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

Benjie Ezeh,[*,††] Marina Haiman,[*] Hannes F. Alber,[†] Birgit Kunz,[*] Bernhard Paulweber,[§] Arno Lingenhel,[*] Hans-Georg Kraft,[*] Franz Weidinger,[†] Otmar Pachinger,[†] Hans Dieplinger,[*] and Florian Kronenberg[1,*,]**

Institute of Medical Biology and Human Genetics,[*] University of Innsbruck, Schöpfstr. 41, A-6020, Innsbruck, Austria; Innsbruck University Hospital,[†] Department of Cardiology, Innsbruck, Austria; Department of Internal Medicine,[§] Landeskrankenhaus, Salzburg, Austria; GSF-National Research Center for Environment and Health,[**] Institute of Epidemiology, Neuherberg, Germany; and Vitateq Biotechnology GmbH,[††] Innsbruck, Austria

Abstract **Recent studies showed lower apolipoprotein A-IV (apoA-IV) plasma concentrations in patients with coronary artery disease (CAD). The actual distribution of the antiatherogenic apoA-IV in human plasma, however, is discussed controversially and it was never investigated in CAD patients. We therefore developed a gentle technique to separate the various apoA-IV-containing plasma fractions. Using a combination of precipitation of all lipoproteins with 40% phosphotungstic acid and 4 M MgCl2, as well as immunoprecipitation of all apoA-I-containing particles with an anti-apoA-I antibody, 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%). 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 mg/dl vs. 11.85 ± 2.82 mg/dl, $P = 0.029$), but no major differences for the three plasma apoA-IV fractions. We conclude that our gentle separation method reveals a different distribution of apoA-IV than in many earlier studies. No major differences exist in the apoA-IV plasma distribution pattern between CAD patients and controls. Therefore, the antiatherogenic effect of apoA-IV has to be explained by other functional properties of apoA-IV (e.g., the antioxidative characteristics).**—Ezeh, B., M. Haiman, H. F. Alber, B. Kunz, B. Paulweber, A. Lingenhel, H-G. Kraft, F. Weidinger, O. Pachinger, H. Dieplinger, and F. Kronenberg. **Plasma distribution of apoA-IV in patients with coronary artery disease and healthy controls.** ***J. Lipid Res.*** **2003.** 44: **1523–1529.**

Supplementary key words apolipoprotein A-IV • lipoprotein A-I:pA-IV • LpA-IV • lipid-free apoA-IV • antiatherogenic

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, 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 in vitro 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 apoA-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 (CAD) (16). This finding was confirmed in a Chinese study population (17), as well as in patients with kidney impairment (18).

Manuscript received 4 February 2003 and in revised form 18 April 2003.

Published, JLR Papers in Press, June 1, 2003.
DOI 10.1194/jlr.M300060-JLR200

Abbreviation: CAD, coronary artery disease.

[1] To whom correspondence should be addressed.
e-mail: florian.kronenberg@uibk.ac.at

Reports about the distribution of apoA-IV in human plasma are contradictory. They described a wide variety of apoA-IV distributions 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 clear already 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 that 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 CAD and controls are responsible for the antiatherogenic 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 in inducing cholesterol efflux from macrophages (28). Our study aimed, therefore, to investigate whether there are clinical correlations to these in vitro findings.

MATERIALS AND METHODS

Patients

A total of 52 patients (44 men and eight women) with a history of 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 with 52 healthy controls one-to-one 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 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 apoA-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 apoA-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 intraassay and interassay coefficients of variation of 4.5% and 6.6%, respectively (29).

Determination of lipid-free apoA-IV

The lipoproteins from 100 μl plasma were precipitated by adding 5 μl of 40% phosphotungstic acid (PTA, pH = 7.6) and 5 μl of 4M $MgCl_2$. This method was shown to completely remove all lipoproteins, as demonstrated by Bartholome et al. (31). After 2 h of incubation at room temperature, the precipitated plasma was centrifuged for 15 min at 15,000 rpm. The clear supernatant containing lipoprotein-depleted plasma was used for measurement of lipid-free apoA-IV (**Fig. 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 $MgCl_2$. The dissolved pellet was dialyzed overnight against PBS at 4°C in Spectra/Por® molecularporous membrane (MWCO 6,000–8,000) and subjected to immunoblot analysis.

TABLE 1. Earlier studies investigating the plasma distribution of apolipoprotein A-IV

Author	Method	Observation
Beisiegel and Utermann (19)	Ultracentrifugation	ApoA-IV present in d < 1.006 g/ml (=chylomicrons and VLDL)
Beisiegel and Utermann (19)	Immunoelectrophoresis	Most apoA-IV present in d > 1.21 g/ml (=unassociated with major lipoprotein fractions)
Green et al. (3)	Sequential ultracentrifugation	98% apoA-IV present in lipid-free fractions
Green et al. (3)	Agarose column chromatography	77% apoA-IV is unassociated ("free") with lipoproteins, while 23% is associated
Bisgaier et al. (22)	Agarose gel chromatography and immunoprecipitation	15–25% of apoA-IV is associated with HDL
Lagrost et al. (23)	Immunoprecipitation with anti-apoA-I	Majority of apoA-IV is associated with HDL
Malmendier et al. (24)	Affinity chromatography	93% apoA-IV is associated with apoA-I
Dieplinger et al. (25)	Gel filtration chromatography	25% of apoA-IV is associated with HDL, 75% is not associated
Duverger et al. (26)	Sequential immunoaffinity chromatography	Identified LpA-I:A-IV and LpA-IV; about 6% apoA-IV associated apoA-I, 70% is not apoA-I-associated
Von Eckardstein et al. (27)	Sequential immunoaffinity chromatography and nondenaturating 2D-polyacrylamide gradient gel electrophoresis (2D-NDGE)	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
Böttcher et al. (21)	Preparative free-solution isotachophoresis and 2D-NDGE	ApoA-IV found in fractions with slow-α- and pre-β mobility, mainly unassociated with apo A-I
Asztalos et al. (20)	2D-NDGE	ApoA-IV found between α- and pre-β HDL subfractions

apoA-IV, apolipoprotein A-IV; LpA-IV, lipoprotein particles containing apoA-IV without apoA-I; LpA-I:A-IV, lipoprotein particles containing apoA-IV with apoA-I.

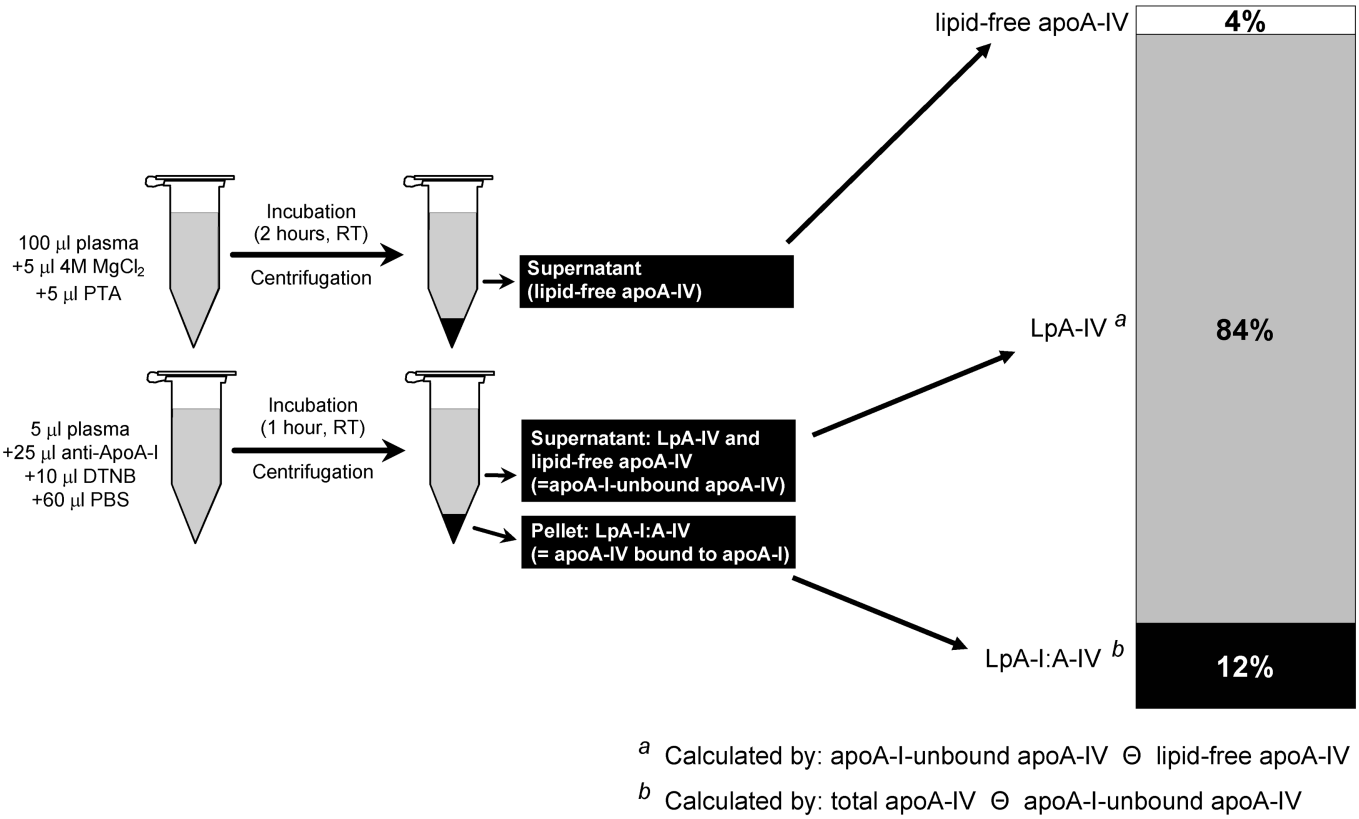

Fig. 1. A schematic illustration of the experimental procedure to investigate the apolipoprotein A-IV (apoA-IV) plasma distribution. The right bar provides the results of the apoA-IV plasma distribution from a healthy control group.

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 5 µl of plasma to 25 µl of rabbit anti-human apoA-I γ-globulin fraction (Behring Diagnostics GmbH, Marburg, Germany), and 10 µl 1 mmol/l DTNB solution and 60 µl 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 down 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 10,000 rpm. ApoA-IV was quantified by ELISA in the clear supernatant containing both LpA-IV and lipid-free apoA-IV (Fig. 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 5 µl plasma with 10 µl DTNB and 85 µl of PBS in order to ensure 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 transfered to cellulose nitrate membranes. Rabbit anti-human apoA-IV and rabbit anti-human apoA-I (Behring Diagnostics GmbH) served as the first antibody, and HRPO-conjugated swine anti-rabbit IgG (DAKO, Denmark) as the 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. $P < 0.05$ 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/$MgCl_2$ 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 (**Fig. 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, 34–36). 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 (Fig. 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 5 µl plasma with 25 µl anti-apoA-I. This amount of antibody was

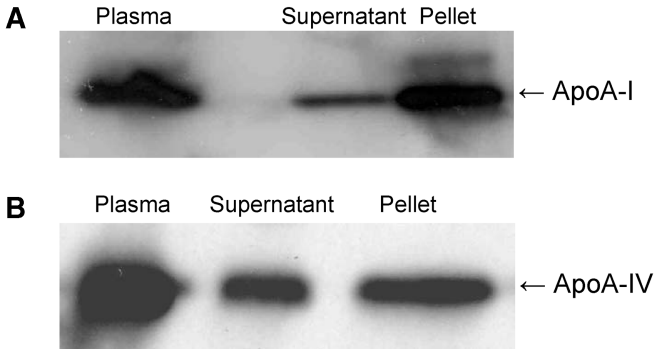

Fig. 2. Analysis of supernatants and pellets after precipitation of lipoproteins by adding 5 μl of 40% phosphotungstic acid (PTA) and 5 μl of 4 M $MgCl_2$ to 100 μl of plasma. A, B: The bands obtained after immunoblotting with anti-apoA-I and anti-apoA-IV, respectively. Native plasma is shown as a control. B: The proportion of material subjected to the gel between supernatant and pellet is 1:6.

necessary to obtain a complete precipitation of apoA-I (**Fig. 3**). In order to ensure 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 the 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 (Fig. 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 nonspecific precipitation of apoA-IV could be excluded (**Fig. 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/$MgCl_2$, we found only small amounts of apoA-IV in the supernatant (Fig. 4B).

TABLE 2. Comparison of total apoA-IV and various apoA-IV plasma fractions as well as lipids and renal function parameters between coronary artery disease patients and age- and sex-matched healthy controls

	CAD Patients (n = 52)	Healthy Controls (n = 52)	*P*
Total apoA-IV (mg/dl)[a]	10.28 ± 3.67	11.85 ± 2.82	0.029
Lipid-free apoA-IV (%)	4.79 ± 1.94	4.22 ± 1.50	0.044
LpA-I:A-IV (%)	12.38 ± 7.69	11.79 ± 7.04	0.64
LpA-IV (%)	82.70 ± 7.35	84.07 ± 6.69	0.25
Total cholesterol (mg/dl)	208 ± 36	230 ± 41	0.003
HDL cholesterol (mg/dl)	40 ± 11	62 ± 16	<0.001
LDL cholesterol (mg/dl)	133 ± 33	144 ± 40	0.10
Trigylcerides (mg/dl)	114 ± 65	157 ± 87	0.16
Creatinine values (mg/dl)	0.99 ± 0.13	0.94 ± 0.11	0.034
Urea (mg/dl)	36.37 ± 9.57	33.76 ± 6.90	0.076

CAD, coronary artery disease. Values are presented as mean ± SD.
[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.

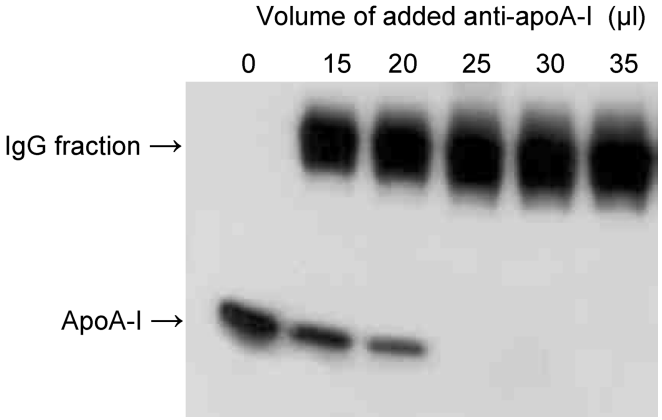

Fig. 3. Immunoblot analysis of supernatants from plasma after immunoprecipitation with anti-apoA-I antibody. Lane 1 shows plasma, and lanes 2–6 investigate supernatants after incubation of plasma from the same donor with increasing amounts of anti-apoA-I. The higher-molecular-weight band originates from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

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 (Fig. 1, 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 with controls

The mean total plasma apoA-IV concentrations in CAD patients were slightly but significantly lower when compared with 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

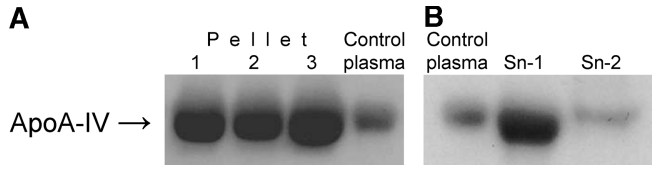

Fig. 4. Characterization of supernatant and pellet after immunoprecipitation of apoA-I-containing particles with an anti-apoA-I antibody. A: The specific association of apoA-IV with apoA-I in the pellet. Pellets from three samples (pellet 1–3) were washed twice intensively with PBS, dissolved, and subjected to immunoblot analysis with anti-apoA-IV. B: 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/$MgCl_2$ (Sn-2).

TABLE 3. Multiple linear regression analysis: influence of selected variables on various apolipoprotein A-IV plasma fractions

Variable	Coefficient	SE	*P*	Change in R^2
Lipid-free apoA-IV				
Total apoA-IV	0.049	0.009	<0.001	0.305
HDL cholesterol	−0.004	0.002	0.019	0.074
LpA-IV				
Total apoA-IV	0.826	0.049	<0.001	0.812
HDL cholesterol	−0.026	0.009	0.004	0.030
Triglycerides	0.008	0.003	0.02	0.017
LpA-I:A-IV				
HDL cholesterol	0.036	0.008	<0.001	0.293
Total apoA-IV	—	—	0.077	—

Total apoA-IV, total cholesterol, HDL and LDL cholesterol, and triglycerides were offered to the model. Only significant or borderline significant variables are listed.

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 attributable to different techniques of plasma separation (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 overestimation 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 nonfasting 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 are in fact associated with lipoproteins (3). This is in agreement with another study using the gel filtration chromatography method that reported 25% of total plasma apoA-IV to be associated with HDL (as determined by coelution with apoA-I). The remaining 75% were not apoA-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 that used 2D nondenaturating polyacrylamide gel electrophoresis found apoA-IV mainly in the α- and pre-β-HDL subfractions, mainly unassociated with apoA-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 to develop a gentle separation technique that allows the analysis of the physiological apoA-IV distribution in human plasma suitable for analysis of large sample numbers. The use of chemical PTA/$MgCl_2$ 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 techniques, 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 that represent the

majority of plasma apoA-IV still belong to the HDL class because they contain lipids.

Interestingly, HDL cholesterol shows a negative association with lipid-free apoA-IV and LpA-IV, which can be interpretated 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 of 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 that 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 in 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 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 the 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 differences 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 on 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 Von 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 rather than distributional properties of apoA-IV; for example its antioxidative properties. In this regard, the observation of Qin et al. (40) that, in rats, 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 apoA-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 cannot completely exclude that the precipitation of lipoproteins with PTA and MgCl2 induces artifactial 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 that used sequential immunoaffinity chromatography or the nondenaturing 2D gel-electrophoresis method (20, 21, 27).

It is at first glance surprising that we observed lower total cholesterol levels in our CAD patient group when compared with 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 fractions (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 rely less 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).

This study was supported by grants from the Österreichischer Herzfonds, the Austrian Science Fund (P-14717 to F.K. and P-14040 to H-G.K.), and the Austrian National Bank (Project #9331 to F.K.). The study was also supported by Dr. Legerlotz Stiftung to F.K. and H.D., the "MFF Tirol" (Project #8) to H.D., and by the Genomics of Lipid-associated Disorders (Gold) of the Austrian Genome Research programme (GEN-AU). The authors acknowledge Dr. Münscher (Behring Diagnostics GmbH, Marburg, Germany) for the generous gift of affinity-purified anti-apoA-I.

REFERENCES

1. Utermann, G., and U. Beisiegel. 1979. Apolipoprotein A-IV: a protein occurring in human mesenteric lymph chylomicrons and free in plasma. Isolation and quantification. *Eur. J. Biochem.* **99:** 333–343.
2. Weisgraber, K. H., T. P. Bersot, and R. W. Mahley. 1978. Isolation and characterization of an apoprotein from the d less than 1.006 lipoproteins of human and canine lymph homologous with the rat A-IV apoprotein. *Biochem. Biophys. Res. Commun.* **85:** 287–292.
3. Green, P. H. R., R. M. Glickman, J. W. Riley, and E. Qinet. 1980. Human apolipoprotein A-IV. Intestinal origin and distribution in plasma. *J. Clin. Invest.* **65:** 911–919.

4. Kalogeris, T. J., F. Monroe, S. J. Demichele, and P. Tso. 1996. Intestinal synthesis and lymphatic secretion of apolipoprotein A-IV vary with chain length of intestinally infused fatty acids in rats. *J. Nutr.* **126:** 2720–2729.
5. Rodriguez, M. D., T. J. Kalogeris, X. L. Wang, R. Wolf, and P. Tso. 1997. Rapid synthesis and secretion of intestinal apolipoprotein A-IV after gastric fat loading in rats. *Am. J. Physiol.* **272:** R1170–R1177.
6. Stein, O., Y. Stein, M. Lefevre, and P. S. Roheim. 1986. The role of apolipoprotein A-IV in reverse cholesterol transport studied with cultured cells and liposomes derived from another analog of phosphatidylcholine. *Biochim. Biophys. Acta.* **878:** 7–13.
7. Steinmetz, A., R. Barbaras, N. Ghalim, V. Clavey, J-C. Fruchart, and G. Ailhaud. 1990. Human apolipoprotein A-IV binds to apolipoprotein A-I/A- II receptor sites and promotes cholesterol efflux from adipose cells. *J. Biol. Chem.* **265:** 7859–7863.
8. Steinmetz, A., and G. Utermann. 1985. Activation of lecithin:cholesterol acyltransferase by human apolipoprotein A-IV. *J. Biol. Chem.* **260:** 2258–2264.
9. Chen, C. H., and J. J. Albers. 1985. Activation of lecithin:cholesterol acyltransferase by apolipoproteins E-2, E-3 and A-IV isolated from human plasma. *Biochim. Biophys. Acta.* **836:** 279–285.
10. Dvorin, E., N. L. Gorder, D. M. Benson, and A. M. Gotto, Jr. 1986. Apolipoprotein A-IV. A determinant for binding and uptake of high density lipoproteins by rat hepatocytes. *J. Biol. Chem.* **261:** 15714–15718.
11. Goldberg, I. J., C. A. Scheraldi, L. K. Yacoub, U. Saxena, and C. L. Bisgaier. 1990. Lipoprotein ApoC-II activation of lipoprotein lipase. Modulation by apolipoprotein A-IV. *J. Biol. Chem.* **265:** 4266–4272.
12. Guyard-Dangremont, V., L. Lagrost, and P. Gambert. 1994. Comparative effects of purified apolipoproteins A-I, A-II, and A- IV on cholesteryl ester transfer protein activity. *J. Lipid Res.* **35:** 982–992.
13. Duverger, N., G. Tremp, J. M. Caillaud, F. Emmanuel, G. Castro, J. C. Fruchart, A. Steinmetz, and P. Denèfle. 1996. Protection against atherogenesis in mice mediated by human apolipoprotein A-IV. *Science.* **273:** 966–968.
14. Cohen, R. D., L. W. Castellani, J. H. Qiao, B. J. Van Lenten, A. J. Lusis, and K. Reue. 1997. Reduced aortic lesions and elevated high density lipoprotein levels in transgenic mice overexpressing mouse apolipoprotein A-IV. *J. Clin. Invest.* **99:** 1906–1916.
15. Ostos, M. A., M. Conconi, L. Vergnes, N. Baroukh, J. Ribalta, J. Girona, J. M. Caillaud, A. Ochoa, and M. M. Zakin. 2001. Antioxidative and antiatherosclerotic effects of human apolipoprotein A-IV in apolipoprotein E-deficient mice. *Arterioscler. Thromb. Vasc. Biol.* **21:** 1023–1028.
16. Kronenberg, F., M. Stühlinger, E. Trenkwalder, F. S. Geethanjali, O. Pachinger, A. Von Eckardstein, and H. Dieplinger. 2000. Low apolipoprotein A-IV plasma concentrations in men with coronary artery disease. *J. Am. Coll. Cardiol.* **36:** 751–757.
17. Warner, M. M., J. Guo, and Y. Zhao. 2001. The relationship between plasma apolipoprotein A-IV levels and coronary heart disease. *Chin Med. J. (Engl.).* **114:** 275–279.
18. Kronenberg, F., E. Kuen, E. Ritz, R. Junker, P. König, G. Kraatz, K. Lhotta, J. F. E. Mann, G. A. Müller, U. Neyer, W. Riegel, P. Riegler, V. Schwenger, and A. Von Eckardstein. 2002. Apolipoprotein A-IV serum concentrations are elevated in mild and moderate renal failure. *J. Am. Soc. Nephrol.* **13:** 461–469.
19. Beisiegel, U., and G. Utermann. 1979. An apolipoprotein homology of rat apolipoprotein A-IV in human plasma: isolation and partial characterization. *Eur. J. Biochem.* **93:** 601–608.
20. Asztalos, B. F., M. E. Brousseau, J. R. McNamara, K. V. Horvath, P. S. Roheim, and E. J. Schaefer. 2001. Subpopulations of high density lipoproteins in homozygous and heterozygous Tangier disease. *Atherosclerosis.* **156:** 217–225.
21. Böttcher, A., J. Schlosser, F. Kronenberg, H. Dieplinger, G. Knipping, K. J. Lackner, and G. Schmitz. 2000. Preparative free-solution isotachophoresis for separation of human plasma lipoproteins: apolipoprotein and lipid composition of HDL subfractions. *J. Lipid Res.* **41:** 905–915.
22. Bisgaier, C. L., O. P. Sachdev, L. Megna, and R. M. Glickman. 1985. Distribution of apolipoprotein A-IV in human plasma. *J. Lipid Res.* **26:** 11–25.
23. Lagrost, P., P. Gambert, M. Boquillon, and C. Lallemant. 1989. Evidence for high density lipoproteins as the major apolipoprotein A-IV-containing fraction in normal human serum. *J. Lipid Res.* **30:** 1525–1534.
24. Malmendier, C. L., J-F. Lontie, L. Lagrost, C. Delcroix, D. Y. Dubois, and P. Gambert. 1991. In vivo metabolism of apolipoproteins A-IV and A-I associated with high density lipoprotein in normolipidemic subjects. *J. Lipid Res.* **32:** 801–808.
25. Dieplinger, H., E-M. Lobentanz, P. König, H. Graf, C. Sandholzer, E. Matthys, M. Rosseneu, and G. Utermann. 1992. Plasma apolipoprotein A-IV metabolism in patients with chronic renal disease. *Eur. J. Clin. Invest.* **22:** 166–174.
26. Duverger, N., N. Ghalim, G. Ailhaud, A. Steinmetz, J-C. Fruchart, and G. Castro. 1993. Characterization of apoA-IV-containing lipoprotein particles isolated from human plasma and interstitial fluid. *Arterioscler. Thromb.* **13:** 126–132.
27. Von Eckardstein, A., Y. Huang, S. Wu, A. S. Sarmadi, S. Schwarz, A. Steinmetz, and G. Assmann. 1995. Lipoproteins containing apolipoprotein A-IV but not apolipoprotein A-I take up and esterify cell-derived cholesterol in plasma. *Arterioscler. Thromb. Vasc. Biol.* **15:** 1755–1763.
28. Fournier, N., V. Atger, J. L. Paul, M. Sturm, N. Duverger, G. H. Rothblat, and N. Moatti. 2000. Human apoA-IV overexpression in transgenic mice induces cAMP-stimulated cholesterol efflux from J774 macrophages to whole serum. *Arterioscler. Thromb. Vasc. Biol.* **20:** 1283–1292.
29. Kronenberg, F., E-M. Lobentanz, P. König, G. Utermann, and H. Dieplinger. 1994. Effect of sample storage on the measurement of lipoprotein(a), apolipoproteins B and A-IV, total and high-density lipoprotein cholesterol and triglycerides. *J. Lipid Res.* **35:** 1318–1328.
30. Rosseneu, M., G. Michiels, W. Dekeersgieter, J. B. Bury, J. P. De Slypere, H. Dieplinger, and G. Utermann. 1988. Human apolipoprotein A-IV quantitation by sandwich enzyme linked immunosorbent assay. *Clin. Chem.* **34:** 739–743.
31. Bartholome, M., D. Niedmann, H. Wieland, and D. Seidel. 1981. An optimized method for measuring lecithin:cholesterol acyltransferase activity, independent of the concentration and quality of the physiological substrate. *Biochim. Biophys. Acta.* **664:** 327–334.
32. Bisgaier, C. L., O. P. Sachdev, E. S. Lee, K. J. Williams, C. B. Blum, and R. M. Glickman. 1987. Effects of lecithin:cholesterol acyltransferase on distribution of apolipoprotein A-IV among lipoproteins of human plasma. *J. Lipid Res.* **28:** 693–703.
33. Frank, P. G., and Y. L. Marcel. 2000. Apolipoprotein A-I: structure-function relationships. *J. Lipid Res.* **41:** 853–872.
34. Forte, T. M., and M. R. McCall. 1994. The role of apolipoprotein A-I-containing lipoproteins in atherosclerosis. *Curr. Opin. Lipidol.* **5:** 354–364.
35. Mooser, V., S. M. Marcovina, A. L. White, and H. H. Hobbs. 1996. Kringle-containing fragments of apolipoprotein(a) circulate in human plasma and are excreted into the urine. *J. Clin. Invest.* **98:** 2414–2424.
36. Trenkwalder, E., A. Gruber, P. König, H. Dieplinger, and F. Kronenberg. 1997. Increased plasma concentrations of LDL-unbound apo(a) in patients with end-stage renal disease. *Kidney Int.* **52:** 1685–1692.
37. Dallinga-Thie, G. M., P. H. E. Groot, and A. van Tol. 1985. Distribution of apolipoprotein A-IV among lipoprotein subclasses in rat serum. *J. Lipid Res.* **26:** 970–976.
38. Lefevre, M., M-Y. Chuang, and P. S. Roheim. 1986. ApoA-IV metabolism in the rat: role of lipoprotein lipase and apolipoprotein transfer. *J. Lipid Res.* **27:** 1163–1173.
39. Skinner, E. R. 1994. High-density lipoprotein subclasses. *Curr. Opin. Lipidol.* **5:** 241–247.
40. Qin, X. F., D. K. Swertfeger, S. Q. Zheng, D. Y. Hui, and P. Tso. 1998. Apolipoprotein AIV: A potent endogenous inhibitor of lipid oxidation. *Am. J. Physiol.* **274:** H1836–H1840.