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Distribution of apolipoprotein AI-containing lipoprotein subclasses in plasma of normolipidemic subjects*

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The distribution of apoA-I among apoA-I-containing lipoprotein (AI-Lp) subclasses in plasma was studied by immunoblotting utilizing agarose gel matrix incorporating anti-apoA-I as the transfer medium. Nine AI-Lp subclasses were detected in the plasma of normolipidemics, with relative molecular masses ranging from 70 000 to ≥ 354 000 and diameters from 7.12 to ≥ 11.6 nm. The mass distribution of AI-Lp subclasses was significantly different between males and females, and some subclasses increased gradually with age while others decreased. There was a significant strong positive correlation between subclass 1 (M_r 70 000–75 000) and subclass 3 (M_r 105 000–126 000) in all subjects and age groups. Analysis of similar AI-Lp or HDL subclasses reported in the literature showed variability in the sizes reported by various workers. This stresses the need for a unified classification of such subclasses, and this work contributes to this direction. The quantitative nature of the method used in this work compared with the semiquantitative approaches used earlier makes it a better method for the study of the quantitative changes of the subclasses in various physiological and pathological states. The method helps to generate ideas for *in vitro* and *in vivo* studies of apoA-I exchange among subclasses and *in vivo* kinetic studies. Conclusion. Plasma level of the AI-Lp subclasses varied quantitatively with age and gender, and strong correlations were detected between some subclasses. This work contributes to a better classification of AI-Lp subclasses according to their size. Comparison of the method used here with the methods reported in the literature revealed its advantages.

Keywords: immunoblotting, stability of apoA-I-containing lipoproteins on storage, HDL subclasses, microheterogeneity of HDL, ApoA-I exchange, ApoA-I kinetics

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INTRODUCTION

An inverse relation between high density lipoprotein (HDL) abundance and cardiovascular diseases (CVD) has been concluded from epidemiological studies. HDL is measured in the plasma as its cholesterol content (HDL-C), which simply quantitates the amount of cholesterol contained within the HDL fraction and does not necessarily correlate with the number of particles nor with their net anti-atherogenic properties (Joy & Hegele, 2008a; Cavigiolio *et al.,* 2008). Moreover, measurement of plasma HDL-C is of low predictive value and its use as a biomarker in assessing the individual's risk of CVD is now questionable (Ansell *et al.*, 2005; Navab *et al.,* 2006; Cavigiolio *et al.,* 2008). Nonetheless, despite the preponderance of evidence linking low HDL-C levels with cardiovascular morbidity and mortality, there is no definitive evidence proving that increasing HDL-C levels reduces the incidence of major cardiovascular events (Singh *et al.,* 2007; Vickers, *et al.,* 2007; Barter *et al.,* 2007; Hsia *et al.,* 2008; Joy & Hegele, 2008a; 2008b).

While HDL has other functions in addition to its role in the reverse cholesterol transport (RCT) (Ansell *et al.,* 2003; 2005; Barter *et al.,* 2004; Navab *et al.,* 2006; Chapman, 2006, and references therein), many factors have adverse effects on HDL function. Modifications of HDL proteins and lipids markedly affect their function (Zheng *et al.,* 2004; Brock *et al.,* 2008; Jayaraman *et al.,* 2008; Shao *et al.,* 2008; Yu *et al.,* 2008), and the functionality of different HDL subfractions appears to vary substantially, hence attention has shifted toward strategies for targeting HDL composition as adjunctive therapy to prevent and treat CVD (Singh *et al.,* 2007); therefore, a deeper understanding of the different functions of HDL and their associated biomarkers is badly needed (Kontush & Chapman, 2006; Tall, 2007).

Despite intense research, the underlying mechanisms of HDL atheroprotection remain incompletely understood (Reilly & Tall, 2007). This reflects complex relationships between HDL and CVD, and the situation is further aggravated by the heterogeneity of HDL and the different methods used for its subfractionation (Reschly *et al.,* 2002; Maiorano *et al.,* 2004; Kontush & Chapman, 2006). Sophisticated separation techniques have revealed more HDL complexity than previously thought, and the apolipoprotein content, the HDL-associated proteins and enzymes, the composition and type of phospholipids, the size, charge, and configuration of HDL particles are crucial in determining their various roles (Alenezi *et al.,* 2004). Due to this heterogeneity, apoA-I possibly exists in diverse conformations (Phillips *et al.,* 1997), and its native structure has not yet been amenable to study. Therefore, detailed study of the chemical composition of HDL subclasses is essential to get deeper insight into the mode of lipid–protein interaction and the resulting

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Abbreviations: AI-Lp, AI-containing lipoprotein; ApoA-I, apolipoproein A-I; CV, coefficient of variation; CVD, cardiovascular disease; DNBA, 5,5-dithiobis-2-nitrobenzoic acid; gPAGE, gradient polyacrylamide gel electrophoresis; HDL, high density lipoprotein; HDL-C, HDL-cholesterol; LDL-C, low density lipoprotein-cholesterol; NC, nitrocellulose; RCT, reverse cholesterol transport; SPSS, Statistical Package for Social Sciences; TAGs, triacylglycerols; VLDL-C, very low density lipoprotein-cholesterol

Table 1. Subfractionation of HDL by different methods

Method of separation	Subfractions reported		References	Type of separation
	No.	Designation		
Ultracentrifugation	5	HDL_{2b}, HDL_{2a}, HDL_{3a}, HDL_{3b}, HDL_{3c}	Tall *et al.,* 1982; Kontush *et al.,* 2003	Isolation
Electrophoresis 1) gPAGE 2) 2-dimensional	5	HDL_{2b}, HDL_{2a}, HDL_{3a}, HDL_{3b}, HDL_{3c}	Anderson *et al.,* 1977; Blanche *et al.,* 1981; Verdery *et al.,* 1989; Li *et al.,* 1994	Detection
	10	apoA-I-containing subfractions	Vézina *et al.,* 1988	Detection
	12	3 of α mobility, 4 of pre-α mobility, 2 of pre-β_1 mobility, 3 of pre-β_2 mobility	Asztalos *et al.,* 1993	Detection
NMR	3	Large, intermediate, small HDL	Freedman *et al.,* 1998	Detection
Chromatography 1) Gel filtration	3	Large, intermediate, small LpA-I	Duverger *et al.,* 1993	Isolation
2) High-performance size-exclusion	3	Large, intermediate, small LpA-I	Nanjee & Brinton, 2000	Isolation
3) HPLC	5	VLHDL, LHDL, MHDL, SHDL, VSHDL	Okazaki *et al.,* 2005	Detection
Immunological	4	(A-I)HDL_2, (A-I+A-II)HDL_2, (A-I)HDL_3, (A-I+A-II)HDL_3	Atmeh *et al.,* 1983	Isolation
	5	Lp(A-I without A-II)$_1$, Lp(A-I without A-II)$_2$, Lp(A-I with A-II)$_1$, Lp(A-I with A-II)$_2$, Lp(A-I with A-II)$_3$	Cheung & Albers, 1984	Isolation
Ultrafiltration	2	Small (A-I)HDL, small (A-I+A-II)HDL	Atmeh, 1990	Isolation

conformation of apoA-I in each subclass and its relation to the function. Nevertheless, the number of HDL subclasses that exist in the circulation is not established yet due to differences in properties utilized for their separation and because subpopulations of HDL have different chemical composition, size, charge, and function (Suenram *et al.,* 1979; Tall *et al.,* 1982; Castro & Fielding, 1988; Braschi *et al.,* 1999).

Detection and isolation of HDL subclasses is done by various techniques that depend on differences in their physico-chemical or immunological properties. The different methods of separation of HDL and the subfractions reported are shown in Table 1. These numerous and weakly related subfractionations show the need for a unified classification of HDL subclasses. At present, it is known that plasma levels of various HDL subclasses vary differently in various physiological and pathological states (Cavallero *et al.,* 1995; Atmeh & Robenek, 1996; Asztalos *et al.,* 2000). The two main subclasses, LpA-I and Lp(A-I+A-II), vary with age (Ohta *et al.,* 1989; Srinivasan *et al.,* 1998), sex and race (Ohta *et al.,* 1988; 1989a; Li *et al.,* 1996; Srinivasan *et al.,* 1998), and in different diseases (Puchois *et al.,* 1987; Ohta *et al.,* 1989b; Stampfer *et al.,* 1991). Moreover, *in vitro* studies have shown that LpA-I can remove cholesterol from cells (Barbaras *et al.,* 1987) and prevent LDL oxidation by copper ion (Ohta *et al.,* 1989c) more efficiently than Lp(A-I+A-II). Nonetheless, the clinical relevance of circulating levels of individual HDL subfractions to atherosclerosis and cardiovascular disease (CVD) is unclear, and conflicting results have been obtained; therefore, a new trend has emerged where the measurement of the quality and novel functions of HDL are expected to provide an improved means of identifying subjects at increased risk of CVD (Navab *et al.,* 2006).

In fact, the term HDL does not represent a physiologically relevant entity, rather, it is an operational term used to denote a class of lipoproteins that can be isolated in the laboratory by ultracentrifugation within the density range of 1.063–1.210 g/mL, hence, more physiologically relevant species should be sought. In the light of the heterogeneity of HDL particles and the lack of consensus on the number and type of HDL subclasses present in the blood due to the different methodology used to identify and quantitate these subclasses, it is difficult to compare them and define the function of each subclass. At present, five approaches are used that can detect and measure the relative distribution of the numerous HDL subclasses in the plasma: 1) separation of HDL by high performance liquid chromatography (HPLC) as one band, then resolving five subclasses by curve fitting using Gaussian summation method (Okazaki *et al.,* 2005); 2) separation of plasma on nondenaturing gPAGE followed by lipid staining (Li *et al.,* 1994); 3) separation of plasma on nondenaturing gPAGE followed by electrotransfer onto nitrocellulose (NC) membrane and treatment with anti-apoA-I (Vézina *et al.,* 1988); 4) separation of plasma on agarose gel in one dimension then electrophoresis in the second dimension on nondenaturing gPAG followed by electrotransfer onto NC or similar membranes, and treatment with anti-apoA-I (Miida *et al.,* 1990; 1997; Asztalos *et al.,* 1993; Xu & Fu, 2003); 5) separation of plasma on nondenaturing gPAGE followed by electrotransfer onto agarose gel matrix in which anti-apoA-I was incorporated (Atmeh & Robenek, 1996). The first approach does not detect subclasses of diameter less than 7.6 nm, and the advantages and disadvantages of the remaining four approaches are summarized in Table 2. A comparison of these methods reveals advantages of the quantitative method of the fifth approach (Atmeh & Robenek, 1996), whereas the

Table 2. Comparison between three methods for analysis of HDL subclasses

Method of analysis	Number of steps	Time (d)	Advantages	Disadvantages
Plasma separated by agarose gel electrophoresis, then in second dimension by gPAGE followed by electrotransfer onto nitrocellulose (NC) or nylon membrane, then treatment with anti-apoA-I (Miida *et al.,* 1990; Asztalos *et al.,* 1993; 1997; Xu & Fu, 2003)	17	3	1, Good separation 2, Measures distribution of apoA-I in subclasses 3, Detects smallest subclasses	1, Semi-quantitative, NC membrane has limited and different capture capacities for proteins and lipoproteins (Vézina *et al.,* 1988; Atmeh & Robenek, 1996) 2, Subclasses appear as diffused spots 4, One gradient gel for each sample 5, Use of foreign proteins for saturating NC membrane may affect antigenicity of apolipoproteins (Atmeh & Robenek, 1996) 6, Use of radioactive isotopes (Miida *et al.,* 1990, 1997; Asztalos *et al.,* 1993)
Plasma separated by gPAGE followed by staining with lipid stain (Li *et al.,* 1994) or by electrotransfer onto NC membrane, then treatment with anti-apoA-I (Vézina *et al.,* 1988)	5	6	1, Good separation 2, Up to 15 samples/gel 3, Measures distribution of lipid components of subclasses (Li *et al.,* 1994) or their apoA-I content (Vézina *et al.,* 1988)	1, Semi-quantitative due to disproportionate staining of subclasses with variable lipid contents (Li *et al.,* 1994), or due to limited capture capacity of NC membrane (Vézina *et al.,* 1988) 2, Does not detect smallest subclasses due to their low content of lipids (Li *et al.,* 1994) 3, gel preservation may be difficult (Li *et al.,* 1994)
Plasma separated by gPAGE followed by immunoblotting against agarose gel containing anti-apoA-I (Atmeh & Robenek, 1996)	6	2	1, Good separation 2, Measures distribution of apoA-I in subclasses 3, Quantitative, detects smallest subclasses (Atmeh & Robenek, 1996) 4, Subclasses appear as bands 5, Up to 15 samples/gel 6, Dried agarose gel can be kept permanently	1, Requires delicate handling of agarose gel after electrotransfer 2, Amount of antibody used is critical, therefore, should be optimized in each lab

other three approaches (Vézina *et al.,* 1988; Miida *et al.,* 1990; 1997; Asztalos *et al.,* 1993; Li *et al.,* 1994; Xu & Fu, 2003) are semi-quantitative, therefore, this approach (Atmeh & Robenek, 1996) is promising for detection, quantitation, and classification of the more physiologically relevant AI-containing lipoprotein (AI-Lp) subclasses directly from fresh plasma. By this method, discrete native AI-Lp subclasses can be identified and all the subclasses present in the sample can be detected as bands on one plate, which enables the determination of their molecular size and mass.

In the present work we utilized this method to study the distribution of apoA-I among the AI-Lp subclasses in fresh plasma from normolipidemic subjects.

MATERIALS AND METHODS

Materials. All chemicals used were of biochemical grade. Anti-apoA-I antibody and apoA-I control serum were purchased from Meridian Life Sciences (USA). Enzymatic methods as commercial kits were used for the determination of triacylglycerols (TAGs) (Sigma, USA), total cholesterol and HDL-C (Acromex, Jordan).

Subjects. Ninety fasting apparently healthy subjects were divided in two ways: 1) segregated into males (n = 42) and females (n = 48); 2) segregated into three age groups comprising both males and females: group 1 (G1) (≤ 25 yrs), group 2 (G2) (26–40 yrs), and group 3 (G3) (≥ 41 yrs) (Table 4). The subjects were healthy and consumed diets rich in vegetables and olive oil (the Mediterranean diet) and did not drink alcohol. All samples were run in duplicates and the average was used.

Samples. Venous blood was withdrawn from the subjects into EDTA-containing tubes and plasma was isolated promptly by low-speed centrifugation at 4 °C. Forty microliters of the LCAT inhibitor, 5,5-dithiobis-2-nitrobenzoic acid (DNBA) (37.5 mM), was added immediately to each milliliter of plasma in addition to the following preservatives: gentamycin final concentration (0.08 mg • mL^{-1}), sodium azide (0.1 mg • mL^{-1}), and chloramphenicol (0.08 mg • mL^{-1}).

Table 3. Total plasma apoA-I and lipid profile of all subjects

Parameter	All subjects	Males	Females	% Diff[a]
n	90	42	48	
Age (yrs)	31.6 ± 12.9[b]	32.5 ± 13.4	30.8 ± 12.7	
Total A-I (mg/dL)	140 ± 27	133 ± 29	147 ± 23	10[c]
Total cholesterol (mg/dL)	159 ± 40	152 ± 36	166 ± 42	8
HDL-cholesterol (mg/dL)	34 ± 18	28 ± 12	39 ± 21	28[d]
LDL-cholesterol (mg/dL)	109 ± 40	103 ± 40	113 ± 40	9
VLDL-cholesterol (mg/dL)	18 ± 10	20 ± 11	15 ± 9	−33[c]
TAGs (mg/dL)	88 ± 49	100 ± 54	77 ± 43	−30[c]

[a]Percentage difference of means = [(females−males/males) × 100%]; [b]Mean ± S.D.; [c]$P < 0.05$; [d]$P < 0.01$

Preparation and running of 4–25% nondenaturing polyacrylamide gel. A linear nondenaturing 4–25% gPAGE was prepared and cast in (11.1 × 7.3 cm) glass plates and run in a vertical electrophoresis system (model: Mini-PROTEAN®3, Bio-Rad, USA). Three microliters of plasma (expected to contain about 4 μg apoA-I) was applied to the gel wells and 12 μL of a solution of protein markers containing 4 μg of each of ovalbumin (45 000), bovine serum albumin monomer (66 5000), bovine serum albumin dimer (133 000), urease trimer (272 000), and urease hexamer (545 000) was applied to one well. The gel

Table 4. Total plasma A-I and lipid profile of subjects separated into age groups

Parameter	Group 1 (≤25 yrs)	Group 2 (26–40 yrs)	Group 3 (≥41 yrs)	% Difference between groups		
				Groups 1 & 2[a]	Groups 1 & 3[b]	Groups 2 & 3[c]
n	36	28	26			
Age (yrs)	19.4 ± 4.1[d]	32.5 ± 4.9	48.9 ± 7.8			
Total A-I (mg/dL)	136 ± 22	142 ± 26	144 ± 34	–4	–6	–1
Total-cholesterol (mg/dL)	149 ± 37	155 ± 42	180 ± 34[c, d]	–4	–20[f]	–16[f]
HDL-cholesterol (mg/dL)	34 ± 14	33 ± 15	36 ± 26	3	6	9
LDL-cholesterol (mg/dL)	100 ± 40	108 ± 41	124 ± 38[c]	–8	–24[e]	–15
VLDL-cholesterol (mg/dL)	15 ± 8	16 ± 10	23 ± 11[c, d]	–7	–53[f]	–43[f]
TAGs (mg/dL)	75 ± 41	81 ± 47	116 ± 55[c, d]	–8	–55[f]	–43[f]

[a]Percentage difference of means of groups 1 and 2 = [(group 2 — group 1/group 1) × 100%]; [b]Percentage difference of means of groups 1 and 3 = [(group 3 — group 1/group 1) × 100%]; [c]Percentage difference of means of groups 2 and 3 = [(group 3 — group 2/group 2) × 100%]; [d]Mean ± S.D.; [e]$P < 0.05$; [f]$P < 0.01$.

was run at 70 V for 30 min and then at 120 V for 4.5 h in 14 mM Tris and 110 mM glycine buffer, pH 8.3. After the end of the run, the part containing the molecular mass markers was cut, fixed in 10% sulfosalicylic acid for 1 h and stained in Coomassie Brilliant Blue staining solution (0.04% Coomassie Brilliant Blue R-250 in 3.5% perchloric acid), and de-stained in 5% acetic acid solution until clear background was obtained. The rest of the gel was immediately placed in an electrotransfer cell to electrotransfer the lipoproteins onto agarose gel matrix containing anti-apoA-I.

Preparation of agarose gel matrix. One percent agarose gel solution containing anti-apoA-I (1.5% of the gel solution volume) was prepared in the Tris/glycine buffer, pH 8.3, and treated as described elsewhere (Atmeh & Robenek, 1996). In brief, the antibody was mixed with the warm agarose solution (56 °C) containing 4% polyethylene glycol (PEG 6000) and poured (1.5 mm thick) on glass plates of the required area, then kept in a moist chamber until use.

Immunoelectroblotting of plasma. The gPAG that contained the separated subclasses was placed in the transfer cell as follows (Atmeh & Robenek, 1996): the gel was placed on a wet filter paper, supported by a piece of wet sponge, then the solidified agarose gel was slipped down from the glass plate slowly over the gPAG, covered by another wet filter paper and wet sponge. The two gels were placed in the transfer cassette of the transfer cell (Trans-Blot Cell, Bio-Rad, USA) filled with the buffer, the agarose gel layer facing the anode. The transfer was done overnight at 100 mA constant current. After completion of the transfer, the agarose gel was carefully removed and placed on a glass plate or a hydrophilic plastic film, wrapped with a piece of soft medical gauze, and washed several times overnight in 0.9% normal saline to remove the un-reacted antibody. The agarose gel was then dried and stained with 1% Coomassie Brilliant Blue R-250 in 50% methanol and 7% acetic acid, and de-stained with a fresh solution of 10% methanol and 7% acetic acid. The dried gel was scanned and the bands were analyzed by the software UN-SCAN-IT (Silk Scientific Inc., USA) to calculate the percentage of AI-Lp subclasses. The mass of apoA-I in each AI-Lp subclass was calculated by multiplying the percentage of the subclass by the plasma concentration of apoA-I.

Study of the stability of AI-Lp subclasses in plasma on storage. Fresh plasma samples (n = 7) were collected and each sample was divided into two parts as follows: to one part the LCAT inhibitor, 5,5-dithiobis-2-dinitrobenzoic acid (DNBA), was added promptly after plasma separation to a final concentration of 1.5 mM; the other part of plasma was kept without DNBA. Both parts were stored at 4–8 °C, and portions of the two parts were subjected to immunoblotting at day 0 (fresh plasma), day 1, and day 2.

Chemical tests. The concentration of apoA-I in plasma was determined by electroimmunoassay using the method of Laurell (1972).

Data analysis. Calculation of the coefficient of variation from duplicate measurements. Due to the low stability of some of the AI-Lp subclasses, we calculated the coefficient of variation (CV) from duplicate measurements of the AI-Lp subclasses. The standard deviation (S.D.) was calculated from the relation:

$S.D. = SQRT(\Sigma d^2/2n)$ where d is the difference between the two measurements. The grand mean was calculated from the two means (X_1, X_2): grand mean $= (X_1 + X_2)/2$.

The CV% was calculated from the relation: CV% = (S.D./grand mean) × 100%.

Statistical analysis. Statistical analysis of data was done by using the Statistical Package for Social Sciences (SPSS). The Student's *t*-test was used to calculate the significance of difference between two variables. Correlations were calculated as linear correlation coefficient between two variables; therefore, comparison was done between two variables at a time by calculating the simple (bivariate) correlation coefficient and the level of significance of correlation was calculated.

RESULTS

Lipid profiles of the subjects

Since the subjects were healthy and consumed rich diets in vegetables and olive oil (the Mediterranean diet) and did not drink alcohol, their plasma lipid profiles were relatively low, as shown in Table 3. There was statistically significant differences between males and females, where females had higher plasma levels of total apoA-I and HDL-C, and lower levels of VLDL-C and TAGs (Table 3). In the age groups, there was a gradual increase in plasma total apoA-I, total cholesterol, LDL-C, VLDL-C, and TAGs with age (Table 4).

AI-Lp subclasses

At least nine AI-Lp subclasses were detected in the fresh plasma of the subjects with the relative molecular mass range from 70000 to >354000 and Stokes' radii in the range of 3.56 to >5.80 nm (Table 5). Due to minor variations in the positions of the subclasses' bands in different subjects, in addition to the appearance of many subclasses

Table 5. Lipoprotein AI subclasses detected in fresh plasma

Subclass number	Relative molecular mass (M_r)	Stokes' radius (nm)
1	70000–75000	3.56–3.61
2	89000–105000	3.80–4.00
3	105000–126000	4.00–4.23
4	126000–158000	4.23–4.53
5	158000–200000	4.53–4.87
6	200000–240000	4.87–5.15
7	240000–282000	5.15–5.41
8	282000–354000	5.41–5.80
9	>354000	>5.80

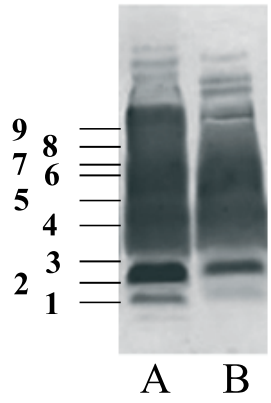

Figure 1. ApoA-I-containing lipoprotein subclasses in plasma of two normolipidemic subjects
Fresh plasma was separated by 4–20% gPAGE followed by electrotransfer onto agarose gel matrix containing anti-apoA-I, then the agarose gel was washed, dried, stained, and de-stained. Band numbers 1–9 represent molecular mass borders between the subclasses as follows: 1, 70000; 2, 89000; 3, 105000; 4, 126000; 5, 158000; 6, 200000; 7, 240000; 8, 282000; 9, 354000. Bands of higher molecular masses were not detected in all subjects at the same positions, therefore, they were considered as subclasses of molecular mass > 354000.

as broad bands, we considered the molecular masses and Stokes' radii of the broad bands as ranges (Fig. 1, Table 5). This is expected since HDL subclasses separated by gPAGE usually appear as a broad band representing a spectrum or a continuum of particles (Vézina *et al.,* 1988; Atmeh *et al.,* 2009) due to the microheterogeneity of these subclasses.

Stability of AI-Lp subclasses in plasma on storage

When the plasma (fresh, after one day, and 2 days after collection) without the LCAT inhibitor DNBA was immunoblotted against anti-apoA-I, there were statistically significant changes in the percentage of the subclasses 1, 2, 4, and 6 with time (Table 6). When the plasma with DNBA was run similarly, there was a gradual decrease in subclasses 4 and 5, whereas subclasses 1, 2, and 6 showed gradual increase with time (Table 6).

Distribution of AI-Lp subclasses in plasma

The CV of mass distribution of apoA-I among the AI-Lp subclasses was less than 7%. The mass distribution of the AI-Lp subclasses was in the range of 9.0–21.7 mg/dL (6.5–16.4%) in males, and 7.7–25.0 mg/dL (5.1–16.7%) in females (Tables 7 and 8). The mass of apoA-I in subclasses 5–9 was statistically significantly higher in females than males (Table 7). Comparing the age groups, there was no statistically significant differences in the mass of AI-Lp subclasses between group 2 and group 1, whereas subclasses 2 and 3 were higher and subclass 5 was lower in group 2 than in group 1 (Table 7). Subclass 2 in group 3 subjects was significantly higher than in group 1, and subclass 7 was significantly lower in group 3 than in group 2.

When the mass of apoA-I among the AI-Lp subclasses was correlated with the lipid profile of the subjects, subclass 1 showed a statistically significant negative correlation with LDL-C in all subjects, males, and group 1, while the correlation was significantly positive with VLDL-C and TAGs in all subjects, males, females, and group 1 (Table 9). Similar trend can be seen in subclass 3 in all subjects, and at a lower level of significance in males and group 1. HDL-C showed statistically significant positive correlation with subclasses 5 and 9 in all subjects and group 3, and with subclass 5 in females alone (Table 9). Interestingly,

Table 6. Effect of plasma storage on distribution of AI-containing lipoproteins
Plasma samples were incubated for 1 or 2 days without or with LCAT inhibitor DNBA[a], then separated by 4–20% gPAGE followed by electrotransfer onto agarose gel matrix containing anti-apoA-I, then agarose gel was washed, dried, stained, and de-stained. The significance of difference is related to day 0. Values are expressed as mean ± S.D. (n = 4).

AI-Lp subclass	Without DNBA				With DNBA			
	Time of incubation			General trend	Time of incubation			General trend
	Day 0	Day 1	Day 2		Day 0	Day 1	Day 2	
1	3.4	8.5[b]	10.6[b]	Increase	3.4	7.2	9.9[b]	Increase
2	12.4	16.5[b]	18.1[b]	Increase	12.2	17.4	19.4[b]	Increase
3	9.6	9.4	8.4	Decrease	9.4	10.1	8.5	Variable
4	16.1	8.9[b]	9.5[b]	Variable	16.4	11.1[b]	9.9[b]	Decrease
5	21.3	16.7	14.6	Decrease	21.4	17.5	12.2[b]	Decrease
6	9.7	12.0[b]	12.8[b]	Increase	9.8	10.2	13.3[b]	Increase
7	10.1	11.3	9.3	Variable	10.3	11.1	10.2	Variable
8	10.1	10.6	9.3	Variable	10.5	9.0	8.6	Decrease

Table 7. Distribution of AI-containing lipoprotein subclasses in all subjects, gender and age groups
Fresh plasma was separated by 4–20% gPAGE followed by electrotransfer onto agarose gel matrix containing anti-apoA-I, then agarose gel was washed, dried, stained, and de-stained. Values are expressed as mg/dL (mean ± S.D.).

	AI-Lp subclass								
	1	2	3	4	5	6	7	8	9
All All	8.3 ± 4.5	21.6 ± 7.7	13.9 ± 5.4	17.3 ± 6.9	23.5 ± 7.0	19.5 ± 8.5	14.5 ± 5.0	12.8 ± 4.5	12.0 ± 4.3
Males	9.0 ± 5.1	21.2 ± 7.5	14.2 ± 6.2	17.1 ± 7.5	21.7 ± 5.7	16.9 ± 7.5	12.6 ± 4.2	11.6 ± 4.5	10.5 ± 3.3
Females	7.7 ± 3.8	22.0 ± 8.1	13.5 ± 4.7	117.4 ± 6.4	25.0 ± 7.7	21.7 ± 8.8	16.1 ± 5.2	14.0 ± 4.3	13.3 ± 4.6
%Diff.[a]	−15	4	−5	2	15[b]	29[c]	28[d]	21[b]	27[c]
Group 1	8.0 ± 4.6	18.8 ± 5.4	12.5 ± 4.8	16.2 ± 6.0	24.0 ± 6.4	19.9 ± 9.1	14.0 ± 4.2	12.3 ± 3.3	12.5 ± 4.5
Group 2	7.8 ± 4.2	22.2 ± 8.6	14.1 ± 5.0	18.1 ± 7.3	24.9 ± 8.1	20.5 ± 9.4	16.1 ± 5.6	13.8 ± 5.5	11.3 ± 3.8
Group 3	9.5 ± 4.6	25.1 ± 8.2	15.6 ± 6.3	17.7 ± 7.5	21.0 ± 5.7	17.4 ± 6.0	12.9 ± 4.9	12.4 ± 4.8	12.1 ± 4.6
G2–G1[e]	−3	18	13	12	4	3	15	12	−10
G3–G1[f]	19	34[c]	25[b]	9	−13	−13	−8	1	−3
G3–G2[g]	22	13	11	−2	−16	−15	−20[b]	−10	7

[a]%Difference = [(Females–Males)/Males] × 100; [b]$P < 0.05$; [c]$P < 0.01$; [d]$P < 0.001$; [e]Percentage difference = [(G2–G1)/G1]*100; [f]Percentage difference = [(G3–G1)/G1] × 100; Percentage difference = [(G3–G2)/G2] × 100.

total cholesterol showed no significant correlation with the subclasses except in group 1, where it was negatively correlated with subclasses 1–3 and positively correlated with subclass 7 (Table 9).

Correlating the mass of apoA-I among the AI-Lp subclasses showed a very strong positive correlation between subclass 1 and 2 in all subjects, males, females, group 1, and group 3, but not in group 2 (Table 11). Significant positive correlation was detected between subclasses 1 and 3, and between subclasses 3 and 4 in all subjects, males, females, and all age groups; moreover, all the other significant correlations between subclasses were positive except between subclasses 1 and 6 in females and between subclasses 4 and 6 in females and group 2 (Table 11).

When the percentage distribution of the AI-Lp subclasses was inter-correlated, there was a general trend of significantly negative correlation between the lager subclasses 5–9 and the smaller subclasses 1–4 (Table 12).

DISCUSSION

The traditional CVD risk factors, HDL-C and LDL-C, are no longer considered as good predictors of coronary atherosclerosis (Third Report of National Cholesterol

Table 8. Percentage distribution of AI-containing lipoprotein subclasses in all subjects, gender and age groups
Fresh plasma was separated by 4–20% gPAGE followed by electrotransfer onto agarose gel matrix containing anti-apoA-I, then agarose gel was washed, dried, stained, and de-stained. Values are expressed as mean ± S.D.

	AI-Lp subclass								
	1	2	3	4	5	6	7	8	9
All	5.8 ± 2.7	14.9 ± 3.5	9.6 ± 2.8	12.1 ± 4.3	16.6 ± 3.7	13.6 ± 5.2	10.2 ± 2.6	9.1 ± 2.3	8.5 ± 2.9
Males	6.5 ± 2.8	15.7 ± 3.2	10.4 ± 2.9	12.8 ± 4.8	16.4 ± 3.4	12.6 ± 4.4	9.5 ± 2.4	8.8 ± 2.3	7.9 ± 2.4
Females	5.1 ± 2.5	14.1 ± 3.6	8.8 ± 2.6	11.5 ± 3.7	16.7 ± 4.0	14.6 ± 5.6	10.7 ± 2.8	9.3 ± 2.4	9.1 ± 3.1
%Diff.[a]	−22[b]	−11[b]	−15[b]	−10	2	16	13[b]	6	15
Group 1	5.7 ± 2.5	13.8 ± 3.2	8.9 ± 2.7	11.8 ± 3.5	17.6 ± 3.7	14.4 ± 5.5	10.2 ± 2.5	9.0 ± 2.3	9.3 ± 3.4
Group 2	5.4 ± 3.1	14.4 ± 3.8	9.6 ± 2.8	12.6 ± 5.6	16.8 ± 3.4	13.9 ± 5.3	10.9 ± 2.8	9.3 ± 2.5	7.9 ± 2.5
Group 3	6.5 ± 2.4	17.1 ± 2.6	10.5 ± 2.8	12.0 ± 3.4	14.7 ± 3.4	12.1 ± 4.3	9.1 ± 2.4	8.8 ± 2.2	8.3 ± 2.2
G2–G1[e]	−5	4	8	7	−5	−3	7	3	−15
G3–G1[f]	14	24[d]	18	2	−16[c]	−16	−11	−2	−11
G3–G2[g]	20	19[c]	9	−5	−13[b]	−13	−17[b]	−5	5

[a]%Difference = [(Females–Males)/Males] × 100; [b]$P < 0.05$; [c]$P < 0.01$; [d]$P < 0.001$; [e]Percentage difference = [(G2–G1)/G1] × 100; [f]Percentage difference = [(G3 – G1)/G1] × 100; [g]Percentage difference = [((G3–G2)/G2] × 100

Table 9. Correlation of mass of AI-Lp subclasses with plasma lipids in males (M), females (F), and groups

	Plasma component (mg/dL)	AI-Lp subclass								
		1	2	3	4	5	6	7	8	9
All subjects (M+F) n=90	Total apoA-I	0.29[b]	0.28[a]	0.44[b]	0.22[d]	0.49[c]	0.31[b]	0.42[c]	0.41[c]	0.17[d]
	Total C									
	HDL C					0.28[a]				0.30[b]
	LDL C	–0.28[a]		–0.23[a]						
	VLDL C	0.41[c]		0.28[a]		–0.36[c]		–0.40[c]		
	TAG	0.41[c]		0.29[a]		–0.36[c]		–0.40[c]		
Males n=42	Total apoA-I			0.40[a]		0.54[b]		0.34[a]		
	Total C									
	HDL C									
	LDL C	–0.35[a]								
	VLDL C	0.38[a]		0.35[a]				–0.45[b]		
	TAG	0.38[a]		0.36[a]				–0.44[b]		
Females n=48	Total apoA-I	0.44[b]		0.58[c]		0.42[b]		0.41[b]	0.51[b]	
	Total C									
	HDL C					0.33[a]				
	LDL C									
	VLDL C	0.40[b]				–0.42[b]				
	TAG	0.40[b]				–0.41[b]				
Group 1 (M+F) n=36	Total apoA-I	0.56[c]	0.56[c]	0.67[c]	0.37[a]	0.55[c]	0.43[a]	0.39[a]	0.37[a]	
	Total C	–0.47[b]	–0.37[a]	–0.45[b]				0.41[a]		
	HDL C									
	LDL C	–0.57[c]	–0.46[b]	–0.49[b]				0.40[a]		
	VLDL C	0.50[b]								
	TAG	0.51[b]								
Group 2 (M+F) n=28	Total apoA-I			0.56[b]		0.77[c]	0.43[a]	0.49[b]	0.69[c]	
	Total C									
	HDL C									
	LDL C							–0.43[a]		
	VLDL C					–0.45[a]		–0.52[b]		
	TAG	0.35[d]				–0.45[a]		–0.52[b]		
Group 3 (M+F) n=26	Total apoA-I							0.43[a]		
	Total C									
	HDL C					0.54[a]				0.60[b]
	LDL C									
	VLDL C						–0.47[a]	–0.60[b]		–0.44[a]
	TAG	0.29[d]					–0.47[a]	–0.60[b]		–0.44[a]

[a]$P < 0.05$; [b]$P < 0.01$; [c]$P < 0.001$; [d]Not significant

Education Program (NCEP), 2002; Alenezi *et al.,* 2004; Cavigiolio *et al.,* 2008; Chung *et al.,* 2009). Recently, the American Diabetes Association and the American College of Cardiology (Brunzell *et al.,* 2008) recommended non-HDL cholesterol and apoB as targets of therapy in patients with cardiovascular risk, but HDL-C was not considered as a primary treatment target for CVD (Third Report of National Cholesterol Education Program (NCEP), 2002; Singh *et al.,* 2007). There is also a strong need for biomarkers that reflect the anti-atherogenecity of HDL better than do plasma concentrations of HDL cholesterol or apoA-I (von Eckardstein, 2008). However, HDL is an operational term and does not represent a physiologically relevant entity, although it has an important role in the RCT. Despite the pleiotropic potentially antiatherogenic functions of HDL, the causality of the inverse association between HDL cholesterol and cardiovascular risk has not been definitely proven, because several data from epidemiological, clinical, as well as human and animal genetic studies are contradictory or can be differently interpreted

Table 10. Correlation of abundance of AI-Lp subclasses with plasma lipids in males (M), females (F), and groups

	Plasma component (mg/dL)	AI-Lp subclass								
		1	2	3	4	5	6	7	8	9
All subjects (M+F) n = 90	Total apoA-I									
	Total C									0.25[a]
	HDL C									0.24[a]
	LDL C									0.23[a]
	VLDL C	0.45[c]	0.34[b]	0.42[c]	0.26[a]	−0.39[c]	−0.24[a]	−0.44[c]		−0.24[a]
	TAG	0.45[c]	0.34[b]	0.42[c]	0.26[a]	−0.39[c]	−0.24[a]	−0.44[c]		−0.25[a]
Males n = 42	Total apoA-I									
	Total C									
	HDL C									
	LDL C									
	VLDL C	0.40[a]		0.53[c]	0.36[a]	−0.37[a]		−0.56[c]		
	TAG	0.41[a]		0.53[c]	0.35[a]	−0.37[a]		−0.56[c]		
Females n = 48	Total apoA-I									−0.31[a]
	Total C									−0.32[a]
	HDL C									
	LDL C									
	VLDL C	0.43[b]	0.45[b]			−0.43[b]				
	TAG	0.43[b]	0.45[b]			−0.42[b]				
Group1 (M+F) n = 36	Total apoA-I									
	Total C	−0.51[b]		−0.47[b]				0.59[c]		
	HDL C									
	LDL C	−0.61[c]	−0.38[a]	−0.49[b]				0.64[c]		
	VLDL C	0.40[a]								
	TAG	0.41[a]								
Group2 (M+F) n = 28	Total apoA-I									
	Total C							−0.38[a]		0.38[a]
	HDL C									
	LDL C									0.45[a]
	VLDL C	0.41[a]		0.52[b]	0.46[a]	−0.41[a]		−0.46[a]		
	TG	0.41[a]		0.51[b]	0.45[a]	−0.41[a]		−0.46[a]		
Group3 (M+F) n = 26	Total apoA-I									
	Total C									0.50[a]
	HDL C									0.48[a]
	LDL C									
	VLDL C	0.53[a]	0.53[a]	0.44[a]				−0.58[b]		−0.45[a]
	TAG	0.53[a]	0.53[a]	0.45[a]				−0.59[b]		−0.46[a]

[a]$P < 0.05$; [b]$P < 0.01$; [c]$P < 0.001$

(von Eckardstein, 2008). The dilemma was clearly expressed by Nofer *et al.* (2002): "In our view, these and other contradictions point to the fact that not the concentration of HDL cholesterol *per se* but rather concentration of HDL subclasses are important determinants of the anti-atherogenic potential of these lipoproteins. The diverse actions of HDL observed *in vitro* and *in vivo* could be explained by variation in the content of particles with completely different composition and properties, for example, α-HDL and pre-β-HDL. The assignment of various cellular effects to HDL subclasses and the clarification of their role in prevention of atherosclerosis will be important challenges of lipidology".

Indeed, different efficacies of HDL subclasses in the interactions with cells have been reported (Brewer *et al.,* 2004; Nakamura *et al.,* 2004), and the plasma levels of different subclasses were shown to vary appreciably in various pathological conditions (Puchois *et al.,* 1987; Wilson *et al.,* 1993; Montali *et al.,* 1994; Rader *et al.,* 1994; Syvänne *et al.,* 1995; Asztalos *et al.,* 2001; 2005; Ji *et al.,* 2006). Therefore, the quantitative study of "HDL" sub-

Table 11. Inter-correlation of AI-Lp subclasses

	Subclass	2	3	4	5	6	7	8	9
All subjects	1	0.57[c]	0.63[c]	0.22[d]					
	2		0.50[c]						
	3			0.58[c]	0.22[a]			0.30[b]	
	5					0.31[b]	0.32[b]	0.45[c]	
	6							0.34[b]	
	7						0.53[c]	0.43[c]	0.26[a]
	8								0.24[a]
Males	1	0.72[c]	0.63[c]			0.38[a]		0.35[a]	
	2		0.74[c]	0.48[b]				0.35[a]	
	3			0.66[c]	0.36[a]			0.51[c]	
	4							0.34[a]	
	5					0.45[b]	0.52[c]	0.59[c]	
	6						0.41[a]	0.41[a]	0.33[a]
	7							0.56[c]	0.45[b]
	8								0.37[a]
Females	1	0.47[c]	0.62[c]			–0.31[a]			
	2		0.27[d]						
	3			0.50[c]					
	4					–0.37[a]			
	6						0.52[c]		
Group 1	1	0.61[c]	0.56[c]						
	2		0.57[c]		0.47[b]				-0.40[a]
	3			0.50[b]					
	4						0.37[a]		
	6							0.40[a]	
	7							0.52[a]	
Group 2	1	0.36[d]	0.45[a]						
	3			0.45[a]					
	4					–0.40[a]			
	5						0.41[a]	0.64[c]	
	6						0.71[c]		
	8								0.44[a]
Group 3	1	0.86[c]	0.88[c]	0.55[b]				0.61[b]	
	2		0.81[c]	0.67[c]				0.59[b]	
	3			0.80[c]				0.60[b]	
	4							0.48[a]	
	5								0.63[b]
	6						0.58[b]		0.52[a]
	7							0.47[a]	0.68[c]

[a]$P < 0.05$; [b]$P < 0.01$; [c] $P < 0.001$; [d]Not significant

classes in terms of their apoA-I content is the best way, so far, to study their distribution in fresh plasma. Since the other reported methods for the study of the distribution of HDL subclasses are semiquantitative and may not detect the smallest subclasses (Table 2), the results reported here contribute to a better classification of AI-Lp particles and quantitative determination of the subclasses.

We report here the identification of nine different AI-Lp subclasses, with relative molecular masses ranging from 70 000 to more than 354 000 (Stokes' radii ranging from 3.56 to more than 5.80 nm). No smaller subclasses

Table 12. Inter-correlation of abundance of AI-Lp subclasses

	Subclass	2	3	4	5	6	7	8	9
All subjects	1	0.60[c]	0.47[c]		−0.31[b]	−0.44[c]	−0.51[c]	−0.28[a]	−0.22[a]
	2		0.38[c]		−0.35[b]	−0.39[c]	−0.51[c]	−0.30[b]	−0.33[b]
	3			0.37[c]	−0.30[b]	−0.61[c]	−0.49[c]	−0.25[a]	−0.31[b]
	4					−0.51[c]			
	5								
	6						0.36[c]		
	7								
	8								
Males	1	0.50[b]	0.36[a]		−0.36[a]		−0.55[c]		
	2		0.38[a]			−0.33[a]	−0.58[c]	−0.35[a]	
	3			0.34[a]	−0.40[a]	−0.65[c]	−0.54[c]		−0.41[a]
	4					−0.44[b]			
	5								
	6								
	7								
	8								
Females	1	0.65[c]	0.51[c]			−0.54[c]	−0.42[b]		
	2		0.31[a]		−0.37[a]	−0.38[a]	−0.42[b]		
	3			0.35[a]		−0.55[c]	−0.40[b]		
	4					−0.57[c]			
	5								
	6						0.41[b]		
	7								
	8								
Group 1	1	0.44[a]	0.37[a]			−0.35[a]	0.54[b]		
	2					−0.36[a]	−0.50[b]		−0.44[a]
	3					−0.43[a]	−0.39[a]	−0.57[c]	
	4					−0.51[b]			
	5								
	6								
	7							0.37[a]	
	8								
Group 2	1	0.73[c]	0.43[a]		−0.49[b]	−0.47[a]	−0.46[a]		
	2				−0.60[c]	−0.41[a]	−0.48[b]	−0.39[a]	
	3			0.42[a]		−0.67[c]	−0.59[c]		
	4					−0.54[b]			
	5								
	6						0.57[c]		
	7								
	8								
Group 3	1	0.59[b]	0.69[c]			−0.52[a]	−0.47[a]		
	2				−0.44[a]		−0.44[a]		−0.65[b]
	3			0.46[a]	−0.45[a]	−0.81[c]	−0.45[a]		−0.47[a]
	4					−0.54[a]	−0.45[a]		
	5								
	6								
	7								
	8								

[a]$P < 0.05$; [b]$P < 0.01$; [c]$P < 0.001$

Table 13. AI-lipoprotein subclasses compared with HDL subclasses reported in literature

Subclasses number	Diameter (nm)	Reported HDL subclasses with their diameters (nm)
1	7.12	Small (AI)HDL and small (AI+AII)HDL (7.12) (Atmeh, 1990) HDL subclass (7.12) (Atmeh *et al.,* 2009)
2	7.60–8.00	HDL_{3b} and HDL_{3c} (7.62–7.97) (Blanche *et al.,* 1981) HDL_{3c} (7.80) (Verdery *et al.,* 1989); HDL-14 (7.86) (Li *et al.,* 1994) VSHDL (7.60) (Okazaki *et al.,* 2005); Sm-LpAI (7.50) (Duverger *et al.,* 1993) LpAI/AII (8.00) (Leroy *et al.,* 1993) α_3-HDL (7.62) and pre-α_4-HDL (7.67) (Asztalos *et al.,* 1993) HDL subclass (7.96) (Atmeh *et al.,* 2009)
3	8.00–8.46	HDL_{3b} (8.40) (Verdery *et al.,* 1989); HDL_{3a} (8.44) (Blanche *et al.,* 1981) HDL-12 (8.30) and HDL-13 (8.14) (Li *et al.,* 1994) pre-α_3-HDL (8.40) (Asztalos *et al.,* 1993); HDL subclass (8.42) (Atmeh *et al.,* 2009) $Lp(AI+AII)_3$ (8.00) (Cheung & Albers, 1984); Lp(AI+AII) (8.10) (Ohta *et al.,* 1988)
4	8.46–9.06	HDL_{3a} (9.00) (Verdery *et al.,* 1989); HDL subclass (8.92) (Atmeh *et al.,* 2009) HDL-9 (8.90), HDL-10 (8.73), and HDL-11 (8.53) (Li *et al.,* 1994) VSHDL (8.80) (Okazaki *et al.,* 2005); Md-LpAI (8.9) (Duverger *et al.,* 1993) Lp(AI) (8.80) (Ohta *et al.,* 1988; Leroy *et al.,* 1993); $Lp(AI)_2$ (8.50) (Cheung & Albers, 1984) $Lp(AI+AII)_2$ (8.90) (Cheung & Albers, 1984); Lp(AI+AII) (8.70) (Leroy *et al.,* 1993) Lp(AI+AII) (9.00) (Ohta *et al.,* 1988)
5	9.06–9.74	HDL_{2a} (9.16) (Blanche *et al.,* 1981); HDL_{2a} (9.60) (Verdery *et al.,* 1989) HDL-7 (9.57) and HDL-8 (9.24) (Li *et al.,* 1994); HDL subclass (9.46) (Atmeh *et al.,* 2009) α_2-HDL (9.20) and pre-α_2-HDL (9.42) (Asztalos *et al.,* 1993) Lp(AI+AII) (9.60) (Leroy *et al.,* 1993); $Lp(AI+AII)_1$ (9.60) (Cheung & Albers, 1984)
6	9.74–10.30	SHDL (9.80) (Okazaki *et al.,* 2005); HDL-6 (10.00) (Li *et al.,* 1994) Lp(AI+AII) (10.10) (Ohta *et al.,* 1988); HDL subclass (10.02) (Atmeh *et al.,* 2009)
7	10.30–10.82	HDL_{2b} (10s.57) (Blanche *et al.,* 1981); HDL_{2b} (10.80) (Verdery *et al.,* 1989) HDL-5 (10.55) (Li *et al.,* 1994); LpAI (10.60) (Leroy *et al.,* 1993)
8	10.82–11.60	HDL-3 (11.25) and HDL-4 (10.96) (Li *et al.,* 1994) MHDL (10.90) (Okazaki *et al.,* 2005) pre-α_1-HDL (11.05) and α_1-HDL (10.97) (Asztalos *et al.,* 1993)
9	>11.6	HDL_1 (12.00) (Verdery *et al.,* 1989) HDL-1 (12.46) and HDL-2 (11.74) (Li *et al.,* 1994) LHDL (12.10) and VLHDL (13.50) (Okazaki *et al.,* 2005) pre-β_2-HDL (12.17–13.74) (Asztalos *et al.,* 1993)

were detected in the subjects studied, in accordance with a previous finding that the concentration of the smallest subclasses, of 42 000–50 000, in healthy subjects was very low (Atmeh & Robenek, 1996). Subclasses of relative molecular mass greater than 354 000 were not detected in all subjects and did not have the same mobility in different subjects, moreover, some of them appeared as sharp bands. These large particles may represent apoA-I complexes with LDL (Ogasawara *et al.,* 2008), proteins (Vézina, 1988; Vaisar *et al.,* 2007), or albumin (Atmeh *et al.,* 2009).

The sizes of the AI-Lp subclasses were compared with similar HDL subclasses reported in the literature (Table 13). Data in the table show large variability in the subclassification of HDL particles and the different sizes given to the same subclass. As an example, the subclasses HDL_{2a} and HDL_{3a} were reported by Blanche *et al.* (1981) with the diameters of 9.16 and 8.44 nm, respectively, whereas Verdery *et al.* (1989) reported the diameters as 9.60 nm and 9.00 nm, respectively. This discrepancy may have resulted from differences in methodology used and/or differences in the subjects studied. In fact, from our experience, the position of HDL or AI-Lp subclasses on gPAGE can vary slightly or substantially from subject to subject. These variations can be explained by the microheterogeneity of HDL subclasses, where subclasses of similar size may have different combinations of apolipoproteins and phospholipids (Atmeh *et al.,* 2009), moreover, each lipid component (phospholipids, cholesteryl ester, TAGs) of the subclass contains several types of fatty acids (Atmeh, unpublished). This means that combinations between these variables, apolipoproteins and lipids with different fatty acids, will give rise to a large number of possible compositions, i.e., microheterogeneity; consequently, this will be reflected in a small change in the molecular mass and size of the particles which, in turn, will affect their migration in gPAGE. This is in accordance with the reported changes in the electrophoretic mobility of LpAI and LpAI/AII subclasses as a result of changes in the ratio of neutral to polar lipids (Ohta *et al.,* 1994). Furthermore, another contributing factor to the microheterogeneity is the presence of small peptides (Hortin *et al.,* 2006; Vaisar *et al.,* 2007) or proteins such as albumin (Atmeh *et al.,* 2009) associated with HDL. However, the presence of several particles with slight differences in composition within a particular subclass will give rise to broad bands in the gPAGE. For this particular reason, we prefer to consider the molecular mass and radius as ranges rather than fixed values, unless the band is sharp, as in the case of the small subclasses of relative molecular mass of 42 000, 45 000, and 50 000 (Atmeh & Robenek, 1996; Atmeh & Issa, 2005).

When plasma was stored at 4 °C in the presence of the LCAT inhibitor DNBA, or in its absence, the proportion of some subclasses changed with time (Table 6). This low stability on storage is expected, since in a previous report isolated pure small LpAI subclasses (42 000–70 000) underwent large changes with the appearance of several fractions of higher molecular mass on storage at 4 °C for 3–5 days with as well as without the LCAT inhibitor (Atmeh & Issa, 2005). From these

data we can conclude that such rearrangements of the AI-Lp subclasses, whether in a pure form or in plasma, appear LCAT-independent. It seems that this low stability and easy remodeling of the small subclasses may be essential for their function, which is in accordance with the report of Guha *et al.* (2008) who stated that "HDL stability must be delicately balanced to maintain the structural integrity of the lipoprotein assembly and ensure structural specificity necessary for interactions of HDL with its metabolic partners, while facilitating rapid HDL remodelling and turnover at key junctures of cholesterol transport" and "HDLs are stabilized by kinetic barriers that decelerate protein dissociation and lipoprotein fusion".

The lipid profiles of males and females were different, where females had statistically significantly higher plasma apoA-I and HDL-C than males, while the TAGs were lower, with no significant differences in total cholesterol or LDL-C (Table 3); these results are similar to those reported by Li *et al.* (1996). Moreover, there were differences in the plasma level of the AI-Lp subclasses between males and females, where the subclasses 5–9 were statistically significantly lower in males, subclasses 1 and 3 were non-significantly higher and subclass 2 lower in males (Table 7). Differences in similar subclasses have been reported between males and females (Li *et al.*, 1996). In the age groups there was a non-significant gradual increase in total cholesterol, LDL-C, VLDL-C, and TAGs with age, however, there were no significant differences in the lipid profile between groups 1 and 2, whereas group 3 showed significantly higher total cholesterol, VLDL-C, and TAGs than both groups 1 and 2, and higher LDL-C than group 1 (Table 4). Interestingly, there was a gradual increase in the level of the subclasses 2 and 3 with age, and they were statistically significantly higher in group 3 than in group 1, while subclass 7 was significantly lower in group 3 than in group 2 (Table 7).

The data was grouped as bivariate tables and bivariate analysis was performed. The correlation between subclasses 3 and 2 was significantly positive in all subjects, males, group 1, and group 3, and non-significant in females, while the correlation between subclasses 1 and 3 was significant and strongly positive in all subjects and groups (Table 11). This can be understood in the light of the report of Cavigiolio *et al.* (2008) showing that reconstituted HDL subclass of diameter of 8.4 nm, equivalent to our subclass 3, undergoes conversion to a particle of 7.8 nm diameter, equivalent to our subclass 2, and lipid-poor apoA-I is formed. This phenomenon may contribute to the concept of *in vivo* remodeling of AI-Lp (or HDL) subclasses. Other significant correlations between AI-Lp subclasses can be seen in Tables 11 and 12. Furthermore, the significant negative correlation between percentages of subclasses 5, 6, 7, 8, and 9 with the rest of the subclasses may suggest a reciprocal relationship where the smaller subclasses may have been formed from the transformation of the larger ones (Cavigiolio *et al.,* 2008).

Plasma apoA-I was significantly positively correlated with the mass of all AI-Lp subclasses in all subjects except subclasses 4 and 9 where the correlation was non-significant, in accordance with similar correlations reported by Tian *et al.* (2007), whereas the associations in males, females, and the other groups were variable (Table 9). Moreover, the plasma level of the smallest AI-Lp subclass (subclass 1) was significantly positively correlated with the plasma TAGs in all subjects, males, females, group 1, and non-significantly positively correlated in groups 2 and 3 (Table 9). This can be explained in the light of the preferential hepatic lipase (HL) effect on the large subclasses where it was reported that HL is most effective in hydrolyzing HDL if it is enriched with TAGs (Lewis & Rader, 2005) producing small particles, the so-called lipid-poor apoA-I. This small particle can be filtered through the renal glomeruli and be degraded by the renal tubular cells (Rader, 2006). This enhanced clearance may explain the absence of the smallest LpAI subclasses (42 000, 45 000, 50 000) or their presence at low concentration (Atmeh & Robenek, 1996) in healthy normolipidemic subjects. Since the plasma TAGs level is low in our subjects (Tables 3 and 4), it is not unexpected to have low level or absence of the smallest subclasses.

The immunoblotting method used in this work can aid in the study of exchange of apolipoproteins between the subclasses both *in vitro* and *in vivo* by isolating the small subclasses of molecular mass $\leq$50 000 that contain apoA-I as the sole protein (Atmeh *et al.,* 2009), labeling them, incubating them with plasma or other subclasses, or given to volunteers. The distribution of label among the subclasses can be quantitatively analyzed by the method described here. Isolation of the small HDL subclasses in a pure form can be done by the recently reported method of Atmeh *et al.* (2009), however, due to the low level of the small LpAI subclasses in fasting healthy subjects, a postprandial sample after a fatty meal could be obtained where the level of the small subclasses increases (Atmeh & Robenek, 1996). The statement of Rader (2006) "There remains tremendous need for new *in vivo* kinetic approaches to reliably assess the effects of new therapies on RCT and HDL function and predict their effect on atherosclerosis" asserts the need for new kinetic approaches. Such an approach can be achieved by a combination of the immunoblotting method described here and the mathematical approach reported previously by Atmeh (1987). In that approach exponential equations were derived from differential equations which enable the calculation of the amount of label from HDL components in any of the intravascular, extravascular, and urine compartments as a fraction of the original dose of the label at any time point, beyond the timely collected blood and urine samples, from the plasma concentration of the labeled subclass, and without the need for 24 h urine collection. If the small LpAI is labeled and injected to a subject, the distribution of the label among the subclasses with time can be measured from the dried agarose gel after immunoblotting. These data can be used to study the maturation of the small LpAI subclasses and to calculate the amount of label from each subclass in the extravascular and urine compartments. The obtained decay data can be further analyzed by the appropriate kinetic model to calculate the kinetic parameters of each subclass.

CONCLUSIONS

The results of this work: 1) revealed a quantitative change in the mass of AI-Lp subclasses in plasma samples on storage, 2) detected variations in the distribution of AI-Lp subclasses related to gender and age, 3) showed significant correlations between some of the subclasses and between some subclasses and the lipid profile of the subjects, 4) contribute to a unified classification of AI-Lp subclasses according to their size.

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