A-I assays 022–454 (in admission and discharge samples, P < 0.0001 and P = 0.004) and 109–121 (in admission and discharge samples, P = 0.04 and 0.0009) was significantly lower in MI group than in non-MI group. By further classifying the MI patients into STEMI and NSTEMI groups, we found that the assays were able to discriminate between the

**Table 2**
Baseline characteristics of cardiac patients from cohort-II.

A. Background information.

| | Data available | Non-MI (n = 109) | Data available | MI (n = 86) | P value | Data available | All (n = 195) |
|---|---|---|---|---|---|---|---|
| Gender, Men[a] | 109 | 65 (59.6) | 86 | 57 (66.28) | 0.37 | 195 | 122 (62.56) |
| Age[b] | 109 | 66 (55–76) | 86 | 71.5 (59–78) | 0.21 | 195 | 69 (57–77) |
| **Risk factors** | | | | | | | |
| Familiy history of CAD[a] | 100 | 24 (24) | 85 | 33 (38.82) | *0.02 | 185 | 57 (30.81) |
| Smoking[a] | 98 | 20 (20.41) | 85 | 23 (27.06) | 0.30 | 183 | 43 (23.5) |
| Diabetes[a] | 109 | 13 (11.93) | 86 | 15 (17.44) | 0.30 | 195 | 28 (14.36) |
| Hypercholesterolemia[a] | 109 | 43 (39.45) | 86 | 54 (62.79) | *0.001 | 195 | 97 (49.74) |
| Hypertension[a] | 109 | 43 (39.45) | 86 | 54 (62.79) | *0.001 | 195 | 97 (49.74) |
| Previous CAD[a] | 109 | 9 (8.26) | 86 | 29 (33.72) | *<0.0001 | 195 | 38 (19.49) |
| **Outocome (1.5 years)** | | | | | | | |
| Death | 109 | 7 (6.4) | 86 | 20 (23.25) | *0.0007 | 195 | 27 (13.8) |
| MI | 109 | 7 (6.4) | 86 | 21 (24.41) | *0.0003 | 195 | 28 (14.3) |

B Lipid parameters and two-site apo A-I assays from admission and discharge samples.

| | Admission samples | | | | | Discharge samples | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Data available (n) | Non-MI (n = 109) | Data available (n) | MI (n = 86) | P value | Data available (n) | Non-MI (n = 48) | Data avaliable (n) | MI (n = 86) | P value |
| **Lipid paramters** | | | | | | | | | | |
| TC[c], mg/dL | 90 | 193.9(184.7–203.6) | 67 | 187.4(175.4–200.2) | 0.39 | 48 | 186.2(171.9–201.7) | 84 | 157.9(149.6–166.7) | *0.0006 |
| TG[c], mg/dL | 90 | 94.3(87.2–101.9) | 67 | 98.2(90.4–106.7) | 0.47 | 48 | 55.7(50.3–61.5) | 84 | 46.4(43.4–49.6) | *0.002 |
| FFA[c], mmol/L | 86 | 0.35(0.31–0.4) | 64 | 0.58(0.49–0.69) | *<0.0001 | 48 | 0.27(0.23–0.32) | 83 | 0.24(0.22–0.27) | 0.23 |
| HDL-C[c], mg/dL | 90 | 46.3(43.2–49.5) | 66 | 40.8(40.4–42.9) | *0.002 | 48 | 41.1(38.1–44.3) | 84 | 36.3(33.1–38.5) | *0.01 |
| LDL-C[c], mg/dL | 90 | 123.1(114.6–132.2) | 66 | 123.2(109.9–135.8) | 0.98 | 47 | 115(99.5–132.9) | 84 | 96.8(90.0–104.5) | *0.02 |
| ApoA–I[c], mg/dL | 105 | 142.7(137.2–149) | 84 | 132.2(121.5–136.9) | *0.008 | 48 | 126.9(119.2–135) | 86 | 108.2(99.5–113.3) | *<0.0001 |
| ApoB[c], mg/dL | 105 | 94(89.1–99.2) | 84 | 100.7(99.5–107) | 0.09 | 48 | 96.7(89.8–104.1) | 86 | 86.6(81.5–91.5) | *0.02 |
| **Two-site apoA–I assays (apoA–I; mg/dL)** | | | | | | | | | | |
| Assay 022–454[c] | 109 | 123.5(121.5–131.4) | 86 | 103.1(99.5–109.7) | *<0.0001 | 48 | 95.1(90–104.3) | 86 | 79.8(73.6–86) | *0.004 |
| Assay 109–121[c] | 109 | 77.6(73.7–84.1) | 86 | 69.2(66.8–74.111) | *0.04 | 48 | 63.2(56.5–70.7) | 86 | 49.9(45.9–54.2) | *0.0009 |
| Assay 110–525[c] | 109 | 73.4(66.8–80.8) | 86 | 82.5(73.7–92.4) | 0.19 | 48 | 50.5(44.7–56.9) | 86 | 47.6(41.6–54.4) | 0.56 |

Categorical variables are compared between MI and non-MI patients by Chi-sqaure test or Fisher's exact test and P values are reported.
Lipid parameters and two-site apo A-I assays were measured in samples collected at the time of admission (admission samples) and discharge (discharge samples) from hospital. Measured values were naturally log transformed and compared between MI and non-MI patients. Comparison was done by *t*-test assuming equal variance in all parameters, except for HDL-C (admission samples) and assay 110–525 (discharge samples) which were tested by *t*-test assuming unequal variance.
MI, myocardial infarction; CAD, coronary artery disease; TC, total cholesterol; TG, triglyceride; FFA, free Fatty acid; HDL-C, high density lipoprotein cholesterol; LDL-C, low density lipoprotein cholesterol; Apo A-I, apolipoprotein A-I ; ApoB, apolipoprotein B.
[a] N (%); [b] Median (Quantiles25 - Quantiles75); [c] Geometric mean (95 % CI). * Statistically significant values (<0.05).

patients without MI (non-MI) and with STEMI (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), and, between non-MI and NSTEMI patients (assay 022−454: in admission samples, P = 0.02) (see supplementary Figure S5). However, the assay 110−525 (in admission and discharge samples, P = 0.19 and 0.56) did not significantly discriminated between non-MI and MI subjects. Concentration of HDL-C (in admission samples, P = 0.002; in discharge samples = 0.01) and conventionally determined apo A-I (in admission samples, P = 0.008; in discharge samples, P = <0.0001) were significantly lower in MI patients as compared to individuals without MI. Conventional ELISA apo A-I assay was able to discriminate between non-MI and STEMI (in discharge samples, P = 0.0008), between non-MI and NSTEMI (in admission samples, P = 0.03 and in discharge samples, P = 0.004); however HDL-C could discriminate only between non-MI and STEMI patients (in admission and discharge samples, P = 0.02 and 0.04) (see supplementary figure S5).

*3.3.2.2. Correlation between apo A-I assays.* Conventional ELISA apo A-I assay results demonstrated a strong positive correlation with apo A-I concentration measured with the phage based two-site apo A-I assays 022−454 (Pearson's r = 0.82, P < 0.0001) and 109−121 (Pearson's r = 0.66, P < 0.0001), however, with the assay 110−525 there was only a moderate correlation (Pearson's r = 0.44, P < 0.0001). Analysis of the correlations among the three two-site apo A-I assays showed a weak correlation between assays 110−525 and 109−121 (Pearson's r = 0.26, P < 0.0001), a moderate correlation between the assays 110−525 and 022−454 (Pearson's r = 0.50, P < 0.0001), and, a strong correlation between the assays 109−121 and 022−454 (Pearson's r = 0.68, P < 0.0001).

*3.3.2.3. Clinical Outcome during follow-up time of 1.5 years and survival analysis.* The patients were followed-up for 1.5 years post admission to the hospital for death and MI (See Fig. 1). For K–M survival analysis, all the patients were divided into tertiles (lowest concentration group being the 1st tertile) based on the concentration of apo A-I obtained from the two-site apo A-I assays: assay 022−454 (<97.4, 97.4–123, >123 mg/dL in admission samples; <75.3, 75.3–102, >102 mg/dL in discharge samples), assay 109−121(<63.5, 63.5–84.3, >84.3 mg/dL in admission samples; <46.9, 46.9–61.7, >61.7 mg/dL in discharge samples) and assay 110−525 (<64.1, 64.1–87.6, >87.6 mg /dL in admission samples; <38.7, 38.7–59.4, >59.4 mg/dL in discharge samples). In addition, the patients were divided into tertiles based on HDL-C (<37.4, 37.4–47, >47 mg/dL in admission samples; 34.3, 34.3–43.6, >43.6 mg/dL in discharge samples) and apoA–I determined with conventional method (<130, 130–150, >150 mg/dL in admission samples; 110, 110–120, >120 mg/dL in discharge sample). Number of death and MI events in the tertiles of two-site apo A-I assays, conventional apo A-I assay and HDL-C in all the patients altogether, and, in patients categorized into MI and non-MI groups are shown in Table S3 and S4.

K–M survival analysis of two-site apo A-I assays, conventional apo A-I test and HDL-C with admission samples (Fig. 5) and discharge samples (Fig. 6) for mortality in whole cohort, and, separately in MI and non-MI group was done. The survival curve for mortality in non-MI patients is not shown because of very few death events (7 deaths). Within 1.5yrs of follow-up, according to assay 109−121 there was a significant difference in survival between the admission tertiles in whole cohort (P = 0.01, Fig. 5b i) and borderline significance between the admission tertiles in MI group (P = 0.05, Fig. 5 b ii). Even within shorter follow-up period of 6 months, the assay showed significant difference between the tertiles in whole cohort (P = 0.04, Fig. 5b i) and in MI patients (P = 0.04, Fig. 5b ii). On the other hand, the assay 022−454 (Fig. 5a i-ii) and assay110−525 (Fig. 5c i-ii) showed no significant difference between the admission tertiles of whole cohort and separately in MI patient within 6 months and 1.5 yrs. The conventional apo A-I assay showed a significant difference in the death survival between the admission tertiles in whole cohort within 1.5 yrs (P = 0.03), but did not showed any significant difference in the death survival of MI patients (P = 0.51) separately, and, in shorter follow-up period of 6 months (in all patients and separately in MI group, P > 0.05) (Fig. 5d i-ii). The death survival was not significantly different in admission tertiles of HDL-C within 6 months and 1.5 yrs (P > 0.05) in whole cohort and separately in MI patients (Fig. 5e i-ii). From non-MI category, among the three two-site apo A-I assays, conventional apo A-I and HDL-C method, only two-site apo A-I assay 110−525 (Supplementary Figure S6) showed a significant difference in the death survival between admission tertiles of individuals within 1.5 years (P = 0.01) and within 6 month (P = 0.02). In discharge samples, the two-site apo A-I assays 022−454 and 109−121, conventional apo A-I assay and HDL-C did not show any significant difference in death survival (within 6 months and 1.5 years) between the tertiles in whole cohort and separately in MI patients (P > 0.05) (Fig. 6). On the other hand, with assay 110−525 there was significant difference in survival between the discharge tertiles in whole cohort within 6 months (P = 0.04), but not within 1.5 yrs (P = 0.06) (Fig. 6c i). The assay 110−525 did not show any significant difference in survival between the discharge tertiles of MI patients separately within 6 months (P = 0.27) and 1.5 yrs (P = 0.38) (Fig. 6c ii). In non-MI patients separately, none of the two-site apo A-I assays, conventional apo A-I and HDL method showed any significant difference in the discharge tertiles of death survival within 6 months or 1.5 yrs (P > 0.05).

K–M survival analysis for MI with admission samples and discharge samples in whole cohort (Supplementary Figure S7), and, separately in MI and non-MI patients was done. There was no significant (P > 0.05) difference in MI survival (within 1.5 years and 6 months) between the admission tertiles and discharge tertiles in whole cohort, and separately in MI and non-MI patients with any of the two-site apo A-I assays, HDL-C and conventional apo A-I method (P > 0.05).

## 4. Discussion

This study describes the development and clinical evaluation of three two-site apo A-I immunoassays (022−454, 109−121 and 110−525) that were designed using apo A-I antibodies generated against the intact HDL particles isolated from plasma of CAD subjects. The assays utilize apo A-I antibodies as scFv fragments fused to bacterial alkaline phosphatase (scFv-APs) and filamentous phage (scFv-phage) which imparts better avidity and solubility to the aggregation prone scFv fragments. These properties altogether provide easier way of improving the analytical performance and robustness of scFv antibodies in immunoassays.

Conventional apo A-I methods, whether turbidimetric or ELISA-based are certainly easier and more robust in practical terms as compared to the phage based two-site apo A-I assays described here, however, the purpose of this study was to investigate the potential clinical value of HDL and/or apo A-I -containing subpopulations of HDL particles as recognized by the the generated novel apo A-I antibodies. Conventional apo A-I methods are based on the use of polyclonal apo A-I antibodies, which means that they most likely reflect the sum of all apo A-I forms in different HDL subpopulations thus failing to recognize the heterogeneity of apo A-I or HDL particles that may carry important clinically relevant information related to atherosclerosis. Our two-site apo A-I assays make use of monospecific antibodies that were generated against purified preparations of CAD associated HDL particles. Since the

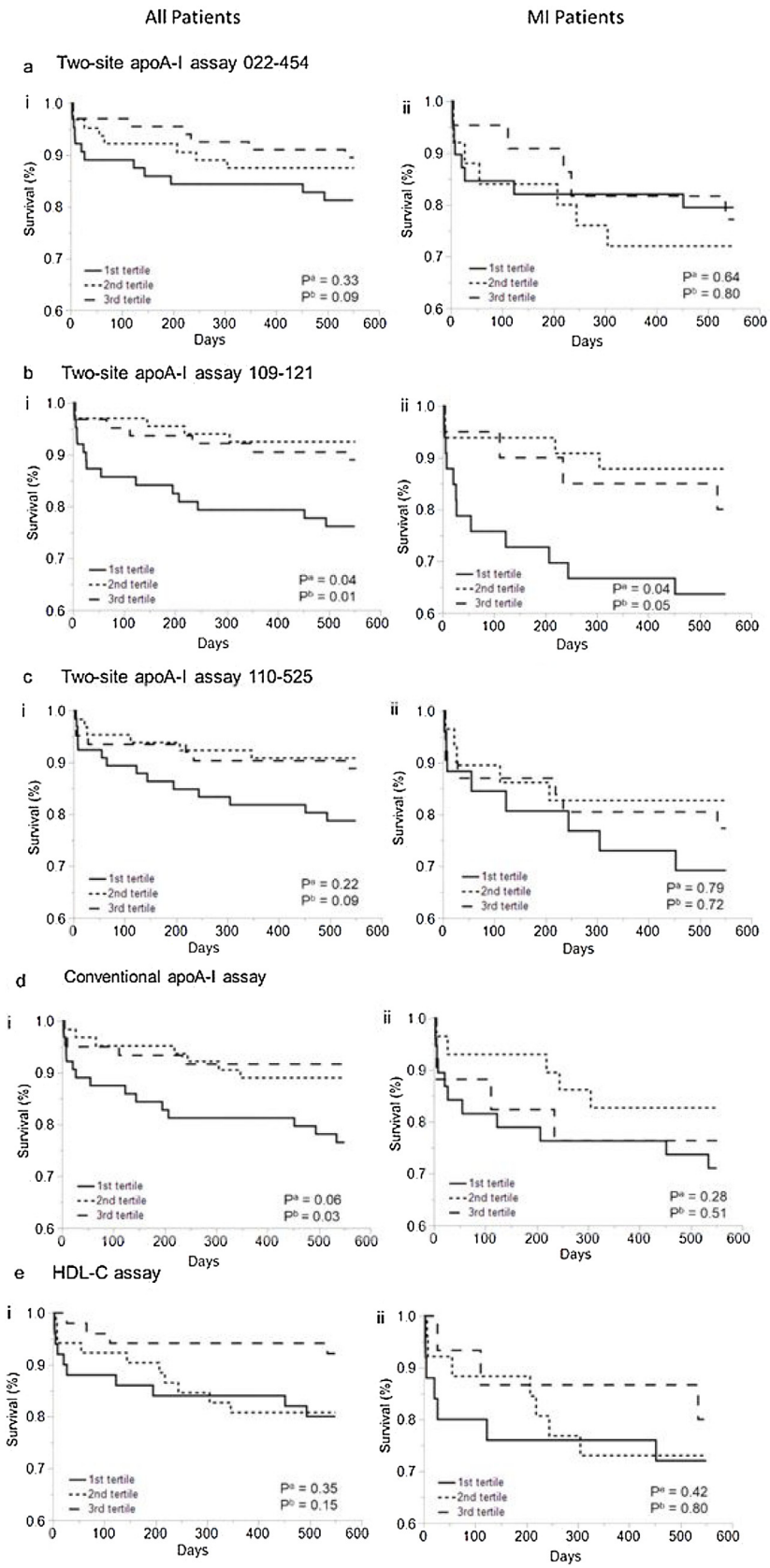


**Fig. 5.** Kaplan-Meier survival curves for mortality (within 1.5yrs) based on admission samples measured from all the cardiac patients (n = 195) and the patients categorised into MI (n = 86) (cohort-II). Patients were stratified according to apoA-I concentration obtained from two-site apoA-I assay 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.

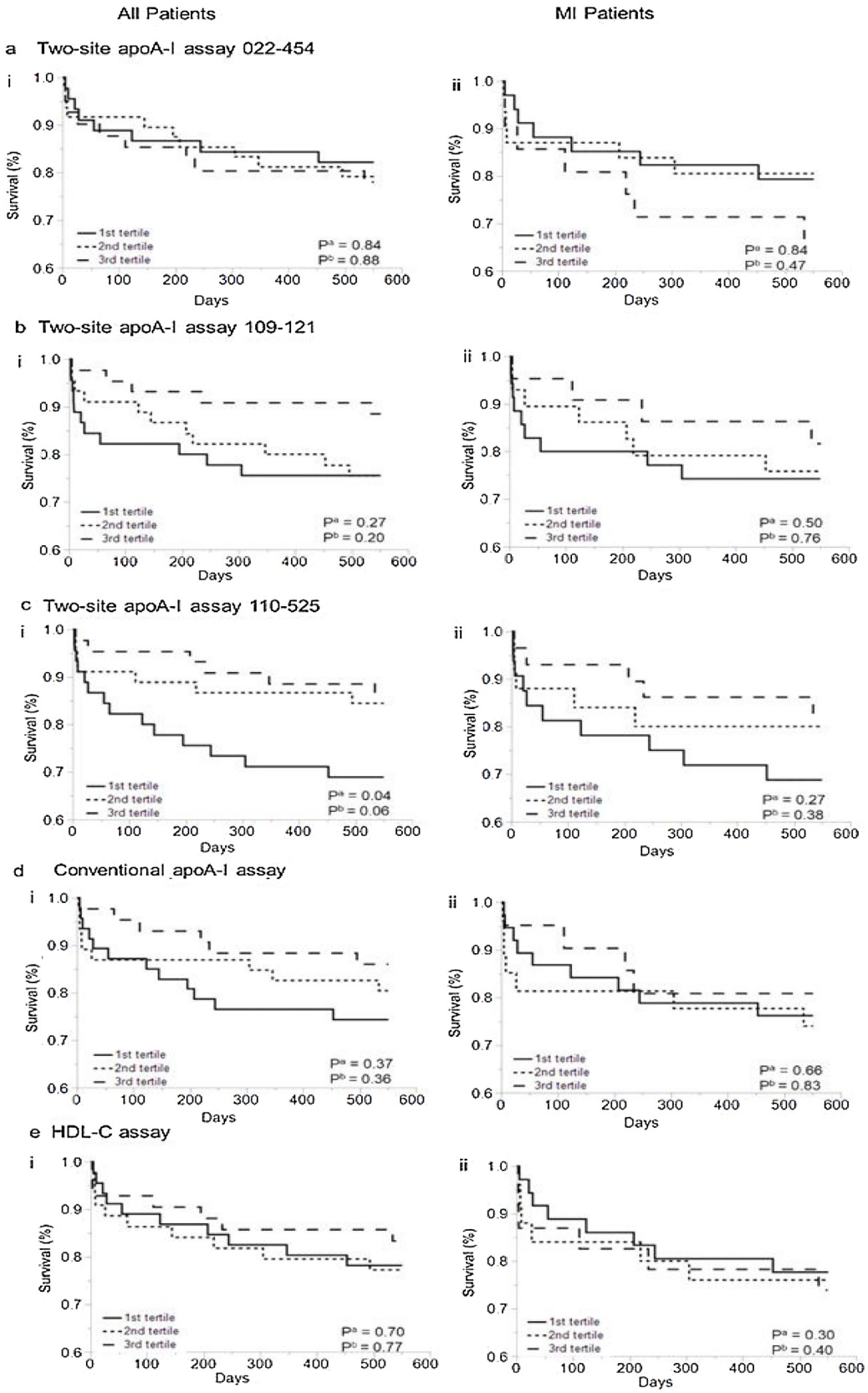


**Fig. 6.** Kaplan-Meier survival curves for mortality (within 1.5yrs) based on discharge samples measured from all the cardiac patients (n = 134) and the patients categorised into MI (n = 86) (cohort-II). Patients were stratified according to apoA-I concentration obtained from two-site apoA-I assay 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.

epitopes of these antibodies were not known therefore in these two-site apo A-I assays total HDL (= HDL2 + HDL3 subclasses) was used as calibrator. The two-site apo A-I assays could reliably measure sample with moderately elevated plasma TG concentration (2 mg/mL = about 2.3 mmol/L) with the exception of assay 110−525 being slightly affected. The two-site apo A-I assays seemed to be affected by freeze-thawing cycles of samples, the assay 110−525 being the most sensitive in this respect while the assays 022−454 and 109−121 were found to be more resistant to this sample treatment. These observations are in agreement with previous reports of HDL particles being sensitive to repeated freeze-thaw [20]. Clinical validation of new immunoassay technologies are based on archival samples, where the sample quality may be questionable to accurately reflect fresh samples to be used for routine purposes. We selected a chest pain clinical sample cohort having multiple samples per patients to guarantee identical sample storage history and used future cardiac events from the clinical follow-up to indicate differences between the different immunoassays.

There are some noticeable attributes of these two-site apo A-I assays in quantitative terms. First, the concentration of apo A-I determined with these assays (particularly the assay 110−525) was very different in samples from the two different clinical panels the reason for that might be due to the long storage periods of samples (cohort-I and cohort-II samples were collected between year 2006–2008 [16] and year 2000–2001 [17], respectively). A recent report indicates that structure and function of HDL are affected by long term storage due to shedding off apo A-I from HDL particles [21]. Second, the concentration of apo A-I determined with the two-site apo A-I assays was very different from apo A-I determined with the immunoturbidimetric and ELISA methods, which conceivably is a combined effect of these two-site apo A-I assays using monospecific scFv antibodies, a completely different two-site assay design and the use of particular HDL particle preparation with unknown densities of the epitopes recognized by the monospecific scFvs. The conventional immunoturbidimetric/ELISA apo A-I method uses purified apo A-I as calibrator and is based on the detection of broad number of apo A-I immunocomplexes between the antigen and the polyclonal apo A-I antibody.

HDL from atherosclerotic patients not only exhibit impaired atheroprotective functions but also acquire pro-atherogenic properties and are referred to as “dysfunctional” HDL; this occurs even in the presence of normal or elevated HDL-C levels. Factors responsible for modifying HDL and turning the atheroprotective HDL forms into dysfunctional form are related to change in HDL protein composition, alteration in HDL-associated lipids via apo A-I and apo A-II, post translational modifications or myeloperoxidase (MPO) induced changes [22]. Dysfunctional HDL particles demonstrate increased content of oxidized phospholipids, triacylglycerols [23], serum amyloid A (SAA), complement factor C3, and other inflammatory proteins [24]. In addition, MPO mediates oxidation of lipid-poor apo A-I in the arterial wall thus generating a dysfunctional HDL particle which is able to activate nuclear factor-κB (NF-κB) and promote arterial inflammation [25]. It has been suggested that the profile of HDL changes or evolves during the ACS process [26] and dysfunctional alteration in HDL is more pronounced in ACS patients than in individuals with stable CAD [27]. Therefore, this feature of HDL can be helpful in ACS risk stratification. In many studies, higher apo A-I level has been found to be associated with lower risk of chronic heart disease (CHD) [28], MI, MI or CV death [26,29,30]). In our study, for patients in cohort-I, we found that both two-site apo A-I assay 110−525 and conventional apo A-I measured significantly lower concentrations in ACS compared to non-CAD patients, whereas no significant difference between individuals without CAD and with stable CAD was seen. HDL-C, on the other hand could not discriminate between ACS and non-CAD individuals. However, it must be noted that the number of samples in cohort-I was only 36 patients, which is a limitation of this study. In more extensive chest pain patient’s cohort-II, the two-site apo A-I assays 022−454 and 109−121 demonstrated their ability to statistically differentiate the MI patients from non-MI individuals with admission and discharge samples alike. Similarly, in the same population, also the concentration of apo A-I determined with conventional assay and concentration of HDL-C was significantly lower in MI cases as compared to non-MI cases. In addition, the distinction between non-MI and STEMI patients was much more pronounced than the difference between non-MI and NSTEMI patients (supplementary figure S5) which is in support of the prior study where cholesterol efflux function of HDL has been stated to be impaired more in STEMI patients than in non-cardiac or NSTEMI patients [31]. The samples of cohort-II were previously used in many other studies elsewhere and therefore the samples had gone through many freeze-thawing cycles (especially the admission samples) which might have affected the performance of our two-site apo A-I assays. Therefore, to minimize the confounding effect of repeated sample freeze-thawing we measured the admission samples as well as the discharge samples which had not gone through freeze-thawings so often. Although the authors are aware that the result from cohort-I and cohort-II are not in full accordance with each other, it is argued that the available samples cohorts are relevant for a preliminary evaluation of the clinical groups using the novel monospecific scFv antibody based two site assays against the conventional apo A-I and HDL methods.

In the survival analysis of chest pain patients from cohort-II (all patients, and, MI and non-MI patients separately) for event of MI within follow-up of 1.5 yrs, we found no significant difference in MI free survival of patients (all patients, and, MI and non-MI patients separately) falling into different tertiles of HDL-C and, apo A-I determined either with the two-site apo A-I assays or conventional apo A-I assay. It is important here to mention that in this cohort, 19.4 % individuals (38 out of 195) had a history of CAD prior to admission to hospital. Therefore, it is possible that due previous manifestation of CAD in some patients, 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 [32], while in contrast some of the previous studies have failed to show the relation between increased level of HDL-C and mortality [4,33,34]. In our study, there was no significant difference in the survival of patients according to HDL-C tertiles. Few studies have shown the relation between apo A-I and mortality [35,30]. With regard to mortality within follow-up of 1.5 yrs, there was difference in survival of individuals belonging to different tertiles of apo A-I in the chest pain patients altogether (two-site apo A-I assay 109−121 and 110−525, and conventional apo A-I assay) and in MI patients separately (two-site apo A-I assay 109−121), *i.e.,* individuals in lower tertiles died earlier. Interestingly in patients without MI, assay 110−525 was able to significantly differentiate between the tertiles of individuals who died, with 6 deaths out of the 7 deaths in this group falling into the lowest tertile. The short-term survival during 6 months was significantly different according to the tertiles of two-site apo A-I assay 109–121 (all patient and MI patients) and assay 110−525 (all patient) but not according to conventional apo A-I assay. We can anticipate from our clinical evaluation here that the corresponding CAD specific recombinant apo A-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 patients within short period of six months and 1.5yrs. However, their ability to predict the future risk of MI or CAD and stratify the type of CAD (stable CAD or ACS) or type of MI (NSTEMI or STEMI) needs to be further evaluated.

In summary, three novel two-site apo A-I assays were developed by making use of recombinant apo A-I antibodies derived from phage-display based scFv-antibody library against the CAD

HDL particles and clinically evaluated in cardiac disease related patients. These results although based on limited archival sample cohorts are clearly indicative of association of the novel apo A-I antibodies (recognizing apo A-I on the surface of a variety of HDL subpopulations) based assays with variable prognosis of atherosclerosis. The assays 109−121 and 110−525 seem to have a possibility to add to the diagnostic and predictive value for cardiac conditions, however, this proposition needs to be extensively and carefully evaluated in future. In addition, these antibodies could be implemented in studying the functional aspects of HDL to understand the atherosclerotic status in a better way. The phage-based assay format used to evaluate these recombinant apo A-I antibodies in this study is rather complex and deviates from routinely used enzyme-immunoassays and immunoturbidimetric assays and therefore may not as such be ideal for use in laboratory routines. Hence the aim of the future studies will be to simplify the format of these apo A-I assays and to further evaluate their clinical and diagnostic relevance in well characterized clinical cohort. In addition, it would also be interesting to characterize in detail the epitopes in apo A-I protein that the recombinant apo A-I antibodies are recognizing, once proved clinically important.

## Author statement

We wish to submit the revised manuscript entitled “Three two-site apo A-I immunoassays using phage expressed detector antibodies - preliminary clinical evaluation with cardiac patients” to Journal of Pharmaceutical and Biomedical analysis. We have made changes in the original manuscript based on reviewers’ comments.

We confirm that this work is original and has not been published elsewhere and is not currently under consideration for publication elsewhere. There are no conflicts of interest associated with this article. All the listed authors have approved the article submission.

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## Author contributions

All authors confirmed they have contributed to the intellectual content of this paper and have met the following 3 requirements: (a) significant contributions to the conception and design, acquisition of data, or analysis and interpretation of data; (b) drafting or revising the article for intellectual content; and (c) final approval of the published article.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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## Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jpba.2020.113772.

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