Plasma distribution of apolipoprotein A-IV in patients with coronary artery disease and healthy controls

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Abbreviated title: Plasma distribution of apoA-IV in CAD patients and controls

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Abbreviation: CAD, coronary artery disease
**Abstract**

Recent studies observed lower apolipoprotein A-IV (apoA-IV) plasma concentrations in patients with coronary artery disease (CAD). The actual distribution of the anti-atherogenic apoA-IV in human plasma, however, is discussed controversially and it was never investigated in CAD patients. We therefore developed a new and gentle technique to separate the various apoA-IV-containing plasma fractions. Using a combination of precipitation of all lipoproteins with 40% phosphotungstic acid (PTA, pH=7.6) and 4M MgCl$_2$ on the one hand and immunoprecipitation of all apoA-I-containing particles with an anti-apoA-I antibody on the other hand, we obtained three fractions of apoA-IV: lipid-free apoA-IV (about 4% of total apoA-IV), apoA-IV associated with apoA-I (LpA-I:A-IV, 12%) and apoA-I-unbound but lipoprotein-containing apoA-IV (LpA-IV, 84%).

Finally, we compared these three apoA-IV fractions between 52 patients with a history of CAD and 52 age- and sex-matched healthy controls. Patients had significantly lower apoA-IV levels when compared to controls (10.28±3.67 vs. 11.85±2.82 mg/dl, p=0.029). However, we observed no major differences for the three plasma apoA-IV fractions between patients and controls.

We conclude that the use of a gentle method to separate the various apoA-IV-containing plasma fractions reveals a different distribution of apoA-IV than in many earlier studies. Furthermore, there exist no major differences in the apoA-IV plasma distribution pattern between CAD patients and controls. Therefore, the anti-atherogenic effect of apoA-IV has to be explained by other functional properties of apoA-IV (e.g. the antioxidative characteristics).

**Supplementary keywords:** apoA-IV, LpA-I:A-IV, LpA-IV, lipid-free apoA-IV, anti-atherogenic
**Introduction**

Human apolipoprotein A-IV (apoA-IV) is a 46 kDa glycoprotein (1,2). It is produced in the epithelial cells of the small intestine (3) and synthesis and secretion of apoA-IV in rats are stimulated by ingestion of lipids (4). Studies in rats showed that the produced apoA-IV is released by the small intestine into the mesenteric lymph and enters the plasma compartment as a structural protein of chylomicrons, VLDL and HDL or unassociated with lipoproteins (5).

There exists accumulating evidence from in vitro studies that apoA-IV plays an important role in reverse cholesterol transport. This pathway removes cholesterol from peripheral cells and transports it to the liver or steroidogenic organs where cholesterol can be metabolized to bile acids and hormones, respectively. ApoA-IV binds to peripheral cells, promotes cholesterol efflux and enhances the formation of small HDL particles (6,7) by activating LCAT (8,9). In addition, apoA-IV may participate in the binding and uptake of HDL by rat hepatocytes (10). Moreover, apoA-IV modulates the activation of lipoprotein lipase (11) and the CETP-mediated transfer of cholesteryl esters from HDL to LDL in tissue culture studies (12). These investigations are in line with studies of Duverger et al. and Cohen et al. who demonstrated that genetically modified fat-fed mice carrying several copies of the human or mouse apoA-IV gene developed markedly less atherosclerosis than control mice (13,14). It was even demonstrated that atherosclerosis-prone apoE knockout mice showed considerable protection against atherosclerotic lesions when the human apo A-IV gene was overexpressed either in liver or in the intestinal tract (13,15). In humans, a case-control study in two different ethnic populations revealed an inverse association between plasma apoA-IV concentrations and coronary artery disease (16). This finding was confirmed in a Chinese study population (17) as well as in patients with kidney impairment (18).

Reports about the distribution of apoA-IV in human plasma are contradictory. They described a wide variety of apoA-IV distribution ranging from apoA-IV almost entirely bound to HDL to mostly unassociated with major lipoprotein fractions (1,3,19-27) (Table 1). From these studies it becomes already clear that the most important factor that may account for these discrepancies is the procedure for isolating apoA-IV-containing lipoproteins itself. Some of these techniques are drastic and
destructive resulting in stripping off significant amounts of apoA-IV from HDL and thereby artificially increasing the lipoprotein-unbound apoA-IV.

The first aim of this study was therefore to elucidate the actual distribution of apoA-IV in human plasma by developing a new and gentle technique which minimizes the possibility of artifactual dissociation of this apolipoprotein. The second aim was to investigate whether differences in the distribution of apoA-IV between patients with coronary artery disease (CAD) and controls are responsible for the anti-atherogenic properties of apoA-IV. Various apoA-IV-containing subfractions have been shown in vitro to exhibit reversed cholesterol transport activities with different efficiencies (26,27). Recently, lipid-free apoA-IV was shown to be particularly effective inducing cholesterol efflux from macrophages (28). Our study aimed therefore to investigate whether there are clinical correlates to these in vitro findings.

Subjects and Methods

Patients

A total of 52 patients (44 men and 8 women) with a history of coronary artery disease (CAD) were investigated. Twenty-eight of them had already suffered a myocardial infarction weeks or, in most cases, years before this study. The other patients showed significant stenoses during angiography of the coronary arteries. Their mean age was 55±9 years. Due to the exclusion criteria, none of the patients was taking lipid-lowering drugs. Fifty-seven percent of the patients were taking antihypertensive medications and 72% were taking thrombocyte aggregation inhibitors. These patients were compared to 52 healthy controls 1-to-1 matched for sex and age with an average age of 54±7 years who were recruited from the "Salzburg Atherosclerosis Prevention Program in Subjects with High Stroke Risk" (SAPHIR) study. The randomly selected control subjects underwent a basic check-up program involving all parameters of insulin resistance-syndrome. Hepatic and renal impairment and therapy with lipid-lowering drugs were exclusion criteria in both, patients and controls. The study was approved by the institutional ethic committees and subjects gave informed consent.

Measurement of plasma apo A-IV concentration

Venous blood was collected in tubes containing EDTA after an overnight fasting period. The plasma was isolated and frozen at −80°C prior to analysis. Freezing of
the material did not significantly influence the measurement of the various plasma apoA-IV fractions. Plasma apoA-IV concentrations were determined using an enzyme-linked immunoabsorbent assay (ELISA) that employs affinity-purified rabbit anti-human apoA-IV polyclonal antiserum as the capture antibody and the same antibody coupled to horseradish peroxidase as detection antibody (29,30). Plasma with known content of apo A-IV served as calibration standard. The lower detection limit of this assay is 0.002 mg/dL. Each patient-control pair was analyzed in triplicate within the same assay run resulting in an intraassay and interassay coefficients of variation of 4.5% and 6.6%, respectively (29).

**Determination of lipid-free apoA-IV**

The lipoproteins from 100µl plasma were precipitated by adding 5µl of 40% phosphotungstic acid (PTA, pH=7.6)) and 5µl of 4M MgCl₂. This method was shown to completely remove all lipoproteins as demonstrated by Bartholome et al. (31). After 2 hours of incubation at room temperature, the precipitated plasma was centrifuged for 15 minutes at 15,000 rpm. The clear supernatant containing lipoprotein-depleted plasma was used for measurement of lipid-free apoA-IV (Figure 1). To control whether apoA-I and apoA-IV were precipitated by this procedure, we dissolved the lipoprotein-containing pellet in PBS by adding equimolar amounts of tri-sodium citrate dihydrate with respect to MgCl₂. The dissolved pellet was dialyzed over night against PBS at 4°C in Spectra/Por® molecularporous membrane (MWCO 6.000-8.000) and subjected to immunoblot analysis.

**Determination of LpA-I:A-IV and LpA-IV**

To measure these two apoA-IV-containing fractions, we started with an immunoprecipitation step of human plasma with anti-apoA-I followed by the quantification of apoA-IV in the supernatant. For immunoprecipitation we added 5µl of plasma to 25µl rabbit anti-human apoA-I γ-globulin fraction (Behring Diagnostics GmbH, Marburg, Germany) and 10µl 1 mmol/l DTNB solution and 60µl PBS pH 7.3. DTNB blocks the activity of LCAT which is known to influence the plasma lipoprotein distribution of apoA-IV (32). The temperature of the mixture was kept in ice-water prior to the addition of DTNB. After vortexing, the mixture was incubated for 1 h at room temperature and centrifuged for 15 min at 10000 rpm. ApoA-IV was quantified by ELISA in the clear supernatant containing both, LpA-IV and lipid-free apoA-IV (Figure 1). This measured value was used to calculate i) the amount of apoA-IV
associated with apoA-I (LpA-I:A-IV) by subtracting it from the total apoA-IV plasma concentrations and ii) the amount of apoA-I-unbound but lipoprotein-bound apoA-IV (LpA-IV) which corresponds to this value minus the amount of lipid-free apoA-IV. The total amount of plasma apoA-IV was determined after incubation of 5µl plasma with 10µl DTNB and 85µl of PBS in order to assure the same conditions as in the immunoprecipitation experiment.

Immunoblot analysis

Immunoblot analysis of apoA-IV and apoA-I were performed on samples separated by SDS polyacrylamide gel electrophoresis and transfer to cellulose nitrate membranes. Rabbit anti-human apoA-IV and rabbit anti-human apoA-I (Behring Diagnostics GmbH Marburg Germany) served as first antibodies and HRPO-conjugated swine anti-rabbit IgG (DAKO, Denmark) as second antibody. The final detection was performed by chemiluminescence reaction with DuPont Reagent ECL Western blotting reagent.

Statistical analysis

A paired t-test was used to compare continuous variables between matched patients and controls. We used a multiple linear regression analysis to examine the influence of plasma concentrations of total apoA-IV, total and HDL cholesterol and triglycerides on the various plasma fractions of apoA-IV in healthy controls. Statistical analysis was performed with SPSS for Windows 11.0. A p value <0.05 was considered significant.

Results

Methodological approaches for studying apoA-IV plasma distribution

To investigate whether lipid-free apoA-IV indeed exists, we removed all lipoproteins by PTA/MgCl₂-precipitation of plasma. Immunoblot analysis revealed that apoA-I was almost exclusively found in the pellet and almost no apoA-I was left in the supernatant (Figure 2). The faint band seen in the supernatant represents lipid-free apoA-I. Such forms have been described for apoA-I and also for other apolipoproteins (33,34)(35,36). When the immunoblot of the supernatant and pellet was analyzed with anti-apoA-IV, apoA-IV was mainly detected in the pellet and smaller amounts in the supernatant (Figure 2). Quantification of apoA-IV in the
supernatant by ELISA demonstrated a proportion of 4%-5% of apoA-IV to be lipid-free (Table 2).

To measure LpA-I:A-IV and LpA-IV we incubated 5µl plasma with 25µl anti-ApoA-I. This amount of antibody was necessary to obtain a complete precipitation of apoA-I (Figure 3). In order to assure that apoA-I was completely precipitated in each sample, each supernatant in subsequent experiments was controlled by immunoblot analysis with anti-apoA-I. No trace of apoA-I in the supernatant could be seen on the blots after immunoprecipitation in all investigated samples. We then measured apoA-IV in the supernatant by ELISA. LpA-I:A-IV was calculated as difference between total plasma apoA-IV and apoA-IV in the supernatant and corresponds to the apoA-IV captured in the respective pellet. To obtain the LpA-IV fraction we subtracted the lipid-free fraction of apoA-IV from the amount of apoA-IV in the supernatant of the anti-apoA-I immunoprecipitation (Figure 1).

To demonstrate that apoA-IV in the pellet after precipitation of apoA-I was indeed associated with apoA-I, we extensively washed the pellet with PBS. Since apoA-IV was still found in the pellet, a non-specific precipitation of apoA-IV could be excluded (Figure 4A). Furthermore, we showed that apoA-IV in the supernatant after precipitation of apoA-I was still lipoprotein-bound: after precipitation of the supernatant with PTA/MgCl₂ we found only small amounts of apoA-IV in the supernatant (Figure 4B).

**ApoA-IV Distribution in controls**

When we investigated the plasma distribution of apoA-IV by the indicated procedure in healthy controls, we found that about 4% of apoA-IV existed as lipid-free apoA-IV, 12% as LpA-I:A-IV and 84% as LpA-IV (Figure 1 and Table 2). Table 3 presents the results from a multiple linear regression analysis investigating the variables associated with the various apoA-IV-containing plasma fractions. Lipid-free apoA-IV was associated with the total amount of plasma apoA-IV and with HDL cholesterol. Since the LpA-IV fraction accounts for 84% of the total amount of apoA-IV concentrations, the latter explained about 81% of the former. Further small amounts were explained by HDL cholesterol and triglycerides. HDL cholesterol explained about 29% of the LpA-I:A-IV fraction.
ApoA-IV distribution in patients with CAD compared to controls

The mean total plasma apoA-IV concentrations in CAD patients were slightly but significantly lower when compared to the healthy controls (10.28±3.67 mg/dl vs. 11.85±2.82 mg/dl, p=0.029, Table 2). The percentage of lipid-free apoA-IV was slightly but significantly higher in CAD patients than in healthy controls (4.79±1.94 % vs. 4.22±1.50 %, p=0.044). No differences were observed for the percentage of LpA-I:A-IV or LpA-IV (Table 2). We also did not observe differences in the apoA-IV distribution between patients who had already suffered a myocardial infarction and those who showed a positive coronary angiography.

Discussion
Previous findings on apoA-IV distribution

Previous investigations about the distribution of apoA-IV in human plasma led to very contradictory results, most likely attributed to different techniques of plasma separation (see Table 1) (1,3,19-27). In earlier studies, ultracentrifugation methods were frequently used for characterizing apoA-IV-containing plasma fractions. These methods however, have been shown to be inadequate for studying the apoA-IV distribution, because they result in partial redistribution of apoA-IV. It has been observed that ultracentrifugation causes apoA-IV to be removed from lipoproteins and results in over-estimation of lipid-free apoA-IV (1,3,19,23,37). Various chromatographic methods provided a better estimation of apoA-IV distribution. However, the number of samples that can be processed at one time is limited, making studies of apoA-IV distribution very difficult (38). Moreover, the elution pattern of such chromatographic methods depends to some extent on the column pressure, a parameter that could be a source of artificial association as well.

Beisiegel and Utermann found that human apoA-IV is present in all fractions of d<1.006 g/ml (=chylomicrons and VLDL) when isolated by a single ultracentrifugal spin from non-fasting subjects, but is lost during recentrifugation from this density fraction. With immunoelectrophoretic methods, however, they observed that most of human apoA-IV is unassociated with the major lipoprotein fractions in serum (19). Green et al. found 98% of apoA-IV in the lipid-free fraction after ultracentrifugation of plasma. With agarose column chromatography of fasting plasma they observed the majority (about 77%) of plasma apoA-IV to be unassociated with lipoproteins,
whereas 23% eluted with lipoproteins suggesting that substantial amounts of apoA-IV is in fact associated with lipoproteins (3). This is in agreement with another study using gel filtration chromatography method which reported 25% of total plasma apoA-IV to be associated with HDL (as determined by coelution with apo A-I). The remaining 75% were not apo A-I-associated (25). Similarly, Bisgaier et al. found 15-25% of apoA-IV to be associated with HDL (22). In contrast, Lagrost et al. reported that a majority of serum apoA-IV was associated with HDL and that this could not have been the result of an artifactual association or known in vitro redistribution of apoA-IV (23). They further demonstrated by immunoprecipitation experiments that, upon serum incubation with anti-apoA-I antibodies, most of serum apoA-IV coprecipitated with apoA-I (23). Similarly, using affinity chromatography, they observed 93% to be associated with apoA-I (24). Studies which used two-dimensional (2D) non-denaturating polyacrylamide gel electrophoresis found apoA-IV mainly in the α- and pre-β HDL subfractions, mainly unassociated with apo A-I (20,21,27). Most of the mentioned techniques allow a detailed qualitative analysis but are hardly suitable for quantitative analysis of larger sample numbers.

**Findings with the new method**

Amidst all these contradictions, our major task was therefore, to develop a gentle separation technique which allows the analysis of the physiological apoA-IV distribution in human plasma suitable for analysis of large sample numbers. The use of chemical PTA/MgCl₂-precipitation (31) under the conditions stipulated, enabled the precipitation of all lipoproteins from human plasma resulting in lipid-free apoA-IV in the supernatant. Likewise, the immunoprecipitation with anti-apoA-I antibody carried out under the described conditions, resulted in a quantitative precipitation of LpA-I:A-IV. With these two analytical methods we were able to show that plasma apoA-IV exists in three different subclasses in plasma: 1) LpA-I:A-IV, 2) LpA-IV and 3) lipid-free apoA-IV. In line with reports that apoA-IV is partly associated with HDL (3), we observed substantial amounts of apoA-IV (about 12 %) associated with apoA-I (=LpA-I:A-IV ) and 84% to be associated with lipoproteins that do not contain apoA-I (=LpA-IV). This is in agreement with some previous studies by our and other groups (22,25) which reported a somewhat higher amount of HDL-association of apoA-IV than presented in this work. This can probably be attributed to the difference in separation technique but also to different definitions of HDL particle classes.
Whereas historically (and also technically derived) HDL was defined as a particle density class prepared by ultracentrifugation of plasma, modern more or less gentle separation techniques have "produced" a large number of major and minor HDL subclasses (39). Thus a 25% associated HDL-apoA-IV moiety (obtained after gel filtration of plasma) is not necessarily identical with apoA-I-containing apoA-IV particles obtained by the immunoprecipitation described in this study. Accordingly, the LpA-IV particles which represent the majority of plasma apoA-IV yet belong to the high density lipoprotein class since they contain lipids.

Interestingly, HDL cholesterol shows a negative association with lipid-free apoA-IV and LpA-IV which can be interpreted that HDL particles (apoA-I-containing or -free) are saturable "targets" for apoA-IV binding (32) (Table 3). This is supported by a positive correlation of LpA-I:A-IV with HDL cholesterol.

**ApoA-IV distribution in CAD patients**

Finally, we addressed the question whether the distribution of apoA-IV in CAD patients differs from control groups. Studies in mice overexpressing human or mouse apoA-IV showed an anti-atherogenic effect of apoA-IV (14)(13,15). These experiments, however, were done in murine animals and have to be considered with caution due to a lipoprotein profile in these animals which differs in several aspects from the human profile. However, in line with these results, we previously demonstrated that decreased plasma concentrations of apoA-IV in CAD patients are associated with atherosclerotic diseases (16), which was confirmed by two other studies (17,18). Therefore, we were interested whether the distribution of apoA-IV in human plasma is associated with atherosclerosis as well or, in other words, whether the different plasma fractions of apoA-IV have a different atherogenic potential. For this reason, we compared the distribution of apoA-IV in plasma with CAD and matched controls. There was a small but significant difference in the distributional pattern of the lipid-free apoA-IV between CAD patients and control group (4.79% vs. 4.22%, p=0.044). However, it is unlikely that this small difference is responsible for the atherogenic potential of apoA-IV. There were no differences in the percentage of LpA-I:A-IV between CAD patients and controls, nor were there any difference in the distributional pattern of LpA-IV. Therefore, the anti-atherogenicity of apoA-IV is presumably not the result of the extent of association of apoA-IV with lipoproteins, nor is it to be explained on the basis of lipid-free apoA-IV that circulates in human
plasma, but rather of other concomitant roles of this apolipoprotein in lipid metabolism and reverse cholesterol transport.

The observation that LpA-IV constitutes the major subpopulation of apoA-IV is partly consistent with the observation made by Duverger et al., and Eckardstein et al. (26,27) who identified two subclasses of apoA-IV that are free from apoA-I but play an important role in the reverse cholesterol transport process. The LpA-IV could indeed play a salient role in reverse cholesterol transport, but it does not explain the anti-atherogenic effect of apoA-IV in CAD patients. Our observation that the plasma apoA-IV levels of control groups were significantly higher in comparison to CAD patients, is consistent with previous results (16-18). On the other hand, the anti-atherogenicity and the protective effect of apoA-IV is to be explained by other functional than distributional properties of apoA-IV, for example its antioxidative properties. In this regard, the observation of Qin et al. (40) in rats, that the increase in apoA-IV levels may represent a natural response in the body to guard against lipid oxidation and the generation of deleterious lipid peroxidation products seems very plausible. Observations from studies in mice overexpressing apoA-IV suggest that apo A-IV acts by increasing the potential of HDL to promote cholesterol efflux from cholesterol-loaded cells (14) and/or by exerting antioxidative properties within the arterial wall (15). This elevates the putative function of apoA-IV as a potent endogenous antioxidant (15).

Limitations of the study

We can not completely exclude that the precipitation of lipoproteins with PTA and MgCl2 induces artifactual association of apoA-IV with lipoproteins by increasing the ionic strength of the assay solution. The main evidence that apoA-IV is partially bound to lipoproteins, however, comes from studies which used sequential immunoaffinity chromatography or the non-denaturing two-dimensional gel-electrophoresis method (20,21,27).

It is at the first glance surprising that we observed lower total cholesterol levels in our CAD patient group when compared to age- and sex-matched controls (Table 2). This can be explained by the selection of patients without lipid-lowering drug treatment to avoid an influence by this intervention on the apoA-IV distribution. Since we did not observe a correlation between total or LDL cholesterol and the various apoA-IV fraction (data not shown), we do not expect that this could have influenced
our findings. On the other hand, this selection criterion could have resulted in a overrepresentation of patients whose atherogenic potential does less rely on lipoprotein disturbances than on other pathogenetic mechanisms such as inflammation or immunological components. In line with this is the observation of a smaller difference in apoA-IV concentrations between CAD patients and controls than in previous studies (16-18).

Acknowledgments

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References


Figure legends

Figure 1: Schematic illustration of the experimental procedure to investigate the apoA-IV plasma distribution. The right bar provides the results of the apoA-IV plasma distribution from a healthy control group.

Figure 2: Analysis of supernatants and pellets after precipitation of lipoproteins by adding 5µl of 40% PTA and 5µl of 4M MgCl₂ to 100µl of plasma. Panel A and B show the bands obtained after immunoblotting with anti-apoA-I and anti-apoA-IV, respectively. Native plasma is shown as a control. In panel B the proportion of material subjected to the gel between supernatant and pellet is 1:6.

Figure 3: Immunoblot analysis of supernatants from plasma after immunoprecipitation with anti-apoA-I antibody. Lane 1 shows plasma, lanes 2-6 investigate supernatants after incubation of plasma from the same donor with increasing amounts of anti-apoA-I. The higher molecular weight band originates from immunoglobulins of the precipitating anti-apoA-I antibody. Complete immunoprecipitation of apoA-I was obtained with 25µl of anti-ApoA-I.

Figure 4: Characterization of supernatant and pellet after immunoprecipitation of apoA-I-containing particles with an anti-apoA-I antibody. Panel A demonstrates the specific association of apoA-IV with apoA-I in the pellet. Pellets from three samples (pellet 1-3) were washed twice intensively with PBS, dissolved and subjected to immunoblot analysis with anti-apoA-IV. Panel B shows the immunoblot analysis with anti-apoA-IV in a control plasma, the supernatant after precipitation of apoA-I (Sn-1) and after precipitation of this supernatant with PTA/MgCl₂ (Sn-2).
Table 1: Earlier studies investigating the plasma distribution of apolipoprotein A-IV

<table>
<thead>
<tr>
<th>Author</th>
<th>Method</th>
<th>Observation</th>
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<tbody>
<tr>
<td>Beisiegel and Utermann (19)</td>
<td>Ultracentrifugation</td>
<td>ApoA-IV present in d &lt;1.006 g/ml (=chylomicrons and VLDL)</td>
</tr>
<tr>
<td>Beisiegel and Utermann (19)</td>
<td>Immunoelectrophoresis</td>
<td>Most apoA-IV present in d &gt;1.21 g/ml (=unassociated with major lipoprotein fractions)</td>
</tr>
<tr>
<td>Green et al. (3)</td>
<td>Sequential ultracentrifugation</td>
<td>98 % apoA-IV present in lipid-free fractions</td>
</tr>
<tr>
<td>Green et al. (3)</td>
<td>Agarose column chromatography</td>
<td>77 % apoA-IV is unassociated (&quot;free&quot;) with lipoproteins, while 23% is associated</td>
</tr>
<tr>
<td>Bisgaier et al. (22)</td>
<td>Agarose gel chromatography and immunoprecipitation</td>
<td>15–25 % of apoA-IV is associated with HDL</td>
</tr>
<tr>
<td>Lagrost et al. (23)</td>
<td>Immunoprecipitation with anti-ApoA-I</td>
<td>Majority of apoA-IV is associated with HDL</td>
</tr>
<tr>
<td>Malmendier et al. (24)</td>
<td>Affinity chromatography</td>
<td>93% apoA-IV is associated with apoA-I</td>
</tr>
<tr>
<td>Dieplinger et al. (25)</td>
<td>Gel filtration chromatography</td>
<td>25 % of apoA-IV is associated with HDL, 75 % is not associated</td>
</tr>
<tr>
<td>Duverger et al. (26)</td>
<td>Sequential immunoaffinity chromatography</td>
<td>Identified LpA-I:A-IV(^a) and LpA-IV(^b); About 6 % apoA-IV associated apoA-I, 70% is not apoA-I-associated</td>
</tr>
<tr>
<td>Von Eckardstein et al. (27)</td>
<td>Sequential immunoaffinity chromatography and non-denaturating 2D-polyacrylamide gradient gel electrophoresis (2D-NDGE)</td>
<td>Three subclasses: LpA-IV-1, -2 and -3; LpA-IV-1 and -2 with slow α-mobility do not contain apoA-I; LpA-IV-3 only in 10% of the samples.</td>
</tr>
<tr>
<td>Böttcher et al. (21)</td>
<td>Preparative free-solution isotachophoresis and 2D-NDGE</td>
<td>ApoA-IV found in fractions with slow-α- and pre-β-mobility, mainly unassociated with apo A-I</td>
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<tr>
<td>Aszatalos et al. (20)</td>
<td>2D-NDGE</td>
<td>ApoA-IV found between α- and pre-β HDL subfractions</td>
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</table>

\(^a\) LpA-I:A-IV = Lipoprotein particles containing apoA-IV with apoA-I.  
\(^b\) LpA-IV = Lipoprotein particles containing apoA-IV without apoA-I
Table 2: Comparison of total apoA-IV and various apoA-IV plasma fractions as well as lipids and renal function parameters between CAD patients and age- and sex-matched healthy controls.

<table>
<thead>
<tr>
<th></th>
<th>CAD Patients (n=52)</th>
<th>Healthy Controls (n=52)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total apoA-IV (mg/dl)</td>
<td>10.28 ± 3.67</td>
<td>11.85 ± 2.82</td>
<td>0.029</td>
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<tr>
<td>Lipid-free apoA-IV (%)</td>
<td>4.79 ± 1.94</td>
<td>4.22 ± 1.50</td>
<td>0.044</td>
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<td>LpA-I:A-IV (%)</td>
<td>12.38 ± 7.69</td>
<td>11.79 ± 7.04</td>
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<td>LpA-IV (%)</td>
<td>82.70 ± 7.35</td>
<td>84.07 ± 6.69</td>
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<tr>
<td>Total cholesterol (mg/dL)</td>
<td>208 ± 36</td>
<td>230 ± 41</td>
<td>0.003</td>
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<tr>
<td>HDL cholesterol (mg/dL)</td>
<td>40 ± 11</td>
<td>62 ± 16</td>
<td>&lt;0.001</td>
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<td>LDL cholesterol (mg/dL)</td>
<td>133 ± 33</td>
<td>144 ± 40</td>
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<tr>
<td>Triglycerides (mg/dL)</td>
<td>114 ± 65</td>
<td>157 ± 87</td>
<td>0.16</td>
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<tr>
<td>Creatinine values (mg/dl)</td>
<td>0.99 ± 0.13</td>
<td>0.94 ± 0.11</td>
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<td>Urea (mg/dl)</td>
<td>36.37 ± 9.57</td>
<td>33.76 ± 6.90</td>
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</tbody>
</table>

a ApoA-IV levels in CAD patients were adjusted for creatinine, since a significant correlation was observed with creatinine. This was not observed in the control group.

Values are presented as mean ± SD
Table 3: Multiple linear regression analysis: Influence of selected variables on various apolipoprotein A-IV plasma fractions.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Coefficient</th>
<th>SE</th>
<th>P</th>
<th>Change in $R^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Lipid-free ApoA-IV</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total apoA-IV</td>
<td>0.049</td>
<td>0.009</td>
<td>&lt;0.001</td>
<td>0.305</td>
</tr>
<tr>
<td>HDL cholesterol</td>
<td>-0.004</td>
<td>0.002</td>
<td>0.019</td>
<td>0.074</td>
</tr>
<tr>
<td><strong>LpA-IV</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total apoA-IV</td>
<td>0.826</td>
<td>0.049</td>
<td>&lt;0.001</td>
<td>0.812</td>
</tr>
<tr>
<td>HDL cholesterol</td>
<td>-0.026</td>
<td>0.009</td>
<td>0.004</td>
<td>0.030</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>0.008</td>
<td>0.003</td>
<td>0.02</td>
<td>0.017</td>
</tr>
<tr>
<td><strong>LpA-I:A-IV</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HDL cholesterol</td>
<td>0.036</td>
<td>0.008</td>
<td>&lt;0.001</td>
<td>0.293</td>
</tr>
<tr>
<td>Total apoA-IV</td>
<td>-</td>
<td>-</td>
<td>0.077</td>
<td>-</td>
</tr>
</tbody>
</table>

Total apoA-IV, total cholesterol, HDL and LDL cholesterol and triglycerides were offered to the model. Only significant or borderline significant variables are listed.
100 µl plasma + 5 µl 4M MgCl₂ + 5 µl PTA

Incubation (2 hours, RT)

Centrifugation

Supernatant (lipid-free apoA-IV)

LpA-IV

84%

100 µl plasma + 5 µl 4M MgCl₂ + 5 µl PTA

Incubation (1 hour, RT)

Centrifugation


Pellet: LpA-I:A-IV (= apoA-IV bound to apoA-I)

5 µl plasma + 25 µl anti-ApoA-I + 10 µl DTNB + 60 µl PBS

Incubation (2 hours, RT)

Centrifugation

Supernatant (lipid-free apoA-IV)

LpA-I:A-IV

12%

Calculated by: apoA-I-unbound apoA-IV ÷ lipid-free apoA-IV

Calculated by: total apoA-IV ÷ apoA-I-unbound apoA-IV
Ezeh et al.: Figure 2

**A**

Plasma  
Supernatant  
Pellet  

---

ApoA-I

**B**

Plasma  
Supernatant  
Pellet  

---

ApoA-IV
Ezeh et al.: Figure 4